Cellular and biochemical mechanisms underlying circadian rhythms in vertebrates

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Circadian clocks organize neural processes, such as motor activities, into near 24-hour oscillations and adaptively synchronize these rhythms to the solar cycle. Recently, the first mammalian clock genes have been found. Unpredicted diversity in signaling pathways and clock-controlled gating of signals that modulate timekeeping has been discovered. A diffusible clock output has been found to control some behavioral rhythms. Consensus is emerging that circadian mechanisms are conserved across phylogeny, but that mammals have developed a great complexity of controls.

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Abbreviations

CLK	protein product of the circadian clock gene *clock*
CREB	Ca^{2+}/cAMP response element binding protein
FRQ	protein product of the clock gene *frequency*
ICER	inducible cAMP early repressor
IGL	intergeniculate leaflet
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide
NOS	NO synthase
NPY	neuropeptide Y
PACAP	pituitary adenylyl cyclase-activating peptide
PER	protein product of the clock gene *period*
PKG	cGMP-dependent protein kinase
SCN	suprachiasmatic nucleus
TIM	protein product of the clock gene *timeless*
WC-2	protein product of the clock gene *white collar-2*

Introduction

Organismic behaviors and activities of their underlying motor systems do not occur at random, nor are they constant. Rather, they vary in quality and amplitude with near 24-hour periods. Thus, these daily oscillations in behaviors are termed 'circadian rhythms'. Locomotory activity, sleep/wake states, feeding, drinking and vocalizations are behaviors that exhibit circadian rhythmicity. Each circadian behavior oscillates with a fixed phase relationship to the major environmental oscillation on earth, the cycle of light and darkness. If light falls within usual daylight hours, rhythms continue unperturbed. However, when light occurs at night, it signals a mismatch between external and internal time. Organisms respond by adjusting the phasing of their behaviors, forward or backward, so that they reassume their normal phase relationship to day and night in the environment.

Circadian rhythms of behavior are characteristic of the broad range of life, including cyanobacteria and molds; therefore, a nervous system is not required for 24-hour timekeeping or phase adjustment. With centralization of sensory processing and motor commands, the primary locus of circadian control became cephalic. Exactly 25 years ago, discrete lesions of rat brain identified a brain site controlling circadian rhythms of corticosterone [1], drinking behavior and locomotor activity [2]. Remarkably, the tiny suprachiasmatic nucleus (SCN), which lies paired at the base of the hypothalamus and nested in the optic chiasm, was found to be essential. Locomotory behavior continued to occur in animals with SCN lesions, but activity no longer was patterned into daily oscillations. In addition, the SCN was found to receive from the eye a direct projection, the retinohypothalamic tract, that is essential for synchronization to light/dark cycles [3]. Therefore, the SCN fulfilled the major criteria for a circadian clock, in that it was necessary both for generating and transmitting a timebase for circadian patterning (i.e. it is a timekeeper) and for channeling clock sensitivity so as to recognize signals of temporal desynchronization, such as nocturnal light, and orchestrating appropriate, resynchronizing phase adjustments (i.e. it is a gatekeeper).

The bilateral SCN compose the site of the primary circadian clock in mammals and some birds, whereas in other vertebrates, circadian behavioral control lies in the pineal gland or is shared between the pineal and SCN [4]. In addition, a circadian clock is contained within the retina in lower vertebrates [5] and, recently, has been identified in mammals as well [6••]. Although the phase control that the retinal clock exerts may be limited to retinal functions, it has the potential to modulate signals from the retina to the SCN by shaping light capture and efferent information flow, as well as to be itself shaped by feedback from the SCN. These issues have not yet been addressed.

Over the quarter of a century since its discovery, the SCN has been the subject of increasing attention, until, presently, the number of publications focusing upon the SCN is in a logarithmic phase of growth [7•,8•]. Although the past year has seen unparalleled progress in understanding mechanisms underlying circadian rhythms in a wide range of organisms, the emphasis of this review is on the remarkable insights that have been gained into the circadian clock in the vertebrate brain. I will focus upon new insights into SCN regulatory mechanisms in mammals, including clock genes that participate in

timekeeping, clock-controlled gating of input signals and output mechanisms.

Clock genes in the SCN

A large literature from genetically tractable organisms, such as the fruit fly (*Drosophila*) and the bread mold (*Neurospora*), has elegantly demonstrated that circadian rhythms are encoded in the genome. Specific 'clock genes' essential for timing and resetting circadian rhythms have been identified. When *Drosophila* are maintained under constant conditions, the *period* (*per*) and *timeless* (*tim*) genes undergo alternating cycles of transcription and translation over each near 24-hour period. Alterations in these genes can shorten, lengthen or abolish periodicity of complex behaviors under circadian control, such as locomotory activity and eclosion of pupae into adults [9].

The genes *per* and *tim* encode PER and TIM, proteins with unusual properties. When PER and TIM reach adequate concentrations, they heterodimerize [10••]. The heterodimeric conformation marks them for translocation from cytoplasm to nucleus, where they inhibit transcription of *per* and *tim* mRNA. Over the next hours, they undergo extensive post-translational modifications, including hyperphosphorylation and degradation [11••,12••]. These modifications represent a point at which cellular biochemistry and/or signal transduction pathways can intersect with the molecular machinery, although mediators have not yet been identified. The programmed decay of PER and TIM permits reinitiation of their transcription. Thus, PER and TIM generate a negative feedback loop: by inhibiting their own transcription for about one quarter of the circadian cycle, they contribute significantly to the length of the circadian period. So strong was the genetic evidence from invertebrates, as well as from mold *Neurospora* [13••,14•], that it has been surprising that no clock genes had been isolated in mammals sooner.

A genetic basis for mammalian clocks had been demonstrated by the spontaneous appearance of a heritable mutation in hamster that shortened the circadian period of locomotory activity [15], but the gene had not been identified. Suddenly, within recent months, a novel gene, *clock* (*clk*), has been cloned from mouse [16••], after it had been identified by screening mutagenized mice for defects in their behavioral rhythms and localized by chromosomal mapping [17]. *clk* encodes a DNA-binding protein, CLK, which suggests that it may directly regulate transcription.

Most intriguingly, CLK also contains an unusual protein–protein binding region, the PAS domain. Among the very few proteins that contain PAS domains are the products of two clock genes, *per* (cloned from *Drosophila* [10••]) and *wc-2* (cloned from *Neurospora* [18••]). Therefore, it is possible that CLK could interact with a PER- or WC-2-like protein, should one exist in mammals. No mammalian gene functionally equivalent to *per* had been found, despite intense interest in such a possibility. Just as this review was written, a *per* homologue was cloned from mouse and human [19••,20••]. It is expressed in the SCN, in which it cycles.

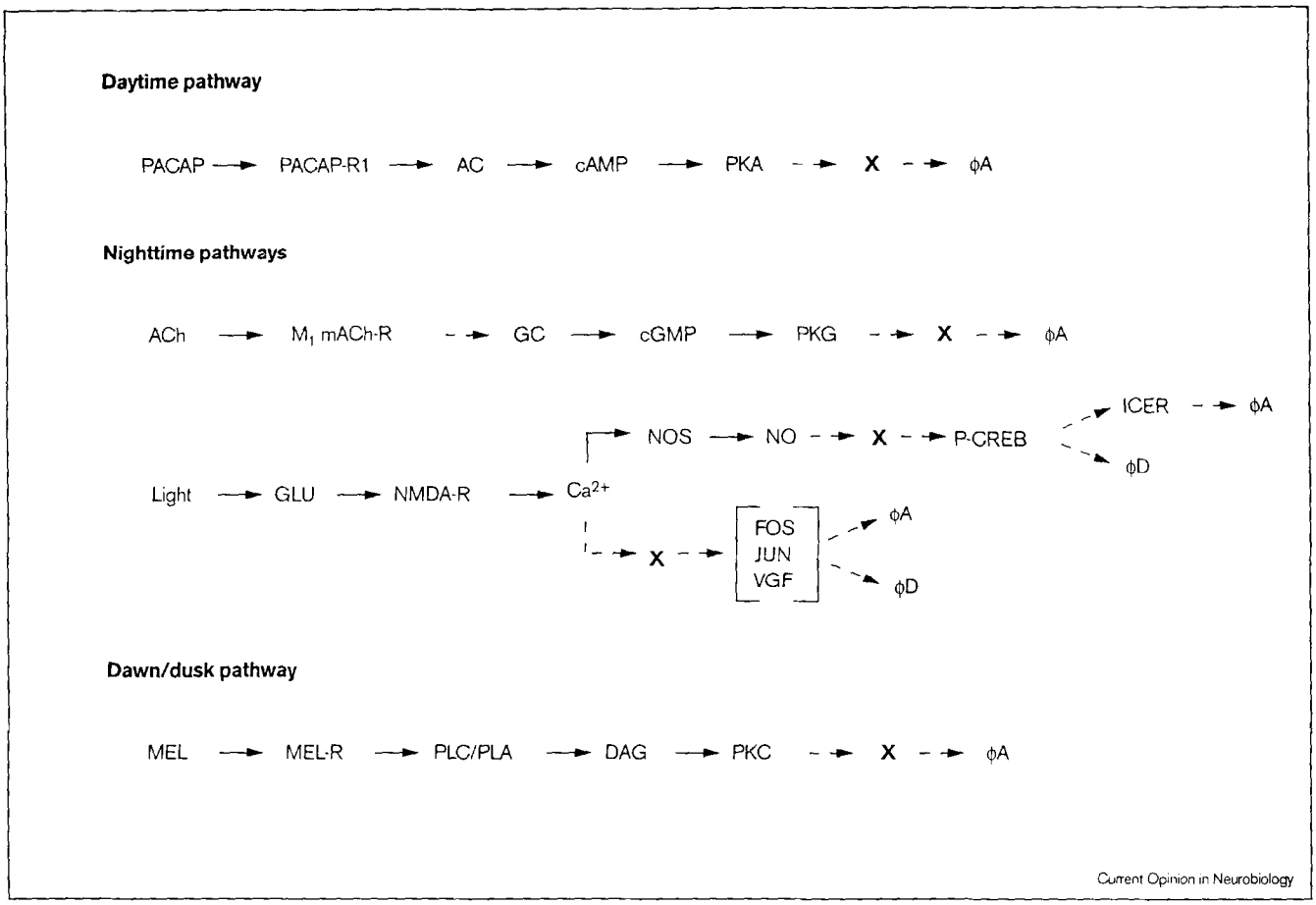
These exceptional findings will focus attention upon both the molecular and biochemical mechanisms by which these genes and their proteins are regulated and by which they contribute to circadian timing in the SCN. The exciting possibility exists that mammalian PER may regulate circadian period through interactions with a CLK- and/or TIM-like protein. The nearly simultaneous discoveries of two mammalian clock genes, both with PAS domains and the potential thereby to heterodimerize, can be predicted to spur intense activity and progress in molecular and biochemical aspects of clock mechanisms.

Clock-controlled gating of signaling pathways that regulate phase

Structurally, the SCN is the most complex of biological clocks. In the rat, it contains ~8000 neurons that lie in close apposition with a nearly equal number of glia, in a volume of about 0.068 mm^3 [21]. SCN neurons are among the smallest in the brain (7–12 μm) and exhibit broad phenotypic heterogeneity. Nevertheless, there is a consensus that even in such a complex clock structure, timekeeping is a cellular process. Indeed, this was demonstrated compellingly when individual neurons dissociated from the SCN of neonatal rat expressed independently phased circadian firing rhythms while cultured on an electrode array [22]. It follows that gating of sensitivity to specific stimuli and phase resetting must be cellular properties. Moreover, the clock must be able to restrict the range of responses in the cellular repertoire so that activation of select signaling pathways can occur only at the appropriate time in the circadian cycle. How does the clock control signaling pathways to produce differential sensitivity?

This issue has been vigorously addressed using two major paradigms. Classically, substances have been tested *in vivo*, where the effects of reagents injected into the SCN or cerebral ventricles can be compared with the effect of the natural environmental stimulus in inducing phase-adjustment in behaviors. Increasingly, signaling pathways have been studied in a brain slice preparation, where either agonists and antagonists can be applied to discrete sites and their effects on the endogenous circadian rhythm of SCN neuronal activity can be assessed directly. Both approaches have proven useful, but can produce different results that then need to be reconciled. Over the past year, technical refinements have made it possible to apply NMDA (the potent agonist of ionotropic glutamate receptors) near the SCN *in vivo*, which generates a light-like, phase-response curve [23•]. This result appeared after many unsuccessful attempts *in vivo*. It validated a range of well-controlled studies [24•] supporting the observations that injection of NMDA antagonist *in vivo* blocks the phase-resetting effect of light

Figure 1



Summary of signaling pathways regulated by the clock in four temporal domains. This figure incorporates new data reported this year and discussed in this review. Dashed arrows indicate points with an unknown number of steps. Relative positions of critical gating sites in each pathway are designated by an '**X**'. AC, adenylyl cyclase; ACh, acetylcholine; DAG, diacylglycerol; GC, guanylate cyclase; GLU, glutamate; M1 mACh-R, muscarinic subtype 1 ACh receptor; MEL, melatonin; MEL-R, MEL receptor; NMDA-R, NMDA receptor; ϕA, phase advance; ϕD, phase delay; PACAP-R1, type 1 PACAP receptor; PKA, protein kinase A; PKC, protein kinase C; PLA, phospholipase A; PLC, phospholipase C; VGF, vesicular protein regulated by growth factors.

[25] and that very localized application of NMDA directly to the SCN *in vitro* induces light-like phase shifts [26].

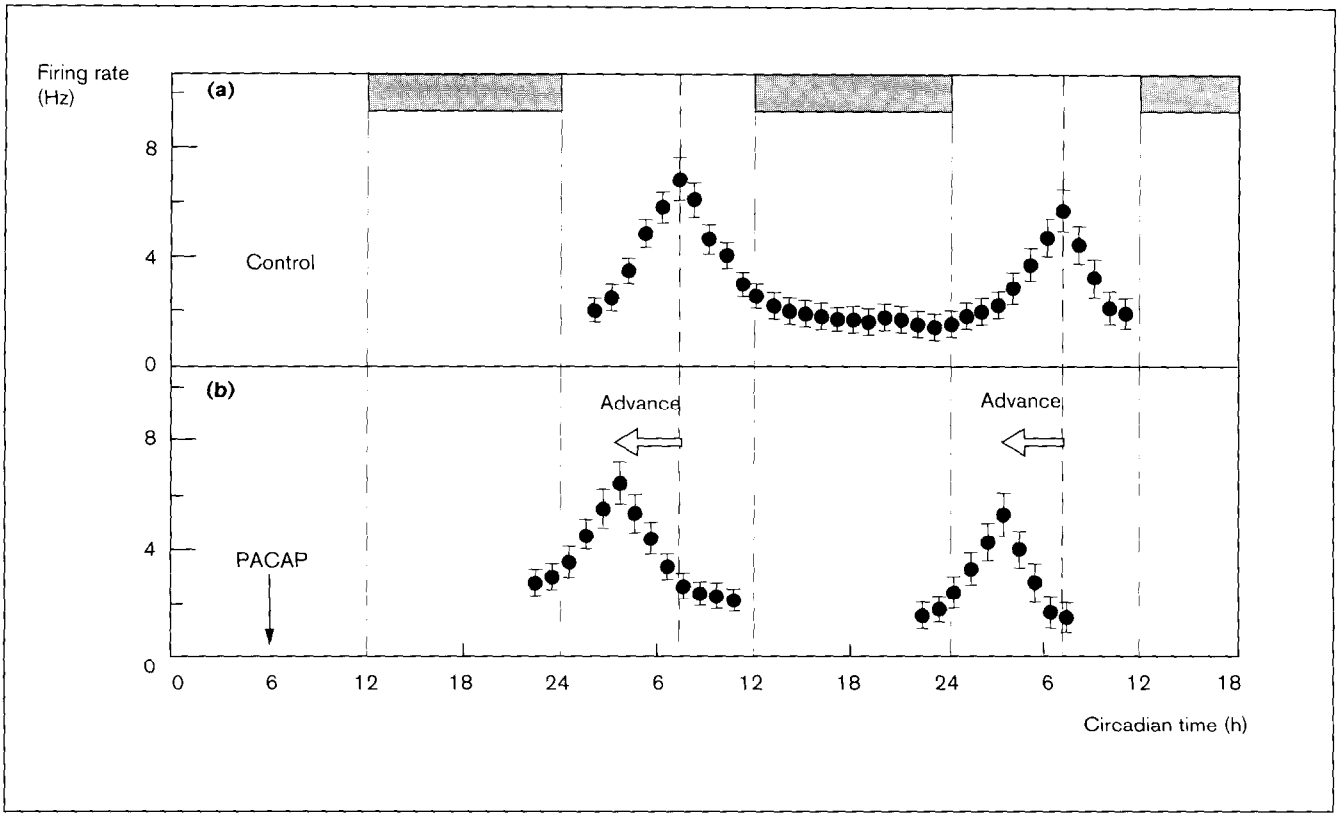
The recent year has witnessed intense work on clock regulatory pathways, which are activatable only during specific periods in the 24-hour cycle. On the basis of these temporal restrictions, the circadian cycle can be divided into discrete temporal states, termed domains of the clock: day, night, dusk and dawn [27••]. The new studies not only contribute to defining the properties of these clock domains, they also emphasize the complexity of control that the clock exerts over signal integration and phase resetting within the SCN. These new data have been incorporated into the putative clock-gated regulatory pathways depicted in Figure 1—they will be discussed below in terms of the clock domain that they regulate.

Daytime regulators

Even in the constant conditions of the brain slice chamber, the SCN exhibits differential sensitivities to resetting agents, including intracellular messengers. During the day, stimuli that activate the cAMP signaling pathway prevail [28]. While a variety of evidence suggests that daytime behavioral arousal can alter clock phase via serotonin or neuropeptide Y (NPY), two papers recently appeared suggesting that pituitary adenylyl cyclase-activating peptide (PACAP) may be the first messenger for cAMP [29,30••]. PACAP is localized in retinal ganglion cells that project to the retinorecipient region of the rat SCN, where they can intertwine with serotonergic fibers from the raphe nucleus [31•]. They also project to a second site, the intergeniculate leaflet of the thalamus (IGL), which, in turn, innervates the retinorecipient SCN via NPY-containing fibers. This pattern of innervation suggests an integrative role for PACAP in communication involving eye, IGL and SCN.

When applied via microdrop to the SCN *in vitro*, PACAP induces a robust (6-hour) phase advance during the subjective midday, but not during the subjective night

Figure 2



PACAP, a cAMP stimulating neuroactive peptide stored in terminals of the retinal ganglion cells that innervate the SCN, can adjust phasing of the clock in daytime. **(a)** Spontaneous activity of SCN neurons, maintained in a brain slice under constant conditions, oscillates with a 24-hour period and peaks midday (dashed line) at circadian time 7 (CT 7=7 hours into the light phase of the light/dark cycle of the animal from which the SCN was derived). **(b)** When applied to the SCN at midday, PACAP advances the phase of the rhythm measured at the peak by 3.5 hours. The SCN were maintained *in vitro* under constant conditions. Subjective night is marked by shaded horizontal bars. Replotted from [30••].

(Figure 2) [30••]. This effect requires activation of cAMP-dependent processes. The mRNA for the type 1 PACAP receptor, which couples positively to adenylyl cyclase, is localized to SCN neurons in the retinorecipient area. It will be important to determine which, if any, of the other daytime signaling agents can modulate the PACAP/cAMP response, so as to identify the next steps in activation and to understand why the SCN appears to respond to PACAP in the day and to glutamate at night, even though both are localized to retinal ganglion cells that project to the SCN.

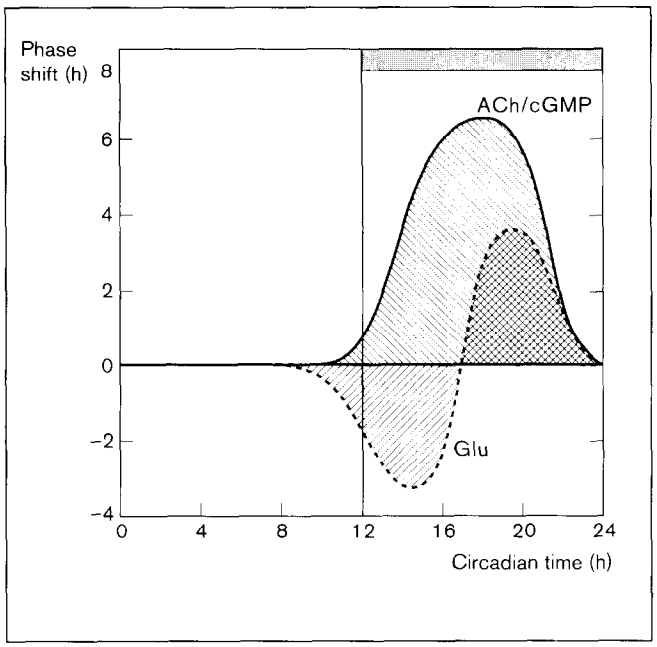
Nighttime regulators

Light, glutamate and NO

The night is the best understood, yet most paradoxical domain of the clock. In animals maintained in constant darkness, stimulation by a pulse of light causes phase-resetting of behavioral rhythms throughout the subjective night, but not during subjective day. The nocturnal response is curiously biphasic, so that during the early night, the response is a delay in phase of subsequent rhythms, whereas during late night, the response is a phase advance. Because this response pattern is present during constant conditions, the clock must gate both its sensitivity to the stimulus and the direction of the phase change. This complex response involves common elements of a signaling pathway that comprises glutamate→NMDA receptor activation→stimulation of nitric oxide synthase (NOS)→intercellular movement of nitric oxide (NO) [26].

That the light signaling pathway should be based upon NO seemed surprising, as scant NOS had been detected in SCN by diaphorase staining. This issue was recently resolved [32••,33•]. The SCN was demonstrated by biochemical assays to possess ample NOS activity, with expression nearly as high as in the cerebellum [32••]. Further, under confocal microscopy of rat SCN stained with a highly specific antibody, neuronal NOS was observed to localize extensively in presynaptic terminals and fine processes throughout the SCN. Together, they form a nitrergic plexus that invests the entire nucleus so that no cell is far from a potential source of NO. Interestingly, in mouse, NOS appears to be largely within SCN neurons, suggesting diversity in NOS localization sites. In each case, NO has the potential to contribute to intracellular signal transduction as well as to intercellular signal transmission, but how it signals a delay at one time of night and an advance at another remains an enigma.

Figure 3



Clock-controlled gating of signaling by glutamate (Glu), acetylcholine (ACh) and cGMP. Application of glutamate, the cholinergic agonist carbachol or an analog of cGMP to the SCN in a brain slice has no effect on phasing of the circadian rhythm of neuronal activities in the subjective daytime, but causes robust resetting at night. To express this phase–response relationship graphically, the lack of effect on phase by treatment midday at circadian time 7 (CT 7) is plotted as zero change, while the phase advance to the cholinergic agonist, carbachol, applied at midnight (CT 18) is plotted at +6.5 hours. Although the sensitive periods to these three substances overlap, the patterns of responses differ. Glutamate induces phase delays in early night (CT 14 = –3.0 hours) and advances (CT 20 = +3.5 hours) in late night, whereas cholinergic and cGMP stimulation induce phase advances throughout the night. Replotted from [26,34,37••].

Acetylcholine and cGMP

Overlapping with the nocturnal sensitivity of the SCN to light, glutamate/NMDA receptor activation and NO, the SCN expresses sensitivity to cGMP analogs [34] and to cholinergic activation via muscarinic receptors [35,36•]. Indeed, a recent paper [37••] has demonstrated convincingly that muscarinic activation of the SCN at midnight rapidly induces a transient rise in cGMP levels and protein kinase G (PKG) phosphotransferase activity. These studies establish acetylcholine as a first messenger for cGMP in the SCN. While timing of sensitivity to both glutamatergic and cholinergic activation is overlapping, the responses are opposite in early night whereas they match in late night (Figure 3). This suggests that at late night, glutamatergic and cholinergic signaling pathways may be related through activation of NOS, with concomitant production of cGMP. The cholinergic pathway may mediate transmission from other brain sites that interact with retinal stimuli at the level of the SCN.

Transcriptional activation by light and glutamate

Accumulating evidence indicates that light-induced phase shifts involve signal transduction events that lead to transcriptional activation. A mechanism by which ligand binding to a membrane receptor induces changes in transcription involves a cascade of changes that activate protein kinases, phosphotransferases that can translocate to the nucleus. There they may phosphorylate transcription factors, such as the Ca^{2+}/cAMP response element binding protein (CREB). When CREB becomes phosphorylated at a single regulatory site (Ser133) forming P-CREB, it increases the transcription rate of the genes it regulates by fourfold [38]. A recent report demonstrated that, like light [39], both glutamate and NO induce P-CREB [40••]. Surprisingly, the CREB protein is present at relatively constant levels in midday and midnight. Because CREB is present in both day and night, but glutamate and NO induce P-CREB only at night, a gate that regulates clock-resetting by light, glutamate, NMDA receptors and NO must lie between NO and CREB.

An interesting report demonstrated induction of a CREB relative, ICER (inducible cAMP early repressor), which can also be induced by Ca^{2+} signaling, for up to 3 hours after exposure to 1 hour of light in the late night [41•]. ICER is the first transcriptional inhibitor to be associated with light-induced phase shifts and is speculated to inhibit cAMP-inducible genes via its trans-repressing potency. While a role for cAMP has not yet been demonstrated in nighttime phase regulation, increased intracellular Ca^{2+} must certainly accompany NMDA receptor opening. Which second messenger leads to P-CREB formation in response to a light stimulus needs to be determined carefully. An important role for ICER is a strong hypothesis because phase shifts can, on theoretical grounds, be predicted to involve both activations of genes involved in the new temporal domains to which the clock is shifted and in inhibitions of genes associated with the old temporal state and/or the dynamic phase-shifting process.

While a host of immediate-early genes, including members of the *fos* and *jun* families, have been known to be induced by light, recent, more thorough studies have found temporal and spatial differences in their expression patterns. FOS has been found to colocalize with different and various peptidergic neurons during delays versus advances (see Figure 3) [42•]. Furthermore, when *junB* and *fosB* mRNAs were examined over a range of circadian times, they were found to be expressed spontaneously in the dorsal region of the nucleus [43•,44•]. In contrast, light-induced phase shifts were preceded by induction of immediate early genes only in the ventral SCN [43•,44•]. The first late response gene to be induced by light has been identified as *vgf* [45•], but its function is unknown.

Whenever FOS expression has been evaluated in the context of the light/glutamate/NO signal transduction pathway, inconsistencies have appeared. In late night, intracerebroventricular injection of L-NAME, a competitive NOS inhibitor, blocks light-induced phase advances, but not the induction of FOS [46]. A recent report [47•] examined the SCN of hamsters exposed to light in early night and found that L-NAME significantly attenuated FOS. These data suggest that different signaling mechanisms, possibly located in different cell types, lie downstream from NO in the pathways by which light induces phase delays and advances. This subject deserves careful scrutiny.

Together these data emphasize the complexity of transcriptional activations that mediate phase shifts by light. Sequential transcriptional activations almost certainly extensively remodel the biochemistry and cell physiology of clock neurons as they make the transition from one temporal domain to the next.

Regulatory pathways at dusk and dawn

The regulatory domains described above cover nearly the entire 24-hour period of the circadian cycle. However, they exclude two obvious regions, one at the day-to-night transition (dusk) and another at the night-to-day (dawn) transition. Melatonin was considered as a candidate clock regulator for these crepuscular domains because it has been shown to affect SCN neuronal firing rates acutely in brain slices and to cause entrainment of locomotory behaviors when injected into rats at dusk [48•]. This was recently confirmed by the demonstration that the SCN in the brain slice expresses sensitivity to phase-resetting by melatonin during the transitional domains. Melatonin administration to the SCN *in vitro* advances the phase of the neuronal activity rhythm at both subjective dusk and dawn, but has no effect during the day or night [49••]. This phase-resetting is mediated via G protein-linked activation of protein kinase C. Thus, melatonin, whose production by the pineal is restricted to night via an efferent pathway from the SCN, can feedback to directly regulate the circadian clock.

The subtype of melatonin receptor that mediates phase-resetting is unknown. The melatonin 1a receptor (Mel1a) can lead to activation of protein kinase C when transfected into fibroblasts [50•]. However, when the SCN of mice with targeted disruption of the Mel1a receptor were studied in the brain slice paradigm, no loss of phase-resetting to melatonin was found [51••]. As the response was blocked by pertussis toxin, the signal was transduced by G proteins, presumably at a membrane receptor. Nevertheless, acute suppression of SCN firing rate was lost. No binding of 2-^{125}I-melatonin could be found in the membranes of the SCN from these knock-out mice. While this may seem perplexing, it supports an observation from the study of melatonin phase-resetting of rat SCN in the brain slice, which examined the dose–response relationships at dusk and found that as little as 2×10^{-13} M melatonin can induce phase-shifts of maximum amplitude [49••]. Thus, two very different studies [49••,51••] have found that only very low concentrations of melatonin are required for clock regulatory effects. The aggregate data demonstrate that melatonin has unusual signaling characteristics that are not presently understood.

Mechanisms coupling clock output to control of circadian behaviors

Until recently, little was known regarding the mechanisms coupling SCN timekeeping to the sites of motor control. It seemed intuitive that specific neural pathways exiting the SCN should contribute signals gating behavioral output at nearby hypothalamic regions known to regulate food intake, locomotory activity or reproduction. Yet, the mediators and modes of transmission to sites controlling specific behaviors had not been established. This notion was shaken with the report that fetal SCN transplanted in semipermeable polymeric capsules successfully restore some motor functions in SCN-lesioned hosts [52••]. The capsule excluded neural outgrowths from the transplant but allowed diffusion of molecules <500 kDa across the porous membrane. Therefore, some substance(s) that diffuse(s) from the encapsulated SCN can signal circadian rhythmicity. Rhythmicity in locomotor activity, feeding and related behaviors, but not neuroendocrine or reproductive functions, could be restored by encapsulated SCN. This suggests that the SCN may have a paracrine relationship, rather than the anticipated synaptic interaction, with some efferent targets. It follows that SCN influence on receptive brain sites through which behaviors are controlled may function as a gated output that is rather generally permissive or inhibitory.

Conclusions

Rapidly accumulating evidence emphasizes the breadth of the role that the circadian clock in the SCN performs as it integrates brain and body systems temporally and synchronizes them with external time. Although we as yet know little about the individual component cells of the brain's clock, progress is being made in defining processes and elements of the mechanisms that generate timekeeping, gating and output signals. Perhaps not surprisingly, fundamental properties of the mammalian clock appear to be very similar to those of circadian clocks in less complex organisms. However, the number of regulators appears considerably greater and more diverse. Will the pathways by which the SCN gates responses likewise show an amplification of control mechanisms? What is clear is that this fascinating puzzle box has many secrets left.

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26. Ding JM, Chen D, Weber ET, Faiman LE, Rea MA, Gillette MU: **Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO.** *Science* 1994, **266**:1713-1717.

27. •• Gillette MU: **Regulation of entrainment pathways by the suprachiasmatic circadian clock: sensitivities to second messengers.** *Prog Brain Res* 1996, 111:121-132.
This synthetic paper evaluates potential gating sites for phase-resetting stimuli in day versus night and concludes that critical clock-controlled gating sites are located downstream from intracellular second messengers.

28. Prosser RA, Gillette MU: **The mammalian circadian clock in the suprachiasmatic nuclei is reset *in vitro* by cAMP.** *J Neurosci* 1989, **9**:1073-1081.

29. Piggins HD, Stamp JA, Burns J, Rusak B, Semba K: **Distribution of pituitary adenylate cyclase activating polypeptide [PACAP] immunoreactivity in the hypothalamus and extended amygdala of the rat.** *J Comp Neurol* 1996, **376**:278-294.

30. •• Hannibal J, Ding JM, Chen D, Fahrenkrug J, Larsen PJ, Gillette MU, Mikkelsen JD: **Pituitary adenylate cyclase-activating peptide [PACAP] in the retinohypothalamic tract: a potential daytime regulator of the biological clock.** *J Neurosci* 1997, **17**:2637-2644.
This provocative paper reports that a cAMP-activating peptide, PACAP, is localized in retinal projections to both the suprachiasmatic nucleus (SCN) and intergeniculate leaflet (IGL). It provides evidence that PACAP regulates clock phase during daytime via a type 1 PACAP receptor and cAMP signaling. These findings raise the possibility of two types of retinal projections to the SCN and the potential for interaction among neurotransmissions from the retina, IGL and raphe nucleus.

31. • Meyer-Bernstein EL, Blanchard JH, Morin LP: **The serotonergic projection from the median raphe nucleus to the suprachiasmatic nucleus modulates activity phase onset, but not other circadian rhythm parameters.** *Brain Res* 1997, **755**:112-120.

This report demonstrates that serotonergic innervation of the suprachiasmatic nucleus (SCN) from medial raphe interacts with the SCN clock.

32. •• Chen D, Hurst WJ, Ding JM, Faiman LE, Mayer B, Gillette MU: **Localization and characterization of nitric oxide synthase in the rat suprachiasmatic nucleus: evidence for a nitrergic plexus in the biological clock.** *J Neurochem* 1997, **68**:855-861.

See annotation [33•].

33. • Wang H, Morris JF: **Presence of neuronal nitric oxide synthase in the suprachiasmatic nuclei of mouse and rat.** *Neuroscience* 1996, **74**:1059-1068.

These two papers [32••,33•] report the localization of neuronal nitric oxide synthase (NOS) in SCN of rat and mouse. The first study used confocal imaging and demonstrates that in rat NOS is primarily in fibers, possibly from other brain sites, that invest the suprachiasmatic nucleus (SCN). This study also evaluates the biochemical properties of SCN NOS.

34. Prosser RA, McArthur AJ, Gillette MU: **cGMP induces phase shifts of a mammalian circadian pacemaker at night, in antiphase to cAMP effects.** *Proc Natl Acad Sci USA* 1989, **86**:6812-6815.

35. Liu C, Gillette MU: **Cholinergic regulation of the suprachiasmatic nucleus circadian rhythm via a muscarinic mechanism at night.** *J Neurosci* 1996, **16**:744-751.

36. • Bina KG, Rusak B: **Muscarinic receptors mediate carbachol-induced phase shifts of circadian activity rhythms in Syrian hamsters.** *Brain Res* 1996, **743**:202-211.

This study convincingly demonstrates that stimulation of brain muscarinic receptors mediates cholinergic phase resetting *in vivo*.

37. •• Liu C, Ding JM, Faiman LE, Gillette MU: **Coupling of muscarinic cholinergic receptors and cGMP in nocturnal regulation of the suprachiasmatic circadian clock.** *J Neurosci* 1997, **17**:659-666.

This study provides one of the first demonstrations of functional linkages between muscarinic cholinergic stimulation and cGMP production in the central nervous system. These data establish a 'cause and effect' relationship between muscarinic receptor stimulation, activation of the cGMP/PKG pathway and nocturnal phase-resetting of the suprachiasmatic nucleus (SCN).

38. Hunter T, Karin M: **The regulation of transcription by phosphorylation.** *Cell* 1992, **70**:375-387.

39. Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, Takahashi JS, Greenberg ME: **Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock.** *Science* 1993, **260**:238-241.

40. •• Ding JM, Faiman LE, Hurst WJ, Kuriashkina LR, Gillette MU: **Resetting the biological clock: mediation of nocturnal CREB phosphorylation via light, glutamate, and nitric oxide.** *J Neurosci* 1997, **17**:667-675.

This study convincingly provides a link between P-CREB formation, phase resetting and nocturnal light, glutamate and NO stimulation.

41. • Stehle JH, Pfeffer M, Kuhn R, Korf HW: **Light-induced expression of transcription factor ICER [inducible cAMP early repressor] in rat suprachiasmatic nucleus is phase-restricted.** *Neurosci Lett* 1996, **217**:169-172.

The first report of a trans-repressor (ICER) to be induced by light.

42. • Romijn HJ, Sluiter AA, Pool CW, Wortel J, Buijs RM: **Differences in colocalization between Fos and PHI, GRP, VIP and VP in neurons of the rat suprachiasmatic nucleus after a light stimulus during the phase delay versus the phase advance period of the night.** *J Comp Neurol* 1996, **372**:1-8.

Light induces FOS in different sets of peptidergic neurons during phase delays compared with phase advances.

43. • Guido ME, Goguen D, Robertson HA, Rusak B: **Spontaneous and light-evoked expression of JunB-like protein in the hamster suprachiasmatic nucleus near subjective dawn.** *Neurosci Lett* 1996, **217**:9-12.

See annotation [44•].

44. • Guido ME, Rusak B, Robertson HA: **Expression of FOSB mRNA in the hamster suprachiasmatic nucleus is induced at only selected circadian phases.** *Brain Res* 1996, **739**:132-138.

These two papers [43•,44•] suggest that there is both clock- and light-controlled expression of immediate early genes in the suprachiasmatic nucleus (SCN).

45. • Wisor JP, Takahashi JS: **Regulation of the *vgf* gene in the golden hamster suprachiasmatic nucleus by light and by the circadian clock.** *J Comp Neurol* 1997, **378**:229-238.

The first late response gene induced by a light-stimulus was found to be the vesicular factor *vgf*.

46. Weber ET, Gannon RL, Michel AM, Gillette MU, Rea MA: **Nitric oxide synthase inhibitor blocks light-induced phase shifts of the circadian activity rhythm, but not c-*fos* expression in the suprachiasmatic nucleus of the Syrian hamster.** *Brain Res* 1995, **692**:137-142.

47. • Amir S, Edelstein K: **A blocker of nitric oxide synthase, N^G-nitro-L-arginine methyl ester, attenuates light-induced Fos protein expression in rat suprachiasmatic nucleus.** *Neurosci Lett* 1997, **224**:29-32.

This study reports that L-NAME, a NOS inhibitor, blocks light-induced FOS expression during early night, whereas an earlier study [46] found that during late night the same inhibitor does not block FOS induction, while inhibiting the phase advance. Together, these data indicate that light activates distinct signaling pathways to induce FOS during phase delay versus advance.

48. • Gillette MU, McArthur AJ: **Circadian actions of melatonin at the suprachiasmatic nucleus.** *Behav Brain Res* 1996, **73**:135-139.

An up-to-date review of temporal aspects of melatonin interaction with the suprachiasmatic nucleus (SCN).

49. •• McArthur AJ, Hunt AE, Gillette MU: **Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn.** *Endocrinology* 1997, **138**:627-634.

This study demonstrates that the suprachiasmatic nucleus (SCN) is sensitive to phase control by melatonin, the pineal hormone whose synthesis is under clock control, only at dusk and dawn. Activation of a pertussis toxin-sensitive G protein leads to transient stimulation of protein kinase C activity only at these times.

50. • Godson C, Reppert SM: **The Mel1a melatonin receptor is coupled to parallel signal transduction pathways.** *Endocrinology* 1997, **138**:397-404.

Ectopic expression of the Mel1a receptor in fibroblasts produces a receptor that, upon binding ligand, activates phospholipase A_2 and protein kinase C.

51. •• Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VK, Reppert SM: **Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock.** *Neuron* 1997, **19**:91-102.

Mice in which the Mel1a receptor was deleted genetically lack an acute inhibition of firing in suprachiasmatic nucleus (SCN) neurons in response to melatonin, but express melatonin-induced phase-resetting of SCN rhythms.

52. •• Silver R, LeSauter J, Tresco PA, Lehman MN: **A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms.** *Nature* 1996, **382**:810-813.

This surprising report demonstrates that the suprachiasmatic nucleus (SCN) encapsulated in a polymer that excludes cell processes and molecules >500 kDa can regulate the circadian rhythm of wheel-running rhythms in hamsters.