Journal of Biological Rhythms, Vol. 8, Supplement, 1993, pp. S53–S58

The Organization of the Suprachiasmatic Circadian Pacemaker of the Rat and Its Regulation by Neurotransmitters and Modulators

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Abstract The long-term goal of our research is to understand how cells of the suprachiasmatic nucleus (SCN) are organized to form a 24-hr biological clock, and what roles specific neurotransmitters and modulators play in timekeeping and resetting processes. We have been addressing these questions by assessing the pattern of spontaneous neuronal activity, using extracellular and whole-cell patch recording techniques in long-lived SCN brain slices from rats. We have observed that a robust pacemaker persists in the ventrolateral region of microdissected SCN, and have begun to define the electrophysiological properties of neurons in this region. Furthermore, we are investigating changing sensitivities of the SCN to resetting by exogenous neurotransmitters, such as glutamate, serotonin, and neuropeptide Y, across the circadian cycle. Our findings emphasize the complexity of organization and control of mammalian circadian timing.

Key words brain slice, glutamate, neuropeptide Y, rat, serotonin, suprachiasmatic nucleus, ventrolateral SCN, whole-cell patch recording

The central role of the suprachiasmatic nuclei (SCN) in the mammalian circadian system is well established. An endogenously pacemaking tissue, the SCN exhibit a near-24-hr period in intrinsic rhythms of electrical activity and vasopressin secretion (Earnest and Sladek, 1987; Gillette and Reppert, 1987; Prosser and Gillette, 1989). Outputs from this central pacemaker supply a time base for circadian rhythms in cellular, tissue, and organismic functions. Behavioral circadian rhythms are reset differentially over the 24-hr circadian cycle by variables that include environmental lighting (DeCoursey, 1964; Boulos and Rusak, 1982) and activity state (Mrosovsky and Salmon, 1987). These phase-resetting stimuli must affect the SCN through input pathways, such as those from the retina (Moore and Lenn, 1972), intergeniculate leaflet (Swanson et al., 1974; Card and Moore, 1982), or dorsal raphe (Aghajanian et al., 1969; Moore et al., 1978). However, little is known about the way in which the cellular components of the SCN are organized to carry out timekeeping or to analyze phase-resetting information. We have been seeking to determine (1) the functional organization of the SCN through electrophysiological analyses of regional distribution of pacemaking properties and neuronal characteristics, as well as (2) the circadian nature of SCN pacemaker regulation by neurotransmitters and modulators.

We have been using the hypothalamic brain slice (Hatton et al., 1980) to study the functional organization of the SCN directly. Slices are prepared from Long–Evans rats,

raised to 5–10 weeks of age on a light–dark cycle (LD 12:12) in our inbred colony. Our previous work established that circadian pacemaking and resetting properties are endogenous to the SCN in slices and can be analyzed *in vitro* (for a review, see Gillette, 1991). The circadian rhythm of SCN electrical activity was recorded extracellularly in intact and microdissected slices of rat hypothalamus for 1–3 days after slice preparation. Persistence of a rhythm in microdissected subregions was examined. Whole-cell patch recording in slices (Blanton et al., 1989) of single SCN neurons was performed over the circadian cycle to assess the range of electrophysiological features of SCN neurons, together with diurnal changes in electrical properties. Neurotransmitters and neuromodulators were applied focally with a micropipette to their SCN projection sites; effects on the phase of the electrical activity rhythm were determined from the behavior of the ensemble of single units. In addition, the levels of glutamic acid decarboxylase (GAD, the biosynthetic enzyme for the inhibitory neurotransmitter γ-aminobutyric acid [GABA]) in SCN micropunched from brain slices were assessed over the circadian cycle by Western blotting. In experiments aimed at understanding regulation by retinohypothalamic afferents, Dr. Michael Rea's laboratory has examined several parameters after stimulation of the optic nerve (the release of excitatory amino acids, field potential activity, and sensitivity to pharmacological blockade of SCN *in vitro*); these results are discussed in the paper by Rea and colleagues in this issue.

Hypotheses tested in our research include the following: (1) Pacemaking properties are distributed throughout the SCN; (2) the neurons of the SCN are homogeneous with respect to their electrical and pacemaking properties; (3) neuromodulators from inputs implicated in phase shifts of behavior by dark pulses (serotonin [5-HT] from the raphe, neuropeptide Y [NPY] from the intergeniculate leaflet) are effective phase-shifting agents for SCN during the circadian day; (4) GAD levels are constant over the circadian cycle; and (5) light information carried by the retinohypothalamic tract affects the SCN via excitatory amino acids (viz., glutamate). Our progress toward evaluating these hypotheses is presented here.

Regarding the first hypothesis, cellular organization of the SCN pacemaker was examined by determining whether regional variation in the circadian oscillation in ensemble neuronal activity was expressed in the intact SCN brain slice. Post hoc analysis of the pattern of activity recorded on day 2 *in vitro* revealed that, indeed, both the ventrolateral (VL) and dorsomedial (DM) subregions of the SCN exhibited pronounced activity peaks (Tcheng et al., 1989). These were not apparently altered by bisecting the brain slice at the base of the third ventricle, which severs connections between the bilaterally paired SCN. In other words, each SCN appears to be an autonomous pacemaker.

The rat SCN pacemaker has two natural anatomical subdivisions: (1) the VL SCN, site of the relatively larger neurons (mean diameter 9.6 ± 1.5 μm), including those containing vasoactive intestinal peptide (VIP) upon which afferents from the retina, intergeniculate leaflet, and dorsal raphe form synapses (van den Pol and Tsujimoto, 1985; Guy et al., 1987); and (2) the DM SCN, which is composed of relatively smaller vasopressin-containing neurons (mean diameter 7.8 ± 0.9 μm) that give rise to numerous efferent fibers (van den Pol, 1980). We hemisected each SCN so as to separate the VL SCN from the DM SCN, in order to determine the pacemaking ability of each region. When activity in a single intact SCN was monitored continuously for 32 hr, the characteristic sinusoidal circadian pattern in firing frequency (Gillette and Prosser, 1988) was observed with high-amplitude peaks at 24-hr intervals (Gillette et al., 1992). Surprisingly, hemisection did not affect this pattern in the VL SCN, whereas amplitude and shape of the neuronal activity rhythm appeared altered in

the DM SCN after surgical isolation (Tcheng and Gillette, 1990; Gillette et al., 1992). These preliminary results are consistent with the alternative to the hypothesis tested—namely, that there may be localization of pacemaking function within the SCN.

In order to address the second hypothesis (the issue of neuronal heterogeneity at the cellular level), we have begun to examine individual neurons of the SCN, using the whole-cell patch recording technique (Blanton et al., 1989) in the rat brain slice. Initial efforts were concentrated in the VL SCN, although we have recently begun to extend our observations to the DM SCN. By using this approach, we have found the SCN to be composed of a variety of electrophysiologically distinct cell types (Gallman et al., 1991; Gallman and Gillette, 1993). Our observations challenge the hypothesis, which had been suggested by early workers in the field (Wheal and Thompson, 1984), that the SCN is electrically homogeneous. Furthermore, preliminary findings concerning DM SCN neurons do not contradict the alternative hypothesis—that there *are* regional differences in properties of neurons of the VL and DM SCN.

The third hypothesis concerns the potential role of 5-HT and NPY in mediating phase shifts induced by behavioral arousal and dark pulses, respectively. This was tested by applying a 30-fl droplet of either 10^{-6} M 5-HT or NPY to the VL SCN in brain slices. Effects upon the rhythms of neuronal activity of 5-min microdrop applications at various points in the circadian cycle were assessed over 1–2 days posttreatment. These experiments were designed to evaluate the permanence, receptor specificity, and dose dependency of phase changes, compared to the responses of controls treated with microdrops of medium. SCN sensitivity to 5-HT was restricted to the subjective day (circadian times 2–11 [CTs 2–11], with peak phase advance occurring at CT 7 (Fig. 1). Both 5-carboxyamidotryptamine (5-CT), a 5-HT_1 receptor agonist, and 8-hydroxy-2-*n*-propylamino)-tetralin (8-OH-DPAT), an agonist specific for the 5-HT_{1A} receptor subtype, also induced large advances in the peak

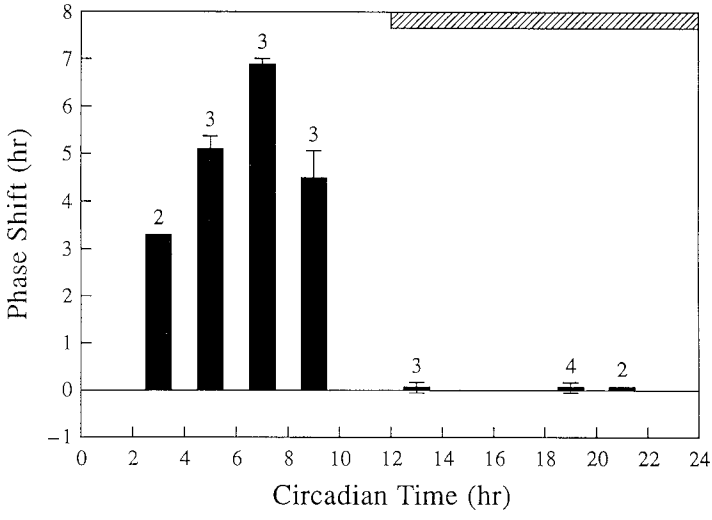

FIGURE 1. Phase response curve relating the time of 5-HT treatment to the time of appearance of the peak in ensemble neuronal firing rate in the next circadian cycle. For treatments at CT 7, the time of maximal phase advance, activity was monitored for 2 days posttreatment. The peak on day 3 appeared at CT 0, 24 hr after that on day 2, demonstrating that the phase shift measured on day 2 was complete. The hatched horizontal bar represents subjective night. Adapted from Medanic and Gillette (1992a.)

of the electrical activity rhythm in SCN *in vitro*; these treatments were without effect during the subjective night (Medanic and Gillette, 1992a). Thus, the regulatory effect of 5-HT on the neurons of the VL SCN appears to be mediated through a 5-HT_{1A} receptor subtype. Interestingly, the SCN in slices shows both late-day and late-night windows of sensitivity to NPY-induced phase resetting (Medanic and Gillette, 1993). The daytime period of sensitivity to NPY is distinct from, but overlaps with, the latter portion of 5-HT sensitivity (Medanic and Gillette, 1992b). These results are consistent with roles for 5-HT and/or NPY in nonphotic (Mrosovsky and Salmon, 1987) and/or dark-pulse (Boulos and Rusak, 1982; Ellis et al., 1982; Albers and Ferris, 1984) entrainment processes.

Next, we examined the hypothesis that levels of GAD remain constant over the circadian cycle. GAD is the biosynthetic enzyme for the most abundant inhibitory neurotransmitter in the SCN, GABA. Between 50% and 100% of SCN neurons are GAD-positive (van den Pol and Moore, personal communication). GABA-ergic neurons, those containing GAD, are distributed throughout the nucleus (van den Pol and Tsujimoto, 1985), and GABA administration inhibits 65% of SCN neurons (Liou et al., 1990). The amplitude of the daily oscillation of mean spontaneous firing frequency for rat SCN neurons shows an excursion between 8 Hz at midday and 2 Hz at midnight. This may be regulated, at least in part, by changing GABA levels, which in turn would be controlled by changing GAD levels and/or GAD activity over the course of the circadian cycle. To enable us to evaluate this issue, SCN from brain slices maintained *in vitro* were rapidly frozen on dry ice, and the SCN were removed by micropunch. Western blot analysis of the two major GAD isozymes, GAD_{65} and GAD_{67}, demonstrated that both were present in SCN at the four 6-hr intervals examined, and that the levels underwent a significant circadian variation (Richard et al., 1991). Whether circadian modulation of GAD activity also affects GABA biosynthesis in the SCN is currently under investigation.

The fifth hypothesis tested in this research regards the potential role of excitatory amino acids in mediating the effects of light at night in this system. These experiments have been addressed primarily by Dr. Michael Rea's laboratory, as discussed in Rea et al.'s paper in this issue. Our laboratory has begun to evaluate the effect on the circadian rhythm of ensemble neuronal activity of the excitatory amino acid glutamate (GLU). GLU (at 10^{-2} M in a 1-μl droplet of medium) was applied for 10 min to one SCN *in vitro*. Preliminary results suggest that this brief GLU application can induce both phase delays and advances at night, and strengthens the possibility that GLU may mediate the phase-shifting effects of light on the SCN pacemaker.

With the finding that pacemaking properties reside in the VL SCN (at least), we can proceed to focus upon the electrophysiological properties of this region. Differences between the VL and DM SCN will be interesting to document. Circadian periods of sensitivity to 5-HT, NPY, and GLU, tentatively described in this report, differ from those we have described for cyclic adenosine monophosphate (Prosser and Gillette, 1989), cyclic guanosine monophosphate (Prosser et al., 1989) and melatonin (McArthur et al., 1991), emphasizing the complexity of SCN regulatory processes. Because the SCN integrate most circadian behaviors and metabolic fluxes, this research has basic relevance to understanding circadian dysfunction induced by transmeridian travel and sustained irregular work schedules, with application to improving human performance under conditions that induce circadian desynchronization.

ACKNOWLEDGMENT

Generous support from the University Research Initiative of the Air Force Office of Scientific Research, Grant No. AFOSR 90-205, is gratefully acknowledged.

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