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Differential Responses of Hypothalamus-Pituitary-Adrenal Axis Immediate Early Genes to Corticosterone and Circadian Drive

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The hypothalamus-pituitary-adrenal (HPA) axis diurnal cycle of activity is manifest in circadian rhythms of ACTH and corticosterone secretion, which in the rat peak around the onset of the dark period. This cycle is thought to be driven by daily fluctuations in activity of CRH neurons within the paraventricular nucleus of the hypothalamus (PVN), controlled by suprachiasmatic nucleus inputs. In this study we examined whether the circadian drive that regulates ACTH and corticosterone basal secretion in the rat is reflected in PVN immediate early gene expression and, if so, whether different genes respond uniformly or uniquely to circadian stimulatory input. In addition, we examined how circadian drive and acute stress, two categories of stimuli that induce HPA axis activation, comparatively affect gene expression within different components of the HPA axis (c-*fos* mRNA, CRH heteronuclear RNA, and zif268 mRNA in PVN; c-*fos* mRNA, proopiomelanocortin heteronuclear RNA, and zinc finger 268 mRNA in anterior pituitary; c-*fos* mRNA and nerve growth factor I-B mRNA in adrenal cortex). Finally, we examined whether circadian differences in gene expression depend on endogenous glucocorticoids and, if so, whether the dependence is on an acute or permissive influence of the hormone. We found that a circadian drive that regulates HPA axis basal hormone secretion is also manifest on basal c-*fos* gene expression in the PVN. Moreover, we show that different immediate early genes within the HPA axis anatomical components display different diurnal patterns of gene expression. These differential patterns result, in part, from gene-specific responses to circadian signals and acute and/or permissive glucocorticoid actions. (*Endocrinology* 148: 2542–2552, 2007)

THE CIRCADIAN RHYTHM of basal hypothalamus-pituitary-adrenal (HPA) axis secretory activity is essential for maintaining normal energy balance (1). A peak in glucocorticoid release is observed at the end of the resting period (just before the onset of darkness in nocturnal animals) in preparation for the increased metabolic demands of the active state, and a minimum is reached when the animal returns to sleep. This pattern is chiefly regulated by a rhythmic secretion of ACTH from corticotrope cells of the anterior pituitary. The rhythm in ACTH secretion, in turn, is driven by CRH secretion from parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN), as indicated by the block of this rhythm after passive immunization with CRH antiserum (2, 3). Increased CRH secretion in the evening is thought to depend on active stimulation by suprachiasmatic nucleus (SCN) afferents (4, 5) that project to PVN neurons. Both direct (6, 7) and indirect projections, the latter passing through the ventral subparaventricular zone and the dorsomedial hypothalamic nucleus (8, 9), have been described. In the rat, glucocorticoid feedback regulation of ACTH secretion appears to require a higher level of corticosterone output in the evening (PM) than the morning (AM) (10).

Based on the above evidence, it was hypothesized that in the AM, CRH neuron stimulatory input to the pituitary is negligible and low plasma ACTH levels reflect constitutive secretion of corticotropes. In the PM, a stimulatory drive from PVN CRH neurons increases ACTH secretion (hypothalamic circadian drive). Therefore, in the AM a tonic-negative feedback effect of low concentration corticosterone is sufficient to maintain ACTH levels in the normal range. In the PM, a higher concentration of corticosterone, likely acting at both pituitary and hypothalamic sites, is required to exert the same effect (11). If the hypothesized circadian drive is present only at one time of day, then it is expected that the basal (and perhaps also stress induced) activity of the CRH neuron changes across the 24-h day. We postulated that immediate early gene (IEG) expression in the PVN would provide a good measure for investigation of the presence of circadian drive upstream of the HPA hormonal output. IEGs are rapidly induced by a change in intracellular state, after cellular stimulation from intercellular signals (12, 13). To determine whether circadian inputs result in selective or global effects on IEG expression, we examined more than one IEG within each anatomical component of the HPA axis. Thus, we measured c-*fos*, zif268 (also known as NGFI-A or egr-1) and CRH gene expression in the PVN at two times of the day, during the resting phase (2 h after lights on, AM) and just before the initiation of activity (1 h before lights off, PM). Because the circadian rise in corticosterone secretion precedes (anticipates) the onset of the dark period, the choice of these two measurement times ensures that the photoperiod

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Abbreviations: ADX, Adrenalectomy/adrenalectomized; AM, morning; Bw, corticosterone drinking water; HPA, hypothalamus-pituitary-adrenal; IEG, immediate early genes; hnRNA, heteronuclear RNA; NGFI-B, nerve growth factor I-B; PM, evening; POMC, proopiomelanocortin; PVN, paraventricular nucleus of the hypothalamus; SCN, suprachiasmatic nucleus; zif268, zinc finger 268; ZT, Zeitgeber time.

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(lights on) and rat's activity period (inactive) were the same for all measures, whereas basal corticosterone was quite different between the AM and PM measures. To examine whether patterns of IEG expression observed in the PVN are similar in downstream components of the HPA axis, we also examined the basal expression of c-*fos*, zif268, and POMC genes in the anterior pituitary. In the adrenal cortex, we examined basal and stress-induced expression of c-*fos* and another widely expressed IEG, NGFI-B (14).

In addition to the circadian rhythm of basal HPA axis hormonal secretion, an acute activation of the axis and release of glucocorticoids is observed in response to stress. It is not known to what extent the circadian corticosterone rhythm directly or indirectly affects basal and stress-induced IEG expression in the axis. The surge in corticosterone that occurs around the onset of the dark photoperiod could directly affect the basal and stress-induced gene expression levels during the ensuing several hours (evening time levels). In this paper we refer to such glucocorticoid effects on HPA axis hormone levels and gene expression changes as acute effects of glucocorticoids. On the other hand, there may also be an influence of this circadian variation of corticosterone secretion such that its prior occurrence affects subsequent cellular activity and gene expression irrespective of recent circulating levels of corticosterone. For example, Jacobson *et al.* (15) found that giving adrenalectomized (ADX) rats a corticosterone replacement regimen that produced a diurnal variation in corticosterone levels (corticosterone in the drinking water) was sufficient to normalize the duration of an ACTH response to acute stress challenge in the morning. In contrast, replacement with constant levels of corticosterone (sc corticosterone pellets) was ineffective on this measure. An important feature of providing ADX rats with corticosterone in the drinking water is that almost all of the drinking occurs during the first several hours after lights off as the rats engage in feeding (15). Consequently, these ADX rats have a PM surge in circulating corticosterone. However, due to the very short half-life of corticosterone [approximately 20 min in rats; (16)], circulating corticosterone levels are nearly undetectable during the next morning when the rats are inactive. The Jacobson study indicates that a surge of corticosterone during the evening was sufficient to normalize the temporal pattern of the ACTH response to acute stress the following morning. In this study we refer to these relatively long-lasting delayed effects of glucocorticoids as permissive effects.

To determine possible glucocorticoid effects due to either the ongoing or previous diurnal phasic surge in corticosterone levels, we compared dependent measures in ADX rats, ADX rats given corticosterone in their drinking water, or sham-operated rats. The choice of a PM measurement time in this study that precedes the onset of the dark period minimizes the extent to which ADX rats given corticosterone in the drinking water have already begun their daily feeding and water intake on the test day. Consequently, these rats, as opposed to sham-ADX rats, lack the concurrent PM surge in corticosterone. On the other hand, these rats, in contrast to noncorticosterone-replaced ADX rats, had a surge in corticosterone approximately 20–22 h earlier as a result of feeding and drinking during the previous night.

Materials and Methods

Animals

Male Sprague Dawley rats (10 wk old, weight range 270–290 g at experimental onset; Harlan Labs, Indianapolis, IN) were allowed a 2-wk acclimation period before experimental use. Animals were housed two per polycarbonate tub with wood shavings and were allowed food and tap water *ad libitum*. The colony room lights were regulated on a 12-h light, 12-h dark cycle, with lights on at 0700 h. Procedures for ethical treatment of animals conformed to the guidelines found in the Guide for the Care and Use of Laboratory Animals, Department of Health and Human Services Publication (National Institutes of Health) no. 80-23 (revised 1996 edition) and were approved by the University of Colorado Institutional Animal Care and Use Committee.

Restraint stress procedure

Restraint involved taking rats from the home cage and placing them in adjustable length (15.5 ± 2.5 cm long and 6.3 cm diameter) Plexiglass tubes with air holes in the front, top, and back. This stressor is considered to be primarily psychological in nature because it does not produce pain or direct physical insult (17). Restraint took place in a separate room adjacent to the home cage room.

Adrenalectomy and corticosterone replacement

Adrenalectomies were performed under halothane-induced anesthesia via the dorsolateral approach. Control animals were operated under the same anesthesia, but the adrenals were not removed (sham animals). Adrenalectomized animals were given immediate access to 0.9% saline (ADX animals) or 0.9% saline containing 25 μg/ml corticosterone (Sigma, St. Louis, MO) (ADX + Bw) and were allowed to recover from surgery for 5 d before testing. There was no detectable plasma corticosterone as determined by our corticosterone assay (see below) in ADX rats.

In situ hybridization histochemistry and image analysis

Tissue sections (10 μm thick) were cut on a cryostat (model 1850; Leica Microsystems, Nussloch, Germany) through the extent of the PVN [~ 1.80 mm posterior to bregma (63)] or through the middle portion of the adrenal gland, thaw mounted onto poly-L-lysine-coated slides and stored at −80 C.

In situ hybridization for nerve growth factor I-B (NGFI-B), c-*fos*, and zinc finger 268 (zif268) mRNAs and CRH heteronuclear RNA (hnRNA) and image analysis of digitalized images from x-ray films were performed as previously described (18). For the generation of the riboprobes, plasmids containing a fragment of c-*fos* cDNA (courtesy of Dr. T. Curran, St. Jude Children's Research Hospital, Memphis, TN) or zif268 cDNA (courtesy of Dr. J. Milbrandt, Washington University School of Medicine, St. Louis, MO) or a portion of the CRH intron (kindly provided by Dr. R. Thompson, University of Michigan, Ann Arbor, MI) were used. The NGFI-B plasmid harboring a 839-bp fragment of the NGFI-B gene spanning nucleotides 188-1027 (GenBank accession no. NM_024388) was generated in house. Briefly, total RNA from rat hippocampus was isolated using the SV Total RNA extraction system (Promega, Fitchburg, WI) and first-strand cDNA was produced using SuperScript II reverse transcriptase reagents (Invitrogen, Carlsbad, CA). After PCR with NGFI-B-specific oligonucleotides, the amplified product was cloned into pCRII-TOPO vector (Invitrogen) according to manufacturer instructions. The identity of the cloned DNA was verified by DNA sequencing (University of Colorado Molecular, Cellular, and Developmental Biology sequencing facility).

ACTH RIA

Blood samples for the ACTH assay were collected into EDTA-coated tubes and stored at −80 C. Plasma concentrations of ACTH were determined by RIA procedures described elsewhere (19) using antiserum (rabbit antibody Rb7, courtesy of Dr. Bill Engeland, University of Minnesota, Minneapolis, MN). The detection limit for this assay was 15 pg/ml for a 50-μl sample; the intraassay coefficient of variability was 6–10% and the interassay coefficient of variability was 9%.

Corticosterone enzyme immunoassay

Measurement of plasma corticosterone was conducted on 20 μl of plasma with an enzyme immunoassay kit (Assay Design, Ann Arbor, MI) according to manufacturer's instructions. The sensitivity for this assay was 130 ng/100 ml. The intraassay coefficient of variability was 8–10% and the interassay coefficient of variability was 10%.

Semiquantitative PCR

Relative levels of gene expression from dissected anterior pituitaries were measured by semiquantitative PCR as previously described (20). Briefly, RNA was extracted from anterior pituitary tissue according to the method of Chomczynski and Sacchi (21), using Trizol (Invitrogen). First-strand cDNA was generated with random hexamers using SuperScript II reverse transcriptase (Invitrogen). Hot-start PCR, using Taq-Bead hot start polymerase (Promega) was used to generate fragments of the POMC gene spanning intron 1 and exon 2 and c-*fos* and zif268 coding regions. Oligonucleotide sequences were as follows: POMC forward, 5′-GAGGCATAAACTGGCACC-3′, POMC reverse, 5′-GCTGT-TCATCTCCGTTGC-3′; c-*fos* forward, 5′-CTTCCTTTGTCTTCAC-CTACC-3′, c-*fos* reverse, 5′-CCTTCTCTGACTGCTCACA-3′; and zif268 forward, 5′-TCACTCACCCACCATGGACAA-3′, zif268 reverse, 5′-TCAGCAGCATCATCTCCTCCA-3′. Linear amplifications were obtained at 30 cycles for POMC and c-*fos* and 25 cycles for zif268. Duplicate reactions for each sample were run on 3–3.5% agarose gels, and digital images of the UV-visualized bands were quantified using the National Institutes of Health Image program. Relative levels of expression are indicated as ratios between the densitometric measure of the test gene and a housekeeping gene (GAPDH).

Experiment 1: diurnal comparison of basal and acute stress activity in adrenal-intact rats

Animals (N = 24) were separated into four groups (n = 6); two groups were subjected to restraint stress for 15 min before rapid decapitation that occurred in the morning, 2 h after lights on [AM = zeitgeber time (ZT) 2], or in the evening, 1 h before lights off (PM = ZT 11). The other two groups were killed at the same times of day, but they were left undisturbed in their cages until death.

Experiment 2: diurnal comparison of basal and acute stress activity in sham and ADX rats

Animals (N = 48) were separated into eight groups (n = 6). Half of the animals were adrenalectomized and half were sham operated. Five days after operation, animals from each group were then subjected to either 15 min restraint stress in the AM or PM and immediately killed or killed at the same times without restraint stress experience.

Experiment 3: diurnal comparison of basal activity in sham, ADX, and ADX + corticosterone drinking water (Bw)-replaced rats

Animals (N = 48) were separated into six groups (n = 8). One third of the animals were ADX, one third were ADX and given corticosterone (25 μg/ml) in the drinking fluid (ADX + Bw), and one third was sham operated. Five days after operation animals from each group were either killed in the AM or PM without further manipulations.

Statistical analysis

ANOVA was used to examine overall main effects and interactions of time of day and stress condition (experiment 1; 2 × 2 factorial design); time of day, stress condition, and glucocorticoid state (experiment 2; 2 × 2 × 2 factorial design); or time of day and glucocorticoid state (experiment 3; 2 × 3 factorial design). Data were log transformed before applying ANOVA because of the greater variability associated with the measures in ADX and stressed rats, compared with basal measures in adrenal-intact rats. Within this framework, our criterion to establish the significance of circadian effects was to apply unpaired Student's t tests in *post hoc* pairwise comparisons only in those instances in which the ANOVA yielded a significant main effect for time of day or a significant interaction of time of day with any other factor. The program StatView (SAS Institute Inc., Cary, NC) was used in all the analyses. An alpha level of $P < 0.05$ was used to determine statistical significance. Data presented represent group means ± SEM.

Results

Experiment 1: diurnal differences in basal and stress-induced HPA axis hormone levels and PVN gene expression of adrenal-intact rats

Basal levels

There was a strong diurnal rhythm of basal corticosterone secretion in adrenal-intact animals killed 2 h after lights on (AM) or 1 h before lights off (PM) ($P < 0.001$; Fig. 1A). There was also a trend for an increase in basal ACTH in the PM (Fig. 1B, *inset*); however, neither the overall time of day main effect nor time of day by stress condition interaction was statistically significant for this measure. Thus, as previously described (11, 22), the diurnal amplitude was substantially larger for corticosterone than ACTH.

Basal c-*fos* mRNA in the PVN (Fig. 1C) showed an increase in the PM ($P < 0.05$), suggesting an increase in activation state in PVN neurons as the animal approaches the time of awakening. Unlike c-*fos*, CRH hnRNA showed a significant decrease in the PM ($P < 0.01$; Fig. 1D). Finally, zif268 mRNA basal expression in the PVN was low and did not significantly change with time of day (Fig. 1E).

Stress-induced levels

Fifteen minutes of restraint stress produced rapid elevations in plasma hormones levels (corticosterone and ACTH) and PVN gene expression (c-*fos* mRNA, CRH hnRNA, and zif268 mRNA; Fig. 1, A–E). Stress-induced changes were not significantly different between the two times of day for any of the parameters analyzed, except for CRH hnRNA (Fig. 1D). Stress-induced CRH hnRNA levels were significantly lower in the PM than AM ($P < 0.01$). In the case of c-*fos* mRNA, there was a significant time of day by stress condition interaction ($P = 0.01$), lending support to the notion that the induction of the c-*fos* gene by acute stress overrides the diurnal difference in basal expression.

Experiment 2: diurnal differences in basal and stress-induced HPA axis hormone levels, PVN gene expression of adrenal-intact (sham) and ADX rats, and adrenal cortical gene expression of sham rats

The second experiment was essentially a replication of the first experiment with the added dimension of comparing the responses of adrenal-intact rats 5 d after sham surgery (sham) with rats 5 d after ADX.

Sham rats: basal and stress-induced plasma hormone and PVN gene expression levels

As expected, the diurnal pattern of basal and stress-induced plasma hormone and PVN gene expression levels of sham rats was very similar to that seen in experiment 1 (Fig. 2, A–E). Importantly, this experiment replicated the PVN gene expression patterns of adrenal-intact rats observed in experiment 1. Specifically, basal c-*fos* mRNA levels were

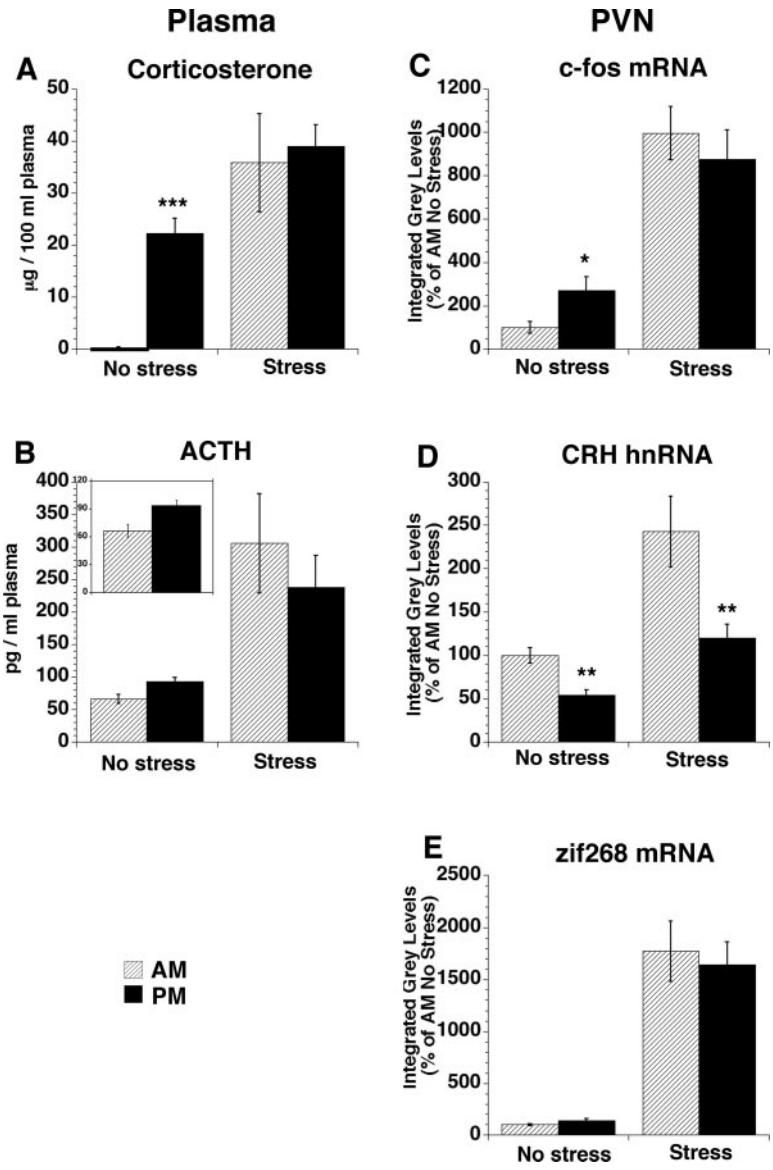

FIG. 1. Effect of circadian drive and 15 min restraint stress on HPA axis hormone levels and gene expression. Plasma corticosterone (A) and ACTH (B) measures were taken from trunk blood, and c-*fos* mRNA (C), CRH hnRNA (D), and zif268 mRNA (E) levels were measured in the PVN by *in situ* hybridization. Samples were collected either in the morning (AM, ZT2) or evening (PM, ZT11). (*, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ *vs.* respective AM time point, unpaired *t* test; all no stress *vs.* stress comparisons at a given time of day condition were significant, $P < 0.05$; n = 6).

higher in the PM than AM ($P < 0.05$; Fig. 2C), basal CRH hnRNA levels were lower in the PM than AM ($P < 0.001$; Fig. 2D), and basal zif268 mRNA levels did not differ between the two times of day (Fig. 2E). Furthermore, stress-induced CRH hnRNA levels were lower in the PM, compared with the AM ($P < 0.05$; Fig. 2D).

Sham rats: basal and stress-induced adrenal cortical gene expression levels

It is known that stress produces changes in adrenal gland immediate early gene expression (23, 24). c-*fos* Diurnal changes in the adrenal gland have been observed (25); however, parallel measures of IEG transcriptional activation in adrenal gland and PVN under basal and stress-induced conditions are lacking. Therefore, in experiment 2, along with PVN gene activation, we measured the pattern of diurnal basal and stress-induced c-*fos* and NGFI-B mRNA in the cortical layers of the adrenal gland from sham rats (Fig. 2, F–H). Under basal conditions, adrenocortical c-*fos* mRNA showed a trend to increase in the evening (Fig. 2F, *inset*), although two-way ANOVA indicated no main effect of time of day nor a time of day by stress condition interaction. Acute stress produced a substantial increase in c-*fos* expression in the adrenal cortex at both times of day, although perhaps to a lesser extent in the PM than AM (Fig. 2, F and H). In the case of NGFI-B gene expression in the adrenal cortex, there was a clear increase in basal mRNA in the evening ($P < 0.01$), and NGFI-B mRNA was markedly elevated by stress at both times of day ($P < 0.001$ in AM and $P < 0.05$ in PM; Fig. 2, G and H).

ADX rats: basal and stress-induced plasma hormone and PVN gene expression levels

ADX dramatically increased basal AM and PM ACTH levels (Fig. 2B), in agreement with previous observations (26). Moreover, in ADX animals, basal ACTH levels were higher in the PM than AM ($P < 0.01$). In the PVN, ADX

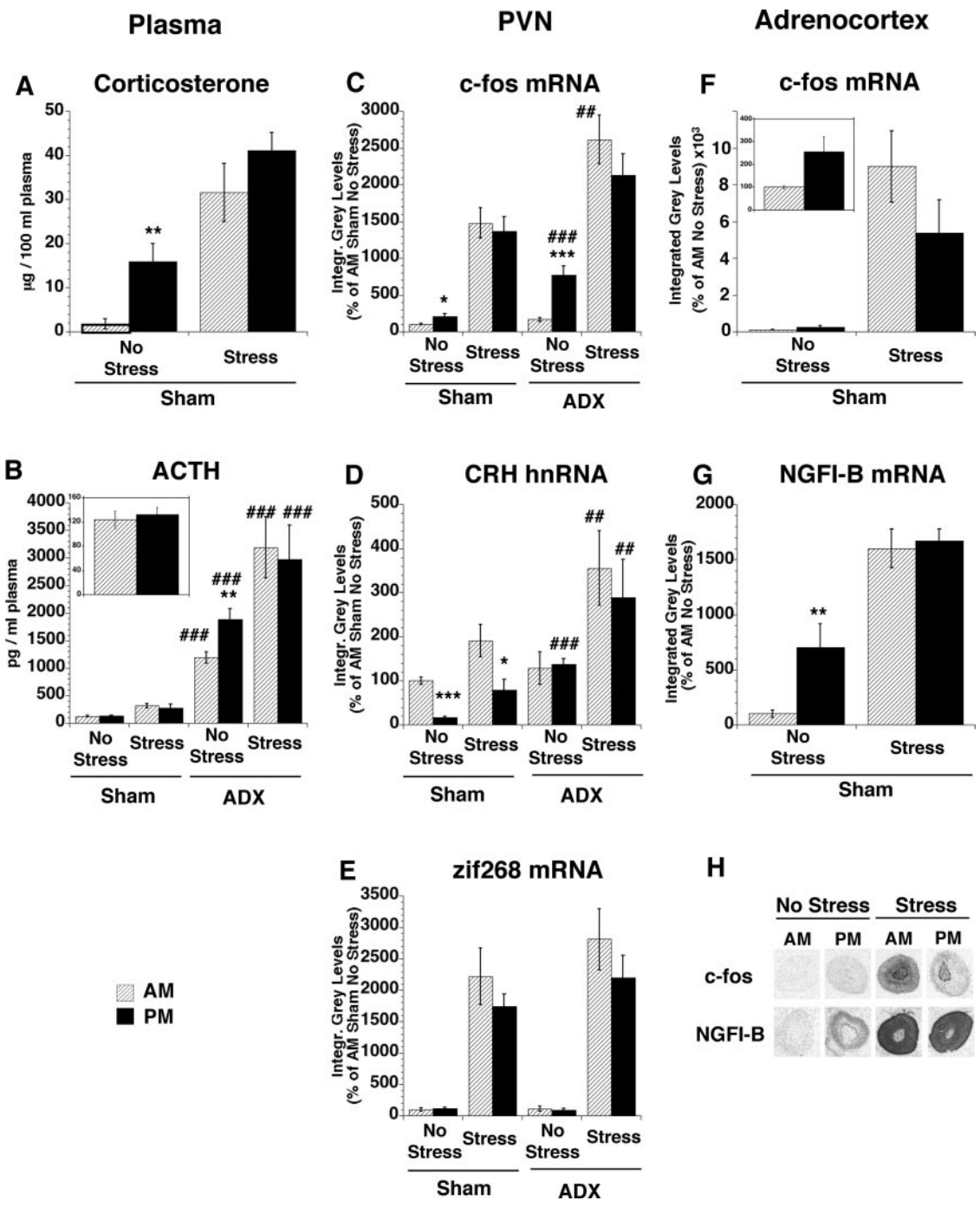

FIG. 2. Effect of 5 d ADX and 15 min restraint stress on HPA axis hormone levels and gene expression in PVN and adrenocortex. Plasma corticosterone (A) and ACTH (B) measures were taken from trunk blood. c-*fos* mRNA (C), CRH hnRNA (D), and zif268 mRNA (E) levels were determined in the PVN and c-*fos* mRNA (F) and NGFI-B mRNA (G) in the adrenocortex by *in situ* hybridization. Samples were collected in the morning (AM, ZT2) or evening (PM, ZT11). *Insets* show an enlarged view of the no stress ACTH levels and c-*fos* mRNA levels in the adrenocortex. H, Representative autoradiograms of *in situ* hybridization signals for c-*fos* mRNA and NGFI-B mRNA in the adrenal gland (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ *vs.* respective AM time point; #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$ *vs.* sham rats for same time of day and stress condition, unpaired *t* test; all no stress *vs.* stress comparisons at a given time of day and glucocorticoid state condition were significant, $P < 0.05$, except for ACTH and CRH hnRNA ADX PM; n = 6).

increased evening but not morning basal levels of c-*fos* mRNA ($P < 0.001$; Fig. 2C), thus producing an increase in the basal diurnal difference amplitude compared with sham animals. In this experiment, ADX did not significantly affect basal levels of CRH hnRNA in the morning (Fig. 2D), but it did increase CRH hnRNA basal evening levels ($P < 0.001$), bringing them close to the ADX AM basal levels and thereby abolishing a diurnal difference in basal CRH hnRNA levels at these two times of day. There was no significant effect of ADX on basal levels of zif268 mRNA (Fig. 2E).

As in adrenal-intact rats, 15 min of restraint produced a large increase of all measured parameters in ADX rats (Fig. 2, B–E). There was no diurnal difference in the stress-induced levels of these parameters, including CRH hnRNA. Overall levels, however, of stress-induced ACTH, c-*fos* mRNA (only AM reached significance) and CRH hnRNA (but not zif268 mRNA) were higher in ADX rats than sham rats ($P < 0.01$).

Experiment 3: effect of ADX ± Bw replacement on diurnal differences in basal HPA axis hormone levels, PVN gene expression, and anterior pituitary gene expression

To discriminate which of the above effects brought about by ADX were due to removal of a permissive corticosterone effect or removal of an acute phasic influence of PM elevations in basal corticosterone, experiment 3 included a group of ADX rats that had corticosterone in their drinking water (ADX + Bw). Permissive corticosterone effects would be manifest in both sham and ADX + Bw groups, whereas phasic influences of corticosterone would affect only the sham group. As shown in Fig. 3A, AM corticosterone levels in ADX + Bw rats were less than sham rats and barely detectable. PM corticosterone levels were slightly higher, averaging around 4 μg/dl, probably as a result of an early start (before lights out) in daily activity and drinking for some of the animals in this group. The average corticosterone levels for this group were, however, well below those measured in sham rats at the same time.

Sham rats: basal plasma hormone and PVN gene expression levels

Plasma hormone and PVN gene expression patterns in sham rats were the same as those observed for adrenal-intact rats in experiment 1 and sham rats in experiment 2 (Fig. 3, A–E). Thus, there was a large PM increase in basal corticosterone ($P < 0.001$) and a small statistically nonsignificant PM increase in basal ACTH. For gene expression in the PVN, there was a PM increase in basal c-*fos* mRNA ($P < 0.05$), a PM decrease in basal CRH hnRNA ($P < 0.001$), and no diurnal difference in zif268 mRNA.

ADX ± Bw: basal plasma hormone, thymus weights, and PVN gene expression levels

As observed in experiment 2, ADX produced a large increase in basal ACTH levels, and there was a significant diurnal difference with higher PM basal ACTH levels ($P < 0.01$; Fig. 3B). AM ACTH in the ADX + Bw group was low and similar to sham levels, indicating a permissive influence of prior nocturnal intake of corticosterone. Interestingly, PM basal ACTH levels of ADX + Bw rats were elevated to nearly ADX levels in the PM. Measure of thymus weights confirmed that corticosterone replacement was appropriate to reduce thymus weight toward adrenal-intact values without producing signs of higher than normal corticosterone exposure (thymus weights: sham, 414 ± 20 g; ADX, 555 ± 22 g; ADX + Bw, 485 ± 20 g; significance of *post hoc t* test: $P < 0.05$ for ADX +Bw, compared with either ADX or sham).

The effects of ADX ± Bw on PVN gene expression are summarized in Figs. 3 and 4. As in experiment 2, ADX produced a large increase in PM PVN basal c-*fos* mRNA ($P < 0.01$) but had no effect on AM levels. Corticosterone drinking water replacement normalized the PM levels of c-*fos* expression, thereby eliminating the increased circadian amplitude difference observed in ADX animals (Figs. 3C and 4A). In this experiment, in contrast to the previous experiment, ADX produced a significantly elevated level of basal CRH hnRNA at both times of day, compared with sham rats ($P < 0.001$; Figs. 3D and 4A). Nevertheless, as in the previous experiment, ADX eliminated the diurnal difference in basal CRH hnRNA. Corticosterone replacement reduced basal CRH hnRNA levels to within range of the sham levels; however, it failed to reinstate the diurnal difference between AM and PM CRH hnRNA levels observed in sham rats. As in experiment 2, there was no corticosterone-dependent effect observed on zif268 mRNA levels (Fig. 3E).

Sham and ADX ± Bw rats: basal anterior pituitary gene expression

In this third experiment, we also determined the effects of ADX ± Bw on IEG expression in the pituitary gland (Figs. 3, F–H, and 4B). Anterior pituitaries were dissected and mRNA extracts used in semiquantitative reverse transcriptase PCR analysis with primers specific for c-*fos* mRNA, proopiomelanocortin (POMC) hnRNA, and zif268 mRNA.

The expression pattern of c-*fos* mRNA in the anterior pituitary showed a marked diurnal difference in ADX + Bw rats that was not evident in sham or ADX rats ($P < 0.01$; Fig. 3F). There was also a large PM increase in POMC hnRNA levels of ADX + Bw rats ($P < 0.001$; Figs. 3G and 4B). In addition, sham rats showed a small but significant increase in POMC hnRNA levels in the PM ($P < 0.01$) and a similar trend, although not significant, was observed in ADX animals. Levels of zif268 mRNA in the anterior pituitary were similar for all groups; however, there was a small but significant decrease in the PM of sham rats ($P < 0.05$; Fig. 3H).

Discussion

Evidence for a circadian drive that modulates basal c-fos gene expression in the PVN

In accordance with the hypothesis that the increased secretion of ACTH and corticosterone observed in the evening in rodents is the result of increased CRH secretion (11), we investigated potential changes in PVN activation status between morning and evening by examining IEG expression in the PVN. We examined two IEGs, c-*fos* and zif268, which have been used extensively in neuroscience to provide evidence for recent cell activation events (12, 13). Both genes are rapidly induced in the PVN by acute stress (27). Previous work has shown that in the PVN a large percent of stress-induced c-*fos* mRNA colocalizes with CRH-containing neurons (28, 29). The CRH gene is also rapidly induced by stress in these cells as detected when measuring primary transcript (30, 31) and serves as a phenotypic-specific marker with activity-dependent properties.

We found that the diurnal difference in HPA axis hormone secretion was also manifest by a selective elevation of PVN basal c-*fos* expression in the evening. This was

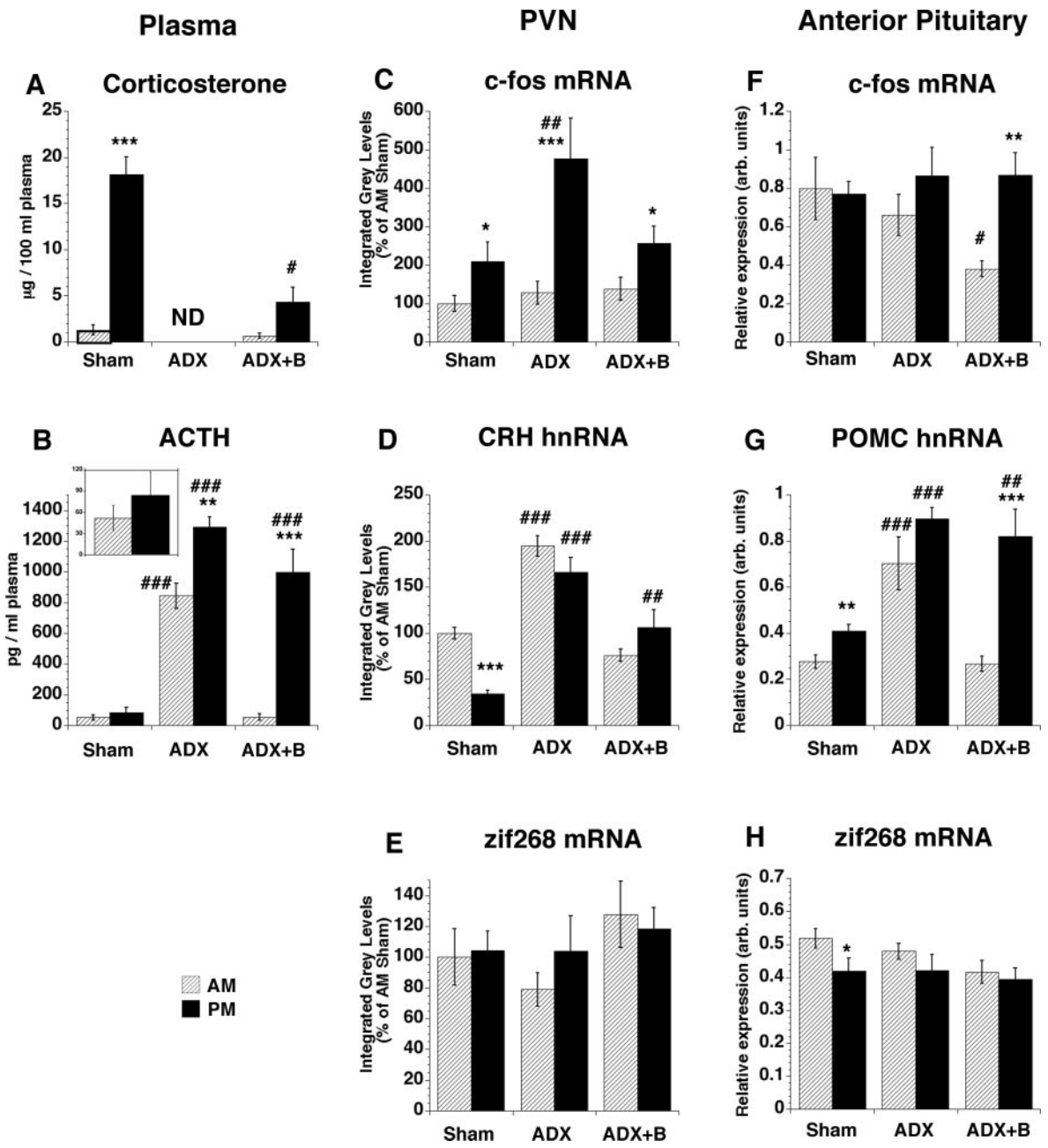

FIG. 3. Effect of 5 d ADX ± corticosterone replacement in the drinking water (ADX + Bw) on basal HPA axis hormone levels and gene expression in PVN and anterior pituitary. Plasma corticosterone (A) and ACTH (B) measures were taken from trunk blood. c-*fos* mRNA (C), CRH hnRNA (D), and zif268 mRNA (E) levels in the PVN were determined by *in situ* hybridization, and c-*fos* mRNA (F), POMC hnRNA (G), and zif268 mRNA (H) were measured by semiquantitative PCR in the anterior pituitary. Samples were taken in the morning (AM, ZT2) or evening (PM, ZT11). *Inset* shows an enlarged view of the no stress ACTH levels. (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ *vs.* respective AM time point; #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$ *vs.* sham rats for same time of day, unpaired *t* test; *bars* represent means ± SEM, n = 8). ND, Non detectable.

observed in all three experiments (Figs. 1–4). In line with the idea that the increased c-*fos* gene expression in the PM was associated with increased CRH neuron activity and hence CRH secretion, a parallel increase in corticotrope POMC hnRNA (Figs. 3 and 4) was also observed in the evening. The POMC gene acts as a corticotrope-specific activity marker when examining levels of the primary transcript (POMC hnRNA) (32). Thus, PVN basal c-*fos* expression may reflect a circadian central drive that marks the activity of the CRH neuron during the 24 h. Interestingly, despite evidence for increased PVN neuronal activity, other genes in the PVN (CRH and zif268) did not display the same trend of increased evening expression observed with c-*fos* (discussed later).

In adrenal-intact rats, there was only a small increase in basal ACTH levels at the PM time point sampled. However, in ADX rats, there was a strong diurnal increase in basal ACTH in the PM. The fact that this diurnal difference was

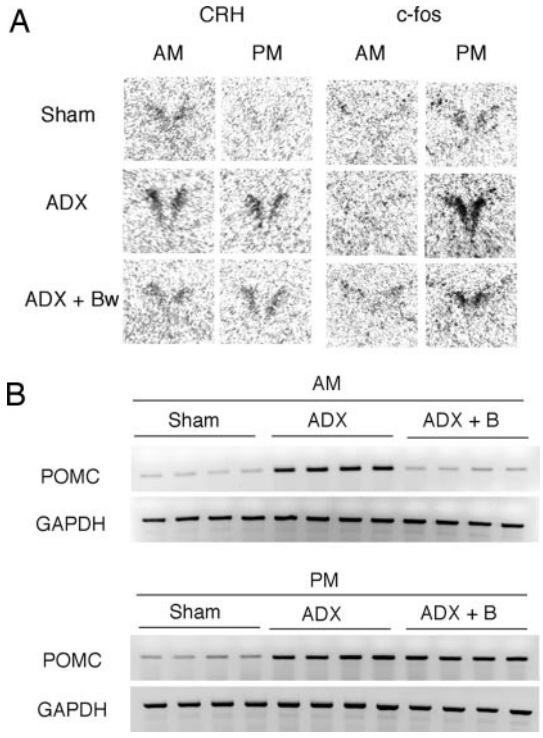

FIG. 4. A, Representative autoradiographic images of *in situ* hybridization signals showing the effects of adrenalectomy (ADX) and corticosterone replacement (ADX + B) on expression of CRH hnRNA and c-*fos* mRNA in the PVN, in the morning (AM) and evening (PM). B, Representative images of duplicate samples (two samples per treatment) of POMC hnRNA amplified using semiquantitative PCR and separated by agarose gel electrophoresis, in the AM (*top panel*), and PM (*bottom panel*). For each panel, underneath the POMC images, we show the mRNA levels of the housekeeping gene GAPDH used to normalize the expression of POMC hnRNA in the same samples.

still present (and even enhanced) in ADX rats given corticosterone in the drinking water suggests that the normal diurnal surge of endogenous corticosterone in the evening largely limits the concurrent secretion of ACTH. Perhaps consistent with this conclusion is the observation from other studies that replacement of ADX rats with moderately low constant levels of corticosterone (sc corticosterone pellets) is sufficient to normalize basal ACTH in the AM but not PM (22, 33). Interestingly, the large PM elevation of ACTH in ADX rats with corticosterone pellets is substantially delayed after the onset of the dark period.

Differential gene expression response to circadian drive, stress, and glucocorticoid effects in the PVN

Each of the three genes examined in the PVN had a unique response profile to the diurnal, acute stress and glucocorticoid conditions of this study. Thus, each gene reported different aspects of PVN activation and regulation. Here we summarize the features of each gene expression pattern.

c-fos Gene expression patterns. c-fos Gene expression patterns. As described As described above, c-*fos* mRNA expression in the PVN was elevated in the evening under basal conditions, in phase with diurnal HPA axis hormone secretion and pituitary POMC hnRNA levels. Stress caused an even greater elevation of c-*fos* levels at both times of day, with no diurnal difference in this gene's peak stress-induced expression. Basal PVN c-*fos* mRNA levels in ADX animals were sensitive to removal of glucocorticoids but only in the evening. Consequently, basal c-*fos* mRNA levels in the PVN of ADX rats were indistinguishable from those of sham rats in the AM but considerably elevated in the PM. This suggests that, whereas a constant low level of c-*fos* expression in the AM is not modified by changes in corticosterone status, endogenous corticosterone normally constrains the extent to which c-*fos* gene expression is increased in the PM in response to increased circadian drive. Administration of corticosterone in the drinking water to ADX rats normalized PM levels of c-*fos* mRNA. Thus, corticosterone has a permissive effect on PM basal c-*fos* expression that does not require the concomitant presence of high levels of circulating corticosterone but rather a surge in circulating corticosterone the previous evening. Glucocorticoid receptor is highly expressed within CRH neurons of the PVN (34); it remains to be determined, however, whether this circadian-dependent effect of glucocorticoids on c-*fos* expression is a result of a direct action of glucocorticoids in the PVN.

We also found that ADX increased stress-induced c-*fos* in the PVN, compared with adrenal-intact animals (experiment 2, Fig. 2). This result confirmed previous studies conducted in the AM (30, 35–37). The results have extended previous analysis by demonstrating a similar enhancement of stress-induced c-*fos* in the PM of ADX rats and an absence of a diurnal difference in the extent of c-*fos* induction by stress. Taken together these results suggest that: 1) the relatively recent (within the past 24 h), but not concurrent, presence of glucocorticoid is sufficient to exert an inhibitory influence on either the magnitude of a net neural stimulatory circadian drive or the intrinsic PVN response to that drive, and 2) activation of stress neurocircuitry overrides the diurnal difference in PVN basal c-*fos* expression, irrespective of adrenal status.

zif268 Gene expression patterns. A somewhat unexpected finding of this study was that zif268 gene expression displayed no significant diurnal change in the PVN (Figs. 1–3). However, such lack of response was not due to a generalized insensitivity of this gene to neural stimulation because zif268 gene expression in the PVN reacted promptly and prominently to acute stress in a manner similar to c-*fos* (Figs. 1 and 2). The fact that these two IEGs display such different patterns of expression in response to circadian input is interesting, given the fact that generally c-*fos* and zif268 have been shown to share very similar activation patterns and to be induced by similar sets of stimuli (38). It is possible that zif268 and c-*fos* are expressed in different subpopulations of parvocellular PVN neurons, and further colocalization studies are required to address this point. If, on the other hand, the two genes are expressed in the same cells, these observations indicate that whereas stress activates a signaling cascade that converges on some common regulator, circadian drive can selectively activate signaling to c-*fos* but not to zif268 (or CRH gene expression; see below). The neurochemical connections between SCN outputs and PVN are not yet completely mapped out and appear to include both direct

and indirect connections, with perhaps a predominance of the latter on parvocellular CRH neurons (6, 7, 9, 39, 40). Within this framework, it is likely that neurochemical signals that relay circadian information to the PVN vary between AM and PM, triggering differential signaling cascades and selective responses in gene expression.

Finally, zif268 response in the PVN differed from c-*fos* in that it was insensitive to glucocorticoid status, under both basal and stress conditions. This is in agreement with previous studies showing that stress-induced c-*fos* gene expression in the PVN can be suppressed by long-term elevation of glucocorticoids, whereas stress-induced zif268 expression is relatively insensitive to glucocorticoid elevation (27).

CRH gene expression patterns. Whereas c-*fos* expression rose in the PM under basal conditions, CRH primary transcript showed a decline at this time of day. This pattern agrees with previous observations that the rhythm of CRH basal gene expression (primary and mature transcript accumulation) is in opposite phase to corticosterone and ACTH diurnal secretion patterns (22, 41). These observations have been taken to indicate that basal CRH gene transcription and peptide secretion are under separate control and are relatively uncoupled (22, 42).

It is striking that not only basal CRH hnRNA levels but also stress-induced levels were lower in the PM than AM. This is in contrast to the lack of a diurnal difference in stress-induced plasma hormone, c-*fos* mRNA, and zif268 mRNA levels. The fact that stress produced equal degrees of c-*fos* and zif268 activation at both times of day suggests that a similar amount of restraint-induced neural stimulation was imparted to the CRH neuron in the morning and evening. Thus, one or more factors restrict basal and stress-induced CRH gene expression in the evening. One possible candidate for this effect is corticosterone, the levels of which increase in the PM. This prospect is supported by the fact that the long-term absence (5 d) of glucocorticoids not only increased basal CRH hnRNA levels, compared with sham levels (particularly evident in Fig. 3), but also completely abolished the AM *vs.* PM difference in basal (Figs. 2–4) and stress-induced (Fig. 2) CRH hnRNA levels. Placing corticosterone in the drinking water of ADX rats normalized the overall basal CRH hnRNA levels but did not reinstate the AM/PM difference observed in sham animals (Fig. 3). This suggests that the acute elevation of basal corticosterone levels in the evening probably suppressed both basal and stress-induced CRH hnRNA levels at this time of day. This conclusion is also supported by the observation that within the corticosterone replaced ADX group some animals had slightly higher corticosterone levels than others, maybe as a result of some early diurnal drinking before the lights out transition. Interestingly, in this group corticosterone levels and CRH expression showed a strong inverse correlation ($r = -0.81$, $P = 0.01$). Thus, an acute elevation of corticosterone levels can inhibit CRH gene expression under basal conditions and can limit its stress-dependent induction. There is support for glucocorticoids to have a direct effect on CRH gene expression. Glucocorticoids have been shown to inhibit transcriptional activation of the human CRH promoter through a negative glucocorticoid response element (43, 44). Glucocorticoids may also regulate CRH mRNA stability in the rat (45).

Contrary to our observations, other studies have reported a lack of dependence of basal CRH gene expression circadian fluctuations on glucocorticoids (22, 46). Whereas the reason for this discrepancy with the results of our study remains to be established, it is possible that because our experiment examined only two time points, we missed a potentially shifted peak and trough of CRH expression in the ADX animals. If this is the case, then glucocorticoids may interact with circadian stimuli in setting the phase of circadian CRH gene expression changes. In this respect it is interesting to note that Watts *et al.* (22), using a regimen of constant corticosterone replacement via sc pellets in ADX rats, still observed a circadian difference in CRH expression but with a marked phase shift from sham animals. Thus, it is also possible that different outcomes may be expected, depending on the type of corticosterone replacement used (in drinking water, as in our study, *vs.* constant release pellets, as in Refs. 22 and 46).

Finally, although not examined in this study, it is worth noting for comparison purposes that arginine vasopressin hnRNA in the parvocellular PVN has been shown to display a circadian rhythm of expression in ADX rats and ADX rats supplemented with constant release corticosterone pellets (22) but not adrenal-intact animals. This result raises the intriguing possibility that the normal diurnal rhythm in corticosterone secretion masks an endogenous rhythm in arginine vasopressin gene expression in the PVN.

Anterior pituitary gene expression

In the anterior pituitary, relative POMC gene expression displayed a similar profile to HPA axis hormone secretion (Fig. 3), suggesting that transcriptional regulation of ACTH production and ACTH secretion may be relatively well coupled. Levels of POMC hnRNA exhibited a clear diurnal pattern under basal conditions, with increased levels in the evening, in line with presumed increased CRH peptide secretion at this time of day. POMC transcription can be induced by a CRH receptor-dependent signaling cascade that involves protein kinase A and calcium (47–49), whereas glucocorticoids have the potential to prevent transcriptional activation of the POMC gene by interfering with positive regulators on its promoter (50, 51). In our experiment, ADX induced elevated basal POMC hnRNA expression at both times of day, suggesting a tonic suppressive effect of glucocorticoids on this gene. Very likely, the tonic inhibition that glucocorticoids exert on ACTH plasma levels, observed in the present (Figs. 2 and 3) and previous studies (11), is, at least in part, due to an effect at the level of POMC gene expression. Interestingly, whereas placement of corticosterone in the drinking water of ADX rats normalized the morning levels of POMC hnRNA, it did not correct the large increase in basal POMC hnRNA levels observed in the PM of ADX animals (Fig. 3G). A very similar pattern of ACTH secretion was evident in the same rats (Fig. 3B). This suggests that the acute presence of high concentrations of corticosterone in the PM suppresses the levels of POMC hnRNA and

ACTH secretion at this time of day, when presumably POMC transcription and ACTH secretion are otherwise enhanced by a sustained increase in CRH secretion. In a previous report (46), PM levels of POMC mRNA were still elevated, compared with sham rats, in ADX rats replaced with pellets at low doses of corticosterone (~1.5 μg/dl) but not with higher doses of corticosterone (~2.7 μg/dl). It is noteworthy that there was also a significant diurnal difference in anterior pituitary c-*fos* mRNA of ADX rats given corticosterone replacement (Fig. 3F), even though there was no diurnal difference in c-*fos* mRNA in the anterior pituitary of sham and ADX rats. It is possible that the presence of several c-*fos* expressing cell types in the anterior pituitary, most of which do not respond to CRH stimulation, masked small diurnal CRH-dependent stimulatory effects on c-*fos* expression in corticotropes of sham and ADX rats.

In contrast to the other pituitary-related measures, there was little evidence for a diurnal difference in zif268 mRNA expression in the anterior pituitary. A previous study has shown that increases in zif268 mRNA levels can be detected in the anterior pituitary after acute stress (32).

Adrenal gland gene expression

Besides gene expression changes in the PVN and anterior pituitary, we examined IEG expression within the adrenocortex. We chose to analyze c-*fos* and NGFI-B, a transcription factor that plays a role in the ACTH-dependent regulation of some adrenocortical steroidogenic enzymes (52, 53). NGFI-B expression in cortical areas of the adrenal gland (zona fasciculata and reticularis) has been previously shown to increase after stress (24); however, no direct measure of diurnal expression levels of this gene has been reported. Overall, c-*fos* mRNA pattern of expression had a similar trend to plasma ACTH, with small basal differences, and therefore seemed to reflect the acute stimulation of the adrenocortex by ACTH (Fig. 2). NGFI-B gene expression in the adrenal cortex showed a much more pronounced diurnal change under basal conditions, and it was markedly elevated by stress. Thus, NGFI-B gene expression was reminiscent of the profile of corticosterone secretion. These data suggest the possibility that the expression of different IEGs in the adrenocortex may reflect different aspects of adrenal function. Changes in adrenal gland sensitivity with time of day have long been proposed (54, 55); however, the mechanism remains obscure. Recent evidence for a direct role of SCN in controlling adrenal sensitivity has been produced (4, 56) and corroborated by anatomical evidence of SCN-derived autonomic inputs to the gland (57–61). In addition, it appears that light stimulation is capable of inducing steroidogenic enzymes and the NGFI-B family of transcription factors, as well as corticosterone secretion in the absence of ACTH secretion, via an SCN-dependent mechanism that requires intact autonomic innervations (62).

Conclusion

In brief, we have shown that a circadian drive that regulates HPA axis basal hormone secretion is also manifest on basal c-*fos* gene expression in the PVN. We show that different IEGs within the HPA axis anatomical components display different patterns of diurnal rhythmicity; in part, these differential patterns of gene expression are the result of gene-selective responses to diurnal-dependent intercellular signals and acute and/or permissive glucocorticoid actions.

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