Suprachiasmatic Nucleus: The Brain's Circadian Clock

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ABSTRACT

The tiny suprachiasmatic nucleus (SCN) of the hypothalamus plays a central role in the daily programming of organismic functions by regulating day-to-day oscillations of the internal milieu and synchronizing them to the changing cycles of day and night and of body state. This biological clock drives the daily expression of vital homeostatic functions as diverse as feeding, drinking, body temperature, and neurohormone secretion. It adaptively organizes these body functions into near-24-hour oscillations termed *circadian rhythms.* The SCN imposes temporal order 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. Each of these properties, derived from the timebase of the SCN's endogenous near-24-hour pacemaker, persists when the SCN is maintained in a hypothalamic brain slice *in vitro.* Single-unit recording experiments demonstrate a spontaneous peak in the electrical activity of the ensemble of SCN neurons near midday. By utilizing this time of peak as a "pulse" of the clock, we have characterized a series of time domains, or windows of sensitivity, in which the SCN restricts its own sensitivity to stimuli that are capable of adjusting clock phase. Pituitary adenylyl cyclase-activating peptide (PACAP) and cAMP comprise agents that reset clock phase during the day time domain; both PACAP and membrane-permeable cAMP analogs cause phase advances only when applied during the day. In direct contrast to PACAP and cAMP, acetylcholine and cGMP analogs phase advance the clock only when applied during the night. Sensitivity to light and glutamate arises concomitant with sensitivity to acetylcholine and cGMP. Light and glutamate cause phase delays in the early night, by acting through elevation of intracellular Ca^{2+}, mediated by activation of a neuronal ryanodine receptor. In late night, light and glutamate utilize a cGMP-mediated mechanism to induce phase advances. Finally, crepuscular domains, or dusk and dawn, are characterized by sensitivity to phase resetting by the pineal hormone, melatonin, acting through protein kinase C. Our findings indicate that the gates to both daytime and nighttime phase resetting lie beyond the level of membrane receptors; they point to critical gating within the cell, downstream from second messengers. The changing patterns of sensitivities *in vitro* demonstrate that the circadian clock controls multiple molecular gates at the intracellular level, to assure that they are selectively opened in a permissive fashion only at specific points in the circadian cycle. Discerning the molecular mechanisms that generate these changes is fundamental to understanding the integrative and regulatory role of the SCN in hypothalamic control of organismic rhythms.

I. Introduction

Behaviors fundamental to life—such as reproduction, locomotion, sleep, feeding, drinking, intraspecific communication, and memory formation—exhibit

regular cyclic variations that recur with characteristic phase relationships to cyclic environmental changes (Aschoff and Honma, 1959). Since the beginning of life, alternation of day and night attending the rotation of the Earth has been the major environmental variable to which organisms have been exposed. Superimposed upon the daily cycle is a seasonal influence that modifies the relative durations of day and night over the course of a year. Considering the importance of environmental changes in illumination, it is not surprising that animals exhibit day-night oscillations in the appearance of their most fundamental behaviors. But why do frogs call at night, while birds sing in the morning? And why do primates hunt during the day, while lemurs do so at night? What is the driving force behind cyclic changes in behavior?

The answer would seem obvious: changes in behavior could be cued by light and darkness. Yet, perhaps surprisingly, behaviors continue to oscillate when animals are placed in an environment devoid of timing information (constant darkness, temperature, food, and water). However, the period of oscillation deviates from 24 hours 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; De Coursey, 1960a). A stable circadian rhythm is expressed for weeks in the absence of measurable environmental signals. This demonstrates that periodicity is not an after-effect of the day-night condition, which has a period of precisely 24 hours. As time passes in constant conditions, the active phase drifts away from its relative position in the cycle of day and night under which the animal had been entrained (Figure 1). In fact, as the onset of activity migrates by the same small amount each day, it will eventually occur at a time in direct antiphase to its original position. Furthermore, under free-running conditions, exposure to light during subjective night shifts behavioral rhythms such that they realign their usual phase relationship with day and night (Rawson, 1959; De Coursey, 1960b). These discoveries raised the distinct possibility that circadian behaviors are *innate* characteristics of organisms and are not determined simply as a consequence of environmental driving forces.

The relative importance of exogenous versus endogenous contributions to behavioral rhythmicity was vigorously debated at the inception of the search for mechanisms regulating circadian changes (Aschoff, 1960; Brown, 1960; De Coursey, 1960a; Pittendrigh, 1960). The persistence of behavioral oscillations with a circadian, rather than 24-hour, period under constant conditions suggested that these behaviors were not simply derived from the environmental solar cycle. Thus began the search for the endogenous circadian rhythm generator. If circadian rhythmicity is an innate property, where is the locus of control, and how do biological systems produce oscillations with such a long period? Furthermore, how does exposure to the day-night cycle alter endogenous rhythms to maintain biological synchrony with the 24-hour cycle of environmental light and darkness?

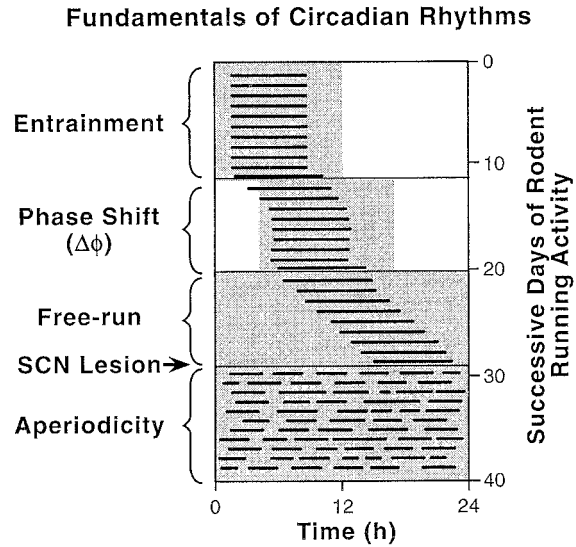

FIG. 1. The unusual properties of the circadian system are prominently displayed by the circadian organization of rodent wheel-running behavior. Nocturnal rodents begin running at the time of lights-off when maintained on a light-dark cycle. Shifting the time of lights-on and lights-off will cause the animal's behavioral rhythm to shift so that it re-establishes an appropriate relationship to the light-dark cycle. The circadian rhythm of wheel-running activity persists when the animal is placed into constant darkness. Under constant conditions, the period of wheel-running activity changes to express that of the animal's endogenous clock; this is defined as the free-running period. Wheel running continues in SCN-lesioned animals but is no longer coordinated into a distinct circadian rhythm.

Endogenous circadian rhythmicity is not simply a behavioral phenomenon. Rather, rhythmicity is a ubiquitous feature of the biochemical and physiological functioning of organisms. A variety of circadian rhythms have been described in humans. Among these, the most easily measured is the body temperature rhythm (Figure 2). Because this rhythm has a consistently stable circadian period of approximately 25 hours, it is often utilized as a marker for the human circadian pacemaker.

Hormonal rhythmicity has been at the forefront of recent endocrine research (reviewed by Van Cauter, 1990). Although hormonal rhythms exhibit complex waveforms due to combined effects of the circadian pacemaker, organismic state (e.g., activity level, sleep, and feeding), and the pulsatile nature of secretion, clear diurnal patterns of secretion have been reported (Figure 2; Schwartz, 1993). Plasma melatonin (Arendt *et al.,* 1989; van Cauter and Turek, 1993), growth hormone (Takahashi *et al.,* 1983), prolactin (Robyn, 1981), thyrotropin releasing hormone (van Coevorden *et al.,* 1989), luteinizing hormone (Kapen *et al.,* 1976),

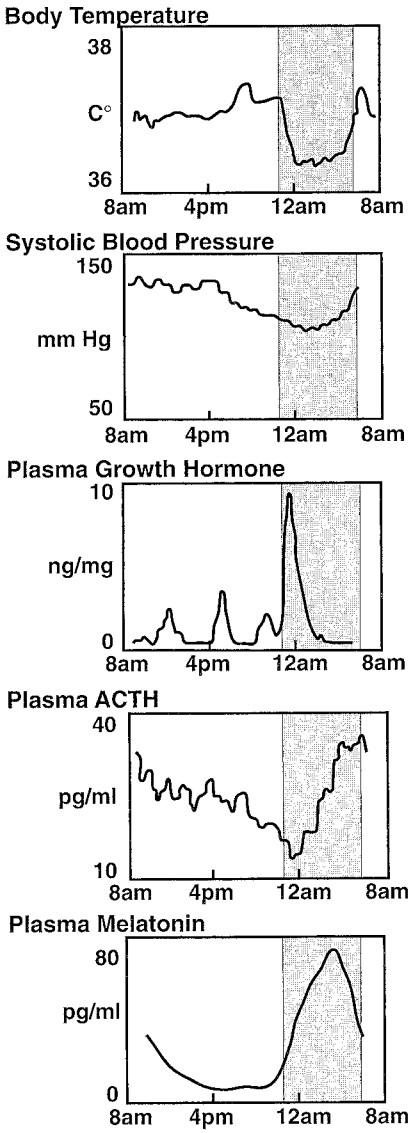

FIG. 2. A variety of human physiological functions, including body temperature, blood pressure, and many endocrine parameters, are expressed in a near-24-hour oscillatory pattern driven by the circadian pacemaker. Body temperature and blood pressure are low at night and peak during the day. Each endocrine rhythm has its own unique profile. Growth hormone peaks just after lights-off in a light-dark cycle. Plasma adrenocorticotropic hormone (ACTH) rises throughout night and peaks at the time of lights-on. Plasma melatonin peaks during the middle of night. [Data replotted from Schwartz, W.J. *Adv. Intern. Med.* **38,** 81–106, 1993.]

and leptin (Sinha *et al.,* 1996; Licinio *et al.,* 1997,1998) are all elevated during the night, in antiphase to adrenocorticotropic hormone and cortisol (Weitzman *et al.,* 1983; Jejeune-Lenain *et al.,* 1987). These oscillations in hormone secretion continue in a constant environment (Figure 3) and, therefore, are clock regulated. Circadian rhythmicity appears to pervade virtually every level of the organization of life. In fact, maintenance of a constant *milieu interior* may be a consequence of a balance among rhythmic, mutually opposed control mechanisms (Schwartz, 1993).

This chapter will summarize present understanding of neural mechanisms underlying the temporal regulation of behavioral and physiological circadian rhythmicity. It will focus primarily upon research utilizing a brain slice preparation to study the clock tissue in isolation. First, it will review evidence that a discrete locus in the mammalian brain serves as the biological clock. Second, it will discuss the unique timekeeping and gatekeeping properties of this biological clock. Third, it will consider the complex nature of temporal gating of clock sensitivity to incoming stimuli in the context of interplay between endogenous and exogenous control. Collectively, it will provide insight into neural substrates

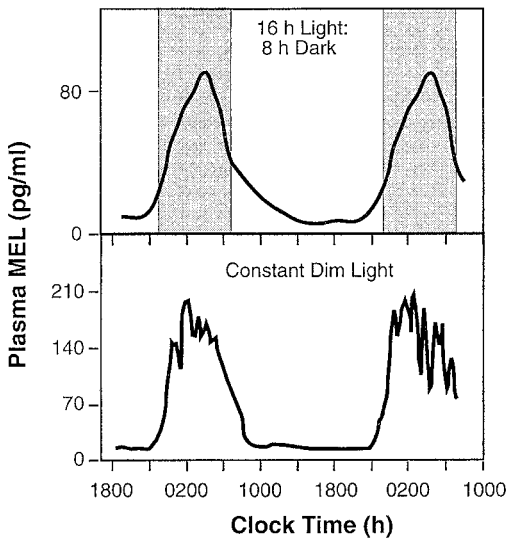

FIG. 3. Oscillations in the pineal hormone melatonin persist under constant environmental conditions. This example displays the persistence of the human melatonin rhythm for a subject maintained in constant dim light. Thus, the secretion of melatonin is driven by the endogenous circadian pacemaker. [Top panel is reprinted with permission from Arendt, J., Minors, D.S., and Waterhouse, J.M. (eds.). "Biological Rhythms in Clinical Practice." 1989, John Wright. Bottom panel is reprinted with permission from van Cauter, E., and Turek, F.W. In "Endocrinology" (L.J. DeGroot, ed.). 1993, W.B. Saunders.]

and cellular mechanisms of temporal organization that pace behaviors to a rhythm of 24-hour time.

II. A Circadian Clock in the Suprachiasmatic Nucleus

Circadian rhythms of behavior and physiology are characteristic of the broad range of organisms, including cyanobacteria and molds. Thus, a nervous system is not required for 24-hour timekeeping or phase adjustment. As sensory processing and motor commands are centralized, cephalic sites have become the primary loci for control of behavioral, physiological, and endocrine functions. The first evidence for a role of the central nervous system in regulation of the mammalian circadian system came from experiments that demonstrated an effect of light in adjusting the phase of locomotor rhythms. It is now well established that exposure to light in the early night sets the clock back several hours, while late-night light exposure moves the clock forward. Deficit of circadian function after damage to the hypothalamus revealed a requirement for ventral medial area (Richter, 1967). Discrete lesions of rat brain identified a brain site controlling circadian rhythms of drinking behavior and locomotor activity (Stephan and Zucker, 1972) and corticosterone (Moore and Eichler, 1972). Remarkably, the tiny suprachiasmatic nucleus (SCN), which lies paired at the base of the hypothalamus and nestled in the optic chiasm, was proven essential for generation of circadian rhythms. Animals with SCN lesions continued to display locomotory behavior but activity was no longer organized into predictable, 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 fulfills the major criteria for a circadian clock: it 1) generates and transmits a timebase for circadian patterning (a timekeeper), and 2) channels clock sensitivity so as to recognize signals of temporal desynchronization (e.g., nocturnal light) and orchestrates appropriate, resynchronizing phase adjustments (a gatekeeper).

The spontaneous appearance of a heritable mutation that shortened the circadian period of locomotory activity provided the first evidence of a genetic component for mammalian circadian rhythmicity (Ralph and Menaker, 1988). Transplantation of the SCN from a short-period mutant hamster into wild-type animals rendered arrhythmic by SCN lesion restored rhythmicity with the period of the mutant SCN. Wild-type transplants into SCN-lesioned, mutant hamsters generated a near-24-hour rhythm in hamsters of the mutant genotype. This solidified the evidence that the SCN is the primary circadian pacemaker in mammals and demonstrated that properties encoded by genes expressed in SCN underlay circadian rhythm generation. While the genetic basis of this mutation in the hamster has not been identified, there has recently been an explosion of new information regarding the molecular mechanisms regulating circadian function. Three

mammalian homologs of the well-studied clock gene, *period (per)* of *Drosophila,* have recently been identified in the SCN (Sun *et al.,* 1997; Tei *et al.,* 1997; Shearman *et al.,* 1997). While the mRNAs of the *mper* family all cycle in the SCN, only *mper1* and *mper2* are responsive to nocturnal light; light at night induces expression of *mper1* and *mper2*. The first putative mammalian clock gene, *clock* (King *et al.,* 1997; Antoch *et al.,* 1997), is also found in *Drosophila* (Darlington *et al.,* 1998; Allada *et al.,* 1998). CLOCK heterodimerizes with the protein product of another recently discovered clock gene, BMAL (Hogenesch *et al.,* 1998; Rutila *et al.,* 1998) to upregulate transcription of *per* (Gekakis *et al.,* 1998). The genetic basis for circadian timing is not only ubiquitous across phylogeny but mechanisms appear highly conserved.

The demonstration that the SCN is essential for the circadian patterning and endogenous period of circadian behaviors has established the central importance of this brain site in coordination of the physiology and behavior of organisms with the omnipresent day/night cycle. The foundation studies assessed the effects of altering the SCN on the behaving animal *in vivo.* Concurrently, a complementary approach developed in which SCN properties were evaluated *in vitro.* Additional, unanticipated clock properties were revealed by studying the SCN in isolation. The remainder of this chapter will focus on SCN properties ascertained by studying the "clock in a dish."

III. Properties of the Clock in the Dish

Our studies focus on the temporal organization of the rat SCN in a hypothalamic brain slice preparation (Gillette, 1991). This approach has enabled us to probe endogenous SCN properties in an environment where the SCN is relatively devoid of feedback loops or inputs from structures outside the hypothalamus. 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, provided food and water *ad libitum.* The rats have been inbred for > 33 generations and therefore exceed the standards for genetic homogeneity. Coronal slices of the SCN-bearing hypothalamus are cut 500 μm thick at room temperature with a mechanical chopper (Figure 4). The slice containing the medial SCN is maintained in 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 *et al.,* 1995). Slices maintained in this manner are viable for experimentation for at least 3 days.

Perhaps surprisingly, the SCN, when sectioned in a 500 μm coronal slice, generates near-24-hour oscillations in ensemble neuronal firing rate *in vitro* (Prosser and Gillette, 1989), similar to those *in vivo* (Inouye and Kawamura, 1979).

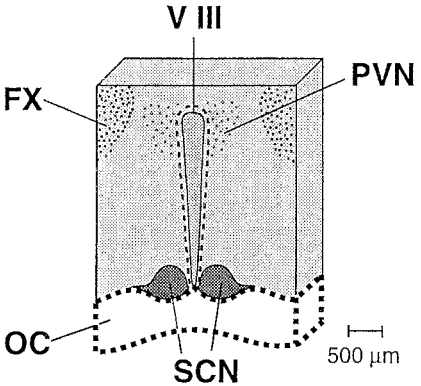

FIG. 4. A diagrammatical representation of a hypothalamic brain slice containing the SCN. SCN continues to oscillate in this preparation and can be used for experimentation for at least 3 days *in vitro*.

Spontaneous activity of neuronal ensemble is responsible for rhythm generation. Single units are sampled extracellularly over a 4-minute epoch. The mean firing rate is calculated for that cell at the circadian time of the measurement. The electrode then is repositioned and another unit is isolated and recorded, so as to sample throughout the nucleus. From these data, a running average is produced and the time of peak for each daily oscillation over the course of successive days *in vitro* is evaluated as a measure of clock phasing (Ding *et al.*, 1994) (Figure 5). The robustness of the rhythm 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 of much of the SCN within the slice is intact. Because the rhythm repeats *in vitro* with minimal maintenance, it also follows that the SCN itself synthesizes necessary proteins and factors to maintain circadian functions.

The aggregate data demonstrate that the SCN imposes temporal order upon an organism in two ways: 1) through generating signals that relay time-of-day information via output pathways, 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. Two prominent SCN output 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. As these signals wax and wane gradually over the circadian cycle, they convey information regarding both the passage of time and the phase of the clock. Such signals orchestrate the

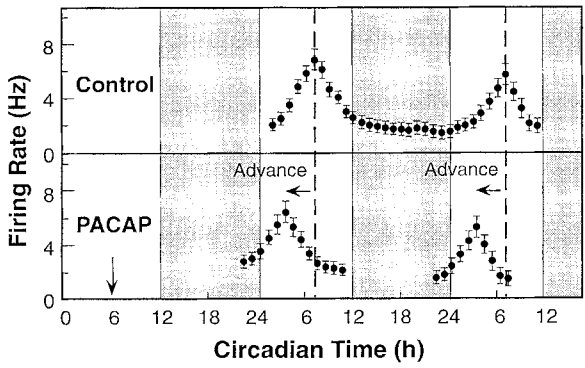

FIG. 5. The circadian rhythm of spontaneous electrical activity of the ensemble of SCN neurons of a hypothalamic brain slice from rat (top panel). Plotted (filled circles) are the 2-hour means ± S.E.M. of firing rate of SCN neurons sampled. Successive 2-hour means are offset by 15 minutes to produce a running average; only the 1-hour offsets are plotted, so that individual values can be discerned. Means were derived from 124 individual neurons sampled from a single SCN over 38 hours on days 2 and 3 *in vitro*, after preparing the slice on day 1. The gray area marks subjective nighttime, CT 12–24. The vertical dashed lines mark the times of the peak in the oscillations, which was at CT 7 on both days sampled. This peak time is characteristic of both untreated and media-treated SCN in brain slices. The bottom panel shows a phase advance of the electrical activity rhythm induced by administration of pituitary adenylyl cyclase-activating peptide (PACAP) between CT 5 and 6, midsubjective day, by exchanging the bath in the brain slice chamber. On days 2 and 3, the peak (marked by the tip of the arrow) appeared 3 hours earlier than in controls (dashed lines). [Data replotted from Hannibal, J., *et al. J. Neurosci.* **17,** 2637–2644, 1997.]

circadian rhythms of surrounding hypothalamic areas as well as of multiple other brain, autonomic, and peripheral sites (Inouye and Kawamura, 1982).

A second, more subtle but equally important form of temporal organization imposed by the biological clock resides in the regulation of its own sensitivity to stimuli that adjust phasing. The SCN receives and integrates signals regarding environmental and organismic state. However, input signals alter clock timing only if they occur during specific phases of the circadian cycle, when the clock is receptive to them. Altering phasing of the host of behavioral, metabolic, and hormonal rhythms under clock control is adaptive only if the phase-adjusting stimulus is perceived by the clock as an "error" signal. In this context, the error signal would convey asynchrony between environmental or organismic state and clock time. For example, a light stimulus provided during the day is entirely appropriate to a biological clock that is synchronized to the cycle of day and night. In contrast, light encountered at night would represent an error signal, an inappropriate correspondence in timing between the clock cycle and the environmental light-dark cycle.

A programmed, near-24-hour sequence of sensitivities to stimuli that can adjust its phase is preserved in the hypothalamic brain slice preparation (Gillette, 1986,1996; Gillette *et al.,* 1995). Periods of sensitivity to phase resetting by specific input variables correlate with discrete intervals in the clock's entrained cycle: some inputs 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 a message indicating desynchrony between the clock and organismic or environmental state. In this way, the clock temporally filters, or gates, the information that can access its timekeeping mechanism across the circadian cycle.

We have successfully used the stable circadian rhythm of firing rate of the ensemble of SCN neurons as a bioassay to evaluate gating of specific windows of clock sensitivity to various stimuli. By probing elements in various cell signaling pathways, we can understand mechanisms by which certain stimuli reach the "gears" of the clock. Extracellular signaling molecules, such as neurotransmitters from afferent projections, are termed *first messengers.* Binding of extracellular molecules to specific integral membrane receptor 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. Our objective is to determine the gating sites; gating could take place within either or both levels of signaling cascades.

IV. Temporal Domains in Clock-Controlled Gating of Signaling Pathways

The SCN is structurally the most complex of all biological clocks. Each SCN contains ~ 10,000 neurons, which lie in close apposition with a nearly equal number of glia in a volume of about 0.068 mm^3 (van den Pol, 1980). These cells, which are among the smallest in the brain (7–12 μm), exhibit broad phenotypic heterogeneity. Nevertheless, there is a consensus that, even in such a complex clock structure, timekeeping is a cellular process. Indeed, the expression of independently phased circadian firing rhythms from individual neurons dissociated from neonatal rat SCN cultured on an electrode array provides compelling evidence for the cellular nature of this clock (Welsh *et al.,* 1995). It follows that gating of sensitivity to resetting 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. We have endeavored to determine how the clock temporally regulates the responsiveness of specific signaling pathways.

In an attempt to define and understand the underlying control mechanisms subserving clock-gated windows of sensitivity, we exposed the SCN *in vitro* to treatments that activate elements of specific signaling pathways. Treatments were administered at various discrete points in the circadian cycle and the time of the peak in the neuronal activity rhythm was assessed over the next one or two circadian cycles *in vitro*. If the time of peak appeared earlier during cycle(s) after treatment compared to controls, the phase of the rhythm had been advanced (Figure 5). If the time of peak appeared later than in controls, then the phase had been delayed by the treatment. By assessing the changing relationship between the circadian time of treatment and its effect on phase, a phase-response curve (PRC) was generated. This relationship graphically presents the temporal pattern of SCN sensitivity to activation of specific signaling pathways and, in fact, defines the window of sensitivity to phase resetting via this pathway. The permanence of the phase shift was examined by evaluating the time of the peak in neuronal activity over 1 or 2 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 spheres identified as sensitive to phase resetting via specific first and second messenger pathways coincide with discrete portions of the circadian cycle. In terms of these temporal restrictions, the circadian cycle can be divided into several discrete temporal states, or domains, of the clock: day, night, dusk, and dawn (Gillette, 1996). Our studies not only contribute to defining the properties of the clock's temporal domains, they also emphasize the complexity of control that the clock exerts over signal integration and phase resetting within the SCN. These properties have been incorporated into putative clock-gated regulatory pathways, as shown in Figure 6. Each will be discussed in the context of the clock domain that is regulated.

Subjective day and night are distinct with respect to their sensitivities and response characteristics. Furthermore, each correlates with discrete periods of sensitivity to specific neurotransmitter systems that are demonstrated to impinge upon this hypothalamic site, as evidenced by a large body of neuroanatomical studies (Moore, 1996). This permits speculation regarding the nature of pathways that gain access to and regulate the biological clock at different points in the circadian cycle. We will now consider, in turn, the major identified domains of clock sensitivity.

A. THE DAYTIME DOMAIN

During the subjective day, the SCN clock exhibits a well-characterized sensitivity to phase shifting by treatments that affect pathways regulated by the ubiquitous second messenger, cAMP (Figure 6). Application in midsubjective daytime (CT 7) of any of a range of treatments that share a common site of action in

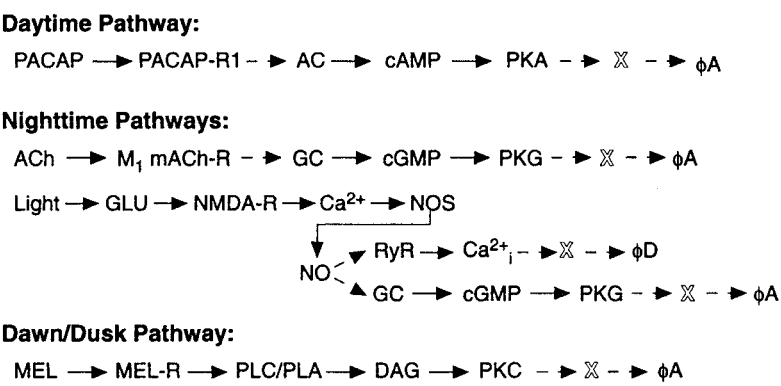

FIG. 6. Summary of signaling pathways that can access the clock in daytime and nighttime. Dashed arrows indicate points with an unknown number of steps. Relative position of a critical gating site in each pathway is designated by an -*x*-.

stimulating cAMP-dependent protein kinase (PKA), either directly or indirectly, by elevating endogenous cAMP levels, cause an advance in the phase of the neuronal activity rhythm by 4.0 to 4.6 hours. Effective agents include cAMP analogs, forskolin, and a specific cAMP phosphodiesterase inhibitor. The cAMP analogs, 8-benzylamino cAMP (BA-cAMP) (Gillette and Prosser, 1988; Prosser and Gillette, 1989), 8-bromo cAMP (Br-cAMP) (Prosser and Gillette, 1989), and 8-chlorophenylthio cAMP (CPT-cAMP) (Gillette and Prosser, 1988) penetrate into the cell and resist degradation to directly activate PKA. Forskolin (Prosser and Gillette, 1989), which directly stimulates adenylyl cyclase, results in enhanced synthesis of native cAMP and, thus, indirect activation of PKA. RO 20–1724 (Prosser and Gillette, 1989) specifically inhibits the phosphodiesterase that degrades cAMP, permitting endogenous cAMP accumulation and subsequent activation of PKA. The concordance of the responses to this range of treatments suggests that the clock mechanism can be accessed by cAMP-activated pathways during the subjective daytime domain.

The cAMP-mediated response is highly selective and temporally restrictive. The SCN clock is not responsive to the degradation product of Br-cAMP, Br-5′-AMP (Prosser and Gillette, 1989), nor to the analogs formed by 8-bromo- or $N^2O^{2'}$ dibutyryl-modifications of another purine cyclic nucleotide, cGMP, when applied at midsubjective day (Prosser *et al.,* 1989). Each of these treaments has no effect on the timing of neuronal activity; the SCN rhythm continues unperturbed, with a daily peak near CT 7. At this time, it is also insensitive to tetraphorbol acetate (TPA), which activates protein kinase C by mimicking membrane fatty acids (McArthur *et al.,* 1997), as well as to stimuli that mediate Ca^{2+} influx

and NO production (Ding *et al.,* 1994,1998). Therefore, this time domain is selective for activation of a cAMP/PKA pathway.

The phase-response relationship between the time of application of BA-cAMP and the phase-shifting response of the SCN reveals that sensitivity is restricted to subjective daytime (Figure 7). Sensitivity to phase shifting via activation of the cAMP pathway first appears early in the daytime domain, between CT 2 and 3, representing 2 to 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 and 7, when phase advances of 4–6 hours are induced by BA-cAMP. Then, responsiveness of the SCN 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. The range of other treatments listed above that elevate endogenous cAMP also are ineffective at midsubjective night, CT 18. Therefore, we can conclude that 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. The same magnitude of phase advance in time-of-peak activities is measured on both the second and third day *in vitro,* following administration of the cAMP analog on the first day (as in Figure 5) (Prosser and Gillette, 1989). This indicates that the process leading to phase advance of the clock mechanism is completed between the time of treatment and the appearance of the next peak in activity, so that a stable new phase is assumed. Therefore, our data support the hypothesis that the clock mechanism shifts rapidly, within the first hours after stimulation *in*

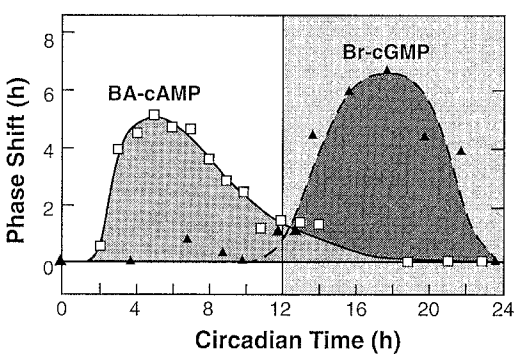

FIG. 7. Phase-response curves demonstrate different temporal sensitivities of the SCN to cAMP and cGMP analogs. Each data point was derived from a single experiment (as in panel B of Figure 5) and represents the shift in phase of the SCN rhythm (in hours) in response to a 1-hour exposure to the analog initiated at the circadian time denoted. The domain of clock sensitivity to BA-cAMP is during subjective daytime, while sensitivity to Br-cGMP occurs in antiphase, during subjective nighttime. [Data replotted from Prosser, R.A., McArthur, A.J., and Gillette, M.U. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 6812–6815, 1989.]

vitro. This is distinct from phase shifts *in vivo,* which can take several days to completely restabilize (De Coursey, 1960b; Daan and Pittendrigh, 1976).

In the studies detailed above, stimuli were bath applied to brain slices containing the SCN together with several cubic millimeters of anterior hypothalamic tissue surrounding the third ventricle and extending toward the supraoptic nuclei (Gillette, 1991). Test compounds were dissolved in medium, equilibrated to match conditions within the brain slice chamber, and then used to replace the normal EBSS within the chamber, bathing the brain slices for 1 hour, after which the test solution was replaced by normal EBSS. Thus, all cells in the slice were exposed to the treatment. In these experiments, the possibility that the site activated by the treatment was in a non-SCN region of the brain slice could not be excluded. However, in subsequent experiments, non-SCN hypothalamus was effectively removed by trimming slices to the edges of the SCN (Gillette and Reppert, 1987). In these reduced slices, the SCN responded to bath application of BA-cAMP with the same amplitude shift as when the whole slice was bathed in the treatment (R.A. Prosser and M.U. Gillette, unpublished data). This finding is consistent with the idea that cAMP-induced phase shifts result from action of cAMP within the SCN itself.

The basis of the daytime domain's sensitivity and temporal selectivity to activation of the cAMP pathway is presently unknown. The obvious first level of control would be the cell membrane. Gating could occur through mediation of ligand binding to a receptor linked to a cAMP-dependent pathway, at the G protein(s) whose activation leads to stimulation of adenylyl cyclase, or at adenylyl cyclase itself. While a variety of evidence suggests that daytime behavioral arousal can alter clock phase via serotonin, possibly through a cAMP pathway (Medanic and Gillette, 1992; 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 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.

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 5) (Hannibal *et al.,* 1997). This effect parallels the effects of direct stimulation of the cAMP pathway (Figure 7). The PACAP 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. It will be important to determine which, if any, other daytime signaling agents can modulate the PACAP/cAMP response, to identify

the next steps in activation and to understand the mechanism behind the SCN's restricted gating that allows a response to PACAP in the day and to glutamate at night, even though both are localized to retinal ganglion cells that project to the SCN.

While the clock may regulate the constituency of the membrane components so that they vary and, thus, could restrict activation via receptor-mediated processes, restricted clock sensitivity to cAMP analogs demonstrates unequivocally that the clock controls the open state of *intracellular* gates. The fact that cAMP analogs, which partition through the membrane and directly activate cAMP-dependent intracellular processes, induce phase resetting only in daytime obviates the existence of a critical gate positioned at some point within the cell, downstream from cAMP production. Intracellular gating could occur at several levels: 1) through circadian modulation of the regulatory and catalytic subunits of cAMP-dependent protein kinase (PKA), 2) among non-PKA regulatory molecules in the signaling cascade, or 3) at substrate molecules whose phosphorylation by PKA is required to generate the phase shift. Further, considering the redundancy in biological control systems, multiple control points within a single pathway are likely. The identities of these gating sites and molecules are presently a subject of intense interest.

B. THE NIGHTTIME DOMAIN

The onset of subjective night is marked by a dramatic alteration in SCN sensitivity to phase-resetting stimuli. Sensitivity to stimulation via cAMP pathways wanes and, simultaneously, robust sensitivities to stimulation via two different signaling pathways develop. The first is the pathway utilized by environmental light, acting through retinohypothalamic projections mediated by glutamate/NMDA receptor activation, Ca^{2+}, and nitric oxide (NO) (Ding *et al.,* 1994). The other is channeled through muscarinic cholinergic activation of another cyclic nucleotide second messenger, cGMP. Cholinergic innervation of the SCN most likely originates in the basal forebrain and pontine tegmental nuclei of the brainstem (Bina *et al.,* 1993). Both of these regions contribute to regulation of a well-defined circadian behavior, sleep. While timing of nocturnal sensitivity to both glutamate and carbachol is overlapping, the responses are opposite in early night, whereas they match in late night (Figure 8). This suggests that glutamatergic and cholinergic signaling pathways in the late night may be related through activation of NOS with concomitant production of cGMP. In contrast, the diametric directionality of phase shifts induced by GLU vs. CARB/cGMP in the early night indicates a divergence in signaling pathways downstream from NO. Further, this phenomenon suggests that the GLU-induced phase delay is independent of cGMP activation. Determining the bases for both the similarities and the differences has been the focus of intense investigation in our laboratory.

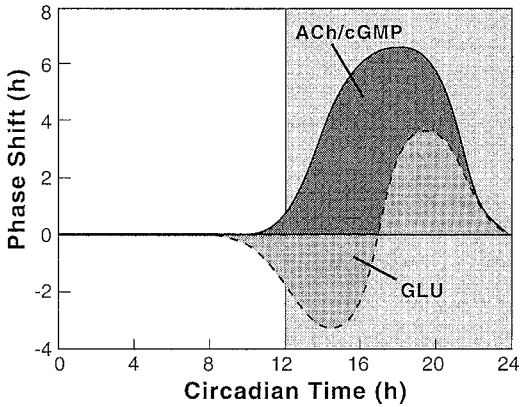

FIG. 8. Clock sensitivities to pathways stimulated by Br-cGMP and glutamate appear during subjective night. Phase-response curves for Br-cGMP and glutamate demonstrate that the timing of sensitivity to glutamate coincides with the timing of sensitivity to Br-cGMP; however, the patterns of the responses differ. Administration of the 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.

1. Light/Glutamate/NO

The night domain 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 are transmitted from the retina to the SCN via the retinohypothalamic tract. The phase-response relationship between a brief pulse of light administered to animals maintained in constant darkness and the resulting shift in the behavioral locomotor activity is curiously biphasic (Figure 9) (Summers *et al.,* 1984). Daytime light has no effect on the phase of the rhythm. With the onset of subjective night, light causes a delay in phase of subsequent rhythms. Curiously, as night proceeds, the phase-delaying effect of light wanes until, at approximately CT 17, the response of the clock switches to a phase advance. As subjective day approaches, the phase-advancing effects of light abate, completing the circadian cycle. Because this response pattern is present in constant conditions, the clock must gate both its sensitivity to the stimulus and the direction of the phase change.

The light PRC for behavioral rhythms is virtually identical to the response of the SCN to direct application of microdrops of GLU (Figure 9). The bimodal effects of GLU applied to the SCN *in vitro* can be replicated using NMDA, an agonist of the ionotropic GLU receptor, and donors of the membrane-soluble

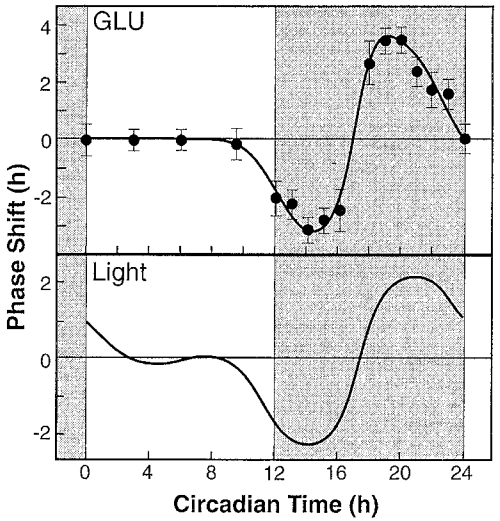

FIG. 9. The phase-response curve of the effects of GLU on SCN electrical activity *in vitro* closely resembles the PRC of the effects of light pulses on wheel-running activity *in vivo*. GLU and light cause phase delays in the early night and phase advances in the late night. [Replotted from Ding, J.M., *et al. Science* **266,** 1713–1717, 1994.]

signaling agent, nitric oxide (NO) (Figure 10). Competitive inhibitors of nitric oxide synthase (NOS), L-nitro-arginine-methyl ester (L-NAME) and L-N-arginine, block the effects of GLU both at CT 14 and 20; the inactive stereoisomer, D-NAME, is without effect (Ding *et al.,* 1994). Collectively, the data indicate that this complex, bimodal response involves common elements of a signaling pathway comprising glutamate → NMDA receptor activation → stimulation of NOS → intercellular movement of NO (Ding *et al.,* 1994).

The elements of this pathway have largely been corroborated *in vivo* (Ebling *et al.,* 1991; Rea *et al.,* 1993; Weber *et al.,* 1995b). Very localized injection of NMDA near the SCN can induce light-like phase advances (Mintz and Albers, 1997). Furthermore, intracerebroventricular injection of antagonists of the NMDA receptor (Ebling *et al.,* 1991; Rea *et al.,* 1993) or of NOS (Weber *et al.,* 1995b) blocks phase shifts of hamsters to light pulses administered at night. The concordance of both the timing of sensitivities and patterns of the bimodal responses supports the notion that these stimuli are elements in the signaling pathway leading from light to clock resetting.

That the light signaling pathway should be based upon NO seemed surprising, since scant NOS is detected in SCN by diaphorase staining. However, biochemical assays have determined that the SCN produces ample NOS activity; SCN expression is nearly equal to the cerebellum (Chen *et al.,* 1997). Further,

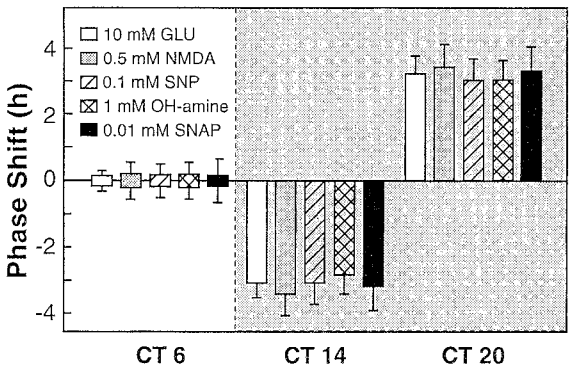

FIG. 10. SCN sensitivities to glutamate, NMDA, and NO donors (SNP, hydroxylamine, or SNAP) are temporally overlapping. Each was applied to the surface of the SCN *in vitro* for 10 minutes as a 0.2 μl drop at the concentration indicated. In each case, application midday had no effect, whereas, in early night (CT 14), each induced a phase delay and, in late night (CT 20), each induced a phase advance. Plotted are the means ± S.D. (n = 3–6 in each case). Results did not vary significantly among the treatments at each time point (unbalanced ANOVA). [Data from Ding, J.M., *et al. Science* **266,** 1713–1717, 1994.]

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 formed 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.

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 NOS (Garthwaite and Boulton, 1995; Bredt and Snyder, 1992). NOS is present in the SCN (Decker and Reuss, 1994) and 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). Furthermore, bath application of hemoglobin, which avidly binds NO, also blocks phase shifts induced by microdrops

of GLU. Because the large hemoglobin protein is only slowly imbibed by cells, its interference in a physiological process involving NO is evidence for a requirement for intercellular movement of NO to affect the response. Because the temporal sensitivity and pattern of the response to NO, which is downstream from NOS, is identical to that for light and GLU, a critical gate for reversal of the directionality of phase shifting via this pathway must lie within the cell, downstream from NO.

Studies focusing upon transcriptional activation initiated via signal transduction pathways provide additional insight as to the location of nighttime gates to phase-shifting stimuli. 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). Significantly, although light, GLU, and NO induce serine[133] phospho-CREB (P-CREB) only at night, levels of the CREB protein itself do 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.

Recently, we have discovered a significant bifurcation between the GLU signaling pathway that causes phase delays in the early night and the pathway leading to phase advances in the late night. The fact that NO donors mimic the effects of light and GLU demonstrates that the difference between a phase delay and phase advance must lie in clock-controlled gating downstream from NO. In contrast, stimulation of cGMP-dependent pathways leads to phase advances throughout the night (Figures 7 and 8), suggesting that cGMP is not involved in GLU-induced phase delays. These data are supported by evidence that light-induced phase advances *in vivo* (Weber *et al.,* 1995a; Mathur *et al.,* 1996) and GLU-induced phase advances *in vitro* (Ding *et al.,* 1998) are blocked by a specific isoquinoline inhibitor of PKG, KT5823. KT5823 does not block light- or GLU-induced phase delays nor does it affect clock phase when applied alone at either of these times. Thus, we hypothesized that cGMP is not required for phase delays and, more importantly, that gating of the directionality of the phase shift lies between NO and cGMP. Recently, we demonstrated that GLU-induced phase delays are a consequence of Ca^{2+}-induced intracellular Ca^{2+} release. Thapsigargin, which depletes $Ca^{2+}{}_i$ by blocking the Ca^{2+}-ATPase required to replenish endoplasmic reticulum $Ca^{2+}{}_i$ stores (Thastrup *et al.,* 1990), completely blocked GLU-induced phase delays but had only a small effect on GLU-induced phase advances (Ding *et al.,* 1998). These data indicate that the GLU signaling pathway leading to phase delays requires $Ca^{2+}{}_i$ release. Specifically, activation of a neuronal ryanodine receptor by caffeine, or immunosuppressive agents (FK506 or rapamycin) that stabilize the ryanodine receptor in the open position, mimics the effects of light/GLU in the early night but does not effect phase in the late night

(Ding *et al.,* 1998). Furthermore, light- and GLU-induced phase delays are blocked by the ryanodine receptor antagonist, dantrolene. Together with preliminary evidence for a circadian pattern of SCN ryanodine receptor binding, our data are the first demonstration of the mechanism behind clock-gated divergence in GLU signaling in the SCN.

2. Acetylcholine and cGMP

In contrast to the biphasic response of the SCN to nocturnal light/GLU/NO, the SCN expresses a robust, monophasic sensitivity to cGMP analogs (Prosser *et al.,* 1989) and cholinergic activation via muscarinic receptors (Liu and Gillette, 1996; Liu *et al.,* 1997) (Figure 8). Analogs of cGMP, which activate protein kinase G (PKG, the cGMP-dependent protein kinase), applied throughout most of subjective night, induce robust phase advances in clock phase; a maximal phase advance of 6.5 hours appears at CT 18. This advance is nearly twice the maximal advance induced by GLU at CT 20. The SCN is insensitive to this treatment after CT 22 and throughout the subjective daytime. Interestingly, the cGMP-sensitive period overlaps completely in amplitude and duration the window of sensitivity to phase shifting by the acetylcholine agonist, carbachol (CARB). The pharmacological profile for SCN responsiveness to CARB is consistent with activation of M_1-like receptors. Each of the following cholinergic muscarinic receptor agonists induces nocturnal phase shifts with a rank order of potency of ACh > McN-A-343 > CARB > muscarine (Liu and Gillette, 1996). These studies establish acetylcholine as a first messenger for cGMP in the SCN. Furthermore, the extensive overlap of the PRCs for cGMP and CARB indicates that access to this cholinergic pathway is gated by the clock downstream from cGMP.

M_1 receptors have been shown to couple to cGMP activation (Hu and El-Fakahany, 1993); thus, binding of the cholinergic stimulus to an M_1 muscarinic cholinergic receptor could activate the cGMP pathway in the SCN. Furthermore, it has been reported that, at CT 18, carbachol stimulates cGMP production and activates PKG within SCN in the reduced slice (Liu *et al.,* 1997). Thus, acetylcholine, which is localized in afferents to the SCN from brainstem and basal forebrain sleep structures (Bina *et al.,* 1993), is a strong candidate for the first messenger of cGMP. It is noteworthy that gating of the cholinergic response matches that of 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 within the cell, downstream from cGMP.

3. Integrated Nocturnal Responses

Both the pattern and amplitude of the responses induced by glutamate, NMDA, and NO differ from the responses to carbachol and 8-bromo cGMP (Figures 8 and 10). Yet, 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). Paradoxically, cGMP may contribute to the phase advance induced by light in that injection of KT5823, a specific PKG inhibitor, blocks light-induced phase advances but not delays *in vivo* (Weber *et al.,* 1995a; Mathur *et al.,* 1996) as well as *in vitro* (Ding *et al.,* 1998). We hypothesize, therefore, that in the pathway stimulated by light in the last half of the night, NMDA and NO may activate the cGMP/PKG pathway. However, since the phase shift induced by the light/Glu/NO pathway is smaller in amplitude than that stimulated by the cGMP/PKG pathway alone, it is likely that Glu activates additional, as yet unidentified, signaling pathways. Also, differences may lie in the source of cGMP. It is likely that NMDA receptor activation leading to cGMP elevation occurs within a specific subcellular microdomain that is isolated from the cGMP pool activated by muscarinic acetylcholine stimulation. These fundamental differences in the phase shifts induced by each of these classes of nocturnal stimuli suggest that different clock-controlled gates regulate these two pathways.

C. CREPUSCULAR DOMAINS

The regulatory domains described above cover nearly the entire 24-hour period of the circadian cycle. However, we must also consider clock gating during the day-to-night and night-to-day transitions. Because pineal melatonin secretion, driven by the SCN, is restricted to subjective night, this hormone may serve as an endocrine signal relaying timing information to the entire organism. The presence of melatonin receptors in the SCN suggests that melatonin feeds back to regulate SCN function. The duration of melatonin secretion changes in parallel with alterations in the relative durations of light and dark in the 24-hour cycle. Thus, it is not surprising that the circadian clock might be sensitive to phase resetting by melatonin during the crepuscular domains, times where information concerning altered daylength must be communicated to the organism. Melatonin affects SCN neuronal firing rates acutely in brain slices and causes entrainment of locomotory behaviors when injected into rats (Mason and Brooks, 1988; Shibata *et al.,* 1989; Armstrong, 1989; reviewed in Gillette and McArthur, 1996). Melatonin's central role as a mediator of phase shifting during crepuscular domains was recently confirmed by the demonstration that the SCN in the brain slice expresses sensitivity to phase resetting by melatonin during these transitional periods. Melatonin administration to the SCN *in vitro* advanced the phase of the neuronal activity rhythm at both subjective dusk and dawn but was without effect in day or night (Figure 11) (McArthur *et al.,* 1991,1997). Furthermore, melatonin phase resetting is mediated via G protein-linked activation of protein kinase C (Hunt *et al.,* 1995). Activation of protein kinase C is itself gated by the clock. These studies have established a functional role for SCN-driven melatonin production to feed back and modulate activity of the circadian clock.

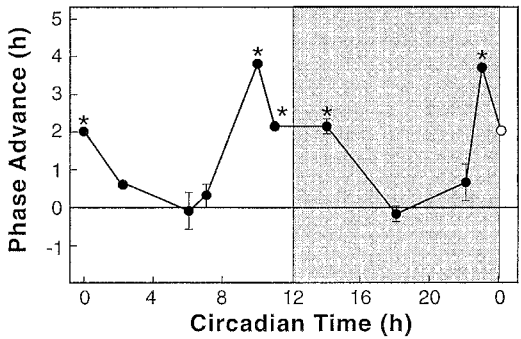

FIG. 11. The phase-response curve for melatonin bath applied *in vitro* to the SCN slice. Melatonin-induced phase advances are restricted to the dawn and dusk periods. [Data from McArthur, A.J., Hunt, A.E., and Gillette, M.U. *Endocrinology* **138,** 627–634, 1997. Copyright The Endocrine Society.]

Subjective dusk and dawn, the period surrounding the light-to-dark and dark-to-light transitions in the entraining lighting cycle, mark temporal domains during which the clock mechanism is relatively insensitive to stimulation by cAMP, cGMP, Ca^{2+} influx, or NO. While compelling evidence indicates that protein kinase C activation is an essential element in melatonin signal transduction, the gate for melatonin's effectiveness in phase shifting has not been established. Based upon our findings that gating of sensitivities to daytime and nighttime resetting stimuli is downstream from second messengers, we hypothesize that the gate for melatonin lies at a parallel level in its signaling cascade.

V. Conclusions

Together, our findings demonstrate that the generation of both a 24-hour timebase and a programmed pattern of sensitivities to phase-adjusting stimuli survive in the SCN *in vitro* and, therefore, are fundamental properties of the circadian clock. The SCN exhibits temporally restricted sensitivities to phase resetting. On the basis of these temporal restrictions, the circadian cycle can be divided into discrete temporal states, or *time domains,* of the clock: day, night, dusk, and dawn (Gillette, 1996). At any given time, the circadian clock is receptive to only a subset of the various pathways that can reset it. Gates are open to phase-shifting effects of PACAP and cAMP during the subjective daytime, while these same gates are closed during subjective night. In direct contrast, muscarinic cholinergic agonists and cGMP have access to the clock mechanism only during subjective night. The gates are open to NMDA receptor-mediated glutamate neurotransmission throughout most of subjective night. However, the signal transduction pathways accessed by glutamate are dramatically different in early night,

compared to late night. In fact, late-night glutamate-induced signaling shares many features with the muscarinic cholinergic pathway. Alternatively, Ca^{2+}-mediated intracellular Ca^{2+} release activated through a neuronal ryanodine receptor is a unique characteristic of the phase-delaying effects of glutamate. Thus, for the first time, clearly defined differences in the mechanisms leading to phase advances, compared to those regulating phase delays, have been identified. Finally, the crepuscular domains are windows of time sensitive to the effects of melatonin acting through protein kinase C. Each of these sensitivities (summarized in Figure 6) is presently defined in the context of extracellular signaling molecules in SCN afferents. Nevertheless, our findings demonstrate that gates to both daytime and nighttime clock resetting lie beyond the level of membrane receptors; they point to critical gating sites within the cell, downstream from their second messengers. The changing patterns of sensitivities *in vitro* to these second messengers demonstrate that the circadian clock controls multiple molecular gates at the intracellular level 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 the SCN's role in integration and regulation of endogenous and exogenous rhythms. Gating allows the clock to prime itself to receive exogenous signals, instilling them with temporal relevance. Accordingly, the endogenous timepiece anticipates significant exogenous cues that provide the clock with information critical for maintenance of synchrony with the changes in darkness and light inherent to daily rotations of the Earth. In this manner, the clock restricts and orders physiological and behavioral functions. Thus, by integrating exogenous and endogenous time cues, the clock synchronizes song, foraging, reproduction, and sleep into patterns adapted to each organism, to secure its niche within nature's ecosystem.

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DISCUSSION

Patrick Casey: In *Drosophila* development, there is a link between PACAP signaling and a molecule termed NF1, a GAP for Ras-like proteins. In fact, NF1 function seems to be required for PACAP signaling. Has anyone looked at NF1-knockout mice to see if circadian rhythms are compromised?

Martha Gillette: No one has examined the potential link between PACAP signaling and NF1 and GTPase-activating protein. Should this pathway be activatible in the SCN, it is likely to work via Ras mediators, PKC, and/or MAPK. I do not know of any studies on the NF1-knockout mouse that might contribute insights along these lines.

Jennie Mathers: Has anyone looked at cave-dwelling organisms to see if they maintain biological rhythms in the absence of light?

Martha Gillette: Cave-dwelling organisms don't, very well. Many cave-dwelling species have lost their eyes or are functionally blind. Some of the arthropods and fishes that have evolved in such aperiodic environments show no evidence of circadian rhythms in behavior or physiological measures (i.e., oxygen consumption) when studied under constant conditions. Some are aperiodic in light:dark cycles as well. Others follow the light:dark cycle with weak oscillations that do not persist in constant conditions. These observations can be integrated as due to loss of the central circadian clock because of lack of selective pressures to maintain it.

Stephen Marx: Your elegant model is very complex and yet very simple in many ways. As endocrinologists, we focus often on extracellular ligands. How much have you and others tested the effects of the whole "kitchen shelf" of potential ligands on these systems, including at different times?

Martha Gillette: The SCN is indeed responsive to a remarkable range of ligands. There are few substances that have been tested without observing an effect on phase. Vasopressin, a major output signal, is one exception. The range of ligands that can cause phase resetting include most classical neurotransmitters, neuromodulators, and several trophic factors. Effects of steroid hormones are presently under study. Preliminary evidence is that they may affect the acute activity state and period length. On the receptor side of the equation, an NIH data base has more receptors catalogued as expressed in the SCN than ligands that have been tested. All of this suggests that the SCN is a highly integrative signal processor!

Howard Fox: Excitotoxic cell death, mediated through glutamatergic pathways, has been proposed as a final common pathway in neuronal death for a number of neurodegenerative conditions.

First, are the neurons in the SCN sensitive to glutamate-mediated excitotoxic death, and second, are there neurodegenerative diseases in which SCN neurons are affected?

Martha Gillette: Although glutamate is the primary messenger of light at SCN neurons, this brain region is remarkably resistant to excitotoxicity. This statement derives both from *in vivo* and *in vitro* studies. While rhythms have not been assessed in all neurodegenerative diseases, there is compelling evidence that a subset of individuals with senile dementia, Alzheimer's type (SDAT), exhibit disruption of their rest-activity cycles. This has been correlated with degenerative changes in the SCN, post mortem. Although not a neurodegenerative disease, sleeping sickness has been shown to alter the physiology of SCN neurons and their response to light. It would be interesting to know whether AIDS-associated dementia affects the SCN.

Michael Young: Are the changes in cell structure in SCN that accompany exposure to constant light reversible upon transfer to constant darkness or light/dark cycles?

Martha Gillette: I do not recall.

William W. Chin: What is the nature of the neurons in the SCN? Is there a homogeneous neuron cell population? Given the heterogeneous nature of the cells in the SCN, what is the possibility that there are major paracrine/autocrine regulators of the timekeeper functions of the SCN? What is the effect of different culture/perfusion paradigms in the firing biology of the SCN hypothalamic slices?

Martha Gillette: The population of neurons in the SCN is heterogeneous with respect to neuropeptide composition, firing patterns, and morphology. While all SCN neurons appear to express GABA, they co-express one of five neuropeptides: 1) vasoactive intestinal peptide (VIP), 2) somatostatin (SS), 3) calbindin/calretinin, 4) gastrin-releasing peptide (GRP), and 5) vasopressin (VP). Yes, it is likely that there are paracrine/autocrine regulators of timekeeping within the heterogeneous cell population. The general consensus within the field is that most/all cells are clocks but they need paracrine signals to maintain synchrony. The diffusible messenger, NO, is a likely candidate for such a synchronizer. SCN slices have been studied in acute conditions with minimal salts/glucose/bicarbonate perifusion as well as in static/acute culture or organotypic culture. In each case, circadian rhythmicity of neuronal firing rate and vasopressin secretion is maintained, with the highest activities/levels measured in subjective day. This peak is independent of the time of slice preparation, as long as it occurs in daytime of the donor's cycle.

Sandra Raff: Have you had an opportunity to look at neonates and whether the pathways are intact? Are there any maturational pathway issues?

Martha Gillette: The ontogeny of rhythmicity and maternal-fetal entrainment has been extensively studied by Drs. S. Reppert and F. Davis. I refer you to their papers for insight on this interesting subject. Neonates certainly have functioning SCN clocks. There is evidence that entraining pathways change during the neonatal period, although the precise timing of sensitivities to signals meaningful in postnatal life has not yet been carefully studied.