vesicles and therefore unable to activate membrane transport of K (*13*). Moreover, the increase in Na and K permeabilities induced by short periods of deoxygenation has no effect on the water content and hemoglobin S concentration (*14*), and it is probably produced by membrane damage induced by hemoglobin S polymerization, rather than by activation of specific transport pathways.

Earlier studies have not systematically addressed the problem of regulation of cell volume and cation transport in oxygenated SS cells. In one report (*6*), the oxygenated, low-density, reticulocyte-rich fraction of SS cells was found to have a large ouabain-resistant K efflux. We have now shown that this increased K transport takes place through a *p*H- and volume-dependent system, maximally active in the least dense fraction but present also in other fractions of SS cells. When SS erythrocytes are swollen at *p*H 7.4 or incubated in acid medium, they can effectively decrease their water content and increase their MCHC. Because cellular dehydration makes a major contribution to the abnormal rheology of SS cells (*15*), it will be important to identify the role of this K movement in the formation of irreversibly sickled cells. The effect of deoxygenation on the *p*H- and volume-dependent K transport pathway and the relation to the increased K and Na permeabilities induced by sickling await further investigation. Our findings suggest that in SS erythrocytes a reduction in cell volume (with a parallel reduction in cell deformability) can be accomplished not only by repetitive cycles of polymerization and depolymerization, but also by a decrease in internal *p*H when hemoglobin is in the fully ligated state.

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Suprachiasmatic Nucleus Vasopressin Messenger RNA: Circadian Variation in Normal and Brattleboro Rats

GEORGE R. UHL AND STEVEN M. REPPERT

In situ hybridization of an oligonucleotide probe complementary to vasopressin messenger RNA (mRNA) in sections from normal or Brattleboro rat hypothalami revealed hybridization densities in each of three vasopressin-rich nuclei: the supraoptic, paraventricular, and suprachiasmatic. When entrained to a daily light-dark cycle, each rat strain displayed diurnal variation in hybridizable mRNA in the suprachiasmatic, but not in the supraoptic or paraventricular nuclei. The higher values for suprachiasmatic mRNA in the morning correlate well with previously elucidated morning increases in vasopressin immunoreactivity in the cerebrospinal fluid. These results (i) support the utility of in situ hybridization techniques for elucidating physiological influences on regional peptidergic function, (ii) are consistent with a prominent role for vasopressinergic suprachiasmatic neurons in generating the cerebrospinal fluid vasopressin rhythm, and (iii) suggest that regulation of this mRNA rhythm is not dependent on release of intact peptide.

CONCENTRATIONS OF THE NEUROpeptide vasopressin in the cerebrospinal fluid (CSF) vary in a circadian rhythm, with morning levels three to ten times those at night (*1, 2*). The persistence of this rhythm in blinded animals indicates that it is generated by an endogenous circadian pacemaker; it is also independent of the osmotic regulation of plasma vasopressin (*3*). Vasopressin-containing neurons in three hypothalamic zones—the paraventricular (PVN), supraoptic (SON), and suprachiasmatic nuclei (SCN)—are the major candidates for the generation of this CSF vasopressin rhythmicity (*4*).

In situ hybridization methods can allow assessment of peptide messenger RNA (mRNA) densities in various brain regions (*5*). We used a recently developed method to study diurnal vasopressin (prepropressophysin) mRNA rhythms and to ascertain whether the cellular contents of transmitter-specific mRNA's reflect the functional activity of a particular neuronal population. This appears to be the case for several other peptidergic endocrine systems, in which variations in a cell's secretory activities parallel alterations in the expression of the gene for its secreted product (*6*).

In addition, this method could provide insight into the identity of the cell groups responsible for the generation of CSF vasopressin rhythmicity. Cells in the SCN are heavily implicated in circadian cyclicity by a variety of lesion, metabolic, and electrophysiologic studies (*7*). However, these neurons contain only a modest fraction of total hypothalamic vasopressin (*8*). The suprachiasmatic pacemaker could therefore act either directly by regulating vasopressin release from SCN neurons or indirectly by modulating other vasopressin-containing nuclei receiving SCN input, such as the PVN.

Finally, we could examine Brattleboro rats for regional diurnal vasopressin mRNA cyclicity. These animals display diabetes insipidus, lack hypothalamic vasopressin, and have a deletion mutation in a structural portion of their vasopressin gene (*9*). Several groups have also searched for regulatory defects in the Brattleboro gene, and have found either reduced or normal hypothalamic vasopressin mRNA levels in the Brattleboro animals (*10–12*). Interpretation of these studies is complicated, however, by the difficulty in matching the degrees of dehydration in Brattleboro and control groups. Examination of the diurnal vasopressin rhythm in the SCN can obviate this problem, since vasopressin mRNA in this

G. R. Uhl, Department of Neurology and Howard Hughes Neuroscience Group, Massachusetts General Hospital, and Harvard Medical School, Wellman 405, Massachusetts General Hospital, 50 Blossom Street, Boston, MA 02114.

S. M. Reppert, Children's Service and Laboratory of Developmental Chronobiology, Massachusetts General Hospital, and Harvard Medical School, Boston, MA 02114.

nucleus is not sensitive to dehydration (*13*). In addition, the ability to assess physiological mRNA regulation in these vasopressin-poor rats also allows assessment of the extent to which release of intact vasopressin peptide is required to maintain proper regulation of vasopressin mRNA.

We determined morning and evening hybridizable vasopressin mRNA densities in SON, PVN, and SCN in normal and homozygous Brattleboro rats housed under a rigidly controlled light-dark cycle. Two Sprague-Dawley (Charles River), or homozygous Brattleboro (Blue Spruce Farms) male rats (200 g) were housed in each sound-attenuated cage under diurnal lighting (12 hours of light per day), with lights on at 6:00 a.m. Light intensity at mid-cage was 700 lux. After 10 days of acclimation to this lighting cycle, animals were blinded by enucleation under ether anesthesia and returned to their cages (*14*). The animals were killed 3 days later by intracardiac perfusion with PLPG (0.5 percent depolymerized paraformaldehyde, 1 percent glutaraldehyde, 75 m*M* lysine, 37.5 m*M* $NaPO_4$, *p*H 7.4, 10 m*M* sodium periodate) to 1 ml per gram of body weight (*12, 15*). The time of killing was based on the zenith and nadir of the daily CSF vasopressin rhythm: 9:45 to 11:30 a.m. (morning group) and 9:45 to 11:45 p.m. (evening group) (*3*). Brains were postfixed in PLPG for 90 minutes, soaked in buffered 7 percent sucrose for 30 minutes, cut into slabs, and frozen on cryostat chucks. Ten-micrometer sections were cut through the SON, PVN, and SCN with assessment of anatomic features by dark-field microscopy.

In situ hybridization was performed by a modification of standard methods (*5*) as described (*12*). Sections were first treated with HCl and proteinase K as described (*12*). A ^{35}S-labeled, single-stranded, 40-base oligonucleotide probe "VP-J" was prepared by chemical and enzymatic means and gel-purified (*12*). This probe was complementary to the vassopressin-specific exon C of the prepropressophysin gene. The specific activity was approximately 15,000 Ci/mmol.

The ^{35}S-labeled oligonucleotide probe was hybridized with tissue sections overnight at 37°C in 0.8*M* NaCl and 60 percent deionized formamide in a complex hybridization buffer (*12*). In control experiments, 500 fmol of a 45-base unlabeled oligonucleotide which was complementary to VPJ were added to each microliter of hybridization mixture. Sections were washed, dehydrated, dried, exposed to Ultrofilm (LKB), and then exposed to emulsion-coated cover slips (Kodak NTB2) (*12, 16*). Standards (frozen sections of ^{35}S-containing brain homogenate) of known radioactivity were exposed to film and emulsion in parallel with experimental sections. Film and emulsions were developed, and the tissue underlying the emulsion was stained with toluidine blue (*12, 16*).

Autoradiograms were analyzed by an observer unaware of the animals' status. Regional optical densities on films were assessed using a digitized image analysis system (RAAS; Amersham/Loats Associates, Westminster, Maryland) (*17*). Grain densities in autoradiograms were counted manually over two 1000-μm^2 regions of each medial SCN. Statistical analysis was performed with a two-tailed Student's *t* test.

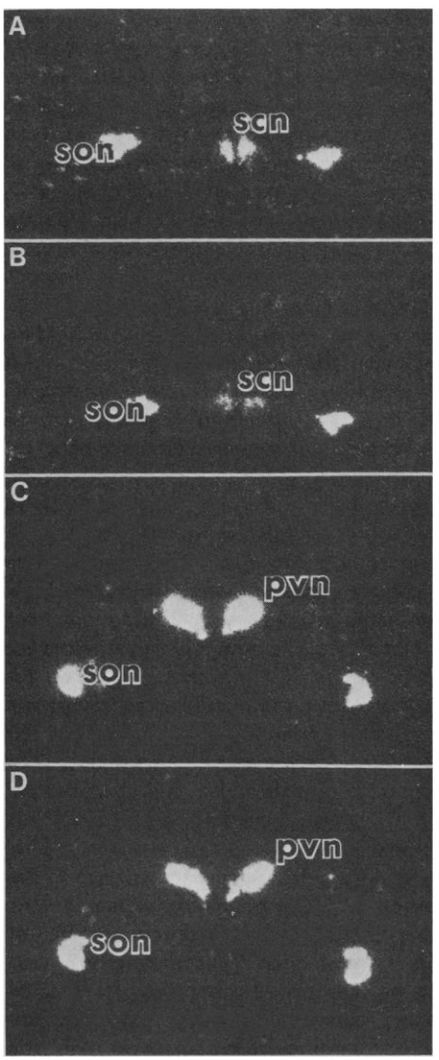

Fig. 1. Prints of film autoradiograms of sections from Sprague-Dawley rats after hybridization with ^{35}S-labeled VP-J probe. (A) Rat killed at 10 a.m. The section is at the level of the suprachiasmatic nucleus (SCN) and supraoptic nucleus (SON). (B) Rat killed at 10 p.m. The level is as in (A). (C) Rat killed at 10 a.m. The section is at the level of the paraventricular (PVN) and supraoptic (SON) nuclei. (D) Rat killed at 10 p.m. The level is as in (C). Magnification is ×14 before reduction. Increased whiteness corresponds to increased grain density.

This technique provided a very dense hybridization signal over the entire SON and the magnocellular portion of the PVN in each of the rat strains examined (*12*). The medial portion of the SCN and the parvocellular portion of the paraventricular nucleus both displayed hybridization of moderate density (Figs. 1 and 2). Competition experiments with excess unlabeled complementary probe resulted in virtual elimination of hybridization in each of these three hypothalamic nuclei. This anatomic distribution of hybridization and the results of competition experiments, as well as reductions of hybridization densities when the sections were first treated with ribonuclease (*12*), suggested that we were detecting authentic vasopressin mRNA in these tissues.

Regional analysis of autoradiograms from hybridization to sections from normal Sprague-Dawley rats revealed morning SCN ^{35}S-labeled VP-J hybridization densities which were more than twice those observed in the evening (Figs. 1 and 3). Autoradiographic standards displayed a linear relation between radioactivity and optical density over this density range. Film autoradiograms showed that these SCN morning values were 213 ± 12 percent (mean ± SEM) of evening densities ($P < 0.005$). Analysis of emulsion autoradiograms over the SCN provided increased confidence in the anatomic localization and confirmed this finding; morning grain densities were 216 ± 13 percent of evening values ($P < 0.01$). In contrast, hybridization in the SON and PVN showed no such alteration. Morning values were 99 ± 5 percent and 104 ± 3 percent of evening results, respectively ($P > 0.1$ in each case) (Fig. 3).

A virtually identical pattern was noted in the homozygous Brattleboro rats. Brattleboro rat SON hybridization in the morning was 95 ± 6 percent of evening values, and morning densities in the PVN were 90 ± 6 percent of evening densities. However, morning SCN densities were 220 ± 17 percent of evening values (Fig. 3).

Our findings provide correlative evidence for selective participation of a single hypothalamic vasopressin-containing nucleus, the SCN, in rhythmic modulation of one aspect of vasopressin synthesis. Of the three major hypothalamic vasopressin-rich nuclei, the SCN contains the lowest levels of the peptide (*8*). Nevertheless, our observation that only SCN vasopressin mRNA fluctuates diurnally and the temporal correlation between changes in mRNA level and known daily dynamics in CSF vasopressin concentrations are consistent with a role for these vasopressin-containing cells in producing rhythmicity in CSF vasopressin content.

Conversely, PVN and SON cells supply blood-borne vasopressin through their projections to the posterior pituitary (*18*). Our inability to detect diurnal vasopressin mRNA fluctuations in either the SON or PVN fits well with failures by others to observe a dramatic daily rhythm of blood vasopressin in rats (*1*).

The results of our study are also in accord with lesion experiments and with studies of vasopressin release from cultured SCN explants. Vasopressin rhythmicity in the CSF remains, although with reduced amplitude, after lesions of the PVN or isolation of the SCN from the rest of the brain (*19*). Preliminary reports also suggest that cyclic vasopressin release can be detected during maintenance of SCN explants in culture (*20*).

These results provide an example of parallelism between changes in region-specific functional brain peptidergic activity and localized alterations in peptide mRNA content, as has been reported in a variety of endocrine and neuroendocrine systems. Changes in peptide production could be coupled to fluctuations in cellular peptide secretory activity by alterations in transcriptional rates, mRNA stability, or a variety of posttranscriptional processing steps (*21*). The hybridization techniques used here reflect steady-state mRNA levels and do not allow us to separate alterations in transcriptional rates from changes in RNA stability. Nevertheless, these results are consistent with a role for mRNA fluctuations in generating or replacing the varying amounts of SCN vasopressin released at different times of the day.

The demonstration of an SCN vasopressin mRNA rhythm in Brattleboro rats supports the concept that regulation of vasopressin gene transcription can occur in these animals in a setting that is unlikely to be contaminated by effects of their long-term dehydration (*13*). This finding is in accord with the vasopressin (prepropressophysin) structural gene deletion mutation and subsequent frameshift noted by Schmale *et al.* (*10*). Together these data support the presence of vasopressin gene transcriptional regulation in the Brattleboro homozygotes; the posttranscriptional defect postulated in these animals could thus lead to their vasopressin deficiency.

The diurnal cyclicity of vasopressin mRNA in the SCN may provide an interesting model neuronal system for improved understanding of the relationship between peptide gene regulation and cellular function. For instance, one possible means of generating the diurnal oscillation in activity of these neurons could involve feedback of large amounts of vasopressin onto SCN cell surface receptors, with subsequent diminution of peptide production and release. Maintenance of vasopressin mRNA rhythm in Brattleboro rats, whose neurons produce virtually no biologically active vasopressin, suggests that this aspect of the "biological clock" does not require such feedback control for function.

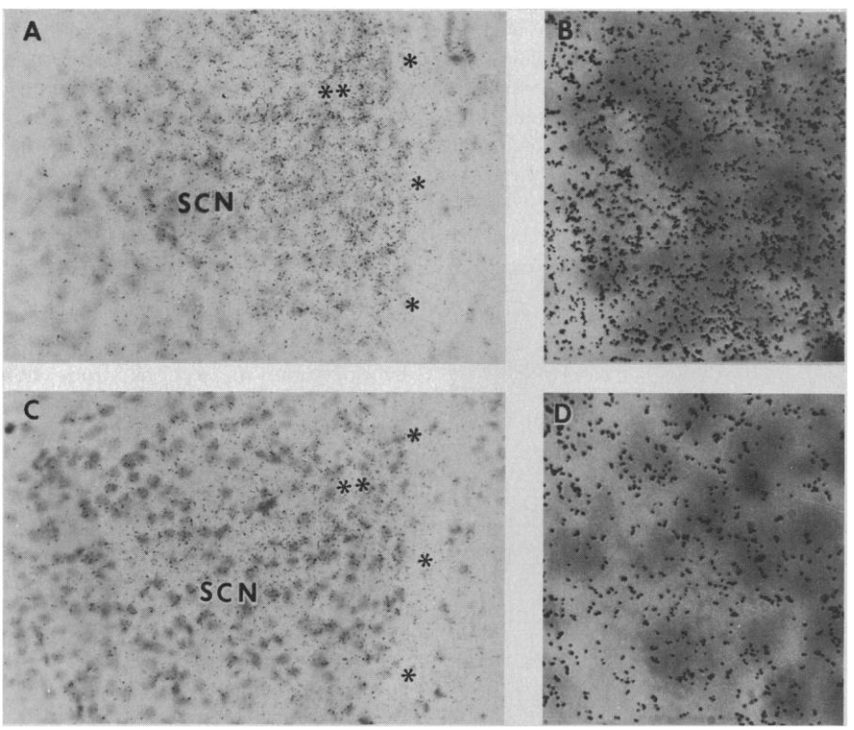

Fig. 2. Bright-field images of autoradiograms from emulsion-coated cover slips of the suprachiasmatic nuclei (SCN) of Sprague-Dawley rats. Single asterisks in (A) and (C) mark the medial border of the SCN, double asterisks mark the area where (B) and (D) were taken. (A) Nissl-stained section demonstrating the area sampled in (B) (×25 objective magnification, focus on neurons). (B) Rats killed at 10 a.m.; hybridization density over the dorso-medial SCN (×100 objective magnification, focus on autoradiographic grains. (C) Nissl-stained section demonstrating the area sampled in (D). (D) Rat killed at 10 p.m.; hybridization density over the dorso-medial SCN.

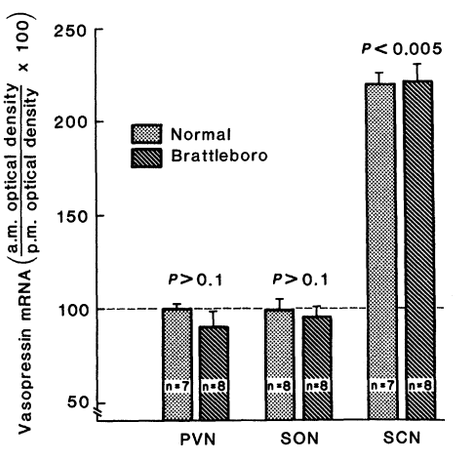

Fig. 3. Regional ^{35}S-labeled VP-J hybridization densities quantitated from film autoradiograms of normal rats (stippled bars) or Brattleboro rats (cross-hatched bars). Optical densities over the paraventricular nucleus (PVN), supraoptic nucleus (SON), and suprachiasmatic nucleus (SCN) of rats sacrificed in the morning are expressed as percent of the optical densities over the same nuclei in rats sacrificed in the evening in the same experiment. Data presented here are from four experiments. In the first study of normal animals mean densities for PVN, SON, and SCN were: 0.584, 0.530, 0.115 (p.m.) and 0.564, 0.503, and 0.263 (a.m.); in the second study they were 1.961, 2.076, 0.3624 (p.m.) and 2.107, 2.113, and 0.7380 (a.m.). In the first study of homozygous Brattleboro animals, values were 0.481, 0.487, 0.049 (p.m.), and 0.408, 0.428, and 0.115 (a.m.); in the second study they were 0.49, 0.59, 0.063 (p.m.) and 0.480, 0.576, and 0.156 (a.m.). In order to allow valid comparisons between experiments, the optical density for each nucleus in each individual animal was expressed as a percentage of the mean evening optical density for that nucleus in each experiment. Mean and standard error of the mean for these results in PVN, SON, and SCN were: normal rats: 100 ± 3.7, 100 ± 6.3, 100 ± 4.9 (p.m.); 104 ± 2.1, 101 ± 5.6, 213 ± 12.4 (a.m.); Brattleboro rats: 100 ± 10.3, 100 ± 6.2, 100 ± 3.4 (p.m.); 92 ± 6.9, 95 ± 3.0, 221 ± 17.5 (a.m.). To allow graphic representation, data are presented as the mean ± averaged SEM from morning and evening groups. The number of animals shown on each bar were assessed in both morning and evening groups. *P* values shown were obtained from Student's *t* test.

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Existence of High Abundance Antiproliferative mRNA's in Senescent Human Diploid Fibroblasts

C. K. LUMPKIN, JR., J. KEITH MCCLUNG, OLIVIA M. PEREIRA-SMITH, JAMES R. SMITH

Polyadenylated RNA isolated from senescent human diploid fibroblasts (HDF) inhibited DNA synthesis in proliferation-competent cells after microinjection, whereas polyadenylated RNA from young HDF had no inhibitory effect. Polyadenylated RNA from young cells made quiescent by removal of serum growth factors had a slight inhibitory effect on DNA synthesis. The abundance level of inhibitor messenger RNA (mRNA) from senescent cells was estimated at 0.8 and that of quiescent cells at 0.005 percent. These results demonstrate the existence of one or more antiproliferative mRNA's in nonproliferating normal human cells; these RNA's code for factors that either work antagonistically to initiators of DNA synthesis or regulate the expression of the initiators in some way. The abundance level of the inhibitory mRNA in senescent cells indicates the feasibility of developing a complementary DNA probe that will be useful in studying cell cycle control mechanisms.

LIMITED PROLIFERATIVE POTENTIAL is an inherent property of normal cells grown in culture (*1, 2*) and leads to cellular senescence. The mechanisms responsible for this phenomenon and the events that result in loss of proliferative control and cellular immortalization are not known. However, cell fusion studies have demonstrated that the phenotype of limited proliferation is dominant over the phenotype of immortality (*3–5*). We have found that senescent cells produce a membrane-associated protein that inhibits initiation of DNA synthesis (*6*). It is likely that production of this inhibitory activity is part of the mechanism resulting in cellular senescence. Conversely, modification in the protein itself or in regulation of its production would result in immortalization, which is necessary for tumor progression. Identification of the protein or the nucleic acid sequence coding for the protein would be important in understanding the regulation of cell division. As a first step in the identification of the RNA coding for this inhibitor protein, we microinjected polyadenylated [poly(A)$^+$] RNA isolated from senescent and from young cycling human diploid fibroblasts (HDF) into proliferation-competent cells.

Proliferation-competent cells were obtained by arresting young HDF in the G_1 phase of the cell cycle by maintenance in medium containing a low concentration of serum (0.5 percent) for at least 1 week (*7*). Polyadenylated RNA was isolated from young cells having at least 50 population doublings remaining (*8*) and from senescent cells that could not grow to confluence in 4 weeks after subculture and had a labeling index of <1 percent (after 24 hours of labeling). The poly(A)$^+$ RNA, at a concentration of 1 mg/ml, was microinjected into the cytoplasms (4×10^{-11} ml per cell) of the proliferation-competent cells (*8, 9*). Immediately after injection, the medium was replaced with medium containing 10 percent fetal bovine serum (FBS) and [^{3}H]thymidine (0.1 μCi/ml). The cells were incubated for approximately 16 hours, at which time most of the uninjected cells were labeled. They were then fixed in 95 percent ethanol and processed for autoradiography. The senescent cell poly(A)$^+$ RNA had a dramatic inhibitory effect while the young-cell poly(A)$^+$ RNA produced no significant inhibition (Table 1). To test whether the RNA preparation from senescent cells contained any nonspecific inhibitory activity, the material not bound to the oligo-deoxythymidylate column [non-poly(A)$^+$ RNA] was injected. This RNA did not inhibit DNA synthesis. The effect of the microinjection procedure itself on the incorporation of [^{3}H]thymidine was tested by microinjection of buffer. This resulted in a slight stimulation. The inhibitory effect of poly(A)$^+$ RNA from senescent cells was lost after incubation with ribonuclease A. Thus, intact poly(A)$^+$ RNA from senescent cells is responsible for inhibition of DNA synthesis. About 10 hours after the uninjected cells began to synthesize DNA, the cells injected with senescent cell poly(A)$^+$ RNA also entered S phase. We assume that this is due to intracellular degradation of the injected RNA.

Young cells prevented from proliferating for longer than 2 weeks by maintenance in medium containing only 0.5 percent serum (quiescent cells) also produce a membrane-associated protein that inhibits DNA syn-

C. K. Lumpkin, Jr., V.A. Medical Center, North Little Rock, AR 72114.
J. K. McClung, O. M. Pereira-Smith, J. R. Smith, Department of Virology and Epidemiology, Baylor College of Medicine, Houston, TX 77030.