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Chapter 6

SCN ELECTROPHYSIOLOGY IN VITRO: RHYTHMIC ACTIVITY AND ENDOGENOUS CLOCK PROPERTIES

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Accumulated evidence from a wide range of in vivo studies over the last 30 years supports a role for the suprachiasmatic nucleus (SCN) as the primary circadian clock in mammals. These studies indicate that the SCN generates a long period oscillation that is close to but significantly different from 24 hours and that the clock mechanism undergoes circadian changes in its sensitivity to resetting stimuli. Abolition of circadian behavioral rhythms by lesion of the SCN and persistence of rhythms only within the SCN region when it is semi-isolated by knife cuts suggest that all elements of the circadian pacemaker may be intrinsic to this region. This conjecture cannot be proven from these results, however, because of the complex environment in vivo.

Over the last 7 years the brain slice technique has enabled researchers to remove the putative clock from the central nervous system and to examine it in isolation. This technical development enabled rapid progress in determining endogenous clock properties of the cluster of neurons and glia that comprise the SCN. The in vitro approach measures clock activity differently from in vivo studies: rather than marking phase at the time of onset in behavioral activity rhythms, the time of the peak in electrical activity of the ensemble of SCN neurons or in vasopressin secretion is determined. Thus, in vitro studies simplify the complexity of the "black box" that is the clock in studies in vivo by measuring primary outputs of the SCN without interference from afferents or feedback loops from other brain regions.

Study of the SCN in vitro enables the researcher to determine which of the properties attributed to it from in vivo studies are truly endogenous; that is, generated within the SCN. In addition, this approach allows us to dissect the intrinsic organization of the SCN, the physiology and biochemistry of the time-keeping mechanism, and SCN regulation by neurotransmitters and modulators. However, in order to use this approach successfully to understand the clock, one should first account for clock characteristics that have been deduced from animal studies. That is, a spontaneous, near 24-hour oscillation should persist in vitro, and the SCN brain slice ought to demonstrate an endogenous circadian change in sensitivity to resetting.

This field is not quite yet at the threshold of understanding; however, significant progress has been made along each of these lines of investigation. This chapter presents (1) the historical and methodological context of electrophysiological study

of rhythmic SCN activity, (2) rhythmic electrophysiological characteristics of the SCN in vitro, (3) the intrinsic functional organization of the SCN clock, (4) endogenous clock properties determined from in vitro studies, and (5) future directions in this field.

HISTORICAL AND METHODOLOGICAL CONTEXT OF THE ELECTROPHYSIOLOGICAL STUDY OF RHYTHMIC ACTIVITY

Localization of a Circadian Clock in the SCN In Vivo

The existence of an endogenous biological clock that organizes mammalian behaviors and physiology around the daily solar cycle of light and darkness has been difficult to prove. Circadian behaviors driven by clocks are manifestly similar to environmentally driven changes in behavior. However, in 1960 DeCoursey reported that the activity rhythm of the flying squirrel continues in constant darkness with remarkable regularity such that the free-running rhythm differs from the 24-hour solar cycle by a small but predictable degree. DeCoursey's observation (1960*a*) was the first of many that have supported the notion that circadian rhythms of mammalian behavior and physiology are driven by an endogenous clock. Circadian behavioral rhythms of nearly all species have been found to continue under constant conditions with a characteristic period τ (tau) that differs significantly from 24 hours (reviewed by Moore-Ede et al. 1982).

Richter reported in 1967 that ablation of the ventromedial hypothalamus eliminated multiple behavioral rhythms. This finding implicated a brain region as a site for such a clock. Selective lesioning of discrete brain loci in the early 1970s identified the suprachiasmatic nuclei (SCN) at the base of the hypothalamus as critical to organizing rhythms of behavior (Stephan and Zucker 1972) and neurosecretion (Moore and Eichler 1972) around the period of the day. Because loss of this region has no effect on the execution of the events themselves, the SCN region must provide timing information only. This temporal organizer appears to permit execution of behavior at some periods of the day but not at others. The phasing of its time is fixed by the ambient cycle of light and dark (DeCoursey 1960*b*.)

An elegant study by Inouye and Kawamura in 1979 demonstrated that SCN neurons generate a circadian rhythm of electrical activity in situ. Freely behaving rats were implanted with electrodes that measured the summed activity of roughly 1000 neurons at the base of the 200-μm tungsten electrode, comparing that with activity summed adjacent to but outside the SCN. This differential multiunit activity (MUA) demonstrated a circadian pattern of rhythmic activity that was high in the day and low at night. Circadian rhythms of MUA also were measured in nearby brain regions: these rhythms were in antiphase to that within the SCN. When the SCN were nearly isolated from the brain by dorsolateral knife cuts, the pattern continued within the SCN region; however, it was abolished in regions outside the cut.

Although neither rhythmic humoral nor diffusible factors nor remaining neural connections could be excluded as affecting the SCN from without, Inouye and Kawamura's work was the first nondeletion study to provide strong evidence for a time-keeping mechanism within the SCN. Because the MUA in other brain regions was 180° out-of-phase to the SCN pattern until the intervening knife cut abolished it, electrical signals carried by SCN efferents must provide the timing information, probably inhibitory in these nocturnal rodents, to the other brain regions.

Development of the SCN Brain Slice

The above studies pointed to the possible central importance of the SCN in the generation of mammalian circadian rhythms. This hypothesis could be tested if the SCN could be studied in isolation. Thus, the advent of the brain slice technique offered unusual potential. Central nervous system tissue had been found to survive up to about 6 minutes of hypoxia without mea-

surable damage (Yamamato 1972). This allowed the investigator adequate time to dissect out the brain region of interest in a tissue slice not greater than 650 μm thick (Alger et al. 1984) and to place it in a life-support system where its endogenous properties could be monitored in comparative isolation from afferents. Illustrations of the morphology of the hypothalamic brain slice and the brain slice chamber currently used in our laboratory to study circadian rhythms of SCN electrical activity appear in Figure 6-1.

In 1982 three laboratories independently published papers using electrical signals produced by SCN in brain slices to address the nature of endogenous SCN activity (Green and Gillette 1982, Groos and Hendricks 1982, Shibata et al. 1982). All three used a coronal slice, 300–500 μm thick, of the hypothalamic region containing the SCN. The amount of extra-SCN hypothalamic tissue included varied in the three studies. Although the precise amount of extra-SCN tissue in each slice was not detailed, it was significantly more extensive than that in Figure 6-1 and included at least the paraventricular and supraoptic nuclei. Each group maintained slices at 37° C at the interface of a glucose/bicarbonate-supplemented salt solution of slightly different composition and flow rate, exposed to a humidified atmosphere of 95% O_2/5% CO_2. All measured extracellular activity of single neuronal units, although the region of SCN as well as neuronal type sampled differed.

Each group formulated their experiments slightly differently. Shibata, Oomura, and their co-workers at Kyushu University initiated experiments at six points evenly spaced throughout the 24-hour day. For a single experiment they prepared hypothalamic slices from rats at one of these times, held the slice for 1 hour then recorded for an 8-hour session during which each cell was monitored for at least 1 hour. Recordings from 134 neurons in the ventrolateral SCN area, the results of at least 17 experiments initiated by sacrifice at six different points, were plotted together. The 2-hour grand means of neuronal firing rate showed significant difference between midday and midnight. The resulting day-night difference in electrical activity of individual cells from the ventrolateral region reflects the day-night difference recorded for MUA in vivo, with

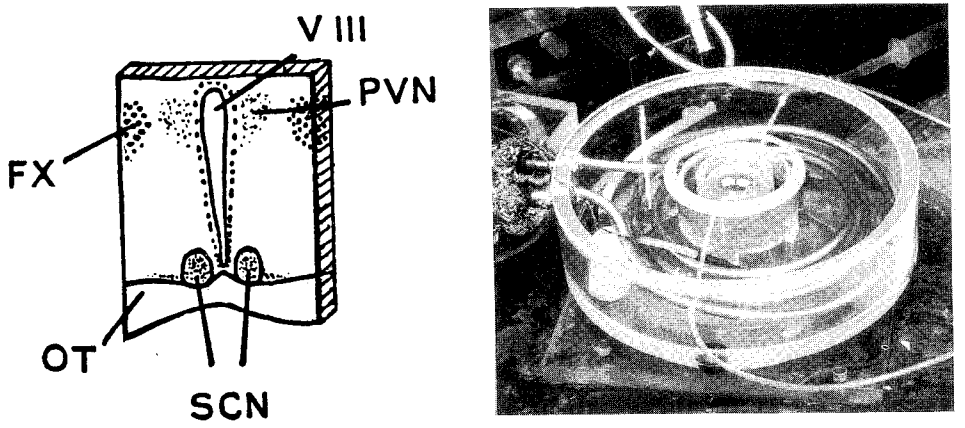

Figure 6-1. Left, Morphology of a coronal hypothalamic slice containing the SCN, as well as the optic chiasm (OT), paraventricular nucleus (PVN), fornix (FX), and third ventricle (VIII). **Right,** Brain slice chamber currently used in our laboratory for electrical recording (for details, *see* Hatton et al. 1980). Brain slices (*small squares within the inner circle*) rest upon mesh within the inner well at the interface of Earle's balanced salt solution (supplemented with glucose and bicarbonate, pH 7.4 at 37°C, and perifused at 34 ml/h) and a humid atmosphere of 95% O_2:5% CO_2 bubbled through the outer well of heated H_2O.

higher activity in slices prepared during the day than the night. Some single cells monitored for as long as 4 hours were observed to undergo large changes in firing rate, although not always in concert with the changing level of grand mean activity. These results suggest that the spontaneous activity levels recorded in vivo are sustained in the ventrolateral SCN in the brain slice; however, the recording periods were not sufficiently long to demonstrate that the SCN can generate a circadian rhythm of electrical activity in vitro.

Green and R. Gillette, at the University of Illinois, succeeded in recording single units in rat SCN in a single slice over 24 hours. The sampling procedure differed from the other two studies in that all cells that could be clearly discriminated as single units were counted, recordings were made at random throughout the SCN, and units were counted for relatively brief periods (~2 minutes) and averaged in order to assess the population activity; if sampling were truly random, this procedure should produce a pattern of activity similar to MUA in vivo.

Two-hour means of the population of single unit firing rates showed a robust rhythm of activity, the major periodicity of which was 24 hours. Activity peaked in the early afternoon of the donor's day; the trough, roughly one-third the amplitude of the peak, occurred near midnight. This pattern appeared whether the experiment was initiated in the morning or evening and even to the extent that SCN from animals whose lighting cycles were 12 hours out-of-phase maintained their respective phases when co-incubated during the recording session. This finding demonstrated that the SCN is capable of maintaining a near 24-hour rhythm of single unit firing rate in vitro that matches the pattern of MUA in vivo. The phase of the oscillation appeared to be determined by the prior lighting cycle of the donor, rather than the point in the circadian cycle at which the experiment was initiated.

Groos and Hendriks, working at the University of Leiden, The Netherlands, reported a similar result for slices studied over a 6- to 30-hour recording period. This study analyzed only the ventral and medial SCN. Only cells exhibiting a random discharge pattern over 10–120 minutes, excluding beating and bursting cells, were counted. These SCN cells showed a high amplitude oscillation in discharge rate over the circadian cycle, whereas those of the arcuate hypothalamus and retrochiasmatic area exhibited no circadian pattern of activity in vitro. The finding of Green and Gillette—that the SCN could maintain an oscillation in vitro—was confirmed for the ventromedial subregion. Further, this oscillation in firing rate of the ensemble of SCN neurons was correlated with changes over the course of the day in the firing rates of individual neurons recorded for as long as 14.3 hours, thereby corroborating data of the Shibata group. Thus, each of these three studies complemented the others and established firmly the feasibility of studying the SCN clock in vitro.

RHYTHMIC ELECTROPHYSIOLOGICAL CHARACTERISTICS OF SCN IN VITRO

Methods of Measurements of Rhythmic Electrical Activity In Vitro

The experiments reported in the remainder of this chapter come primarily from my laboratory. In these studies, slices were made from 2- to 5-month-old Long-Evans rats that had been inbred in our laboratory over the last 7 years to reduce individual differences. The donors were reared on a 12-hour light : 12-hour dark cycle. Any experimental lighting cycle different from that of the colony was maintained for at least 3 weeks before sacrifice.

Slices 500 μm thick and containing the medial SCN region are made on a mechanical chopper. The Earle's balanced salt solution that perifuses the slices is similar in composition to that used by other laboratories except for its higher glucose concentration (24.6 mM). Slices are maintained in constant environmental conditions, including constant cool light transmitted by fiber optics from a tungsten source.

We use a sampling technique similar to that of Green and Gillette (1982). It is

based upon rapid sampling of single units to determine the neuronal firing rate pattern of the ensemble of SCN neurons, rather than the long-term activity of a single cell. Stable recordings of multiunit activity are difficult to achieve in vitro (Gillette, unpublished observations), primarily because the slice is suspended on mesh at the interface of the perifused medium. Random sampling throughout the SCN at a rate that is rapid enough to determine the representative behavior of the population while accurately assessing the firing characteristics of each neuron sampled in the slice should generate an oscillation very similar to that measured by Inouye and Kawamura (1979) with the large multiunit electrode in vivo.

We sample extracellular activity with a glass microelectrode pulled to a tip diameter of 2–4 μm and filled with 5 M NaCl. The electrode is advanced slowly by a hydraulic microdrive so it can detect any cell at a distance and will not mechanically stimulate the cell. After a 2-minute period of observation to determine the firing pattern and the stability of the electrode's relationship to the cell, a computer makes two 2-minute counts of activity in 10-second bins, which enables us to assess both the acute firing pattern and rate. The electrode is then moved deeper along the electrode track until another unit is detected. After measuring all cells encountered during that pass through the slice, the electrode is moved at random to a new position within the SCN visible in the fresh tissue of the slice, and the sampling process is begun again. Data are averaged over 2 hours and then plotted as the 1-hour running average.

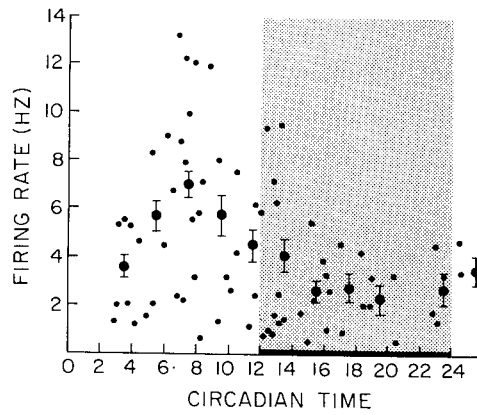

Figure 6-2. SCN neuronal activity during the first 24 hours in vitro. Spontaneous activities of individual neurons randomly encountered and recorded extracellularly (*small dots*). Two-hour means of single unit activities recorded in a single SCN over a circadian cycle ± SEM (*large dots*). Slices were maintained in constant light. Shading distinguishes subjective night (the dark phase of the donor's lighting cycle) from day.

Circadian Patterns of Neuronal Activity In Vitro

When the range of spontaneous activities in individual neurons is examined over the course of the 24-hour cycle in vitro, a much greater range is seen during the subjective day than the subjective night (small dots, Fig. 6-2). The subjective day—the period of "lights on" in the donor's lighting cycle—is characterized by very rapidly firing units, with activities up to 14 Hz and occasionally higher. Very slow units with activities below 1 Hz are observed, as well as a range of intermediate activities. It is the predominance of fast-firing neurons during midday that accounts for the rise in mean activity. Both Shibata et al. (1982) and Groos and Hendricks (1982), who followed individual units for as long as 14 hours, observed neuronal firing rates to rise or fall by as much as threefold during the day, although the direction of change did not correlate absolutely with the direction of change in the population mean activity.

During the subjective night, in contrast, activity is generally low. Units with activities greater than 5 Hz are rare, and most activity is in the 0.5–3.0 Hz range. Additionally, the number of silent units increases; fewer spontaneously active units are sampled per hour at night because they become difficult to find (Gillette, unpublished observations.)

The ensemble single unit firing rate is determined by taking the 2-hour means of all single units sampled during that interval. The pattern of the ensemble firing rate for a

single SCN in vitro sampled over 24 hours (large dots, Fig. 6-2) is very similar to the pattern of multiunit activity measured in vivo by Inouye and Kawamura (1979, 1982). The mean firing rate for the ensemble of neurons oscillates in a circadian pattern. This near 24-hour oscillation is sinusoidal in form: activity is high in the donor's day, peaking at 7–8 Hz near circadian time 7 (CT 7, where circadian time begins at lights-on in the entrained lighting cycle of the slice donor and continues for 24 hours before starting over), and is low at night with a trough near CT 19. The peak is relatively sharp so that the time of the symmetrically highest point in the curve is identified easily. Because the peak is so well defined under these conditions, we use the time-of-peak to mark the phase of the circadian oscillation in ensemble firing rate. Thus, the time-of-peak can be used to measure stability or perturbation of the oscillation with time or treatment.

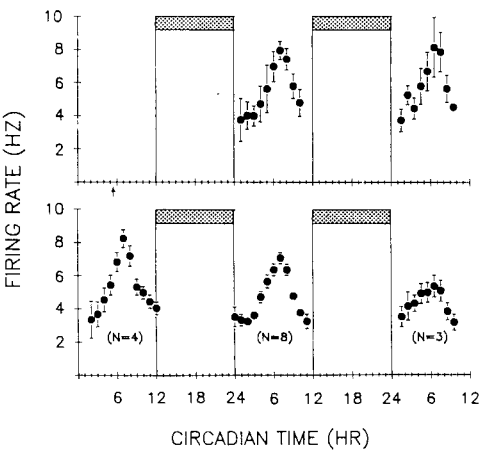

Figure 6-3. Circadian patterns of ensemble neuronal firing rate over three cycles in vitro. Recording was performed only to define the time-of-peak activity. Two-hour means ± SEM of all single units recorded are plotted with 1-hour lags. **Top panel,** Recording from a single SCN on day 2 (peak = CT 7.0) and day 3 (peak = CT 6.5). **Bottom panel,** Grand means for SCN recorded on successive days showing the stability of the time-of-peak (*see* Table 6-1 for CTs of peaks.) N, number of experiments averaged for each day's mean; horizontal bar, subjective night; arrow, time of slice preparation. (Adapted from Prosser and Gillette 1989*a*.)

Stability of the Peak in the Electrical Activity Rhythm

The time of peak electrical activity in vitro shows little variation between animals from our inbred strain of rats. During the first 24-hour period in vitro, ensemble firing rate peaks at CT 7.1 ± 0.1 hr (n = 4) in SCN from 2 month-old rats (Prosser and Gillette 1989*a*). The low degree of variability in the time-of-peak between animals from our colony enables us to use the peak as a reliable measure of phase of the pacemaker or clock within the SCN.

When slices are maintained for longer periods, up to 72 hours in vitro in the presence of antibiotic, electrical activity continues with a robust rhythm for at least three cycles (Fig. 6-3, Prosser and Gillette 1989*a*). (We have not yet studied SCN maintained in vitro for longer periods.) The time-of-peak is remarkably stable over this period of time in vitro, showing no significant deviation from 24 hours (Table 6-1). When the time-of-peak on day 1 and day 3 are compared, the data suggest that the free-running period under these conditions is slightly shorter than 24 hours. The fact that the 500-μm brain slice contains less than the entire anteroposterior extent of the SCN and that the perifusion medium contains only glucose/bicarbonate-supplemented salts may contribute to this result. Nevertheless, the ability of SCN to generate stable, robust circadian rhythms of neuronal firing rate while maintained for 3 days in vitro in this minimal medium of glucose and salts emphasizes the endogenous nature of the SCN clock. Additionally, it enables us to investigate resetting

Table 6-1. Daily Time-of-Peak in the Rhythm of Neuronal Firing Rate In Vitro*

Day 1	Day 2	Day 3
CT 7.1 ± 0.1 (n = 4)	CT 6.9 ± 0.2 (n = 8)	CT 6.6 ± 0.4 (n = 3)

* From Prosser and Gillette, 1989*a*.

properties of the pacemaker using this preparation.

Effect of the Time of Slice Preparation

Timing of the peak is independent of the time of slice preparation, as long as it occurs during the donor's day (Gillette 1985). When slices are made at various times over the entrained light period of the donor's light-dark cycle, the rhythm picks up at a point predicted from our other experiments and continues the circadian pattern until units are no longer detected. The pattern and timing of the peak are determined by the lighting cycle of the donor. When compared in terms of circadian time, which reckons time according to the 24 hour lighting cycle, they are the same for SCN from donors from the parent colony (lights on 7 a.m.–7 p.m.) and those housed in separate care facilities with a lighting cycle reversed from that of the parent colony (Gillette and Reppert 1987).

Although timing of this oscillation is uniform for slices prepared at various times during the donor's day, it is altered in a predictable way by sacrifice and slice preparation during the night. This change can be seen in the phase-response relationship in Figure 6-4, in which the circadian time of these manipulations is plotted against the response of the clock, measured as the change in the time-of-peak (Gillette 1986). During the donor's night, the timing of the peak is shifted in a stimulus-response relationship very like that between the time of light pulses to the eyes (DeCoursey 1964) or of direct electrical stimulation to the SCN (Rusak and Groos 1982) and the effect on rodent behavioral rhythms. The difference is that large amplitude shifts in the time of peak electrical activity (up to 5 hours) are observed in vitro in the first cycle after slice preparation at night (Fig. 6-4). The shifts in behavior induced by stimulation in vivo are of less than half this amplitude after the first circadian cycle (DeCoursey 1964); large amplitude shifts take several days to develop (Rusak and Groos 1982).

The SCN is exposed to several potential phase-shifting stimuli during slice preparation, including stimulation of optic nerve afferents, both by light and cutting, and elevated extracellular K^+ caused by damage and activity during the slicing procedure. Nevertheless, these stimuli are only effective in resetting the SCN clock when they occur during the entrained night period. Thus, the clock is able to respond in vitro to a resetting stimulus experienced at the time of slice preparation in a manner predicted from in vivo studies.

Surprisingly, the shift that occurs appears larger in amplitude and more rapid in its development than would have been anticipated from in vivo studies (Fig. 6-4). This observation suggests that feedback circuits from other CNS sites normally

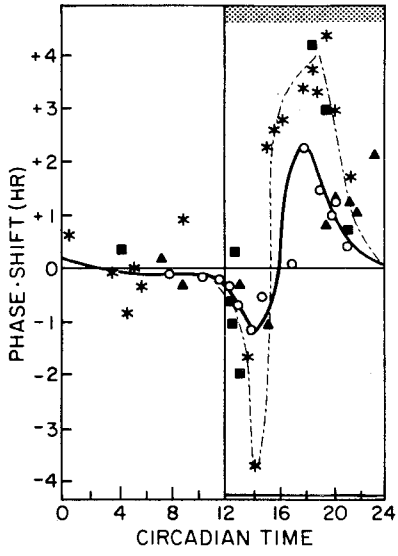

Figure 6-4. Phase-response relationship between the time at which the donor was sacrificed and the brain slice prepared versus the time of the peak in the next cycle of SCN neuronal firing rate in vitro (*). The *dashed line* is the predicted phase-response curve. This phase-response curve is similar to that of DeCoursey (1964) for the effect of a 10-minute light pulse on the next cycle of wheel running (*circles, solid line*), as well as the data of Rusak and Groos (1982) for the effect of direct electrical stimulation of the SCN on equilibrated behavioral rhythms (*squares, triangles*). Shaded bar designates subjective night for all studies. (Adapted from Gillette 1986.)

may damp phase-shifting responses in the SCN. Brain regions that oscillate out-of-phase from the SCN due to inhibitory inputs from the SCN (Inouye and Kawamura 1979) would be effective sources of feedback. The SCN in the hypothalamic slice has been removed from these inertial forces and shifts rapidly, perhaps immediately (as predicted by Pittendrigh 1974 and *see* below).

This study demonstrated that the effect of a resetting stimulus that occurs at the time of slice preparation is preserved in vitro. The concordance between the neuronal phase-response curve (for the effects of time of slice preparation on the peak in the SCN electrical activity rhythm in vitro) and the behavioral phase-response curve (for the effect of light or SCN stimulation in intact animals on the time of onset of the activity rhythms) suggests that the mechanism of readjustment of the clock mechanism is fundamentally the same. This particular study suggested that the SCN brain slice could be used for study of the resetting mechanism, and it emphasized the importance of initiating in vitro experiments during the entrained day.

Relationship Between Rhythmic Electrical Activity and Vasopressin Secretion

The electrical activity rhythm must be primarily an output of the time-keeping mechanism. Possibly this rhythm drives the rhythms in MUA in other brain regions measured by Inouye and Kawamura (1979), which in turn could control behavioral rhythms. An additional consideration, first raised by Reppert and his coworkers in 1981, has been whether the rhythm of vasopressin measured in the cerebrospinal fluid is also an output of the SCN. Vasopressin is the major peptidergic component of the SCN (Vandesande et al. 1975; *see* Chapter 9), and integrity of the SCN is essential for the circadian rhythmic pattern of vasopressin in the cerebrospinal fluid (Schwartz and Reppert 1985).

To examine whether the SCN releases vasopressin in a rhythmic pattern and how this rhythm might relate to the rhythm of neuronal firing rate, we measured both rhythms simultaneously in vitro (Gillette and Reppert 1987). The glucose-supplemented salt solution perifused through the brain slice chamber was collected for vasopressin analysis by radioimmunoassay while the ongoing electrical activity of incubated SCN was recorded. The results show that SCN in a brain slice from which other vasopressinergic nuclei have been removed does secrete vasopressin in a circadian rhythmic pattern (Fig. 6-5A), corroborating results measuring vasopressin release from an SCN explant cultured in enriched medium (Earnest and Sladek 1986, 1987). The pattern resembles that for vasopressin measured by Reppert et al. (1981) in the cerebrospinal fluid in freely behaving rats (Fig. 6-5C), suggesting that the SCN might be a source of vasopressin.

When the vasopressin pattern (Fig. 6-5B) is compared with the electrical activity pattern for the same experiment (Fig. 6-5A), the peak in vasopressin release precedes the peak in electrical activity by roughly 2 hours. Because vasopressin release falls sharply after it peaks, the relative amount of vasopressin released has dropped to the daily mean level at the time of the peak in neuronal firing and within 3 hours reaches its daily nadir. This daily low in vasopressin secretion precedes the trough in the electrical activity rhythm by 6–8 hours.

Although the number of experiments reported in Gillette and Reppert (1987) was small, displacement of the peaks and troughs between the rhythms of vasopressin release and electrical activity was consistent. This suggests that the SCN clock in some way times these two putative outputs separately. Each may represent a separate mode by which the SCN transmits temporal information to other brain regions—one by specialized circuits and the other by neuromodulatory effects on regions bathed by cerebrospinal fluid. How they might be coupled to the SCN time-keeping mechanism is unknown. These results suggest further that the rapidly firing neurons, usually tonic in their activity pattern (Gillette, unpublished observa-

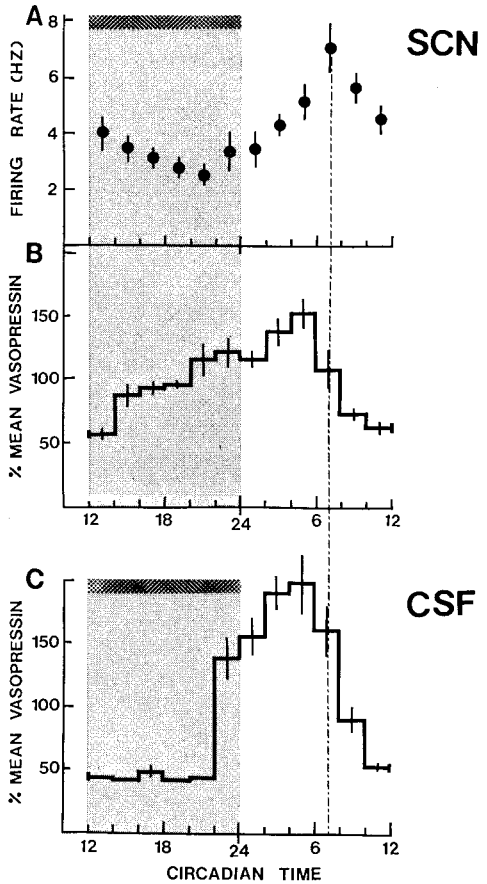

Figure 6-5. Population profiles of circadian patterns of firing rate (**A**) and vasopressin release (**B**) from SCN slices with PVN removed. Each value is the 2-hour mean ± SEM of all samples at each point. **C**, Vasopressin levels in cerebrospinal fluid sampled from freely behaving Long-Evans rats (replotted from Schwartz et al. 1983). Time-of-peak in the rhythm of firing rate is marked by the *dashed line*. Slices were maintained in constant light; subjective night is designated by *shaded horizontal bar*. (Adapted from Gillette and Reppert 1987.)

tions), that characterize the peak in the ensemble neuronal firing rhythm do not contribute to the peak in vasopressin secretion. By analogy with vasopressinergic neurons from other hypothalamic regions (Cobbett et al. 1986), SCN vasopressin neurons might be intense periodic bursters.

Day-Night Differences in Acute Responses of SCN Neurons In Vitro to Neurotransmitters/Modulators

The diversity of neuronal firing rates at any one time (Fig. 6-2) and of neuroactive substances and receptors localized to the SCN (*see* Chapters 2 and 3) suggests that intercellular communication plays a critical role within the SCN. If the action of neurotransmitters and modulators is related to the circadian rhythmic function of the SCN, circadian change in responsiveness or concentration might be expected. Although the responsiveness of SCN neurons to numerous neuropharmacological substances has been documented (Liou et al. 1983, 1986*b*, Meijer and Groos 1988, Nishino and Koizumi 1977, Shibata et al. 1983*a*,*b*), little is known about how responsiveness relates to the phase of the circadian cycle. Those studies that examined circadian variation in transmitter/modulator sensitivity in the SCN are reviewed here.

Five neuroactive substances have been examined for circadian changes in SCN neuronal responsiveness: 5-hydroxytryptamine (serotonin), γ-amino butyric acid (GABA), neuropeptide Y, vasopressin, and melatonin. Most studies compared few time points; therefore, the reported lack of circadian change in sensitivity cannot be accepted as fact, and further studies are needed to substantiate this claim fully. In a study of anesthetized rats throughout the light-dark cycle, single units recorded extracellularly responded to iontophoretically applied 5-hydroxytryptamine with a two- to threefold increased sensitivity during the subjective dark phase (Mason 1986). In contrast, no circadian variation in response to iontophoresed GABA was detected between the day and night points examined.

Daytime application of neuropeptide Y to SCN neurons of hamster in vitro is excitatory (Mason et al. 1987); in the rat it is either excitatory or transiently excitatory and then inhibitory (Shibata and Moore 1988). At night fewer neuropeptide Y-responsive cells were recorded, and both

acute and prolonged exposure had no effect on firing rate. This differs from the excitatory response to vasopressin, which is the same in day and night (Shibata and Moore 1988).

The pineal hormone melatonin, applied in vitro either topically (Mason and Brooks 1988) or by superfusion (Shibata et al. 1989), caused an abrupt, reversible decrease in spontaneous activity of SCN neurons from rat. This inhibitory effect was limited to the late subjective day, roughly CT 9-14; at other times, no response was observed.

The relationship between these circadian differences in responsiveness to neurotransmitters and modulators and the variety of firing rates observed over the circadian cycle (Fig. 6-2) is unknown. In fact, the short incubation periods in the above studies of SCN in vitro do not permit us to determine whether the changes in responsiveness are endogenous or were driven by afferents before dissection. Interestingly, neuropeptide Y (Albers et al. 1984) and melatonin (Cassone et al. 1986) have been implicated in phase shifting from in vivo studies, whereas vasopressin has proven ineffective (Albers et al. 1984.) Mechanisms underlying differential responsiveness, as well as the transduction mechanisms by which ligand binding might contribute to the clock procession or resetting, will need to be elucidated as our understanding of integrated SCN function grows.

INTRINSIC FUNCTIONAL ORGANIZATION OF THE SCN CLOCK

The body of research discussed above argues against the possibility that the SCN is composed of a homogeneous class of pacemaker cells firing in synchrony. These electrophysiological data, together with morphological and immunocytochemical data largely from van den Pol's laboratory (van den Pol 1980, van den Pol and Tsujimoto 1985, van den Pol and Gorcs 1986), provide compelling evidence that the SCN is a complex nucleus containing diverse types of neurons. The role of the various cell types in SCN function is a mystery at present. One of the most important issues to address is the location and organization of the pacemaker within the SCN. Is there a class of pacemaker or clock cells? This would suggest that a single cell might be capable of sustaining a 24-hour oscillation in its own activity. Or, is the 24-hour clock an emergent property of the circuitry within the SCN? Although we cannot as yet answer these questions, we have made progress in localizing the pacemaker that generates the circadian rhythm of neuronal firing within the SCN brain slice.

Because the thickness of CNS tissue that can be sustained in vitro is limited by the diffusion rate of O_2, one cannot include the entire 950-μm length of the SCN in coronal brain slices. Thus, the laboratories that have examined the ability of the SCN in a coronal slice to generate at least one cycle of neuronal firing rate in vitro have used less than the rostrocaudal extent of the SCN. The slicing procedure cuts the SCN at different rostrocaudal levels, ranging from cutting through the most central point to retaining the middle 500 μm, without apparent effect on the phase or period of the rhythm of electrical activity (Gillette, unpublished). Since a complete 24-hour oscillation has been demonstrated by several groups (Gillette 1986, Green and Gillette 1982, Groos and Hendricks, 1982), it is apparent that there is redundancy of the SCN pacemaker.

An important issue to resolve is whether the oscillation in the firing rate of SCN neurons is generated within the SCN or whether there is contribution from other hypothalamic regions within the slice. We have addressed this issue by surgically reducing the coronal slice that we usually use to within 50–100 μm of the SCN borders on the dorsal and lateral sides; the optic chiasm ventral to the SCN was left undisturbed (Fig. 6-6). As indicated in our preliminary report (Gillette and Prosser 1988*a*), this reduced slice continues to generate a robust circadian rhythm of firing rate. The times of peak activity on both days 1 and 2 in vitro overlap with those in the larger hypothalamic slice. Because the cut surrounding the SCN damages the surface to a depth of about 50 μm (Alger et al.

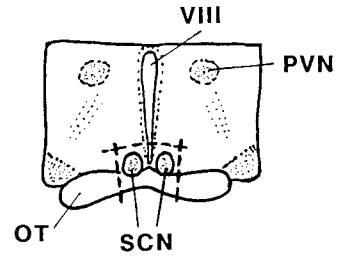

Figure 6-6. A coronal hypothalamic slice surgically reduced (cut along dashed lines) to within 50 μm of the SCN. Abbreviations as in Figure 6-1.

1984), the viable tissue remaining in this reduced slice is almost entirely within the SCN. Thus, the information to keep circadian time indeed appears to be within the SCN.

No one has as yet recorded long-term activity in SCN sliced in other than the coronal plane. It is not known whether horizontal or sagittal slicing of the SCN will permit the clock to continue to run and to express itself as an oscillation in electrical activity. These experiments will be important and may prove surprising.

To begin to probe the intrinsic functional organization of the SCN, we have performed a number of manipulations in addition to surgical reduction on the coronal slice. The results are still preliminary (Tcheng et al. 1989), but have yielded sufficiently interesting data so as to underscore the usefulness of this approach. Hemisection of the usual slice by a vertical cut severing the optic chiasm, the connections between the two SCN, and the region dorsal to the third ventricle produces two halves, each contain one SCN. This cut does not appear to affect the time or amplitude of the peak measured on day 2, suggesting that each SCN may potentially be an autonomous pacemaker. Post hoc analysis of data of an unperturbed oscillation in firing rate on day 2 (Fig. 6-3) indicates that ensemble neuronal activity oscillates in both anatomical subdivisions—the dorsomedial and ventrolateral SCN regions—of the intact coronal slice.

We are now performing further microsurgery on the hemisected slice, bisecting the SCN along a 45° angle from the base of the third ventricle. This severs the connections between the natural anatomical subdivisions. Both dorsomedial and ventrolateral portions of the SCN survive this surgery and appear healthy on day 2 in vitro. Our laboratory is now investigating the ability of each half to generate a circadian oscillation in neuronal activity with a normal time-of-peak. Preliminary results (Tcheng and Gillette, in preparation) suggest that bisection changes the ability of one of the regions to oscillate; however, further experiments are necessary to characterize this change.

ENDOGENOUS CLOCK PROPERTIES DETERMINED FROM IN VITRO STUDIES

The finding that SCN generate a stable rhythm of ensemble firing rates for several cycles in vitro led us to attempt to perturb the oscillation with agents affecting identified biochemical pathways. We could treat the slice during the first cycle and then observe any effect on the timing of the peak on days 2 and 3. The reasoning behind these experiments is that agents that reset the oscillation must gain access to the time-keeping mechanism, either through an input pathway or because they are themselves elements of the clock mechanism. These two possibilities can be distinguished. If a phase-shifting substance is part of the clock mechanism, the level of the substance should itself oscillate with the procession of the clock. If it does not, then that phase-shifting substance must be part of an input pathway.

cAMP and the SCN Clock

Because the clock must reside within cells and resetting must involve some alteration in the ongoing physiology of the cell, we approached resetting by thinking of it as a change in cell state. One key point in cellular regulation, especially in changes in cell state, is control of the ubiquitous second messenger, cyclic adenosine 3′,5′-monophosphate (cAMP). Furthermore, cAMP had been shown to affect two long period oscillators in molluscan nervous

systems (Eskin et al. 1982, Gillette et al. 1982). Therefore, we began the search for clock resetting agents by determining the effect of various treatments that specifically stimulate cAMP pathways on the SCN rhythm of electrical activity in vitro.

Our first experiments tested membrane-soluble analogues of cAMP that are potent stimulators of cAMP-dependent kinases (Meyer and Miller 1974), the effector enzymes that mediate changes in cell state by phosphorylation of specific regulatory proteins. Additionally, these analogues are resistant to degradation by phosphodiesterases. Thus, they are extremely effective in revealing the consequences of activating cAMP pathways. The effect of 1-hour bath application at CT 7-8 of 5×10^{-4} M 8-benzyl amino cAMP (BA-cAMP) upon the subsequent oscillations in firing rate is dramatic (Gillette and Prosser 1988*b*). The time-of-peak in the next circadian cycle is advanced by ~4.5 hours compared with controls, and this advance is perpetuated without decrement on day 3 (Fig. 6-7). Although occasionally damping of the amplitude was seen on day 3, this was not a usual characteristic of BA-cAMP, but rather was related to the health of the tissue on the third day (Prosser and Gillette 1989*a*). We have obtained similar phase shifts with bath application of other analogues, 8-chlorophenylthio cAMP and 8-bromo cAMP; simple media changes were ineffective. These results support the hypothesis that stimulation of cAMP-dependent pathways causes a permanent resetting of the SCN clock.

When the effect of cAMP analogues applied during subjective midday was compared with the effect of application during the subjective night, a striking circadian difference in response was observed (Fig. 6-8). In contrast to the daytime response, the 1-hour treatment had no effect on the timing of the peak in subsequent cycles when applied during the night. Detailed analysis of the phase-response relationship for cAMP stimulation (Prosser and Gillette 1989*a*) revealed a sensitive period during most of the subjective day (CT 3-12; Fig. 6-9). Peak sensitivity to phase advance occurred during CT 4-6. No period sensitive to phase delay was observed, even at lower concentrations of BA-cAMP.

The specificity of the daytime phase-advancing effect was tested further (Prosser and Gillette 1989*a*) by application of (1) forskolin, a diterpine stimulator of adenylate cyclase, the enzyme that synthesizes cAMP, and of (2) two inhibitors of phosphodiesterase, the degradative enzyme for

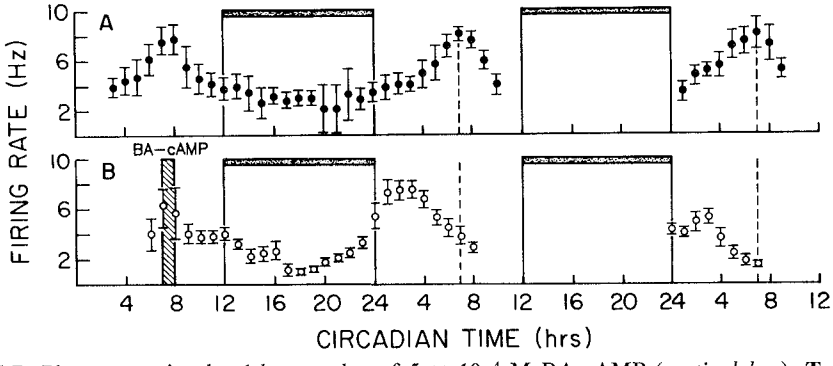

Figure 6-7. Phase resetting by 1-hour pulse of 5×10^{-4} M BA-cAMP (*vertical bar*). **Top panel,** Unperturbed control, a composite of one 24-hour recording session on day 1 with a separate experiment recording activity on days 2 and 3. Control times-of-peak are marked with *dashed line* for comparison with experimental treatments. **Bottom panel,** Composite of one 24-hour recording session on day 1 with a separate experiment recording activity on days 2 and 3. Treatment at CT 7-8 induces a 4.5-hour advance in the time of the peak in firing rate on day 2 compared with controls. The peak on day 3 occurs 24 hours later and is still advanced by 4.5 hours with respect to the control. (Adapted from Gillette and Prosser 1988*b*.)

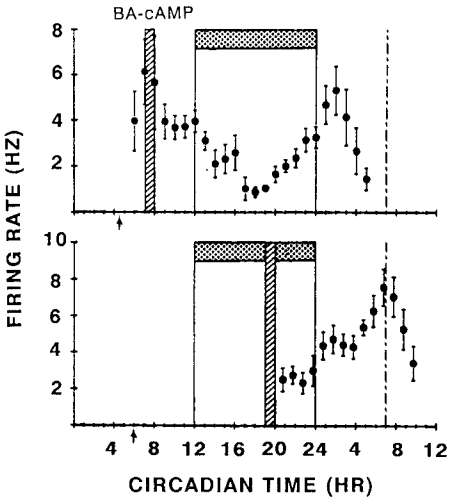

Figure 6-8. Phase-dependent effect of BA-cAMP on the circadian rhythm of neuronal firing rate. **Top,** A 1-hour treatment during the subjective day between CT 7-8 induces a 5.0-hour advance in the peak. **Bottom,** Treatment during the subjective night between CT 19-20 has no effect on the timing of the peak. *See* Figure 6-3 for explanation of the symbols. (Adapted from Prosser and Gillette 1989*a*).

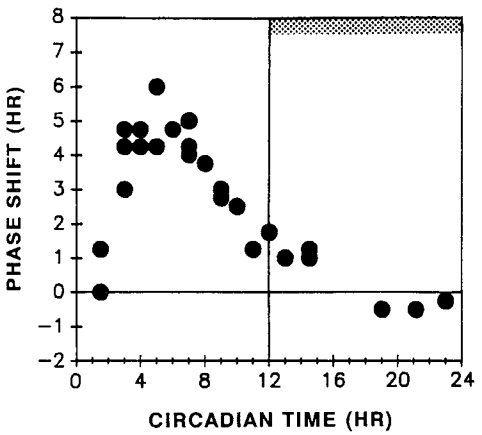

Figure 6-9. Phase-response relationship between the time of a 1-hour pulse of 5×10^{-4} M BA-cAMP and the time of the peak in neuronal firing rate. The sensitive period extends through most of the subjective day, from CT 3-12. Treatments during the subjective night have little effect on the timing of the peak. *Filled circles,* time-of-peak in the cycle after treatment. (Adapted from Prosser and Gillette 1989*a*.)

cAMP. Both types of treatment augment endogenous cAMP and thus are important tests of the hypothesis that stimulation of cAMP pathways is critical to the effect. These agents induced phase advances of similar magnitude to those stimulated by the specific analogues, and the sensitive period again occurred during the subjective day. Inactive cAMP analogues, as well as the cAMP breakdown product, 5′-AMP, were ineffective. The dose-response relationship for BA-cAMP showed that a half-maximal phase advance was induced at about 10^{-10} M. Thus, the specificity and sensitivity of the SCN to a variety of cAMP-enhancing treatments reinforce the original hypothesis that cAMP is an effector of daytime phase advances.

These results are important on three levels. First, they suggest that the SCN is specifically sensitive to stimulation of cAMP-dependent pathways. This leads to the question of which extracellular events mediated by which primary messenger(s) elevate(s) cAMP and whether such neuromodulators are endogenous to the SCN or are part of afferent pathways.

Second, sensitivity to cAMP is temporally limited to the day. This finding demonstrates that the SCN in the brain slice undergoes a circadian change in sensitivity to resetting agents, as was predicted from in vivo studies. Thus, a second clock property—circadian change in the clock mechanism—that was ascribed to the SCN from studies of resetting of behavioral rhythms in animals is maintained in vitro. Further, the period of temporal sensitivity mirrors the sensitivity of animals to daytime phase-advancing stimuli, such as dark pulses on a background of light (Boulos and Rusak 1982, Ellis et al. 1982) and induced locomotor activity (Mrosovsky and Salmon 1987). These stimuli appear to be mediated by neuropeptide Y (Albers and Ferris 1984) from the intergeniculate leaflet (Moore et al. 1984) and by serotonergic inputs from the raphe (Aghajanian et al. 1969), respectively. Perhaps these stimuli converge upon cAMP pathways within the SCN.

Third, the phase shift induced by cAMP is complete by the time the peak appears in the next cycle, and most probably much

earlier. This supports an additional property that was hypothesized to characterize circadian clocks by Pittendrigh (1974) from studies of *Drosophila* and was suggested by our earlier work (Gillette 1986): clocks rapidly, perhaps instantly, reset.

Once we had established that cAMP was part of a phase-shifting pathway, it became important to determine whether cAMP might be an element of the clock mechanism. This role would be suggested if, in the absence of afferent input, the levels of cAMP themselves oscillate within the SCN and this oscillation showed a temporal correlation to the period of sensitivity to cAMP-induced phase shifting. If a rise in cAMP is part of the normal procession of the clock, we might expect a peak in cAMP levels to occur just after the period sensitive to cAMP-induced phase shifting (Fig. 6-10). Such a peak in cAMP levels has been reported for freshly dissected SCN (Murakami and Takahashi 1983). According to this line of thinking, treatments stimulating cAMP pathways induce phase shifts by prematurely activating substrates critical to procession of the clock that are normally activated by a later rise in cAMP at the end of the period sensitive to cAMP phase shifting.

We approached this issue using the brain slice because doing so allowed us to examine cAMP levels without the possibility of afferent modulation or the effects of anoxia during dissection. Slices were incubated for several hours and then rapidly frozen on dry ice. SCN were punched from the frozen tissue, and cAMP levels were determined by radioimmunoassay. This procedure enabled us to determine the endogenous levels associated only with procession of the clock, which we know occurs normally in vitro.

The results show a robust oscillation in cAMP in SCN in vitro, with peaks shortly before dusk and dawn (Prosser and Gillette 1989*b*). Because the predusk peak near CT 10 was predicted by our phase-shifting results (Fig. 6-10), the finding strongly suggests that a regulated change in cAMP is part of the SCN clock mechanism. The second peak before dawn does not correlate with the temporal sensitivity to phase shifting, suggesting that it is part of an output pathway. It will be important to examine the levels of the synthetic and degradative enzymes that regulate these endogenous changes (Prosser and Gillette, submitted), as well as the temporal regulation of phosphoprotein substrates (Gillette et al. 1986), to elucidate the mechanism of cAMP action during phase shifting and normal time keeping. An additional consideration will be determining whether the cAMP oscillations occur in the cells that are sites of phase shifts stimulated by cAMP.

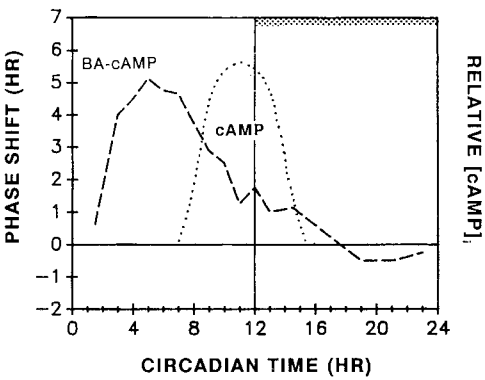

Figure 6-10. Phase-response curve for BA-cAMP (*dashed line*) derived from Figure 6-9 plotted with hypothesized time-of-peak in endogenous cAMP (*dashed line,* relative $[cAMP]_i$).

cGMP and the SCN Clock

In the course of examining the sensitivity of the SCN clock to cAMP, cyclic guanosine 3′,5′-monophosphate (cGMP), the structurally similar purine cyclic nucleotide, was tested as a control. SCN slices were treated for 1 hour with bath-applied cGMP analogues, 8-bromo cGMP (Br-cGMP), or N^2,$O^{2\prime}$-dibutyryl cGMP, and the following cycles were observed for changes in the time of the peak in ensemble firing rate. Treatment between CT 7-8, the period when cAMP analogues induced phase advances of 4.5 hours, had little effect upon the time-of-peak (Fig. 6-11; Prosser et al. 1989). In fact, 1-hour Br-

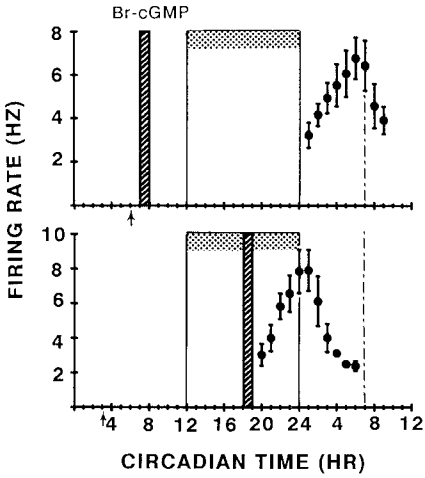

Figure 6-11. Phase-dependent effect of a 1-hour pulse of Br-cGMP on the timing of the peak in SCN electrical activity. **Top panel,** Treatment during subjective day, CT 7-8, has little effect on the time-of-peak. **Bottom panel,** Treatment during subjective night, CT 18-19, induces a 6.5-hour phase advance. See Figure 6-3 for explanation of the symbols. (Adapted from Prosser et al. 1989.)

cGMP treatments at a range of time points during the subjective day were without effect.

Surprisingly, in contrast to cAMP effects, we found that nighttime application of either cGMP analogue for 1 hour induced robust phase advances—up to 6.5 hours at CT 18 (Fig. 6-11; Prosser et al. 1989.) These phase advances were stable during the second cycle after treatment, indicating that the phase change represented resetting of the underlying pacemaker. The magnitude of the phase change depended upon the nocturnal time of treatment: smaller advances occurred before and after the period of maximal sensitivity, CT 16-18; no periods sensitive to phase delay were observed.

The differing temporal sensitivity of the SCN clock to analogues of cAMP and cGMP is apparent in Figure 6-12 in which the phase-response curves are plotted together. This figure summarizes the results of 28 experiments at 16 different 1-hour periods with BA-cAMP and 18 experiments at 13 different times with Br-cGMP (Prosser et al. 1989). The sensitive periods to the two cyclic nucleotides are 12 hours out-of-phase and virtually nonoverlapping. The sensitive period to cAMP is during the subjective day and that to cGMP is during the subjective night.

These results, like those regarding cAMP, are important on several levels. First, they indicate that at night, the SCN is specifically sensitive to stimulation of cGMP-dependent pathways. Which neuromodulators lead to increased cGMP within the SCN is unknown. However, the temporal sensitivity to cGMP stimulation is concordant with the period of rodent sensitivity to phase advance by nighttime phase-shifting agents, such as light pulses on a background of dark (DeCoursey 1964) or direct electrical stimulation of the SCN (Rusak and Groos 1982). Further, phase advances stimulated by cGMP analogues are similar to those induced in vitro by pulses of 50 mM K^+ at CT 17 (Gillette 1987), which should provide a potent depolarizing stimulus at a time when neuronal activity is low (Fig. 6-2).

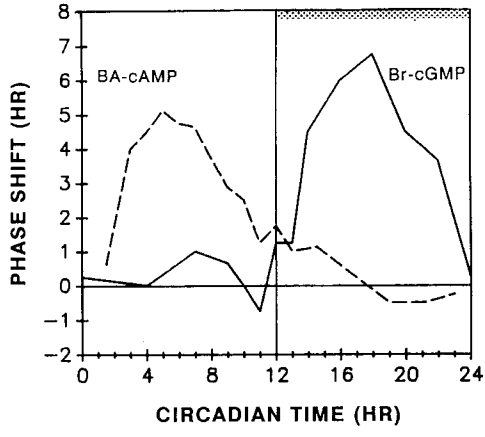

Figure 6-12. Superimposed phase-response curves for 1-hour treatments with BA-cAMP (*dashed line*) and Br-cGMP (*solid line*). Changes in the time-of-peak in the electrical activity rhythm induced by these treatments are plotted against circadian time of treatment. SCN clock sensitivity to the two second messengers is roughly 180° out-of-phase. (Adapted from Prosser et al. 1989.)

Interestingly, Liou et al. (1986*a*) reported that iontophoretic application of cGMP analogue to SCN neurons in brain slices generally resulted in increased spontaneous activity, whereas application of cAMP analogue most often resulted in decreased activity. Although the time of application was not specified, these results, when considered with the data presented above, suggest that nighttime phase-shifting agents might act through elevating cGMP and stimulating depolarization events. On the other hand, daytime phase-shifting agents might act through elevating cAMP and stimulating hypopolarizing events.

Second, our finding that the SCN clock in vitro is sensitive to a separate second messenger system in antiphase to its sensitivity to cAMP reveals that the circadian changes in sensitivity to resetting observed in vivo must be due to fundamental molecular and cellular changes within the SCN itself. There may be diurnally changing sensitivities in peripheral sensory systems, as well as changes in afferent pathways impinging upon SCN neurons that would serve to modulate the efficacy of phase-shifting stimuli in vivo. Changes of this sort would be secondary in effect, however, compared with primary changes in the biochemical substrates within the SCN and, most likely, clock neurons.

Third, because the sensitive period to treatments with cGMP analogues is during the night, the phase-advanced peak of firing rate occurs only 6 hours after initial exposure. The ensemble firing rate actually starts to rise long before this time; it is significantly elevated at CT 22, the earliest time point measured, to a level observed near CT 2 in the day-treated SCN. The 6-hour phase advance in the time-of-peak is perpetuated without alteration 24 hours later. This finding underscores the rapidity with which the SCN clock can be reset.

Melatonin Phase Shifts In Vitro

A recent study addresses the ability of the pineal hormone to induce phase shifts of the SCN rhythm of neuronal firing in vitro (McArthur et al. 1989). Chapter 16 evaluates thoroughly the evidence from a variety of in vivo studies that melatonin feeds back upon the SCN; this brief discussion addresses only in vitro data. Our approach was to bath-apply 10^{-9} M melatonin to the SCN brain slice and then to determine whether there was a phase perturbation in the circadian rhythm of neuronal firing in the next two cycles. This approach relies upon the ability of melatonin receptors to survive the trauma of slice preparation and, indeed, would be the first attempt to test the ability of the clock in vitro to respond to a ligand neuromodulator.

The results are clear. The SCN is reset in vitro by physiological concentrations of melatonin when they are applied near dusk (CT 10 and 14); it is unresponsive at other phases of the circadian cycle (McArthur et al. 1989.) The rhythm of the firing rate is phase advanced by 6 hours within the first cycle after treatment, and the new phase is perpetuated in the following cycle. The temporal sensitivity is exactly what would be predicted from in vivo work (Cassone et al. 1988). If this result is upheld when the specificity of the response to melatonin is verified, it will provide definitive evidence that melatonin acts directly at the level of the SCN to reset the clock. Most interestingly, the period of sensitivity in the rat is at dusk, the phase of the circadian cycle directly impinged upon by seasonal changes in night length (Pittendrigh 1988).

FUTURE DIRECTIONS OF IN VITRO STUDY OF THE SCN

We now are armed with the knowledge that the elements of the clock are intrinsic to the SCN and survive in vitro for days, changing in a predictable way over the circadian cycle. A recent report suggests that the distinctive morphology of the SCN is maintained for weeks in organotypic culture (Gainer et al. 1989). This raises the intriguing possibility that circadian functions are maintained over this longer period in vitro as well, which would enable the researcher to collect an abundance of information over many successive cycles.

The organotypic slice thins as it flattens upon the substratum, allowing individual cells to be discerned with appropriate optics while performing electrophysiological measurements. Such technical developments as these, coupled with methods currently in hand, will provide new insights into SCN function. Future directions of in vitro study of the SCN are summarized in Table 6-2.

The area that I most expect to flourish, and which cannot be studied equivalently in vivo, is elucidation of the intrinsic functional organization of the SCN. In particular, as the tools of modern neurophysiology are brought to bear on SCN neurons in vitro, our understanding of the physiological rôles of the number of neuronal types in the SCN will grow. The nature of circuit interactions and changes in cellular and membrane properties of individual neurons with circadian time will be established. From this knowledge will come an answer to a central question: what is the nature of the pacemaker? Is it a property of cells or of circuits?

At the same time, understanding of the regulation of the SCN clock by inputs will grow. Currently, melatonin is the only exogenous substance that has been shown to reset multiple cycles of SCN circadian rhythms in vitro. However, many neurotransmitters and modulators have been implicated in phase-shifting organismic rhythms in vivo (*see* Chapter 5). These must be tested in the in vitro system to demonstrate that they, like melatonin, act directly upon the SCN, rather than through stimulating circuits that act upon the SCN through another transmitter/modulator system. This line of inquiry must be put in perspective by identification of phase-shifting ligands with afferent pathways known to innervate the SCN.

Additionally, the mechanism by which these neurochemical inputs are transduced into signals that affect the clock—from the level of second messengers to regulatory molecules in the membrane, cytosol, and nucleus—can be addressed through parallel studies of the biochemistry and molecular biology of the SCN and physiological studies in vitro. In this way in vitro studies will contribute to elucidation of the most basic question: how is a circadian clock built out of cells and molecules?

Eventually, study of the SCN in vitro must encompass the organization of clock output. Do clock neurons also form output lines? Or are other neurons driven to produce rhythmic outputs? Known output lines will need to be identified with cells, the physiology of which has been studied in vitro. Thus, in vitro study must eventually lead to understanding of the SCN in its rightful place, integrated within the central nervous system.

The future directions of this approach are limited only by techniques available—nearly all of the questions we can formulate remain to be answered. This approach is less than 10 years old; where we will be after the next decade can only be imagined.

Table 6-2. Future Directions of Studies of SCN In Vitro

1. Elucidation of intrinsic SCN organization
a. Roles of various types of neurons
b. Nature of circuit interactions
c. Cell and membrane properties vs time
d. Nature of the pacemaker: cell or circuit?
2. Regulation of the clock by inputs
a. Neurotransmitter/modulator effects
b. Identification with input pathways
c. Transduction mechanism
d. Biochemical nature of the clock mechanism
3. Organization of SCN outputs
a. Pacemaker vs output
b. Identification of output lines

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REFERENCES

Aghajanian, G.K.; Bloom, F.E.; Sheard, M.H. Brain Res. **13:**266–273; 1969.

Albers, H.E.; Ferris, C.F. Neurosci. Lett. **50:**163–168; 1984.

Albers, H.E.; Ferris, C.F.; Leeman, S.E.; Goldman, B.D. Science **223:**833–835; 1984.

Alger, B.E.; Dhanjal, S.S.; Dingledine, R.; Garthwaite, J.; Henderson, G.; King, G.L.; Lipton, P.; North, A.; Schwartzkroin, P.A.; Sears, T.A.; Segal, M.; Whittingham, T.S.; Williams, J. In Dingledine, R. ed. Brain slices. New York: Plenum Press; 1984:381–437.

Boulos, Z.; Rusak, B. J. Comp. Physiol. **146:**411–417; 1982.

Cassone, V.M.; Chesworth, M.J.; Armstrong, S.M. Physiol. Behav. **36:**1111–1121; 1986.

Cassone, V.M.; Roberts, M.H.; Moore, R.Y. Am. J. Physiol. **255:**R332–R337; 1988.

Cobbett, P.; Smithson, K.G.; Hatton, G.I. Brain Res. **362:**7–16; 1986.

DeCoursey, P.J. Cold Spring Harbor Symp. Quant. Biol. **25:**49–55; 1960*a*.

DeCoursey, P.J. Science **131:**33–35; 1960*b*.

DeCoursey, P.J. J. Comp. Physiol. **63:**189–196; 1964.

Earnest, D.J.; Sladek, C.D. Brain Res. **382:**129–133; 1986.

Earnest, D.J.; Sladek, C.D. Brain Res. **422:**398–402; 1987.

Ellis, G.B.; McKlveen, E.; Turek, F.W. Am. J. Physiol. **242:**R44–R50; 1982.

Gainer, H.; Castel, M.; Wray, S. Soc. Neurosci. Abstr. **15:**1087; 1989.

Gillette, M.U. J. Physiol. **369:**55P; 1985.

Gillette, M.U. Brain Res. **379:**176–181; 1986.

Gillette, M.U. Soc. Neurosci. Abstr. **13:**51; 1987.

Gillette, M.U.; Prosser, R.A. Soc. Neurosci. Abstr. **14:**385; 1988*a*.

Gillette, M.U.; Prosser, R.A. Brain Res. **474:**348–352; 1988*b*.

Gillette, M.U.; Reppert, S.M. Brain Res. Bull. **19:**135–139; 1987.

Gillette, M.U.; Reiman, A.M.; Lipeski, L.E. Soc. Neurosci. Abstr. **12:**845; 1986.

Gillette, R.; Gillette, M.U.; Davis, W.J. J. Comp. Physiol. **146:**461–470; 1982.

Green, D.J.; Gillette, R. Brain Res. **245:**198–200; 1982.

Groos, G.; Hendricks, J. Neurosci. Lett. **34:**283–288; 1982.

Hatton, G.I.; Doran, A.D.; Salm, A.K.; Tweedle, D.C. Brain Res. Bull. **5:**405–414; 1980.

Inouye, S-I.T.; Kawamura, H. Proc. Natl. Acad. Sci. USA **76:**5962–5966; 1979.

Inouye, S-I.T.; Kawamura, H. J. Comp. Physiol., **146:**153–160; 1982.

Liou, S.Y.; Shibata, S.; Yamakawa, K.; Ueki, S. Neurosci. Lett. **41:**109–113; 1983.

Liou, S.Y.; Shibata, S.; Shiratsuchi, A.; Ueki, S. Neurosci. Lett. **67:**339–343; 1986*a*.

Liou, S.Y.; Shibata, S.; Ueki, S. Neuropharmocology **25:**1009–1014; 1986*b*.

Mason, R. J. Physiol. **377:**1–13; 1986.

Mason, R.; Brooks, A. Neurosci. Lett. **95:**296–301; 1988.

Mason, R.; Harrington, M.E.; Rusak, B. Neurosci. Lett. **80:**173–179; 1987.

McArthur, A.J.; Prosser, R.A.; Gillette, M.U. Soc. Neurosci. Abstr. **15:**1060; 1989.

Meijer, J.H.; Groos, G.A. Brain Res. Bull. **20:**89–96; 1988.

Meyer, R.B.; Miller, J.P. Life Sci. **14:**1019–1040; 1974.

Moore, R.Y.; Eichler, V.B. Brain Res. **42:**201–206; 1972.

Moore, R.Y.; Gustafson, E.L.; Card, J.P. Cell Tiss. Res. **236:**41–46; 1984.

Moore-Ede, M.C.; Sluzman, F.M.; Fuller, C.A. The clocks that time us. Cambridge, MA: Harvard University Press; 1982.

Mrosovsky, N.; Salmon, P.A. Nature **330:**372–373; 1987.

Murakami, N.; Takahashi, K. Brain Res. **276:**297–304; 1983.

Nishino, H.; Koizumi, K. Brain Res. **120:**167–172; 1977.

Pittendrigh, C.S. In Schmidt, F.O.; Worden, F.G. eds. The Neurosciences, Third Study Program. Cambridge, MA: Massachusetts Institute of Technology Press; 1974; 437–458.

Pittendrigh, C.S. J. Biol. Rhythms **3:**173–188; 1988.

Prosser, R.A.; Gillette, M.U. J. Neurosci. **9:**1073–1081; 1989*a*.

Prosser, R.A.; Gillette, M.U. Soc. Neurosci. Abstr. **15:**1060; 1989*b*.

Prosser, R.A.; McArthur, A.J.; Gillette, M.U. Proc. Natl. Acad. Sci. USA **86:**6812–6815; 1989.

Reppert, S.M.; Artman, H.G.; Swaminathan, S.; Fisher, D.A. Science **213:**1256–1257; 1981.

Richter, C.P. Proc. Assoc. Res. Nerv. Ment. Dis. **45:**8–27; 1967.

Rusak, B.; Groos, G. Science **215:**1407–1409; 1982.

Schwartz, W.J.; Reppert, S.M. J. Neurosci. **5:**2771–2778; 1985.

Schwartz, W.J.; Coleman, R.J.; Reppert, S.M. Brain Res. **263:**105–112; 1983.

Shibata, S.; Moore, R.Y. J. Biol. Rhythms **3:**265–276; 1988.
Shibata, S.; Oomura, Y.; Kita, H.; Hattori, K. Brain Res. **247:**154–157; 1982.
Shibata, S.; Liou, S.Y.; Ueki, S. Jap. J. Pharm. **33:**1225–1231; 1983*a*.
Shibata, S.; Liou, S.Y.; Ueki, S. Neurosci. Lett. **39:**187–192; 1983*b*.
Shibata, S.; Cassone, V.M.; Moore, R.Y. Neurosci. Lett. **97:**140–144; 1989.
Stephan, F.K.; Zucker, I. Proc. Natl. Acad. Sci. USA **69:**1583–1586; 1972.
Tcheng, T.K.; Gillette, M.U.; Prosser, R.A. Soc. Neurosci. Abstr. **15:**1059; 1989.
van den Pol, A.N. J. Comp. Neurol. **191:**661–702; 1980.
van den Pol, A.N.; Gorcs, T. J. Comp. Neurol. **252:**507–521; 1986.
van den Pol, A.N.; Tsujimoto, K.L. Neuroscience **15:**1049–1086; 1985.
Vandesande, F.; Dierickx, K.; DeMey, J. Cell Tiss. Res. **156:**377–380; 1975.
Yamamoto, C. Exp. Brain Res. **14:**423–435; 1972.