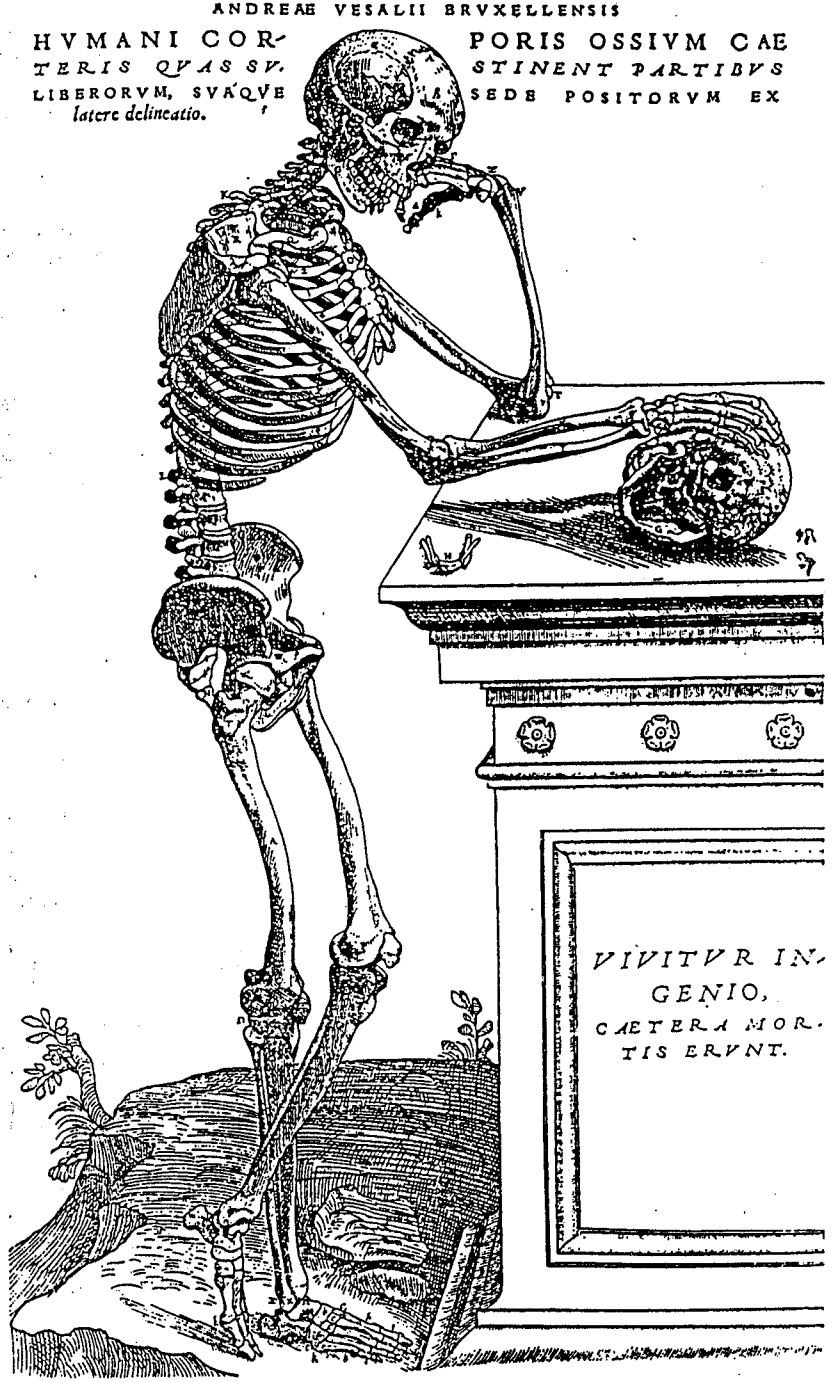


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# THE NEUROETHOLOGY OF TIME: REGULATION OF THE BRAIN'S CIRCADIAN CLOCK

**Martha U. Gillette**

Dept. of Cell & Structural Biology, Chemistry & Life Sciences Lab., University of Illinois, 601 S. Goodwin Avenue, Urbana, IL, U.S.A. 61801

## Introduction

Behaviors do not occur at random, nor are they constant. Rather, they vary in quality and amplitude over time. When assessed longitudinally, behaviors fundamental to life, such as locomotion, sleep, feeding, drinking, intraspecific communication, memory formation and reproduction, exhibit regular cyclic variations (Aschoff and Honma, 1959). These changes occur with characteristic phase-relationships to cyclic environmental variations. Alternation of day and night is the major environmental variable to which organisms have been exposed as life evolved over a trillion rotations of the earth. Superimposed upon the daily cycle is modification of the relative durations of day and night by the seasons. In view of the relative dominance of this class of environmental variables, it is not surprising that animals exhibit day-night oscillations in the peaks and troughs of occurrence of their most fundamental behaviors. But, what makes birds sing at the morning and frogs call at night? Why do lemurs hunt at night while primates do so in day? What drives these cyclic changes in behavior?

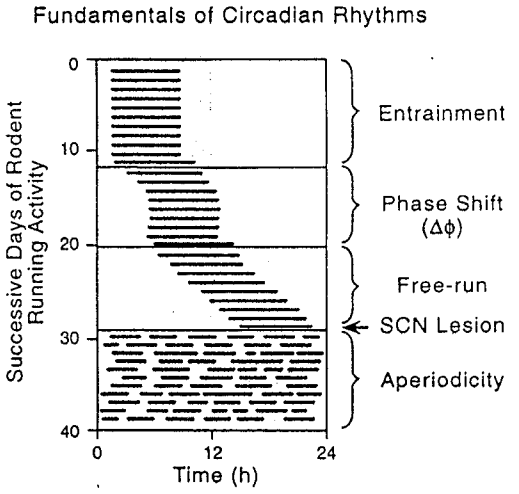



*Figure 1. Schematic presentation of wheel-running activity of a hamster illustrates fundamental circadian principles. Event marks representing turns of the wheel merge during intense bouts of activity so that they appear as a solid horizontal line. Activity begins shortly after the onset of night (shaded area) in the light:dark cycle. A shift in the lighting cycle induces a delay in the phase of the activity rhythm. In constant darkness the rhythmic patterning of activity continues, but with a period slightly longer than 24 hours. SCN lesion abolishes the periodic organization of this behavior.*

The answer would seem obvious: light and darkness must cue the changes in behavior. This can be tested easily by placing the animal in an aperiodic environment (constant darkness, temperature, food and water). Surprisingly, behaviors continue to oscillate under these conditions as under the light-dark cycle (Fig. 1). Careful repeated measures reveal that the period of oscillation is no longer precisely 24 hours. Rather, it deviates by a small but constant amount each cycle so that the period of the free-running oscillation is *circadian*, about a day in length (Rawson, 1959; DeCoursey, 1960a). This demonstrates that periodicity is not an after-effect of the day-night condition, wherein the period is precisely 24 hours. Behaviors continue to oscillate with a stable circadian rhythm for weeks in the absence of measureable environmental signals. Over this time, the active phase moves away from its relative position in the cycle of day and night under which the animal had been entrained (Fig.1). Before long, the active phase can pass through a time when it is inverted from its original position, as it keeps on migrating by the same small amount each day. Light exposure during the subjective night of the free-running rhythm effectively readjusts behavioral rhythms to their usual phase relationship with respect to day and night (Rawson, 1959; DeCoursey, 1960b). These unanticipated findings raised the possibility that the patterning of circadian behaviors is not determined solely by environmental forces.

Development of the field of animal behavior has witnessed recurrent discussion of the relative importance of exogenous versus endogenous contributions to behavior. This issue was vigorously debated at the inception of the search for mechanisms regulating circadian rhythms (Aschoff, 1960; Brown, 1960; DeCoursey, 1960a; Pittendrigh, 1960). The persistence of rhythmicity with a circadian, rather than 24-hour, period under constant conditions suggested that these behaviors were not driven by the solar cycle. This observation provided impetus to determine whether circadian rhythms might be generated endogenously. If so, where did the locus of control lie, and how did a biological system produce oscillations with such a long period? Furthermore, if the rhythms were endogenous, how did exposure to the day-night cycle alter the internal system so that rhythms remained synchronized to the 24-hour cycle of environmental light and darkness?

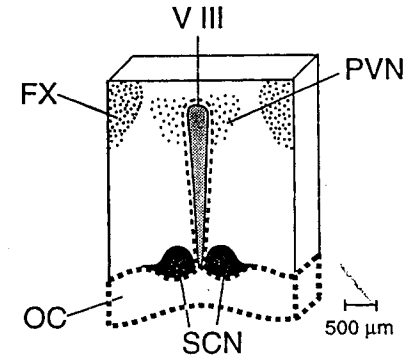
This review will summarize present understanding of neural mechanisms underlying the temporal regulation of circadian behavioral rhythms. It will focus primarily upon research from my laboratory in which a brain slice preparation was used to study the clock tissue in isolation. First, it will review evidence that there is a biological clock in the mammalian brain. Second, it will consider the unusual timekeeping and gatekeeping properties of this circadian clock. Third, it will evaluate temporal gating over the circadian cycle of clock sensitivity to resetting stimuli in the context of interplay between endogenous and exogenous control. Overall, it should provide insight as to neural substrates and cellular mechanisms of temporal organization that pace behaviors to a rhythm of 24-hour time.



**The circadian clock in the suprachiasmatic nucleus**

Circadian rhythms of behavior are characteristic of the broad range of life, including cyanobacteria and molds, so 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 control of many complex behaviors became cephalic. Experiments demonstrating an effect of light in regulating phasing of locomotory rhythms suggested that sensory targets in the central nervous system might perform an important role in circadian control. Rodents in which the basal hypothalamus was damaged showed deficits in behavioral rhythmicity; this finding focused the search for a brain clock on the ventral medial area (Richter, 1967). Localized lesion of rat brain identified a brain site controlling circadian rhythms of corticosterone (Moore and Eichler, 1972) and drinking behavior and locomotor activity (Stephan and Zucher, 1972). Remarkably, the tiny suprachiasmatic nucleus (SCN), which lies paired at the base of the hypothalamus and nested in the optic chiasm (Fig. 2), was essential. Locomotory behavior continued to occur in animals with SCN lesions, but activity no longer was patterned into daily oscillations. Additionally, the SCN was found to receive from the eye a direct projection, the retinohypothalamic tract, that is essential for synchronization to light-dark cycles (Moore and Lenn, 1972). Therefore, the SCN fulfilled the major criteria for a circadian clock: it was necessary both for 1) generating and transmitting a timebase for circadian patterning (a timekeeper) and 2) channeling clock sensitivity so as to recognize signals of temporal desynchronization, *e.g.,* nocturnal light, and orchestrating appropriate, resynchronizing phase adjustments (a gatekeeper).

*Figure 2. The SCN hypothalamic brain slice. The paired SCN lie at the base of the third ventricle (V III), closely apposed to the optic chiasm (OC). The SCN are clearly discernable in the fresh coronal slice. Included in the slice are the paraventricular nucleus (PVN) and the fornix (FX). Clock properties preserved in this hypothalamic slice are maintained in slices surgically reduced to the edges of the SCN.*




A genetic basis for mammalian clocks was demonstrated by the spontaneous appearance of a heritable mutation in hamster that shortened the circadian period of locomotory activity (Ralph and Menaker, 1988). Both SCN in wild type animals (circadian period, $\tau$ = 24.1 hours) were lesioned, rendering the animals arrhythmic (Fig. 1). This was followed by transplantation of the SCN from a short period mutant hamster ($\tau$ = 20.2 hours) into the brain of a lesioned wildtype. Not only did the SCN transplant restore rhythmicity in wheel-running, the period was that of the $\tau$ mutant. The inverse experiment produced 24.1-hour rhythms in hamsters of mutant genotype. This re-

vealed that properties encoded in genes expressed in the SCN underlie circadian rhythms. The genetic basis of this mutation in hamster has not been determined. However, mRNA homologous to that of the well-studied clock gene, *period*, of *Drosophila* has recently been identified in the SCN of mammals, where it cycles (Sun *et al.*, 1997; Tei *et al.*, 1997). Genetic control of circadian timing is thus ubiquitous across phylogeny.

With these demonstrations that the SCN is essential for the circadian patterning and endogenous period of circadian behaviors, the central importance of this brain site in timing behavioral occurrence was established. These studies assessed the effects on the behaving animal of altering the SCN *in vivo*. Concurrently, a complementary approach developed in which SCN properties were evaluated *in vitro*. Additional unanticipated clock properties have been revealed by studying the SCN in isolation. Such studies from my laboratory will be the subject of the remainder of this review.

## Clock Properties in the Dish

We study the temporal organization of the SCN of the rat in a hypothalamic brain slice preparation (Fig. 2) (Green and Gillette, 1982; Gillette, 1991). This approach enables us to directly evaluate properties endogenous to the SCN. Experiments are performed on tissue from 7- to 9-week-old inbred Long Evans rats, reared in a 12-hour light:12-hour dark schedule and fed *ad libitum*. Our rats have been inbred for >33 generations and, thus, meeting the criterion for genetic homogeneity. Coronal slices of SCN-bearing hypothalamus are cut 500 μm thick at room temperature with a mechanical chopper. The slice containing the medial SCN is maintained a large-volume perifusion-interface chamber (Hatton *et al.*, 1980). The chamber is perfused with Earle's Balanced Salt Solution (EBSS, Sigma), supplemented to a final concentration of 24.6 mM glucose, 26.2 mM bicarbonate (pH 7.4) and gentamicin (0.0005%, Sigma) and exposed to a moist atmosphere of 95% $O_2$:5% $CO_2$ (Gillette, 1991; Gillette *et al.*, 1995).

Perhaps surprisingly, the SCN, when sectioned in a coronal slice, generates near 24-hour oscillations in ensemble neuronal firing rate *in vitro* (Prosser and Gillette, 1989), like those *in vivo* (Inouye and Kawamura, 1979). The rhythm emerges from the spontaneous activity of neuronal ensemble. Single units are sampled extracellularly over two 2-minute epochs. The mean is taken as the firing rate for that cell at the circadian time of the measurement. The electrode then is repositioned so as to sample throughout the nucleus. From the running average, the time-of-peak in each daily oscillation over the course of successive days *in vitro* is evaluated as a measure of clock phasing (Fig. 3A) (Ding *et al.*, 1994). The robustness of SCN activity over successive days in buffered saline solution is remarkable among brain slice preparations. It is likely that the SCN lends itself well to this type of preparation because the cells are very small (7-12 μm), forming primarily local circuits (van den Pol, 1980), and thus the integrity



f much of the SCN within the slice is intact. It also follows that the SCN must itself synthesize proteins and factors necessary to maintain circadian functions.

The aggregate data suggest that the SCN imposes temporal order upon the body in two ways: 1) through generating output signals that relay time-of-day information, and 2) through gating its own sensitivity to incoming signals that adjust clock timing. Both properties are derived from the timebase of the SCN's endogenous, near-24-hour clock. Regarding the first property, the clock generates signals that communicate timing cues beyond the SCN. The most prominent of these signals, neuronal firing rate (Inouye and Kawamura, 1979; Green and Gillette, 1982) and vasopressin secretion (Earnest and Sladek, 1986; Gillette and Reppert, 1987), are oscillatory in nature. These signals wax and wane gradually over the circadian cycle (Fig. 3A), and, thus, they can convey information about both the passage of time and the phase of the clock. Such signals orchestrate the circadian rhythms of surrounding hypothalamic areas, as well as of multiple other brain, autonomic and peripheral sites (Inouye and Kawamura, 1982).

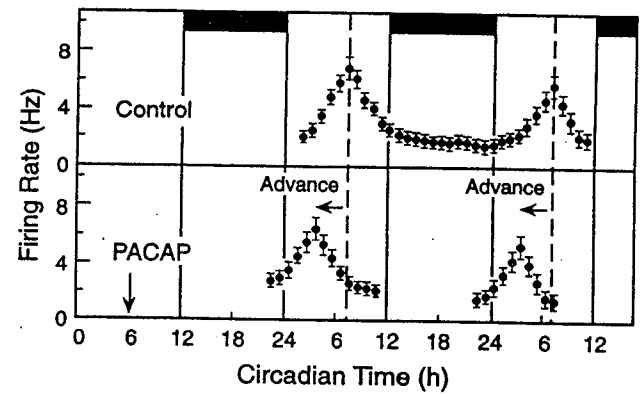



*Figure 3. Circadian clock properties are maintained in vitro. A) Spontaneous activity of SCN neurons, maintained in a brain slice under constant conditions, oscillates with a 24-hour period that peaks midday (dashed line) at CT 7. Plotted is the running average of the 2-hour means ± SEM of firing rate of neuronal ensemble sampled. Means were derived from 124 individual neurons sampled from a single SCN over 38 hours on days 2 and 3 in vitro. A horizontal bar marks subjective nighttime, CT 12-24. The vertical dashed lines mark the times of the peak (CT 7) in control oscillations. B) PACAP, a cAMP-stimulating neuroactive peptide stored in terminals of the retinal ganglion cells that innervate the SCN, was applied at CT 6 on day 1. This advanced the phase of the rhythms, as measured at the peaks, by 3.5 hours. Data from Hannibal et al., 1997.*

A second form of temporal organization imposed by the biological clock lies in the regulation of its own sensitivity to stimuli that adjust phasing. Signals relaying environmental and organismic state impinge upon the SCN and are integrated there. However, they alter clock timing only if they occur during specific phases of the circadian cycle, when the clock is receptive to them. Altering the phase of the host of behavioral, metabolic and hormonal rhythms under clock control would be adaptive only if the phase-adjusting stimulus were an 'error' signal. In this context, the error signal would convey asynchrony between environmental or organismic state and clock time. For example, light occurring in the day is appropriate while the biological clock is synchronized to the cycle of day and night. However, light encountered at night would represent an error signal, an inappropriate correspondence in timing between the clock cycle and the environmental light-dark cycle.

Even in the brain slice, the SCN undergoes a programmed, near-24-hour sequence of sensitivities to stimuli that can adjust its phase (Gillette, 1986; Gillette *et al.*, 1995; Gillette, 1996). Sensitive periods correlate with discrete periods in the clock's entrained cycle: some are restricted to subjective day and others to subjective night, while a distinct set appear only at dusk and dawn. These programmed changes in sensitivity significantly restrict the ability of specific signals to alter clock timing, and effectively determine whether the clock recognizes a stimulus as an error signal. In this way, the clock temporally filters, or gates, the information that can access its timekeeping mechanism across the circadian cycle.

The stable circadian rhythm of firing rate of the ensemble of SCN neurons can be used as a bioassay to probe for gating sites (Fig. 3). We have evaluate elements in various cell signaling pathways. Extracellular signaling molecules, such as neurotransmitters from afferent projections, are termed *first messengers*. Binding of extracellular molecules to selective receptors that are integral membrane proteins initiates a series of protein-protein interactions that result in the intracellular production of secondary signaling molecules. These small intracellular molecules produced via signal transduction across the membrane are *second messengers* that activate specific molecular cascades within the cell. Gating could take place within either or both levels of signaling cascades.

**Clock gating of signaling pathways defines temporal domains**

Structurally, the SCN is the most complex of biological clocks. One SCN contains ~8,000 neurons, which lie in close apposition with a nearly equal number of glia in a 950 μm-long ovoid structure (van den Pol, 1980). These cells are among the smallest (7-12 μm) in the brain 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 (Welsh *et al.*, 1995). It follows that gating of sensitivity to resetting stimuli and the phase resetting process 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?

We probed these sensitivities by briefly exposing the SCN *in vitro* to treatments that activate elements of specific signaling pathways. We administered treatments at various discrete points in the circadian cycle, and then assessed the time of the peak in the neuronal activity rhythm over the next one or two days *in vitro*. If the peak appeared earlier than in controls during the cycle(s) after treatment, the phase of the rhythm had been advanced (Fig. 3B). If it appeared later than in controls, then the phase had been delayed by the treatment. The changing relationship between the circadian time of treatment and its effect on phase was assessed using a phase-response curve (PRC). This relationship graphically presents the temporal pattern of SCN sensitivity to activation of specific signaling pathways. The permanence of the phase-shift was examined by evaluating the time of the peak in neuronal activity over one or two days after a treatment. Timing of the peak after experimental reagents had been administered at the maximal point of sensitivity was compared with the time of the peak in media-treated controls.

Temporal domains that we have identified as sensitive to phase resetting via specific first and second messenger pathways coincide with discrete portions of the circadian cycle. Based on these temporal restrictions, the circadian cycle can be divided into temporal states, termed *domains* of the clock: day, night, dusk and dawn (Gillette, 1997). These studies not only contribute to defining the properties of these clock domains, they emphasize the complexity of control that the clock exerts over signal integration and phase resetting within the SCN. They have been incorporated into putative clock-gated regulatory pathways in Figure 4 and will be discussed in terms of the clock domain that they regulate.

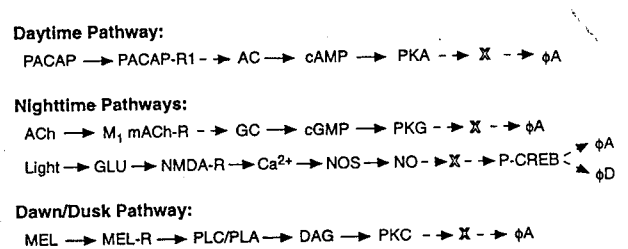



*Figure 4. Summary of signaling pathways regulated by the SCN clock in four temporal domains. This figure is based upon data discussed in this review. Notice the differences in the pathways that can access the clock in daytime, nighttime or at dusk and dawn. Dashed arrows indicate points with an unknown number of steps. Relative position of a critical gating site in each pathway is designated by -x-.*

Subjective day and night have distinct sensitivities and response characteristics. Furthermore, they correlate with periods of sensitivity to specific neurotransmitter systems that a large body of neuroanatomical studies have identified as impinging upon this hypothalamic site (Moore, 1996). Neurotransmitters localized to these afferent pathways can be tested *in vitro* to identify domains of SCN sensitivity and their signaling elements. From these data we can speculate as to mechanisms that can access and regulate the biological clock at different points in the circadian cycle. The major identified domains of clock sensitivity will be considered in turn.

*The Daytime Domain*

Even in the constant conditions in 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 (Fig. 5). Application in mid-subjective daytime at circadian time 7 (CT 7, 7 hours after light onset in the rat colony) of any treatment that stimulates cAMP-dependent protein kinase (PKA) causes a 4-6 hour advance in the phase of the rhythm of neuronal activity. Effective agents include analogs of cAMP, forskolin, which directly stimulates synthesis of native cAMP, and RO 20-1724, which inhibits cAMP degradation cAMP (Gillette and Prosser, 1988; Prosser and Gillette, 1989). The concordance of the responses to this range of treatments suggests that the clock mechanism can be accessed by cAMP-activated pathways during the daytime domain.

This response is highly selective. The SCN clock does not respond to the degradation product of analog 8-Br-cAMP, 8-Br-5'-AMP (Prosser and Gillette, 1989), nor to analogs of a structurally related purine cyclic nucleotide, cGMP, when applied at mid-subjective day (Prosser *et al.*, 1989). After these treatments, the SCN rhythm continues unperturbed with a daily peak near CT 7. At this time it is also insensitive to tetraphorbol acetate (TPA), which mimics membrane fatty acids in activating protein kinase C (McArthur *et al.*, 1997), as well as to stimuli that mediate $Ca^{+2}$ influx and NO production (Ding *et al.*, 1994). Therefore, this sensitivity is selective for activation of a cAMP/PKA pathway.

The phase response relationship between the time of application of 8-BA-cAMP and the phase-shifting response of the SCN reveals that sensitivity is temporally restricted to subjective daytime (Fig. 5). Sensitivity to activation of the cAMP pathway first appears early in the daytime domain, between CT 2-3, representing 2-3 hours after the initiation of the light portion of the entrained 12 hour light:12 hour dark cycle (Prosser and Gillette, 1989). The response rapidly peaks between CT 4-7, when phase advances of 4-6 hours are induced by 8-BA-cAMP. Then, it slowly wanes until phase is altered by $\leq 1$ hour when the cAMP analog treatment is administered at CT 11 or later into the subjective night. Treatments listed above that elevate endogenous cAMP also are ineffective at mid-subjective night, CT 18. Therefore, the molecular gate to the clock accessed by cAMP in the daytime must be closed at night.



The phase adjustments stimulated by cAMP *in vitro* are rapid and stable. Peak activities measured on the second and third day *in vitro*, after administration of the cAMP analog on the first day, show the same degree of phase advance (as in Fig. 3) (Prosser and Gillette, 1989). This indicates that the process leading to phase advance of the clock mechanism has been completed between the time of treatment and the appearance of the next peak in activity, so that a stable new phase is assumed and continued in subsequent cycles. Therefore, our data support the hypothesis that the clock mechanism shifts rapidly, within the first hours after stimulation *in vitro*. This is in distinction from phase shifts *in vivo*, which can take several days to restablize (De Coursey, 1960b; Daan and Pittendrigh, 1976).

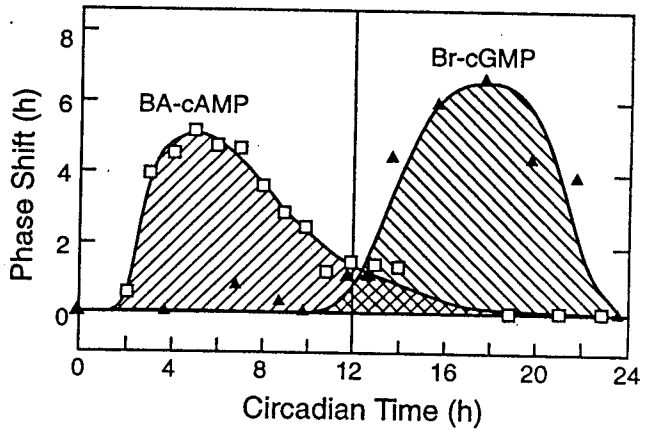



*Figure 5. Two phase response curves (PRCs) demonstrating different temporal sensitivities of the SCN to cAMP and cGMP analogs. Each data point was derived from a single experiment (as in panel B of Fig. 3) and represents the shift in phase of the SCN rhythm (in hours) in response to a 1-hour exposure to the analog that was initiated at the circadian time denoted. The domain of clock sensitivity to 8- BA-cAMP is during subjective daytime while sensitivity to 8-Br-cGMP occurs in antiphase, during subjective nighttime. Data replotted from Prosser et al., 1989.*

While a variety of evidence suggests that daytime behavioral arousal can alter clock phase via serotonin, possibly through a cAMP pathway (Prosser *et al.*, 1990; Medanic and Gillette, 1992; Lovenberg *et al.*, 1993; Prosser *et al.*, 1994), recent evidence argues strongly that pituitary adenylyl cyclase activating peptide (PACAP) may be the first messenger for cAMP (Hannibal *et al.*, 1997). PACAP induces a robust, 6-hour phase advance during the subjective midday, but not during the subjective night (Fig. 3B) (Hannibal *et al.*, 1997). This effect requires activation of cAMP-dependent processes, and the mRNA for the PACAP-type 1 receptor, which couples positively to adenylyl cyclase, is localized to SCN neurons in the retinorecipient area. 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. 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. Why the SCN responds to PACAP in the day and to glutamate at night, even though these neurotransmitters both localize to retinal ganglion cells that project to the SCN, is unknown.

The basis of the daytime domain's sensitivity and temporal selectivity to activation of the cAMP pathway is presently unknown. A possible first level of control would be the cell membrane. Gating could take place at the receptor where ligand binding initiates this response, at the G protein(s) whose activation leads to stimulation of adenylyl cyclase, or at adenylyl cyclase itself. While the clock may regulate membrane components so that they vary and, thus, restrict activation via receptor-mediated processes, restricted clock sensitivity to membrane permeant cAMP analogs demonstrates unequivocally that the clock does control the open-state of *intracellular* gates, downstream from cAMP production. This gating could occur at several levels: 1) through circadian modulation of the levels of the regulatory and catalytic subunits of cAMP-dependent protein kinase (PKA), 2) among non-PKA regulatory molecules in the signaling cascade, and 3) at substrate molecules whose phosphorylation by PKA is required to generate the phase shift. Given the redundancy in biological control systems, there may be multiple control points within a single pathway. The identities of these molecular gates is presently a subject of intense interest.

*The Nighttime Domain*

With the onset of subjective night, SCN sensitivity to phase resetting stimuli changes remarkably. Sensitivity to stimulation via cAMP pathways wanes and, simultaneously, robust sensitivities to stimulation via two signaling pathways develop. One is the pathway by which environmental light adjusts phase by way of retinohypothalamic projections that signal to the SCN via glutamate/NMDA receptor activation/nitric oxide (NO). The second pathway comprises acetylcholine/muscarinic receptor activation/cGMP. Candidate sources for cholinergic innervation of the SCN are basal forebrain and pontine tegmental nuclei of the brain stem (Bina *et al.*, 1993). Both of these regions contribute to the regulation of a circadian behavior, sleep. Timing of nocturnal sensitivity to these pathways is overlapping, but the directions and amplitudes of the phase shifts differ (Fig. 6). What is the basis of this difference?

***Light/Glutamate/NMDA/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 in subjective day. Photic stimuli impinging upon the retina are transmitted directly to the SCN via the retinohypothalamic tract. The phase response relationship between a brief pulse of light to animals in constant darkness and the resulting shift in their locomotor rhythms is biphasic (Fig. 7) (Summers *et al.*, 1984). In



animals in constant darkness, the circadian system is unresponsive to light in the subjective daytime, but with the onset of subjective night, brief light exposure causes circadian rhythms to be delayed. About midway through this sensitive period, near CT 17, the response of the circadian clock reverses, so that circadian rhythms are advanced, rather than delayed, by light. As the transition to daytime approaches, sensitivity to light disappears and the cycle is complete.

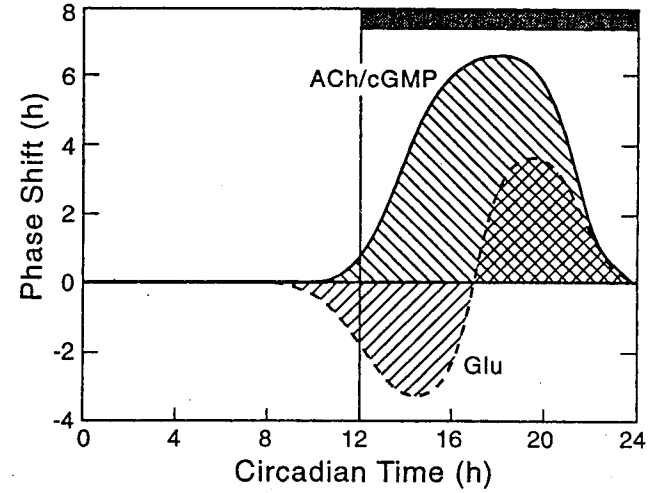



*Figure 6. PRCs for glutamate, acetylcholine and cGMP reveal that sensitivity is restricted to subjective night. While these reagents appear to act during the same nocturnal domain of SCN sensitivity, the patterns of the responses differ. Administration of the carbachol or cGMP analog to the SCN induces only phase advances during subjective night; maximal advances of up to 6.5 hours occur in response to stimulation between CT 16-18. Glutamate, on the other hand, induces phase delays early during subjective night, with a maximal delay of ~ 3 hours at CT 14, and phase advances late at night, with the largest advance of 3.5 hours between CT 19-20. Derived from Prosser et al., 1989; Ding et al., 1994; Liu and Gillette, 1996.*

This pattern is virtually identical to the pattern of the phase shifting response of the SCN neural activity rhythm to direct application of microdrops of glutamate (Fig. 7) (Ding *et al.*, 1994). The bimodal response elicited by brief application of glutamate to the SCN *in vitro* is also produced by NMDA, agonist of the ionotropic glutamate receptor, and donors of the gaseous signaling agent, nitric oxide (NO) (Fig. 8) (Ding *et al.*, 1994). Specific antagonists of the NMDA receptor or of nitric oxide synthase (NOS) block both phase delays and advances stimulated by microdrops of glutamate. Therefore, this signaling pathway can be hypothesized to include light → glutamate →

NMDA receptor activation → $Ca^{+2}$ influx → NOS activation → NO production → → phase shift (Fig. 4). Elements of this pathway have been corroborated *in vivo* (Ebling, *et al.*, 1991; Rea *et al.*, 1993; Weber *et al.*, 1995b; Ebling, 1996).

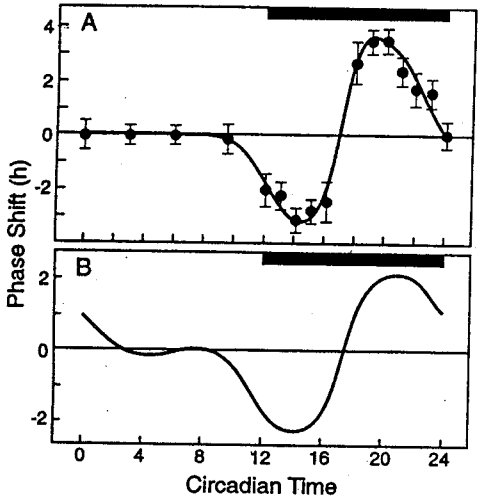



*Figure 7. The PRCs for glutamate in vitro and light in vivo are strikingly similar. A) The effect on phasing of circadian rhythms of SCN neuronal activity of a 10-minute drop of glutamate applied directly to the SCN (Ding et al., 1994). B) The PRC for the effect of a 1-hour light pulse of 150 lux on the circadian rhythm of locomotor activity of rats in constant darkness (B, Summer et al., 1984).*

This complex signaling pathway contains several points at which the clock could restrict access. NMDA receptors may be absent or unresponsive to glutamate in the daytime. However, NMDA-mediated synaptic responses from the optic nerve to SCN neurons have been rigorously demonstrated in the subjective daytime (Kim and Dudek, 1991); the extent to which they are similar to nighttime responses has not been determined. In hippocampal cells, ligand binding to the NMDA receptor leads to a rise in $Ca^{+2}$ influx (Ghosh and Greenberg, 1995), which combines with calmodulin and activates nitric oxide synthase (NOS) (Bredt and Snyder, 1992; Garthwaite and Boulton, 1995) . NOS is present in the rat SCN (Decker and Reuss, 1994; Chen *et al.*, 1997). NOS specific activity does not vary significantly over the circadian cycle (Chen *et al.*, 1997), while the response to NO changes in a pattern like that to light and glutamate (Ding *et al.*, 1994). NO must move intercellularly, for exogenous hemoglobin, which avidly binds extracellular NO, blocks phase shifts induced by glutamate (Ding *et al.*, 1994). Because the temporal sensitivity and pattern of response to NO is identical to that for light and glutamate, a critical gate for this pathway must lie within the cell, in the steps downstream from NO.

Additional insight as to the location of nighttime gates has been contributed by studies focusing upon transcriptional activation initiated via signal transduction pathways. A gate restricting the phase shifting response of hamsters to light lies upstream from the transactivation factor, $Ca^{+2}$/cAMP response element binding protein (CREB) (Ginty *et al.*, 1993). Phosphorylation at serine$^{133}$ of this transcription factor is a common node



for multiple signaling pathways activated by extracellular signals that initiate transcription (Hunter, 1995). Like light, glutamate and NO induce serine$^{133}$ phospho-CREB (P-CREB) only at night (Chen *et al.*, 1997). The amount of CREB protein itself does not vary between night and day (Ginty *et al.*, 1993; Ding *et al.*, 1997). Therefore, a gate to nocturnal phase shifting in response to light must lie between NO and CREB.

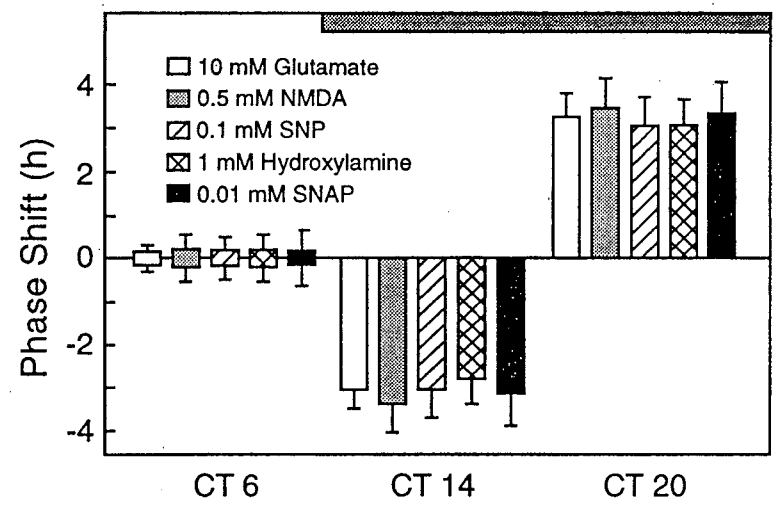



*Figure 8. SCN sensitivites to glutamate, NMDA and NO donors (SNP, hydroxylamine or SNAP) are temporally overlapping. Each was applied to the surface of the SCN in vitro for 10 min as a 0.2 µl drop at the concentration indicated. In each case, application midday had no effect, whereas in early night (CT 14) each induced a phase delay and in late night (CT 20) each induced a phase advance. Results did not vary significantly among the treatments at each time point (unbalanced ANOVA). Data from Ding et al., 1994.*

***Acetylcholine and cGMP.*** Overlapping with the nocturnal sensitivity of the SCN to light/ glutamate/ NMDA receptor activation /NO, the SCN expresses sensitivity to cGMP analogs (Prosser *et al.*, 1989) and cholinergic activation (Liu and Gillette, 1996). Bath application of cGMP analogs induces phase advances of >1 hour between CT 14 and 22 (Fig. 5) (Prosser *et al.*, 1989). The maximal advance, 6.5 hours, appears at CT 18. This advance is nearly twice the maximal advance induced by glutamate at its peak efficacy, CT 20. The SCN is insensitive to activation of the cGMP pathway after CT 22, throughout the subjective daytime until early night. This circadian pattern of sensitivity to cGMP analogs applied either in the bath or by microdrop to only the SCN (Liu and Gillette, 1994) matches the response to the general cholinergic agonist,

carbachol (Fig. 6). Thus, while the temporal domain of these sensitivities is the same is for glutamate signaling, the direction of response is strictly phase advance.

The response to carbachol shows a pharmacological profile consistent with an $M_1$-like muscarinic response. Agonists of cholinergic muscarinic receptors induce phase shifts with a rank order potency of acetylcholine > McN-A-343 > carbachol = muscarine (Liu and Gillette, 1996). Muscarinic activation at midnight (CT 18) rapidly induces a transient increase in cGMP level and cGMP-dependent protein kinase (PKG) phosphotransferase activity within the SCN (Liu *et al.*, 1997). These studies establish acetylcholine as a first messenger for cGMP in the SCN. It is noteworthy that gating of the cholinergic response matches that to cGMP analogs, which like cAMP analogs act intracellularly. It follows that the SCN clock restricts access via this cholinergic pathway to the nighttime domain at a gating point again within the cell, downstream from cGMP.

***Integrated nocturnal response.*** Both the pattern and amplitude of the responses induced by glutamate, NMDA and NO differ from the responses to cholinergics and cGMP analogs (Fig. 6). An exception appears in the late night, after CT 20, where they overlap. This may indicate that the two responses are related at this time. $M_1$ receptors have been demonstrated to activate cGMP pathways in SCN and other neurons (Chen *et al.*, 1997; Hu and El-Fakahany, 1993). In many systems, NO binds to an intracellular receptor in the form of the heme moiety of guanylyl cyclase, stimulating the production of cGMP (Lincoln and Cornwell, 1993; Bredt and Snyder, 1992). Intracerebroventricular injection of hamsters with KT5823, a specific PKG inhibitor, blocks light induced phase advances, but not delays (Weber *et al.*, 1995; Mathur *et al.*, 1996).

These data suggest that cGMP may contribute to the phase advance induced by light via glutamate. Thus, glutamatergic and cholinergic signaling pathways may converge in the late night through activation of NOS, production of NO, and, subsequently, cGMP. At this time, the cholinergic pathway may mediate transmission from other brain sites, such as the basal forebrain and brainstem nuclei involved in the regulation of sleep (Bina *et al.*, 1993), that could interact with retinal stimuli at the level of the SCN. Intracellular mediators of the delay portion of the light response are unknown, but critical to identify. This pathway is likely to diverge from the phase-advancing response at a point downstream from NO and to be gated within an intracellular signaling pathway near the level of the gate(s) for cGMP (Fig. 4).

*The Crepuscular Domains*

The regulatory domains described above cover nearly the entire 24-hour period of the circadian cycle. However, they exclude two regions, those at the day-to-night and night-to-day transitions. Dusk is the circadian point when the pineal hormone, melatonin, had been shown to affect SCN neuronal firing rates acutely in brain slices and to cause entrainment of locomotor behaviors when injected into rats (Mason and Brooks,



1988; Shibata *et al.*, 1989; Armstrong, 1989; reviewed in Gillette and McArthur, 1996). Therefore, melatonin was a candidate clock regulator for these crepuscular domains. This role of melatonin was recently confirmed by the demonstration that the SCN in the brain slice expresses sensitivity to phase-resetting by melatonin at these transitional temporal domains. Melatonin administration to the SCN *in vitro* significantly advanced the phase of the neuronal activity rhythm at both subjective dusk and dawn, but was without effect in day or night (Fig. 9) (McArthur *et al.*, 1991; McArthur *et al.*, 1997). Further, melatonin signaling is mediated via G protein-linked activation of protein kinase C (PKC). Activation of PKC is itself gated. This established that melatonin, whose production by the pineal is regulated via an efferent pathway from the SCN, can feedback to directly regulate the circadian clock. The mechanism by which SCN sensitivity to melatonin is gated has not been established. Based upon our findings that gating of sensitivities to daytime and nighttime resetting stimuli occurs downstream from second messengers, we hypothesize that the gate for melatonin lies at a parallel level in its signaling cascade.

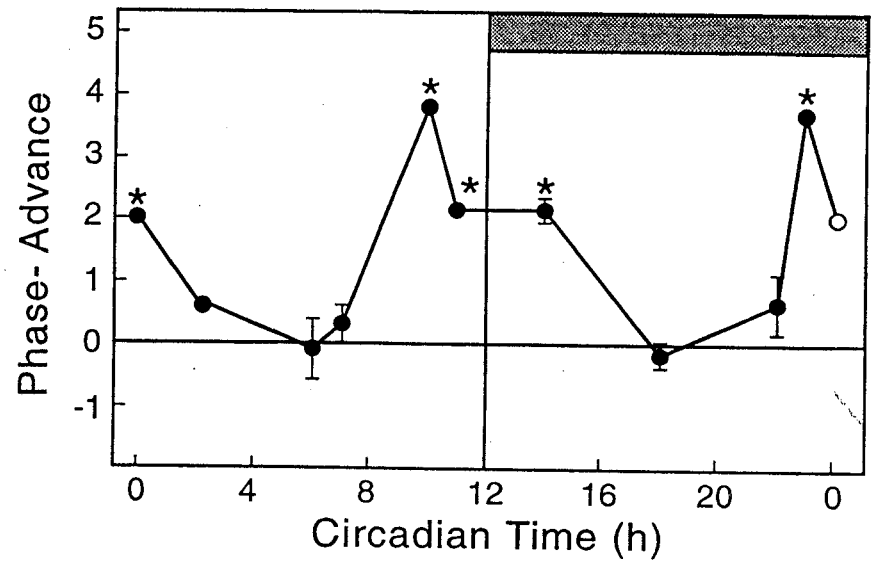



*Figure 9. The PRC for the pineal hormone melatonin. Melatonin was bath-applied to the SCN brain slice for 1 hour at nine points in the circadian cycle. Significant phase advances were observed only at times surrounding the subjective dusk and dawn domains of the clock. Brain slices were maintained under constant conditions. Data from McArthur et al., 1997.*

## Conclusions

These findings demonstrate that the generation of both a 24-hour time base and a programmed pattern of changing temporal sensitivities to phase-resetting stimuli persist in the SCN *in vitro* and, therefore, are fundamental properties of the circadian clock. Agents that selectively stimulate PACAP receptors/cAMP are effective in the subjective daytime, those that activate muscarinic receptors/cGMP or NMDA receptors/$Ca^{+2}$/NO pathways are effective at night, while stimulation of melatonin receptors/PKC is effective at both dusk and dawn (Fig. 4). While membrane-associated gates have not yet been rigorously evaluated, our findings demonstrate that in all temporal domains, gates to clock-resetting lie beyond the level of membrane receptors; they point to critical gating sites lying within the cell, downstream from second messengers. The changing patterns of sensitivities *in vitro* to intracellular messengers reveal that the circadian clock controls multiple molecular gates in a way that assures that they can be selectively opened in a permissive fashion only at specific points in the clock cycle.

Understanding the molecular mechanisms that generate these changes is fundamental to understanding integration of endogenous and exogenous regulators of circadian rhythms. Through gating, the clock primes itself for exogenous signals, imbuing them with temporal relevance. In this way, the endogenous timepiece anticipates significant exogenous cues that provide the clock with information critical to maintaining synchrony with the external cycle of day and night. This restricts and orders behavioral occurrence. Thus, by integrating exogenous and endogenous time, the circadian clock adaptively synchronizes song, foraging, reproduction and sleep to the important temporal features of the animal's life on earth.

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