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Corticotropin-Releasing Hormone and Arginine Vasopressin Gene Transcription in the Hypothalamic Paraventricular Nucleus of Unstressed Rats: Daily Rhythms and Their Interactions with Corticosterone

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To drive the daily ACTH secretory rhythm from anterior pituitary corticotropes signals from the circadian clock schedule CRH and, to lesser extent, arginine vasopressin (AVP) release from neuroendocrine terminals. In turn, releasable pools of CRH and AVP in neuroendocrine terminals are sustained by synthetic mechanisms in the medial parvicellular paraventricular nucleus, a critical component of which involves transcribing primary (heteronuclear (hn)) RNA transcripts from their cognate genes. To determine the fundamental daily patterns of ACTH secretagogue gene transcription in unstressed rats, we measured CRH and AVP hnRNA levels at 1- to 4-h intervals throughout the day using *in situ* hybridization. *Crh* gene transcription is readily detectable throughout the day, and shows a pronounced rhythm that is temporally correlated with CRH mRNA levels, but is uncoupled from ACTH release. However, *avp* gene transcription is barely detectable and shows no discernable rhythm. We then performed similar experiments in adrenalectomized rats with or without corticosterone replacement. Corticosterone-dependent mechanisms regulate CRH hnRNA levels at the nadir and peak as well as the onset of nocturnal *crh* gene transcription. A prominent rhythm of *avp* hnRNA seen in adrenalectomized animals was dampened by corticosterone. This study shows, first, CRH synthesis in intact animals is maintained by a nocturnal episode of *crh* gene transcription, parameters of which are modulated by corticosterone-dependent mechanisms; second, circulating corticosterone is sufficient to completely inhibit a daily rhythm of *avp* gene transcription present in adrenalectomized rats; third, the neural systems that activate *crh* gene transcription can be uncoupled from those driving ACTH release. (*Endocrinology* 145: 529–540, 2004)

SECRETION FROM THE hypothalamo-pituitary-adrenal (HPA) axis exhibits two distinct activation patterns: circadian-dependent release, which is driven by the suprachiasmatic nucleus (SCH) and is essential for maintaining normal energy balance (1, 2); and stress-dependent release, which follows internal or external challenges. Numerous studies have characterized HPA synthetic and secretory patterns after stress, but the neural basis of circadian-dependent release remains very poorly understood despite its importance for normal metabolism.

CRH is the principal neural signal controlling diurnal ACTH and glucocorticoid rhythms (3–7). CRH is synthesized in neuroendocrine neurons in the medial parvicellular (mp) part of the hypothalamic paraventricular nucleus (PVH). Some neuroendocrine CRH neurons also synthesize arginine vasopressin (AVP) (8, 9), the secretion of which is thought to contribute to ACTH secretion during some types of stress (9). *Crh* gene transcription and its attendant regulatory mechanisms are central to all aspects of ACTH secretion and constitute prime targets for agents controlling CRH synthesis, including glucocorticoids and stress (10–13). Despite this central role for *crh* gene transcription, how it behaves or is controlled throughout the normal 24-h period is unknown. Similarly, although barely detectable in intact animals during the early to mid part of the light phase, it is unclear whether *avp* gene transcription occurs in parvicellular neuroendocrine neurons at other times of the day.

Measuring steady state CRH mRNA levels has provided fundamental insights about the actions of major regulatory influences on CRH neurons (14, 15). However, CRH heteronuclear RNA (hnRNA) is considered a better index of *crh* gene transcription and provides clearer insights about the regulatory processes influencing CRH neurons (10–12, 16–19). Using CRH mRNA to delineate daily patterns of gene expression in unstressed animals has yielded only equivocal results (20–23), most likely because tracking subtle changes in the abundant amounts of CRH mRNA is difficult. Alternatively, monitoring transcription rates has been more challenging because CRH hnRNA levels are reportedly either extremely low or undetectable in unstressed animals (10–12, 16–19). As maintaining CRH synthesis requires transcription, there are at least two explanations for these findings: either synthesis is maintained by continuous low level transcription, or there is at least one significant, but as yet unidentified, episode of *crh* gene transcription. Distinguishing between these two possibilities will characterize a key regulatory component of the neural mechanisms controlling

Abbreviations: ADX, Adrenalectomized; AVP, arginine vasopressin; hnRNA, heteronuclear RNA; HPA, hypothalamo-pituitary-adrenal; KPBS, potassium PBS; mp, medial parvicellular; pm, posterior magnocellular; PVH, paraventricular nucleus; SCH, suprachiasmatic nucleus; ZT, zeitgeber time.

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CRH neuroendocrine neurons and, consequently, normal energy metabolism.

To this end, we measured CRH hnRNA and mRNA, and AVP hnRNA levels over a 24-h period in the PVHmp using a fine-grained temporal *in situ* hybridization analysis. Correlations were also made in these same animals between the RNAs and plasma ACTH, corticosterone, and leptin concentrations. We then used adrenalectomized (ADX) rats with or without corticosterone replacement to determine whether corticosterone-dependent mechanisms contribute to the dynamics of daily *crh* and *avp* gene transcription. Some aspects of this study have been reported in abstract form (24).

Materials and Methods

Animals and treatments

Adult male Sprague Dawley rats (225–250 g body weight at the beginning of the experiment) were housed two to a cage on a 12-h light, 12-h dark photoperiod [lights on at 0600 h = zeitgeber time (ZT) 0000 h] with unlimited access to water and rat chow, and were allowed at least 7-d acclimation to the animal quarters.

To investigate the effect of the varying dynamics of corticosterone secretion on the temporal organization of *crh* gene transcription, some animals were bilaterally ADX under halothane anesthesia using flank incisions. At this time they were given a sc pellet weighing approximately 100 mg containing either 100% paraffin wax or 40% corticosterone/60% cholesterol (25, 26). Based on results from preliminary experiments, 40% corticosterone pellets were chosen because of their ability to normalize thymus weights, plasma ACTH concentrations, and CRH mRNA levels in the PVHmp when sampled around the midpoint of the light phase.

All animal procedures were approved by the institutional animal care and use committee of University of Southern California.

Tissue preparation

Animals were killed by rapid decapitation at a variety of assigned time points throughout the day. To minimize disturbance in the animal quarters, care was taken not to kill groups of animals at intervals of less than 6 h within any 1 d. For animals killed during the dark phase, decapitation was performed immediately adjacent to the housing facilities in subdued light. Time from cage removal to decapitation was less than 2 min in all cases. Brains were rapidly removed from each animal and immediately placed in ice-cold 4% paraformaldehyde in 0.1 M borate buffer, pH 9.5. At the same time, trunk blood was collected into two cooled vials, coated with either EDTA-saline for ACTH assay or heparin-saline for corticosterone and leptin assays. Plasma was separated after centrifugation and stored at −70 C for plasma ACTH, corticosterone, and leptin determinations at a later date. Finally, in some animals thymuses were removed, dissected free of adipose and connective tissue, blotted dry, and weighed.

Brains were fixed in the borate/paraformaldehyde fixative for 20–24 h. Sucrose was then added to the 4% paraformaldehyde solution to attain a 12% (wt/vol) sucrose concentration, and fixation was continued for a further 2 d at 4 C. At this stage brains were frozen in hexanes cooled with powdered dry ice and immediately stored at −70 C until sectioning at a later date. Eight series of one-in-eight 15-μm-thick frozen coronal sections were cut through the rostral hypothalamus using a sliding microtome and saved in ice-cold potassium PBS (KPBS) containing 0.25% paraformaldehyde. Sections were mounted the same day on SuperFrost Plus (Fisher Scientific, Pittsburgh, PA) slides, vacuum-desiccated overnight, postfixed in KPBS-4% paraformaldehyde for 1 h at room temperature, rinsed five times for 5 min each time in clean KPBS, air-dried, and then stored at −70 C in air-tight containers containing silica gel desiccant for hybridization at a later date. Serial sections were saved for thionine staining.

In situ hybridization

Sections were hybridized with either a [^{35}S]UTP-labeled cRNA probe transcribed from a 700-bp cDNA sequence encoding RNA for part of the prepro-CRH sequence, a 700-bp *Pvu*II fragment of intron 1 of the *avp* gene, or a [^{35}S]UTP/[^{35}S]CTP-labeled cRNA probe transcribed (11) from a 536-bp *Pvu*II fragment complementary to a sequence within the single CRH intron (27). cRNA probes were synthesized using the Gemini kit (Promega, Madison, WI) and the appropriate RNA polymerase. The characterization of all probes has been reported previously (19, 28, 29).

In situ hybridization with the radiolabeled cRNA probes was performed as described previously (29) with posthybridization modifications to the CRH hnRNA hybridization as follows. After the ribonuclease incubation at 37 C and room temperature washes from 4× to 0.1× standard saline citrate, slides were incubated at 70 C for 30 min with slight agitation every 10 min. Sections were exposed to Microvision C x-ray film (Diagnostic Imaging, Inc., Mira Loma, CA) for appropriate exposure periods (1–42 d), then dipped in nuclear track emulsion (Kodak NTB-2, Eastman Kodak, Rochester, NY; diluted 1:1 with distilled water); exposed for 5 d (CRH mRNA), 21 d (AVP hnRNA and CRH hnRNA in experiment 2), or 42 d (CRH hnRNA in experiment 1); developed; and counterstained with thionine.

Quantitation of [^{35}S]UTP-labeled cRNA hybridization signals

Mean gray levels of the RNA hybridization signals in the Nissl-defined subdivisions of the PVH were measured from images on Microvision C x-ray film using IP-Lab Spectrum imaging software (Scananalytics, Inc., Fairfax, VA) as described previously (19, 29). Hybridization values were expressed on a 0–255 grayscale. Parcellation of the PVH was determined using the scheme and nomenclature of Swanson (30). We have previously demonstrated the linearity of the *in situ* hybridization signal response on the x-ray film and our detection system (19). Those parts of the PVH in which measurements of CRH (PVHmp) or AVP hnRNA [PVHmp and the posterior magnocellular (pm) part of the PVH] were taken were defined using the adjacent Nissl- and CRH mRNA-hybridized sections. When measuring the AVP hnRNA hybridization signal in the PVHmp, we did not attempt to exclude from analysis signal derived from any scattered magnocellular neurons located in this region. We did, however, also report daily variations in AVP hnRNA in the magnocellular neurons in the PVHmp.

RIAs

Plasma corticosterone, ACTH, and leptin concentrations were measured in duplicate unextracted samples from every animal using [^{125}I]antigen-labeled double-antibody RIAs for corticosterone (ICN Biochemicals, Costa Mesa, CA), ACTH (DiaSorin, Inc., Stillwater, MN), and leptin (Linco Research, Inc., St. Charles, MO), all supplied in kit form. To increase the sensitivity of the corticosterone assay, half-volumes of all reagents were used, and the percentage of total binding was adjusted to approximately 29%. The lower sensitivity limits were 5 ng/ml, 19 pg/ml, and 0.5 ng/ml for corticosterone, ACTH, and leptin assays, respectively. Plasma samples from all animals in this study were assayed together in single assays for each hormone. The intraassay coefficients of variation were less than 8% for all assays.

Experimental design

Two experiments were performed. In the first (experiment 1), 13 groups of intact animals (n = 5–8/group) were decapitated at various times to encompass the entire day. Blood and brain tissue were collected and processed from all animals as described. AVP hnRNA, CRH hnRNA, and CRH mRNA hybridization was performed on sections from all time points in single assays. One additional group of four rats was anesthetized for 5 min at ZT 0500 h with halothane and then decapitated 10 min later. Brains were removed and processed as described and were hybridized for CRH hnRNA together with the sections from the groups killed throughout the day.

In the second experiment (experiment 2), two sets of ADX animals implanted with either 0% or 40% corticosterone pellets were decapitated 6–7 d after ADX at 4-h intervals across the day (n = 5–7/time point). Although treated at a later date, these animals were housed in the exact same facilities and under the same conditions as the intact animals used in experiment 1. Blood and tissues were collected and processed from all animals as described. Sections from the ADX animals at all time

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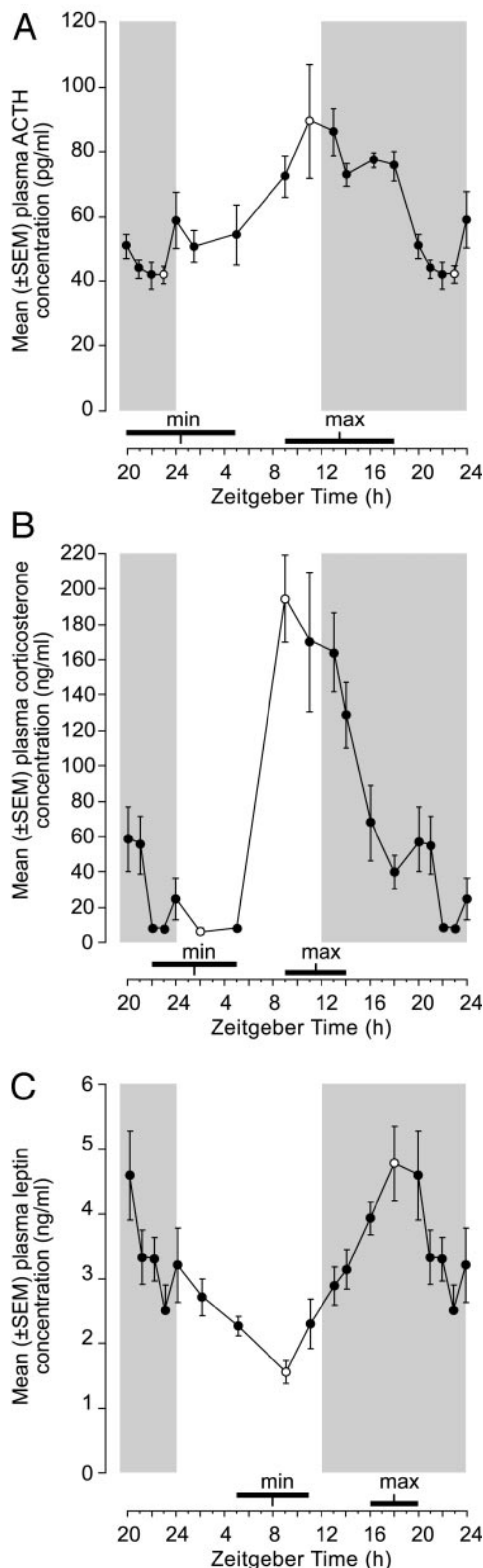

FIG. 1. The mean (±SEM) plasma ACTH (A), corticosterone (B), and leptin (C) concentrations in groups of intact animals killed at various times throughout the day. Hormone concentrations were measured in blood samples taken from the same animals as those in Fig. 2. ○, Time when the minimum and maximum values were measured for each variable. The range of times that were not significantly different from these values is shown by the *bars* above the x-axis. The midpoint of each of these ranges was designated the maximum or minimum of the daily variations. See Table 1 for statistical analysis. Lights were on from ZT 0000–1200 h. *Gray shading* indicates the time of lights off. Values measured at ZT 2000–2400 h were double-plotted to help clarify the variations across the 24-h period.

points were hybridized for CRH hnRNA and mRNA in single assays. These two assays also included selected sets of sections from groups of intact animals from experiment 1 chosen to correspond as closely as possible to the times the ADX animals were killed. This strategy ensured that direct comparisons of the relative RNA values in intact and 0% ADX, and 40% ADX animals could be made. We note that the intact animals from experiment 1 are not the ideal control for this comparison, which would be sham-ADX animals. However, we are confident the 6 d between sham surgery and decapitation minimized potential differences in measured variables from those in unmanipulated intact animals, as we have found in other circumstances (Watts, A. G., unpublished observations).

Statistical analysis

The significance of differences in plasma hormone concentrations and RNA hybridization signals was determined across the day using one-way ANOVA, followed by Fisher's least significant difference *post hoc* test. This analysis was used to determine two parameters: first, the presence of a statistically significant daily variation in each variable, and second, the time of day when each variable became significantly different from the measured minimum or maximum mean value. This second parameter provided a range for the daily maximum and minimum, the midpoint of which was designated as the time when each variable began to increase and decline during the day. ANOVA followed by Fisher's least significant difference *post hoc* test were also used to compare the characteristics of the daily variations in experiment 2. $P < 0.05$ was regarded as being statistically significant. All statistical analyses were performed using Excel (Mac version 5.0, Microsoft, Redmond, WA) and Systat (Mac version 5.2) software.

Results

Experiment 1

Daily variations in plasma hormone concentrations in intact animals. Significant rhythms were seen in the mean plasma hormone concentrations for ACTH, corticosterone, and leptin. Figure 1A and Table 1 show that the minimum plasma ACTH and corticosterone concentrations were found around the time of lights on; maximum values occurred around the time of lights off. The existence of a significant, but low amplitude, daily rhythm in plasma ACTH concentrations is consistent with some (21, 31), but not all (32), previous studies. The characteristics of the corticosterone rhythm correspond to those reported many times by other groups (3, 9), with the time of minimum and maximum values closely corresponding to those of plasma ACTH (Fig. 1B and Table 1). Mean minimum plasma leptin concentrations were found approximately 8 h later than ACTH, whereas maximum values occurred in the middle of the dark phase at ZT 1800 h (Fig. 1C and Table 1). The timing of a plasma leptin rhythm is consistent with previous studies (31–35), although absolute values differ, presumably reflecting the different body weights and strain differences of the animals used in the various studies.

Daily variations in crh and avp gene expression in intact animals. Figures 2A and 3, and Table 1 show that there was a highly

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TABLE 1. The timing parameters and statistical significance of the daily variations measured in experiments 1 and 2 for CRH hnRNA, AVP hnRNA, and CRH mRNA levels in the mp and pm parts of the PVH; and plasma ACTH, corticosterone, and leptin concentrations

Experiment 1	Synthesis				Secretion		
	Parvicellular			*Magnocellular*, AVP hnRNA	ACTH	Corticosterone	Leptin
	CRH hnRNA	CRH mRNA	AVP hnRNA				
Experiment 1							
Intact							
Significant variation?	Yes	Yes	No	Yes	Yes	Yes	Yes
Significance (ANOVA)	F(12,67) = 12.298 $P < 0.0001$	F(12,68) = 2.998 $P < 0.0025$	F(12,56) = 0.927 $P = 0.627$	F (12,56) = 2.495 $P < 0.02$	F(12,69) = 5.932 $P < 0.0001$	F(12,69) = 13.588 $P < 0.0001$	F(12,69) = 4.695 $P < 0.0001$
Time of minimum (ZT)	1230 h	1430 h			0030 h	0130 h	0800 h
Time of maximum (ZT)	0000 h	2230 h			1330 h	1130 h	1800 h
Experiment 2							
Intact							
Significant variation?	Yes	No	Not determined		Yes	Yes	Yes
Significance (ANOVA)	F(6,33) = 10.927 $P < 0.0001$	F(6,34) = 2.075 $P = 0.082$			F(6,35) = 3.835 $P < 0.005$	F(6,35) = 10.714 $P < 0.0001$	F(6,35) = 9.135 $P < 0.0001$
Time of minimum (ZT)	1100 h				2200 h	0200 h	0900 h
Time of maximum (ZT)	0200 h				0900 h	0900 h	1800 h
ADX							
Significant variation?	Yes	No	Yes	No	Yes	Not detectable	No
Significance (ANOVA)	F(5,26) = 4.905 $P < 0.003$	F(5,26) = 1.780 $P = 0.154$	F(5,22) = 14.269 $P < 0.0001$	F(5,26) = 0.1.435 $P = 0.245$	F(5,26) = 9.370 $P < 0.0001$		F(5,28) = 2.295 $P = 0.076$
Time of minimum)ZT)	1400 h		0200 h		0200 h		
Time of maximum (ZT)	2200 h		1400 h		1000 h		
ADX + CORT							
Significant variation?	Yes	No	Yes	No	Yes	Not determined	Yes
Signifcance (ANOVA)	F(5,31) = 6,048 $P < 0.0005$	F (5,30) = 1,223 $P = 0.32$	F(5,26) = 2,800 $P < 0.05$	F(5,22) = 0.628 $P = 0.680$	F(5,31) = 3,465 $P < 0.02$		F(5,31) = 5,508 $P < 0.0015$
Time of minimum (ZT)	0200 h		0200 h		0400 h		1000 h
Time of maximum (ZT)	1800 h		1800 h		1800 h		1800 h

CORT, Corticosterone.

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significant daily rhythm of CRH hnRNA in the PVHmp. At the time of minimum values (ZT 1100–1400 h), the hybridization signal was barely detectable, whereas at the time of maximum values (ZT 2200–0200 h) it was readily apparent (Figs. 3 and 4). Figure 4 shows that CRH hnRNA levels at the time of the daily maximum were substantially lower than those seen after a brief anesthesia stress, illustrating the wide dynamic range of the mechanisms that can activate *crh* gene transcription.

Significant daily variations in the amount of CRH mRNA in the PVHmp showed a much lower amplitude than those of CRH hnRNA and exhibited a more complex shape (Figs. 2B and 3, and Table 1), presumably reflecting the variety of influences in addition to transcription that determine steady mRNA levels (36). However, the times when the minimum and maximum mRNA values were observed coincided with those of hnRNA, with highest values seen around lights on and lowest around lights off.

AVP hnRNA hybridization signal in the PVHmp was consistently low throughout the day, and no significant daily variation was discernable (Figs. 2 and 3, and Table 1). Examination of slide autoradiographs showed that there were occasional magnocellular neurons in the PVHmp (*e.g.* Fig. 3, *lower panels*), which probably accounted for the measurable signal we report in this part of the nucleus.

There was a small, but significant, variation in the AVP hnRNA hybridization signal in the pm part of the PVH across the day (Fig. 2 and Table 1). Lower levels tended to occur during the light, whereas higher levels were evident toward the end of the dark period.

Experiment 2

One possible explanation for the shape of the CRH hnRNA rhythm seen in intact animals is that it is simply the response of a corticosterone-sensitive, servo-type mechanism controlling *crh* gene transcription, as would be predicted by the simple $\log_{10}$ dose-response relationship between corticosterone and CRH mRNA (37, 38). Thus, as plasma corticosterone levels increase during the day, they increasingly inhibit *crh* gene transcription, but as levels fall at night, inhibition is removed, and transcription is activated. To test this possibility, we examined what effect either removing corticosterone entirely or providing corticosterone to ADX animals with constant release pellets had on the characteristics of the various daily gene expression and hormone rhythms.

First, we characterized the effects of a constant release corticosterone pellet in animals killed between 0500 and 0600 h ZT. Table 2 shows that providing ADX animals with a 100-mg 40% corticosterone pellet returned CRH hnRNA, CRH mRNA, plasma ACTH, and thymus weight to values seen in intact animals killed at this time. Pellets of this size were then used in the remaining experiments.

Daily variations in plasma hormone concentrations in intact, ADX, and ADX animals with corticosterone replacement. Table 1 and Fig. 5A show that there were significant daily variations in plasma ACTH concentrations in all three experimental groups. The times of maximum and minimum values were approximately the same in ADX (0%) and intact animals (around the time of lights off), but maximum values occurred approximately 6–8 h later in ADX (40%) animals.

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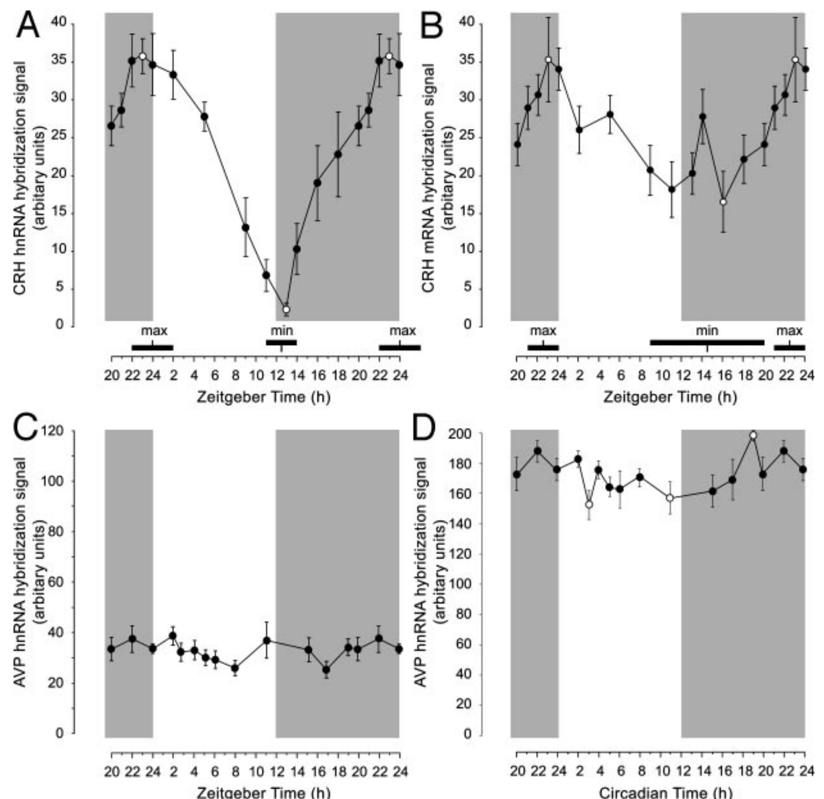

FIG. 2. The mean (±SEM) CRH hnRNA (A), CRH mRNA (B), and AVP hnRNA (C) levels in the mp part and AVP hnRNA levels (D) in the pm part of the hypothalamic PVHmp from groups of intact animals killed at various times throughout the day. Hybridization was measured on sections through the PVH taken from the same animals as those in Fig. 1. ○, Time when the minimum and maximum values were measured for each variable. For A and B, the range of times that were not significantly different from these values is shown by the *horizontal bars* above the x-axis. The midpoint of each of these ranges was designated the maximum or minimum of the daily variations. See Table 1 for statistical analysis. Lights were on from ZT 0000–1200 h. *Gray shading* indicates the time of lights off. Values measured at ZT 2000–2400 h were double-plotted to help illustrate the variations across the 24-h period.

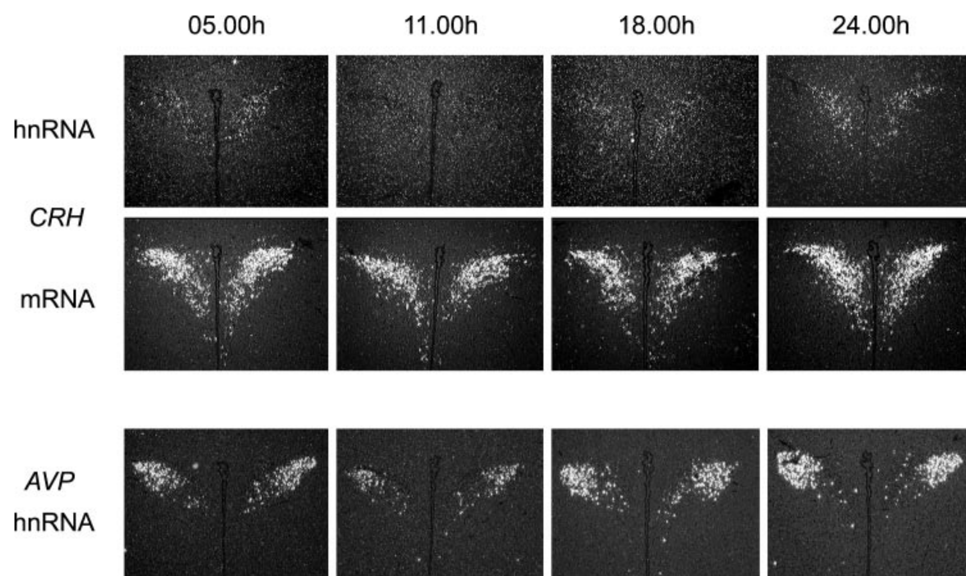

FIG. 3. Dark-field photomicrographs of CRH hnRNA, CRH mRNA, and AVP hnRNA hybridization in the PVHmp in intact animals at various times of the day to illustrate the range of RNA levels seen throughout the day. At any one time, serial sections are illustrated from the same animal.

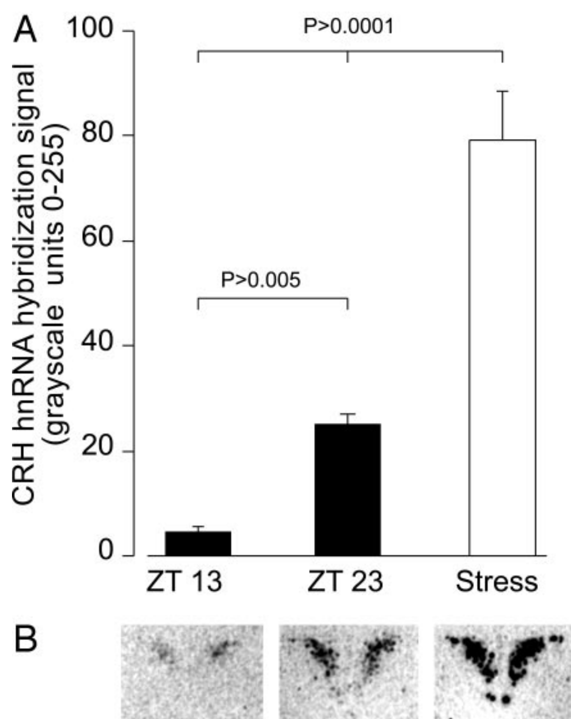

FIG. 4. A, The mean (±SEM) CRH hnRNA hybridization signal in the PVHmp from animals killed at the minimum (ZT 1300 h) and the maximum (ZT 2300 h) and from animals killed 10 min after a 5-min halothane anesthesia stress. B, Photomicrographs of the CRH hnRNA hybridization in the PVH from representative animals from each group exposed to Microvision x-ray film. All sections were hybridized and exposed together.

Plasma leptin concentrations, however, only showed significant daily variations in intact and ADX (40%) animals, not in ADX (0%) animals (Table 1 and Fig. 5B). Although maximum plasma leptin concentrations in ADX (0%) animals were also seen in the middle of the dark period, the differences between groups across the day did not quite reach statistical significance (Table 1). Unlike plasma ACTH concentrations, maximum and minimum plasma leptin values in intact and ADX (40%) animals occurred at the same times in both groups (Table 1).

Daily variations of crh gene expression in intact, ADX, and ADX animals with corticosterone replacement. Table 1 and Fig. 5C show that there were significant daily variations in CRH hnRNA levels in the PVHmp in all three groups of animals. We should point out the that the differences in absolute grayscale brightness values seen between Figs. 2A and 5C are a consequence of the different exposure times used for the CRH hnRNA hybridization in experiments 1 (42 d) and 2 (21 d). Different exposure times were employed to ensure that the signal from ADX (0%) animals remained on the linear part of the response curve of our analysis system, which meant that the absolute CRH hnRNA values from the set of intact animals were different in experiment 1 (Fig. 2A) and experiment 2 (Fig. 5C) even though they were the same samples.

CRH mRNA levels in experiment 2 did not show a statistically significant daily variation in any group (Table 1 and Fig. 5D). However, the tendency of the changes in CRH mRNA levels in ADX (0%) animals was to parallel the changes in hnRNA levels, in a manner similar to the CRH hnRNA/mRNA patterns in experiment 1 (Fig. 2). We interpret the lack of variation in the CRH mRNA data from intact animals in experiment 2 compared with the significant variations seen in experiment 1 (Fig. 2B) as being a consequence of the intrinsically small amplitude of the mRNA rhythm and the smaller number of time points sampled in experiment 2 [6] compared with those in experiment 1 [13].

To determine the effects of manipulating the circulating corticosterone environment on the *crh* gene transcription rhythm in the three experimental groups, we compared four characteristics of the 24-h fluctuation pattern: mean value at the nadir (the low point of the rhythm), mean value at the peak of the rhythm, the amplitude, and time of day when minimum and maximum CRH hnRNA levels occurred.

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TABLE 2. Mean (±SEM) plasma corticosterone, ACTH, CRH hnRNA, and CRH mRNA in the mp part of the PVH, and thymus weights of ADX animals killed at the midpoint of the light phase (0500–0600 h) are all normalized by sc implantation of a 100-mg 40% corticosterone pellet

	Intact	ADX + 40%	ADX + 0%
Corticosterone (ng/ml)	NM	35.6 ± 5.7	ND
ACTH (pg/ml)	54.1 ± 9.3	60.5 ± 6.4	300.4 ± 40.6[a]
CRH hnRNA (gray scale)	21.4 ± 1.6	23.1 ± 3.7	53.1 ± 2.0[a]
CRH mRNA (gray scale)	43.4 ± 2.6	45.1 ± 3.1	74.3 ± 7.8[a]
Thymus weight (mg/100 g body weight)	210.5 ± 12.7	189.8 ± 9.2	303.6 ± 18.8[a]

NM, Not measured; ND, not detectable.
[a] Values are significantly greater ($P < 0.001$ or less) than those in intact animals.

Nadir

Figure 6A shows that there was no significant difference between mean nadir CRH hnRNA levels in intact and ADX (40%) animals, but these were both significantly lower than those from ADX (0%) animals.

Peak

Figure 6B shows that the peak levels attained by CRH hnRNA during the day were significantly higher in ADX (40%) animals than in intact animals, both of which were significantly lower than that in ADX (0%) animals.

Amplitude

The amplitude of the daily fluctuations was calculated in each experimental group by subtracting the value of the mean CRH hnRNA level at the lowest point in the day from the individual values at the time of the mean maximum hnRNA level. Figure 6C shows that the different corticosterone treatments had no significant effect [$F(2,16) = 2.719$; $P = 0.096$] on the amplitude of the daily variations in CRH hnRNA. However, there was a tendency for the amplitude to increase from intact to ADX (0%) animals (Fig. 6C), suggesting that corticosterone-dependent mechanisms may decrease the amplitude of the rhythm if plasma corticosterone were increased significantly above the circadian mean.

Time of day when minimum and maximum CRH hnRNA levels occur

These times were determined in each experimental group by taking the midpoint of the range of times that were not statistically different from the minimum or maximum values, respectively. Table 1 and Fig. 6D show that compared with intact animals, the onset of transcription in ADX (0%) animals was slightly delayed, whereas maximum values occurred about 4 h earlier, approximately 2 h before lights off. However, in ADX (40%) animals, transcription both began and peaked earlier than in either of the other two groups.

Daily variations in avp gene expression in intact, ADX, and ADX animals with corticosterone replacement

Both groups of ADX animals showed significant daily variations in AVP hnRNA levels in the PVHmp (Figs. 5 and 7; Table 1). This was most noticeable in ADX (0%) animals, in which a prominent rhythm was evident. In both ADX groups, minimum levels occurred at the beginning of the light period (ZT 0200 h), whereas maximum levels occurred in the middle of the dark period (ZT 1800 h). This pattern closely matched the variations in plasma ACTH, but were somewhat different from those in CRH hnRNA.

AVP hnRNA levels in ADX (40%) animals were significantly lower at all time points examined compared with those in ADX (0%) animals ($P < 0.02$ or less). Unfortunately, we were unable to directly compare the levels of AVP hnRNA between the ADX and intact animals because all sections from intact animals been used for other assays. However, examining emulsion-coated slides showed that AVP hnRNA levels in the ADX (40%) animals at the beginning of the light phase (Fig. 7) were equivalent, to those in intact animals (Fig. 3). There were no significant daily variations in AVP hnRNA levels in the PVHpm in either ADX group, and no differences were seen between the two experimental groups at any time.

Discussion

We show that in intact rats a prominent increase in CRH hnRNA levels occurs at night when rats are most active. These data strongly suggest that, like the secretory components of the HPA axis, *crh* gene transcription is not constant across a 24-h period, but increases and decreases in a simple rhythm. As CRH hnRNA and mRNA fluctuate in phase, nocturnal gene transcription most likely maintains CRH mRNA, and consequently is a key contributor toward sustaining CRH synthesis and thus normal HPA function in unstressed intact animals. Although CRH hnRNA levels at their maximum are much lower than those seen after a transient stressor, they are detectable at all times of the day using appropriate detection methods.

The daily variation in CRH hnRNA levels in intact animals exhibits a simple rhythm, where transcription begins around lights off when CRH hnRNA levels are extremely low and continues until lights on when CRH hnRNA levels begin to fall. This pattern contrasts sharply with AVP hnRNA, which is expressed at very low levels in the PVHmp of intact animals and shows no variation across the day. With either extremely low circulating corticosterone or chronic stress conditions, AVP is coexpressed with CRH in about half of the CRH neuroendocrine neurons in the rat PVHmp (8, 9). The constant and very low levels of AVP hnRNA we see throughout the day are consistent with the findings of studies using Brattleboro rats (4), CRH knockout mice (7), and CRH immunoneutralization (5), showing that diurnal corticosterone release in intact animals is driven almost exclusively by CRH release.

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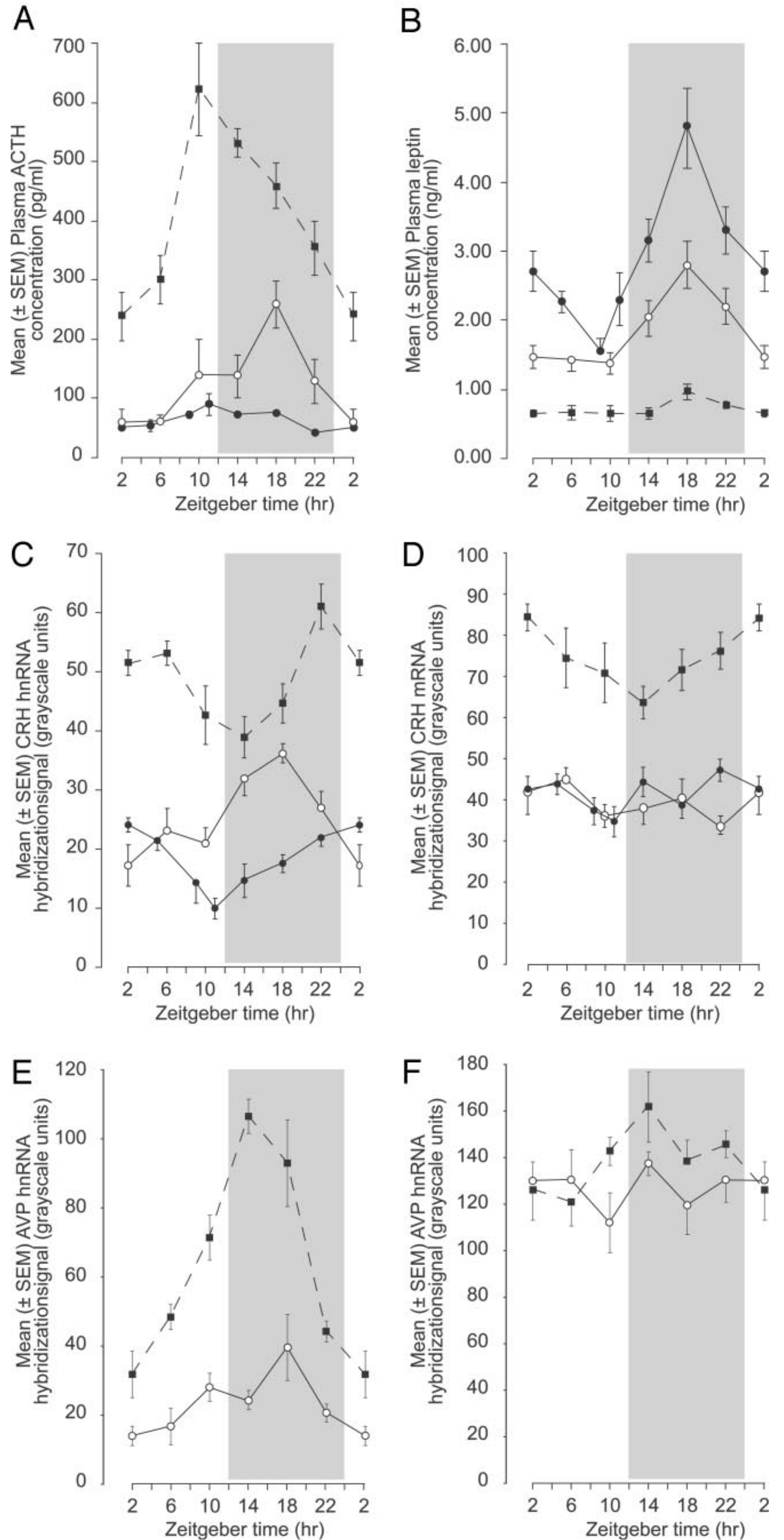

FIG. 5. The mean (±SEM) plasma ACTH (A) and leptin (B) concentrations, and levels in the PVHmp of CRH hnRNA (C), CRH mRNA (D), AVP hnRNA (E), and AVP hnRNA (F) in the PVHpm from groups of intact (●), ADX (40%; ○), and ADX (0%; ■) animals killed at various times throughout the day. See Table 1 for statistical analysis. Lights were on from ZT 0000–1200 h. *Gray shading* indicates the time of lights off. Values measured at ZT 0200 h were double-plotted to help illustrate the variations across the 24-h period.

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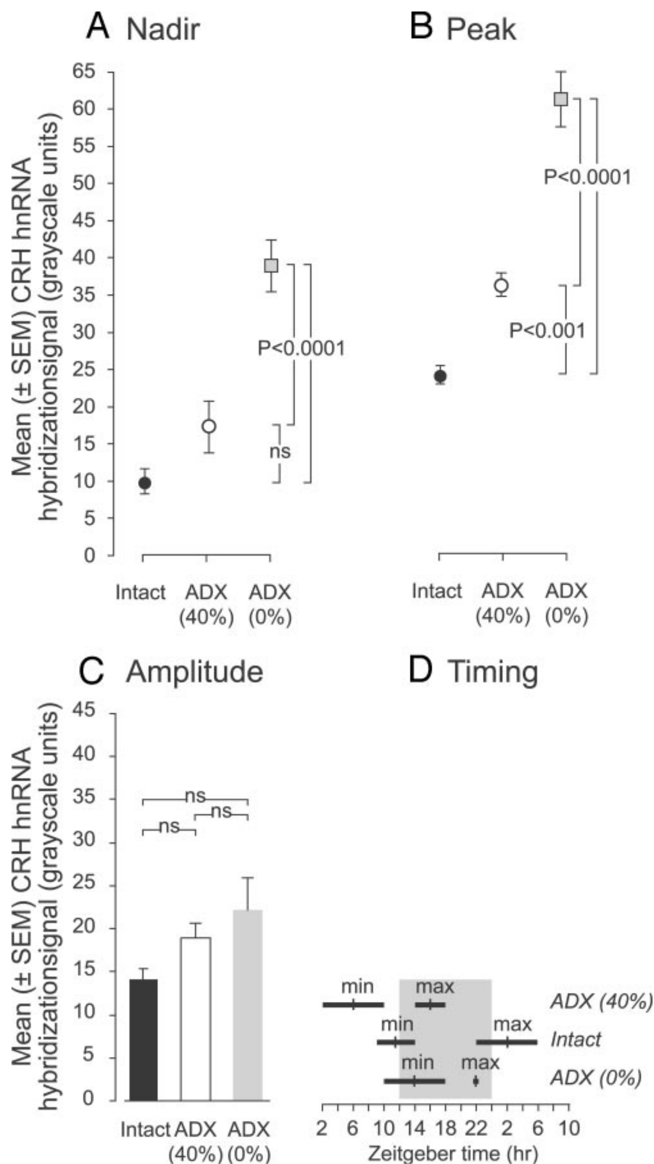

FIG. 6. A, The mean (±SEM) CRH hnRNA levels in the PVHmp at the time of the minimum value in intact (●), ADX (40%; ○), and ADX (0%; ▨) animals. B, The mean (±SEM) CRH hnRNA levels in the PVHmp at the time of the maximum value in intact (●), ADX (40%; ○), and ADX (0%; ▨) animals. C, The mean (±SEM) amplitude of CRH hnRNA levels in the PVHmp in intact (■), ADX (40%; □), and ADX (0%; ▨) animals. D, The times of day when the minimum and maximum CRH hnRNA levels are seen in intact and 40% and 0% ADX animals. The range of times that is not significantly different from these values is shown by the *horizontal bars* within each experimental group. The midpoint of each of these ranges (*vertical line*) is designated the maximum or minimum of the daily variations.

Four parameters are important for defining the daily pattern of *crh* gene transcription: the time of day when transcription begins, the subsequent rate of increase in CRH hnRNA accumulation, the time of day when transcription ceases, and the subsequent rate of decline in CRH hnRNA accumulation. It is likely that different mechanisms determine the value of each of these parameters, which, in turn, involves the integration of neural information encoded by sets of PVHmp afferents and humoral agents, of which corticosterone is the most important (14, 15, 39, 40). We show in experiment 2 that the amount of CRH hnRNA present at both the nadir and peak is a crucial target of corticosterone's long-term actions on CRH synthesis. These observations show that in the absence of stress the overall level of transcription is significantly higher in ADX (0%) animals than in intact or ADX (40%) animals; a finding consistent with studies in which CRH hnRNA levels were measured at single times during the day (13, 17, 41, 42). Although the amplitude of the daily CRH hnRNA variation is statistically indistinguishable among the three groups, its downward trend from ADX (0%) to intact animals suggests that corticosterone will have a significant effect on the amplitude of daily variations if corticosterone's mean value increases above that seen in intact animals. Collectively, these data suggest that when plasma corticosterone is chronically elevated to levels above those seen in unstressed intact animals, all parameters of the daily rhythm in *crh* gene transcription are targeted to reduce CRH mRNA levels and ultimately the amount of CRH peptide available for release in the median eminence.

How corticosterone regulates the overall level of *crh* gene transcription is unknown, but a basic property must involve the time required for transcription to respond to changes in circulating corticosterone. Exactly how long this takes in unstressed animals using physiological levels of corticosterone is unclear, but the consensus is that it is slow (12, 41). Certainly if circulating corticosterone shifts above or below the circadian mean for a significant period (during chronic stress or after adrenalectomy with or without exogenous corticosterone treatment), resultant changes in *crh* gene expression take at least 12 h to become measurable (37, 41). Considered together, these data suggest that one component responsible for the sluggish response of *crh* gene expression to changes in circulating corticosterone is mediated by mechanisms that modify the rate of either increase or decline in *crh* gene transcription depending on whether corticosterone is decreasing or increasing. The fact that the time when transcriptional activation/decline occurs is constrained within daily time windows implies that *crh* gene expression is, like ACTH secretion (43), differentially sensitive to corticosterone across a 24-h period.

A parameter that is related to this timing issue is the time during the day when transcription is activated. In ADX (40%) animals, CRH hnRNA levels clearly begin to increase earlier than in the other two groups. Although the explanation for this observation is unknown, we have previously demonstrated that the corticosterone environment influences the onset and duration of *crh* gene transcription after hypovolemic stress (13, 42), and the present observation may indicate the existence of a similar mechanism here.

The nature of the mechanisms responsible for stimulating *crh* (in all groups) and *avp* (in both ADX groups) gene expression during the dark period is currently unknown, but they must play a critical role in controlling ACTH secretagogue synthesis and, hence, overall HPA function. For *crh* gene expression, we show that the ability of these mechanisms to stimulate and maintain the transcription that starts around the beginning of the dark period in intact animals clearly does not depend upon the absolute level or the dynamics of corticosterone secretion. Many groups have shown

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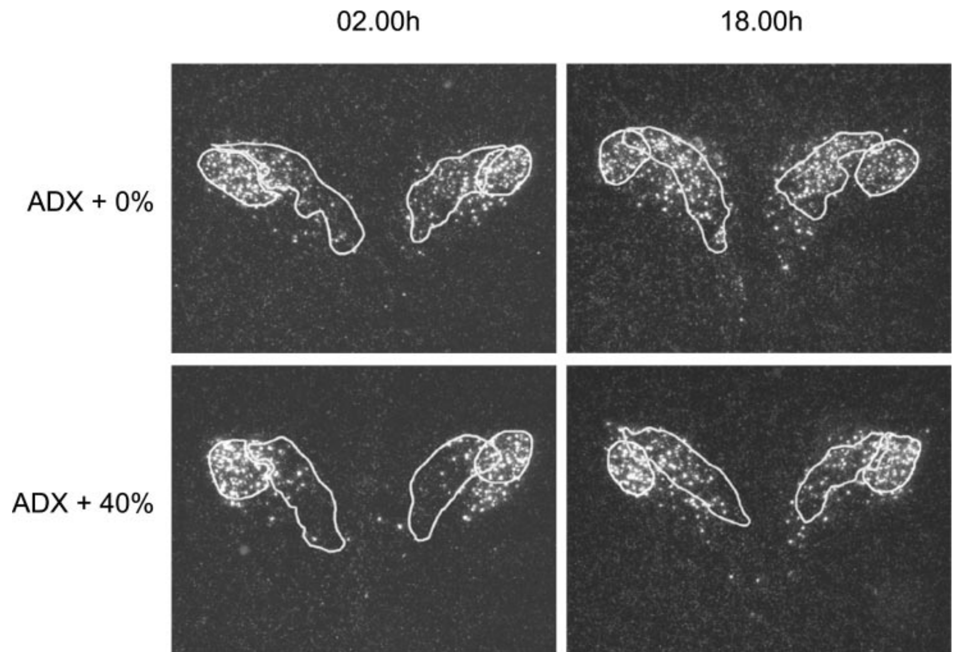

FIG. 7. Dark-field photomicrographs of AVP hnRNA hybridization in the mp and pm parts of the PVHmp of ADX animals with either 0% or 40% corticosterone pellets implanted sc. *White lines* indicate the areas used to quantitate AVP hnRNA hybridization signals in the PVHmp and PVHpm. These areas were derived from adjacent sections hybridized for CRH mRNA or stained with thionine.

that corticosterone suppresses CRH synthesis (8, 37, 38, 44–46), and we show that the pattern of *crh* gene transcription in intact animals is clearly the inverse of corticosterone fluctuations. Thus, the simplest explanation for the daily changes in transcription would be a corticosterone-sensitive, servo-like mechanism that stimulates transcription during the night as circulating corticosterone falls and inhibits transcription during the day as corticosterone levels increase. However, our data show that this explanation is untenable because animals in both experiments show a significant period of *crh* gene transcriptional activation whose amplitude is unaffected by doses of corticosterone up to those equivalent to the mean values seen across the day in intact animals. Although a similar observation regarding part of the daily mRNA rhythm was reported by Kwak *et al.* (22), it is problematic to use mRNA data to infer how corticosterone affects transcriptional mechanisms because corticosterone may affect the half-life of CRH mRNA (36).

Leptin might also be considered a potential humoral regulator of *crh* gene expression across the day. We found that the amplitude of the CRH hnRNA rhythm is indistinguishable in all three treatment groups in experiment 2 despite significant differences in circulating leptin. This observation suggests that leptin is not a critical contributor to the mechanisms responsible for shaping the daily fluctuations in CRH gene expression in unstressed animals. However, we would certainly not exclude the possibility that leptin/corticosterone interactions are important factors in controlling other aspects of *crh* gene expression, a possibility consistent with data from Richard's group (47).

We show that there are significant daily variations in AVP hnRNA levels in the PVHmp of 0% and 40% ADX animals, but not in intact animals. Furthermore, AVP hnRNA levels in ADX (40%) animals are significantly lower than those in ADX (0%) animals at all times of day we examined. Given that the actions of corticosterone on *avp* gene expression are rapid and involve direct actions on the gene, and that the *avp* gene is exquisitely sensitive to circulating corticosterone (12, 17, 41, 48), it would seem that the amounts of corticosterone circulating in intact rats during the latter part of the light and early dark periods are sufficient to completely suppress the mechanism that drives *avp* gene expression. However, if corticosterone is replaced in ADX animals at levels sufficient to normalize thymus weights and CRH mRNA, these are insufficient to completely mask the rhythm. This suggests that, unlike *crh* gene transcription, the dynamics of circulating corticosterone in intact animals are important for blunting daily variations in *avp* gene transcription. The daily variations in AVP hnRNA in both sets of ADX animals show rhythms strikingly coordinated with that of ACTH secretion, but not CRH hnRNA. This suggests that a common, but as yet unknown, mechanism can activate both *avp* gene expression and the release of ACTH secretagogues from CRH neurons in appropriate circumstances.

Given that corticosterone alone cannot account for the daily variations in CRH and AVP hnRNA, a major component in the integrative process that controls *crh* and *avp* gene expression in CRH neurons must be the large group of afferent sets encoding exterosensory and interosensory information. Considering the fact that our data do not support changes in either corticosterone or leptin secretion as being sufficient for driving these nocturnal transcriptional episodes, it would seem reasonable to suggest that at least one of these afferent sets plays a significant role in activating gene transcription during the dark phase. Currently, the detailed architecture of the afferent systems responsible for shaping CRH neuroendocrine function across the day in the absence of stress is poorly understood. However, the circadian timing system controlled by the SCH should be considered as one potential controller of *crh* and *avp* gene activation in these circumstances. The SCH provides the principal timing signal for daily surges of plasma ACTH and corticosterone (2, 49–

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52). Some SCH efferents clearly innervate the PVHmp, but these are more sparse than those that innervate other nearby targets (53–56), particularly the dorsomedial nucleus, which heavily innervates the PVHmp and is heavily implicated in influencing circadian corticosterone output (32, 50, 53–59). The exact nature of the afferent set controlled by the SCH remains to be established.

Finally, an intriguing question addressed by our data is the interplay between those mechanisms controlling *crh* gene transcription and those responsible for releasing CRH at the neuroendocrine terminal, a central issue when considering the overall contribution of gene activation to neuroendocrine function. Synthesis clearly maintains releasable peptide pools in neuroendocrine terminals, but do the afferent and intracellular mechanisms that depolarize the neuronal membrane to release CRH always activate gene transcription? In PC-12 cells transfected with the *crh* gene, membrane depolarization will activate *crh* gene transcription in a cAMP-dependent manner (60), but this coupling may not always be as tight *in vivo*. If it was, one would expect *crh* gene transcription to quickly and invariably follow ACTH secretion. With many types of stress, ACTH release and gene transcription are indeed closely allied (10, 11), but because these stressors rapidly activate both secretion and transcription, it is virtually impossible to discriminate between the onsets of these two processes. We have shown that when stress onset is gradual (19, 40) a looser transcription/secretion coupling is apparent, suggesting that secretion and transcription are to some degree independent. We now show an even greater degree of uncoupling throughout the 24-h period in unstressed animals, where the onset of ACTH secretion, and hence CRH release (4, 7, 10), precedes the onset of *crh* gene transcription by a substantial period in all three experimental groups. Uncoupling is most apparent during the latter part of the light phase in intact and ADX (0%) animals when CRH hnRNA levels and ACTH secretion are moving in opposite directions. An important corollary arising from this loose coupling is that the manner in which neural and humoral information is integrated to drive CRH release is not necessarily the same as that in which it controls *crh* gene expression.

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