Genetic and Molecular Analysis of the Central and Peripheral Circadian Clockwork of Mice

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A hierarchy of interacting, tissue-based clocks controls circadian physiology and behavior in mammals. Preeminent are the suprachiasmatic nuclei (SCN): central hypothalamic pacemakers synchronized to solar time via retinal afferents and in turn responsible for internal synchronization of other clocks present in major organ systems. The SCN and peripheral clocks share essentially the same cellular timing mechanism. This consists of autoregulatory transcriptional/posttranslational feedback loops in which the *Period* (*Per*) and *Cryptochrome* (*Cry*) “clock” genes are negatively regulated by their protein products. Here, we review recent studies directed at understanding the molecular and cellular bases to the mammalian clock. At the cellular level, we demonstrate the role of F-box protein Fbx13 (characterized by the afterhours mutation) in directing the proteasomal degradation of *Cry* and thereby controlling negative feedback and circadian period of the molecular loops. Within SCN neural circuitry, we describe how neuropeptidergic signaling by VIP synchronizes and sustains the cellular clocks. At the hypothalamic level, signaling via a different SCN neuropeptide, prokineticin, is not required for pacemaking but is necessary for control of circadian behavior. Finally, we consider how metabolic pathways are coordinated in time, focusing on liver function and the role of glucocorticoid signals in driving the circadian transcriptome and proteome.

INTRODUCTION

The previous decade has witnessed major, indeed astonishing, advances in our understanding of the molecular genetic and cellular bases to circadian timing in mammals (Reppert and Weaver 2002; Lowrey and Takahashi 2004). The SCN of the hypothalamus were first revealed as the “body clock” more than 30 years ago (Weaver 1998). They remain preeminent as the pacemaker responsible for coordinating circadian physiology across the organism and synchronizing it to solar time by retinally mediated entrainment. But in addition to the SCN, we now appreciate that most major organ systems and diverse cell types contain local circadian clockworks and that SCN-dependent synchronization of these local clocks and their dependent transcriptomes orchestrates daily physiology (Akhtar et al. 2002; Panda et al. 2002). The SCN are therefore no longer thought of as coercing a passive periphery into rhythmic behavior; rather, they harness the intrinsic rhythmicity of organs, both sustaining their amplitude and setting their phase. This precise and elaborate temporal coordination of physiology underlies the marked daily prevalence of morbidity in cardiovascular, metabolic, and other diseases (Hastings et al. 2003). Moreover, circadian disturbance, be it environmental or genetic, is linked to malignancy and metabolic and mental illness. Understanding how the SCN operate as a pacemaker tissue and how their output coordinates circadian physiology are outstanding questions with considerable relevance to human health. They also provide a cardinal example of how regulated gene expression can control complex behavior—the Holy Grail of molecular neurobiology. The purpose of this chapter is to review

recent studies from our laboratories that have addressed these issues. Our broad strategy has been to use real-time imaging of circadian gene expression to explore molecular timekeeping in genetically modified mice. We offer specific examples illustrating how the period of the central and peripheral clocks is determined by the rate of proteasomal degradation of circadian proteins, how neuropeptidergic signaling synchronizes and sustains cellular circadian pacemaking in the SCN, and how behavioral control by the SCN is mediated via neuropeptides not required for pacemaking but necessary for downstream signaling. Finally, we consider how SCN outputs coordinate metabolically relevant pathways, focusing on liver function. It is likely that the relevance of clocks to medicine will expand far beyond sleep disorders to encompass metabolic disturbances of many kinds. Understanding the cellular and molecular genetic bases of peripheral circadian physiology is therefore a strategic aim of the field.

SETTING CELLULAR CIRCADIAN PERIOD BY REGULATED PROTEASOMAL DEGRADATION

The cellular oscillator of the SCN is viewed as a series of interlocked transcriptional/posttranslational feedback loops (Reppert and Weaver 2002; Lowrey and Takahashi 2004). Expression of the genes encoding the negative regulators *Cryptochrome* (*Cry*) and *Period* (*Per*) is *trans*-activated at the start of the circadian day by complexes containing the positive factors, *Clock* and *Bmal1*, that act via E-box *cis*-regulatory elements. This is followed, with

a delay, by progressive accumulation of Per and Cry proteins in the nucleus of SCN neurons (Kume et al. 1999; Field et al. 2000). By the beginning of circadian night, Per and Cry protein abundance is maximal, and negative regulation of the *Per* and *Cry* genes is evidenced by declining levels of the relevant mRNAs, which fall to a nadir by the end of circadian night. In the absence of further transcriptional activity, remaining Per and Cry proteins decline, again tracking the mRNA profile with a delay of about 4 hours. As Per and Cry protein levels reach a minimum around circadian dawn, negative regulation of their genes is released and the cycle starts anew. This core cycle is stabilized by accessory loops involving rhythmic expression of Rev-Erb α the clearance of which at the end of circadian night activates *Bmal1* (by derepression), augmenting the renewed surge in E-box-mediated drive.

The molecular details of these loops, including the nature of the interactions among the proteins, DNA regulatory sequences, and histone modification are described elsewhere in this volume, but a central question remains: What sets the period of the clock to 24 hours? Clearly, this is an emergent property conferred by various factors and processes. For example, a mutation that compromises the *trans*-activational function of Clock slows period to about 28 hours, whereas mutations of Per2 or casein kinase I (CKI) that alter Per2 stability accelerate period to about 20 hours. To identify further determinants of circadian period, and by implication components of the core molecular clockwork, a dominant *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen for period mutations is being conducted in mice (Bacon et al. 2004). In G₁ progeny of ENU-mutagenized animals, a single founder animal was identified with a significantly lengthened period (~0.4 hours) of its rest-activity cycle. Inheritance testing and intercrossing revealed that the mutation, called “after hours” (*Afh*), was semidominant with a period of about 27 hours in homozygotes (Fig. 1A). To test the effect of the mutation on the core molecular loop, *Afh* mice were crossed into the Per2::Luciferase reporter line (Yoo et al. 2004), and bioluminescence was recorded in SCN organotypical slice cultures. The prolongation of period seen for the behavioral rhythm was also evident in the molecular rhythm in vitro, showing that the mutation affected the core SCN clockwork (Fig. 1B,C,D). Moreover, the mutation had a global effect on timekeeping, with significant lengthening of period across major organ systems including kidney, lung, and liver monitored in vitro (Fig. 1E). To assess the molecular basis of the *Afh* phenotype, in vivo expression of circadian proteins and mRNA was examined in SCN and peripheral tissues. Homozygotes exhibited a dramatic reduction in Per protein abundance and Per and Cry mRNA levels in SCN and liver (Fig. 2A,B). At the protein level, however, the mutation had a complex effect on Cry levels. In the SCN, the cycle of Cry immunoreactivity was not different from that of wild type, even though mRNA expression was markedly reduced. In the liver, peak levels of Cry (assessed by western blot) during circadian night were reduced but during circadian day levels were elevated.

The *Afh* mutation was mapped to a gene encoding an F-box protein with leucine-rich repeats, Fbx13 (Godinho et

Figure 1. The afterhours long-period mutant. (A) Representative double-plotted actograms of wheel running from wild-type (*left*) and homozygous *Afh* mutant mice. (B) Representative bioluminescence recordings of PER2::LUC expression in organotypic SCN slice cultures from wild-type (*blue*), heterozygous (*red*) and homozygous (*green*) *Afh* mutants. (C) Mean (+S.E.M.) circadian period of bioluminescence rhythms from SCN of wild-type and *Afh* mutant neonatal SCN. (D) Comparison of circadian periods of rest-activity cycles in vivo, and SCN bioluminescence rhythms in vitro from adult wild-type and *Afh* mutant mice. (E) Mean (+S.E.M.) circadian periods of bioluminescence rhythms from tissue explants of wild-type and *Afh* mutant mice. (Redrawn from Godinho et al. 2007 [AAAS].)

al. 2007). These adapter proteins bind to substrates destined for proteasomal degradation, using the leucine-rich repeat (LRR) to specify substrate and their integral F box to bind to a Skp-Cullin ubiquitin ligase complex (Fig. 2C). These interactions allow assembly of a polyubiquitin chain onto the target, facilitating subsequent proteasomal degradation. The *Afh* mutation is a cysteine-to-serine substitution at residue 358 within a highly conserved region of the LRR which would therefore likely affect substrate affinity of Fbx13. This issue was resolved with the biochemical identification of Cry1 and Cry2 as native substrates for Fbx13 and loss of affinity for these proteins in the mutant (Busino et al. 2007). Moreover, in both cell line and native tissues, using either recombinant or native Cry, it was shown that the consequence of the *Fbx13^{Afh}* mutation was increased stability of Cry proteins, presumably because they were no longer targeted effectively to the proteasome (Fig. 2D).

These biochemical observations provide a ready explanation of the phenotype at both gene expression and behavioral levels. Failure to clear Cry at the end of circa-

Figure 2. Altered dynamics of protein and mRNA expression within the clockwork of *Afh* mutant mice. (A) Abundance of Cry1 and Per2 proteins in liver and SCN (assessed by western blot and immunostaining, respectively) from wild-type (closed symbols) and *Afh* homozygous mutant (open symbols) mice (mean $\pm$ S.E.M.). (B) Expression of Cry1 and Per2 mRNA in SCN (assessed by in situ hybridization) from wild-type (closed symbols) and *Afh* homozygous mutant (open symbols) mice (mean $\pm$ S.E.M.). (C) Western blots of Cry1 levels in organotypic lung slice culture of wild-type and *Afh* homozygous mutant mice following treatment with cycloheximide. (D) Representative bioluminescence recordings from NIH-3T3 cells transfected with *mPer2::Luciferase* reporter and either empty vector (black), wild-type Fbx13 (blue) or *Afh* mutant Fbx13 (red). (E) Schematic representation of the role of Fbx13 in ubiquitination of Cry and the impact of the *Afh* mutation thereon. (Redrawn from Godinho et al. 2007 [AAAS]; and M.H. Hastings et al., unpubl.)

dian night leads to a prolongation of the phase of negative feedback onto E-box-mediated gene expression. Consequently, mRNA levels of *Per* and *Cry* (and other clock-controlled genes) are suppressed for longer and/or more severely so that on the following cycle, peak levels of *Per* and *Cry* proteins are reduced. To test this presumed impact of the *Fbx13^{Afh}* mutation on E-box-mediated gene expression, NIH-3T3 fibroblasts were transfected with either wild-type or mutant Fbx13, and luciferase expression driven either by *Per2* or *Bmal1* promoter fragments was recorded (Ueda et al. 2002). Overexpressed Fbx13^{Afh} markedly repressed the *Per2* reporter (Fig. 2D), whereas

wild-type Fbx13 had no effect. Neither construct suppressed the *Bmal1* reporter, but the mutant extended period from 21.5 ± 0.1 hours (empty vector) and 21.1 ± 0.2 (wild-type Fbx13) to 23.3 ± 0.2 hours (*Fbx13^{Afh}* mutation) (mean $\pm$ S.E.M., $n = 4$). The more pronounced and extended suppression of gene expression in late circadian night arising from the failure to clear *Cry* proteins prolongs the circadian cycle such that the escape from negative feedback takes longer than in wild type. This selective prolongation of negative feedback was evident in the bioluminescence curves recorded from SCN slices, in which the nadir of *Per2* expression was extended in homozygotes, and is sufficient to explain their longer behavioral period.

These studies, and the complementary work on the “overtime” mutation of Fbx13 (Siepka et al. 2007), identify Fbx13 as a regulator of *Cry* protein stability through its role in targeting *Cry* for proteasomal degradation, and hence Fbx13 is a determinant of circadian period. The corresponding F-box adapter protein for *Per* is likely to be β TrCP, and inappropriate phosphorylation of *Per* proteins via mutations in *Per2* or CKI ϵ and CKI δ accelerates their degradation and thereby shortens circadian period (Gallego and Virshup 2007). The contribution of upstream kinases, including forms of CKI, to the licensing of *Cry* for ubiquitination remains to be determined. What is clear from studies in plants, fungi, fruit flies, and now mice is that regulated proteasomal degradation of circadian proteins mediated by F-box-containing ubiquitin ligases is a critical step in tuning circadian period to the solar cycle. As such, circadian clocks join the wider context of biological timing in which oscillatory processes, such as the cell division cycle, depend on very tight temporal control of the production, posttranslational modification, and ultimate clearance of proteins.

NEUROPEPTIDE SIGNALING AND THE SYNCHRONIZATION OF CIRCADIAN CLOCK CELLS IN THE SCN

The demonstration of free-running electrophysiological rhythms in SCN neurons dispersed in culture was instrumental in formulating the cell-autonomous model of circadian timing (Welsh et al. 1995). The natural condition of SCN neurons, however, is to be embedded within circuits, and thus a central question to understanding the SCN as a tissue-based clock is to ask how the individual oscillatory cells synchronize their molecular pacemakers and thereby broadcast a coherent circadian signal throughout the organism. Although early studies implicated GABAergic signaling in this coordination, more recently, the SCN neuropeptide vasoactive intestinal polypeptide (VIP) and its SCN target the VPAC₂ receptor (encoded by the *Vipr2* gene) have been shown to mediate cellular synchronization within the SCN (Harmar et al. 2002; Colwell et al. 2003). In mice lacking the *Vipr2* gene or the VIP peptide, behavioral rhythms appear normal under a regular lighting schedule but are severely perturbed on transfer to free-running conditions (Fig. 3A). Locomotor activity immediately jumps forward into putative circadian day, successive activity bouts are poorly

coordinated, activity onset is irregular from day to day, and multiple but transient periodicities ranging beyond the circadian norm are evident. Loss of effective circadian control to behavior while mice are under the lighting schedule is evidenced by the acute induction of locomotor activity upon unscheduled exposure to darkness during the light phase. These behavioral perturbations are accompanied *in vivo* by suppressed and apparently arrhythmic circadian *Per* and *Cry* expression across the SCN, as assessed by *in situ* hybridization and immunostaining. The origin of the behavioral disturbances lies in disrupted molecular clockwork within the SCN, as revealed by bioluminescence recordings from *Vipr2* knockout SCN slices (Maywood et al. 2006). Not only is circadian coherence lost in the mutant tissue, but the overall level of *Per*-driven luciferase expression is also reduced by an order of magnitude (Fig. 3B). The reduction in mean expression level across the tissue indicates that the phenotype of the *Vipr2*^{-/-} SCN is not merely a desynchronization of otherwise high-amplitude cellular

oscillators; rather, the amplitude of the individual circadian clockworks is reduced. This is confirmed by microscopic imaging, using charge-coupled device (CCD) cameras to monitor circadian gene expression in individual SCN neurons across the slice (Fig. 3C). Whereas cells in the wild-type slice exhibit high amplitude, synchronized, and sustained circadian cycles, in the mutant slices, appreciably fewer cells are detectable by bioluminescence and of those that are, the level of circadian gene expression is appreciably reduced and the cycles are poorly defined and asynchronous across the slice. These effects of the *Vipr2*^{-/-} mutation are also evident with electrical rhythms in both dispersed cultures and acute SCN slices, presumably a consequence of impaired molecular timekeeping (Aton et al. 2005; Brown et al. 2005). Furthermore, in VIP deficient tissues, electrophysiological synchrony can be briefly restored with VIP agonists (Aton et al. 2005, 2006).

It is therefore clear that VIP-mediated signals released by retinorecipient cells of the SCN core both synchronize and sustain the molecular clockwork of neurons right across the SCN. What is the cellular basis for this? VPAC₂ is positively coupled to adenylyl cyclase (AC) and in some tissues can thereby regulate Ca²⁺ signals (Hagen et al. 2006). Compromised SCN gene expression (and hence disrupted circadian behavior) likely arises, therefore, from a deficiency in intracellular cAMP and possibly Ca²⁺ signaling within the SCN. Consistent with this, direct activation of AC with forskolin acutely induces circadian gene expression in *Vipr2*^{-/-} slices (Fig. 4A). Furthermore, acute depolarization of the mutant slice by the elevation of extracellular potassium levels, which would trigger influx of Ca²⁺, or addition of the SCN neuropeptide gastrin-releasing peptide (GRP) transiently activates *Per* expression and synchronizes cellular rhythms (Maywood et al. 2006). In the absence of endogenous VIP signaling, however, this imposed synchrony is progressively lost as oscillator cells free-run and drift out of phase (Fig. 4B). These observations raise several interesting questions about the SCN circuitry and cellular timekeeping. First, what are the relationships between membrane depolarization, intracellular signaling via calcium and cAMP, and circadian gene expression? Given that the SCN cell is a neuron, is electrical activity, membrane depolarization, and Ca²⁺ signaling a necessary condition for the molecular clockwork to run (Lundkvist et al. 2005)? If so, what are the specific roles of particular neuropeptidergic inputs? Does VIP facilitate the cellular clock solely by activating AC or does a simultaneous or consequential change in membrane depolarization and Ca²⁺ signaling further enhance *Per* expression? Certainly, electrical silencing of the SCN by tetrodotoxin severely compromises circadian gene expression within individual cells and also leads to their desynchronization (Yamaguchi et al. 2003; Maywood et al. 2007). This raises a second issue: Are the amplitude of cellular circadian gene expression and interneuronal synchrony interdependent, as predicted by models of cellular timekeeping across the SCN (Bernard et al. 2007)? If so, VIP may be a critical neurochemical mediator of these relationships, representing both the output of one cellular

Figure 3. Disrupted circadian behavior and cellular circadian synchrony in SCN of mice lacking the VPAC₂ receptor for VIP. (A) Double-plotted representative actograms of wheel running in wild-type and homozygous *Vipr2* mutant (KO) mice under a lighting schedule (LD) and then free-running (DD). (B) Recordings of mPer1::luciferase activity in SCN slice cultures from animals depicted in A. (C) Raster plots of circadian luciferase expression in 25 representative cells from wild type and homozygous *Vipr2* mutant SCN slices. Accompanying Rayleigh plots illustrate cellular synchrony in wild type and its absence in mutant SCN. (Redrawn from Maywood et al. 2006 [Elsevier]; and M.H. Hastings et al., unpubl.)

Figure 4. Circadian synchronization in SCN and periphery of VPAC₂ receptor null mice. (A) Representative bioluminescence recordings from *Vipr2*^{-/-} SCN slices treated with either vehicle (black line) or forskolin (10 μ M, gray line). (B) Raster plots of circadian luciferase expression in 25 representative cells from homozygous *Vipr2*^{-/-} SCN slice, before and after treatment with 100 nM GRP. (C) Daily (left) and circadian (right) rhythms of corticosterone levels in wild type (mean $\pm$ S.E.M., closed symbols) and their absence in *Vipr2*^{-/-} mice (open symbols). (D) Circadian gene expression in liver and heart of wild-type (closed symbols, mean $\pm$ S.E.M.) and their phase advance in *Vipr2*^{-/-} mice (open symbols). (E) Double-plotted, representative wheel-running actograms of wild-type and *Vipr2*^{-/-} mice, subjected to restricted feeding under both LD and DD. (Redrawn from Maywood et al. 2006 [Elsevier]; Sheward et al. 2007 [Society for Neuroscience]; and M.H. Hastings et al., unpubl.)

oscillator and the (synchronizing) input to its neighbors in the circuit. Hence, it is the ability of SCN neurons to communicate via VIP (and other?) signals that makes the entire nucleus such a robust and precise timekeeper as cellular synchrony and amplitude are reciprocally supportive. Indeed, the power of these circuit-coupling effects is revealed by their ability to compensate for clock gene mutations which at the level of isolated cells would compromise the clockwork (Liu et al. 2007). The consequence of obligate synchronization and interdependence, however, is that when interneuronal signals are compromised, the cellular clockwork of the SCN becomes far less robust than that of fibroblasts, which oscillate perfectly well in the absence of synchronizing cues (Welsh et al. 2004).

CIRCADIAN ENTRAINMENT IN *VIPR2*^{-/-} MICE

In wild-type animals, light pulses relayed by the retina reset the SCN clockwork by acutely increasing levels of *Per* expression, initially in the retinorecipient core and then in the shell. This involves a glutamatergic signaling cascade via NMDA (*N*-methyl-D-aspartate) receptors and mitogen-activated protein kinase (MAPK), ultimately activating (by phosphorylation) the calcium/cyclic AMP response element (CRE)-binding protein (CREB) (Hastings et al. 2003). Both *Per1* and *Per2* carry CRE sequences through which pCREB can induce their expression. A similar cascade also activates the immediate-early gene *c-fos* in the retinorecipient SCN, but in all cases, these responses are gated so that light is only effective when delivered during circadian night. In the *Vipr2*^{-/-} mouse, however, light pulses activate MAPK, CREB phosphorylation, *Per*, and *c-fos* expression in circadian day just as effectively as they do in circadian night (Hughes et al. 2004; Maywood et al. 2007). Loss of circadian timekeeping arising from the absence of VIP signaling therefore leaves open the gate for retinal activation of the core SCN neurons. Hence, VIP signals are necessary for circadian regulation of the core clockwork but not for its acute regulation by glutamatergic retinal inputs.

Compromised SCN timekeeping in VPAC₂ null mice also has marked consequences for downstream physiological rhythms. Female *Vipr2*^{-/-} mice show irregular estrous cycles on a light/dark cycle, a disruption that is exacerbated by continuous darkness, and accompanied by phase advanced cycles of *Per*, *Cry*, and *Bmal1* expression in the uterus (Dolatschad et al. 2006). Circadian rhythms of corticosterone secretion are also lost, the peak secretion at the start of circadian night being absent and levels remaining permanently low (Fig. 4C). This is not, however, a generic suppression of adrenal activation, because behavioral arousal by restricted feeding acutely increases corticosterone levels (Sheward et al. 2007).

The circadian cycle of corticosteroid secretion is an important internal synchronizer of circadian rhythms in the periphery, especially the liver (Balsalobre et al. 2000; Reddy et al. 2007; see below). In *Vipr2*^{-/-} mice, clock gene expression remains rhythmic in the liver, in contrast to its absence from the SCN. The entire program, however, is phase-advanced by 4–6 hours relative to wild type, reflecting the combined effects of an advanced feeding schedule and the loss of rhythmic corticosterone, which usually opposes phase shifts of the liver clockwork by feeding cycles (Le Minh et al. 2001). The maintenance of rhythmic feeding in these “SCN-disabled” mice is an intriguing finding and is consistent with the view that feeding cycles can be regulated by other hypothalamic regions, especially the dorsomedial nucleus (Gooley et al. 2006). Indeed, there is clear evidence that *Vipr2*^{-/-} mice retain a food-entrainable oscillator. Under a regime of restricted feeding, they exhibit very strong anticipatory behavior, an accompanying surge in circulating corticosterone levels, and rhythmic *Per* expression in the liver peaking with anticipated feeding time. Thus, food intake remains an effective synchronizing agent in *Vipr2*^{-/-} mice, capable of controlling circadian behavior, corticosteroid secretion,

and the clockwork of peripheral tissues, and although VIP signaling is critical for the function of the SCN pacemaker, it is not required for the food entrainable oscillator.

PROKINETICIN SIGNALING AND CIRCADIAN OUTPUT CONTROL BY THE SCN

Effective functioning of the extra-SCN oscillators in brain and peripheral tissues is dependent on their coordination by the SCN. Some of the physiological effects of the *Vipr2* null mutation may arise from impaired signaling by VIP-containing projections from SCN neurons to hypothalamic targets, for example, gonadotrophin hormone-releasing hormone neurons that control the estrous cycle. In addition, neuronal transplantation studies indicate that diffusible paracrine factors may signal circadian time to SCN targets, and transforming growth factor- α (TGF- α) (Kramer et al. 2005) and the neuropeptide cardiotrophin-like cytokine (Kraves and Weitz 2006) have recently been identified as likely mediators. Attention has also focused on Prokineticin 2 (Prok2), an 81-amino-acid secretory peptide first identified in gut but also strongly expressed in the SCN. Prok2 is a typical clock-regulated gene; its transcript levels in the SCN are highly circadian and driven by Clock/Bmal via E boxes. It is also regulated by light, presumably via a glutamatergic MAPK cascade. Pharmacological studies indicated a role for Prok2 in the suppression of daytime activity in nocturnal rodents (Cheng et al. 2002), but in both day-active and night-active species, its peak of expression in the SCN is in circadian daytime, indicating that it signals time rather than activity state (Lambert et al. 2005). Its receptor (Prokr2) is widely expressed in both the SCN and SCN target areas (Fig. 5A), including the medial thalamus, arcuate nucleus, and dorsomedial hypothalamus (Cheng et al. 2002).

To test the potential role of Prokr2 in the mediation of circadian outflow, we created a null mutation (Fig. 5A) (Prosser et al. 2007). Locomotor activity rhythms in null mice were severely disturbed even on a lighting cycle. The onset of activity around lights-off was poorly defined, and there was very little wheel running or general movement in the first part of the dark phase. In contrast, spontaneous activity at the end of the dark phase was comparable to that of wild-type mice. Upon release into continuous dim light, the residual activity remained rhythmic, evidence of an underlying circadian regulation, but it was poorly organized and activity onset lacked definition. This disturbance within circadian night was also evident in telemetric recordings of core body temperature (Fig. 5C). The typical sustained nocturnal elevation observed in wild-type mice was absent from mutants, the profile being bimodal with a brief elevation at the beginning of the night, a prolonged decline, and then finally a small increase at the end of circadian night coincident with the spontaneous surge in locomotor activity. Loss of Prokr2 signaling therefore altered the pattern of circadian behavior and physiology, an effect comparable to that seen with targeted deletion of the gene encoding the Prok2 ligand (Li et al. 2006).

Given the precedent of the *Vipr2*^{-/-} mutant, the Prokr2 null phenotype may have arisen from disordered molecu-

Figure 5. Compromised circadian behavior and thermoregulation, but a functional SCN pacemaker in mice lacking prokineticin receptor 2. (A) Autoradiographic distribution of Prok2 analog binding in mouse forebrain. (PVT) Paraventricular thalamus; (DMN) dorsomedial hypothalamus. (B) Double-plotted, representative wheel-running actograms of wild-type (left) and Prokr2 null (KO) mice entrained to LD (above gray line) and free-running under DD (below gray line). (C) Representative mean daily profile ($\pm$ S.E.M.) of core body temperature of wild-type (red) and Prokr2 null (KO, black) mice. (D) Representative *Per1::luciferase* bioluminescence rhythms from wild-type (left) and Prokr2 null (right) SCN slices. (Redrawn from Prosser et al. 2007 [National Academy of Sciences].)

lar timekeeping in the SCN. Bioluminescence recordings from SCN slices, however, confirmed that the molecular timekeeper is as robust in the null mutant as in the wild type (Fig. 5D). Although these SCN rhythms provide an explanation for the free-running behavioral and temperature rhythms, the coherence of the SCN pacemaker is at odds with the disturbed nocturnal patterning observed *in vivo*, i.e., altered behavioral waveform was not accompanied by changes in the SCN waveform. Prokr2 signaling is not therefore necessary for cellular pacemaking or synchrony in the SCN, despite its high level of expression therein. Rather, the data are consistent with a role for Prokr2 in mediating circadian control by a functional SCN over behavioral and/or physiological rhythms. More specifically, it appears that Prokr2-mediated signals are important for signaling specifically nocturnal events. It remains to be determined how this particular peptidergic signal is integrated into other SCN outputs, although one

possibility is that a series of such signals is released across the circadian cycle, particular circadian times being identified by a phase-specific neurochemical “cocktail.”

CIRCADIAN ORGANIZATION IN PERIPHERAL TISSUES

Understanding the molecular underpinnings of circadian physiology has been revolutionized by genome-based analytical techniques, not the least of which are “gene-chip” microarray methods by which the entire transcriptome of a tissue can be examined across circadian time. For any particular tissue, 5–10% of transcripts are under circadian regulation (Akhtar et al. 2002; Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002), but with the exception of the canonical core clock genes and ubiquitous output genes such as albumin D-element-binding protein (*Dbp*), the circadian genes differ markedly between tissues as they tend to represent key regulators of the particular tissue’s metabolic functions. Moreover, because circadian integration at the level of the organism essentially involves alternation between catabolic and anabolic phases, within any particular tissue, rhythmic genes fall into discrete, functionally coherent circadian clusters (Fig. 6A). Consequently, the transcriptomic profile of a tissue follows a precise temporal program, with particularly marked changes associated with anticipated dawn and dusk.

In the liver, for example, genes associated with glycolysis/gluconeogenesis, nitrogen metabolism, cytoskeleton, xenobiotic metabolism, vesicle trafficking, and solute carrier transporters are circadian, thereby enabling the organ to prepare for anticipated changes in ingestion, nutrient availability, and nutrient mobilization over the rest/activity cycle. Within a functional grouping, for example, various tubulin isoforms, there is clear coordination of their mRNA abundance, emphasizing the elegance and sophistication of circadian synchronization (Fig. 6B). Of particular interest is circadian regulation of cyclins and other cell division factors (Reddy et al. 2005). For example, *Wee-1* expression is circadian in many peripheral tissues, including both liver and kidney (Fig. 6C), contributing to circadian regulation of cell division and growth (Matsuo et al. 2003). This pattern is ultimately dependent on SCN signals because circadian coordination of *Wee-1* (and most other genes) is lost in the livers of SCN-ablated mice (Fig. 6C). The basis for this control lies within functional E boxes in the *Wee-1* gene, which are activated by Clock and Bmal1 and suppressed by Cry (Fig. 6D). *Wee-1* therefore illustrates the hierarchy of circadian regulation for clock-controlled genes in peripheral tissues: Within peripheral cells, the intracellular clockwork drives its expression, but for gene expression to be coherent at the tissue level, these clocks are regulated by SCN-derived cues.

Having established the complexity of circadian gene expression within peripheral tissues, the question arises as to how it is synchronized? The contribution of rhythmic glucocorticoids (GCs) was noted above (Balsalobre et al. 2000), and in circadian mutant mice, including *Vipr2* knockouts, loss of rhythmic corticosterone is associated with altered rhythms of gene expression in peripheral tis-

Figure 6. Circadian transcriptome and circadian metabolism. (A) Representative antiphasic clusters of circadian transcripts from livers of mice sampled across the circadian cycle. (B) Coordinated circadian expression of genes encoding cytoskeletal subunits. (C) *Wee-1* as a clock-controlled gene: Circadian expression in liver and kidney analyzed by in situ hybridization is lost in SCN-ablated mice (Mean $\pm$ S.E.M.). (D) Luciferase reporter assays reveal Clock/Bmal-dependent transcription of *Wee-1* and its suppression by Cry. (Redrawn from Akhtar et al. 2002 [Elsevier]; and M. Hastings et al., unpubl.)

sues (Sheward et al. 2007). It is difficult, however, to test the role of any single factor in intact mice because its action cannot be isolated from those of other SCN-dependent synchronizing cues. We therefore rendered mice arrhythmic by ablation of the SCN, a procedure that would remove all synchronizing cues and cause desynchrony of cellular clocks in peripheral tissues. Mice then received an acute injection of the GC receptor agonist dexamethasone (DEX). Our prediction was that when applied against the arrhythmic background of SCN-ablated mice, an effective GC stimulus would resynchronize multiple cellular clocks in the tissue, driving them to a unique phase and thereby establishing a common temporal pattern in groups of animals sampled at different times following injection. By analyzing samples by DNA microarray, it would be possible to identify which elements of the liver circadian transcriptome are subject to acute regulation by GC. On the basis of our own and other

studies, we identified a target population of 366 circadian transcripts that were rhythmic in tissues from intact mice and showed no circadian variation following SCN ablation. Of these, 57% responded to a single activation by DEX (Reddy et al. 2007), individual genes showing rapid or delayed activation, or suppression (Fig. 7A). This circadian resynchronization included induction of *Per1*, *Cry1*, and *Bmal1*, all of which carry GC response elements (GRE) and so are direct targets for circulating corticosterone. Similarly, two thirds of the responsive circadian genes also carry GREs, whereas a partially overlapping 38% carry E boxes. Consequently, activation of the circadian transcriptome by GC can occur by at least two overlapping routes: direct regulation of genes through GREs and indirect regulation via E boxes following GRE-mediated activation of the core clockwork. Further transcriptional cascades are also clock/GC-regulated, for example, those dependent on Dbp which does not carry GREs but is nevertheless GC responsive, likely an indirect effect mediated through its E-box sequences. To identify further circadian transcriptional cascades sensitive to GC, we screened our set of GC-sensitive circadian

genes for transcription-factor-binding sites and identified a high frequency of targets (39% vs. 7% in noncircadian genes) of hepatocyte nuclear factor 4 α (HNF4 α) (Fig. 7B). HNF4 α is a pivotal liver transcription factor that regulates numerous metabolic pathways, including ureagenesis, the production of serum proteins, and the activity of cytochrome P450 genes (Odom et al. 2004). It carries both GREs and E boxes, and correspondingly, its expression in the liver is circadian, lost on SCN ablation and restored by GC (Fig. 7C,D). Moreover, expression of a number of its downstream targets, including ornithine transcarbamylase (a central factor in nitrogen metabolism), is circadian (Fig. 7E). Rhythmicity of these genes is dependent on the SCN and is also lost in HNF4 α null liver, highlighting the contribution of HNF4 α to circadian coordination of the transcriptome.

Having characterized these circadian transcriptional cascades, a critical question is their relationships to protein expression: Are they faithfully translated? By combining two-dimensional gel electrophoresis with mass spectroscopy, we have analyzed the circadian proteome of liver (Reddy et al. 2006). Although coverage is more limited than with a microarray, this approach revealed that 135 (21%) of 642 “spots” resolved in the gel were under circadian regulation. Of these, it was possible to identify 49 circadian proteins that were the products of 39 unique genes. These were principally enzymes and showed distinct phase clustering between circadian day and circadian night (Fig. 8A). Western blots confirmed circadian regulation for a number of proteins and also showed their rhythmic expression to be sensitive to circadian mutations (Fig. 8B). As with the transcriptomic analysis, a number of circadian factors were rate-limiting and/or vital components of key pathways, including carbohydrate and nitrogen metabolism (Fig. 8C). By comparison with published transcriptomic data and de novo assays of mRNA expression, it is evident that almost half (44%) of circadian proteins do not exhibit a circadian transcript, indicating that posttranscriptional and posttranslational mechanisms are in turn circadian and play a significant part in sculpting the circadian proteome (Reddy et al. 2006). Potential mediators of the former include the deadenylase nocturnin. Encoded by a clock-regulated gene, it is thought to control mRNA stability, and a series of clock-regulated metabolic genes are dys-regulated in nocturnin knockout mice (Green et al. 2007). MicroRNAs provide a further level of posttranscriptional circadian regulation, modifying mRNA expression by altering its stability and sequestration and/or directly impeding translation. MicroRNAs are active in the SCN (Cheng et al. 2007) and likely in peripheral clockworks as well. Evidence of circadian control over posttranslational modification is provided by peroxiredoxin 6. In the liver, this protective antioxidant factor is expressed rhythmically at the mRNA level and is present as a series of phosphoproteins that cycle in their abundance (Reddy et al. 2006). Remarkably, two isoforms cycle in antiphase to each other, with one in phase with the transcript and the other not. Thus, evidence is accumulating that a diversity of posttranscriptional regulatory mechanisms define the circadian proteome and thereby the unique daily metabolic programs of individual tissues.

Figure 7. Coordination of the liver circadian transcriptome by glucocorticoid signaling. (A) Relative expression levels of circadian genes in livers of SCN-ablated mice are arrhythmic with vehicle treatment (left), and activated by DEX (mean $\pm$ S.E.M.) either acutely (middle) or with a delay (right). (B) Frequency of regulatory elements in promoter and introns of circadian GC-regulated genes in liver. (C) Circadian expression of HNF4 α mRNA in mouse liver (mean $\pm$ S.E.M.). (D) HNF4 α mRNA is arrhythmic in liver of SCN-ablated mice and induced by DEX (mean $\pm$ S.E.M., * p < 0.05 vs. vehicle). (E) Circadian expression of HNF4 α target gene *OTC* in liver of wild-type mice and its loss in HNF4 α null liver (green bars) (mean $\pm$ S.E.M.). (Redrawn from Reddy et al. 2007 [AASLD].)

Figure 8. Circadian regulation of the liver proteome. (A) Representative plots of nocturnal and diurnal clusters of circadian proteins. (B) Validation by western blot of circadian expression of liver enzymes and loss of the rhythm in liver from *Clock* mutant mice. (C) Representative metabolically relevant circadian regulated enzymes from mouse liver (mean $\pm$ S.E.M.). (Redrawn from Reddy et al. 2006 [Elsevier].)

CONCLUSION

Notwithstanding the recent progress described here and elsewhere in this volume, major questions remain regarding the molecular and cellular bases of circadian timing in mammals. First, although components of the feedback loops and the cellular factors that determine their behavior are being individually characterized, it is unclear how these components interact at a molecular level, forming multimeric complexes with emergent properties that specify and reflect circadian time. We have no knowledge of circadian protein structure and no biochemically validated quantitative model for the molecular circadian oscillator of mammals. Second, the relationship between the core molecular loops and neural activity in the SCN is unresolved, but one possible outcome is that far from being an autonomous clock cell, SCN neurons are more proficient pacemakers when operating within a circuit. Such necessary properties and circuit functions of the

SCN wait to be determined. Third, we know that the SCN have anatomically and neurochemically diverse outputs, but we do not understand the nature of the temporal signals they convey: Are they graded and continuous, binary “stop” and “start,” specific for a particular target, a property of individual SCN cells, or a population code, etc.? Finally, the major challenge will be to exploit newly acquired knowledge of the circadian control of vital cellular processes—cell division, nutrient metabolism, xenobiotic detoxification, etc.—to better manage and prevent major systemic illness.

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