

Chapter 2

Transcriptional Regulation of Circadian Clocks

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2.1 Introduction

The first chapter of this book has introduced the historical background of the circadian clock, as well as its anatomical organization. It has described how researchers over the past several decades have grappled with the problem of biological timekeeping: how a constantly-changing organism can measure time, and in particular solar time, accurately in a changing environment? In the case of simpler eukaryotes, the desired metric is longer than the lifespan of the organism, and the mechanism must be cell-autonomous and robust to cellular division. Added to this already-daunting problem is the difficulty of temperature: biochemical reactions occur with greater rapidity as temperature increases, and any timekeeping mechanism must be immune to these changes. In this chapter, we shall consider the molecular mechanisms by which metazoan organisms have organized timekeeping mechanisms that fulfill all of these criteria.

A cell-autonomous circadian system is present in nearly all cells of all metazoans studied so far, from flies to man, and its component proteins share high homology from one organism to the next. In fact, the same general mechanism is even conserved in plants and simpler eukaryotes. Though individual components are no longer precisely homologous, identical general lessons can be drawn. For those interested in these interesting comparisons, Chap. 7 is devoted to comparing clocks among different organisms later in this book. In it, similarities and differences among circadian systems in metazoans, in plants, in simple eukaryotes like the bread mold *Neurospora crassa*, and in the evolutionarily ancient clocks of photosynthetic cyanobacteria are considered. The present chapter, however, considers the basic design principles of metazoan clocks, the ways in which they are controlled

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by the environment, and the ways in which they in turn control the vast spectrum of circadian output processes.

2.2 Basic Design Principles: The Transcriptional Feedback Loop

Transcription is necessary to exploit the genetic information stored in the genome of an organism. This information has to be converted into an mRNA copy before it can be used as template for the synthesis of its corresponding gene product. In principle, regulation of this process can be achieved by two opposing mechanisms: transcriptional activation or repression. In this section, we will elaborate the principal concepts how to build stable circadian oscillators from simple transcriptional regulatory loops. From the observation of Hardin, Hall, and Rosbash in 1990 that the product of the circadian clock protein PERIOD regulates its own transcription, a model was proposed that has become the cornerstone of thinking about the circadian clock for the past 20 years – a transcriptional feedback loop of gene expression [1]. Since its origin, the idea possessed an immediate appeal. Without any consideration for biology, it was mathematically apparent that such auto-repression could explain the oscillatory behavior – of genes, of proteins, or of anything else. (For a basic description of the mathematics, see Appendix 1. For a brief introduction to the biology of transcription and translation, see Appendix 2.)

2.2.1 *The Simple Transcriptional Feedback Loop*

Plainly stated, for the circadian clock the basic idea of a feedback loop of gene expression is that the transcription of a “clock gene” is repressed indirectly by its product. Although elegantly simple, this idea has two fundamental problems. Most importantly, it does not explain how the circadian oscillator measures daily time. From the moment a eukaryotic gene is “activated” or switched on, the time taken for its transcription and translation is up to 2 h. Thus, in its simplest form, a transcriptional feedback loop would have a period of between 1 and 2 h, and certainly not 24.

This difficulty is best highlighted by “designed” oscillators of gene expression that have been created by multiple groups in an attempt to mimic the functions of the circadian oscillator. For example, Elowitz and Leibler have created a simple oscillator in *E. coli* by introducing synthetic genes that regulate each other, using three known transcriptional repressors from other systems. In their system, the lacI transcriptional repressor inhibited the transcription of the tetR transcriptional repressor, tetR inhibited transcription of the cI transcriptional repressor, and cI inhibited transcription of the original lacI repressor, thereby “closing” the feedback loop. The basic promoters that turned on each gene in the absence of repressor were strong, but were able to be tightly shut off, and the half-life of each protein was

short (less than 1 h). The resulting oscillator had a period of around 2.5 h [2] (See Fig. 2.1a and b.). Already, this simple design was robust to cellular division (in *E. coli* every 20–60 min depending upon nutrients). In natural systems, a similarly short period can be seen in the clock that directs somite formation during vertebrate development. Here, the HES-7 gene product directly represses its own transcription, and the resultant oscillatory period is 2 h long [3].

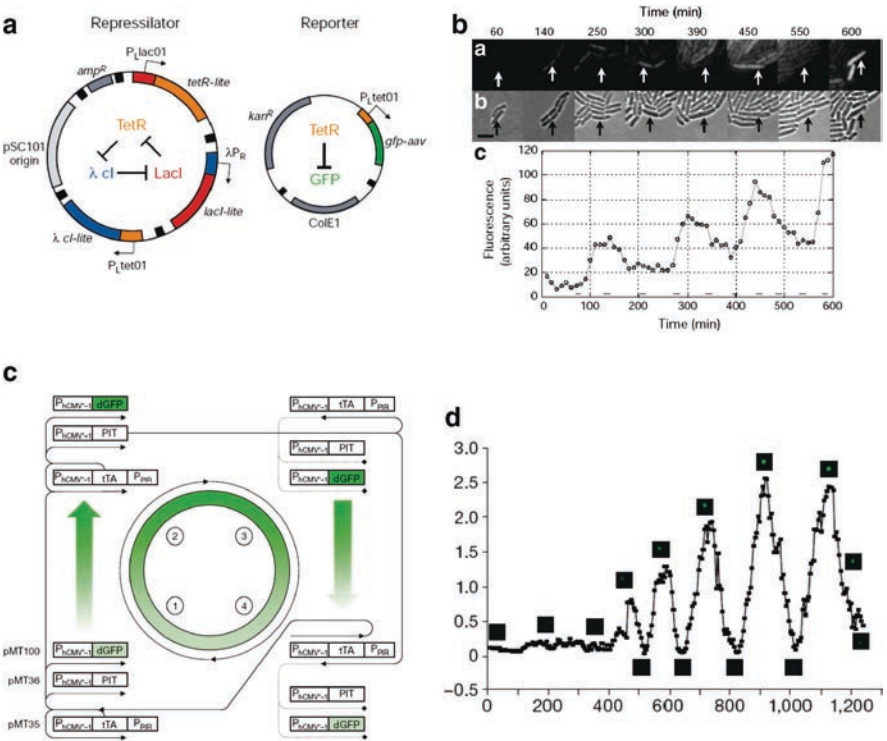


Fig. 2.1 (a) The bacterial “repressilator” of Elowitz and Leibler. It is composed of three repressor genes and their corresponding promoters. It uses p_{lacO1} and p_{tetO1} , which are strong, tightly repressible promoters containing *lac* and *tet* operators, respectively, as well as p_R , the right promoter from phage lambda. The compatible reporter plasmid at right expresses an intermediate-stability GFP variant (*gfp-aav*). (b) Growth and time course of GFP expression of a single cell of *E. coli* strain MC4100 containing the repressilator plasmids. Fluorescent (top) and brightfield (middle) snapshots are shown, along with quantitation of observed fluorescence. (c) The mammalian oscillator of Tigges et al. Autoregulated phCMV-1-driven tTA transcription triggers increasing expression of sense tTA (pMT35), UbV76-GFP (pMT100), and PIT (pMT36) (1). As UbV76-GFP and PIT levels reach a peak (2), PIT steadily induces pPIR-driven tTA antisense expression (3), resulting in a gradual decrease in sense tTA, PIT, and UbV76-GFP (4). (d) Sample output from mammalian CHO cells transfected with equimolar ratios of each of the plasmids of the oscillator system. Text and Figure parts a and b are reproduced from Elowitz and Leibler (2000), parts c and d are reproduced from Tigges et al. [4] with permission

2.2.2 Additional Features Stabilizing Transcriptional Feedback Loops

The second major problem faced by a simple “feedback loop” oscillator is robustness. In the simple form that has been discussed, the period length of the resulting clock – as well as whether it cycled at all – would be highly influenced by the concentration of its components, and could also dampen rapidly. Thus, it would be highly susceptible to “stochastic noise”, the variation of transcription or translation rates from one cell to another based upon random availability of components. Here, again, the ramifications are best illustrated synthetically. The *E. coli* oscillatory system described in the previous paragraph showed both rapid damping and relatively unstable period [2]. To achieve a stable period length, more precise control of nonrepressed transcription – i.e. the transcription of feedback loop components in the “on” state – is required. Such an example can be found in a mammalian synthetic feedback loop designed by Tigges et al. [4]. Here, transcription of the ttA tetracycline-mediated activator was driven by a constitutive strong promoter, the CMV promoter. Antisense transcription of the same gene – i.e. transcription of the other strand of DNA – was driven by the pristamycin-dependent transactivator PIT. Negative feedback was provided at two levels. First, transcription of the PIT gene was itself turned on by the ttA activator; and second, antisense transcription of the ttA locus interferes with ttA production. The activation properties of this network can be modulated by antibiotics, because both the ttA activator and the PIT activator can be potentiated by the presence of antibiotic (tetracycline or pristamycin, respectively), thereby controlling the degree of activation. The resultant oscillator displayed a stable period length in individual cells that was tunable from 2 to 6 h in length, but critically dependent upon activator concentrations for its stability. (See Fig. 2.1c and d) In addition, this synthetic system still displayed significant stochastic variation from cell to cell, with period variations of one-third to one-half the average period length [4]. Overall, based upon this experiment and from others like it, it is likely that two design features aid in robust oscillations: a time delay in the negative feedback loop, and the additional input of positive factors [5].

From these examples, one can conclude that a circadian oscillator based upon a simple feedback loop of gene expression would be very imprecise and only a few hours long. Nevertheless, all circadian oscillators studied so far are remarkably reliable daily timekeepers. Thus, other factors must be operational to aid in their stabilization and in the lengthening of their period. A first clue to these “other factors” is offered by the dazing and evergrowing array of genes that have been shown to be important to the circadian oscillator.

2.3 Clock Genes, Clock Gene Functions

Beginning with the discovery of *Drosophila* mutations that changed the period length of fly activity measured in constant environmental conditions, an ever-increasing array of loci has been shown to influence the circadian clock function.

These genes have been discovered in a variety of different organisms using both genetic and biochemical techniques. Most have been shown to be regulated by other clock gene products, or to interact with them. Set out next is a list of these “clock genes” and their demonstrated or presumed functions within the circadian clock. Subsequently, we shall consider their interactions in a feedback loop model of the circadian oscillator. According to their genetic or biochemical activities, these genes have been classed below as “negative” or “positive” depending upon whether they play a repressive or activating role within this feedback loop. For those wishing to see the interactions more globally while reading about the individual genes, the overall network for mammals is diagrammed in Fig. 2.2, and it will be discussed in detail after the individual genes have been introduced.

2.3.1 *The Period Genes*

These first-discovered of clock genes were initially characterized as mutations of a *Drosophila* gene that affected the period length of fly circadian behavior [6]. All of the mutations cosegregated to the same fly gene, Period (abbreviated Per). Nevertheless, homology-based cloning in mammals has indicated three Period genes, *Per1*, *Per2*, and *Per3* [7]. Because the expression of Per in flies represses its own transcription by direct or indirect means [1], it is traditionally indicated to be at the heart of the circadian “transcriptional feedback loop”, generally in a negative or repressive role. It has also been shown to play an activating role for the *Bmal1* gene [8], discussed below, but this interaction is likely indirect (e.g. the repressor of a repressor).

Genetically, hypomorphic mutations (causing reduction of function) or deletions of one or more *Per* genes have resulted in shorter circadian period length or in arrhythmicity – i.e. the lack of a functional oscillator. Even in humans, a familial mutation mapped to the *Per2* gene causes Familial Advanced Sleep Phase Syndrome, a disease characterized by short circadian period and early behavioral phase [9]. In *Drosophila* mutations can also be found in the *Per* gene that lengthen circadian period [10]. These map to a particular helix believed to be involved in PER protein homo- or heterodimerisation and in temperature compensation, the mechanism by which the circadian clock succeeds in maintaining the same period length at different temperatures [11, 12].

Structurally, the PER proteins contain two PAS (PER-ARNDT-SIM) protein–protein interaction motifs [13], two other C-terminal alpha helices likely involved in interprotein interactions [12], nuclear localization and export signals [14], and sites for post-translational modifications. Hence, it is not surprising that the PERIOD proteins have been shown to interact biochemically with multiple different dedicated members of the circadian oscillator, including Timeless and Cryptochromes. (For a description of these and other mentioned proteins, as well as cited literature, please see their corresponding rubrics below.) The actions of PER proteins are probably facilitated or hindered by a number of nondedicated

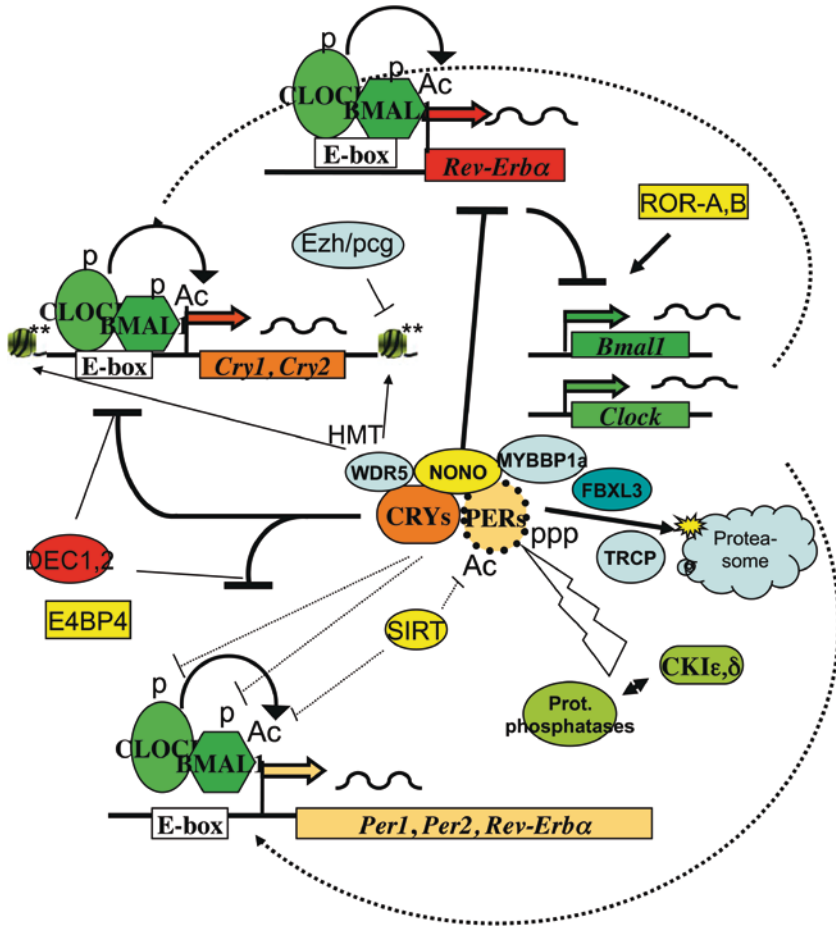


Fig. 2.2 Model of the mammalian circadian oscillator. A pair of transcriptional activators, BMAL1 and CLOCK, activates transcription via E-box motifs of two classes of repressors. In the stabilizing loop, REV-ERB α represses immediately the transcription of the *Bmal1* and *Clock* genes. The transcriptional activators ROR α and ROR β can rhythmically compete with the action of REV-ERB α to fine-tune circadian gene expression. In the core loop, BMAL1 and CLOCK activate the transcription of the *Per* and *Cry* genes. Upon reaching a certain threshold concentrations, these factors counteract the positive factors to repress the *Per*, *Cry*, and *Rev-Erba* genes. This generates two interlocked feedback-loops with their phases separated by about 12 h. Post-translational modifications (p for phosphorylation, e.g. by CKI ϵ,δ , Ac for acetylation) regulate the activity or half-lives of the different proteins. In particular, SIRT may influence the activity of BMAL1 or the half-life of PER2, FBXL3 determines the half-life of the CRY proteins and TRCP determines the half-life of the PER proteins via proteasome-dependent degradation pathways, and various factors (WDR5, Ezh/pcg, and the HAT activity of CLOCK) may regulate the local chromatin structure. Some factors, like NONO and MYBBP1 α , interact with PER or CRY proteins, respectively, but have yet to precise functions. There are additional factors, which are involved in the regulation of circadian genes like the *Dec1* and *Dec2* genes, and E4BP4

proteins – i.e. proteins which play an important circadian function, but additionally play functional roles in other noncircadian systems. These include adaptors for chromatin modifying complexes like WDR5 [15], F-box-containing ubiquitin ligase complex members like β -TRCP in mammals [16] and SLIMB in *Drosophila* [17], corepressors such as MYBBP1a [18] and E4BP4 (a homolog of *Drosophila* Vriille) [19, 20], and RNA-binding proteins such as NONO [15], all of which have been shown to interact with PER protein itself. Another RNA-binding protein, LARK, has been shown to interact with the *Per* mRNA to modulate its stability [21].

Period proteins are modified post-translationally by a number of kinases including casein kinase 1 ϵ , casein kinase 1 δ , and casein kinase 2 [22–26]. In *Drosophila*, the same conserved domain phosphorylated by these kinases in the PER protein has been linked to its nuclear localization and transcriptional repression activity, suggesting that many actions of and upon PER may be inter-related [27, 28]. In mammals, different phosphorylation events have been shown to affect the stabilization of PER and its nuclear localization in different ways (see Chap. 3) [29]. PER protein is also acetylated, and its deacetylation by SIRT1 facilitates its degradation and perhaps also connects PER protein function to cellular metabolism [30].

In mammals, the period genes *Per1* (and possibly *Per2*) are also acutely induced by light in the suprachiasmatic nucleus (SCN) (see also Sect. 2.5.1), and probably play a role in the input of light into the circadian molecular circuit [31, 32]. *Per* genes are also induced in cells by a variety of stimuli that reset the circadian oscillator, and therefore are likely to play a role in clock synchronization at all systemic levels [33, 34]. This role is not completely conserved in all metazoans. In zebra fish, at least one of the (multiple) *Per* genes demonstrates a behavior that is the reverse of the mammalian one, and is repressed by light [35], and in *Drosophila*, the role of PER in light-induced phase shifting is an indirect one: the Timeless and Cryptochrome proteins are likely the direct mediators of light upon the circadian oscillator [36].

2.3.2 *The Timeless Gene*

This gene was also first isolated in *Drosophila*, where its function was shown to be critical to the circadian oscillator, and its presence necessary for the nuclear localization of Period proteins [37, 38]. Since these two proteins dimerize in the cytoplasm prior to translocating to the nucleus, it was largely assumed that TIM and PER translocated as a complex; however, recent FRET studies have disproved this notion, and instead suggest that the two proteins accumulate as dimers together in the cytoplasm and then enter the nucleus separately within the same approximate temporal window [39]. Consistent with this observation, although PER and TIM are both classed as “negative” factors, PER proteins appear capable of directing transcriptional repression in the absence of TIM [40].

TIM also serves as a central regulation point for the effects of light upon the circadian oscillator via its light-dependent degradation mediated through Cryptochromes

[36], discussed next. This degradation also requires proteasome function, probably recruited via the JETLAG protein [41]. In mammals, however, the role of Timeless is highly controversial. The mammalian TIM protein has been shown to interact with other clock proteins in transfection assays [42, 43], and antisense oligo-based loss-of-function experiments in the SCN also suggest a role in the clockwork [44]. Nevertheless, the mammalian TIM is in fact probably the homolog of the distantly-related *Drosophila* Timeout protein important in development, and not of the Timeless protein itself [45]. A mouse *Timeless* knockout perishes early in development at embryonic day 8 [46]. Hence, its direct role in the mammalian circadian clockwork remains a disputed question, and the Timeless protein itself remains one of the most significant differences between insect and mammalian circadian systems.

In insects, however, the importance of Timeless to the circadian oscillator remains unquestioned, and its interaction with PER is important both for PER nuclear localization as discussed earlier, and for the modification of PER by casein kinase 2 [47]. TIM protein is itself post-translationally modified by another kinase crucial to insect circadian function, Shaggy [48]. Shaggy is the *Drosophila* homolog of the mammalian glycogen synthase kinase 3 β kinase, and cellular expression and inhibition studies suggest that this kinase too may play a role in the circadian clockwork [49].

2.3.3 *The Cryptochrome Genes*

The third major dedicated class of circadian genes that play a repressive role in the circadian oscillator are the Cryptochrome genes. These genes were first identified by their homology to blue-light photoreceptors in plants and bacteria, and their effects upon the circadian oscillator were therefore presumed to be light-driven [50]. In fact, mouse knockout studies and numerous functional ones show that in mammals, cryptochromes play an essential role in the inherent mechanism of the circadian oscillator [51], and specifically in transcriptional repression [52]. Surprisingly, they have little or no circadian photoreceptive role at the whole-organism level [53]. Nevertheless, in *Drosophila*, these proteins clearly carry out both functions: on the one hand, they act as blue-light photoreceptors that mediate the light-dependent degradation of the TIM protein [36, 54]; and on the other, they act as direct or indirect transcriptional repressors that play a necessary light-independent role in the circadian clockwork [55].

Structurally, CRY proteins possess an N-terminal domain homologous to bacterial photolyases which is sufficient for phototransduction and also apparently for transcriptional repression [56], and a carboxy-terminal section that is responsible for interaction with other proteins, including TIM and PER [57]. All cryptochrome proteins also bind two cofactors, a pterin (methenyltetrahydrofolate) and a flavin (FADH). In photolyases, the pterin cofactor harvests light and transfers it to the FADH, which in turn interacts with DNA. Although all important residues for photolyase function appear conserved, no photolyase activity has been detected in vertebrate CRY proteins.

Like PER proteins, CRY proteins are implicated in transcriptional repression within the core circadian clock mechanism. In fact, CRY proteins have transcriptional repressive activity independent of PER [58]. It is perhaps due to this potentially redundant function that deletions of one *Cry* gene in mammals can suppress the effects of deletion of a *Per* gene, a hypothesis discussed further below [59]. Finally, tangential to their clock roles, insect CRY proteins also play an important role in sun-compass navigation and magnetosensitivity [60, 61].

2.3.4 The Clock Gene

The *Clock* (Circadian Locomotor Output Cycles **K**aput) gene was first identified via a landmark forward mutagenesis screen in the mouse, followed by positional cloning [62, 63]. A close homolog of similar function exists in *Drosophila* [64]. Together with its partner BMAL1 (described below), CLOCK acts as the principal transcriptional activator of the circadian feedback system. It binds to *cis*-acting elements called E-boxes [65], which are present in the promoter sequences in multiple circadian clock genes of repressive function (including the *Periods* and *Cryptochromes*, and the *Rev-Erb α* repressor gene described below). In some tissues, a second CLOCK-like protein termed NPAS2 is also present [66]. Probably for this reason, the *Clock* gene is dispensable for circadian locomotor activity in mice [67]. Nevertheless, the activity of at least one of these two proteins is essential to circadian function [68, 69]. This activity appears to be that of a traditional transcriptional activator, directly or indirectly recruiting histone-modifying complexes, coactivators/adaptor complexes like p300/CBP, and thus RNA polymerase II itself [70–72].

In several respects, however, CLOCK does not behave as a “traditional” transcriptional activator. In addition to a PAS domain by which it probably interacts with its partner BMAL1, CLOCK possesses an intrinsic acetylase activity [73], which can act not only upon histones but upon its partner BMAL1, and is necessary to its activating function [74]. The same redox-sensitive SIRT1 protein that has been implicated in the deacetylation of PER2 protein has also been ascribed the function of deacetylating CLOCK [75]. Secondly, and in keeping with this connection to redox and cellular metabolism, the heterodimerisation of CLOCK and NPAS2 with BMAL1, and therefore its interaction with its target E-box DNA element, has been found to be redox-sensitive *in vitro* [76].

In mammals, the expression of the *Clock* gene is constant or very weakly circadian, but in *Drosophila* this gene shows a strong circadian amplitude. Its transcription is controlled by a pair of related transcription factors, PDP-1 (PAR-domaine protein 1) and VRILLE. Whereas the former protein activates transcription of *Clock* in flies, the latter represses it. In turn, the transcription of both of these factors is activated by dimers of CLOCK and its partner CYCLE (see below) [77, 78]. Both *Vrille* and *Pdp1* are essential for functional circadian oscillations in flies, and have a mammalian homolog, the E4BP4 protein, that probably plays a role in *Per2* expression [79, 80].

2.3.5 *The Npas2 Gene*

As mentioned in the immediately preceding section, this protein was initially identified as a homolog of the CLOCK protein, and appears to share or assume its functions in many tissues. Unlike CLOCK itself, however, the NPAS2 protein contains a heme-binding domain adjacent to its PAS domain responsible for interaction with the other circadian proteins. This heme-PAS combination is a common regulatory motif in a variety of enzymatic systems including histidine kinase and phosphodiesterase in mammals, as well as oxygen-sensing and nitrogen fixation proteins in plants and bacteria [81]. In the circadian oscillator, heme appears to modulate the activity of NPAS2 by preventing its DNA-binding in response to carbon monoxide [82, 83]. Thus, the NPAS2 protein might play a special role in circulatory or cardiac circadian clocks, but further research is required to clarify the nature of such a role [70].

Both CLOCK and NPAS2 are phosphorylated *in vivo* in circadian fashion. Although the identity of the responsible kinase is not known, this phosphorylation appears to facilitate DNA-binding and to be inhibited by the CRY proteins [84, 85]. Such a mechanism would therefore provide a mechanism for rhythmic transcriptional activation of circadian genes.

2.3.6 *The Bmal1 Gene*

This gene encodes the partner of CLOCK, and was initially identified in a yeast two-hybrid screen for proteins that interact with it [86]. Its fly homolog CYCLE possesses similar function [87]. As mentioned above, in mammals this protein is directly acetylated by its partner CLOCK, and these acetylated residues are critical to its ability to activate transcription [74]. Its interaction with its binding partner is also dictated *in vitro* by the redox potential of the incubation buffer [76]. In the cell, this state would be controlled principally by the concentrations of NAD⁺/NADH, NADP⁺/NADPH, and reduced and oxidized glutathione, opening a tempting link between the circadian clock and cellular metabolism. Although attempts to demonstrate a circadian oscillation of cellular redox state have so far proven unsuccessful, the SIRT1 “sirtuin” protein is a deacetylase activity that modulates circadian function by deacetylating either BMAL1 or PER2, and its activity requires an NAD⁺ cofactor [30, 75]. Thus, two independent lines of evidence could tie the transcriptional activation of this dimer to cellular metabolism, and many more experiments underway in various laboratories will soon clarify this interesting subject.

The CLOCK-BMAL1 heterodimer also interacts physically with PER and CRY proteins [88], and this likely allows the repressive proteins described above to achieve their effects. Chromatin immunoprecipitation studies at clock gene promoters *in vivo* show rhythmic daily binding of CLOCK and BMAL1 to E-boxes, and their

dissociation with these sites concomitant with the transient appearance of PER and CRY proteins [89]. Similarly, CLOCK, NPAS2, and BMAL1 undergo circadian phosphorylation concomitant with DNA-binding, and this phosphorylation appears inhibited by CRY proteins [84, 85]. The simplest model to explain these data would be that direct interaction of PER and CRY proteins with CLOCK/BMAL1 complex provokes their dephosphorylation, the dissociation of this complex from DNA, and the concomitant repression of target genes.

In addition to being phosphorylated and acetylated, the BMAL1 protein is also modified by sumoylation in circadian fashion. Although the effects of this modification for the function of the protein as a whole are not yet clear, overexpression in cells of a mutant BMAL1 protein that cannot be so modified shows altered circadian properties, implying that this post-translational modification also plays a functional role [90].

2.3.7 The *Rev-Erb α* and β Genes

The *Rev-Erb α* gene was originally identified via its binding activity upstream of the clock-gene *Bmal* [91, 92]. For the circadian mechanism itself, the important role of the REV-ERB α protein is its binding to *cis*-acting binding sites (the RREs, or Rev-Erb α -responsive elements) in the promoter of the *Bmal1* gene. This binding is essential to repression of *Bmal1*, and therefore to its rhythmic daily expression. Interestingly, such oscillation is not essential to circadian oscillation, and its disruption in mice results in only a small change in period length [91]. Thus, rhythmic expression of the positively-acting elements of the circadian clock is not essential to clock function. By contrast, overexpression of REV-ERB α has proven an effective genetic tool to silence circadian function, establishing the role of this gene, and of its targets, in the circadian clockwork [93].

The *Rev-Erb α* gene is a part of the nuclear orphan receptor superfamily. Although it lacks a traditional ligand-binding domain, like NPAS2 it is capable of interacting directly with a heme cofactor that is important for its repressive activity [94], and that can phase-shift the circadian oscillator [95]. Repression is likely carried out by the NCoR nuclear receptor corepressor complex [94]. This activity is also directly regulated by lithium ions commonly used to treat bipolar mania [96]. Hence, REV-ERB α may be important for conveying systemic signals from and/or to the circadian clock, and its close homolog REV-ERB β likely plays a redundant role in these effects [97].

The *Rev-Erb α* gene itself contains multiple E-box regions necessary for its circadian transcription [98]. Therefore, it also represents a link in the mammalian circadian oscillator between the proteins controlling the Period and Cryptochrome negative elements and those controlling the positive elements Clock and Bmal1. For example, one likely way in which PER is an activator of *Bmal1* transcription is through its negative regulation of *Rev-Erb α* transcription.

2.3.8 *The Ror α , Ror β , and Ror γ Genes*

The **R**etinoid-related **O**rphan **R**eceptor genes undoubtedly play a significant role in a large amount of nuclear hormone receptor-mediated physiology as well as in development and differentiation, both independently and by dimerising with other nuclear hormone receptor family members. In general, they function as transcriptional activators. Since they bind to the same elements as the REV-ERB α protein, they also affect circadian clock function by competing with REV-ERB α [99, 100]. Nevertheless, this activity appears nonessential to rhythmic *Bmal1* transcription [97]. What may be more important is the potential ability of ROR activators to introduce systemic influences upon the circadian oscillator. For example, PGC-1 is a coactivator of ROR proteins that also regulates energy metabolism, and mice lacking this gene not only show defects in *Bmal1* transcription patterns, but also abnormal diurnal activity patterns [101].

2.3.9 *Clock-Associated Genes I: Kinases and Phosphatases*

The previous paragraphs have discussed all known clock-dedicated proteins that play a transcriptional role within the feedback loop. Equally integral to clock function are an ever-growing number of kinases and phosphatases that modify clock proteins. These include casein kinase 1 ϵ (known as Doubletime in flies) [25, 102], casein kinase 1 δ [103], casein kinase 2 [22, 47], glycogen synthase kinase 3 (known as Shaggy in flies) [48], protein phosphatase 1 [104], protein phosphatase 2A [105], and protein phosphatase 5 [106, 107]. The casein kinase family likely phosphorylates Period and Cryptochrome proteins in multiple places leading to different effects, and the protein phosphatases mentioned above have been implicated in their dephosphorylation. Shaggy is likely the kinase responsible for phosphorylation of Timeless. The functions of most of these modifying proteins are as critical to clock function as the canonical clock-related transcription factors described above: their mutation severely attenuates or eliminates circadian function in metazoans from flies to human beings; and some like casein kinase 1 ϵ appear to be stoichiometric members of clock protein transcription complexes [88, 108]. The first mammalian circadian clock mutation to be identified, the *Tau* mutation in the Syrian hamster, turned out to be in casein kinase 1 ϵ ! [25]. In short, the specific roles of each of these kinases and phosphatases are important enough that they are the subject of Chap. 3 in this book.

2.3.10 *Clock-Associated Genes II: Chaperones*

Even from theoretical grounds, it is easy to see that it would be impossible to have a functional circadian oscillator if its component proteins and RNAs were too long-lived. Hence, it is not surprising that many circadian proteins are targeted for proteasomic

degradation, frequently after their phosphorylation by one of the kinases described above. Research by many labs has shown that clock proteins follow the traditional route to the proteasome: they are recognized by a particular class of chaperones containing an F-box motif, and that recruit a ubiquitin ligase complex. The clock protein is then ubiquitinated and later destroyed. For the most part, these chaperones have been discussed above in the context of their respective targets, and include SLIMB (targeting PER) and JETLAG (targeting TIM) in flies [17, 41], and FBXL3 [109–111], FBXL21 [112], and β -TrCP1 in mammals [16].

A second potentially emerging class of chaperone proteins important to the circadian clock are the heat shock proteins. It was recently discovered that Heat Shock Factor 1 (HSF1) binds to its target genes in circadian fashion and activates transcription at a wide number of chaperone loci at the onset of circadian night. Since mice carrying a mutant *HSF1* gene show an altered circadian period length, it is likely that this binding has functional consequences for the circadian clock [113], but further research is necessary to elucidate its target.

2.3.11 Clock-Associated Genes III: Chromatin-Modifying Proteins

One of the surprising recent discoveries within the circadian oscillator is that rhythmic circadian gene transcription is accompanied by corresponding rhythmic modification and demodification of surrounding chromatin in daily fashion. Thus, histone acetylation and histone methylation accompanies both the activation and the repression of clock genes and clock-controlled genes [70, 72, 89, 114]. It is likely that a large number of chromatin-modifying proteins that have been identified in other systems are also important to the circadian oscillator – histone methylases and demethylases, acetylases and deacetylases, and various classes of ATP-dependent chromatin reorganization machines. For the most part, however, these proteins have not yet been identified in the context of the circadian system. Three notable exceptions are WDR5, which is a histone methyltransferase adapter that interacts with PER proteins and is necessary for circadian histone methylation at multiple circadian loci [15]; the polycomb group protein EZH2, which probably facilitates the organization of a repressive chromatin structure during repressive phases of the circadian cycle [115]; and NCoR, the nuclear receptor corepressor complex that recruits histone deacetylase HDAC3 to clock- and clock-controlled loci [116].

2.3.12 Clock-Associated Genes IV: Coactivators and Corepressors

A growing number of proteins have been isolated that are essential or important to the circadian clock mechanism, and whose actions are important for the transcriptional

repression or activation of clock genes. Nevertheless, their exact functional roles have not yet been fully elucidated. For example, the mammalian CIPC gene appears to play a repressive role by antagonizing the CLOCK-BMAL-mediated activation independent of the cryptochromes. Its depletion results in a shortening of circadian period length [117]. Another repressor, the MYBBP1a protein, has been isolated through interaction with PER2 protein, and can be immunoprecipitated at the promoters of PER-regulated genes, where it appears to aid in transcriptional repression [18]. The NONO protein was also initially isolated via its interaction with PER proteins. Mutation of its homolog *NonA* in *Drosophila* or its depletion in mammalian cells results in arrhythmicity, confirming its importance to the circadian oscillator [15]. Nevertheless, the exact function of this protein remains unknown. Its two RNA-binding domains and previous implications in many different aspects of transcription and RNA processing, in both activating and repressing roles, leave many possibilities open.

In *Drosophila*, another important “mystery” repressor is encoded by the *Clockwork Orange (cwo)* gene. It was initially identified as a corepressor that acts together with PER to repress CLOCK-CYCLE-driven transcription of a large number of clock- and clock-controlled genes [118, 119]. Recent research suggests that at the same time that genes regulated by CWO show reduced peak expression levels, they show elevated trough levels, suggesting direct or indirect effects on both the activation and repression of clock genes [120]. Mammals possess two genes that are possible homologs of *Cwo*: *Dec1* and *Dec2*, which play a nonessential role in the repression of *Per1* and other clock-controlled genes [121].

2.3.13 *Relating Clock Genes Together: Interlocking Feedback Loops*

From the above description, exhausting but far from exhaustive, an idea of the various players of the circadian clock can be gleaned. In mammals, these proteins are organized into two major interlocking feedback loops, summarized in Fig. 2.2 In the first, *Cry*, *Per*, and *Rev-Erb α* transcription is activated by CLOCK or NPAS2 and BMAL1, and repressed by the CRY-PER complex. In the second, *Bmal1* transcription is repressed by REV-ERB proteins and activated by ROR proteins. *Clock* gene transcription is not rhythmic in the mammalian system. In *Drosophila*, a similar architecture exists, with CLOCK-BMAL1 substituted by CLOCK-CYCLE, and PER-CRY complexes probably substituted by PER-TIM complexes, with CRY playing an auxiliary role. Although the *Bmal1*-Rev *Erb α* interlocked loop does not exist in flies, a new feedback loop replaces it. The transcription of the *Clock* gene is strongly rhythmic, and is driven by an insect-specific second feedback loop in which *Clock* transcription is activated by PDP1 and repressed by VRILLE protein. In turn, the transcription of both *PDP1* and *Vrille* is activated by the CLOCK-CYCLE heterodimer [77]. Thus, the fundamental architecture of two interlocked loops is conserved across metazoans.

Given this complex structure, it is tempting to ask what within it is essential to circadian function. This question has assumed additional importance since the discovery of a circadian oscillator in cyanobacteria that is based entirely upon feedback loops of phosphorylation – i.e. in this organism the transcriptional feedback loops deemed essential to the metazoan oscillator are not necessary, since the entire clock can function in vitro in the absence of transcription. It has been speculated that a similar situation exists in mammals, and that transcriptional feedback is an “epiphenomenon” of an underlying ancient phosphorylation oscillator. Although post-translational modifications of clock proteins undoubtedly play a crucial role in all metazoans, absolutely no evidence exists to date to support a “post-translational-only” hypothesis, and a great deal against it.

Nevertheless, it is clear that several aspects of the metazoan oscillator are not required for its basic function. Since the *Rev-Erb α* gene can be deleted with only minor effects upon the core circadian oscillator and circadian behavior [91] – even though *Bmal1* transcription is almost constant as a result – rhythmic transcription of positive-limb components must be dispensable in mammals. On the other hand, the abundance of the positive-limb components CLOCK and BMAL1 is still critically important to circadian function, as well as to the overall period and amplitude of the circadian system. Inducible overexpression of wild-type CLOCK protein results in a shortening of period length in mice, and overexpression of a dominant negative mutant does the opposite [122]. The same is true for BMAL1, since reduction of its level in genetically engineered mice via REV-ERB α dampens or eliminates circadian rhythmicity [93]. Although the *Clock* gene displays rhythmic expression in flies, its protein level is constant [123]. Therefore, it is difficult to imagine that the cyclical nature of its transcription is a crucial feature of the circadian oscillator in flies, either. As in mammals, however, overall levels are important: elimination of either the repressor of this gene *Trille* or its activator *PDP-1* results in behavioral arrhythmicity [20, 77]. Since overexpression of *Clock* RNA per se does not affect circadian rhythms, some of this effect may be indirect [124].

Overall, for both mammals and flies, it is clear that the cyclical expression of positive elements within the circadian oscillator is dispensable, though their presence and abundance remains important. Negative elements pose a different question altogether. Mathematic modeling and experimental evidence all points to a crucial and necessary role of repressive components within the circadian oscillator. An excellent formal proof of this idea in mammals is provided by the fact that mutations in CLOCK and BMAL1 proteins that reduce their interaction with CRY proteins result in arrhythmicity at a cellular level [125]. Some studies have suggested in particular that levels or activities of these repressive components may be particularly important for setting the period length of the circadian oscillator [126]. Certainly, many *Per* mutations exist in flies and even in humans that alter period length, and overexpression of either CRY in mammals or either PER or TIM in flies disturbs the circadian period [127, 128]. Similarly, the expression of a CYCLE-VP16 fusion protein – which elevates the transcription of all CYCLE targets thanks to the strong VP16 transcriptional activation domain – severely

shortens circadian period in flies [129]. Here as well, though, it is possible that cyclic transcription is dispensable. In mammals, expression of constant levels of CRY proteins does not visibly perturb rhythms [130]. In flies, constant transcription of both *Timeless* and *Period* also permits rhythmicity. It is possible, though, that these transcriptional perturbations are being compensated by post-transcriptional effects. In the latter example, PER and TIM protein levels continued to cycle in spite of their constant transcription! [127].

2.3.14 Summary: Redundancy is the Key Important Factor

Because of the interlocked nature of its various elements, it is perhaps not surprising that so many different aspects of circadian clock function can be ablated without abrogation of clock function. As mentioned above, circadian transcription of individual clock genes can be eliminated without serious effects, and these changes may be compensated by post-transcriptional effects. Many other examples of redundancy exist. For example, rhythmic histone methylation accompanies circadian oscillations of transcription in all clock- and clock-controlled genes examined so far. Nevertheless, the reduction of WDR5 protein levels in mammalian cells eliminates many of these oscillations, and has only a modest effect upon the circadian amplitude and none upon period length [15]. Similarly, disruption of the interaction between the NCoR repressor and the HDAC3 histone deacetylase changes the phase of some clock- and clock-controlled genes, but failure to recruit this histone deacetylase does not abrogate clock function [116].

Another example can be found in the redundancy of PER and CRY proteins in mammals. Given that two *Cry* and three *Per* genes exist in mammals, it is not surprising that the disruption of almost any one of these loci has only minor effects upon the clock. The only exception here is the *Per2* locus, which appears to play an essential and nonredundant role in the circadian oscillator. Nevertheless, the nefarious effects of a *Per2* gene disruption can be suppressed. . . .by a *Cry2* deletion! [59] Although *Cry1* gene disruption will not achieve this suppression normally, constant light conditions – which ordinarily degrade circadian rhythms in mice – will now allow such compensation to occur [131]. It is possible that the various PER and CRY proteins have similar roles in the cell – as transcriptional repressors, for example – but different potencies. Therefore, elimination of one member of the PER-CRY complex would change its potency, but elimination of another would change this balance again in a favorable direction. Nevertheless, existing mechanistic data do not argue in favor of functional equivalence of PER and CRY proteins. It is possible, however, that such compensation could also occur kinetically at completely different steps in the same pathway. In this case, a change in the potency of one step (for example, transcriptional repression) might be compensated by changing the effectiveness of a different step. (post-translational modification, nuclear export, etc.).

The overall implication of the redundancy, though, is increased robustness and precision. Perhaps, it is this redundancy that allows the circadian oscillator to continue to function indifferent of temperature and cell division. Most spectacularly, the circadian clock has even been shown to demonstrate transcriptional compensation: overall inhibition of RNA polymerase in a variety of ways does not alter the circadian period significantly! [132] How might such compensation work? Many models have been put forward, and their workings are the subject of Chap. 11 of this book. We shall close this section, though, by noting that temperature compensation and precision was also a problem for mechanical clocks. This inability to tell time accurately outdoors led early sailors repeatedly and tragically to misjudge their longitude. (Celestial indices were inadequate for this purpose due to the earth's rotation). The first reliable solutions were achieved by redundant mechanical gearing that allowed temperature-induced changes to act in opposite directions simultaneously. Perhaps a similar logic might govern the redundant and precise circadian biological clock.

2.4 Input and Phase Shifts

As we have seen in the two previous sections, the mammalian circadian oscillator suffices to generate rhythms with a free-running period of about 24 h. However, to be in resonance with the environment, an organism has to adjust its circadian clock, and consequently the circadian oscillators in the individual cells, every day to the external photoperiod. The flow of information to the circadian oscillator is termed the input. The synchronization of the organism to the environment is the main function of the SCN, which receives the relevant photic signals from the retina. The peripheral oscillators are subsequently synchronized by humoral and neuronal signals derived from the SCN. The readjustment of the circadian clock in response to an input signal is called *phase shift* and was originally investigated in animals (see also Chap. 4). This was useful to elaborate the *phase response curve* for a given Zeitgeber (german; “timing cue” which affects the phase of the circadian clock) but did not provide too much detail on the molecular mechanisms of the input pathways involved.

Solely the identification of clock genes and the recent advances of mammalian circadian in vitro systems allowed the investigation of signaling pathways that have an effect on the phase of the molecular oscillator. In principle, due to the organization of circadian oscillators as transcriptional and post-translational feedback loops, signaling pathways could directly influence the concentration or activity of certain oscillator components and consequently change the phase of the interconnected transcriptional network. Unfortunately, there were so many potential phase shifting agents identified that the overall picture at the moment is more confuse than concise. Therefore, the research nowadays attempts to combine data obtained from the animal and in vitro systems with appropriate computational models to identify the relevant input pathways to the circadian oscillator.

2.4.1 Induction of Genes by Light

A mammalian organism that is exposed to a light pulse at the beginning of its dark phase will adjust the phase of its circadian clock accordingly [133, 134]. Beginning from the next day, the phase of the circadian oscillator will be delayed (Fig. 2.3). In contrast, an animal receiving light information towards the end of its dark phase is forced to advance its circadian clock for the next day. Light will thus affect the phase of the circadian oscillator dependent on the exposure time during the dark phase. The entity of phase shifts of the oscillator in response to light (or any other Zeitgeber) is called a *phase response curve*. Typically, in animals a type-1 phase response curve is observed [134]. During the light phase or subjective light phase under constant dark conditions, it is not possible to provoke a phase shift in animals. This part of the phase response curve is sometimes referred to as the “dead zone”. The light input to the SCN emanates from specialized cells in the retina and reaches the core region of the SCN as a glutamate or *pituitary adenylate cyclase activating peptide* (PACAP) signal (see Chap. 4). During the dead zone the SCN secretes the neuropeptides *Transforming Growth Factor α* (TGF α), *Cardiotropin-Like Cytokine* (CLC), and *Prokineticin 2* (PK2), which suppress the locomotor activity of mice and probably also prevent the inadequate phase shifts by light [135–137].

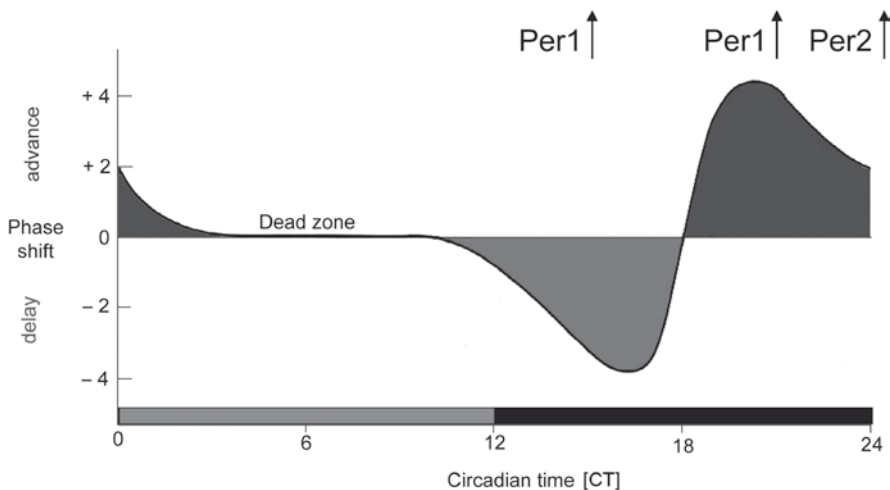


Fig. 2.3 Principles of phase shifting and phase response curves. A light signal (or another specific Zeitgeber) will effect the phase of the circadian oscillator. In a certain period, the oscillator is not responsive to a stimulus. This period is called “Dead zone”. At the beginning of the subjective night phase, a light pulse causes a stable phase delay by up to 4 h. Thereafter, the phase of the oscillator will advance. Concomitant with the behavioral phenotype, a selective induction of the *Per* genes and of other genes like *c-Fos* is observed in the SCN. Courtesy of Isabelle Schmutz, University of Fribourg, Switzerland

This is a difference to the oscillators in the periphery, which can always respond to a resetting signal. The phase response curve for glucocorticoids on the circadian oscillator of the liver, for example, resembles the one shown in Fig. 2.3 but without a dead zone [138]. This is crucial because the periphery should respond to signals from the SCN at any time. Since the circadian oscillator is based on transcriptional feedback loops, the induction and consequently the accumulation of an oscillator component e.g. by light could directly influence the phase of the circadian oscillator.

On the molecular level, *c-fos* was the first gene identified to be induced by light in the SCN [139]. As a typical immediate-early gene, *c-fos* induction had a peak about 30 min after the light pulse and then its expression gradually declined. Most importantly, the induction of *c-fos* strongly correlated with the phase shifting behavior of hamsters by light. The upstream regulator of *c-fos* is the *cAMP response element binding* protein or CREB [140]. After phosphorylation of CREB at its serine residues 133 and 142 in response to light, this protein is capable of binding to CRE-sites within the *c-fos* gene and of activating its transcription [141, 142]. Later on, the binding of ICER, a negative regulator of CREB factors, abolishes the activity of CREB and the transcription of *c-fos* ceases [143–145].

Unexpectedly, mice deficient for the c-FOS protein display a completely normal phase shifting behavior [146]. Therefore, the function of *c-fos* and other immediate-early genes like *junB* and *egr-1*, which were identified in a screen for light-inducible transcripts in the SCN [147], are overall less important for the phase shift behavior of mice but they provide excellent markers to identify the neuronal activity and to reveal a light response in the SCN. Another consequence of a light signal is the drastic increase in serine 10 phosphorylation of histone H3 in the SCN [148]. This specific histone modification correlates with a facilitated accessibility of transcriptional regulatory sites within the chromatin, which may be the reason for the activation of many genes that are not directly involved in the phase shift response.

Shortly after the discovery of the *Period* genes (see Sect. 2.3.1), it was found that those genes were induced in response to a light pulse with a peak 1–2 h after the stimulus [7, 31, 32, 149–152]. The *Per1* gene was induced at the beginning and at the end of the dark phase, while the *Per2* gene was more restricted to the end of the light phase. In spite of this, some research groups also found induction of the *Per2* gene at the beginning of the dark phase. This discrepancy is explained by the different experimental setups employed [153] (genetic backgrounds, light intensities and light conditions used before the experiment, i.e. constant *versus* light-dark conditions). It appears that *Per2* needs more specialized conditions at the beginning of the dark phase for a successful induction by light. Although it appears that the induction of the *Per* genes occurs in different parts of the SCN and with different kinetics [154, 155], in this chapter, we will consider the SCN an entity to facilitate our argumentation.

Similarly, the phenotypes of *Per1* and *Per2* single deficient mice differed. Originally, *Per1* knockout mice were found unable to perform a phase advance in response to a light pulse at the end of the dark phase, while *Per2* knockout mice had a similar problem at the beginning of the dark phase [31]. They were incapable of performing the expected phase delays. This clear distinction between *Per1* and *Per2* was less evident in other mouse strains [156, 157]. Meanwhile, some researchers

interpret the genetic experiments in a way that *Per2* has a more prominent function on the core oscillator, while *Per1* is more important for phase shifts. However, for a definite answer further experiments are necessary.

The induction of the *Per* genes by light appears to be a prerequisite for a phase shift. Interestingly, the *Per1* gene bears a functional CRE-site in its regulatory region and is consequently a target for the activated transcription factor CREB [141, 158]. The induction of *Per1* and *c-fos* occurs with different kinetics in the SCN. This is not completely understood at the moment but suggests that there are other factors that shape the expression of either gene as well. These could be coregulators of the ATF family known to bind together with CREB to CRE-sites or different repressors of the ICER family [159–161]. As a conclusion, the induction of *Per1* or *c-fos* in the SCN by light both rely on CREB binding but the reasons for the different kinetics and the modes of downregulation of both genes are currently unknown. In addition, the induction of the *Per1* gene is sensitive towards inhibitors of histone acetylation and deacetylation but these may be very general processes involved in transcriptional activation and repression, respectively [162]. The induction of the *Per2* gene by light is less well understood. Some experiments suggest a role of either the CREB protein [158] or the PER1 protein in the induction process [163]. Other experiments, mainly in vitro, favor an activation of the *Per* genes by a Ca^{2+} dependent protein kinase C pathway and the direct activation of the CLOCK transcription factor [164].

How would the induction of the *Per* genes cause different phase shifts at different times of the dark phase? This is clearly an unsolved issue. A condition for the different effects is the underlying circadian oscillator. At the beginning of the dark phase, the expression of the *Per* genes in the SCN declines, but there are still high levels of hyperphosphorylated PER proteins and CRY proteins present. In contrast, at the end of the dark phase, the transcription of the *Per* genes recommences but there are only low amounts of hypophosphorylated PER proteins detectable in the SCN. As a speculation, the induction of *Per* genes at the beginning of the dark phase extends the time of active PER proteins being present in the nuclei of the SCN neurons and lengthens the circadian cycle. Therefore, we obtain a stable phase delay for the following days. On the other side, the induction of the *Per* genes at the end of the dark phase mimics the concentrations of PER proteins found later on during the circadian cycle and consequently the following cycles advance. In addition to the *Per* genes, the *Dec1* gene is also light-inducible [121]. This factor was originally identified in a screen to find inhibitors or competitors of BMAL1 and CLOCK-mediated transcriptional activation. Since DEC1 can compete with BMAL1 and CLOCK for binding to regulatory E-box motifs, the induction of the *Dec1* gene by light could immediately modulate the phase of the circadian oscillator in concert with the PER proteins.

2.4.2 Input Signals for Peripheral Oscillators

For a long time, researchers considered the SCN the only real clock generating robust circadian rhythms. The circadian clocks in the periphery were regarded as

“slave oscillators” that were incapable of maintaining rhythms without a permanent input from the SCN. This picture changed with the advances of organ cultures from transgenic rats and mice and with the upcoming mammalian in vitro models [33, 165–168]. The peripheral oscillators are as robust as the oscillator in the SCN [167, 168]. However, the input to both types of oscillators may be different. The major Zeitgeber for the SCN is the environmental light-dark phase but for the periphery, Zeitgebers like food uptake, body temperature, and neuronal and humoral signals have to be taken into consideration.

Explantation studies of different tissues from transgenic *Per2:luc* mice revealed two supplementary facts about peripheral oscillators [166]. First, the period of each tissue varied. This would indicate that there are tissue-specific variants of peripheral oscillators and the regulated transcriptional networks. Secondly, in mice, in which the SCN was ablated and consequently not functional, the organs continued to be rhythmic but they were no longer synchronized amongst each other. This would indicate that the main purpose of the SCN is to synchronize the peripheral oscillators but not to drive circadian rhythms overall. However, there is still evidence for signals that can drive rhythms in peripheral oscillators [93]. In transgenic mice without a functional oscillator in the liver, rhythmic transcripts including those of the *Per2* gene persisted. These rhythms rapidly declined after placing liver slices in culture demonstrating that those rhythms were solely driven by systemic cues.

A considerable progress of our understanding of the input pathways to the peripheral oscillators derived from mammalian circadian in vitro systems. In 1998, Aurelio Balsalobre in Geneva realized that the expression of the *Dbp* gene, an output transcription factor (see Sect. 2.5), transiently decreased after a serum shock in Rat-1 fibroblasts [33]. About 24 h after the shock, the expression levels were up again but continued to decrease thereafter. A careful analysis revealed that this rhythmic behavior proceeded for multiple days and that this was not specific for this gene but that many circadian markers followed the same pattern. The phase differences between all the circadian markers faithfully reflected what was known about the phase differences found in the SCN and peripheral oscillators. In addition, immediately after the serum shock, an induction of *Per1* and *Per2* occurred. Therefore, it was concluded that a serum shock induced free-running circadian rhythms with a period length of 22 h in Rat-1 fibroblasts, which have not been in contact with the SCN for at least 20 years.

Subsequent experiments demonstrated that free-running circadian rhythms could also be induced in *mouse embryonic fibroblasts* (MEF) derived from different genetic backgrounds [169]. Under these experimental conditions, the period of the MEFs in vitro resembled the period of the different mutant mouse strains. For that reason, the mammalian in vitro systems closely reflect the animal models. One major question remained. Are the circadian rhythms in the tissue culture cells newly induced, or are the circadian oscillations of each single cell synchronized? This question was answered by the inspection of individual cells in culture using rhythmically expressed, short-lived fluorescent protein [167]. Under normal culture conditions, the individual cells display circadian rhythms in different phases. After a serum shock, all the different cells become synchronized. This is possible because

tissue culture cells show a typical type-0 phase response. Independent of the position of the oscillator within the circadian cycle a strong signal resets the oscillator always to the same point. Therefore, the oscillators in a culture start cycling from the same point after a serum shock. Using a similar culturing system expressing rhythmically luciferase protein and computer derived simulations, it was proven that the oscillators in cultured fibroblasts were capable of generating robust circadian rhythms similar to the SCN neurons [167, 168].

From early on, the mammalian in vitro systems were used to identify input pathways to the circadian oscillator. One of the first applications was to monitor the influence of dexamethasone, a glucocorticoid hormone analog, on the circadian oscillator. This drug is a potent means to synchronize the circadian oscillators in fibroblasts [34]. These data were compared to the influence of dexamethasone on the livers of animals [138]. As mentioned above, dexamethasone shifts the circadian oscillator of the liver without the presence of a dead zone. However, in tissue culture cells, the phase response to dexamethasone was a typical type-0 phase response. The discrepancy between the effects of dexamethasone on both experimental systems is not known. It is tempting to speculate that due to the absence of moderating hormonal inputs to the cells in the tissue culture, their circadian oscillators are more sensitive to a resetting stimulus. A further reduction of the concentration of dexamethasone to synchronize the tissue culture cells probably will provoke a type-1 phase response.

Interestingly, corticosterone, the natural compound of dexamethasone found in rats and mice, has a direct effect on the phase shift response of the liver circadian oscillator but not on the SCN [138]. The phases of the oscillators in the SCN and in the livers can be separated by up to 12 h using an inverted feeding regimen, a process during which the adaptation of the liver oscillator to the new feeding schedule takes about a week [170, 171]. In mice deficient for the glucocorticoid receptor in the liver or adrenalectomized mice without the capability to secrete corticosterones into the bloodstream, this readjustment occurs in about 2 days suggesting that the signals mediated by the glucocorticoid receptor normally prevent large phase shifts of the liver circadian oscillator [172]. In contrast, after the reconstitution of normal feeding conditions, the liver oscillator requires a couple of days to resynchronize to the phase dictated by the SCN, which is completely independent of the glucocorticoid hormone signaling.

The signaling pathways that were associated with the synchronization of circadian oscillators in vitro were manifold. In addition to a serum shock or glucocorticoids, researchers found an impact of activators of cAMP/CREB signaling (forskolin, dibutyryl cAMP), protein kinase A and C signaling (e.g. phorbol-12-myristate-13-acetate), Ca^{2+} signaling, IL-6 signaling, MAP kinase signaling, and of PPAR α agonists (fenofibrate) on *Per1* induction and/ or the subsequent synchronization of the circadian oscillators in various tissue culture cell models [33, 34, 138, 173–177]. A further breakthrough was the coupling of the mammalian in vitro systems with real-time bioluminescence monitoring. In these systems, a luciferase reporter gene is driven by a circadian regulatory element. Different systems exploit the regulatory region of the *Per1*, *Per2*, *Bmal1*,

Dbp or *Rev-Erba* gene. After the synchronization of the circadian oscillators, it is possible to measure the effect of a given treatment on the magnitude, amplitude or phase of a given reporter gene over the course of multiple circadian cycles. It is possible to exploit these techniques for the high-throughput screening of compounds [178, 179]. The experiments can easily be converted into cotransfection assays to reveal the function of a certain protein on the oscillator, or coupled to RNA interference to monitor the effect of the lack of certain protein on the oscillator (e.g. as described in Brown 2005 [15]). A recent variation of this technique is the transfer of circadian reporter genes by lentiviral-mediated infection. This allows the stable integration of circadian reporter genes even in cells that are normally not easy to transfect. In this manner, it was possible to measure the period of human fibroblasts derived from skin biopsies indicating that the human fibroblasts behave similarly as mouse and rat fibroblasts [180].

Are the input pathways used by light fundamentally different from the ones immersing into the peripheral oscillators? Surprisingly, the answer is no. In an elegant series of experiments, fibroblasts were stably transfected with an expression vector for the photoreceptor melanopsin [181]. These fibroblasts displayed a type-1 phase shift behavior in response to low light intensities and a type-0 phase shift behavior in response to higher light intensities. The phase shift behavior could be blocked by inhibitors of Ca^{2+} signaling or phospholipase C. This indicates that the signaling pathways in fibroblasts mediating the light or hormonal (e.g. a serum shock) input are very similar but specific receptors for a light response are normally missing. Nevertheless, it is tempting to speculate that the process of phase shifting by both types of phase shifting agents in general is essentially the same. Both kinds of phase shifting agents use the induction of specific components to affect the phase of the oscillator for the next circadian cycle.

2.4.3 Integration of the Input Signals

The major Zeitgeber for the SCN is light. The light signal activates the transcription factor CREB by phosphorylation. Upon binding of activated CREB to its relevant binding elements in the *Per1* and *Dec1* genes, these become transiently induced. In addition, under certain circumstances, the *Per2* gene is also induced. Depending on the phase of the underlying circadian oscillator in the SCN, a stable phase advance or phase delay results for the next circadian cycles. For sure, this is a very simplified summary of the processes that occur during the phase shift of a mammalian organism in response to light. Many more signaling molecules and pathways have been characterized to affect the circadian oscillator in the SCN including Vasoactive intestinal polypeptide, Neuropeptide Y, calcium/calmodulin-protein kinase, cGMP-dependent protein kinase II, GABA, glutamate, Gastrin-releasing peptide, and Pituitary adenylate cyclase-activating polypeptide [182–194]. However, this is a rapidly evolving field and it is too early to draw definite conclusions.

Some specific aspects will be further elaborated in Chap. 4. The phase shift is communicated to the various peripheral oscillators via signaling cues some of which remain to be verified *in vivo*.

One potent Zeitgeber for peripheral circadian oscillators is feeding. As mentioned above, it is possible to completely uncouple the liver circadian oscillator from the SCN by an inverted feeding regimen. Restricting the food access to the light phase (when rodents are normally inactive) is sufficient for the uncoupling of both types of circadian oscillators. It is currently unknown whether the feeding behavior of mammals under normal conditions is dictated by the SCN. If this is true, it would provide an elegant link between the SCN and the periphery allowing a tight coupling of the two different systems on one hand but a rapid uncoupling in the case of a limited food access on the other hand.

Another potent Zeitgeber for the periphery is temperature. The body temperature of mammals varies in a circadian fashion. When exactly these kinds of temperature variations were simulated in tissue culture, these temperature rhythms were sufficient to maintain circadian rhythms in Rat-1 fibroblasts [195]. Meanwhile, researchers chose even conditions to synchronize the circadian oscillators of primary human fibroblasts by temperature ramping indicating that rhythmic changes in the body temperature could be a general Zeitgeber for the periphery [180].

How is it possible to integrate the impact of all the different Zeitgebers on the circadian oscillators? To address this question the circadian *transcriptomes* of different tissues were compared [196]. The subsets of genes that were rhythmic in multiple tissues were analyzed for similarities in their regulation. Finally, the proteins expressed by these genes were arranged into regulatory cascades. The overall picture of these theoretical regulatory networks is shown in Fig. 2.4. A light signal to the SCN would activate the protein kinase A. This enzyme would phosphorylate CREB and some other regulatory components of the circadian oscillator. CREB in turn would induce the *Per1* gene, whose gene product (together with PER2 when feasible) would interfere with BMAL1 and CLOCK-mediated transcription to provoke a phase shift.

In response to food uptake, the adrenal gland would produce and secrete glucocorticoid, which would bind to and activate the glucocorticoid receptor. This activated protein can induce both *Per1* and *Per2* and therefore exert the same function as CREB. In the temperature response, the modeling suggests that the transcription factor HSF1 is activated and induces the transcription of many heat shock genes including Hsp90aa1. This protein and others form complexes to inactivate the glucocorticoid receptor, which in parallel could be activated by glucocorticoid due to a general stress response. The rapid inactivation of the glucocorticoid receptor would modulate the induction of the *Per1* and *Per2* genes. Similar to these examples, other signaling pathways could feed into the circadian oscillator by the induction or repression of the genes coding for oscillator components, or directly via the stabilization or degradation of some oscillator components. Only further work will tell us how the circadian oscillator can respond to so many different phase shifting cues at the same time.

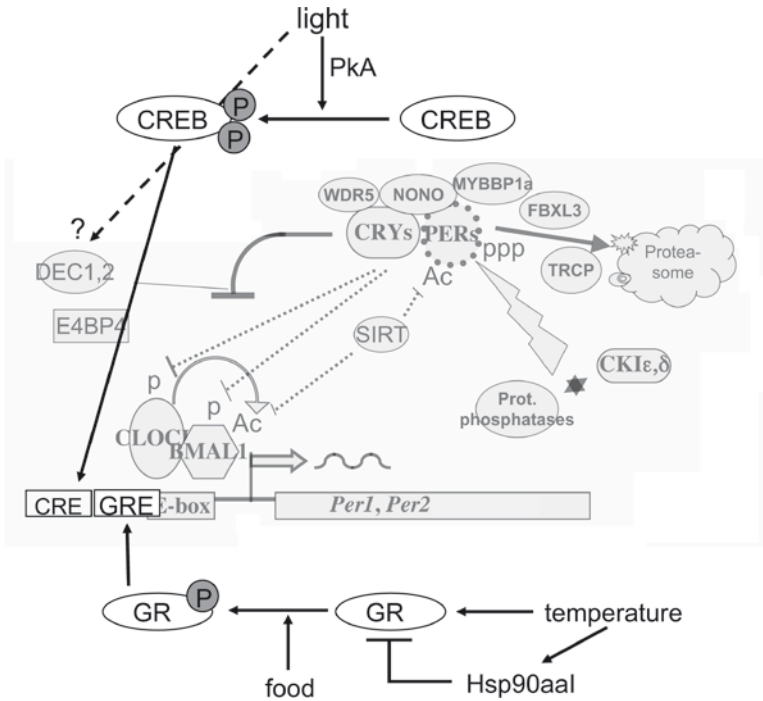


Fig. 2.4 Input to the mammalian circadian oscillator. The input pathways effect the phase of the circadian oscillator in different ways. A light pulse activates the transcription factor CREB by phosphorylation via PkA. This factor can subsequently activate the *Per1* gene. Light also induces under certain circumstances the *Per2* and *Dec* genes. A food-derived signal activates the glucocorticoid receptor (GR), which can activate both *Per* genes. Temperature uses a similar strategy but there is a modulating activity mediated by Hsp90aaI, which appears to inhibit the GR and therefore modulates the response

2.5 Output and Clock Regulated Genes

In contrast to the input, the output is the effector part of the circadian oscillator. All circadian changes in the physiology, metabolism, and behavior are probably linked more or less directly to rhythmic gene expression. Many target genes are hardwired to the circadian oscillator and subsequently expressed in a rhythmic fashion. The organization of the molecular oscillator facilitates the direct coupling of circadian target genes to the transcriptional network. In principle, those rhythmic genes could be activated directly by the BMAL1 and CLOCK or BMAL1 and NPAS2 transcriptional activators, or repressed by the nuclear hormone receptor REV-ERB α (see Sect. 2.3). Indeed, many response elements for these kinds of transcriptional regulators are found in the circadian regulatory regions of rhythmic target genes.

Nevertheless, the situation is more complicated. Many target genes are regulated by rhythmically expressed transcription factors as intermediaries. These factors

appear to be preferentially members of the PAR-bZip or nuclear hormone receptor families but examples are found in nearly all kinds of transcriptional regulator families. Using rhythmically expressed transcription factors as intermediaries allows an amplification of the output, the expression of genes in different phases, and tissue specific gene expression. Therefore, it is not surprising that up to 10% of a given transcriptome (>3,000 genes) is linked to the circadian oscillator but the overlap of rhythmically expressed genes in two different tissues may be less than 100. The next challenge will be the understanding of tissue specific circadian networks.

2.5.1 Regulation of Circadian Target Genes

There are now many genes known to be expressed in a circadian manner. The characterization of these genes unraveled many regulatory mechanisms responsible for the rhythmic transcription of these specific genes. Due to the vast number of circadian target genes, we will present here only a very limited number of examples. Interested readers may refer to the original work done by the different research groups. Here, we would like to focus on some basic principles of the regulation of circadian target genes.

The first example of a circadian target gene directly regulated by the circadian oscillator was the *arginine vasopressin* gene [197]. Originally, this hormone was characterized as a regulator of the salt and water balance in mammals. It is predominantly expressed in the vasopressinergic neurons of the paraventricular nuclei and the supraoptical nuclei, and the final hormone is stored in vesicles in the posterior pituitary. During hypertonic conditions, it is released into the bloodstream to increase water reabsorption in the kidneys. In the SCN, however, this hormone acts as a local neuropeptide. It is released from some SCN neurons to modulate the firing rate of other SCN neurons in the vicinity bearing the V1a receptor. In mice with a homozygous, dominant-negative mutation of the CLOCK protein, the expression of the *vasopressin arginine* gene in the SCN was abolished. Subsequent analysis revealed the existence of an E-box motif (see Sect. 2.3) in its promoter region. In cotransfection experiments, BMAL1 and CLOCK were capable to activate transcription via this E-box motif. Taken together, the genetic and biochemical experiments showed that this gene is hardwired to the circadian oscillator.

The question remains, why the expression of this gene is circadian in the SCN but regulated differently in the other regions of brain like the supraoptical nuclei, where its expression is constant over the day. The mutant CLOCK protein, for example, did not affect the *vasopressin arginine* expression in the supraoptical nuclei. This may be due to the fact that in this region there are only very low levels of BMAL1 detectable and consequently not enough heterodimers are formed to interfere with the expression of this gene. Nevertheless, we learn one important point about gene regulation: one gene can be expressed in a circadian manner, in a tissue specific manner, or in a combination of both. Specific regulatory elements in the promoters and enhancers of the genes have to govern this diversity.

Although there are now many examples of genes regulated by BMAL1 and CLOCK, the effect of a defect of the circadian oscillator on gene expression may be not as prominent as the effect observed for the *vasopressin arginine* gene. Though, there may be interesting phenotypes after all. In *Per2* mutant mice, there is an increase in the intracerebral dopamine levels in the ventral tegmental area and the nucleus accumbens area [198]. As a consequence, these mice display a more depression-resistant like phenotype. In this case, the increase in dopamine levels was associated with a slight downregulation of the *monoamine oxidase A* gene in the *Per2* mutant mice. The monoamine oxidase A is involved in the degradation of dopamine in the mitochondria. Downregulation of this gene indirectly augments the concentration of dopamine. In the regulatory region of this gene, there was again an E-box motif mediating the effects of BMAL1 and NPAS2 in vitro. In addition, in chromatin-immunoprecipitation assays was observed a rhythmic binding of BMAL1 to the promoter region of the *monoamine oxidase A* gene in the ventral tegmental area region. Again, the combination of genetic and biochemical experiments suggests that the *monoamine oxidase A* gene is hardwired to the circadian oscillator in a very defined brain region.

In the same mice, two more phenotypes have been discovered. The PER2 protein acts as a tumor suppressor gene [199]. Mice deficient for the PER2 protein, when irradiated with γ -rays, developed more tumors than their wild-type littermates. This particular phenotype was linked to a deregulation of genes involved in cell-cycle regulation and tumor suppression. In the brains of these mice, also a hyperglutamergic state was observed within the central nervous system [200]. This effect was probably due to a slight downregulation of an astrocyte-specific transporter for glutamate. The resulting phenotype was quite complex. The animals consumed more ethanol and were more resistant to the health-hazardous effects of ethanol. This phenotype could be reverted by the administration of acamprostate, a drug that regulates the intracerebral glutamate levels. Therefore, this neurotransmitter is involved in this phenotype. However, in contrast to the *monoamine oxidase A* gene, it is not known yet, whether this glutamate transporter is a direct target of the circadian oscillator. We have selected these examples to demonstrate that the phenotypes of mutant mice for specific oscillator components may be linked to the circadian oscillator itself or to specific functions of this component independent of the clock. In general, both options are difficult to distinguish.

The mouse *Dbp* gene represents a model system to understand the expression of target genes that are hardwired to the circadian oscillator. It was previously identified as a transcriptional regulator of the *albumin* gene in the liver [201]. Later on, its expression was found to occur with high circadian amplitude in multiple tissues including the liver, the brain, and the SCN [202]. Expression of this gene was abolished in mice with a homozygous, dominant-negative mutation of the CLOCK protein [203]. In addition, the gene contained multiple E-box motifs as potential targets of BMAL1 and CLOCK. A careful analysis of the regulatory region of *Dbp* revealed rhythmic binding of BMAL1 and CLOCK to three distinct regulatory regions [89]. Concomitant with the rhythmic binding of both transcriptional activators, the local chromatin structure changed accordingly. During the activity phase

of this gene, the chromatin was in an open state, while during inactivity the chromatin resembled a heterochromatic, inaccessible state. The oscillator may direct the reversible acetylation of histone H3. The histone acetyl transferase activity of CLOCK [73], upon binding of this factor, may directly modify the local nucleosomes, while the NAD⁺ dependent histone deacetylase SIRT1 may counteract the activity of CLOCK either on the level of histone acetylation or on the acetylation of the BMAL1 protein [75]. Recently, it was described that in the SCN of CLOCK deficient mice the *Dbp* gene was amongst a very limited number of genes, whose expression was abolished [67, 68]. Taken together, the genetic and biochemical experiments strongly suggest that *Dbp* is a genuine target gene for BMAL1 and CLOCK in essentially all the cells with functional oscillator.

Mice deficient for DBP and the related transcription factors TEF and HLF have only subtle effects on the circadian oscillator. However, similar to the *Per2* mutant mice, they exert an interesting phenotype again due to the deregulation of an enzymatic activity. In the brains of these mice, there occurs a slight downregulation of the expression of the *pyridoxal kinase* gene [204]. Its gene product is necessary for the phosphorylation of vitamin B6 derivatives to generate pyridoxal phosphate. Pyridoxal phosphate is a cofactor required by many enzymes, some of which are involved in the metabolism of neurotransmitters, e.g. in the synthesis of dopamine from DOPA, or in the conversion of the excitatory neurotransmitter glutamate to the inhibitory neurotransmitter GABA. It appears that a diminution of the pyridoxal kinase-activity in the brain causes a concomitant reduction of serotonin and dopamine levels and consequently spontaneous epileptic seizures in these mice. In the context of this paper, also an interesting hypothesis was posed. In the brain, there occur only slight oscillations as compared to the other tissues. This may be associated with the fact that large variation of neurotransmitter concentrations would have a harmful impact on brain function.

At the same time, these mice also had a reduced life expectancy due to the deregulation of other enzymes in the liver [205]. Here, mainly enzymes involved in the detoxification pathways for xenobiotic compounds and in drug metabolism were affected. As a result, these mice were very sensitive to the toxic effects of xenobiotic compounds and this may play a part to their reduced life expectancy. Why would detoxification enzymes be expressed in a circadian manner? It is known that cytochrome P450-containing detoxification enzymes produce reactive oxygen species in the absence of their suitable substrates. This would cause severe damage to the enzyme itself and potentially also to the entire cell. The liver cells choose two ways to cope with this problem: first, there is a circadian basal expression of the detoxification enzymes to anticipate the beginning activity phase and the potential uptake of food and xenobiotics. Secondly, there are inducible mechanisms, which can drastically upregulate detoxification enzymes in the presence of higher quantities of xenobiotic compounds. Taken together, here we described an example of rhythmically expressed genes that are regulated by transcription factors, which themselves are hardwired to the circadian oscillator. However, on top of this circadian regulation, there may be inducible regulation to bolster up the expression of these genes, as well.

There exist many more classes of transcriptional regulators that can connect the circadian oscillator to the rhythmic output. A conclusion from DNA-microarray experiments, for example, was that REV-ERB α response elements were identified in the promoter regions of many rhythmic genes expressed in the SCN during the subjective night phase [92]. In the same kind of analysis, cyclic AMP response elements for CREB were found in many genes expressed in the SCN during the subjective light phase. Therefore, it was concluded that these particular kinds of response elements mediate rhythmic transcription of target genes in different phases. In later studies, this range of response elements was extended by E boxes as binding sites for BMAL1 and CLOCK, and D-elements as binding sites for the PAR-zip transcription factors and E4BP4 (Fig. 2.5) [128, 206–209]. However, circadian gene regulation may be even more complicated. The gene for the *Cholesterol 7 α -hydroxylase*, for example, has in vitro binding sites for DEC2, E4BP4/DBP, PPAR α and REV-ERB α and β [210]. All of these factors have to collaborate to fine-tune the expression of this particular gene in the liver.

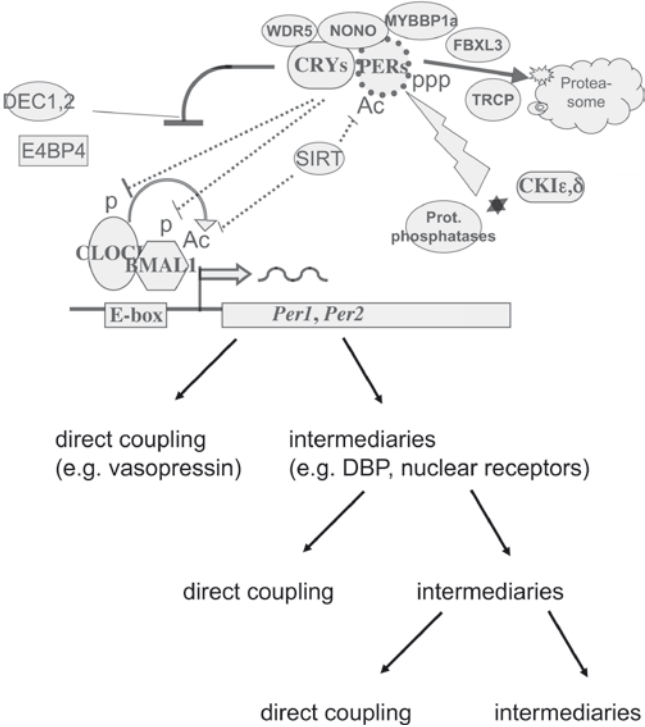


Fig. 2.5 Output from the mammalian circadian oscillator. Some target genes are directly hard-wired to the circadian oscillator, either via BMAL1 and CLOCK, or REV-ERB α . Others rely on rhythmically expressed transcription factors like DBP, or nuclear receptors. Note that this simple make-up facilitates tissue-specific gene expression. Rhythmic signals are easily amplified by tissue-specific transcription factors expressed in a rhythmic fashion

If we combine all the possible ways to regulate the circadian target genes, we end up with very complicated networks of rhythmic gene expression (Fig. 2.5). At the center of these networks, we have the circadian oscillator. To this oscillator are connected a couple of direct target genes including transcriptional regulators. In the next layer, we have target genes that are indirectly regulated by the circadian oscillator with the help of these transcription factors as intermediaries. Since amongst those indirectly regulated genes there are other transcriptional regulators as well, the system creates more and more layers of rhythmically expressed genes. Interestingly, these networks establish also many additional feedback loops, which allow an even more precise regulation of gene activity and, last but not least, may feedback to the circadian oscillator. However, because these networks have so many dynamic layers of gene expression, it becomes increasingly complicated to distinguish between direct or real target genes of the circadian oscillator and a plethora of bystanders, which are rhythmically regulated but do not have any consequence for circadian changes in the physiology or metabolism. In the next section, we will have a closer look on some of these circadian networks and their interconnections. For the particularly well-characterized interaction between the circadian oscillator and the metabolism we have dedicated an entire chapter later on (see Chap. 5).

2.5.2 *Analysis of Circadian Transcriptional Networks*

How is it possible to have a glimpse on circadian transcriptional networks? The method of choice is the use of DNA microarrays. Briefly, RNA is extracted from a given tissue. This RNA is copied into complementary DNA using standard molecular biology methods. During this process the resulting DNA is normally amplified and marked with radioactivity or fluorescence and can be used as a probe. This pool of labeled DNA fragments, which still represents the proportions of the original pool of mRNA, is now hybridized to a DNA microarray. On the surface of a DNA microarray gene specific probes are fixed in well-defined patterns. In this fashion, it is possible to identify a specific transcript out of the pool, and since hybridization is a quantitative process, we also obtain information regarding the amount of a given transcript.

At the beginning, it was possible to analyze for the presence of a couple of thousand different transcripts in a single experiment. Nowadays, DNA microarrays cover the entire potential transcriptome of an organism, even with multiple probe sets. The data for the analysis of circadian networks are normally grouped into time frames, filtered and evaluated for rhythmic patterns. In the early days, this “data-mining” was not very standardized. Therefore, the overlap of rhythmic transcripts found by different research groups in the same tissue was limited. Again, there was a lot of progress achieved and the results from DNA microarrays are much more robust today. For instance, increasing the number of time points allowed a more detailed analysis of the circadian transcripts in the pituitary gland [211]. As another example, in the first experiments the expression of circadian genes in the liver showed a clear

enrichment towards the transitions from the subjective light-to-dark and dark-to-light phases [212]. In subsequent experiments with higher-capacity microarrays, a very even distribution of the expression peaks of circadian mRNA accumulation was observed in the same tissue [213]. Even so, the percentage of rhythmically expressed genes in both studies was always about 10% of the expressed genes.

The first DNA microarray experiments were already conducted in 2002 [212, 214, 215]. However, most of the conclusions drawn at that time are still valid today. A major surprise was the discovery of tissue-specific gene networks. Depending on the experiment performed, between 5 and 10% of rhythmic transcripts in the transcriptome of a given tissue were identified. Tissue-to-tissue comparisons revealed only a restraint number of common genes. In the first experiments, there were a couple of dozen common transcripts, nowadays, there are close to 100. The implications of these findings are that there is a general circadian mechanism at the base of all the different kinds of circadian oscillators but depending on the cell type, these are linked to tissue-specific networks. The use of a general circadian mechanism does not incriminate that all of the circadian oscillators have to be in the same phase. In the brain, for example, circadian oscillations are observed in many regions albeit with different phases. This can probably be achieved either by modulating the input to the circadian oscillator by tissue-specific receptors or transcriptional regulators, or by tissue-specific regulatory feedback loops.

How are these tissue-specific regulatory networks organized? Again we will focus here only on one set of data. Interested readers may refer to the original work published by other groups, too. In addition, you will find a detailed description of the connections between the circadian clock and the metabolism in Chap. 5. The expression of rhythmic transcripts in the SCN was one of the first to be investigated [214, 215]. It was established then that the circadian oscillator could coordinate intracellular processes. One of the major functions of the SCN is the secretion of neuropeptides and neurotransmitters. Consequently the synthesis, processing, and degradation pathways for some neurotransmitters are temporarily optimized in the SCN neurons. This is also reflected in a coordinated expression of components of the ribosomes. Increasing the number of functional ribosomes can augment translation and consequently the production of peptide hormones. Interestingly, there was also observed a temporarily switch of the expression of components of the nascent polypeptide-associated complex and of the signal recognition particle. The first complex directs translation into the cytoplasm, the latter redirects and fixes the ribosomes to the membrane of the endoplasmatic reticulum, a prerequisite for protein secretion. Concomitant with the increase of translation through the membrane of the endoplasmatic reticulum, there was an upregulation of proteins involved in vesicle formation and trafficking. Taken together, the microarray data uncovered a step-by-step organization of neurotransmitter synthesis, starting from the transcription of these genes per se to the secretion of the final product. This may be the advantage of the circadian oscillator: to allow the temporal organization of synthesizing and degrading processes within a single cell.

To sum up, the circadian output is organized in tissue-specific transcriptional networks. It will be very interesting in the near future, to solve all the connections

of the transcriptional networks in the different cell types. At the same token, it will become possible to analyze the interaction of the circadian oscillators between different tissues. However, data and models based solely on mRNA accumulations have to be granted with caution. There exists a second layer of complexity in the organization of circadian oscillators. This layer is made up of post-translational modifications of the gene products. The nature of these modifications and how these modifications affect the circadian oscillator will be in the focus of the next chapter.

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