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Intrinsic neuronal rhythms in the suprachiasmatic nuclei and their adjustment

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Abstract. The central role of the suprachiasmatic nuclei in regulating mammalian circadian rhythms is well established. We study the temporal organization of neuronal properties in the suprachiasmatic nucleus (SCN) using a rat hypothalamic brain slice preparation. Electrical properties of single neurons are monitored by extracellular and whole-cell patch recording techniques. The ensemble of neurons in the SCN undergoes circadian changes in spontaneous activity, membrane properties and sensitivity to phase adjustment. At any point in this cycle, diversity is observed in individual neurons' electrical properties, including firing rate, firing pattern and response to injected current. Nevertheless, the SCN generate stable, near 24 h oscillations in ensemble neuronal firing rate for at least three days *in vitro*. The rhythm is sinusoidal, with peak activity, a marker of phase, appearing near midday. In addition to these electrophysiological changes, the SCN undergoes sequential changes *in vitro* in sensitivities to adjustment. During subjective day, the SCN progresses through periods of sensitivity to cyclic AMP, serotonin, neuropeptide Y, and then to melatonin at dusk. During the subjective night, sensitivities to glutamate, cyclic GMP and then neuropeptide Y are followed by a second period of sensitivity to melatonin at dawn. Because the SCN, when maintained *in vitro*, is under constant conditions and isolated from afferents, these changes must be generated within the clock in the SCN. The changing sensitivities reflect underlying temporal domains that are characterized by specific sets of biochemical and molecular relationships which occur in an ordered sequence over the circadian cycle.

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The paired suprachiasmatic nuclei of the hypothalamus are the seat of the primary circadian timekeeping mechanism of mammals. They mark the passage of time in near 24 h cycles. It is here that entraining signals, initiated by environmental change, are integrated so as to adjust the phase of the pacemaker.

Efferent signals from the suprachiasmatic nucleus (SCN) organize and regulate metabolic, physiological and behavioural functions that occur in circadian patterns. Other chapters in this volume address aspects of the mammalian circadian system studied in whole organisms, whereas we shall examine the neuronal and pacemaker properties of the SCN that are expressed in a hypothalamic brain slice. Because our studies evaluate the SCN isolated *in vitro*, we directly assess intrinsic properties. We have found that each SCN is composed of an electrophysiologically diverse group of neurons that function remarkably autonomously: the SCN generates stable, near 24 h rhythms of ensemble neuronal activity *in vitro* and undergoes an orderly sequence of changes in sensitivity to stimuli that adjust phase. The pattern of responsiveness to neurochemicals known to be contained in SCN afferents demonstrates that the organization of the clock is more complex than simply night-versus-day processes; rather, our findings suggest there is a continuously changing series of sensitivities to multiple phase-adjusting stimuli. This sequence must reflect changes in underlying cellular processes, or 'temporal domains'. These domains are functional epochs, characterized by specific biochemical or molecular substrates and their interactions, which are linked together to generate the daily order of the circadian clock's cycle.

The experimental system that we use is a hypothalamic slice from inbred Long-Evans rats 5–10 weeks old. It differs from whole organism studies of clock function in several important ways.

First, we have dissected the SCN out of the central nervous system into a 500 μm coronal slice of hypothalamus. Therefore, the activities and circadian properties that we measure must be generated spontaneously within the tissue slice. The slice contains less than the 800 μm rostrocaudal extent of each nucleus; we study properties of the medial portion, primarily. Any circadian patterns that we observe therefore are the result of activity in less than the entire nucleus and would indicate that there is redundancy in pacemaker organization.

Second, the suprachiasmatic slice is studied in a defined, constant environment with minimal supplementation. The fresh slice is placed in a brain slice chamber at the interface of a moist, 95% O_2/5% CO_2 atmosphere and the glucose–bicarbonate-supplemented medium (Earle's balanced salt solution, 37 °C), maintained at pH 7.4 and perfused at 34 ml/h (see Prosser & Gillette 1989 for complete methods). The two suprachiasmatic nuclei are clearly visible in the slice, which gives the investigator precise control over the sites of measurement and drug treatment. Thus, with the *in vitro* preparation we have the advantage of a high degree of control over the chemical and physical environment while making manipulations under clear visual guidance.

Third, the suprachiasmatic nuclei that we study within the brain slice are removed from the influence of other brain regions and humoral factors. After the suprachiasmatic slice has been cut, the base of the hypothalamus is surgically reduced to exclude the supraoptic nuclei, although part of the paraventricular

nuclei may be included in the slice because the third ventricle is intact dorsally. Thus, in these studies, the suprachiasmatic nuclei are free of the primary brain regions that project to and regulate them, as well as most feedback loops. Electrical properties, spontaneous activities and sensitivities to chemical perturbations are assessed directly in the free-running clock with little, if any, contamination by signals from other sites. Our measure of phase is the peak of the circadian rhythm of firing activity of the neuronal ensemble, which most probably represents both intrinsic signalling and the primary neural output of the clock. Any circadian changes that we measure *in vitro* are those generated spontaneously within the SCN, that is, those which are components of or are driven by the timekeeping mechanism.

A primary property attributed to the SCN from organismic studies is that of endogenous oscillation with a period about that of the day, i.e. circadian. This behaviour can be studied in our brain slices because they survive under the conditions we use for at least three days *in vitro*. At any one time point within the daily cycle, SCN neurons show diversity in rates and patterns of spontaneous activity. Circadian measurements made with whole-cell patch recording methods in the brain slice (Blanton et al 1989) demonstrate that, electrophysiologically, the SCN may be composed of considerably more cell types than first reported (Wheal & Thompson 1984). Preliminary determinations found that 75% of over 50 neurons sampled were spontaneously active, with firing patterns ranging from very regular to irregular random and burst patterns (Gallman & Gillette 1992). Neurons of the various types could be found across the circadian cycle, but circadian patterns of firing frequency, membrane potential and specific membrane conductance were apparent (Gallman & Gillette 1993). Circadian changes in firing frequency of the sample measured with whole-cell patch recording resemble the sinusoidal oscillation recorded extracellularly for the neuronal ensemble (shown in Fig. 1; Gillette 1991): mean firing rate peaks at mid subjective day, alternating with a trough during subjective night.

The pattern of oscillation in spontaneous activity of the ensemble of single units monitored extracellularly is remarkably stable over consecutive 24 h cycles, so that the time of peak activity is a reliable marker of phase (Prosser & Gillette 1989). Despite the fact that the tissue contains significantly less than the entire SCN and is maintained in glucose-supplemented minimal salt solution, the period of the daily oscillation is near 24 h. These observations reveal that isolated suprachiasmatic nuclei are able to regenerate circadian rhythmic neuronal activity cycles spontaneously given only an exogenous energy source. Further, because this occurs regularly in coronal slices which inevitably have been cut through some portion of the SCN, there must be redundancy of the pacemaker within the entire rostrocaudal extent of the SCN (Gillette 1991).

A second property attributed to circadian clocks from the study of organisms' behaviour is differential sensitivity to phase adjustment. This was tested in the SCN *in vitro* with structural analogues of the ubiquitous second messenger cyclic

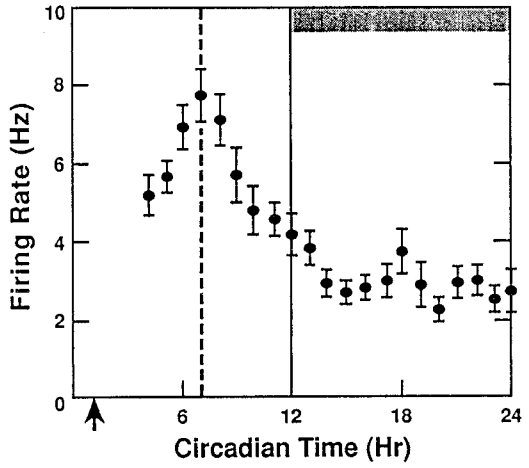

FIG. 1. The circadian oscillation in spontaneous firing rate of the ensemble of neurons of the suprachiasmatic nucleus in a rat hypothalamic brain slice. Mean firing rate (Hz) for all units encountered with an extracellular electrode and sampled within a two-hour interval is plotted against circadian time (CT) of the lighting cycle to which the rat had been entrained, where CT 0 is the time of the dark–light transition. The slice was prepared at CT 1 (arrow) from an 8-week-old rat reared in a 12 h light : 12 h dark lighting cycle and placed in constant light in the interface brain slice chamber; recording commenced at CT 4. Activity peaked at CT 7 (dashed line), seven hours into the entrained light cycle, and was generally low at night (shaded horizontal bar). Error bars, SEM; $n = 6$–15 neurons per two-hour bin (each two-hour bin was offset by 15 min to generate a running average).

AMP (cAMP). Several 8-substituted cAMP analogues, including 8-benzylamino-cAMP (BA-cAMP), 8-bromo-cAMP (Br-cAMP) and 8-chlorophenylthio-cAMP, when applied in a bath for up to one hour during subjective daytime, permanently advanced the time of peak neuronal activity; non-cyclic 8-BA-5′-AMP was ineffective (Gillette & Prosser 1988, Prosser & Gillette 1989). When the SCN was treated at midday, peak firing appeared 4.5 h before the expected time in the two cycles monitored thereafter. These analogues did not produce phase shifts when given during subjective night (Fig. 2). Likewise, agents that increase endogenous cAMP, such as forskolin or RO20-1724, were effective only in the daytime. Conversely, Br-cGMP, a structurally related purine cyclic nucleotide analogue, was effective in antiphase to Br-cAMP and BA-cAMP; it adjusted phase in the SCN only when applied at night-time (Fig. 2, Prosser et al 1989). These were the first demonstrations not only of phase adjustment in the isolated SCN, but also of spontaneous waxing and waning of one sensitivity followed by another. These findings established that SCN clock properties persist *in vitro*.

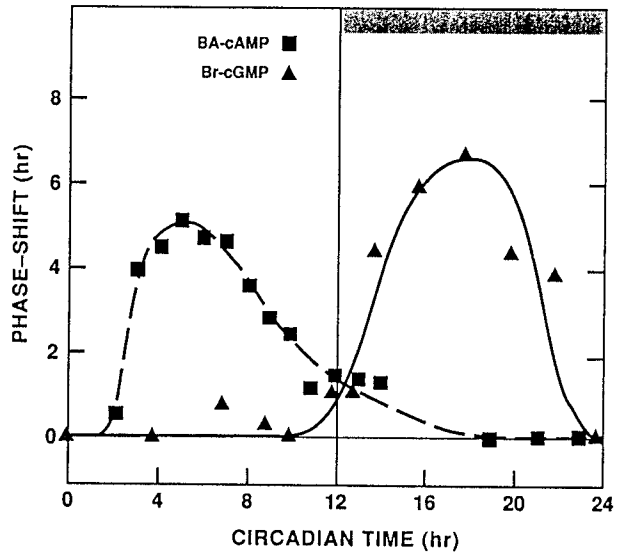

FIG. 2. The sensitivity of the rat SCN to cAMP is in antiphase to that to cGMP. Phase shifts in the time of the peak in the electrical activity rhythm are plotted against the time at which the SCN was treated *in vitro* for one hour with analogues, 8-benzylamino-cAMP (BA-cAMP) or 8-bromo-cGMP (Br-cGMP), applied in a bath. Bromo-5′-AMP was without effect. The rats were entrained to the same light–dark cycle as in Fig. 1. Shaded horizontal bar represents subjective night. Adapted from Prosser et al (1989).

Both cAMP and cGMP are potent regulators of cell state. Their 8′-analogues are exceptional activators of the protein kinases regulated by these cyclic nucleotides and are degraded only slowly (Meyer & Miller 1974). Differential sensitivity of the clock to each nucleotide over the circadian cycle may be modulated at many levels. Although the concentrations of the kinases do not appear to change, the concentrations of their regulatory cyclic nucleotides, as well as the phosphorylation states of the kinases and some substrates, oscillate over the 24 h cycle (Prosser & Gillette 1991, Faiman & Gillette 1991, Weber & Gillette 1991). The critical point at which these changes adjust the clock mechanism has yet to be determined.

The identity of the synaptic neuromodulators mediating phase adjustment, including those activating these second messengers, is not yet known; however, candidates are neuroactive substances known to be localized in afferent fibres and those with ligand-binding sites within the SCN. Major projections to the SCN include those from: (1) the dorsal raphe, containing serotonin (5-hydroxytryptamine, 5-HT); (2) the intergeniculate leaflet of the lateral geniculate nucleus, containing neuropeptide Y (NPY) and γ-aminobutyric acid (GABA);

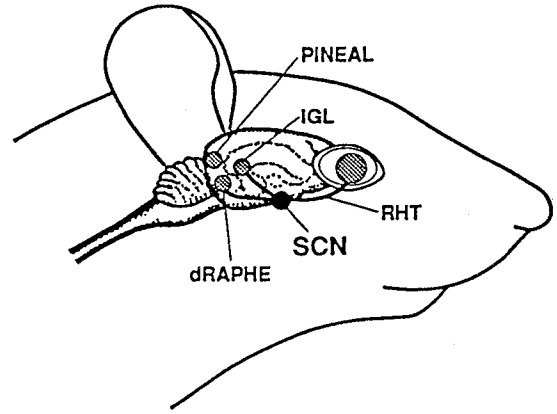

FIG. 3. Schematic of the head of a rat showing major brain sites that regulate the SCN. The suprachiasmatic nuclei are positioned at the base of the hypothalamus, directly over the optic chiasm. They receive serotonergic projections from the dorsal raphe (dRAPHE), NPY/GABAergic projections from the intergeniculate leaflet (IGL), and glutamate-liberating axons from the retina via the retinohypothalamic tract (RHT). Additionally, the pineal is the source of circulating melatonin.

and (3) the retina, via the retinohypothalamic tract (RHT), containing a glutamate precursor (Fig. 3). Additionally, the SCN is one of the few brain regions that bind significant amounts of melatonin, the indoleamine produced nocturnally by the pineal. We have explored the sensitivities of the SCN to candidate modulators.

Neurons of the ventrolateral SCN (vlSCN) of the rat receive serotonergic fibres from the dorsal raphe (Azmitia & Segal 1978, Moore et al 1978, van de Kar & Lorens 1979) as well as NPY- and glutamate-containing terminals from the IGL and retina, respectively (Card & Moore 1989, Mikkelsen & O'Hare 1991, Castel et al 1993). This indicates that the vlSCN in the rat is a major site of signal integration; furthermore, because these fibre types may synapse both on each other and on the same SCN neuron (van den Pol & Gorcs 1986, Guy et al 1987), this integration may have both presynaptic and postsynaptic components. Cognizant of the localized nature of the termination sites of these projections, we designed these experiments to examine the effects of localized application of test substances in microdrops applied directly to the vlSCN.

Both 5-HT and NPY adjust phasing in the SCN when applied *in vitro* during subjective daytime. The period of sensitivities to these two neuromodulators encompasses the period of phase advance induced by non-photic stimuli (Mrosovsky et al 1989; see Mrosovsky 1995, this volume). 10^{-6} M 5-HT applied to the vlSCN in a 10^{-11} ml droplet for five minutes between CT 2 and CT 10 caused significant phase adjustments (>1 h) (Fig. 4; Medanic & Gillette 1992).

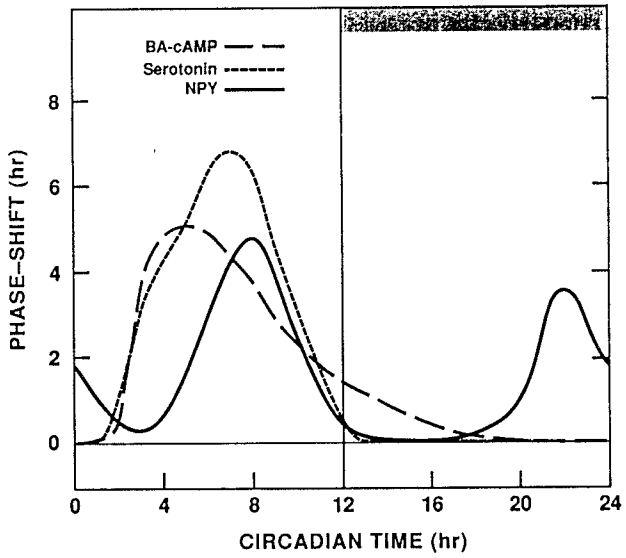

FIG. 4. The family of phase response curves generated in response to three daytime phase-shifting agents, 8-benzylamino-cAMP (BA-cAMP), 5-HT and NPY. BA-cAMP was applied in a bath for one hour at 5×10^{-5} M, whereas 5-HT and NPY were applied at 10^{-6} M in microdrops to the vlSCN for five minutes. Notice the similarities and differences within this family of curves. Curves were fitted by eye to results from the following: BA-cAMP, 25 experiments at 16 CTs from Prosser & Gillette (1989); serotonin, 20 experiments at 7 CTs from Medanic & Gillette (1992); NPY, 30 experiments at 11 CTs from Medanic & Gillette (1993). The rats were entrained as in Fig. 1. Subjective night is represented by the shaded horizontal bar.

The maximal response occurred to application of 5-HT at CT 7 which induced a 7.0 ± 0.1 h phase advance. This period of sensitivity to 5-HT applied as a microdrop overlaps with but is not identical to that for cAMP applied in the bath (Fig. 4). Although the differences in the details of the phase responses to 5-HT and cAMP could be a result of the differing extent of exposure to the stimulus between microdrop and bath application, the similarities, especially between CT 2 and CT 5, are striking and suggestive of a common pathway. Studies with agonists and antagonists have implicated a 5-HT_{1A}-like receptor (Medanic & Gillette 1992, Shibata et al 1992, Prosser et al 1993). A new cAMP-coupled 5-HT receptor (5-HT_7) has recently been cloned from rat brain (Lovenberg et al 1993); although it has not been localized to the SCN, should this receptor reside either in vlSCN neurons or in presynaptic terminals, it could modulate the early phase of the 5-HT-induced shift by a cAMP-dependent mechanism. The disparity between the cAMP- and 5-HT-induced phase response curves after this point would suggest that the latter response, from CT 6 to CT 11

and including the maximal effect, is mediated at least partially by a non-cAMP-dependent mechanism.

Daytime sensitivity to NPY microdrops applied to the vlSCN significantly lags behind the sensitivities to cAMP and 5-HT (Fig. 4; Medanic & Gillette 1993). It first appears at CT 5 and peaks at CT 8 with a maximal phase shift of 4.5 h—about 2.5 h less than the 5-HT peak—and then dissipates in synchrony with the waning 5-HT sensitivity. This temporal pattern of sensitivity of the SCN to NPY in subjective day makes it unlikely that NPY utilizes a cAMP-stimulating signal transduction mechanism. A second period of sensitivity to NPY anticipates dawn, peaking at CT 22, a time when the suprachiasmatic nuclei are insensitive to regulation by both cAMP and cGMP. This bimodal pattern of sensitivity of the free-running SCN to NPY raises questions about the mechanism(s) regulating sensitivity. Is the receptor itself disappearing, then reappearing, or is regulation at the level of intracellular signal transduction elements or at the level of cellular substrates of the transduction cascade? Our results regarding changing sensitivity to cAMP stimulation would suggest that regulation of cellular substrates is the most likely. Whatever the level(s) of regulation, these changes must be close to or driven by the clock, and thus understanding them should lead us toward a better understanding of the timekeeping elements.

Nocturnal sensitivity of the SCN to phase adjustment must include the neural substrates of photic entrainment. Because many lines of evidence support a role for glutamate in mediating light signals from the environment at RHT synapses at the vlSCN (Rusak & Bina 1990, Kim & Dudek 1991), we examined temporal changes in the effect of focal application of glutamate to the SCN. One μl drops, which effectively covered one SCN with 10 mM glutamate for 10 min, produced phase delays and phase advances which are remarkably like those produced by light in time, shape and amplitude, with a maximal delay at CT 14, and maximal advance at CT 19 (Ding & Gillette 1993). Interestingly, although this period of sensitivity spans that to cGMP, the biphasic shape and lesser amplitude of the response to glutamate suggest that activation of cGMP pathways cannot wholly explain the result with glutamate.

Interspersed between the daytime and night-time sequences of sensitivities of the SCN to these neuromodulators are periods that represent the entrained light–dark and dark–light transition zones. The first of these, which surrounds environmental dusk, coincides with a period when injections of the pineal hormone melatonin have been shown to entrain rats and alter energy utilization in the SCN (Redman et al 1983, Cassone et al 1986, 1988). Not surprisingly, bath application of 10^{-9} M melatonin for 60 min adjusts the subsequent cycles of firing rate in the SCN (McArthur et al 1991) by advancing the clock by up to 4.5 h. Careful examination of sensitivity surrounding dawn revealed a second, narrow period of sensitivity to melatonin that appeared sharply at CT 23 and then decayed over the next two hours (McArthur & Gillette 1992). Both of these sensitive periods closely follow those to NPY. In this respect, melatonin

resembles NPY: both show two windows of sensitivity, separated by many hours, which appear spontaneously in constant conditions *in vitro* without exposure to the phase-adjusting agent. In the case of melatonin, these sensitive periods occur at times when seasonal changes in night-length can be expected to stretch the duration of pineal melatonin synthesis into these temporal domains of the SCN.

Overall, we have accumulated evidence that the properties of a circadian clock persist in the SCN *in vitro*. The suprachiasmatic nuclei generate multiple, near 24 h cycles of ensemble neuronal firing rate and show differential sensitivity to phase-adjusting stimuli. The patterns of membrane properties and neuronal activity are circadian, even though less than the entire SCN is present in the brain slice. The SCN *in vitro* continues to generate a stable circadian oscillation in the ensemble electrical activity, even though component neurons show diversity in electrophysiological properties. The contribution of the various electrophysiological types of neurons to integrative or timekeeping functions of the SCN is presently unknown. Further, the isolated SCN proceeds through an orderly sequence of sensitivities to resetting stimuli. This occurs without prior exposure to the stimuli during that circadian cycle. The onset of each sensitive period is generally more rapid than its decay. The neuromodulatory stimuli identified so far act through different signal transduction pathways which are insensitive to direct activation during non-sensitive periods. This suggests that the processes that regulate sensitivity operate beyond the level of the receptor, at least one step into the cell. The cellular substrates that determine the sensitivity and the characteristics of the response to extracellular regulators define separate temporal domains, which appear as an orderly sequence of biochemical and molecular relationships that together make up the circadian cycle. Understanding the components of each domain, its positive and negative regulators and the linkages between successive domains, should permit fine control of phase adjustment.

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DISCUSSION

Armstrong: Although we have looked very carefully, we find that melatonin injections given around dawn to freely moving rats have no effects on locomotor activity (Armstrong et al 1989). We find just a narrow window of sensitivity between CT 9 and CT 12. Recently, we even tried giving injections on three consecutive days at the same CT and this did not work either (unpublished work). So, although the SCN in your preparation seems to be sensitive to melatonin at subjective dawn, we cannot pick up that sensitivity in overt locomotor behaviour.

Gillette: All I would ever claim is that we are showing the sensitivities the SCN is capable of when it is treated with one stimulus in an isolated situation. It will be interesting to start treating the SCN with different stimuli stimultaneously, because that's undoubtedly what would happen normally. We've begun by treating the SCN simultaneously with serotonin and NPY. Much to our surprise, we don't get an intermediate response. The response to NPY predominates, unless its concentration is low. All we have is a skeleton of

response capabilities. At dawn there may normally be a number of stimuli coming in regulating the sensitivity to melatonin. It would be interesting to put the animal on different photoperiods and see if the sensitivity changed.

Cassone: Of course, in the real world, there are changes according to the time of year. Equally important is the fact that the light cycle of the real world is not a square wave made up of 300 lux or nothing. The various neural and endocrine inputs to the SCN might modulate pacemaker functioning in addition to the effects demonstrated on phase. They may also regulate the response to the ambiguous light intensities and colours that occur in the natural world.

Gillette: I would agree with that. We've described the pathways which have been best studied, but they are by no means the only pathways. Dr Moore spoke earlier about substance P as a potential input, and Cote & Harrington (1993) have evidence that the SCN can respond to histamine. The suprachiasmatic nuclei express many other receptors, which may or may not be functional. This is going to turn out to be a system whose regulation is complex.

Reppert: I gather that you think melatonin is activating protein kinase C. How do you think it does this?

Gillette: We think it's working through the classic phospholipase C pathway (McArthur & Gillette 1992), although we haven't been able to measure changes in inositol 1,4,5-trisphosphate ($InsP_3$). This pathway usually involves a receptor interacting through a heterotrimeric G protein with phospholipase C, which then cleaves membrane phospholipids into diacylglycerol, which activates protein kinase C in the membrane, and $InsP_3$, which liberates intracellular Ca^{2+}. Often, $InsP_3$ has a short half-life in the cell and is moved on into other phosphoinositol intermediates, so you need to look not only for $InsP_3$, but also all its various breakdown products.

Reppert: We too have not found melatonin-induced $InsP_3$ changes in the rat SCN (L. L. Carlson & S. M. Reppert, unpublished work). You could also look for changes in intracellular Ca^{2+} by imaging.

Gillette: We haven't done that.

Reppert: Vanecek & Klein (1992) have shown in another system, the neonatal rat pituitary gland, that melatonin actually *inhibits* the gonadotropin-releasing hormone-induced increase in intracellular Ca^{2+}.

Hastings: In the ovine pars tuberalis we've been unable to find any effects of melatonin on phospholipase C, phospholipase D or phospholipase A, but that's a different tissue (McNulty et al 1994).

Gillette: We have looked at this very carefully, to see if we can show the same effects as your group (Hazelrigg et al 1991) and Dr Reppert's (Carlson et al 1989) with melatonin blocking forskolin-stimulated cAMP production. We could not. I would suggest the signal transduction pathway in the rat SCN may be different from that in the median eminence/pars tuberalis.

Reppert: I disagree with your assessment of the results with cAMP. The concentration of melatonin receptors in the SCN is low. Thus, an SCN slice

or explant will have only a small proportion of the cAMP pool coupled to melatonin receptors. When you activate all the cAMP in SCN-containing tissue with forskolin, you would not expect to see a decreased cAMP response with melatonin. In the hypophysial pars tuberalis, the situation is different. The melatonin receptor concentration is high in that tissue such that melatonin inhibits most of the forskolin-stimulated cAMP (Carlson et al 1989).

Gillette: Our results with TPA (12-*O*-tetradecanoylphorbol 13-acetate) and staurosporine correlate surprisingly closely with those with melatonin in the magnitude of the phase advance at both dusk and dawn (McArthur & Gillette 1992). Furthermore, the protein kinase C inhibitor staurosporine completely blocks the phase shifts induced at dawn and dusk by melatonin and TPA. The SCN is sensitive to neither earlier in the day. Thus, protein kinase C is implicated by agonists and antagonists at both windows of sensitivity to melatonin.

Turek: A few years ago there was some interest in the fact that the SCN has functionally distinct regions. You mentioned redundancy in the SCN, but most of your results are from the ventrolateral SCN. Do you think there are differences between the dorsomedial and the ventrolateral SCN?

Gillette: The redundancy lies, I believe, anterioposteriorially. We and others have been saying that for several years. Before we were good at making brain slices, we were happy to see half of the SCN in our slice, but we always, even new students, got the same pattern of activity. That suggests to me that when you are sampling in a coronal slice, no matter whether you have the rostral or caudal half or the medial portion, you get the same pattern. Recordings in the intact SCN slice, whether dorsomedial or ventrolateral, gave a nice oscillation (Tcheng & Gillette 1991). The difference came when we then took that coronal slice and cut it at an angle of 45°. The ventrolateral SCN behaved as though we hadn't done anything to it, as though it were in the intact slice. We were left with much less than half of the SCN yet we got a strong 24 h rhythm. However, there was a change in the dorsomedial portion. If we made a high dorsomedial cut, we could not see a clear oscillation. If we moved the cut down towards the ventrolateral portion, the high amplitude, 24 h oscillation returned in the dorsomedial piece (Gillette et al 1992). This suggests there is something unusual about the intermediate zone that can confer rhythmicity on the dorsomedial region. What surprised us was that the dorsomedial cells were not primarily the pacemaker region, which was our original hypothesis. The ventrolateral portion not only gets the signals and integrates them, but can also generate the rhythm.

Miller: The effect of melatonin in late subjective night is interesting in the light of Kilduff et al's (1992) report that c-*fos* is expressed by the SCN in rats injected subcutaneously with melatonin at that time. We have no idea what behavioural relevance that has.

Gillette: This may be involved in shaping entrainment patterns at the end of long nights in the annual solar cycle.

Miller: There is at least one indole, serotonin, that does seem to mobilize Ca^{2+} in the SCN, at least in fluorescence studies (van den Pol et al 1992).

One problem here is that you are looking at a whole mishmash of SCN cells, within which there is a population of clock cells. We have strong evidence that the 5-HT receptor of relevance here increases the concentration of cAMP yet we have never been able to demonstrate an increase in cAMP concentration by biochemical methodology. Perhaps because only certain cells, a small proportion, are involved, we cannot visualize the increase. Similarly, you may be losing the $InsP_3$ change in the noise.

You have to be phenomenally careful in talking about time domains when you're also mixing paradigms. Part of your conclusions are based on the microdrop methodology and part on bath administration. As you know, our results with serotonin are very different from yours, with respect to the phase delay we see with bath-administered 5-HT or quipazine in the SCN slice at CT 18 (Prosser et al 1993).

Gillette: You have to be even more careful about whether you are using serotonin itself or an agonist which can act as an antagonist at some receptors.

Miller: We have used both quipazine and 5-HT at CT 18, though we haven't generated a full phase response curve (PRC) for serotonin.

Gillette: My point is that you cannot equate serotonin with quipazine, because they can act differently. We have found that agonists of different receptor subtypes induce phase shifts of different magnitudes (Medanic & Gillette 1992).

Miller: The effects of serotonin were identical to the effects of quipazine, and we blocked both effects with metergoline. Although the serotonergic innervation of the SCN is primarily in the ventrolateral division, there is no doubt that there is serotonergic innervation above and beyond the ventrolateral division, so it is quite possible that bath application and microdrop application only to the ventrolateral portion could produce different responses. One of us has to do the corresponding study to straighten this out. The lack of a delay portion in the PRC may not represent a difference in time domain but a difference in technique.

Gillette: Perhaps we should clarify this for the benefit of others. With bath application of serotonin or quipazine in the early portion of the night Dr Miller gets a phase delay. He does not get this with 8-OH-DPAT (8-hydroxy-2-[di-*n*-propylamino]tetralin), so the event is not mediated by a $5\text{-}HT_{1A}$-like receptor, which is interesting. With microdrop application of serotonin or 8-OH-DPAT to the ventrolateral SCN, we do not observe this phase delay at night. We each used different methods of drug application and different agents, and, provocatively, have slightly different results. Interestingly, Mike Rea's group has found that serotonergic agonists can block the optic nerve-stimulated volley of depolarization in the SCN *in vitro* and light-stimulated phase shifts *in vivo* (without themselves stimulating phase shifts) (Rea et al 1994). These findings

suggest that there is an interaction between the serotonin and glutamate systems in the SCN, as in other brain regions. The pharmacology suggests a 5-HT_1-like receptor mediates this effect. Yet, there are other interesting serotonin receptor subtypes found in the SCN which apparently don't mediate the phase advance late in the day or the blocking effect on photic stimuli at night. The serotonin story is going to be complex. This is not surprising considering the diversity of the serotonin system, in terms of both receptor subtypes and signal transduction pathways, and subsequent intersection with other signalling systems.

Waterhouse: This discussion has raised a more general point, that the exact method by which the study is done will influence the kind of result which is obtained. The larger problem to me is the question of how the *in vitro* work relates to the *in vivo* situation. The SCN has many inputs from many brain areas, yet what, essentially, you have described is a similarity between what is found in the total system and what is found in your isolated system. That similarity seems to decrease the importance of what's coming in to the SCN.

Moore: We always hope that the *in vitro* system will provide information about the *in vivo* system, but it's clear that it provides information which is flawed in various ways. Dr Gillette gets nice phase advances but rarely gets phase delays, whereas NPY given *in vivo* produces phase delays, as do dark pulses. The *in vitro* situation is not giving us the same kind of information that the *in vivo* situation is. This system is so tremendously regulated *in vivo* that the *in vitro* system will never approximate it.

Gillette: I agree that there are differences, but we should learn something from them. It's difficult to know that one treatment *in vivo* is really affecting the sites that would normally be stimulated as a consequence of handling or cage changing, for example, and to know that the injected substance is itself the primary player.

Meijer: We have applied glutamate in the *in vivo* situation through cannulae implanted into the SCN. Injection of glutamate produces a PRC like that produced by a dark pulse, whereas you found a light pulse-like PRC *in vitro*. We have always been afraid that glutamate injections in the SCN stimulate the cells at non-physiological sites, and that this was why we obtained this PRC which we did not expect. A possible explanation for our unexpected PRC is that by injecting glutamate into the SCN *in vivo* we also stimulate pathways to the intergeniculate leaflet, for example, and in turn stimulate the projection from the IGL to the SCN which would enhance the release of NPY and GABA, which may produce a dark pulse-like PRC. *In vivo*, you stimulate all these pathways, which in turn stimulate the SCN.

Mrosovsky: Another difference between the *in vivo* and *in vitro* experiments is that the amplitudes you get from slices tend to be large. However, the glutamate-induced phase shifts *in vitro* seem to be an exception. What is the amplitude of the PRC produced by a light pulse in the rat, and how closely does this correspond to what you are getting in the slice with glutamate?

Gillette: We noticed from the beginning that we got phase shifts of large amplitude. One of the first things I did was to apply depolarizing stimuli which induced phase shifts of huge amplitude. We thought this was because when we made the slice we were cutting away the feedback loops which would normally dampen any change. This makes adaptive sense, because there's no reason for an animal suddenly to undergo a phase shift of seven hours. It was quite a surprise to find that dropping glutamate onto the SCN gave us something that looked so much like the photic response. The results suggest that this phase shift is processed primarily within the SCN. Light is one of the strongest entraining stimuli, so perhaps this makes sense. It's really interesting that we see a delay that looks like that produced in response to light and an advance that also has an amplitude of the same order of magnitude as that produced by light.

Mrosovsky: My other point relates to the phase response to NPY, and the apparent differences between Albers & Ferris's (1984) PRC and your bimodal curve. Albers and Ferris's work was a pioneering effort, but their PRC was based on rather few points. In the delay area, near where you found your second advance area, there was one advance point and two delay points, with the average as a delay. That is not enough really for comparisons between different preparations.

Gillette: This finding of two sensitive periods was an unexpected result. This must be interpreted as being what the SCN is capable of when it experiences only NPY in the ventrolateral region. The sensitivity to NPY changes *in vitro*, so it must be regulated by whatever is ongoing and changing over 24 h *within* the SCN.

Shinohara et al (1992) showed that there is increased NPY release in the SCN just after light–dark and dark–light transitions *in vivo*. For either of these NPY releases to affect SCN phase, the photic transitions must intersect the SCN's sensitive periods. Because NPY injected into the SCN *in vivo* causes advances in subjective day, like the *in vitro* response, but at night causes delays, the opposite phase changes from *in vitro* responses, additional regulators must participate nocturnally *in vivo*.

Miller: It is definitely the case that there are conditions in which the *in vivo* situation reflects the *in vitro* situation. Injection of a serotonin agonist into the third ventricle induces phase advances at CT 6 and delays at CT 18 *in vivo* (Edgar et al 1993). It is true that the magnitude of those phase shifts was smaller than we find in SCN slices, but the time course was qualitatively the same.

Daan: Another aspect which is not fully appreciated is that these measurements are made on the first day after the pulse—these are immediate responses. One does not see phase advances in the activity of the whole animal on the first day after a pulse. This is an important difference, which suggests that the pacemaker is reset much more rapidly then the whole system. Do you see differences between Day 1 and Day 2, Dr Gillette, or can you say that there are no transient changes in phase at all in these slices?

Gillette: We have not been able to see what I would call transients. We are cautious about interpreting any change of less than an hour as a significant change. Everything that we have used to induce a phase shift we have checked in two subsequent cycles; in each case, the peak appears 24 h after the first shifted peak, suggesting the system has shifted fully in the first day after the treatment.

Menaker: A classic observation made with *Drosophila* years ago was that whereas phase shifts produced by light are 'permanent', and reset the whole system, others, such as those produced by temperature steps, are transient and decay rapidly (Pittendrigh et al 1958). In your paradigm it is difficult, if not impossible, to distinguish permanent and transient phase shifts and therefore it will be extremely difficult to make the correlation between *in vitro* and *in vivo* results.

Gillette: I don't know to what extent mechanisms could be evaluated from the results with temperature steps, but you might suggest that a permanent resetting involves a fundamental change in the biochemical machinery, whereas a shift in temperature might change the kinetics of something which is not tweaking the clock mechanism, whatever that is.

Menaker: The results in *Drosophila* were explained convincingly and elegantly in terms of the action of a temperature change on one of two coupled oscillators.

Gillette: Was a second treatment given? In Pittendrigh's classical study (1974), the speed of phase resetting was assessed through the effect of double pulses of light on *Drosophila* eclosion rhythms. When two pulses of light were given within one cycle, the response was the sum of the individual responses, suggesting that the pacemaker's state was rapidly shifted to a new time point after the first treatment and it was on this new time state that the second pulse acted. Similarly, Dr Hastings has shown that handling of the animal can change within one hour the ability of light to induce c-*fos* (Mead et al 1992).

Menaker: Two-pulse experiments have been done with *Drosophila*.

Gillette: With the temperature steps?

Edmunds: No, but experiments with temperature pulses were done, which gave steady-state results quite different from those obtained with temperature steps (Zimmerman et al 1968). The results with the pulses could be predicted from the algebraic addition of the separate effects yielded by the steps down and steps up of which the pulses were formally composed.

Lewy: It's impressive that the second day's phase shift is so much less than that after the first day. This suggests that much of the shift occurs within one day. Have you done, or are you considering doing, a two-pulse experiment within one day, to see how instantaneous the phase shift is?

Gillette: That would be a good experiment.

When I gave my first talk on the SCN at the Society for Neuroscience meeting in 1985, Dr Block asked me whether the phase shift *in vitro* was instantaneous; since I was new to the field, I asked what an instant was for a clock. For the first molecular interaction, is it five minutes? We know that our five-minute treatments can produce long-lasting change, but we are setting into motion a cascade of events.

Edmunds: The possible differences between PRCs based on data from Day 1 and data from later on are important, although in your system you are limited, of course, by the time the preparation will survive *in vitro*. Do you find lasting, stable, phase shifts with pulses of cAMP and its agonists or antagonists?

Gillette: They're stable over at least two cycles.

Edmunds: Do you also apply forskolin as a pulse?

Gillette: Yes.

Edmunds: I ask because we have done the same thing in *Euglena*, but we also tried an experiment in which we added saturating concentrations of forskolin to build up the concentration of cAMP (Carré & Edmunds 1993). We reasoned that if we wiped out the normal bimodal cAMP signal, the *rhythm* downstream would be wiped out if it were mediated by the bimodal cAMP signal. Cell division suddenly became essentially arrhythmic, though not stopped. Have you done any experiments like that, blocking not the function but the rhythm?

Gillette: None of the agents I spoke about in my presentation wipes out the rhythm, but some things do. All I can say is that with some treatments we can't measure a rhythm of electrical activity, which is our measure of clock functioning. Several treatments given at night make it impossible to determine phase in the next cycle as there is no clear peak. Since we don't maintain the slices for more than two cycles after treatment, we haven't pursued those agents.

Hastings: Apart from glutamate, most of your treatments seem to have advancing effects. If these influences are operational in the animal's life, are you not surprised that when you take the SCN out and isolate the slice from those influences that the period doesn't lengthen?

Gillette: You might be surprised by that. All I can say is that we haven't been able to observe a lengthened period. The real surprise is that the period we see is so close to 24 h even though we have variable amounts of SCN tissue. Longitudinally in time, the rhythm is running at slightly less than 24 h. We would like to look at this more carefully.

Hastings: Do you know the individual periods of your rats before you prepare the slices?

Gillette: No. It would be useful to know that.

Block: I understand that you have found conductance rhythms (Gallman & Gillette 1993), like those we have observed in *Bulla*, with conductance being low when impulse activity is high, and high when impulse activity is low. Perhaps K^+ channels are closing, causing an increase in activity. The only caveat is that there may be inhibitory synapses that are active at night, which would also give an increase in conductance. Nevertheless, it's reassuring these two systems show the same changes in conductance.

Gillette: There is even more reason to think that conductance changes are basic clock features. A recent study on a plant organ, the monkey pod leaf,

has shown circadian conductance rhythms in K^+ channels in opposing regions of the leaf-movement organ (Kim et al 1993).

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