Recent Insights into Mammalian Circadian Rhythms

Russell N. Van Gelder, MD, PhD

Department of Ophthalmology and Visual Sciences and Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO

Abstract: This short review highlights recent progress in understanding the mammalian circadian clock. Advances in the understanding of the neuroanatomy of circadian rhythms, the molecular biology of the core clock mechanism, mechanisms of light entrainment of the circadian clock, clock synchronization among multiple tissues, and recent work on the relationship of the mouse circadian clock and cancer are discussed. This review is intended as an overview of recent research activity for the interested sleep disorders clinician or researcher.

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INTRODUCTION

SLEEP AND CIRCADIAN RHYTHMICITY ARE INEXTRICABLY INTERTWINED; THE CIRCADIAN CLOCK EXERTS A POWERFUL INFLUENCE ON THE TIMING OF SLEEP AND WAKEFULNESS, WHILE MORE RECENT LITERATURE SUGGESTS THAT SLEEP STATES CAN INFLUENCE THE FUNCTION OF THE CIRCADIAN PACEMAKER. It thus behooves the sleep disorders physician or researcher to maintain familiarity with work from the circadian-clock research community. Unfortunately, the explosion of literature in this field in the past several years (numbering more than 7000 publications listed on PubMed since the year 2000 alone) has made staying current with recent developments a daunting task even for the cognoscenti in the field. This review will attempt to highlight some of the recent research in the field of mammalian biologic rhythms; it is not meant to be a comprehensive review of the field. The interested reader is referred to an excellent recent textbook in the field.[1] The interaction of human circadian rhythms and sleep will be covered in a subsequent review.

BASIC DEFINITIONS

Circadian rhythms are defined as the self-sustained oscillations of living systems that display near–24-hour periodicity when the system is kept away from all external time cues (see Pittendrigh[2] for an excellent overview). Circadian rhythms typically have 4 cardinal properties:

- They persist (by definition) in the absence of external time cues, demonstrating a characteristic *free-running period.*
- The *phase* of the rhythm can be shifted by application of light or drugs. For light, nearly all organisms have a similar phase response to short stimuli, being insensitive to light presented during the subjective day (that is, the times when the animal would normally be exposed to daylight), showing a delay in the phase with light in the early part of the subjective night, and showing an advance in the phase of the rhythm in the late subjective night.
- The *period* (and phase) of the rhythm can be *entrained* by periodic stimuli, such as periodic light-dark cycles, provided their period is near the intrinsic free-running period of the clock.
- The clock is temperature compensated, meaning that its free-running rhythm does not vary markedly with changes in the ambient temperature. This applies primarily to poikilothermic species, not to mammals.

Circadian rhythms have been found in organisms ranging from single-celled photosynthetic prokaryotes[3] through humans, and in nearly every eukaryotic organism in which they have been sought. In mammals, they influence a large number of physiologic, endocrinologic, and biochemical processes, including of course the timing of sleep and wakefulness.

ANATOMIC FEATURES OF THE MAMMALIAN CIRCADIAN CLOCK

While the formal properties of the circadian clock (that is, the nature of the free-running period, the phase response of the clock to light, etc.) had been well described in many species by the late 1960s, the mechanism of circadian timekeeping remained quite mysterious. The first anatomic clues to the circadian-clock mechanism came from ablation studies in rodents, in which specific brain nuclei were destroyed by knife cuts or radiofrequency ablation. In 1972, Stephan and Zucker[4] and, separately, Moore and Eichler[5] demonstrated that ablation of the suprachiasmatic nuclei (SCN) of rats abolished circadian rhythms of drinking activity, locomotion, and hormone release. The circadian clock of the SCN is remarkable; subsequent work has shown that the SCN are capable of sustaining circadian rhythms in cell firing when cultured in vitro.[6-8] Indeed, the individual cells of the SCN appear to maintain rhythmicity when cultured in low-density dispersions,[9-11] suggesting that at least some SCN cells are cell-autonomous oscillators. This notion fits well with the emerging model of the circadian clock being generated by an intracellular genetic mechanism (see below) rather than being an emergent property of cellular-level interactions.

The necessity and sufficiency of the SCN for driving behavioral circadian rhythms have been demonstrated by transplant studies, in which cultured SCN were transplanted into SCN-lesioned hamsters.[12] A naturally occurring hamster mutation (the *tau* mutatation[13]) that shortens the free-running period of the hamster clock from 24 to approximately 20 hours helped researchers demonstrate that the SCN are the "master pacemaker" of the body, at least for locomotor rhythms. Ralph and Menaker[13] demonstrated that after transplantation, the period of the recipient was dependent on the genotype of the donor SCN (that is, a wild-type hamster receiving a *tau* SCN would show the characteristically short free-running period of the *tau* strain). Thus it would appear that the SCN are both necessary and sufficient for organismal circadian rhythms in mammals.

The SCN are well positioned to serve as the master pacemaker (Figure 1). Its location atop the optic chiasm gives it direct access to axons from the retina, which form a dedicated retinohypothalamic tract.[14,15] This tract appears to utilize the neurotransmitter pituitary adenylate cyclase-activating polypeptide as well glutamate.[16,17] The SCN can thus monosynaptically monitor external lighting conditions from the eyes. Additionally, the SCN receive inputs from the thalamus, specifically via a neuropeptide Y-mediated tract from the intergeniculate leaflet, which

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Address correspondence to: Russell N. Van Gelder, MD, PhD, Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, CB 8096, 660 S. Euclid Ave, St. Louis, MO 63110; Tel: 314-747-4251; Fax: 314-747-5537; E-mail: vangelder@vision.wustl.edu

may help nonphotic time cues influence the clock.[18] The outputs of the SCN primarily serve nearby hypothalamic and thalamic nuclei, in particular the medial preoptic nucleus, the anterior part of the paraventricular nucleus of the thalamus, the medial part of the paraventricular nucleus of the hypothalamus, the medial part of the dorsomedial nucleus of the hypothalamus, and principally the subparaventricular zone.[19] The dorsomedial nucleus, preoptic nucleus, and subparaventricular zone all project to the ventrolateral preoptic nucleus, a structure implicated in control of sleep states[20-22]; it is thought that this is the anatomic basis of circadian control of sleep and wakefulness. However, transplantation studies using SCN placed in semipermeable tubing (which cannot form synaptic connections) are capable of rescuing circadian rhythms of rest and activity,[23] so the possibility exists that diffusible factors are sufficient for communicating information from the circadian clock to the centers controlling rest and activity in the brain.

The SCN itself is not a homogeneous tissue but is composed of several cell populations identifiable by their characteristic neuropeptide expression and position within the tear-shaped nucleus. The older literature refers to the "dorsomedial" and "ventrolateral" zones of the SCN, but, because of significant interspecies variability in the anatomy of the SCN, neuroanatomists presently characterize the organization of the SCN as being divided into a "shell" region (in the dorsal SCN), and a "core" region (primarily ventral SCN).[24,25] The core region receives input from the retinohypothalamic tract and contains neurons expressing the neuropeptides vasoactive intestinal polypeptide and gastrin-releasing peptide, while the shell receives input from nonphotic regions and expresses arginine vasopressin and calretinin. The 2 subdivisions have somewhat different output connections. The mechanisms of communication between the 2 divisions, as well as their roles in the generation of circadian rhythmicity, remain an area of active inquiry.

GENETIC AND BIOCHEMICAL BASIS OF THE MAMMALIAN CIRCADIAN CLOCK

These studies led to the (simplified) notion that mammalian circadian rhythmicity pacemaking has an anatomic locus in the SCN. This is remarkable—few other higher neurophysiologic functions have so discrete an anatomic basis. Equally remarkable is the finding that the circadian clock has a discrete genetic basis. More than 30 years ago, Konopka and Benzer[26] identified 3 mutant alleles of a single gene (dubbed *period*) in the fruitfly *Drosophila melanogaster* that had the remarkable properties of speeding up, slowing down, or eliminating the free-running circadian rhythms of both locomotion and hatching (eclosion) in the fly. Much research in the ensuing decades has fleshed out the genetic basis of circadian rhythms in the fly (for review see Van Gelder et al[27] and accompanying website for details, as well as other authors[28-32]). At least 5 genes in the fly (*period, timeless, cycle, Clock,* and *cryptochrome*) seem to be dedicated primarily to the process of setting up a circadian clock, and several additional genes (including *doubletime, vrille,* and *Pdp1*) also have critical roles (but are also required by other biologic processes). The dedicated clock genes are primarily DNA transcriptional activators and repressors, and the core clock mechanism in the fly seems to be primarily driven by an intracellular feedback loop at the level of DNA transcription and translation: the protein products of the *timeless* and *period* loci repress their own transcription with a time delay. This results in fluctuating levels of *period* and *timeless* mRNA (and also protein) during the day. By mechanisms that are not yet understood, the fluctuations in the levels of these gene products produce a clock. Gene microarray experiments, in which the mRNA expression levels of thousands of genes can be monitored simultaneously, have suggested that a relatively small number of genes (approximately 20-400 depending on the study) are rhythmically expressed in the fly's head[33-38]; presumably, the fluctuations in the levels of expression of these genes is somehow transduced into rhythmic behavior. How this is accomplished is presently not known.

While the circadian clocks of other genetically tractable organisms such as the mold *Neurospora crassa* (reviewed in Dunlap et al,[39] Loros et al,[40] and Merrow et al[41]) turned out to be based on similar feedback loops, the underlying mechanisms of mammalian circadian rhythms remained obscure until about 5 years ago, when Sun and colleagues[42] made the seminal discovery that mice harbor a homologue of the mammalian *period* gene. This led to an extraordinarily rapid period of "clock-gene" discovery in the mouse. Ultimately, homologous genes to each of the *Drosophila* clock genes were found as well in the mouse, sometimes in multiple copies. For example, the fly has 1 *period* gene, but the mouse has 3; where there is 1 *cryptochrome* gene in the fly, the mouse has 2. Humans and mice seem to have identical numbers of these genes.

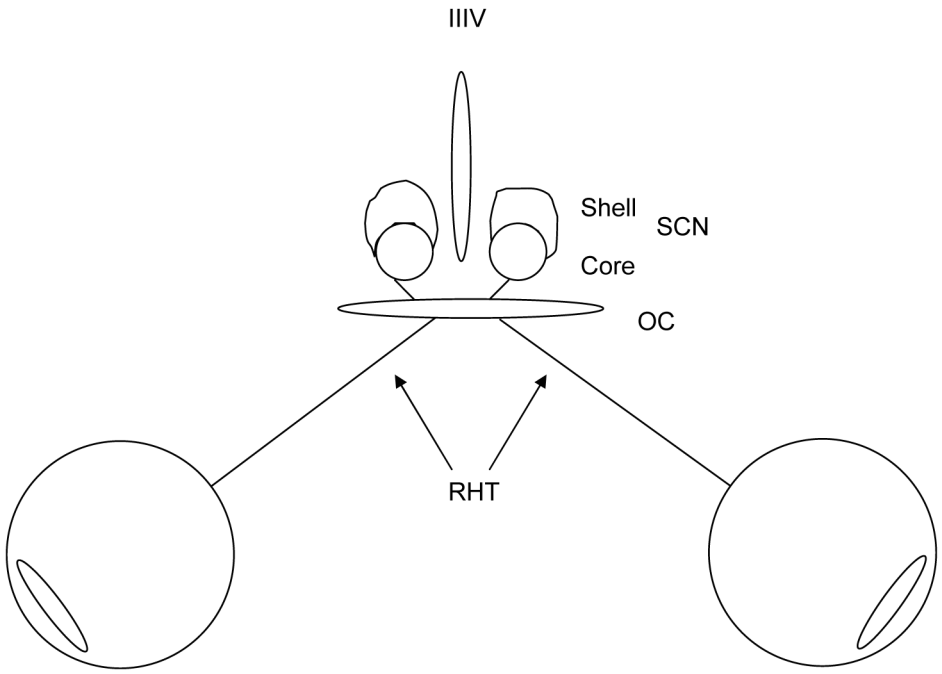

Figure 1—Simplified schematic of the neuroanatomy of the mammalian circadian pacemaker. The suprachiasmatic nuclei (SCN) sit just above the optic chiasm (OC) in the ventral hypothalamus. Neuroanatomists recognize 2 major divisions of the SCN: a "core region" which receives afferent fibers from the retina and expresses the neuropeptide markers vasoactive intestinal polypeptide and gastrin-releasing peptide, and a "shell region," which receives afferent fibers from other brain centers and expresses arginine vasopressin. A dedicated, pituitary adenylate cyclase-activating polypeptide-expressing retinohypothalamic tract (RHT) connects a subset of melanopsin-containing, intrinsically photosensitive retinal ganglion cells to the core region of the suprachiasmatic nuclei. IIIV refers to the third ventricle.

The development in the past several years of reverse genetic technologies (allowing researchers to "knock out" genes of interest by manipulation of the DNA of embryonic stem cells) has allowed investigation of the function of circadian clock genes in the mouse (see Van Gelder et al, [27] Reppert et al,[43] and Takahashi [44]). In general, mice lacking a single *mPeriod* or *mCryptochrome* gene (*m* here for mouse) show alterations in the free-running period, while mice lacking all *mPeriod* or all *mCryptochrome* genes are arrhythmic in free-running conditions.[45-48] These genes function biochemically as repressors of transcription of their own expression. Similarly, mice lacking the genes encoding the transcription factors that basally stimulate *mCryptochrome* and *mPeriod* expression (called *Clock*[49,50] and *Bmal1* [aka *Mop3*][51]) also lose free-running rhythmicity. While there are some differences between the fly clock and the mammalian clock (for instance the exact role of the *timeless* gene in *Drosophila* has been

subsumed by the *mCryptochrome* genes in the mouse), the basic transcription-translation feedback loop appears essentially similar. Ultimately, within the SCN, oscillating levels of these critical clock genes give rise to a timekeeping mechanism. At least 1 rhythmic output of the SCN—the level of the transmitter-hormone arginine vasopressin—is directly controlled by the oscillating transcriptional activity of clock-gene components upon its promoter.[52] Figure 2 summarizes the key genes comprising the mammalian circadian clock and their interactions.

A strong prediction of this model is that naturally occurring mutations in some of the "clock genes" should result in individuals with aberrant circadian rhythms. The familial advanced sleep phase syndrome is a human disease characterized by a heritable tendency toward "lark" status—early morning awakening and early sleep onset—and was mapped genetically to the telomeric end of chromosome 2q. This corresponds to the location of the *hPeriod2* gene; screening of this gene for mutations in patients with familial advanced sleep phase syndrome revealed a conserved serine → glycine mutation in the *hPeriod2* coding region.[53] This serine is one of the main targets of caseine kinase 1 epsilon—which, itself, is a clock gene (responsible for the *tau* mutation in the hamster[54]). Thus humans with mutant *period* genes, like mice and flies, likely show abnormalities in circadian function. In the human, this is directly correlated with an abnormality in the timing of sleep and wakefulness.

HOW DOES LIGHT INFORMATION GET TO THE MAMMALIAN CLOCK?

The human circadian clock has a free-running period of a little more than 24 hours,[55] while the mouse clock runs slightly faster, generally about 23.5 hours. Neither clock would be at all useful unless it were continually reset to local time and thus "entrained" to the 24-hour day. Light has long been known to be the major entraining stimulus for the circadian clock. How lighting information from the external world is transmitted to the SCN, sitting deep in the dark confines of the hypothalamus, has been under intensive investigation over the past several years. Although 1 well-publicized report claimed that light given behind the knees could be an entraining stimulus[56] (the so-called retrogeniculate pathway), subsequent attempts to confirm this result in both animals and humans have not succeeded.[57] In mice and rats, enucleation of the eyes abolishes entrainment of circadian rhythms to light-dark cycles (but leaves those rhythms otherwise intact).[58] Similarly, mice that are genetically modified to lack optic nerves (the *math5-/-* mutant) also show an intact circadian clock that cannot entrain to external light-dark cycles,[59] again demonstrating that the photoreceptor for entrainment resides in the eyes.

Remarkably, although the eyes are apparently required for entrainment of the circadian clock to light-dark cycles, the classic photoreceptors—the rods and cones of the outer retina—are not. As first described by Ebihara and colleagues,[60] and later definitively demonstrated by Foster and colleagues,[61] mice that have lost all their rods and cones (and are thus visually blind) still entrain their clocks to light-dark cycles and can still phase-shift their rhythms to short pulses of light. Reconciling these results required invoking the radical idea that the eye harbors nonrod, noncone photoreceptors. In 2002, Berson and colleagues demonstrated the existence of such photoreceptors through an ingenious experiment.[62] They microinjected a retrograde tracing dye into the SCN of a rat and then identified the retinal ganglion cells projecting directly to the SCN. When they recorded electrical activity from these cells in vitro, they noted that the cells fired action potentials when exposed to light. Cells that were not retrogradely labeled were not photosensitive. This group thus demonstrated the existence of nonvisual photoreceptors in the inner retina; in the rodent, about 3% to 5% of the axons of the optic nerve belong to photosensitive retinal ganglion cells.[63,64] In addition to projecting to the SCN, these cells also project to brain centers involved in pupillary light responses (ie, the olivary pretectal nucleus), the intergeniculate leaflet of the thalamus, and the subparaventricular zone.

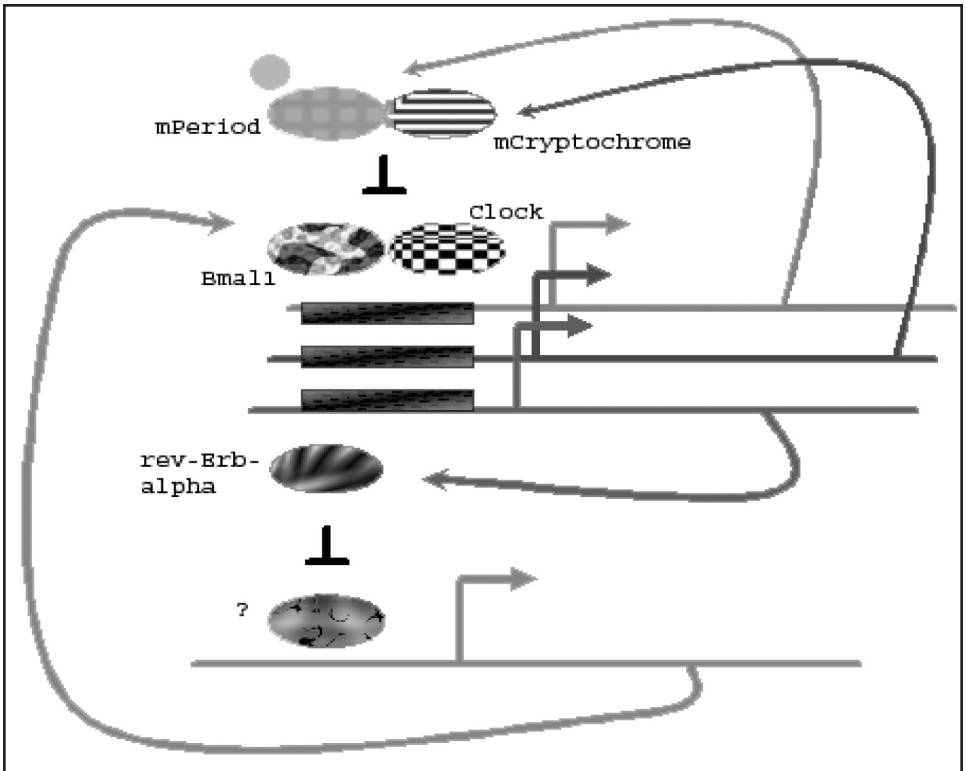

Figure 2—The circular logic of daily timekeeping. The mouse circadian clock requires the function of a set of "clock genes," which are primarily mRNA transcription factors and their repressors. The *Clock* and *Bmal1* genes encode 2 positive helix-loop-helix transcription factors that bind to a recognition sequence, called the "E-box" on the control regions of the genes for *mCryptochrome1* and *mCryptochrome2, mPeriod1* and *mPeriod2*, and the gene *rev-erb-alpha*. mCryptochrome 1 and 2 and mPeriod 1 and 2 proteins form complexes that block Clock:Bmal1 transcription—forming a time-delayed negative feedback loop. Rev-erb-alpha blocks an activator of *Bmal1* transcription, forming a second ("positive") loop, which allows *Bmal1* mRNA to oscillate out of phase with *mCryptochrome* and *mPeriod* mRNAs. Mutations in any of these essential clock genes result in animals with abnormal or absent circadian rhythms. Figure based on Van Gelder et al,[27] where more details and a link to an animation of this system can be found.

The identity of the photopigment subserving these cells has been intensively studied over the past year. Two families of candidate photopigments have been identified. One is the *mCryptochrome* family (*mCry1* and *mCry2*), the murine homologue of the fly *cryptochrome* gene.[65] In flies, *cryptochrome* appears to serve as a primary circadian photopigment,[66,67] where it directly senses light in the pacemaking cells of the fly brain. Cryptochrome protein binds Timeless protein in light (but not dark), leading to rapid degradation of the latter, which is necessary for shifting the phase of the clock.[68] The other candidate for a mammalian photopigment is a novel opsin family member called *melanopsin*.[69,70] Originally identified in the skin of amphibians, a melanopsin homologue was identified in the mouse retina several years ago. Both cryptochrome and melanopsin appear to be expressed in the inner retina (the ganglion cell layer), but melanopsin appears to be expressed exclusively in the photoreceptive cells leaving the optic chiasm and projecting to the nonvisual centers.[63,64]

Knock-out mutants of both cryptochromes and melanopsin have been produced.[45,46,71-74] While mice lacking both *mCry* genes showed loss of their circadian rhythms in total darkness, when these mice were kept in a light-dark cycle, they appeared to have relatively normal behavioral rhythms. Similarly, in a light-dark cycle, mice lacking the melanopsin gene (called

opn4) also showed relatively normal entrainment. When both the cryptochrome and melanopsin-mutant mice were crossed with mice having outer retinal degeneration *(rd)*, however, a very strong phenotype developed in each case.[75,76] The *mCry;rd* mice (that is, the retinal-degenerate mice lacking both cryptochromes) were arrhythmic in light-dark cycles, while the *opn4;rd* mice free ran through light-dark cycles. Similar results were seen crossing the *opn4* mutant gene onto genes resulting in other forms of visual blindness.[77] These results suggest that there is redundancy between the rods and cones and the inner-retinal photoreceptors for transmission of irradiance information to the hypothalamus—only with loss of both does the SCN lose all input from the eye.

These genetic results suggest that both *mCryptochrome* and melanopsin genes play an important role in nonvisual photoreception. Analysis of the pupillary light response has clarified the relative roles of these genes. It has been known for more than 75 years that visually blind mice retain some pupillary light responsiveness.[78] *Opn4;rd* mice lose all pupillary light responsiveness,[76] while *mCry;rd* mice are approximately 5% as sensitive as *rd* mice.[79] Thus, melanopsin is required for inner retinal photoreception, while cryptochrome is not absolutely essential. The relative roles of these 2 genes—for instance, the effect of this pathway on light's direct regulation of sleep and wakefulness—as well the biochemical details of their function in the photoreceptive process, are being actively investigated.

ONE CLOCK OR MANY? EMERGING FEATURES OF THE "MAMMALIAN CLOCKSHOP"

The discovery of the mammalian clock genes has allowed a much more detailed look into the workings of the circadian clock in both the SCN and non-SCN tissue. Investigators can track the rhythmic expression of the "clock genes" as a measure of the clock mechanism in any tissue. One of the most surprising results of these investigations has been the ubiquity of potential circadian oscillators in both SCN and non-SCN tissue.

The signals that maintain synchrony between and within the 2 SCN are presently unknown. The existence of such signals is strongly suggested by experiments studying the "splitting" phenomenon in hamsters. When kept in dim, constant lighting conditions, some hamsters will undergo spontaneous splitting, in which the normal, single major daily activity peak splits into 2 bouts that are one-half cycle (about 12 hours) apart. de la Inglesia et al[80] examined the molecular rhythms of clock-gene expression in hamsters during the episodes of splitting and found that the phases of clock-gene expression on the 2 sides of the brain had dissociated; clock-gene expression rhythms in the left and right SCN were in antiphase to each other. In this unique condition, each suprachiasmatic nucleus is acting as a semiautonomous pacemaker—yet some signal between the SCN maintains the stable phase relationship between the 2. Even finer levels of cell autonomy of the circadian timekeeping mechanism have been suggested by experiments in tissue culture. Balsalobre and colleagues[81] discovered that following treatment in high serum for a brief period of time, rodent fibroblast cells would show circadian oscillations of clock-gene expression for several days, which strongly suggested that these cells contain most of the components necessary for a circadian oscillator.

Subsequent work has revived this older notion that many tissues in the body (and not just the SCN) are capable of sustained rhythmicity in vitro. One of the tricks available in modern mouse genetics is the transgenic animal, in which a synthetic gene is injected into the 1-cell mouse embryo and incorporates into the mouse's germ-line DNA. Several groups have made transgenic animals in which the control elements (promoter and enhancer) of the *period* genes drive a "reporter" gene, such as enzyme luciferase. This enzyme catalyzes the bioluminescent oxidation of the substrate luciferin. In the presence of the luciferin, the amount of luminescence will be proportional to the expression level of the *period* gene. Yamazaki and colleagues generated such a transgenic rat.[82] When they took tissues from these rats and cultured them in vitro, they found that many individual tissues continued to show rhythmic luminescence (indicating a functioning circadian clock) for 2 to 7 days after explantation. Many non-SCN brain regions similarly showed robust rhythmicity for several days when cultured in vitro.[83] While rhythmicity in all non-SCN tissues eventually damped out, rhythmicity in the SCN persisted indefinitely, suggesting that the SCN had greater ability to sustain its rhythms; nonetheless, these results indicate that peripheral tissues retain significant clock properties. Interestingly, the phase of the rhythm—as demarcated by the peak of *Period* gene expression—varied with the tissue being studied. Thus, individual tissues may have their clocks advanced or delayed with respect to the central clock in the SCN. When the researchers applied light to phase shift the animal's rhythms prior to harvest of organs, they found that the SCN clock shifted much faster than the clocks of peripheral tissues, leading to a period of time when the body was internally desynchronized. The signals that bring the peripheral oscillators back into a stable phase relationship with the master oscillator in the SCN remain unknown, but rhythmic feeding has been shown to be able to entrain the oscillator in the rat liver, even dissociating its phase from the SCN.[84-86]

Overall, these results suggest that the mammalian circadian system does not consist of a single clock in the SCN driving protean biochemical and physiologic processes in individual cells but, rather, it is an ensemble of clocks, some more capable than others of sustained independent oscillations. The biochemical basis of the apparently unrivaled ability of the SCN to sustain circadian rhythmicity is unknown but is an area of active inquiry.

NEW FUNCTIONS FOR THE CLOCK

Just as it has been difficult to define the function of sleep, it has been very difficult to understand the functions of the circadian clock that have maintained it through eukaryotic evolution. Animals without clocks still demonstrate rhythmic behavior in light-dark cycles (a phenomenon called *masking*), so the function of a free-running clock in organisms has been a legitimate question. The availability of knockout mice lacking core clock components and, therefore, a functional circadian clock, has—until recently—not shed light on the necessary functions of the circadian clock, as most of these animals have no obvious phenotypes aside from their arrhythmicity in constant-lighting conditions. (The one notable exception to this has been in fecundity—nearly all the murine clock-gene-mutant animals are very poor breeders; some are incapable of breeding as homozygote animals).

Fu et al[87] noted that the life span of their *mPeriod2* knockout mice was less than that of wild-type sibling mice and discovered that these mice developed spontaneous lymphomas at high frequency. Molecular analysis of these mice revealed that a number of cell-cycle and tumor-progression genes were under the control of the *mPeriod2* gene. In particular, the *c-myc* oncogene is under transcriptional control of the *mPeriod2* gene, and its deregulation in the *mPeriod2* knockout mouse may be 1 of the main forces driving tumor formation in these animals. This suggests a deep relationship between the cell-cycle machinery and the circadian-clock machinery and has broad implications for clock control of susceptibility to cancer. A similar conclusion was reached in another set of recent animal experiments. Matsuo and colleagues[88] examined liver regeneration in mice following partial hepatectomy and found that regeneration was tightly controlled by the circadian clock—the liver "preferred" to regenerate only at certain times of the circadian day. These investigators showed that this was also due to direct interactions of some of the core clock genes with genes controlling cell proliferation—in this case, the *wee1* gene that is a major regulator of cyclin function. They then examined genetically arrhythmic mice (lacking *cryptochromes*) and discovered that these mice had reduced overall liver regeneration following hepatectomy, regenerating only approximately 50% to 60% as well as control mice. Thus, the circadian clock seems to control the timing of cellular regeneration in normal tissue.

These findings from animal studies may have very strong repercussions for human cancer risks. Several independent studies in the last several years have suggested that women working swing or graveyard shifts

have significantly higher risks for developing breast cancer than do women working day shifts.[89-91] Similarly, flight attendants flying international routes seem to be at higher risk for developing breast cancer and for developing cutaneous melanoma.[92] Although these epidemiologic studies have many potential confounding factors, they do support the hypothesis that the circadian clock is intimately connected with cell proliferation.

CONCLUSIONS

The past 5 years have seen a phenomenal increase in our understanding of the mechanisms and functions of the mammalian circadian clock. Neuroanatomic knowledge of the SCN has now been complemented by detailed information on the molecular mechanisms of the circadian clock and its entrainment pathways. New fields of inquiry into the mechanisms leading to circadian-clock synchronization within the SCN and between the SCN and peripheral tissues have been made possible by the development of new rodent models for circadian study, including transgenic and knock-out mice and rats. These have allowed new investigations into old unanswered questions such as the selective pressures that have maintained the circadian-clock mechanism. These tools will likely be used to address fundamental questions of how the circadian clock influences the timing of sleep and wakefulness.

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