MORPHOLOGICAL CHANGES IN HYPOTHALAMIC NEUROSECRETORY NUCLEI DURING THE DIURNAL CYCLE

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WILLIAM EARL ARMSTRONG

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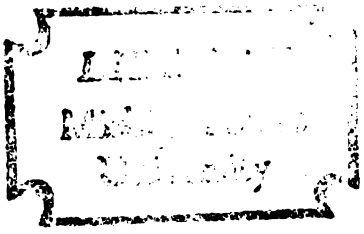

ABSTRACT

MORPHOLOGICAL CHANGES IN HYPOTHALAMIC
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By

William Earl Armstrong

At different times during a 12:12 light-dark cycle, the occurrence of cells with multiple and marginated nucleoli and cell area changes were examined in hypothalamic neurosecretory perikarya known to contain either vasopressin (VP) or oxytocin (OX) in the albino rat. These measures were used as evidence for neurosecretory activity in the suprachiasmatic (SCN), supraoptic (SOa) and paraventricular (PVN)nuclei, and were compared with plasma corticosterone values in order to partially assess the relationship of these nuclei with ACTH regulation. The relationship of these nuclei with neurohypophysial function was gleaned from comparing the morphological responses measured herein with previous descriptions of plasma and pituitary VP.

Fluctuations in plasma VP during the day may underlie urinary excretory rhythms. Rats excrete more urine during the night than during the day. Accordingly, SOa neurons exhibited their highest percentage of cells with multiple nucleoli at the end of the day, indicating this nucleus had been more active when lights were on. The periodicity of this response was in

phase with that of plasma corticosterone concentrations. The changes in this nucleus make a role in both neurohypophysial and adenohypophysial function feasible.

The release of ACTH from the adenohypophysis may be partly regulated by the VP found in the external layer of the median eminence (MEx). It was hypothesized that the source of this VP may be the dorsomedial portion of the SCN (SCdm), shown to contain VP-positive cell bodies using immunocytochemical techniques. However, the percentage of cells with multiple nucleoli did not change in the SCdm, or in the ventrolateral SCN (SCvl) during the day. Cell sizes in both nuclei appeared to vary slightly, with a peak about lights off. This response may represent endocrine activity, or general neuronal activity related to circadian rhythms.

Evidence was provided which indicated there are morphological differences between SCdm and SCvl. Cells in SCvl were larger and more often had an invaginated nucleus than did those in SCdm.

Both lateral (PVl) and medial (PVm) magnocellular divisions of the PVN showed a large increase in cell areas which peaked at 2400 hr in the absence of any fluctuations in the percentage of cells with multiple nucleoli. Recent evidence showing complex extrahypophysial connections of the PVN makes this response difficult to interpret. A role in ACTH regulation for the VP in the PVN has been suggested by research using monkeys. It appears that OX and VP cells were simultaneously active in the PVN, and that these hormones may have central nervous system roles in addition to their known secretion from the neurohypophysis. A dissociation between the SOa and PVN was provided by differences in the nucleolar and areal responses of the two nuclei.

As to the existence of multiple nucleoli, there were two novel findings. Firstly, previous studies on the SOa had shown that, once

nucleolar proliferation was induced, the level of cells with more than one nucleolus remained high for several hours, and even days, after the activating stimuli were terminated. The present study indicates nucleoli may be produced, and subsequently removed from the nucleus in approximately six hours during the diurnal cycle. Secondly, cells with multiple nucleoli were found to be prevalent in the SCN, though their significance in this nucleus remains to be determined.

MORPHOLOGICAL CHANGES IN HYPOTHALAMIC NEUROSECRETORY NUCLEI DURING THE DIURNAL CYCLE

By

William Earl Armstrong

A thesis

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1977

To My Wife, Dee Anne, And

My Parents, Mr. and Mrs.

Earl I. Armstrong

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10. Mean cell areas (in μm^2) from the medial (PVm) (top panel) and lateral (PVl) (bottom panel) divisions of the paraventricular nucleus are graphed as a function of time of day, and represented by solid lines (scale to the left). Also shown are mean difference scores obtained by subtracting the mean cell area of each plane from each animal's mean value, represented by broken lines (scale to the right). The peaks in cell area in both the PVl and the PVm occur at 2400 hr. This change is punctuated further by a similar change in the mean difference scores. H: cells taken from the horizontal plane C: cells taken from the coronal plane.

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INTRODUCTION

Diurnal oscillations characterize a broad spectrum of an organism's behavioral and physiological activities. Rhythmic endocrine activity is of particular interest because of the widespread effects that hormones exert on regulatory and other adaptive functions. Perhaps the best documented of established vertebrate hormonal rhythms are those of plasma corticosteroids, occurring in species ranging from goldfish (Singley and Chavin, 1971) to man (Migeon et al., 1956). Most intensively studied are rats, which exhibit circadian variations in all aspects of the pituitary-adrenal axis, including adrenal (Critchlow, 1963) and plasma (Critchlow et al., 1963; Guillemin et al., 1959; Retienne et al., 1968) corticosterone, plasma and pituitary adrenocorticotropin hormone (ACTH) (Critchlow, 1963; Retienne et al., 1968) and from median eminence extracts, corticotropin-releasing factor (CRF) (Hiroshige, Sakakura and Itoh, 1969). The persistent periodicity in CRF secretion in adrenalectomized (Hiroshige and Sakakura, 1971) and hypophysectomized (Takebe, Sakakura and Mashimo, 1972) rats attests to the rhythm's neural basis and relative independence of negative feedback exerted by ACTH or corticosterone.

Despite the fact that CRF was the first demonstrable hypothalamic releasing factor, attempts at purification have been largely fruitless (see Blackwell and Guillemin, 1973). Schally et al. (1960) did isolate two small polypeptides from neurohypophysial extracts, beta and alpha CRF, and noted their similarities to lysine vasopressin and alpha melanocyte-stimulating hormone, respectively. While this argues for a biochemical separation between vasopressin (VP; the principal mammalian antidiuretic hormone) and CRF, similarities

in physiological functions have been demonstrated and were reviewed by McCann and Dhariwal (1967) and Yates and Maran (1974). Exemplary among these are the common release of VP and ACTH under many of the same, often stressful, conditions, and the ability of VP to release ACTH when injected into the adenohypophysis of the conscious dog.

Relationship of VP with CRF

The interpretations of studies directed at dissociating CRF and VP release to certain stimuli are often confounded by investigators' persistence in drawing conclusions about adenohypophysial VP from its concentration in peripheral plasma. For example, Hedge and De Wied (1971) report that hypothalamic atropine implants inhibit the stress-induced release of ACTH in the rat, but not the rise in plasma VP. Indeed, Zimmerman et al. (1973) were able to measure VP in the long portal vessels of the anesthetized monkey using a sensitive radioimmunoassay, and found concentrations 300 times that obtained concurrently from peripheral blood.

Yates and Maran (1974) also argue that neurohypophysectomized rats with normal ACTH-releasing capabilities may be used as evidence for a separation of VP and CRF. While presumably eliminating the major source of peripheral VP, neurohypophysectomy may not affect concentrations in the pituitary portal system. Terminals from the supraoptic (SON) and paraventricular (PVN) nuclei may exist rostral to the neurohypophysis along the median eminence (see Parry and Livett, 1973). Both of these nuclei synthesize VP and oxytocin (OX) (Burford et al., 1974). There is strong evidence, however, that VP is not the sole CRF, but participates in some conjunctive manner to regulate ACTH secretion. Rats homozygous for hereditary diabetes insipidus, and therefore lacking the ability to synthesize VP (Valtin, 1967), exhibit ACTH release when stressed (see McCann and Dhariwal, 1967, and Yates and Maran, 1974), although this release may be impaired (Wiley et al., 1974).

Recent findings have favored the hypothesis that VP, or some derivative thereof, may act in concert with another substance to release ACTH (Yates and Maran, 1974). Yasuda and Greer (1976) have found that neither arginine or lysine vasopressin is effective in stimulating ACTH secretion from cultured adenohypophysial cells, whereas Pitressin (posterior pituitary extract) induces a dose dependent release. Using gel filtration, two distinct peaks of CRF-active substances from rat median eminence extracts can be separated (Pearlmutter et al., 1975). A combination of the two fractions releases more ACTH *in vitro* than either does alone. Furthermore, derivatives of the lysine vasopressin molecule high in ACTH-stimulating activity have been demonstrated in the rat (Arimura et al., 1969). Perhaps VP is transformed *in vivo* into other biologically active peptides, one of which may be of a potent CRF.

Localization of VP

Information regarding the origin and distribution of hypothalamic hormones has increased tremendously with immunohistochemical data (Zimmerman, 1976). Unfortunately, the inability to identify the amino acid sequence of CRF has in this case precluded the application of these techniques. Immunohistochemical studies have confirmed the presence of VP and OX in both the SON and PVN of several mammals (Zimmerman, 1976). In addition, and unexpectedly, the dorsomedial portions of the rat suprachiasmatic nuclei (SCdm) also stain positive for VP using either immunofluorescent (Burlett and Marchetti, 1975; Swaab et al., 1975) or immunoperoxidase (Vandesande et al., 1975) techniques. In light of these findings, it is intriguing that Lang and his co-workers (1976) have recently found that those nuclei in the rat with significant CRF-activity correspond with nuclei containing VP. This includes the rat arcuate nuclei, shown by George and Jacobowitz (1975) to contain VP when tissue homogenates were analyzed by radioimmunoassay. Immunohistochemical studies have failed to localize VP in this nucleus in the rat, however (Zimmerman, 1976).

It is at this point not unreasonable to speculate that any of the hypothalamic nuclei mentioned above might contribute to the VP, or its associated protein neurophysin, present in the external layer of the rat median eminence (MEx) (see Zimmerman, 1976). This finding obviously gives new impetus to the hypothesis that VP participates in adenohypophysial function. Increases in the amount of neurophysin (Vandesande et al., 1974) and VP (Vandesande et al., 1975) in the rat MEx have been reported following adrenalectomy. In this situation hypersecretion of CRF might be expected in the absence of corticosteroid feedback. A similar phenomenon is thought to occur in the rat SCN (Burlett and Marchetti, 1975; Dierickx et al., 1976; Vandesande et al., 1974) but is still controversial (Zimmerman, 1976). In the monkey, it appears the PVN may be a major source of VP in the MEx (Antunes et al., 1976). Several anatomical characteristics of the SCN and recent physiological reports make this structure a putative source of diurnal fluctuations in CRF in the rat.

SCN and Diurnal Rhythms

Nuclear groups controlling CRF secretion must receive, or themselves contain, some source of oscillation which is entrainable to the imposing light-dark (LD) schedule. This point is well illustrated in experiments where rats are subjected to a sudden alteration in the LD sequence, or blinded. Reversing the LD schedule induces a phase reversal of the corticosterone rhythm (Critchlow et al., 1963) whereas blinding (Moore and Eichler, 1972) and constant darkness (Critchlow, 1963) produce phase shifts in the rhythm without disrupting its circadian periodicity. The SCN receives a bilateral retinal projection in the rat (Moore and Lenn, 1972) and is necessary for the entrainment and generation of several rhythmic functions (Moore and Eichler, 1976), including that displayed by corticosterone secretion (Moore and Eichler, 1972).

A retinohypothalamic projection to the SCN has been found in several vertebrates studied with autoradiographic techniques, including birds (Meier, 1973) and primates (Tigges and O'Steen, 1974) as well as many other mammals (Moore, 1973). Cobalt-filled profiles of small caliber axons have been shown entering the ventral portion of the most posterior aspect of the rat SCN (Mason and Lincoln, 1976). Stimulation of the optic nerve or light excites approximately half of sampled SCN units, while inhibiting a smaller percentage (Nashino et al., 1976). These results give sufficient support to the notion that SCN normally receives information regarding photoperiod. Blinded rats, which show a corticosterone rhythm, would still have intact terminals in the SCN from serotonergic neurons in the brain stem, probably originating in the dorsal and median raphe' nuclei (Fuxe, 1965; Aghajanian et al., 1969). This system may provide periodic input to the SCN which is coupled with that from the eye under normal conditions.

If the SCN is one source of VP found in the MEx, then a connection between the two structures must exist. Studying the rat, Swanson and Cowan (1975) observed silver grains over fibers projecting to the periventricular region of the hypothalamus as well as to an area between the arcuate and ventromedial nuclei after injecting tritiated leucine and proline into SCN. A small contingent of fibers was reliably present in the internal layer of the median eminence, commonly associated with axons from magnocellular neurosecretory nuclei. Terminal degeneration has been reported in the rat MEx subsequent to lesions in the SCN, but not immediately rostral to it (Koritsanszky and Koves, 1976). Ultrastructurally, SCN morphology is compatible with neurosecretion, as dense core vesicles of the 1000-1500 Å range are present in rats (Subaro and Pellegrino de Iraldi, 1969), and rabbits (Clattenburg et at., 1975).

The existence of diurnal neurosecretory rhythms in rats has been suggested on the basis of Gormori-positive staining and somal or nuclear size changes by Rinne and Sonninen (1964), studying the SON and PVN, and by Glantz (1967), studying the SON. Further, daily variations have been reported in the concentration of VP in the neurohypophysis (Retienne, et al., 1968) and the blood (Möhberg and Möhberg, 1976) which may provide an explanation for some of the cellular changes and for periodicities in urine concentration and excretion (Glantz, 1967).

Statement of problem

While diurnal rhythms in the secretion of VP and CRF are well documented, the hypothalamic source(s) of such hormonal oscillations is unknown. This is due to the presence of VP in several discrete nuclei, all of which possess ACTH-releasing activity. The problem, then, is to find the hypothalamic neurosecretory cells whose oscillatory activity (based on anatomical changes) would account for known diurnal changes in VP and CRF in the blood or pituitary gland. Furthermore, would such changes, if they occurred, provide support for the hypothesis that VP participates in diurnal ACTH secretion?

In this regard, the problem requires the cellular response to be related to hormone output. Since corticosterone is easy to measure, and its level in peripheral blood depends on ACTH levels, which depend on CRF secretion from the hypothalamus, this hormone was chosen for comparisons with neurosecretory cell changes.

Magnocellular neurosecretory nuclei contain a proportion of neurons with multiple nucleoli which is dependent upon their activity (Hatton and Walters, 1973; Hoblitzell et al., 1976). Although parvocellular in nature, SCN cell nuclei may also contain more than one nucleolus (Le Beaux, 1971). In an attempt to discern possible secretions of VP from SCN, SON, and PVN

during the diurnal cycle, this nucleolar response, in addition to cell size, was used as an index of activity. Comparisons were made among these VP-containing nuclei in an effort to more precisely determine the origin of neurohypophysial and peripheral vacillations in VP. To quantify neurosecretory activity, the percentage of cells with multiple nucleoli and cell areas were calculated in SCN, SON, and PVN in four groups of four rats each, sacrificed at six hour intervals after being on a 12:12 LD schedule for at least 30 days. For correlational purposes, cardiac blood samples were taken and analyzed for corticosterone, protein concentration, and hematocrit. The rhythm of morphological changes in each nucleus was compared to that of corticosterone. Also, the topographic distribution of VP and OX within the PVN, and of VP within the SCN, was taken into consideration.

METHOD

Subjects

Subjects were 16 male Sprague-Dawley rats (Holtzman Co.), 60 days old and weighing 200-300 g on arrival. The animals were individually housed in wire mesh cages which were placed on a rack and contained in a room under an alternating (12:12) light-dark (LD) cycle. Fluorescent overhead lights provided approximately 6-8 ft.-c. of illumination, as measured by a photometer (American Optical) directed toward the faces of the cages. Ambient temperature ranged from 21-23° C. All rats were provided with food (Wayne Mouse Breeder Blox) and tap water ad libitum throughout the duration of the experiment.

Procedure

Rats were assigned to one of four groups, four animals per group: 1) animals to be killed at lights off (1800); 2) animals to be killed six hours into the dark phase (2400); 3) animals to be killed at lights on (0600); and 4) animals to be killed six hours into the light phase (1200). Animals were allowed to remain on the LD schedule for at least 30 days, but no longer than 32 days, before sacrifice. Groups 1800 and 0600 were run simultaneously as were groups 1200 and 2400. These latter two groups were run six months after groups 1800 and 0600.

Blood sampling. For five days prior to sacrifice, the animal room was entered at the predesignated times and the rats were briefly handled. This was done in order to attenuate stress before blood sampling and perfusion. On the sixth day, each animal was treated in the following way at its scheduled time. The rat was quickly anesthetized with ether and sub-

jected to cardiac puncture with a heparinized glass syringe. A 1-2 cc sample of blood was withdrawn and injected into a glass centrifuge tube. A small amount of this blood was collected in a heparinized glass capillary tube, and both samples were centrifuged. Plasma was drawn off the large sample with a 1 cc tuberculin syringe and a small amount immediately analyzed for protein concentration via refractometry (American Optical). The rest of the plasma was frozen for subsequent fluorometric determinations of corticosterone according to the procedure of Guillemin et al., (1959).[1] Hematocrit readings were taken from the capillary tube and expressed as the percentage of plasma.

Histology. While each rat's samples were being centrifuged, transcardial perfusion was begun with a 0.9% saline solution followed by a solution of one part 37% formaldehyde and nine parts 0.9% saline. With the perfusion complete, another rat was taken and the procedure repeated. Either all four rats from one group or two per group were sacrificed on the same day. In the latter case the remaining rats were handled but not killed until the next day. All rats were sacrificed within the hour following the designated time. The heads of perfused rats were removed and placed in the fixative for a few days. The brains were removed and further soaked in fixative, dehydrated in graded alcohols and methyl benzoate, and embedded in paraffin (Paraplast). Sections (6 μm) were taken on a rotary microtome, floated on warm (52-55°C) water, and mounted on gelatinized glass slides. Following deparaffinization with xylene, the sections were stained for Nissl substance with thionine. Two brains in each group contributed horizontal sections, two coronal sections. The slides were coded by an associate and data were collected blind.

[1]Thanks are due Dr. Ken Moore for corticosterone analysis.

Nuclei examined. Data were collected from the anterior portion of the supraoptic nucleus (SOa), the paraventricular (PVN) and suprachiasmatic nuclei (SNC), and from region CA 4 (Lorente de No', 1934) of the hippocampus (HPC). The PVN was subdivided into lateral (PVl) and medial (PVm) magnocellular divisions according to Hatton et al., (1976). The PVm lies in the anteroventromedial aspect of the PVN, while PVl is situated in the dorsolateral protion of the complex, caudal to PVm. Magnocellular neurons of the SOa lie dorsolateral to the optic chiasm. Parvocellular neurons of the SCN were also further subdived into dorsomedial (SCdm) and ventrolateral (SCvl) portions (see Figure 1). The presence of a few large blood vessels and an apparently greater cell density served to distinguish SCdm from SCvl. The pyramidal neurons in division CA 4 of the HPC are found in the region of the dentate hilus.

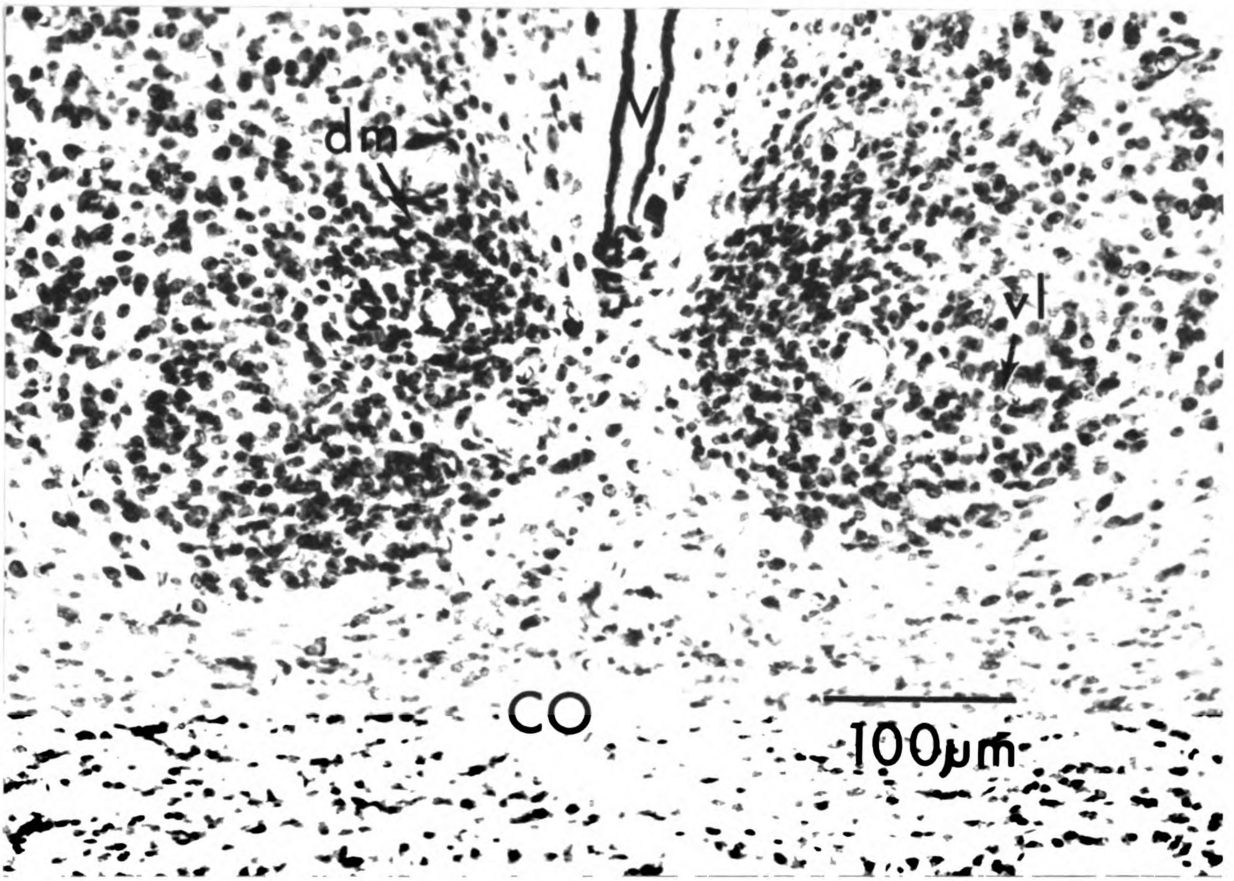
Cell measurements. For cell size determinations, 60 cells from each subnucleus, in each animal, were magnified 2180 times and their perikaryal outlines traced. Only cells with a visible nucleolus were drawn. For each subnucleus, each side of the brain contributed half the total number of cells drawn. In every case, samples were taken from four sections through a particular subnucleus, with each section providing 15 cells. Areal measurements were made by drawing perpendicular lines through the long and short axes of the cell drawing. A weighted average of their lengths

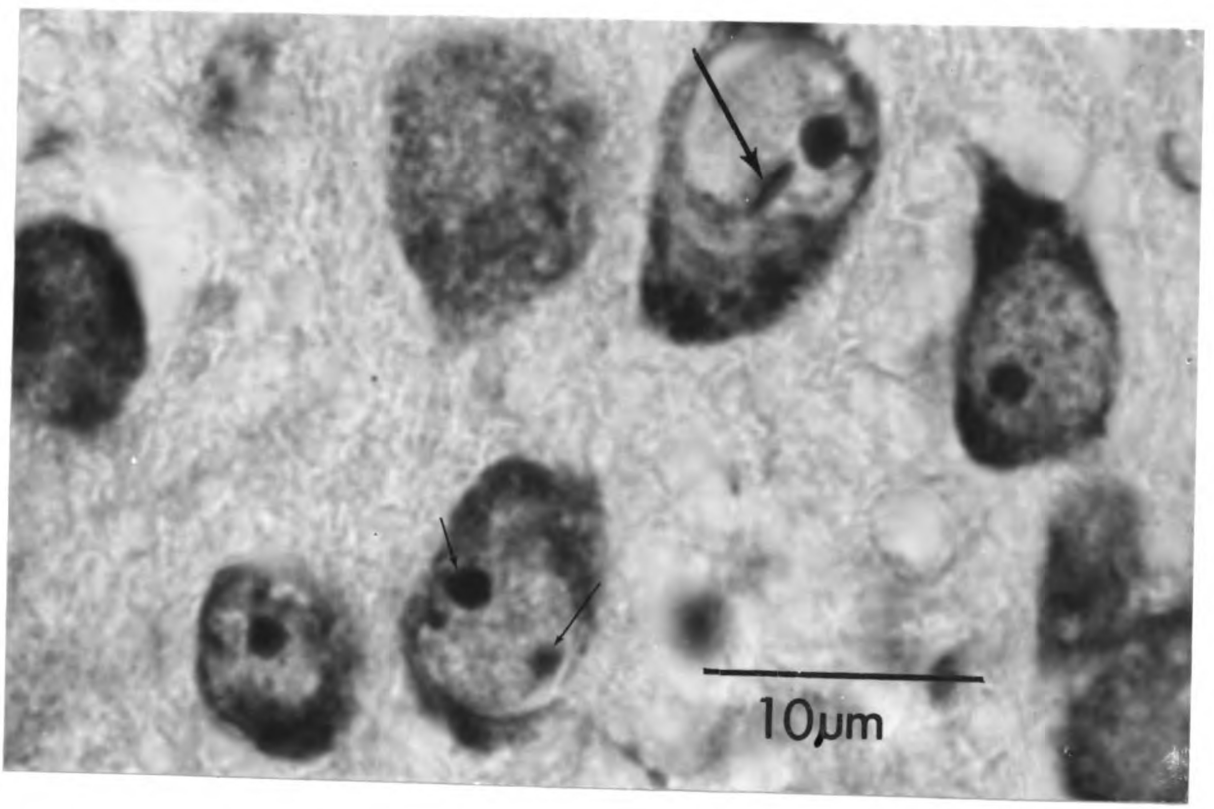
$$\left(\frac{\text{long axis} + 2\text{x short axis}}{2}\right)$$

was then determined. Areas measured by planimetry were obtained from random samples of 80 cells per plane for each subnucleus, collecting these cells from several animals to account for changes in shape and size. A product-moment correlation co-effecient was then computed between the measured areas and the weighted average of the diameters, for each group of 80 cells. A regression equation was finally used to obtain comparable measurements on cells

Figure 1. Photomicrograph of a 22μm coronal section through the suprachiasmatic nuclei, illustrating dorsomedial (dm) and ventrolateral (vl) divisions. The brain was embedded in celloidin and stained with thionine. CO: optic chiasm, V: third ventricle.

Figure 2. Photomicropgraph at high magnification illustrating cell types in the suprachiasmatic nucleus. Large arrow indicates a cell with an invaginated nucleus; small arrows point to two nucleoli in another cell. The brain was embedded in plastic (methyl methacrylate), sectioned at 2 μm, and stained with thionine.


dm
V
vl
CO
100µm


10µm

not measured by planimetry.

Measurements were taken from HPC cells in order that the cell areas from the other nuclei might be expressed as a percentage of HPC cell areas to control for shrinkage. Variation in HPC cell areas over time, however, prevented the use of this manipulation (see Results).

Cell Sampling. Samples of 150 neurons per subnucleus were taken from each animal. In PVl and PVm, 25 cells were sampled in each of six sections chosen randomly from throughout each subnucleus. The same procedure was followed for horizontal sections through SOa, SCdm, and SCvl. For coronal sections through these subnuclei, however, 12 sections were chosen, and 12 or 13 cells were sampled per section. This manipulation insured adequate representation through the extensive rostralcaudal limit of SOa, and compensated for the high rejection rate in SCN, since the nuclear characteristics of many of these cells were not discernable. Sampling was accomplished using a Whipple-Hauser reticule in one eyepiece of a Zeiss microscope. A particular nuclear group was placed under the grid, and before finally focusing a decision was made as to which rows or columns would be sampled. The objective was then focused and cells were counted. A 40x objective with 12.5x eyepieces was used to sample cells from the PVl, PVm and SOa. Samples from SCN were taken under oil immersion with a 100x objective and 12.5x eyepieces. For PVl, PVm and SOa, cells fell into one of the following categories, having either a) one nucleolus, which was not in contact with the nuclear membrane; b) one nucleolus, which was apposed to the nuclear membrane (marginated); c) two or more nucleoli, both of which were unmarginated ('multiples'); or d) two nucleoli, at least one of which was marginated. In addition to these categories, cells of the SCdm and SCvl were further divided into cells with or without a prominent nuclear invagination (i.e., was at least one third of the estimated nuclear diameter; see

Figure 2). Cells with invaginated nuclei were also compared to those without in terms of the occurrence of marginated and multiple nucleoli.

For data analysis, morphological data were transformed to percentages. The non-parametric Mann-Whitney test was used for between group comparisons and the t-test for related means was used for within group comparisons. A product-moment correlational statistic was employed to make further comparisons between subnuclei, and between subnuclei and physiological data, within groups.

RESULTS

Blood measurements

The data for plasma protein concentration and hematocrit are listed in Table 1. Between group comparisons for protein concentration revealed only one reliable difference: group 0600 had a significantly lower mean than did groups 1200 and 2400. While there was no overlap in individual hematocrit values between groups 0600 and 1200, the loss of one sample prevented a meaningful statistical comparison. All other groups were similar to one another on this measure.

Mean plasma corticosterone concentrations are plotted as a function of time of day in Figure 3. A clear diurnal rhythm can be observed, with a peak occurring at 1800 hr and a trough at 2400 and 0600 hr. Groups 2400 and 0600 had significantly lower corticosterone concentrations than did group 1800 ($p < .028$, for each comparison), despite wide variation within groups. While exhibiting a periodicity, these values are higher than the "unstressed" values reported by other investigators, presumably because of the different blood sampling techniques employed (see Discussion).

Suprachiasmatic nucleus

As the photomicrograph in Figure 2 illustrates, cells of the SCN often display multiple nucleoli and one or more marked, nuclear invaginations. While in Nissl-stained preparations these infoldings appear as basophilic stripes, electron microscopic examinations have confirmed the existence of numerous nuclear indentations in SCN neurons (Lebeaux, 1971; Subaro and Pellegrino de Iraldi, 1969). The presence of these membrane extensions, the small size of the cells, and the highly granular appearance of the

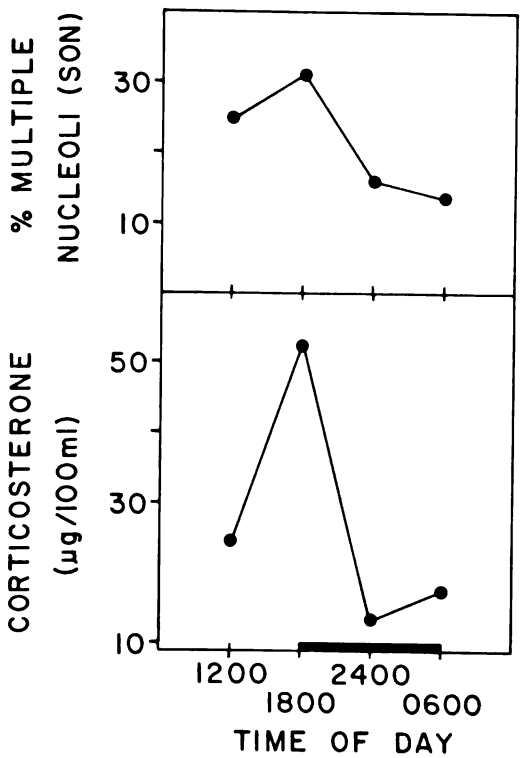
Table 1. Plasma protein and hematocrit values at different times of of the day ($\bar{x} \pm$ SEM)

Time	Hematocrit (% Plasma)	Protein (g/100 ml)
0600 (lights on)	60.3 ± 2.36	5.4 ± .31*
1200	51.3 ± .63	6.5 ± .06
1800 (lights off)	55.0 ± 1.30	5.8 ± .21
2400	56.0 + 2.04	6.4 + .18

* Significantly less than at 1200 or 2400 hrs, $p < .028$ for each comparison.

Figure 3. In the top panel the mean percentages of cells with multiple nucleoli are graphed for the supraoptic nucleus (SOa) as a function of time of day. Mean corticosterone values (in ug/100 ml of plasma) from cardiac blood are similarly plotted in the bottom panel. In this and in all other figures, the dark bar indicates the period of darkness. Note the peak values at 1800 hr, and the troughs at 2400 and 0600 hrs.

Figure 4. The mean percentages of cells exhibiting a nuclear invagination in the dorsomedial (SCdm) and ventrolateral (SCvl) portions of the suprachiasmatic nucleus are plotted as a function of time. SCvl exhibits significantly higher values at all times, except at 0600, where convergence can be observed.


% MULTIPLE NUCLEOLI (SON)
30
10
CORTICOSTERONE (µg/100ml)
50
30
10
1200
2400
1800
0600
TIME OF DAY

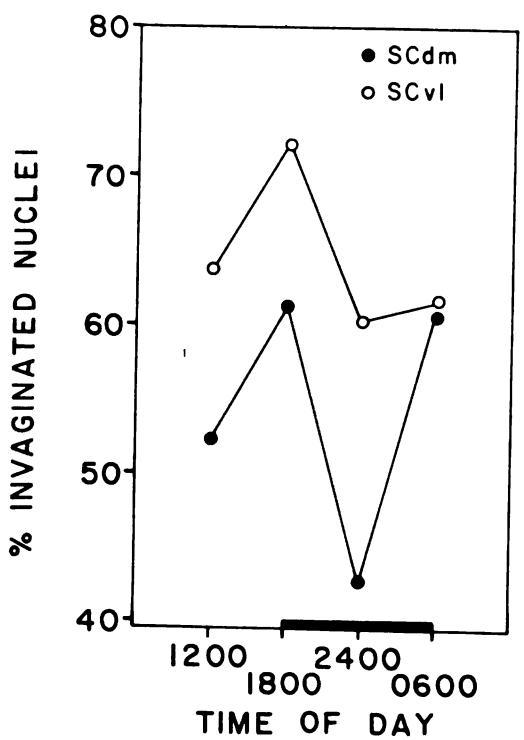
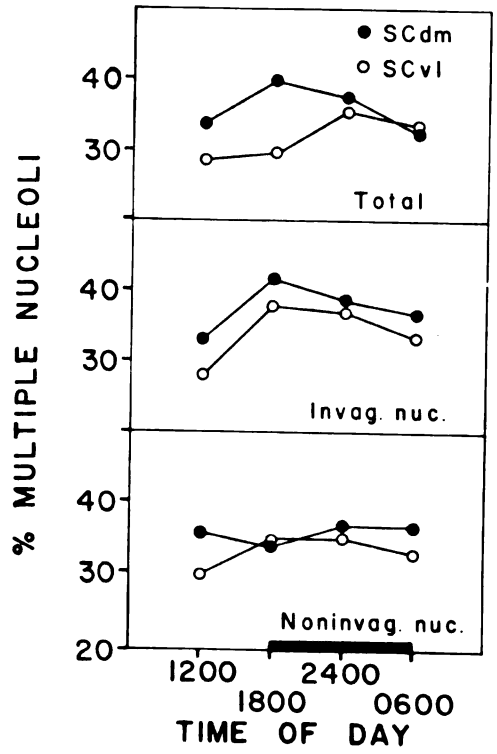
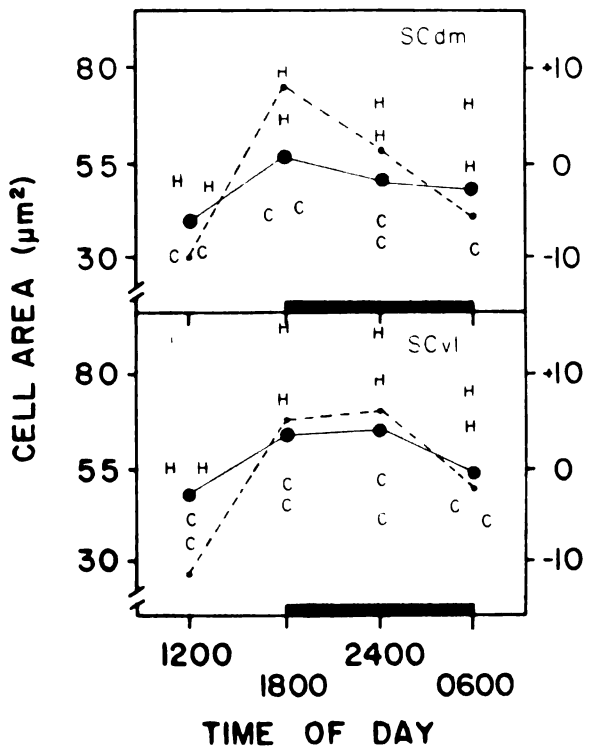

80
70
60
50
40
% INVAGINATED NUCLEI
SCdm
SCvl
1200
2400
1800
0600
TIME OF DAY

Figure 5. In the top panel the mean percentages of 'multiples' for all sampled cells in either the dorsomedial (SCdm) or ventrolateral (SCvl) portion of the suprachiasmatic nucleus are graphed as a function of time of day. Depicted in the middle panel are similar values for cells exhibiting and invaginated nucleus. In the bottom panel the mean percentages for cells without a nuclear invagination are also plotted. Cells with invaginated nuclei exhibit some variation in their percentage of 'multiples,' with a peak 1800. Cells without invaginated nuclei exhibit no such variation.

Figure 6. Depicted are mean cell areas (in μm^2) in the dorsomedial (SCdm) (top panel) and ventrolateral (SCvl) (bottom panel) portions of the suprachiasmatic nucleus as a function of time of day, represented by solid line (scale to the left). Also shown are the mean values of difference scores (in μm^2) from each subnucleus obtained by subtracting the mean cell area of each plane from each animal's mean value, represented by the broken line (scale to the right). Cell areas peak at 1800 hr for both subnuclei. The changes in cell area appear much more profound when the differences between planes are minimized, as can be seen by examining the difference scores. H: cells taken from horizontal plane, C: cells taken from coronal plane.


SCdm
SCvl
40
30
Total
40
30
Invag. nuc.
40
30
Noninvag. nuc.
20
% MULTIPLE NUCLEOLI
1200
2400
1800
0600
TIME OF DAY


SCdm
SCvl
80
55
30
+10
0
-10
CELL AREA (μm²)
1200
2400
1800
0600
TIME OF DAY

nucleoplasm accounted for a high rejection rate during cell sampling.

Invaginated nuclei. In addition to their locations within the SCN, SCdm and SCvl were also distinguished by their respective populations of cells with invaginated nuclei (see Figure 4). Significantly more of these cell types were found in SCvl than in SCdm across all groups ($t=4.39$, d.g.= 15, $p < .001$), though in Figure 4, a convergence can be observed at 0600 hr. A rhythmic occurrence of these neurons is suggested by Figure 4, but variability allowed only one difference to attain statistical significance. In SCvl, the mean percentage of cells exhibiting invaginated nuclei was higher at 1800 than at 0600 hr ($p < .028$).

Multiple nucleoli. As depicted in Figure 5, there were no dramatic fluctuations in the percentage of 'multiples' in either subnucleus as a function of time. Accordingly, no statistical differences were found in this regard. However, when cells with invaginated nuclei were considered separate from those without, some differences emerged. Cells with an infolded nucleus seemed to vary in their percentage of 'multiples' in both SCdm and SCvl. Only SCvl exhibited a significant difference, with more of these cell types present a 2400 hr when compared to 1200 hr ($p < .028$). This response was not significantly correlated with corticosterone concentration in either SCvl ($r= -.13$) or SCdm ($r= .09$). Neurons without invaginated nuclei, as can be seen from Figure 5, did not vary significantly in their percentage of 'multiples'.

Marginated nucleoli. When no distance could be perceived between a nucleolus and the nuclear membrane, the former was considered marginated. Since a nuclear infolding represents a part of the nuclear membrane, nucleoli in close apposition with these basophilic stripes were considered marginated. Listed in Table 2 are the data for marginated nucleoli in SCN. Populations were distinguished on the basis of invagination, number

of nucleoli, and location within the SCN. No significant changes were found across times in the occurrence of margination, regardless of category.

Table 2 also shows significant within group comparisons. When comparing cells with and without invaginated nuclei, it was found that, in cells with only one nucleolus, margination occurred more often in the former for both SCdm ($t = 5.33$, d.f. = 15, $p < .001$) and SCvl ($t = 7.912$, d.f. = 15, $p < .001$). Although not as great a difference was found, the same was true for 'multiples' in SCdm ($t = 2.876$, d.f. = 15, $p < .02$) and SCvl ($t = 2.959$, d.f. = 15, $p < .01$). It thus appears that a nuclear infolding increases the probability of nucleolar margination. Since SCvl contained more cells with invaginated nucleoli, the hypothesis was tested that margination was more prevalent in this subnucleus when compared with SCdm. When all cells with marginated nucleoli were totalled, however, no difference was obtained between the two portions of SCN. This rough equivalence was apparently due to the fact that, in SCdm, more cells without invaginated nuclei contained marginated nucleoli than in SCvl ($t = 2.839$, d.f. = 15, $p < .02$). No other within group differences were noted.

Cell size. Regression equations were obtained for both subdivisions, and for both planes, to predict cell area. In SCdm, the weighted average of the long and short axes was highly correlated with cell areas determined by planimetry, yielding a coefficient of .908 in the coronal plane, and one of .902 in the horizontal plane. From these relationships the equations $y = .2935x - 5.652$ and $y = .2654x - 3.738$ were derived, respectively. A similarly high correlation was obtained for SCvl. In the coronal plane, $r = .844$ and the derived equation was $y = .147x - 1.297$; in the horizontal plane, $r = .848$ and the derived equation was $y = 2.17 - 2.924$.

Table 2. Mean (± SEM) percentage of cells with marginated nucleoli in SCdm and SCvl at different times of the day.

	SCdm				SCvl			
	Singles		Multiples		Singles		Multiples	
	invg.	ninvg.	invg.	ninvg.	invg.	ninvg.	invg.	ninvg.
0600	86.8± .78	71.3± 6.19	99.3± .75	98.3± 1.72	83.8± 1.77	58.7± 7.52	100± .00	90.1± 5.42
1200	82.9± 2.86	68.3± 4.98	98.6± 1.4	96.8± 2.5	84.0± 2.18	64.5± 8.74	97.2± 1.89	96.8± 1.94
1800	80.3± 5.71	70.9± 8.37	97.4± 1.05	84.5± 5.62	82.2± 5.0	58.0± 9.31	95.3± 2.74	79.7± 10.5
2400	79.0± 5.23	60.2± 9.29	99.5± .55	91.7± 4.89	81.8± 5.19	52.9± 5.96	99.4± .63	91.7± 2.88
$\bar{x}$s.	82.3± 1.72*	67.7± 2.58	98.7± 47^{o}	92.8± 3.11	83.0± .56**	58.5± 2.37	98.0± 1.08^{oo}	89.6± 3.59

invg. = cells with an invaginated nucleus
ninvg. = cells without an invaginated nucleus
* significantly greater than SCdm ninvg. singles, $p < .001$.
** significantly greater than SCvl ninvg. singles, $p < .001$.
o significantly greater than SCdm ninvg. multiples, $p < .02$.
oo significantly greater than SCvl ninvg. multiples, $p < .01$.

Scant amounts of cytoplasm were observed when SCN perikarya were projected for tracing. In many cases it was not known whether the nuclear or perikaryal membrane was drawn. There were no differences found between groups in this difficulty, so that all samples represent similar populations of cells in this respect. It was not possible to present cell areas in SCN as a percentage of HPC control cells, since the latter varied in size as a function of time (see Figure 8).

Figure 6 shows the mean predicted cell areas for SCvl and SCdm across time. Both portions of the SCN exhibit fluctuations in the size of their constituent cells. Wide differences between areas in each plane prevented a statistically significant result, however. Horizontally sectioned cells were larger than coronally sectioned cells in SCdm and SCvl ($p < .0001$, for both subnuclei). In an attempt to remove this difference between planes so that between group comparisons could be made, the mean cell area across all groups was calculated for each plane within each subnucleus. This mean was then subtracted from each individual score. This provided a quasi-standardization of scores, and the result of this manipulation can be seen in Figure 6. Similar peaks and troughs occurred in the magnitude of these difference scores as in cell size, but the changes were more dramatic. Difference scores from groups 1800 and 2400 were significantly larger than those at 1200 hr in both subnuclei ($p < .028$, for each comparison).

Cell area proved to be still another basis on which the neurons of SCdm and SCvl could be distinguished. Larger somal areas were found in SCvl in both horizontal ($t = 5.334$, $d.f. = 7$, $p < .001$) and coronal ($t = 8.333$, $d.f. = 7$, $p < .001$) planes.

Since the vacillations in cell area in the SCN were somewhat in phase with the corticosterone rhythm, a correlational measure was applied

to see if this temporal relationship was significant. For coronally sectioned SCdm cells, r= .731 (d.f.= 6, $p < .025$). In horizontally sliced brains, however, cell areas in SCdm were not significantly correlated with corticosterone concentrations (r= .441). A similar trend was observed in SCvl, with a high positive correlation found between cell areas in the coronal plane and plasma corticosterone (r= .787, d.f.= 6, $p < .025$) and a non-significant one between the same two variables in horizontally sectioned brains (r= .248).

Supraoptic nucleus

Magnocellular neurons of SON are different in appearance as well as in size from the parvocellular elements of SCN. Their morphology in Nissl-stained tissue is essentially like that of the PVN perikarya, shown in the photomicrograph in Figure 7. Large amounts of deeply-stained cytoplasm are evident. While the nucleoplasm is somewhat granular, nucleoli stand out as dark spheres in the round or oval nucleus. Invaginations are rare in the nuclear membrane.

Multiple nucleoli. As graphed in the top panel of Figure 3, the percentage of 'multiples' in the SOa showed a distinct diurnal rhythm paralleling that of corticosterone secretion, characterized by a positive correlation coefficient (r= .70, d.f.= 14, $p < .01$). Nucleolar proliferation peaked at 1800 hr, with a precipitous decline by 2400 hr, and a subsequent trough at 0600 hr. Mean values of 'multiples' were significantly lower at 2400 and 0600 hr when compared with group 1800 ($p < .028$, for each comparison).

Marginated nucleoli. As with the parvocellular SCN neurons, nucleoli in SOa were often seen in close proximity with the nuclear membrane. Data for marginated nucleoli in the magnocellular PVN and SOa are listed in Table 3. In the SOa, no differences were found between groups in the per-

Figure 7. Photomicraph of a 6 μm coronal section through the paraventricular nucleus, illustrating magnocellular neurosecretory neurons, some of which contain multiple nucleoli (large arrows). Small arrows point to marginated nucleoli. The brain was embedded in paraffin and stained with thionine.

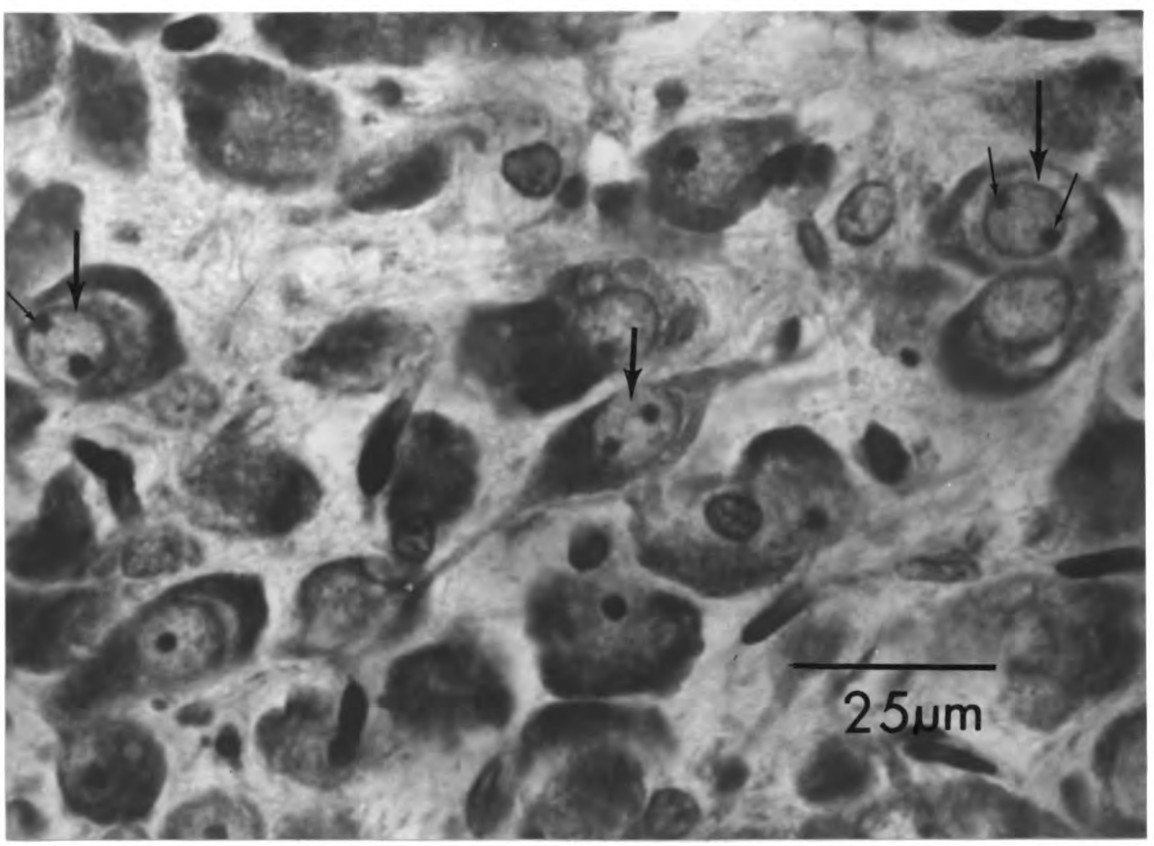

25µm

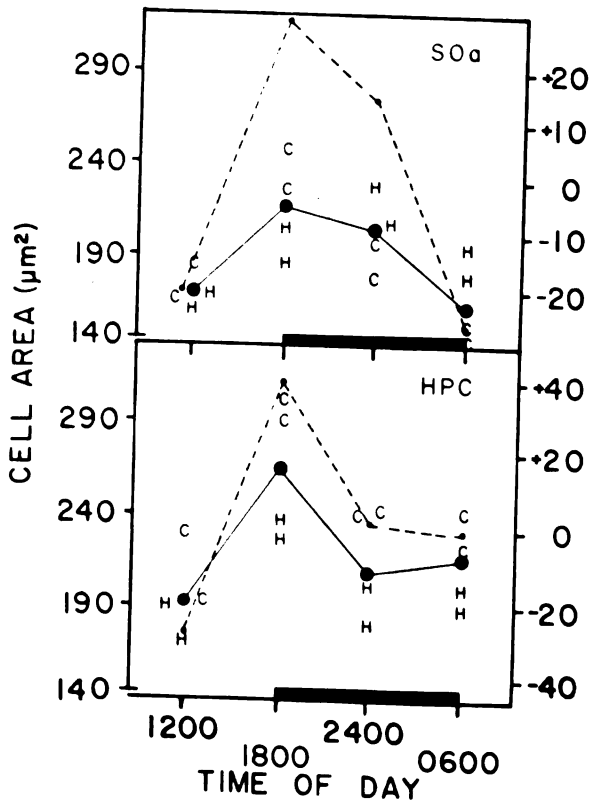
Table 3. Mean (± SEM) percentage of cells with marginated nucleoli in the SON and PVN at different times of the day.

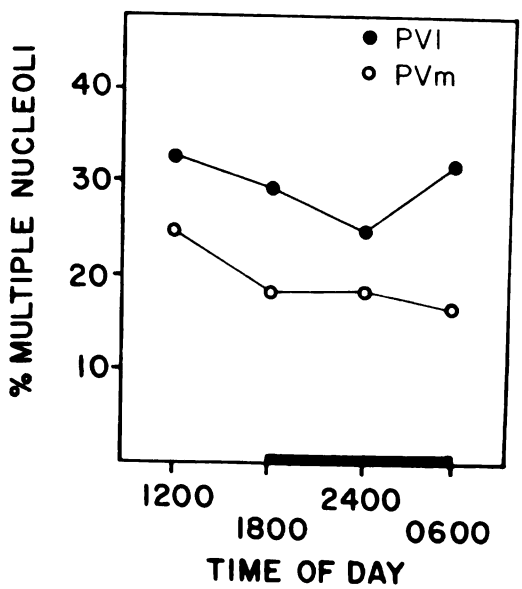
	SOa		PVm		PVl	
Time	Singles	Multiples	Singles	Multiples	Singles	Multiples
0600 (lights on)	34.6± 2.0	93.4± 2.3	23.6± 1.3	92.5± 2.2	34.8± 2.7	90.5± 2.1
1200	30.2± 5.7	92.7± 5.3	21.0± 2.0	94.7± 1.0*	34.2± 3.1	87.0± 2.5
1800 (lights off)	28.6± 3.3	89.2 ± 2.5	28.0± 3.5	82.2± 4.5	33.0± 2.7	89.2± 3.5
2400	26.0± 5.1	80.2± 6.2	19.4± 4.3	85.2± 6.3	31.9± 5.5	76.8± 6.4
total $\bar{x}$s	29.9± 1.8	88.9± 3.0	23.0± 3.8	88.7± 3.0	33.5± 6.0**	85.8± 6.2

* significantly greater than PVm marginated multiples at 1800 hr, $p < .028$.
** significantly greater than total PVm marginated singles, $p < .001$.

Figure 8. Mean cell areas (in μm^2) from the supraoptic nucleus (SOa) (top panel) and hippocampus (HPC) (bottom panel) are plotted against time of day, and represented by solid lines (scale to the left). Also shown are the mean difference scores (in μm^2) from each nucleus, obtained by subtracting the mean cell area of each plane from each animal's mean value, represented by broken lines (scale to the right). In the top panel, note the separate peaks in area for cells in the horizontal (2400 hr) and coronal (1800 hr) planes. Total cell area, as judged by difference scores, peaks at 1800 hr. Note magnitude of the difference scores at 1800 and 2400 hrs. In the bottom panel, HPC cell areas peak at 1800 hr, and quickly drop off at 2400 hr. These changes are similarly reflected in the difference scores. H: cells taken from the horizontal plane, C: cells taken from the coronal plane.

Figure 9. The mean percentages of cells with multiple nucleoli are plotted for the lateral (PVl) and medial (PVm) divisions of the paraventricular nucleus as a function of time of day. The PVl shows a higher percentage of 'multiples' at all times than does the PVm. Neither the PVl or the PVm vary in their percentages of 'multiples' significantly over time.


SOa
HPC
CELL AREA (μm²)
290
240
190
140
+20
+10
0
-10
-20
+40
+20
0
-20
-40
1200
1800
2400
0600
TIME OF DAY


PVI
PVm
% MULTIPLE NUCLEOLI
40
30
20
10
1200
1800
2400
0600
TIME OF DAY

centage of cells with a single, marginated nucleolus. Likewise, the percentage of 'multiples' with at least one marginated nucleolus did not vary across time.

Cell size. The regression equations used to predict cell areas in SOa were as follows. In the coronal plane, y= .368x - .901, where the correlation between areas determined by planimetry and the weighted average of the long and short axes was given by the coefficient .953. In the horizontal plane, this equation was y= .353x - .812, and the correlation coefficient was .936.

Surprisingly, regular fluctuations in cell area were not dramatic in the SOa. As seen in Figure 8, cell size does vary as a function of time. Discrepancies between planes again prevented a valid statistical comparison. Also, Figure 6 illustrates the dissociation between the peak values of cell areas in the two planes. Coronally sectioned brains exhibited a peak in cell area at 1800 hr, or at lights off. In the horizontal plane, however, this peak was observed at 2400 hr. "Standardized" difference scores are also graphed in Figure 8. Despite what appear to be profound differences, no significant deviations between groups were found in mean value of these difference scores. No significant differences were detected in the size of cells between planes.

Paraventricular nucleus

Figure 7 contains a photomicrograph of the lateral division of PVN illustrating the basic characteristics of magnocellular neurosecretory neurons. The description fits that given for SOa.

Multiple nucleoli. That the two subdivisions of PVN differ in more than just their relative locations within the complex is in part demonstrated by comparing their proportions of cells with multiple nucleoli. As previously shown (Hatton et al., 1976), the PVl was found to contain a higher

percentage of 'multiples' than PVm across all groups ($t = 6.19$, $d.f. = 15$, $p < .001$). Between group differences were not detectable, as neither subnucleus changed in its percentage of 'multiples' as a function of the time of sacrifice (see Figure 9). There was a significant correlation between the two portions on this measure ($r = .759$, $d.f. = 14$, $p < .001$).

Marginated nucleoli. A significantly greater percentage of cells in the PV1 had a single, marginated nucleolus when compared with PVm ($t = 4.85$, $d.f. = 15$, $p < .001$; see Table 3). Margination in cells with just one visible nucleolus did not change significantly across times. In the PVm, however, cells with multiple, marginated, nucleoli were more prevalent at 1200 hr than at 1800 hr ($p < .028$). Cells within the PV1 of this type showed no such response. No significant differences were found between the two subnuclei in their relative proportion of 'multiples.'

Cell size. Figure 10 depicts the mean cell areas in the PV1 and PVm over time, predicted from the following equations. For PV1, $y = .299x - 5.577$ in the coronal plane, where $r = .895$; in the horizontal plane, the coefficient of correlation between cell areas and the weighted average of the long and short axes was .949, where $y = .395x - 10.205$. For the PVm, $r = .939$ in the coronal plane and $y = .298x - 5.797$; $r = .945$ and $y = .332x - 7.233$ in the horizontal plane.

Diurnal oscillations in cell areas from both subnuclei were profound, particularly in the horizontal plane where 100 μm^2 separates the cell areas at 2400 and 1200 hr. The uniformity of this cellular response within the PVN complex is the basis for a high correlation between the cell sizes of two divisions ($r = .92$, $d.f. = 15$, $p < .001$). Combining planes, the mean cell areas were greater at 1800 and 2400 hr than at 1200 hr in the PVm and PV1 ($p < .028$, for each comparison). Examining within planes, more differences seemed likely. Scores were thus standardized as described be-

fore for SCN and SOa to ferret out these changes, and are graphed in Figure 10 as well. Difference scores showed a peak at 2400 in both portions of the PVN, with a trough at 1200 hr. In both PV1 and PVm, the following differences were found significant. Groups 1800 and 2400 hr had higher mean difference scores than groups 0600 and 1200 ($p < .028$, for each compariton).

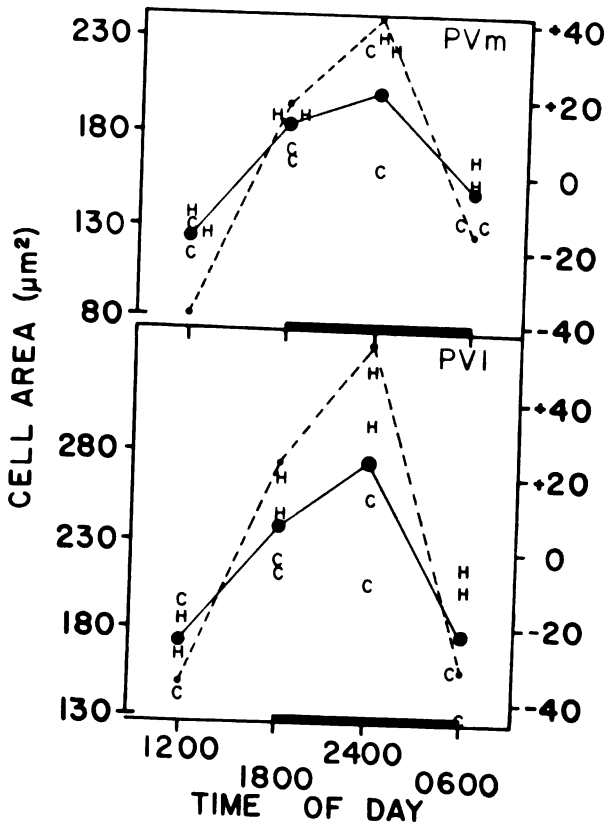
The rhythmic variation in cell size in the PVN is out of phase with plasma corticosterone concentrations. Correlations were thus poor between corticosterone and cell areas in both the PV1 ($r= .44$, $p > .05$) and PVm ($r= .09$, $p > .05$).

Further differences were found in cell areas between the PV1 and PVm. Lateral cells were larger than medials in both the coronal ($t= 4.597$, $d.f.= 7$, $p < .01$) and horizontal ($t= 6.5$, $d.f.= 7$, $p < .001$) planes. Within groups, cells from the horizontally sliced brains appeared larger than those from the coronal plane, but no significant difference was detected.

Hippocampal pyramidal cells

Control cells taken from CA 4 of the hippocampus showed a marked rhythm in their area (see Figure 8), preventing their use as controls for cell shrinkage due to vagaries in embedding. Combining planes, variability precluded any statistical differences from being detected, so these scores were standardized also (for predicted areas, the regression equations used were $y= .294x - 5.652$, $r= .908$ in the coronal plane; for the horizontal plane, $y= .265x - 3.738$, $r= .902$). The difference scores were significantly higher at 1800 than at 1200 and 2400 ($p < .028$, for each comparison). The rhythm for cell area changes was in phase with that of corticosterone secretion, but individual values were not significantly correlated ($r= .441$, $p > .05$).

Figure 10. Mean cell areas (in μm^2) from the medial (PVm) (top panel) and lateral (PVl) (bottom panel) divisions of the paraventricular nucleus are graphed as a function of time of day, and represented by solid lines (scale to the left). Also shown are mean difference scores obtained by subtracting the mean cell area of each plane from each animal's mean value, represented by broken lines (scale to the right). The peaks in cell area in both the PVl and the PVm occur at 2400 hr. This change is punctuated further by a similar change in the mean difference scores. H: cells taken from the horizontal plane, C: cells taken from the coronal plane.


PVm
PVl
CELL AREA (μm²)
230
180
130
80
280
230
180
130
+40
+20
0
-20
-40
+40
+20
0
-20
-40
1200
1800
2400
0600
TIME OF DAY

DISCUSSION

Concerning the results of this investigation, it is clear that a diurnal cyclicity, based on the morphological indices examined, characterizes the function of the hypothalamo-neurohypophysial system. Also, the methodology, in particular the dependent variables one chooses to measure, clearly influences what conclusions can be drawn. For instance, within the SOa and PVN, cell area and nucleolar proliferation were dissociated, and, had either been the sole index of neurosecretory activity, different conclusions would have undoubtedly been reached. Furthermore, discrepancies between this and previous studies of a similar nature may be attributed to methodological differences, and will be discussed where applicable below.

While not the major result of this investigation, it is useful to consider the periodicity of plasma corticosterone first. Changes related to neurosecretion may then be referred back to this important rhythm.

Plasma Corticosterone

Fluorometrically determined concentrations of plasma corticosterone over the 24 hour period closely approximated the well established diurnal rhythm, with a peak at lights off and a trough near lights on (Critchlow, et al., 1963; Guillemin et al., 1959; Retienne, et al., 1968). Absolute values at the peak and trough were noticeably higher than those reported in these previous studies. At the peak, these elevations resembled levels obtained from ether-stressed female rats at lights off (Zimmerman and Critchlow, 1967). Comparable increments in corticosterone secretion were detected by Zimmerman and Critchlow (1967) when rats were stressed at the

peak or trough of the rhythm, whereas here trough levels were only slightly above normal. According to the results of Dunn, et al., (1972), greater stress-induced increases should occur during the trough of the corticosterone rhythm. Divergent results such as these make interpretations of the present data difficult, especially since they were obtained from female rats, whose basal plasma corticosterone concentrations, and elevations to stress, are higher than those seen in males (Critchlow, et al., 1963). Blood samples must be obtained quickly under ether anesthesia (< 3 minutes) to avoid stressed corticosterone levels (Davidson et al., 1968), and it is probable that all rats in the present study were not sampled under the same intensity of stress.

Supraoptic and Paraventricular nuclei

Comparisons with previous studies. Increased cell areas have previously been reported in the rat SOa (Armstrong et al., 1977; Hatton and Walters, 1973) and PVN (Hoblitzell, et al., 1976) under conditions known to induce hormone production. Although choosing only ten of the "largest" cells per animal in an unspecified plane of section, Glantz (1967) found SON cell areas larger at lights off that at lights on. A similar trend is evident in Figure 6, but predicted somal areas varied in their peaks depending on the plane of section. In both the PVl and PVm, the observed peaks in cell area (see Figure 10) match those for estimated nuclear volume reported by Rinne and Sonninen (1964).

Whereas Glantz (1967) was unable to detect fluctuations in the density of staining in Gomori-positive SOa cells in the rat, Rinne and Sonninen (1964) subjectively observed diurnal differences in the density of staining in SON and PVN neurons using aldehyde fuchsin. The SON and PVN were both stained lightly between 2400 and 0400 hrs, a time when nuclear volumes were reportedly largest in both nuclei. Unfortunately, their study was

run when little if any darkness was available. This deficiency detracts from their conclusion that the nuclei were more active at night. Other evidence indicates that plasma levels of VP are higher in the morning (four hours after lights on) than at night (Möhring and Möhring, 1975). It has also been found that albino rats excrete small amounts of concentrated urine during the daytime, in contrast to the larger, more dilute quantities collected at night (Glantz, 1967). Densitometric measurements of neurosecretory material are poor as an index of neurosecretory activity, because they might vary with cell size, regardless of changes in dense core vesicle content, and could thus veil the true dispersion of neurosecretory material within the perikarya. It is possible that such a methodological inadequacy made major contributions to the discrepant findings of Rinne and Sonninen (1964).

Morphological indices and neurosecretion. Proliferation of nucleoli occurs in rat SOa neurons subsequent to osmotic stimulation, either in combination with plasma volume depletion, i.e., during water deprivation (Hatton and Walters, 1973), or alone, as with hypertonic saline ingestion (Armstrong, et al., 1977). The peak level of 'multiples' during the diurnal cycle indicates the SOa has been most active during the day. This response could be less related to secretion per se, than to the restoration of VP (or OX?) in the depleted neural lobe. The fact that pituitary concentrations of VP are lowest during the morning supports the notion that nucleolar multiplication is out of phase with VP secretion by at least six hours. However, the lack of information regarding a diurnal rhythm in OX secretion and a complete description of VP levels in the blood over the day, make a complete assessment of this response impossible. If PVN neurons participate in diurnal VP secretion, then cell areas in this nucleus are also out of phase with circulating and pituitary VP titers. Given the rostro-caudal

distributions of OX and VP within the PVN (Swaab, et al., 1975), the homogeneity of the areal response across both subnuclei implies OX and VP cells were active at the same time.

Origin of rhythmicity. There are few clues as to the origin and control of the daily fluctuations in magnocellular neurosecretory function. Neither the PVN or SON are thought to receive retinal input, and Swanson and Cowan (1975) failed to observe any SCN projections to the two nuclei. According to Nobin, et al., (1972), dopaminergic fibers from the rat periventricular and arcuate regions, both of which receive SCN input (Swanson and Cowan, 1975), innervate the neurohypophysis. Dopamine elicits a graded release of OX and VP from the rat hypothalamus, including the pituitary stalk, in vitro (Bridges, et al., 1976). It is tempting to speculate that neurohypophysial hormone release may be effected by direct inputs on to neurosecretory axons or terminals, and that this release may thus oscillate over the day. The observed changes in nucleoli and cell size may reflect hormone production that is accomodating to neural lobe depletion. Such an hypothesis would account for phase differences between the cell responses in this study and VP levels in pituitary and plasma over the 24 hour period.

Increases in plasma protein concentration accompany blood volume depletion, which is an adequate stimulus for ADH release (Fabian, et al., 1969). Even though changes in plasma protein as measured by refractometry were evident in the present study, it cannot be determined whether or not these reflect blood volume depletion. The data for hematocrit, which can also be used as an index of blood volume, were incomplete where differences were suggested. It is possible that such an internal signal participates in, or results from, the activation of the SOa or PVN during the diurnal cycle.

Relation to corticosterone. From the cycles in SOa and PVN morphology

accounted for in this study, a role in adenohypophysial function is compatible for either nucleus. There are changes in large and small dense core vesicles in the SOa after four hours of water deprivation, when both small (< 1600 Å) and larger (> 1600 Å) vesicles are depleted in the cytoplasm (Tweedle and Hatton, 1976). Electron microscopic investigations have confirmed the existence of neurophysin- and VP-positive granules in the fibers of the MEx which are mostly in the 900-1100 Å range (Silverman and Zimmerman, 1975). Following adrenalectomy in the rat, Dube, et al., (1976) noted an increase in the number of small VP granules within axons abutting portal capillaries. Whether or not these small vesicles originate within the magnocellular neurosecretory perikarya, or perhaps the SCN, remains to be determined. A role in CRF secretion for either the PVN or SOa would again imply that fluctuations in hormone production are out of phase with actual secretion, since increases in CRF secretion would appear prior to those in corticosterone.

Differences between SON and PVN. Demonstrations of OX and VP in near equal proportions have led recent authors to postulate a functional similarity between the two nuclei (George et al., 1976). Such an hypothesis seems untenable with the absence of nucleolar proliferation in the PVN in light of its large cell size changes, while the SOa exhibits much smaller deviation in mean cell area, yet shows a distinct rhythm in its percentage of 'multiples.' Perhaps areal and nucleolar changes are simply the equivalent adjustments cells in each nucleus make, respectively, to produce hormone. This is unlikely since both nuclei respond with increases in somal area and in the percentage of 'multiples' during water deprivation (Hatton and Walters, 1973; Hoblitzell, et al., 1976). Given the elevated levels of corticosterone due to stress, the nucleolar response in the SOa may have been imposed by ether inhalation and cardiac blood sampling. Animals given

subcutaneous injections of isotonic saline when restrained show a short latency increase in the percentage of cells with multiple nucleoli in the SOa (Armstrong, et. al., 1977). However, the possibility that the percentage of 'multiples' in the SOa was stress-induced would still represent a difference between the two nuclei.

Recent anatomical inquiries have demonstrated separate afferent and efferent connections of the two nuclei. In particular, the PVN issues axons to the caudal brain stem and spinal cord, as well as to the neurohypophysis in the rat (Saper, et al., 1976). In the monkey, unilateral lesions of the PVN deplete VP from the ipsilateral MEx (Antunes, et al., 1976). The changes in cell area in the PVN may then reflect extra-neurohypophysial activity, especially that which may be related to adenohypophysial ACTH release.

Suprachiasmatic nucleus

Diurnal changes. No profound changes in any of the variables measured in SCN were witnessed. Between group differences that were evident were small and are open to varying interpretations. Differences between SCdm, whose cells contain VP and its associated neurophysin, and SCvl, were restricted to within subjects, and did not provide clear evidence for a functional separation of the two divisions. Criteria used in this study for neurosecretory activity, derived from responses in the magnocellular neurosecretory system, may not have been sensitive to the secretory capacity of SCdm cells.

Cells with invaginated nuclei do show some variation in their percentage of 'multiples' over time, particularly in the SCvl. These cell types account for approximately half of the sampled cell populations from both subnuclei, but are themselves labile over time. Changes within SCvl on this measure, and in cell size, are probably to related neural activity

in general, as SCN is involved in the maintenance of many bio-behavioral rhythms (Moore and Eichler, 1976). If real, the apparent changes in cell area in the SCdm may reflect neurosecretory activity which could account for CRF secretion.

SCvl and SCdm. The differences between SCdm and SCvl on several measures are interesting butuninterpretable at this time. In the rat, retinal ganglion cell fibers enter the ventromedial protion of the caudal aspect of SCN, and the possibility is slim that these axons terminate on VP or neurophysin producing cells (Mason and Lincoln, 1976). Extensive intrinsic connections have been described within the rat SCN (Guldner, 1976), so a close but indirect photoperiodic modulation of hormone-containing cell bodies in the SCdm may exist. No information is available which might indicate a topographic distribution of terminals from raphe' nuclei within the SCN. Detailed anatomical and physiological investigation, especially with the use of the electron microscope, are needed to determine more precisely the differences between the SCvl and SCdm under different conditions. The synthetic apparatus and product in the cytoplasm of SCN cells may be sensitive to slight increases in secretion. For example, Clattenburg, et al., (1975) have found changes in the Golgi complex, as well as in the dense core vesicle population, in the rabbit SCN after ovariectomy. To what hormone these changes are related is as yet unknown.

Role of VP in SCN. Due to a lack in species generality, the role of the VP in the rat SCN in ACTH regulation has been questioned (Silverman and Zimmerman, 1975). Only the rat and mouse SCN have been found to contain VP or neurophysin (Zimmerman, 1976). As discussed above, the SCN may mediate diurnal neurosecretory rhythms indirectly through its connections with the arcuate, ventromedial, and periventricular regions of the hypothalamus.

It is interesting that Slusher (1964) selectively abolished the corticosterone rhythm in the rat with lesions in the periventricular area, leaving adrenal responses to stress intact. Lesions made more basally only prevented the stress response. The SCN input to the periventricular area may be necessary for the nucleus to affect CRF secretion. Perhaps VP serves as a neurotransmitter in this regard.

Hippocampus

The phase relationship of plasma corticosterone with the area of HPC cells is probably more than coincidental. ^{3}H-corticosterone is taken up in large quantities by the cell nuclei in hippocampus (Warembourg, 1975). The physiological effect of this hormone on these cells has not been fully determined, but in hippocampus some role in modifying unit activity under different behavioral conditions is likely. For example, theta cells (cells intimately associated with theta potentials) increase their firing rates during certain behaviors when awake as well as during paradoxical sleep (Ranck, 1973). In the case of nocturnal animals, corticosterone may participate in preparing the organism for activity, with its peak near lights off.

Although disruption of the corticosterone rhythm has been reported following sections of the fornix by Moberg et al., 1971, others (Wilson and Critchlow, 1974) have failed to replicate this finding. While lesions of the hippocampus have no effect on corticosterone rhythmicity (Lanier et al., 1975), stimulation has been reported to inhibit ATCH release under basal conditions (Mangili et al., 1966). The evidence is as yet inconclusive, but these studies indicate some involvement of the hippocampus in pituitary-adrenal function. The heavy binding of corticosterone to hippocampal cell nuclei suggests this structure may participate in a long feedback loop, with its output affecting hypothalamic function, and perhaps

even modulating CRF release. Proper electrophysiological studies, however, need to be carried out concurrently with hormone assays in order to more precisely determine the effect of corticosterone on hippocampal cell function, and the effect of the latter on CRF secretion.

Nucleoli

In the magnocellular neurosecretory nuclei, demands are met resulting from increased hormone release by adjustments in organelles responsible for RNA and protein synthesis. The nucleolus serves to produce much of the high molecular weight ribosomal RNA vital to protein production (Busch and Smetana, 1970; p. 3). Nucleolar proliferation in SOa (Armstrong et al., 1977; Hatton and Walters, 1973) can be induced by osmotic stress, known to initiate enhanced ^{3}H-leucine incorporation (Norstrom, et al., 1971) and an increase in the uptake of labelled cytidine (George, 1973) in the nucleus. This nucleolar response thus appears correlated with indices of protein or RNA synthesis. The formation of additional nucleoli is an adaptive response, but in mature neurons the mechanism of this multiplication is unknown. A nucleolar chromosome contains a region designated the nucleolus organizer, which houses the DNA template responsible for RNA synthesis as well as nucleolar formation (Busch and Smetana, 1970; pg. 116). No outstanding ultrastructural differences can be seen between two nucleoli within a single nucleus in SOa neurons (Tweedle, personal communication). Studies are now in progress in our laboratory utilizing the uptake of ^{3}H-uridine during stimulated states as a measure of activity. More labelled material in 'multiples' when compared with cells having a single nucleolus may indicate an enhanced protein synthesizing capacity of the former.

In previous investigations of nucleolar proliferation in our laboratory, it has been noted that the increased percentage of 'multiples' in the rat SOa induced by water deprivation (Hatton and Walters, 1973),

stress and hypertonic saline ingestion (Armstrong et al., 1977), remains elevated for some time following termination of the activating stimuli. For example, after 10 days of rehydration following 24 hours of water deprivation, the level of 'multiples' fails to reach that observed in nondeprived control rats (Hatton and Walters, 1973).

In the present study, the percentage of cells with multiple nucleoli in the SOa shows dramatic shifts within six hours. Since in this case the excitatory stimuli are as yet unknown, their onset and termination cannot be determined. Nevertheless, it is interesting that these fluctuations, whereby nucleoli must be removed and produced in relatively large numbers, occur in the normal course of a day. Perhaps the neurosecretory system is "conditioned" to such daily alterations, anticipating the level of synthesis necessary to restore hormone levels in the neural lobe. When under severe challenge, as with water deprivation, the hormone content of the neurohypophysis is undoubtedly depleted beyond the restorative capacity of the SOa (or PVN); all the produced hormone is being secreted. Such a depletion would thus require prolonged synthesis until neurohypophysial levels are returned to baseline levels.

The fate of produced nucleoli is unknown. Expulsion of nucleoli through the nuclear membrane has been noted in nerve cells, but may be a histological artifact (Busch and Smetana, 1970; pg. 429). Structures resembling nucleoli in shape, such as nematosomes, or nucleolus-resembling bodies, have been observed with the electron microscope in the cytoplasm of SOa neurons (see LeBeaux, 1971; Tweedle and Hatton, 1975). The relationship of such elements with nucleoli is poorly understood. The number of such inclusions apparently does not increase during rehydration (Tweedle and Hatton, 1975). Le Beaux (1971) speculates that nucleolus-resembling bodies in the cytoplasm of neurosecretory cells are related to protein

synthesis in their own right, and are sufficiently distinct in their ultrastructure from nucleoli to be considered as separate entities. As can be gleaned from the preceding discussion, the formation and disposal of new nucleoli in mature neurons are phenomena only recently documented; much research is needed to clarify the mechanisms involved in these processes.

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APPENDIX A:

Materials

Materials

Blood Sampling and analysis

1. Refractometer, American Optical, $125.00.
2. Centrifuge, International Equipment Company, $270.00.
3. Capillary Tubes.
4. Micro-capillary Centrifuge, Model MB, International Equipment Company, $303.00.
5. Test tubes, VWR, $4.25.
6. 2cc glass syringes with 26 g needles.
7. 1cc tuberculin syringes, Plastipak.

Histology

1. Rotary Microtome, with knife; Arthur H. Thomas Company, $1600.00.
2. Thionine, Scientific Products, $40.00.
3. Paraffin, Paraplast, $2.10/2 1/2 lb.
4. 2" x 3" Microscope slides, Red label, $9.75/gross. Arthur Thomas Company.
5. Cover glasses, Red label, 1 oz. box, $4.10, Arthur Thomas Company.
6. Jars.
7. Base molds (1" x 1 1/2" x 1/2") $2.00/ea.; and embedding rings; $41.30/100 c.

Microscope equipment

1. Zeiss microscope, Morgan Instruments, $1200.00.
2. Whipple-Hauser reticule.
3. Oil immersion lens, 100x, $900.00, Morgan Instruments.

Photographic Equipment

1. Camera: Dierdorf, 5" x 7" format and optical bench arrangement.
2. Film: Kodak Contrast, Process Ortho.

APPENDIX B:

Histology

Histology

Paraffin embedding

1. Perfuse rat with saline (.90% NaCl) followed by a solution of 10% formalin in .90% saline. Remove brain from skull and soak in the fixative for several days.

2. Place brain in a jar with one layer of cheese cloth covering the mouth, secured by a rubber band. Submerge jar in container (tub or wash basin) with running water overnight.

3. Block the brain to remove undesirable areas.

4. Begin dehydration, following the schedule listed below:

	30% alcohol	overnight
	50% alcohol	2 hrs.
	70% alcohol	2 hrs.
	95% alcohol	2 hrs.
	100% alcohol	1 hr.
fresh	100% alcohol	1 hr.
	Methyl Benzoate + 100% alcohol (equal volumes)	1 hr.
used	Methyl Benzoate	1 hr.
fresh	Methyl Benzoate	1 hr.
	In oven at 60°C	
fresh	paraffin	1 hr.
fresh	paraffin	1 hr.
fresh	paraffin	1 hr.
fresh	paraffin	embed

5. To embed, poor a small amount of paraffin in a greased (vaseline or silicone) metal base mold, and allow it to partially, but not completely, solidfy.

6. Place brain on above layer of paraffin in base mold. Place embedding ring on top of base mold, and fill mold with paraffin.

7. Allow paraffin to harden (an hour at least).

8. Remove block from mold.

Sectioning

1. With a sharp razor blade, block the molded paraffin on all four edges, thus paring down volume of block. Make sure the edges are straight and that the ends of the rectangle are perpendicular to the sides.
2. Place block in rotary microtome chuck; fasten securely.
3. Orient blade flush with block surface.
4. Section at 15μm until tissue is reached. When tissue is reached, section a 6μm.
5. Lay ribbons of sections on brown paper in a tray.
6. Mount sections by floating them in pairs in water (52-56°C) and lifting them off with gelatinized slides.
7. Allow sections to dry vertically.
8. Deparaffinize in 4 changes of xylene, 20 minutes each.
8. Hydrate according to following schedule.

100% alcohol	10 minutes
95% alcohol	10 minutes
70% alcohol	20 minutes
50% alcohol	20 minutes
Distilled Water	20 minutes

10. Stain.

Staining

1. Place slides in 1% acqueous thionine (cold) for 10 minutes.

Thionine
200 ml of 1M NaOH
764 ml of distilled water
10 g of thionine
Must be mixed and heated for one hour at 90° C

2. Rinse briefly in distilled water.
3. Place sections in 70% alcohol for 5 minutes.
4. Differentiate in 95% alcohol. (This happens very fast.)
5. Place in 100% for 5 minutes.
6. Clear in Xylene for 20 minutes.
7. Cover slip.

APPENDIX C:

Raw Data

Plasma Corticosterone Concentrations
(ug/100ml plasma)

Group	Animal #	Value	$\bar{x}$
0600	D_1	12.5	
	D_2	20.0	
	D_3	16.5	17.6
	D_4	21.5	
1200	1 p.m. 1	14.5	
	1 p.m. 2	13.0	
	1 p.m. 3	30.0	
	1 p.m. 4	40.5	24.5
1800	N_1	41.0	
	N_2	81.0	
	N_3	60.0	
	N_4	29.5	52.9
2400	1 a.m. 1	15.5	
	1 a.m. 2	12.5	
	1 a.m. 3	14.5	
	1 a.m. 4	12.0	13.6

Multiple and marginated nucleoli
PVm

Group	Animal #	plane	% multiples	% margination singles	% margination multiples
0600	D_1	(coronal)	9.9	25.7	86.7
	D_2	(horizontal)	16.2	24.0	96.0
	D_3	(coronal)	23.2	25.0	91.4
	D_4	(horizontal)	15.4	19.8	95.7
	$\bar{x}$		16.2	23.6	92.5
1200	1 p.m. 1	(coronal)	23.6	22.1	97.1
	1 p.m. 2	(horizontal)	20.9	15.7	93.8
	1 p.m. 3	(coronal)	15.3	25.4	95.5
	1 p.m. 4	(horizontal)	37.9	20.7	92.5
	$\bar{x}$		24.2	21.0	94.7
1800	N_1	(coronal)	16.2	34.4	86.9
	N_2	(horizontal)	17.0	18.9	84.6
	N_3	(coronal)	10.5	32.6	68.8
	N_4	(horizontal)	29.1	26.2	88.6
	$\bar{x}$		18.2	28.0	82.2
2400	1 a.m. 1	(coronal)	18.8	16.0	75.9
	1 a.m. 2	(coronal)	25.2	31.6	96.9
	1 a.m. 3	(horizontal)	14.6	18.6	95.4
	1 a.m. 4	(horizontal)	14.4	11.4	72.7
	$\bar{x}$		18.3	19.4	85.2

Multiple and Marginated Nucleoli
PV1

Group	Animal #	plane	% miltiples	singles	% margination multiples
0600	D_1	(coronal)	18.4	41.9	96.4
	D_2	(horizontal)	34.4	29.7	86.8
	D_3	(coronal)	41.4	36.0	88.9
	D_4	(horizontal)	33.1	31.7	90.0
	$\bar{x}$		31.8	34.8	90.5
1200	1 p.m. 1	(coronal)	24.0	42.1	91.7
	1 p.m. 2	(horizontal)	40.8	35.6	84.5
	1 p.m. 3	(coronal)	13.5	27.4	90.5
	1 p.m. 4	(horizontal)	42.9	31.8	81.3
	$\bar{x}$		30.3	34.2	87.0
1800	N_1	(coronal)	32.0	36.3	93.8
	N_2	(horizontal)	27.3	25.0	81.0
	N_3	(coronal)	16.3	36.7	96.0
	N_4	(horizontal)	41.3	34.1	85.9
	$\bar{x}$		29.2	33.0	89.2
2400	1 a.m. 1	(coronal)	26.6	28.3	70.7
	1 a.m. 2	(coronal)	32.2	46.6	91.8
	1 a.m. 3	(horizontal)	25.3	32.2	82.1
	1 a.m. 4	(horizontal)	15.9	20.5	62.5
	$\bar{x}$		25.0	31.9	76.8

Multiple and Marginated Nucleoli
SOa

Group	Animal #	plane	% multiples	singles	% margination multiples
0600	D_1	(coronal)	14.6	37.2	93.4
	D_2	(horizontal)	12.6	34.1	89.5
	D_3	(coronal)	17.9	37.9	100.0
	D_4	(horizontal)	9.7	29.3	90.9
	$\bar{x}$		13.7	34.6	93.4
1200	1 p.m. 1	(coronal)	23.2	33.3	92.1
	1 p.m. 2	(horizontal)	26.0	21.7	87.2
	1 p.m. 3	(coronal)	14.6	32.9	100.0
	1 p.m. 4	(horizontal)	38.2	33.0	91.4
	$\bar{x}$		25.2	30.2	92.7
1800	N_1	(coronal)	25.5	36.8	94.9
	N_2	(horizontal)	37.6	27.6	91.5
	N_3	(coronal)	29.6	29.0	86.7
	N_4	(horizontal)	32.6	20.8	83.7
2400	1 a.m. 1	(coronal)	20.1	34.9	80.6
	1 a.m. 2	(coronal)	19.2	30.3	96.6
	1 a.m. 3	(horizontal)	19.6	27.6	76.7
	1 a.m. 4	(horizontal)	6.0	11.3	66.7
	$\bar{x}$		16.2	26.0	80.2

Multiple and Marginated Nucleoli
SCdm

Group	Animal #		% multiples		% margination			
					singles		multiples	
		plane	invg.*	ninvg.**	invg.	ninvg.	invg.	ninvg.
0600	D_1	(coronal)	25.4	46	85.1	78.1	100	93.1
	D_2	(horizontal)	51.6	42.9	86.7	58.3	100	100
	D_3	(coronal)	37.5	38.5	88.9	85	97	100
	D_4	(horizontal)	31.7	17.5	86.6	63.8	100	100
	$\bar{x}$		36.6	36.2	86.8	71.4	99.3	98.3
1200	1 p.m. 1	(coronal)	28.5	28.2	86.4	71.4	94.4	100
	1 p.m. 2	(horizontal)	33.6	40.4	86.3	60.7	100	89.5
	1 p.m. 3	(coronal)	31.8	31.8	84.4	81	100	92.6
	1 p.m. 4	(horizontal)	37.4	40.7	74.4	60	100	100
	$\bar{x}$		32.8	35.3	82.9	68.3	98.6	95.5
1800	N_1	(coronal)	42.7	30.9	85.5	78.9	95	82.3
	N_2	(horizontal)	30.4	27.9	68.3	46.3	96.6	73.1
	N_3	(coronal)	49.5	42.9	93.6	75	100	100
	N_4	(horizontal)	44.1	32.1	73.7	83.3	97.8	82.4
	$\bar{x}$		41.7	33.5	80.3	70.9	97.4	84.5
2400	1 a.m.1	(coronal)	28.1	23.2	78.2	60.5	100	100
	1 a.m. 2	(coronal)	43.7	44	95	83.3	100	97.4
	1 a.m. 3	(horizontal)	36.1	39.5	71.7	59.2	100	78.1
	1 a.m. 4	(horizontal)	47.4	38.3	72	37.8	97.8	91.3
	$\bar{x}$		38.8	36.3	79.2	60.2	99.5	91.7

* invg. = cells with an invaginated nucleus
** ninvg.= cells without an invaginated nucleus

Multiple and Marginated Nucleoli
SCv1

Group	Animal #		% multiples		% margination			
					singles		multiples	
		plane	invg.	ninvg.	invg.	ninvg.	invg.	ninvg.
0600	D_1	(coronal)	22.7	27.6	86.2	74.5	100	95.2
	D_2	(horizontal)	37.1	31.3	83.6	50	100	90
	D_3	(coronal)	41	48.1	86.4	67.9	100	100
	D_4	(horizontal)	33	23.5	78.8	47.3	100	75
	$\bar{x}$		33.5	32.6	83.8	59.9	100	90.1
1200	1 p.m. 1	(coronal)	29.1	26.9	86.9	47.4	92	100
	1 p.m. 2	(horizontal)	27.3	25.7	77.5	53.8	96.7	100
	1 p.m. 3	(coronal)	26.6	24.1	85.8	70.7	100	92.3
	1 p.m. 4	(horizontal)	29	39.6	85.7	86.2	100	94.7
	$\bar{x}$		28	29.1	84	64.5	97.2	96.8
1800	N_1	(coronal)	29.3	28	90	77.8	100	92.9
	N_2	(horizontal)	17.2	32.3	69.3	33.3	90.5	50
	N_3	(coronal)	49.1	59.5	90.2	64.7	100	96
	N_4	(horizontal)	56.8	16.7	79.2	56	90.5	80
	$\bar{x}$		38.1	34.1	82.2	58	95.3	79.7
2400	1 a.m. 1	(coronal)	31.4	22.1	75	59	100	100
	1 a.m. 2	(coronal)	54.3	50	92.9	66.7	100	90
	1 a.m. 3	(horizontal)	29.8	26.3	88.2	42.9	100	90
	1 a.m. 4	(horizontal)	32.5	39.5	71.2	43	97.5	86.7
	$\bar{x}$		37	34.5	81.8	52.9	99.4	91.7

Cell Areas (μm^2)

Group	Animal #	plane	SOa	PVl	PVm	SCdm	SCvl	HPC
0600	D_1	(coronal)	154.0	161.2	138.9	32.8	40.1	222.7
	D_2	(horizontal)	195.5	217.0	170.9	70.2	74.6	214.7
	D_3	(coronal)	126.9	130.9	135.7	35.9	43.2	243.9
	D_4	(horizontal)	179.4	206.6	156.9	52.3	64.3	192.1
	$\bar{x}$		164.0	179.0	150.6	47.8	55.5	218.4
1200	1 p.m. 1	(coronal)	185.1	146.3	119.1	30.4	36.3	194.3
	1 p.m. 2	(horizontal)	159.2	186.7	138.5	50.1	55.9	193.2
	1 p.m. 3	(coronal)	165.7	194.5	132.3	32.3	40.4	232.1
	1 p.m. 4	(horizontal)	168.0	169.8	128.5	48.0	55.7	171.2
	$\bar{x}$		169.5	174.4	129.6	40.2	47.1	197.7
1800	N_1	(coronal)	225.6	251.8	173.4	43.6	50.9	304.9
	N_2	(horizontal)	213.5	207.8	202.6	79.6	84.1	239.9
	N_3	(coronal)	246.4	292.9	167.9	41.5	45.7	292.4
	N_4	(horizontal)	185.3	327.7	192.4	66.6	71.7	229.6
	$\bar{x}$		217.7	270.0	184.1	57.8	63.1	266.7
2400	1 a.m. 1	(coronal)	197.3	212.6	226.6	37.9	51.2	240.9
	1 a.m. 2	(coronal)	175.7	260.8	160.6	33.1	43.2	245.6
	1 a.m. 3	(horizontal)	212.4	217.4	227.3	71.2	84.2	184.7
	1 a.m. 4	(horizontal)	238.4	244.5	232.5	62.1	77.3	215.2
	$\bar{x}$		205.9	233.8	211.8	51.1	64.0	221.6