Vasopressin mRNA in the Suprachiasmatic Nuclei: Daily Regulation of Polyadenylate Tail Length

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Daily variation has been found in the length of the polyadenylate tail attached to vasopressin messenger RNA in the suprachiasmatic nuclei, which is the location of an endogenous circadian pacemaker in mammals. No such variation was found in the supraoptic or paraventricular nuclei. This variation in the length of the polyadenylate tail may underlie the circadian rhythm of vasopressin peptide levels in cerebrospinal fluid and is a unique example of a daily rhythm in messenger RNA structure.

DAILY RHYTHMS IN PLANTS AND animals are the overt manifestation of an innate timekeeping mechanism, that is, a "circadian clock" (*1*). Ordinarily, the alteration of light and darkness synchronizes (entrains) the period and phase of these rhythms to the natural 24-hour day-night cycle. In mammals, there is an endogenous circadian pacemaker in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (*2*). Much effort is now directed to identifying neuroactive substances within the nuclei, the best characterized of which is probably the nonapeptide arginine vasopressin.

Immunoreactive vasopressin is found in parvocellular neurons in the SCN of many species (*3*), and the peptide is synthesized within the nuclei. Vasopressin mRNA is present (*4, 5*), and the hormone and its neurophysin are contained in SCN secretory vesicles (*6*). Vasopressin concentrations in cerebrospinal fluid (CSF) exhibit circadian rhythmicity; levels are high during the light portion of a light-dark (LD) cycle and low during the dark portion in a number of mammals (*7*). Furthermore, CSF vasopressin appears to originate in the SCN. Complete lesions of the nuclei both abolish the rhythm and in most cases reduce the levels of CSF peptide (*8*), but rhythmic release persists when knife cuts sever neural efferents in situ (*8*) or when the nuclei are isolated as hypothalamic explants in vitro (*9*). Determining the molecular basis for this peptide rhythm may help to understand how a circadian pacemaker drives a host of cellular metabolic and functional rhythms. We now show that two distinct species of vasopressin mRNA are present in the SCN and that their expression varies with a daily rhythm.

In our first experiment, adult male Sprague-Dawley rats were maintained on a 12:12 LD cycle and killed by decapitation, either 3 hours after lights on (n = 15) or 3 hours after lights off (n = 15). Each brain was removed and placed in a stereotactic apparatus; a coronal slice, extending from the optic chiasm to the posterior border of the hypothalamus, was cut and frozen on a flat surface at −70°C. The brain slice was sectioned anteroposteriorly in a cryostat until a plane slightly anterior to the SCN was reached. The SCN, the supraoptic nucleic (SON), and the paraventricular nuclei (PVN) were sampled by punching with an 18-gauge needle. Adequacy of sampling was confirmed by histological examination of the remaining tissue. RNA was extracted (*10*) from pooled punch samples of SCN, SON, or PVN and analyzed by RNA blotting with a vasopressin complementary RNA (cRNA) probe (*11*) (Fig. 1, a and b). During lights on, a single vasopressin mRNA species, 740 nucleotides (nt) in length (AVP_{740}), was found in the SCN; during lights off, an additional species, 530 nt long (AVP_{530}), was also detected. In the SON and PVN, AVP_{740} was the only species found during lights on and off.

A difference in the length of the polyadenylate [poly(A)] tail fully accounts for the difference in size of the two mRNA species isolated from the SCN. We removed the poly(A) tail (*12*) by hybridizing mRNA to oligo(dT), digesting with ribonuclease H, and analyzing the resulting deadenylated RNA by RNA blot with a vasopressin cRNA probe (Fig. 1c). The deadenylated body of the vasopressin mRNA that was isolated from all nuclei during lights on and

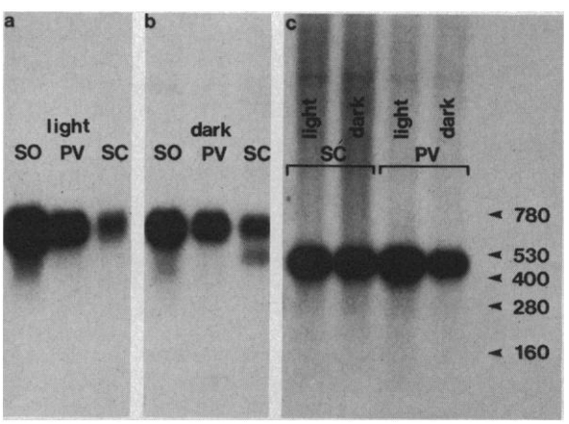

Fig. 1. RNA blot (*26*) of mRNA (5 μg per lane) obtained by micropunching supraoptic (SO) nuclei, paraventricular (PV) nuclei, and suprachiasmatic (SC) nuclei. (**a**) Fifteen animals sacrificed 3 hours after lights on (light). (**b**) Fifteen animals sacrificed 3 hours after lights off (dark). (**c**) Ribonuclease H digestion of poly(A) tail. Blots (a) and (b) were probed with AVP-1, a vasopressin cRNA probe (*11*) complementary to bases 2158 to 2600 of the rat vasopressin gene (*27*). Blot (c) was probed with AVP-2, a cRNA probe complementary to bases 2298 to 2600 of the rat vasopressin gene (*27*). Both AVP-1 and AVP-2 are complementary to portions of exon 3 of the vasopressin gene, which is sufficiently dissimilar to the oxytocin gene to preclude hybridization to oxytocin mRNA. Blots were hybridized with probes at 65°C in 50% formamide and washed at 68°C (*28*). No differences in the size of β-actin mRNA were found after reprobing blots (a) and (b) with a β-actin probe. The sizes, in nucleotides, of RNA molecular size standards (Bethesda Research Laboratories) are at the right of the autoradiogram.

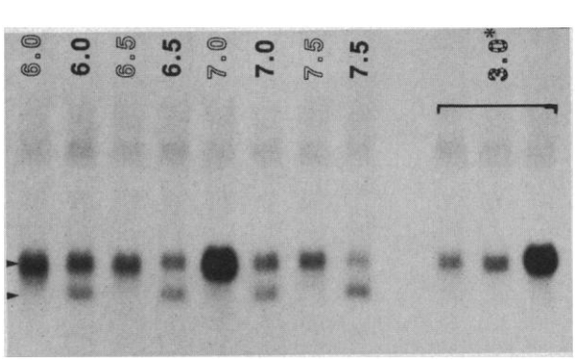

Fig. 2. RNA blot (*26*) of mRNA (20 μg per lane) obtained by micropunching of SCN from individual animals sacrificed 6 to 7.5 hours after lights on (open numerals) or lights off (closed numerals). Positions of the two vasopressin mRNA species are marked (AVP_{740}, upper arrowhead; AVP_{530}, lower arrowhead). Samples were also analyzed from three animals maintained in darkness for 3 hours beyond the usual switch time (numeral with asterisk). The blot was probed with AVP-1.

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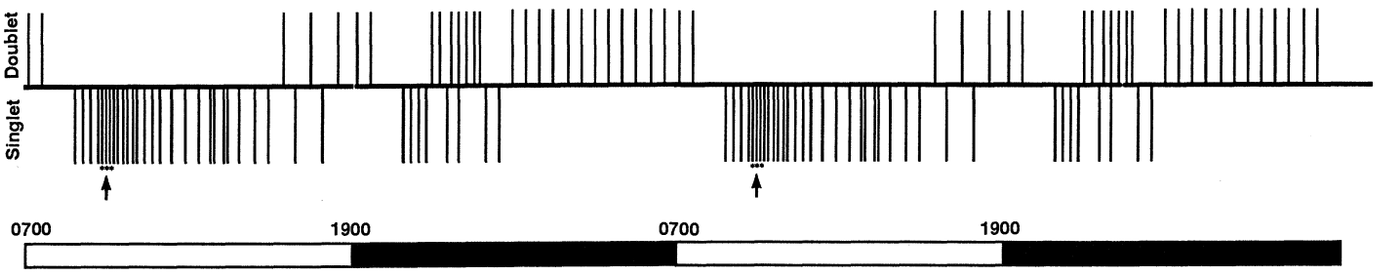

Fig. 3. Results of RNA blot analysis of SCN mRNA from individual animals killed at times indicated (data from one 24-hour period are plotted twice for clarity. The horizontal bar indicates ambient lighting conditions. The presence of both AVP_{740} and AVP_{530} is denoted by "doublet" and of AVP_{740} alone by "singlet." Each vertical line represents one animal. The three animals marked with asterisks were those maintained in darkness for 3 hours beyond the usual switch time (Fig. 2).

lights off migrated as a single 500-nt-long species. Thus, the vasopressin mRNA poly(A) tails in the SCN during lights off were approximately 240 and 30 nt long and accounted for the difference in size of the two vasopressin mRNA species isolated from the nuclei at this time.

To characterize the time course of AVP_{530} appearance in the SCN, we killed additional rats at various times spanning the 24-hour day. In this experiment, two groups of animals were entrained either to a 12:12 LD cycle or to a reversed (DL) cycle. Individual LD-DL pairs were killed at each of 32 time points during the 24-hour day. Punch samples of SCN were obtained, RNA was prepared (*10*) after combining individual punches with skeletal muscle as a carrier tissue, and RNA from each animal was analyzed by RNA blotting. An example of a blot showing RNA from several animals killed in light or dark (6 to 7.5 hours after lights changed) is shown in Fig. 2. The results of RNA blot analysis of all animals are shown graphically in Fig. 3. In all animals AVP_{740} was present at every time examined. However, AVP_{530} appeared 2 to 3 hours before lights off; it was variably expressed during the first half of the dark period but was consistently present during the latter half of the dark period and for the first hour after lights on. No intermediate-sized mRNA species was detected. The abrupt disappearance of AVP_{530} after lights on contrasts with its gradual appearance from 8 to 17 hours later. This may be an artifact of our population data; AVP_{530} may appear abruptly in each animal but at different times within a 9-hour period around the lights off time.

To determine whether the light-induced disappearance of AVP_{530} is an endogenous rhythm entrained to the LD cycle, we entrained three additional rats to the 12:12 LD cycle. In the morning on the day of the experiment, however, lights were not turned on, and the rats remained in darkness until they were killed 3 hours later. As shown in Figs. 2 and 3, the disappearance of AVP_{530} occurred without an environmental lighting cue.

Uhl and Reppert (*5*) used in situ hybridization methods and reported a circadian rhythm of vasopressin mRNA content in the SCN. Although densitometric scans of our RNA blots (*13*) did not show a significant change in mRNA content in the nuclei (3.7 ± 0.5 arbitrary units, mean ± SEM, during lights on and 3.3 ± 0.5 arbitrary units during lights off, $n = 64$), these data were derived from whole SCN punches, whereas the in situ hybridization data (*5*) were obtained by using individual sections of the dorsomedial SCN. In any case, an increase in mRNA content and poly(A) tail length could occur together (*14*), the former perhaps a consequence of the latter.

Our data show a daily alteration of mRNA structure. This variation is restricted to the SCN and appears as an endogenous rhythm entrained to environmental lighting. Its mechanism is unknown; possibilities include separate generation of AVP_{740} and AVP_{530} transcripts from the vasopressin gene, partial deadenylation of AVP_{740} to produce AVP_{530}, or elongation of the poly(A) tract of AVP_{530} by poly(A) polymerase to produce AVP_{740}. Each of these two species might be formed in distinct neuronal populations within the SCN.

Our findings reinforce the concept that the release of vasopressin into the CSF by parvocellular SCN neurons is functionally separate from the secretion of neurohypophyseal vasopressin into the bloodstream by magnocellular SON and PVN neurons. Peptide release into CSF can be dissociated from its secretion into the systemic circulation by various physiological and pharmacological stimuli (*15*). Osmotic stimuli affect levels of vasopressin peptide (*16*) and mRNA (*4, 17*) in the SON and PVN but not in the SCN. Thus, vasopressin may have specific brain functions in addition to its well-established role in the regulation of water balance.

Changes in the size of the poly(A) tail of specific mRNAs occur during embryogenesis (*18–20*) as well as during the stimulation of the expression of specific genes (*14, 21*). These changes in poly(A) tail length may affect mRNA stability (*22*) and translational efficiency (*23*). If the poly(A) tail promotes the stability and enhances the translational efficiency of vasopressin mRNA in the SCN, then its differential expression may underlie the rhythm of vasopressin levels measured in the CSF. Other circadian pacemakers may exert their effects by posttranscriptional control of peptide synthesis. Such a mechanism appears to be responsible for the circadian rhythm of the amounts of luciferin binding protein in the marine dinoflagellate *Gonyaulax* (*24*). Continued molecular dissection of circadian clocks (*25*) may provide new insights to common pacemaker mechanisms.

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12. Total RNA (5 μg) from pooled punch samples was dissolved in 100 m*M* KCl and 0.1 m*M* EDTA and mixed with 10 μg of oligo(dT) (Collaborative Research). The mixture was heated to 65°C for 2 min and incubated at room temperature for 30 min. Ribonuclease H (40 U/ml) (Bethesda Research Laboratories) in 10 m*M* $MgCl_2$, 80 m*M* KCl, 1 m*M* dithiothreitol, bovine serum albumin (0.5 mg/ml), and 50 m*M* tris-HCl, *p*H 7.5, was added and incubated at 37°C for 30 min. RNA was extracted with a mixture of phenol and chloroform, electrophoresed in 1.4% agarose gels containing 2.2*M* formaldehyde, and transferred to GeneScreen (Du Pont).
13. Autoradiograms were scanned with an LKB scan-

ning densitometer. The amount of vasopressin mRNA detected was corrected for the amount of total RNA loaded by quantitating the amount of 18S ribosomal RNA on photographs of ethidium bromide–stained gels.
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29. We thank M. Jacobs for secretarial assistance. Supported in part by grants from the Medical Foundation, University of Sydney and the Royal Australasian College of Physicians, Sydney, Australia (B.R.); the Medical Scientist Training Program (D.F.); National Institute of Neurological and Communicative Disorders and Stroke grant R01NS24542 (W.S.); and NIH grant 1P50HL36568 (J.M.).

18 February 1988; accepted 20 May 1988

Long Lives for Homozygous Trembler Mutant Mice Despite Virtual Absence of Peripheral Nerve Myelin

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Nervous system functions are dependent on point-to-point communication of signals along neuronal axons, and axonal insulation by myelin is thought to speed such conduction. Loss of previously formed myelin or lack of myelin formation can have serious, even fatal, consequences. Mice homozygous for the trembler mutation make virtually no peripheral nervous system myelin, yet have long and functional lives. This result calls into question the view that peripheral nervous system myelin plays a vital role, at least in this species.

AXONS OF CENTRAL AND PERIPHERAL neurons are accompanied along their lengths by supporting glial cells in vertebrates and in many invertebrates, but specialization of those glial cells to form compact myelin is a predominantly vertebrate phenomenon (*1*). Although conduction over axons with glial specializations other than myelin can be quite rapid (*2*), for any given axonal diameter the addition of a myelin sheath, with resultant saltatory conduction, is thought to increase conduction speed markedly (*3, 4*). Loss of previously formed central nervous system (CNS) or peripheral nervous system (PNS) myelin can alter conduction, with devastating consequences, as in the human demyelinating disorders multiple sclerosis (*5*) and the Guillain-Barré syndrome (*6*), respectively. The failure to make an adequate amount of central or peripheral myelin during development is also associated with severe behavioral dysfunction. For example, mice hemizygous for the jimpy mutation, in which CNS but not PNS myelin formation is very deficient, have weakness and seizures and usually die before 1 month of age (*7*). Mice homozygous for the autosomal semidominant trembler-J (Tr^J) mutation have a severe maturational defect such that almost no peripheral myelin sheaths are ever made, although central myelination appears normal (*8*). Such mice have very severe quadriparesis and die before 3 weeks of age. We now report the unexpected observation that mice homozygous for the original dominant trembler (*Tr*) allele (*9*), *Tr/Tr*, also have virtually no PNS myelin, and yet have long and functional lives.

Trembler arose in a noninbred stock (*9*) and is maintained noninbred by ourselves and others. We have placed the *Tr* mutation and the closely linked (1 cM) marker vestigial tail (*vt*) on the same chromosome 11 (*10*). The short tail phenotype expressed by *vt/vt* mice (*11*) serves to distinguish *Tr/Tr* from *Tr*/+ mice, as we have established previously for Tr^J mutants (*12*). With matings of the form *Tr vt*/+ + × *Tr vt*/+ +, short-tailed trembling offspring were *Tr vt/Tr vt* and long-tailed trembling offspring were *Tr vt*/+ + with greater than 95% probability (*12*). Nontrembling offspring were of + +/+ ? genotype. We examined pathological material from 12 *Tr vt/Tr vt* mice ranging in age from 22 days to 10 months, as well as 10 *Tr vt*/+ + and 7 + +/+ ? littermates. Peripheral nerves were examined in all cases; in two littermate trios (*Tr vt/Tr vt*, *Tr vt*/+ +, and + +/+ ?), very extensive sampling was performed, including specimens from proximal and distal hindlimb nerves, autonomic nerves, dorsal and ventral spinal roots, cranial nerves, spinal cord segments and dorsal root ganglia from cervical, thoracic, and lumbar levels, and various parts of the brain. A total of 30 separate PNS sites were examined in the two *Tr vt/Tr vt* mice from these two extensively studied trios. We determined the percentage of large-enough fibers that were myelinated in whole sciatic or sciatic branch sections from all *Tr vt*/+ + mice in samples of at least 200 fibers at ×1000. Because there were so few myelinated fibers in nerves from *Tr vt/Tr vt* mice (see below), we counted the total number of myelinated fibers per nerve cross section in whole sciatic or sciatic branch nerves from all *Tr vt/Tr vt* mice.

We confirmed the findings of Falconer (*9*), with the added confidence offered by use of the *vt* marker, that *Tr* homozygotes and heterozygotes are behaviorally indistinguishable, both having a coarse action tremor and moderate quadriparesis with a waddling gait. The mild behavioral heterogeneity within each of the genotypic classes *Tr vt/Tr vt* and *Tr vt*/+ + in our outcrossed stock did not obscure the classification. Both groups of mice were long-lived. Our *Tr vt*/+ + mice often survived for more than 2 years, and our oldest *Tr vt/Tr vt* mice seemed quite healthy at more than 1 year of age. However, the quadriparesis of some *Tr vt/Tr vt* mice of more than 1 year of age became noticeably worse than that of their *Tr vt*/+ + littermates. Females of each genotype mated and bore progeny easily, but males of both genotypes mated poorly. To make *Tr vt*/+ + × *Tr vt*/+ + matings fruitful, we usually had to give the *Tr vt*/+ + males prior breeding experience with + +/+ + females.

At all ages studied, nearly all nerves of *Tr vt/Tr vt* mice were virtually completely devoid of myelinated fibers (Fig. 1A). In

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