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CHAPTER 9

Regulation of entrainment pathways by the suprachiasmatic circadian clock: sensitivities to second messengers

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Introduction

Seated deep within the hypothalamus, the suprachiasmatic nucleus (SCN) plays a central regulatory role in the daily programming of organismic functions. It regulates the day-to-day oscillations of the internal milieu and synchronizes them to changing cycles of day and night, and of body state. The temporal organization imposed by the SCN is expressed in the daily patterning of vital, homeostatic functions controlled by other hypothalamic nuclei: feeding, drinking, body temperature and neurohormone secretion each vary with a 24-h period. Temporal control extends to metabolic, physiological and behavioral functions as diverse as the timing of transcription in hepatocytes, cell divisions in peripheral organs, cellular composition of the blood, gross muscle strength, blood pressure, computational speed, sleep and wakefulness (Moore-Ede et al., 1982). The SCN adaptively organizes these bodily functions into near 24-h oscillations termed circadian rhythms.

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 SCNs endogenous, near-24-h 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, 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).

A second, more subtle, 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.

The SCN undergoes a programmed, near-24-h sequence of sensitivities to stimuli that can adjust its phase (Gillette et al., 1995). Sensitive periods correlate with discrete periods in the clock's entrained cycle: some are restricted to subjective day and others to subjective night. 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 circadian rhythm of firing rate of the ensemble of SCN neurons can be used as a bioassay to probe for gating sites. We have evaluated elements in various cell signaling pathways. 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. Gating could take place within either or both levels of signaling cascades.

We have found that clock properties predicted from in vivo studies are endogenous to the SCN: they persist when the SCN is isolated in a brain slice and maintained under constant conditions in vitro (Gillette, 1991; Gillette et al., 1995). The SCN continues to generate a stable, near - 24-h rhythm of neuronal activity for up to three cycles in vitro and this rhythm can be reset by a range of stimuli. At specific phases, or time domains, within the 24-h cycle, the circadian clock in vitro is still receptive to only a subset of the various stimuli that can reset it.

Therefore, the circadian clock regulates its own sensitivity to afferent signals. We have demonstrated that this gating is downstream from the second messengers in signaling pathways. It follows that: (1) the states of specific molecular gates regulated by the clock change over the 24-h cycle; and (2) site(s) critical to gating signaling pathways that access the clock lie within the cells of the SCN.

Brain slice preparation, extracellular recording and phase analysis methods

We study the temporal organization of the SCN of the rat in a hypothalamic brain slice preparation (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 Long Evans rats. Rats are born in our inbred colony, reared in a 12-h light/12-h dark schedule and fed ad libitum. Coronal slices of SCN-bearing hypothalamus are cut 500 μm thick at room temperature with a mechanical chopper. The one or two slices containing the SCN are immediately placed in a large-volume perifusion-interface chamber (Hatton et al., 1980). They are maintained at 37°C, bathed in 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).

Spontaneous activity of single units is sampled extracellularly by a glass microelectrode that contains 5 M NaCl (2-μm tip diameter) and is positioned by a microdrive. The firing rate of a neuron, encountered as the electrode is lowered into the slice, is monitored for stability and then counted by computer over two 2-min epochs. The mean is taken as the firing rate for that cell at the circadian time of the measurement. The electrode

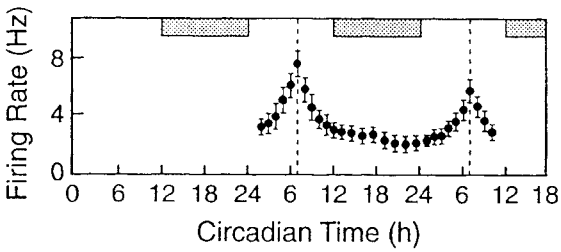

Fig. 1. The circadian rhythm of spontaneous electrical activity of the ensemble of SCN neurons in a hypothalamic brain slice from rat. Plotted (filled circles) are the 2-h means ± SEM of firing rates of SCN neurons sampled. Successive 2-h means are offset by 15 min to produce a running average; only the 1-h offsets are plotted so that individual values can be discerned. Means were derived from 124 individual neurons sampled from a single SCN over 38 h on days 2 and 3 in vitro, after preparing the slice on day 1. A horizontal bar marks subjective nighttime, CT 12–24. The peaks in the oscillations (CT 7 on both days sampled) are marked by the vertical dashed lines. This peak-time is characteristic of both untreated and media-treated SCN in brain slices.

then is repositioned so as to sample throughout the nucleus. Four to eight neurons can be sampled per hour and their mean activities calculated over a 2-h period. These data are filtered to better define the peak of activity: the mean activities of the ensemble of neurons are calculated for sequential 2-h periods, offset by 15 min and then plotted to generate the running average. From this 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 (Ding et al., 1994) (Fig. 1).

Each of the paired SCN is a 950-μm-long ovoid structure positioned near the base of the third ventricle and dorsal to the optic chiasm (van den Pol, 1980). Perhaps surprisingly, the SCN generates near 24-h oscillations in ensemble neuronal firing rate in vitro (Prosser and Gillette, 1989), like those in vivo (Inouye and Kawamura, 1979), when sectioned in a 500-μm coronal slice. This occurs despite surgical deafferentation, subdivision of the rostral-caudal components, and a lack of endocrine and trophic factors. The pattern of activity of the neuronal ensemble is sinusoidal (Gillette, 1986), with mean activity peaking near midday, at CT 7.0 ± 0.1 (Prosser and Gillette, 1989) (CT = circadian time, starting at CT 0 with 'lights on' in the colony, and continuing for 24 h, see Fig. 1). The amplitude of the mean ensemble activity ranges from 8.0 to 1.5 Hz, although a range of neuronal activities can be measured at any timepoint (Gillette, 1986). The time of peak activity of the neuronal population is stable across three cycles and predictable between these inbred animals (Prosser and Gillette, 1989), and, therefore, is a reliable marker of phase. Studies have been performed on SCN slices from outbred rats (Prosser et al., 1990) and hamsters (Margraf et al., 1991) as well; these show somewhat more variation in the time-of-peak, as would be expected from more genetically heterogeneous subjects, but they also are useful for studies of clock properties.

Time domains of sensitivities to second messengers

In addition to modulating its neuronal activity over the 24-h circadian cycle, the SCN undergoes sequential changes in sensitivities to phase adjustment by reagents affecting intracellular signaling pathways. 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 1 or 2 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. 2). 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 activ-

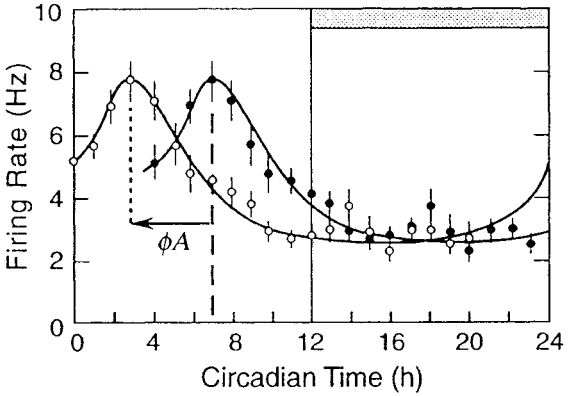

Fig. 2. Demonstration of phase advance of the SCN rhythm of neuronal firing rate. Depicted are smoothed rhythms for a single 24-h period (only 1-h offsets of the 2-h means are presented for clarity). The peak occurs at CT 7 in the unperturbed SCN (closed circles). If this SCN had been treated on the previous day with a phase-advancing treatment, such as a cAMP analog, the rhythm would peak earlier than normal on days 2 and 3 (open circles). In this example, the peak was advanced by about 4 h to CT 3.

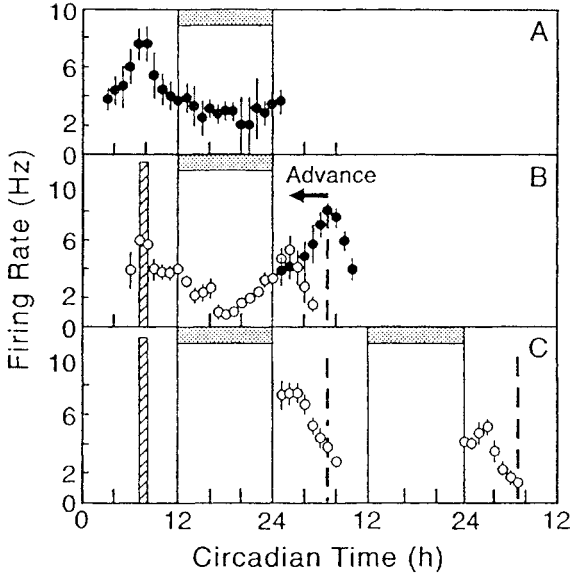

Fig. 3. The SCN rhythm was advanced by administration of an analog of cAMP during subjective daytime. (A) The unperturbed circadian rhythm of neuronal firing rate on day 1 in vitro. (B) BA-cAMP (5×10^{-4} M in EBSS) was applied between CT 7 and 8, mid-subjective day, by exchanging the bath in the brain slice chamber. On day 2, the peak (marked by the tip of the arrow) appeared 4.8 h earlier than in controls (closed circles, dashed line). (C) In another experiment, BA-cAMP was applied as in (B), and only the peaks on days 2 and 3 were defined to evaluate clock phase. Phase advances of similar amplitude were observed on both days. Horizontal bars mark periods of subjective night. Data are replotted from Gillette and Prosser (1988).

ity 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 domains that we have identified as sensitive to phase resetting via specific second messenger pathways coincide with discrete portions of the circadian cycle. 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 has identified as impinging upon this hypothalamic site (see the paper by R.Y. Moore in this volume). This permits us to speculate as to the nature of pathways 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

During the subjective day, the SCN clock expresses a well characterized sensitivity to treatments affecting pathways regulated by the ubiquitous second messenger, cAMP (Fig. 3). Application in mid-subjective daytime (CT 7) of any of a range of treatments that have a common site of action in stimulating cAMP-dependent protein kinase A (PKA) causes an advance in the phase of the rhythm of neuronal activity. PKA can be stimulated either directly, by cAMP analogs, or indirectly, by treatments raising endogenous cAMP. Effective phase shifting agents include: (1) three analogs of cAMP, 8-benzylamino cAMP (BA-cAMP) (Gillette and Prosser, 1988; Prosser and Gillette, 1989), 8-bromo cAMP (Br-cAMP) (Prosser and Gillette, 1989) and 8-chlorophenyl-

thio cAMP (CPT-cAMP) (Gillette and Prosser, 1988), each of which partitions into the cell, directly activates PKA and resists degradation; (2) forskolin (Prosser and Gillette, 1989), which directly stimulates adenylyl cyclase, resulting in enhanced synthesis of native cAMP and, thus, indirect activation of PKA; and (3) RO 20-1724 (Prosser and Gillette, 1989), which inhibits the specific 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.

This response is highly selective and temporally restrictive. The SCN clock does not respond 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 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 and Gillette, 1992a), 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 BA-cAMP and the phase-shifting response of the SCN reveals that sensitivity is restricted to subjective daytime (Fig. 4). Sensitivity to activation of the cAMP pathway first appears early in the daytime domain, between CT 2 and 3, representing 2–3 h after the initiation of the light portion of the entrained 12-h light/12-h dark cycle (Prosser and Gillette, 1989). The response rapidly peaks between CT 4 and 7, when phase advances of 4–6 h are induced by BA-cAMP. Then, it slowly wanes until phase is altered by ≤ 1 h when the cAMP analog treatment is administered at CT 11 or later into the subjective night. The range of other treatments

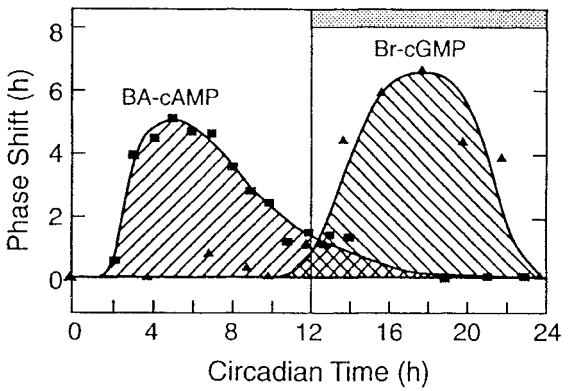

Fig. 4. Phase response curves demonstrate different temporal sensitivities of the SCN to cAMP and cGMP analogs. Each data point is the mean response derived from longitudinal recording experiments (as in panel B of Fig. 3) and represents the mean shift in phase of the SCN rhythm (in hours) in response to a 1-h 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 are replotted from Prosser et al. (1989).

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 (Fig. 3) (Prosser and Gillette, 1989). This indicates that the process leading to phase advance of the clock mechanism was completed between the time of treatment and the appearance of the next peak in activity, so that a stable new phase was 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, 1960; Daan and Pittendrigh, 1976).

The stimuli investigated in the above studies 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 h, 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 some experiments, slices were trimmed to the edges of the SCN, effectively removing non-SCN hypothalamus (Gillette and Reppert, 1987). In these reduced slices, the SCN responded to BA-cAMP application with the same amplitude shift as when the whole slice was bathed in the treatment (Prosser and Gillette, unpublished). This finding indicates that the site sensitive to the action of cAMP lies 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 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 first messenger activating the cAMP pathway is presently unproven, serotonin (5HT) is a candidate. The temporal pattern of sensitivity to serotonin applied to the SCN in microdrops partially overlaps with sensitivity to cAMP analogs (Medanic and Gillette, 1992) and the response to serotonergics requires cAMP production to affect the phase shift (Prosser et al., 1994). Further, an adenylyl cyclase-stimulating receptor, $5HT_7$, participates in the phase shifting response to treatment at CT 7 (Lovenberg et al., 1993). Thus, a first level of gating of this response could lie at 5HT receptors. Whether these membrane components vary temporally to restrict activation from the outside of the cell is unknown; indeed, mRNA for this receptor has not been detected within the SCN.

While the clock may regulate 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 does control the open-state of *intracellular* gates. Because the cAMP analogs partition through the membrane and directly activate cAMP-dependent intracellular processes, yet induce phase-resetting only in daytime, a critical gate lies at some point within the cell, 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; or (3) at substrate molecules whose phosphorylation by PKA is required to generate the phase shift. Further, given the redundancy in biological control systems, there may be multiple control points within a single pathway. The identities of these gating sites and molecules 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 of these pathways is channeled through another cyclic nucleotide second messenger, cGMP. The result of treating the SCN during the night with analogs of cGMP that activate protein kinase G (PKG, the cGMP-dependent protein kinase) is an advance in clock phase; daytime treatment is without effect (Prosser et al., 1989).

Simultaneously, the SCN also becomes sensitive to elements in a pathway stimulated by the glutamatergic agonist, *N*-methyl-D-asparate (NMDA) (Ding et al., 1994). Signal transduction through the NMDA receptor is mediated by a rise in intracellular Ca^{2+}, stimulation of nitric

oxide synthase (NOS) and production of nitric oxide (NO), a gaseous paracrine signal that freely diffuses out of the cell synthesizing it and into nearby cells (Bredt and Snyder, 1992). Thus, the primary signal mediating clock resetting for some cells may be a rise in intracellular NO from diffusional sources. When glutamate, NMDA or NO donors are applied to the SCN, the response depends on the time domain of the circadian clock: each produces a phase delay when applied early at night, a phase advance late at night, but is ineffective when applied in the daytime. Thus, within the nighttime domain, both the cGMP and Ca^{2+}/NO-activated pathways can access the clock, but the response to each differs. What is the basis of this difference?

Let us examine the cGMP-mediated phase shift in greater detail. Bath application of Br-cGMP or dibutyryl-cGMP (each at 5×10^{-4} M) induced phase advances of >1 h between CT 14 and 22 (Fig. 4) (Prosser et al., 1989). The maximal advance, $+6.5 \pm 0.2$ h ($n = 3$), occurred in response to Br-cGMP applied for 1 h between CT 18 and 19. The SCN was insensitive to this treatment after CT 22, as well as 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), is fully overlapping with the response to microdrops of the general cholinergic agonist, carbachol (100 μM applied in a 1 μl drop for 5 min). The response to carbachol shows a pharmacological profile consistent with a muscarinic response. Cholinergic muscarinic receptor agonists induce phase shifts with a rank order potency of acetylcholine > McN-A-343 > carbachol = muscarine (Liu and Gillette, 1996). Differential sensitivity to muscarinic agonists and antagonists suggests that an M_1-like muscarinic receptor mediates the effect of carbachol (Liu and Gillette, 1996).

M_1 receptors have been shown to couple to cGMP activation (Hu and Fl-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 and Gillette, 1994). 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 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 within the cell, downstream from cGMP.

A second type of clock sensitivity develops in parallel to carbachol/cGMP sensitivity. It represents the pathway by which light accesses the clock. 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 locomotory rhythms (Summers et al., 1984) is virtually identical to the pattern of the phase shifting response of the SCN neuronal activity rhythm to direct application of microdrops of glutamate (Fig. 5) (Ding et al., 1994). 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. The same bimodal response is elicited by brief application of glutamate, NMDA or NO donors to the SCN in vitro (Fig. 6, 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 $\rightarrow$ glutamate $\rightarrow$ NMDA receptor activation $\rightarrow$ Ca^{2+} influx $\rightarrow$ NOS activation $\rightarrow$ NO production $\rightarrow$ phase shift.

It must be noted that intrahypothalamic injection of glutamate lateral to the SCN in vivo did

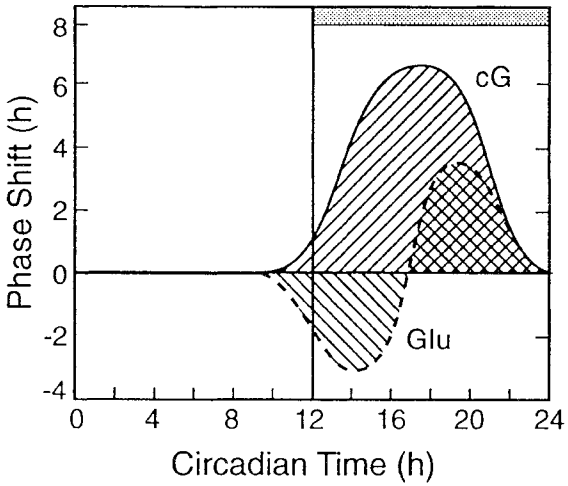

Fig. 5. Clock sensitivities to pathways stimulated by Br-cGMP and glutamate appear during subjective night. Phase response curves for Br-cGMP and glutamate demonstrate that the timing of sensitivity to glutamate coincides with the timing of sensitivity to Br-cGMP, however, the patterns of the responses differ. Administration of the cGMP analog to the SCN induces only phase advances during subjective night; maximal mean advances of 6.5 h occur in response to stimulation between CT 16 and 18. Glutamate, on the other hand, induces phase delays early during subjective night, with a maximal mean delay of 3.0 h at CT 14, and phase advances late at night, with the largest advance of 3.5 h between CT 19 and 20.

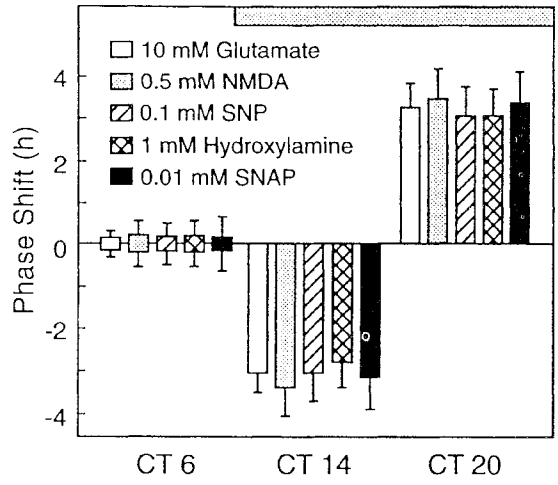

Fig. 6. SCN sensitivites to glutamate, NMDA and NO donors (SNP, hydroxylamine or SNAP) are temporally overlapping. Each was applied to the surface of the SCN in vitro for 10 min as a 0.2-μl drop at the concentration indicated. In each case, application midday had no effect, whereas in early night (CT 14) each induced a phase delay and in late night (CT 20) each induced a phase advance. Plotted are the means $\pm$ SD (n = 3–6 in each case). Results did not vary significantly among the treatments at each time point (unbalanced ANOVA). Data are from Ding et al. (1994).

not phase-shift hamster rhythms in the night, but rather in the day (Meijer et al., 1988). This is likely to be due to stimulation of another glutamatergic pathway distinct from that in the retinohypothalamic tract. Evidence for such a pathway has been reported (Kim and Dudek, 1991). Furthermore, intracerebroventricular injection of antagonists of the NMDA receptor (Ebling et al., 1991; Rea et al., 1993) or of NOS (Weber et al., 1995) blocks phase shifts of hamsters to light pulses administered at night. The concordance of both the timings 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.

This complex signaling pathway contains several points at which the clock could restrict access. Again, the argument can be made that NMDA receptors are 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) (Garthwaite and Boulton, 1995). 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., 1995), while the response to NO changes in a pattern like that to light and glutamate (Ding et al., 1994).

A role for NOS activation in the pathway by which glutamate stimulates nocturnal phase de-

lays and advances has been demonstrated in the SCN: competitive inhibitors of NOS, L-nitro-arginine-methyl ester (L-NAME) and L-*N*-arginine, block the effects of glutamate both at CT 14 and 20; the inactive stereoisomer, D-NAME, is without effect (Ding et al., 1994). Furthermore, bath-application of hemoglobin, which avidly binds NO, also blocks phase-shifts induced by microdrops of glutamate. 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. NO donors, such as sodium nitroprusside (SNP), *S*-nitroso-*N*-acetyl-penicillamine (SNAP) and hydroxylamine, each produce the same result as glutamate and NMDA: no phase shift is observed after treatment in the daytime at CT 7, phase delay results after treatment at CT 14 and phase advance follows when they are applied at CT 20 (Fig. 6) (Ding et al., 1994). Because this temporal sensitivity and pattern of the response to NO, which is downstream from NOS, appears 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 to phase shifting stimuli 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). Significantly, although light, glutamate 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., 1995). Therefore, a gate to nocturnal phase shifting in response to light must lie between NO and CREB.

Both the pattern and amplitude of the responses induced by glutamate, NMDA and NO differ from the responses to carbachol and 8-bromo cGMP (Figs. 5 and 6). 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 (Weber et al., 1995; Mathur et al., 1996). We hypothesize, therefore, that the pathway stimulated by light, NMDA and NO may partially activate the cGMP/PKG pathway. However, since the phase shift induced by the light/glutamate/NO pathway is smaller in amplitude than that stimulated by the cGMP/PKG pathway alone, it is likely to activate additional, as yet unidentified, signaling pathways. Mediators of the delay portion of the response are unknown, but critical to identify. Their gating sites can be projected to lie within an intracellular signaling pathway at a level near those by which cGMP responses are gated. These fundamental differences in the phase shifts induced by each of these classes of nocturnal stimuli suggests that different clock-controlled gates regulate these two pathways.

Other domains

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. The late day/early night period, from CT 10 to 14, is characterized by a well defined window of sensitivity to the pineal hormone melatonin (McArthur et al., 1991). Bath application of 1×10^{-9} M melatonin, for 10 or 60 min to the SCN in vitro results in a phase advance of 3.8 ± 0.1 h ($n = 6$) (Hunt et al., 1995). Preliminary data suggest that at dawn, too, the SCN may express a sensitivity to melatonin (McArthur and Gillette, 1992b). The sensitivity of the SCN to this first messenger has been widely

demonstrated both in vivo and in vitro (Gillette and McArthur, 1996). The mechanism by which melatonin's effectiveness in phase shifting 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 a gate for melatonin lies at a parallel level in its signaling cascade.

4. Conclusions

Together, these findings demonstrate that the generation of both a 24-h time base 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: agents that selectively stimulate cAMP are effective in the subjective daytime, while those that activate cGMP or Ca^{2+}/NO pathways are effective at night (summarized in Fig. 7). These sensitivities are presently being defined in the contexts 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 lying 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 SCNs integrative and regulatory role for hypothalamic control of organismic rhythms.

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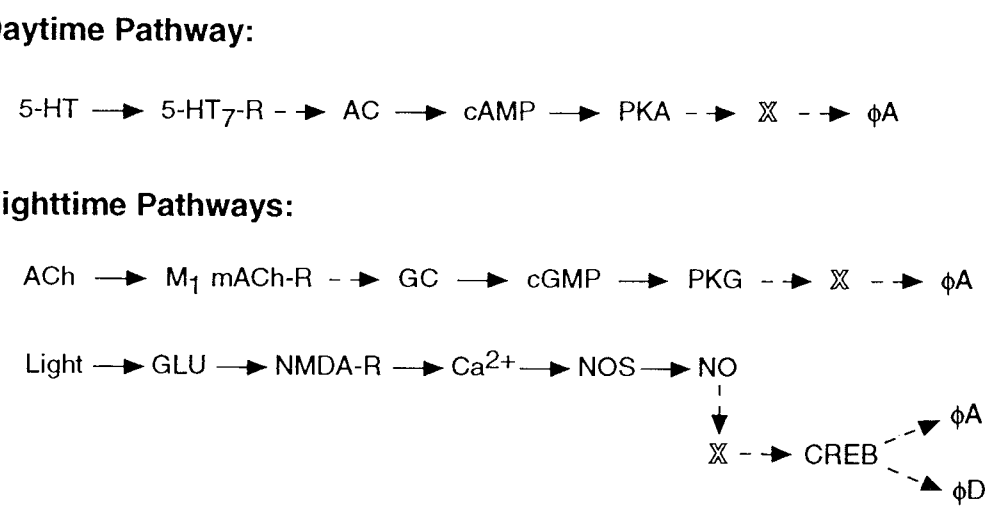

Fig. 7. Summary of signaling pathways that we believe can access the clock in daytime vs. nighttime. Dashed arrows indicate points with an unknown number of steps. Relative position of a critical gating site in each pathway is designated by an —×—.

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