Brain Research Bulletin, Vol. 19, pp. 135–139. © Pergamon Journals Ltd., 1987. Printed in the U.S.A. 0361-9230/87 $3.00 + .00

RAPID COMMUNICATION

The Hypothalamic Suprachiasmatic Nuclei: Circadian Patterns of Vasopressin Secretion and Neuronal Activity *In Vitro*

MARTHA ULBRICK GILLETTE[1]

Department of Physiology and Biophysics and The Neural and Behavioral Biology Program
University of Illinois, Urbana, IL 61801

AND

STEVEN M. REPPERT

Laboratory of Developmental Chronobiology and Children's Service
Massachusetts General Hosptial and Harvard Medical School, Boston, MA 02114

Received 3 April 1987

GILLETTE, M. U. AND S. M. REPPERT. *The hypothalamic suprachiasmatic nuclei: Circadian patterns of vasopressin secretion and neuronal activity* in vitro. BRAIN RES BULL **19**(1) 135–139, 1987.—The suprachiasmatic nuclei (SCN) are intrinsic pacemakers which organize circadian rhythms in mammals. When the SCN of Long-Evans rats are surgically isolated and perifused *in vitro*, they retain the ability to express a 24 hr rhythm of neuronal firing rate. We find that the SCN are also capable of secreting the peptide vasopressin (VP) in a circadian pattern. The pattern of VP secretion is similar to that of SCN neuronal electrical activity measured during perifusate collection. The temporal profile of VP levels in SCN perifusate parallels that seen in cerebrospinal fluid, suggesting that the SCN might be both the pacemaker and a secretory contributor to this rhythm.

Brain slice Circadian rhythms Electrophysiology Hypothalamus Neuronal oscillator Suprachiasmatic nuclei Vasopressin

THE suprachiasmatic nuclei (SCN) of the anterior hypothalamus perform a primary pacemaking function for circadian rhythms in mammals. The intrinsic pacemaking properties of these nuclei were demonstrated when the SCN were surgically isolated from the animal and cultured using the hypothalamic brain slice technique [9–11, 25, 26]. Under these conditions SCN neuronal activity continues the circadian pattern of oscillation previously measured with multiunit electrodes implanted in the intact animal [16]. Thus, the ability to keep 24-hr time is endogenous to the SCN and survives removal from the animal.

The daily oscillation in SCN neuronal firing rate is most probably an expression of a primary cellular pacemaker within the nuclei. This electrical oscillation would serve to transmit temporal information from the SCN to other brain regions which control specific behavioral and physiological rhythms [10, 15, 17]. We undertook to determine whether arginine vasopressin, the major peptidergic component of the SCN [13, 28, 33], might also show circadian rhythmicity, and how this rhythm might be related to the electrical oscillations of the SCN as well as the SCN-driven [23] rhythms of VP levels observed in cerebrospinal fluid (CSF) [20, 21, 24].

Long-Evans rats were bred in our Illinois colony to reduce individual differences and reared to 2–5 months of age. Animals were housed on a 12 hr:12 hr light-dark cycle (lights on from 0700 to 1900 hr), some with a day-reversed schedule (lights on from 2030 to 0830 hr). At the initiation of an experiment, a cage of male litter-mates was removed from the entrained lighting condition. Experiments were initiated at different points in the animals' light period; dark portions of the cycle were avoided [7–9]. Each rat was decapitated, the brain removed, the hypothalamic region blocked and 400 μm slices cut on a manual chopper. The hypothalamic slice containing the SCN was then surgically reduced to exclude the

[1]Requests for reprints should be addressed to Martha U. Gillette, Department of Physiology and Biophysics, 524 Burrill Hall, University of Illinois, 407 South Goodwin Avenue, Urbana, IL 61801.

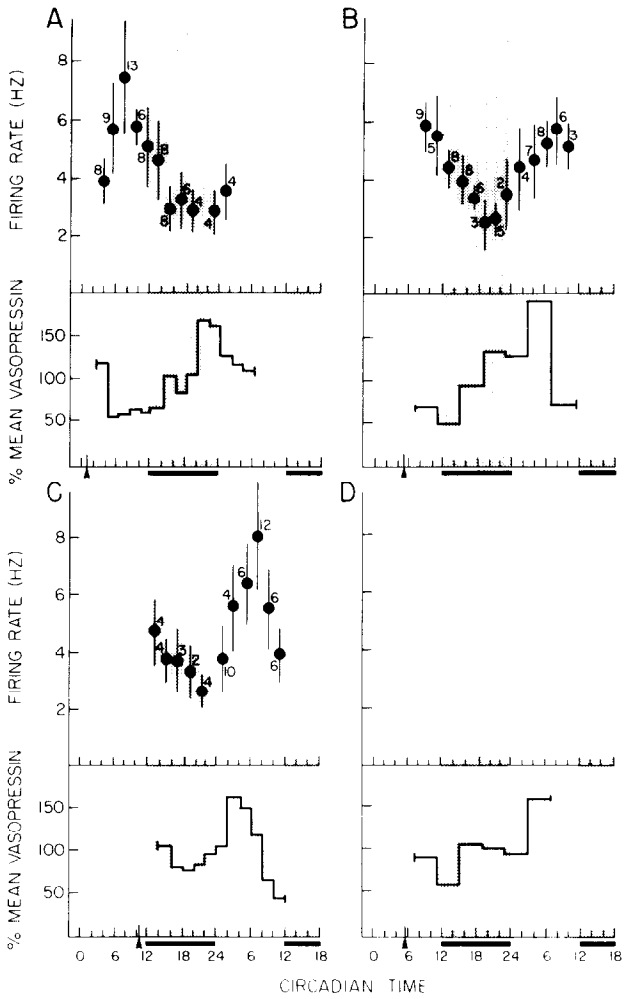

FIG. 1. Circadian patterns of oscillation in single unit firing rate and in vasopressin secreted into perifusate from suprachiasmatic slices maintained *in vitro*. Donor Long-Evans rats had been entrained to a 12 L:12 D lighting cycle with lights on from 0700–1900 hr (circadian time 0–12, where 24 hr circadian time starts at dawn and continues for 24 hr) for all panels except 1C where lights were on from 2030–0830 hr. In each experiment, three sibling Long-Evans rats which had been caged together were used as donors. Mean time of preparing the slices for each experiment is marked by an arrow. After a 1 hr equilibration period of the SCN in the brain slice chamber, perifusate collection and electrical recordings commenced. Neuronal firing rates for single units were analyzed in 2-hr bins ±standard error. Superscripts denote the number of units recorded during that interval. VP data are expressed as percentage change from the daily mean of each experiment. Actual levels of VP release/SCN/4 hr period are: 19–40 μU (A), 24–93 μU (B) 13–45 μU (C) and 9–23 μU (D). Shaded portion designates the subjective night based upon the donor's lighting cycle. (1A) Patterns of single unit firing rate and vasopressin secreted into perifusate for SCN from an experiment initiated at CT 0.5, one half hour after lights on in the colony from 0700–1900. After slice equilibration, perifusate was collected over 2-hr periods for 28 hr. (1B) Patterns of oscillation for SCN electrical activity and VP secretion for SCN from rats sacrificed at CT 5.0–6.4. Perifusate was collected in 4-hr bins for 28 hr. (1C) Patterns of firing rate and perifusate vasopressin of SCN from rats maintained on a reverse lighting cycle. SCN were isolated from three male sibs housed in a different building, in an isolation room with lights on from 2030–0830 (CT 0–12). Animals were sacrificed at CT 10.0–11.0 equilibrated and then electrical recordings were initiated while perifusate was sampled over 2-hr periods for 22 hr. (1D) Pattern of VP secretion from an early experiment in which firing rate was not recorded. Slices were prepared between CT 5.0–6.0 and after 1 hr equilibration VP was collected in 4-hr bins.

supraoptic and paraventricular nuclei. What remained was the SCN, a rim of adjacent hypothalamus and the ventral optic tract.

Three slices at a time were placed in a brain slice chamber [12] in constant light and perifused with Earle's Balanced Salt Solution (GIBCO) supplemented with $NaHCO_3$ (26.2 mM) and glucose (24.5 mM), and bubbled with 95% O_2:5% CO_2. Flow rate was maintained at 34 ml/hr. After a one hour equilibration period, perifusate was collected over 2- or 4-hr intervals into ice cold silanized glass bottles. Immediately after collection, each bottle was transferred from ice to −70°C. Samples were air expressed on dry ice to Boston where they were analyzed blind for VP (see below).

Simultaneous with perifusate collection, firing rate of single neuronal units was recorded extracellularly in one slice by an electrode through a small aperture at the top of the chamber. Single unit firing rates were recorded with glass microelectrodes advanced with an hydraulic microdrive. After stabilization, each unit's firing rate was counted in 24 sequential 10-sec bins. The electrode was than advanced until a new unit was isolated and the counting procedure was repeated. At the end of a pass through the tissue, the electrode was moved at random to a new site in the SCN and the sampling procedure repeated. Units were sampled for at least 24-hr *in vitro* and analyzed in 2-hr bins. We have documented that the population of neuronal activities sampled in this way demonstrates a clear circadian rhythm of firing rate with a well defined peak [9,10].

For VP analysis, a 40-ml portion of each fraction of perifusate was passed through a C18 Sep-Pak column [1]. The peptide was eluted off the column, the elutant was dried and the residue was reconstituted in assay buffer. With this procedure the recovery of vasopressin off of the column was greater than 90% [24]. Vasopressin concentrations were determined (duplicate determinations) by a fully validated [19] RIA system [27].

VP concentrations, expressed in μU, are based on USP posterior pituitary extract reference standard. The limit of assay sensitivity was 0.08 μU/tube. The intra- and inter-assay coefficients of variation were 7% and 15%, respectively.

The results of four separate experiments over a 1½ year period show that vasopressin is released by the SCN in a circadian manner *in vitro* (Fig. 1). Quantitative variation between experiments ranged from 9–23 μU VP/SCN/4 hr in one experiment to 24–93 μU VP/SCN/4 hr in another. Diurnal rates of secretion of VP, analyzed in 4-hr bins, varied from 2.0–4.0 fold. Thus, in order to compare patterns from

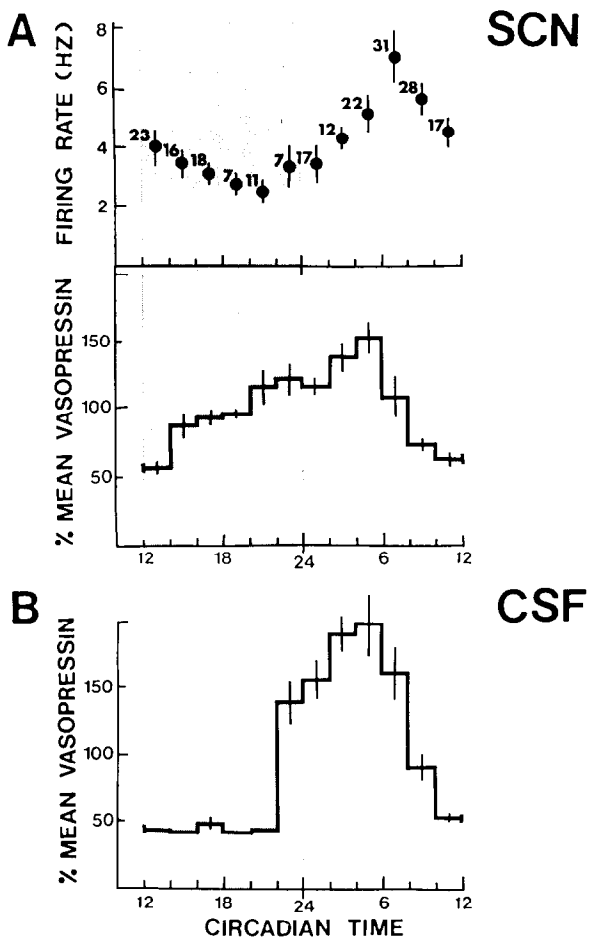

FIG. 2. (A) Population profiles for circadian patterns of single unit firing rates (top) and perifusate vasopressin of isolated SCN *in vitro*. Each value is the mean±standard error of all samples collected in each time period for which there were at least three replications over the course of the study. (B) Population profile for CSF vasopressin from six freely-behaving Long-Evans rats replotted from the study of Schwartz, Coleman and Reppert ([24] Fig. 3). Rats had been implanted with intracisternal cannulae through which CSF was sampled at 2-hr intervals. VP was analyzed as in the present study. Each value is % mean VP ±standard error.

different experiments, data were expressed as percentage of change from the daily mean for each study.

The pattern of VP secretion from SCN *in vitro* was relatively independent of the time at which the experiments were initiated. In the experiments reported in Fig. 1, slices were prepared at CT 0.5–1.5 (1A), CT 5.0–6.4 (1B), CT 10.0–11.0 (1C) and CT 5.0–6.0 (1D). In each case, the continuing course of secretion shows a pattern more closely related to circadian time of the donor than to the time after removal from the animal. These data suggest that secretion is timed by a mechanism endogenous to the SCN.

One-way analysis of variance [30] of the VP population profile (Fig. 2A) indicates the presence of a significant ($p<0.01$) daily rhythm. VP levels are lowest in the late subjective day and early subjective night, CT 10.0–14.0 (Fig. 2A). They increase after CT 14, and stay near the diurnal mean during the rest of the night. A distinct peak occurs early in the subjective day CT 2.0–6.0. Secretion then falls so that by 4–6 hr after the peak, levels have fallen to the diurnal low.

In three of the four experiments, we also examined the relation between the VP oscillation and that of electrical activity in the same reduced piece of tissue (Fig. 1A–C). Within these experiments the patterns of VP secretion and electrical activity from SCN of animals maintained on the 0700–1900 hr lighting schedules (Fig. 1A, B) bear close similarity to those of SCN of animals from the same breeding colony which had been housed for 2 months in a different building, with different caretakers, under reversed day lighting conditions (Fig. 1C, lights on from 2030–0830 hr). This supports the notion that light alone is the primary zeitgeber for both of these intrinsic SCN circadian rhythms.

In these experiments (Figs. 1, 2) the patterns and time of peak of the oscillation in single unit firing rate are very similar to those previously reported for SCN slices *in vitro* [9,10], confirming the health of these slices. There is some variability in the VP patterns we measured, yet general comparisons with the pattern of electrical activity can be made. The patterns are similar in that both activities rise before dawn and peak in the subjective day although there is not a point for point correspondence of VP secretion with the recorded electrical activity of the neuronal population. The maxima and minima of VP secretion appear to lead the maxima and minima of electrical activity. However, more experiments are necessary to elucidate precise phase relationships.

Our data show that rat SCN maintained *in vitro* can express a diurnal pattern of VP release. The pattern is fairly regular and repetitive between experiments: peak levels of VP release in the early subjective day alternate with low levels around subjective dusk. This pattern is not determined by the point at which the experiment was initiated [22], suggesting that the time-keeping and secretory mechanisms of VP release from SCN are affected little by their removal from the animal. The pattern is also independent of real time, demonstrated in animals from a reversed lighting cycle. Thus, SCN secretion of vasopression appears to be timed by the SCN's circadian clock that has entrained to the donor's lighting regime.

Earnest and Sladek report that rat SCN explants maintained in either static or perifused tissue culture systems show a diurnal oscillation of VP in the media for up to four days *in vitro* [5,6]. Strain differences between patterns of VP in CSF of Sprague-Dawley and Long-Evans [24] may account for slight differences in the shapes of the VP curves between their study and ours, as may differences in the culture systems (static culture or low flow rate of enriched medium over a horizontally cut explant vs. high flow rate of a minimal salt solution over a coronally cut SCN slice). Nevertheless, both of these demonstrations show that circadian oscillation in levels of VP secretion is an intrinsic property of SCN tissue.

The diurnal pattern of VP secreted from the isolated SCN into the brain slice perifusate closely resembles that for VP levels in the CSF of Long-Evans rats (see Fig. 2B, redrawn from [24]). Increases in CSF VP also anticipate dawn, reaching peak levels early in the subjective day. That study found some variation between individual rat's CSF VP profiles, but the CSF pattern is generally highly regular, with the mean peak between CT 2.0–6.0 (Fig. 2B), the same period as peak secretion from the SCN (Fig. 2A). Levels in CSF fall to a diurnal low in the late subjective day as they do during secretion from the SCN. However, CSF levels remain sub-

threshold until shortly before dawn whereas secretion from the SCN rises earlier, during the middle of the subjective night; this subtle difference between the two patterns may reflect the period when VP levels diluted by the CSF fall below the assay limits.

The similarity of the VP pattern of CSF *in vivo* to that for VP secreted by the SCN *in vitro* strengthens the notion raised by lesion studies [23] that the SCN may be both the secretory clock and a source of VP which appears in CSF. There is lack of concurrence over anatomical pathways by which vasopressinergic neurons exiting the SCN might access the CSF [2, 3, 14, 18, 29, 31]. Nevertheless, steady-state mRNA levels for VP show circadian fluctuation only in the SCN, not other vasopressinergic nuclei of the hypothalamus [32]. This finding taken with the present study suggests a direct role for vasopressinergic neurons in the SCN in transmitting temporal information from the SCN to the CSF and brain regions bathed by it.

Oscillations in VP secretion and neuronal firing rate potentially represent separate modes by which the SCN transmit time information to other brain regions. The electrical oscillation may relay time-of-day by efferent neuronal output along a limited number of specialized circuits. The VP secretory rhythm potentially modulates daily rhythm in physiology by a generalized neurohormonal effect [4].

ACKNOWLEDGEMENTS

Special thanks are due Dr. Rhanor Gillette for encouragement and discussions during this series of experiments and Bob Coleman, Mark Banister, Daniel Henshaw and Andrew Dowd for expert technical assistance. This study was supported by NSF BNS grants 83-08551 and 84-19342, by PHS-BRSG RR 7030, DK 38116 and NS 22155. S.M.R. is an Established Investigator of the American Heart Association.

REFERENCES

1. Bennett, H. P. J., A. M. Hudson, C. McMartin and G. E. Purdon. Use of octadecasilyl-silica for the extraction and purification of peptides in biological samples. *Biochem J* **168:** 9–13, 1977.
2. Berk, M. L. and J. A. Finkelstein. An autoradiographic determination of the efferent projections of the suprachiasmatic nucleus of the hypothalamus. *Brain Res* **226:** 1–13, 1981.
3. DeVries, G. J., R. M. Buijs, F. W. Van Leeuwan, A. R. Caffé and D. F. Swaak. The vasopressinergic innervation of the brain in normal and castrated rats. *J Comp Neurol* **233:** 236–254, 1985.
4. deWied, D. Central actions of neurohypophyseal hormones. *Prog Brain Res* **60:** 155–167, 1983.
5. Earnest, D. J. and C. D. Sladek. Circadian rhythms of vasopressin release from perifused rat suprachiasmatic explants *in vitro*. *Soc Neurosci Abstr* **11:** 385, 1985.
6. Earnest, D. J. and C. D. Sladek. Circadian rhythms of vasopressin release from individual rat suprachiasmatic explants *in vitro*. *Brain Res* **382:** 129–133, 1986.
7. Gillette, M. U. Preparation of brain slices from the suprachiasmatic nuclei of rat can reset the circadian clock. *J Physiol* **369:** 55P, 1985.
8. Gillette, M. U. Resetting the circadian clock in the suprachiasmatic brain slice. *Soc Neurosci Abstr* **11:** 818, 1985.
9. Gillette, M. U. The suprachiasmatic nuclei: Circadian phase shifts induced at the time of hypothalamic slice preparation are preserved *in vitro*. *Brain Res* **379:** 176–181, 1986.
10. Green, D. J. and R. Gillette. Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic slice. *Brain Res* **245:** 198–200, 1982.
11. Groos, G. and J. Hendricks. Circadian rhythms in electrical discharge of rat suprachiasmatic neurones recorded *in vitro*. *Neurosci Lett* **34:** 283–288, 1982.
12. Hatton, G. I., A. D. Doran, A. K. Salm and C. D. Tweedle. Brain slice preparation: Hypothalamus. *Brain Res Bull* **5:** 405–414, 1980.
13. Hawthorn, J., V. T. Y. Ang and S. J. Jenkins. Localization of vasopressin in the rat brain. *Brain Res* **97:** 75–81, 1980.
14. Hoorneman, E. M. D. and R. M. Buijs. Vasopressin fiber pathways in the rat brain following suprachiasmatic nucleus lesioning. *Brain Res* **243:** 235–241, 1979.
15. Inouye, S.-I. T. and H. Kawamura. Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. *Proc Natl Acad Sci USA* **76:** 5962–5966, 1979.
16. Inouye, S.-I. T. and H. Kawamura. Characteristics of a circadian pacemaker in the suprachiasmatic nucleus. *J Comp Physiol* **146:** 153–160, 1982.
17. Kow, L.-M. and D. W. Pfaff. Suprachiasmatic neurons in tissue slices from ovariectomized rats: Electrophysiological and neuropharmacological characterization and the effects of estrogen treatment. *Brain Res* **297:** 275–286, 1984.
18. Mitchell, J. A. and J. P. Card. Magnocellular neurosecretory axons terminating in the third ventricle of the golden hamster brain: Ultrastructural and immunohistochemical characterization. *Soc Neurosci Abstr* **11:** 387, 1985.
19. Reppert, S. M., H. G. Artman, S. Swaminathan and D. A. Fisher. Vasopressin exhibits a rhythmic daily pattern in cerebrospinal fluid but not in blood. *Science* **213:** 1256–1257, 1981.
20. Reppert, S. M., R. J. Coleman, H. H. Heath and H. T. Keutmann. Circadian properties of vasopressin and melatonin rhythms in cat cerebrospinal fluid. *Am J Physiol* **243:** E489–E498, 1982.
21. Reppert, S. M., J. W. Schwartz, H. G. Artman and D. A. Fisher. Comparison of the temporal profiles of vasopressin and oxytocin in the cerebrospinal fluid of the cat, monkey and rat. *Brain Res* **261:** 341–345, 1983.
22. Reppert, S. M. and M. U. Gillette. Circadian patterns of vasopressin secretion and SCN neuronal activity *in vitro*. *Soc Neurosci Abstr* **11:** 385, 1985.
23. Schwartz, W. J. and S. M. Reppert. Neural regulation of the circadian vasopressin rhythm in cerebrospinal fluid: A preeminent role for the suprachiasmatic nuclei. *J Neurosci* **5:** 2771–2778, 1985.
24. Schwartz, W. J., R. J. Coleman and S. M. Reppert. A daily vasopressin rhythm in rat cerebrospinal fluid. *Brain Res* **263:** 105–112, 1983.
25. Shibata, S., Y. Oomura, H. Kita and K. Hattori. Circadian rhythmic changes of neuronal activity in the suprachiasmatic nucleus of the rat hypothalamic slice. *Brain Res* **247:** 154–157, 1982.
26. Shibata, S., S. Y. Liou, S. Ueki and Y. Oomura. Influence of environmental light-dark cycle and enucleation on activity of suprachiasmatic neurons in slice preparations. *Brain Res* **302:** 75–81, 1984.
27. Skowsky, W. R., A. A. Rosenbloom and D. A. Fisher. Radioimmunoassay measurement of arginine vasopressin in serum: development and application. *J Clin Endocrinol* **38:** 278–287, 1974.
28. Sofroniew, M. V. and A. Weindl. Indentification of parvocellular vasopressin and neurophysin neurons in the suprachiasmatic nucleus of a variety of mammals including primates. *J Comp Neurol* **193:** 659–675, 1980.

29. Sofroniew, M. S. and W. Weindl. Projections from the parvocellular vasopressin- and neurophysin-containing neurones of the suprachiasmatic nucleus. *Am J Anat* **153:** 391–430, 1978.
30. Sokal, R. R. and F. J. Rohlf. *Biometry*. San Francisco: Freeman, 1969.
31. Stephan, F. K., K. J. Berkley and R. L. Moss. Efferent connections of the rat suprachiasmatic nucleus. *Neuroscience* **6:** 2625–2641, 1981.
32. Uhl, G. R. and S. M. Reppert. Suprachiasmatic nucleus vasopressin mRNA: Circadian variation in normal and Brattleboro rats. *Science* **232:** 390–393, 1986.
33. Vandesande, F., K. Dierickx and J. DeMey. Identification of the vasopressin-neurophysin producing neurons of the rat suprachiasmatic nuclei. *Cell Tissue Res* **156:** 377–380, 1975.