Diurnal expression of functional and clock-related genes throughout the rat HPA axis: system-wide shifts in response to a restricted feeding schedule

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Submitted 26 November 2008; accepted in final form 2 February 2009

Girotti M, Weinberg MS, Spencer RL. Diurnal expression of functional and clock-related genes throughout the rat HPA axis: system-wide shifts in response to a restricted feeding schedule. Am J Physiol Endocrinol Metab 296: E888–E897, 2009. First published February 3, 2009; doi:10.1152/ajpendo.90946.2008.—The diurnal rhythm of glucocorticoid secretion depends on the suprachiasmatic (SCN) and dorsomedial (putative food-entrainable oscillator; FEO) nuclei of the hypothalamus, two brain regions critical for coordination of physiological responses to photoperiod and feeding cues, respectively. In both cases, time keeping relies upon diurnal oscillations in clock gene (per1, per2, and bmal) expression. Glucocorticoids may play a key role in synchronization of the rest of the body to photoperiod and food availability. Thus glucocorticoid secretion may be both a target and an important effector of SCN and FEO output. Remarkably little, however, is known about the functional diurnal rhythms of the individual components of the hypothalamic-pituitary-adrenal (HPA) axis. We examined the 24-h pattern of hormonal secretion (ACTH and corticosterone), functional gene expression (c-fos, crh, pomc, star), and clock gene expression (per1, per2 and bmal) in each compartment of the HPA axis under a 12:12-h light-dark cycle and compared with relevant SCN gene expression. We found that each anatomic component of the HPA axis has a unique circadian signature of functional and clock gene expression. We then tested the susceptibility of these measures to nonphotic entrainment cues by restricting food availability to only a portion of the light phase of a 12:12-h light-dark cycle and compared with relevant SCN gene expression. We found that restriction of food availability alters the phase of the circadian nuclear clock gene expression in the HPA axis, despite ongoing photoperiod changes and minor changes in SCN clock gene expression. Thus the HPA axis may be an important mediator of the body entrainment to the FEO.

Diurnal changes in hypothalamic-pituitary-adrenal (HPA) axis activity and glucocorticoid secretion (12, 13, 41, 53, 54) assist in the maintenance of a metabolic balance in the organism. Alterations in this diurnal rhythm are associated with mood disorders (20, 37) and may contribute to a state of altered energy regulation that has been described as a metabolic syndrome (14). The suprachiasmatic nucleus (SCN) (9, 12, 36) is the master neuronal circadian oscillator that synchronizes physiological and behavioral rhythms to the photoperiod (36, 48). The SCN controls the rhythm of glucocorticoid secretion via direct and indirect neural control of the periodic release of corticotropin-releasing hormone [CRH; and subsequently adrenocorticotropic hormone (ACTH) (13–16)] and through autonomic innervation of the adrenal gland (7, 8, 26, 27). A powerful zeitgeber of the glucocorticoid diurnal rhythm, in addition to photoperiod, is the time of meal presentation. If feeding time is restricted in rats by limiting food availability to a few hours during the daytime, then the rats display an anticipatory peak of glucocorticoid release 1–2 h before the availability of food (30). Because this phenomenon is not dependent on a functional SCN, it suggests that there is a distinct food-entrainable oscillator (FEO) (47). Recent studies indicate that function of this FEO critically depends on the dorsomedial hypothalamus (DMH) (18, 21).

The basis of physiological and behavioral circadian rhythms is the periodic expression of clock genes that occurs as a result of an autoregulatory negative feedback loop. Positive transactivator gene products [such as brain and muscle aryl hydrocarbon receptor nuclear translocator-like (Bmal) and circadian locomotor output cycles kaput (Clock)] induce the expression of their own repressors [period (per) and cryptochrome gene families] with a cycle of ~24 h (23, 24, 44). Interestingly, clock gene expression has been reported in non-SCN brain regions (17, 22) and in peripheral tissues (16, 39, 59). However, SCN function is necessary to synchronize the peripheral oscillators to each other and to the photoperiod. In addition, peripheral oscillators can also respond to feeding schedule changes (15, 49), and it appears that glucocorticoids may be involved in the temporal entrainment of peripheral clocks (5, 38, 43). Thus the HPA axis may be an important integrator of various environmental time-keeping signals and a regulator of peripheral tissue synchronization. There is, however, no systematic literature on clock gene expression in the HPA axis to date but rather some reports of individual gene or protein profiles at single levels of the axis obtained from cultured tissue slices or various in vivo models (1, 4, 6, 26, 34, 40, 50–52). Moreover, despite the clear effects of restricted feeding (RF) on corticosteroid secretion, no prior studies have explored the influence of RF on measures of HPA axis function other than circulating hormone levels (32, 33).

To begin to explore the possibility that circadian function of each element of the HPA axis depends on intrinsic clock gene expression, we examined within the HPA axis the diurnal expression of hormone production-related genes (functional genes), crh, the proopiomelanocortin gene (pomc), and steroidogenic acute regulatory protein (StAR; a rate-limiting factor in the de novo synthesis of glucocorticoids)-encoding genes. We also examined the concurrent expression of the clock genes per1 and per2 and their positive transactivator bmal. We previously observed a diurnal variation in basal c-fos expression in the HPA axis (19) and therefore have included measurement of this immediate early gene in our analysis. A primary goal of this study was to determine whether each functional and clock gene examined has a diurnal rhythmic

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expression pattern within the HPA axis. In the case of the clock genes, we also wanted to determine whether there was evidence for coordinated oscillations of clock gene expression within each component of the HPA axis (per1 and/or per2 gene expression in antiphase with bmal gene expression). An additional goal was to examine the phase relationships between functional and clock gene expression within and across each element of the HPA axis and their phase relationship to clock gene expression in the SCN. Finally, we wanted to determine the extent to which daytime RF alters functional and clock gene expression throughout the HPA axis and SCN despite the presence of photoperiodic cues.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (270–290 g; Harlan Laboratories, Indianapolis, IN) were given a 2-wk acclimation period before experimental use. Animals were housed two per cage, and food (Teklad Rodent Diet 8640; Harlan) and tap water were available ad libitum. The colony room lights were regulated on a 12:12-h light-dark (LD) cycle, with lights on at 6:00 A.M. [zeitgeber time (ZT) 0]. 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 No. (National Institutes of Health) 80–23, revised 1996 edition] and were approved by the University of Colorado Institutional Animal Care and Use Committee.

Experiment 1: 24-h Time Course Hormone Secretion and Gene Expression in the SCN and HPA Axis

Groups of rats were decapitated every 4 h over a 24-h period animals per group, (n = 6, total number of animals, N = 36). For the time points of ZT0 (6:00 A.M.) and ZT12 (6:00 P.M.), the rats were decapitated ~15 min before the light transition. The procedure was conducted under dim red light illumination at time points ZT16, ZT18, ZT20, and ZT0. Brains, pituitaries, and adrenal glands were extracted and flash-frozen in a dry ice-isopentene bath at −30°C and then transferred to −80°C. Trunk blood plasma was collected for hormonal assays.

Experiment 2: Effect of RF on SCN and HPA Gene Expression and HPA Hormone Levels

After the 2-wk acclimation period, rats were either maintained on an ad libitum diet (AL rats) or were given restricted access to food between ZT3 and ZT6 (9:00 A.M.-12:00 P.M.) (RF rats). Body weight was monitored at the end of each week. After 24 days, rats from both the AL and RF groups were decapitated at either ZT2 or ZT11 (n = 6, N = 24). These time points were chosen to correspond to 1 h before the expected time of feeding for RF or AL groups, respectively; this allowed for determination of possible anticipatory hormonal and gene expression measures without contamination of those measures by the actual act of feeding. In addition, because both time points occur during the light portion of the LD cycle, any differences in the various measures cannot be attributed to photoperiod differences. Tissues were extracted as described above. Trunk blood plasma was collected for hormonal assays.

In Situ Hybridization Histochemistry and Image Analysis

Coronal brain cryosections (10 µm) were obtained through the extent of the SCN and paraventricular nucleus of the hypothalamus (PVN) [respectively, ~1.30 and 1.80 mm posterior to bregma (42)] or transversely (12 µm) through the middle portion of the pituitary and adrenal glands, thaw-mounted on poly-l-lysine-coated slides, and stored at −80°C.

In situ hybridization for c-fos, star, per1, per2, and bmal mRNA and crh and pomec heteronuclear RNA (hnRNA) and analysis of digitized images from X-ray films were performed as previously described (19). 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), a portion of the crh intron (kindly provided by Dr. R. Thompson, University of Michigan), or a portion of pomec intron A (kindly provided by Dr. S. Watson, University of Michigan) were used.

The per1, per2, bmal, and star expression plasmids were generated in house from either rat cortex total RNA (per1, per2, and bmal) or rat adrenal total RNA (star) using a RT-PCR method described in detail elsewhere (19). The cloned coding portion for each gene is as follows: per1: nuclear transcript (nt) 974–1547, Genebank accession no. NM_001034125; per2: nt 2240–2869, Genebank accession no. NM_031678; bmal: nt 69–1127, Genebank accession no. NM_024362; star: nt 308–757, Genebank accession no. NM_031558. The identity of the cloned DNA was verified by DNA sequencing (University of Colorado Molecular Cellular and Developmental Biology sequencing facility). Tests of sense probes on rat brain slices showed no detectable specific signals above background. Examples of in situ hybridization signals at peak expression for each gene tested in the different tissues are presented in Fig. 1.

ACTH Radioimmunoassay and Corticosterone Enzyme Immunoassay

Plasma ACTH and corticosterone were determined as previously described (19). The detection limit for the ACTH assay was 15 pg/ml for a 50-µl sample; the intra-assay and interassay coefficients of variability were 8–11 and 8%, respectively. The sensitivity for the corticosterone assay was 130 ng/100 ml. The intra-assay and the interassay coefficients of variability were 5–6 and 10%, respectively.

In situ hybridization signals at peak expression for each gene tested in the different tissues are presented in Fig. 1.
Statistical Analysis

In experiment 1, diurnal changes of hormone secretion and gene expression were fitted to a basic sinusoid function \( y = A \times \sin(K(x - x_0)) + \text{const} \) where \( A \) is amplitude, \( K \) is frequency, \( x_0 \) is the phase angle, and const is the y-axis offset. The time of decapitation (x) and phase angle were expressed as ZT. The program ProFit (QuantumSoft) was used to estimate the parameters of best fit, allowing \( A, x_0 \), and const to vary and imposing \( K \) constant (\( K = 0.27 \)). The model fit was tested using a nonlinear procedure (NLIN; SAS statistical software package). The phase angle \( x_0 \) corresponds to the predicted ZT at which the rhythm crosses its midpoint amplitude value as it proceeds from its trough value (minimum) to its peak value (maximum). The \( x_0 \) was estimated with 95% confidence limits. Significant difference in phase angle between curves was assumed for nonoverlapping \( x_0 \) confidence intervals. The predicted values of \( x_0, x_0 \) confidence interval, maximum and minimum, along with the associated \( F \) and \( P \) values for the overall curve fit are reported in Fig. 2.

In experiment 2, ANOVA was used to examine overall main effects and interactions of time of day and feeding condition (2 × 2 factorial design) on log-transformed data. Post hoc pairwise comparisons were examined (Fisher’s least-significant difference) only in those instances where the ANOVA yielded a significant main effect for time of day or feeding condition or a significant interaction between these two factors. SAS was used in all the analyses; \( P < 0.05 \) was considered significant. Data presented represent group means ± SE.

RESULTS

Experiment 1: 24-h Time-Course Hormone Secretion and Gene Expression in the SCN and HPA Axis

In this first experiment, we assessed the periodic profiles of functional and clock gene expression in the PVN, the anterior pituitary, and the adrenal cortex, and we compared the profiles with hormone release and SCN clock gene expression rhythms. Measures were taken at 4-h intervals over 24 h.

HPA axis hormone levels and functional gene expression. We found, as expected (54), that corticosterone secretion peaked around the time of the switch to lights-off (Figs. 2 and 3B). Plasma ACTH levels increased at ZT12–16; however, the overall periodic pattern did not reach statistical significance (Figs. 2 and 3A).

The \( crh \) hnRNA levels in the PVN showed a trough at the time of lights-off and a peak around the time of lights-on (Figs. 2 and 3C). The \( pomc \) hnRNA levels in the anterior pituitary did not display a significant 24-h rhythm (Fig. 2), but instead appeared to have an ultradian rhythm with approximately an 8-h period (Fig. 3D). Finally, \( star \) mRNA levels in the adenocortex progressively increased from the early afternoon, well before the corticosterone peak, with an estimated peak at ZT14 (Figs. 2 and 3E). The phase angle analyses indicated that the profiles of \( star \) expression and corticosterone secretion were not significantly different (Fig. 2).

c-fos expression in the SCN and HPA axis. In the SCN, the highest c-fos mRNA levels occurred immediately preceding the onset of light, steadily decreased throughout the light period, and remained low throughout much of the dark period (Figs. 2 and 3F). There was also a diurnal rhythm of c-fos expression in the PVN; however, peak and trough levels occurred during the opposite times of day from the SCN (Figs. 2 and 3G). The PVN c-fos profile was also in antiphase to \( crh \) expression in the PVN (Figs. 2 and 3, C and G). There appeared to be a diurnal rhythm of c-fos expression in the anterior pituitary; however, because of a large interindividually variability, it did not reach significance (Figs. 2 and 3H). In the adrenal gland, c-fos expression was lowest at ZT1.5 and showed a broad sustained increase between ZT 8 and 20 with an estimated peak at ZT13 (Figs. 2 and 3F). This temporal expression pattern was not significantly different from the profile of \( star \) expression and corticosterone levels (Fig. 2).

Clock gene expression in the SCN and in the HPA axis. As expected, in the SCN, \( per1 \), \( per2 \), and \( bmal1 \) showed very strong rhythmic expression, with \( per1 \) and \( per2 \) peak expression during the light period and \( bmal1 \) peak expression during the dark period (Figs. 2 and 4, A-C). In the PVN, all three clock genes showed a clear rhythmic expression that was in antiphase with their respective expression in the SCN (Figs. 2 and 4, D-F). The temporal expression

Fig. 2. Best-fit circadian rhythm parameters and statistical analyses for the hormone and gene expression measures in experiment 1. The phase angle (\( x_0 \)) with 95% confidence interval (CI) for each hormone and gene expression measure of experiment 1 (also see Fig. 3) were estimated and the values graphically presented (A) and tabulated (B). In B, the estimates of the measured variable’s maximum (max) and minimum (min) and the \( F \) and \( P \) values of the circadian rhythm best fit (24-h sinusoid function) are also presented. The \( x_0, CI, \) max, and min are expressed in zeitgeber time (ZT). *Cases of \( per1 \) or \( per2 \) gene expression that have phase angles that occur 12 h in antiphase with \( bmal1 \) gene expression in the same tissue. In A, the dark bars above the \( x \)-axis represents the period of lights off. AP and Ant Pit, anterior pituitary; Adr and Adrcort, adrenal cortex.
profiles of per1 and per2 in the PVN were not significantly different from the c-fos expression profile in the PVN (Fig. 2).

In the anterior pituitary, we detected rhythmic expression of per2 and bmal, but not per1 (Figs. 2 and 4, G-I). The per2 and bmal expression within the anterior pituitary were in antiphase with each other (Fig. 2). The phase angle of per2 expression in the anterior pituitary was slightly advanced to the SCN per2 expression and was advanced by 6 h with respect to PVN per2 expression (Fig. 2). The phase of bmal expression was delayed by 4 h with respect to SCN bmal expression and advanced by 5 h relative to the PVN gene (Fig. 2).

In the adrenal cortex, we observed a strong rhythmic expression of all three clock genes (Fig. 4, J-L). The mRNA levels of per1 and per2 peaked around the time of lights out, and their phase relationship was similar to per2 expression in the anterior pituitary (Fig. 2). Finally, bmal temporal expression in the adrenocortex was in antiphase with adrenocortical per1 expression and was nearly identical to bmal temporal expression in the anterior pituitary (Fig. 2). Each clock gene also displayed circadian expression in the medulla. There was a slight tendency for per1 and per2 to be phase-delayed with respect to the cortical genes, whereas bmal expression was synchronous to bmal expression in the adrenocortex (data not shown; phase angle analysis: per1, θ0 = 8, θ0 confidence limits 5.7–9.4, P < 0.01; per2, θ0 = 9, θ0 confidence limits 8.6–10.1, P < 0.0001; bmal, θ0 = 17, θ0 confidence limits = 16.4–18, P < 0.0001).

Experiment 2: Effect of RF on SCN and HPA Gene Expression and HPA Hormone Levels

In this experiment, we determined whether restricting the availability of food to 3 h during the light phase would affect...
the relative morning and evening levels of the HPA axis-associated measures. RF animals gained weight over the 3-wk period, albeit at a reduced rate than the AL animals (%body wt change by the 3rd wk: AL rats = +31 ± 1.5%; RF rats = +19 ± 1.6%).

**HPA axis hormone levels and functional gene expression.**

The rhythm of corticosterone secretion in RF rats was reversed from AL controls, as high corticosterone levels were observed 1 h before presentation of the meal (ZT2), and lower levels were measured in the evening [ZT11 (Fig. 5B); time of day F(1,23) = 5.14, P = 0.05; interaction F(1,23) = 22.63, P < 0.01]. ACTH levels did not differ significantly between the two times of day for either feeding condition (Fig. 5A).

In the PVN, the *crh* hnRNA expression pattern, which had lower levels in the evening in AL animals, as expected, was significantly reversed in RF rats [feeding condition F(1,23) = 5.74, P < 0.05; interaction F(1,23) = 9.76, P < 0.01; Fig. 5C]. In the anterior pituitary, we did not observe a significant effect of time of day on the expression of *pomc* hnRNA; however, we observed a significant effect of feeding condition [feeding condition F(1,22) = 9.31, P < 0.01], which was mainly due to an overall higher expression of *pomc* in RF animals that was particularly pronounced in the morning (Fig. 5D).

Finally, adrenocortex *star* mRNA levels were higher in the evening of AL rats than in the morning as expected from the 24-h profile; this was significantly reversed by RF, paralleling the effect observed on corticosterone secretion [interaction F(1,23) = 39.7 P < 0.01; Fig. 5E]. Thus both *crh* expression in the PVN and *star* expression in the adrenocortex showed marked sensitivity to RF schedules.

**c-fos expression in the SCN and HPA axis.** In the SCN, RF led to significantly higher c-fos mRNA levels in the morning and thereby a diurnal difference that was not evident in AL rats [feeding condition F(1,23) = 4.77, P < 0.05; Fig. 5F]. Within the PVN, the clear rhythm of PVN c-fos expression in AL animals was significantly reversed by RF [interaction
RF had no appreciable effect on c-fos expression in the anterior pituitary or adrenal cortex (Fig. 5, H and I).

Clock gene expression in the SCN and HPA axis. The RF regimen affected the clock gene expression profiles at all levels of the HPA axis in a tissue-specific manner but had only a select effect on clock gene expression profiles in the SCN. In the SCN, we observed clear rhythms of per1 and per2 mRNA in AL rats, and these rhythms were not altered by RF [for per1: time of day F(1,23) = 32.1, P < 0.01; for per2: time of day F(1,23) = 13.6, P < 0.01; Fig. 6, A and B]. In AL rats, we observed equal levels of SCN bmal mRNA levels at the two times of day examined (Fig. 6C) in accordance with the fact that these times flank the morning trough of this gene expression (Fig. 4C). RF appeared to lower the morning level and increase the evening level of bmal expression, resulting in an overall significant effect of time of day [F(1,23) = 9.06, P < 0.01] as well as a significant feeding condition by time interaction [F(1,23) = 5.89, P < 0.05].

In the PVN, RF significantly increased the morning levels and decreased the evening levels of per1 mRNA [time of day F(1,23) = 4.68, P < 0.05; interaction F(1,23) = 46.1, P < 0.01; Fig. 6D]. In the PVN, per2 mRNA showed a significant rhythm in AL animals, but, unlike per1, this pattern was not reversed by RF; however, the AM levels of per2 in RF rats were significantly higher than in AL rats [time of day F(1,23) = 12.9, P < 0.01; Fig. 6E]. RF also significantly altered the expression profile of PVN bmal, causing a drastic reduction of the morning levels and a slight increase in the evening levels of this gene [feeding condition F(1,23) = 6.13,
In the anterior pituitary, there was no significant main effect of time of day on per1 gene expression, in line with the lack of rhythm observed in the time-course experiment, and there was no effect of feeding condition (Fig. 6 G). On the other hand, both per2 and bmal expression, which was rhythmic in the anterior pituitary of AL animals, was reversed by RF [for per2: time of day $F(1,22) = 5.78, P < 0.05$, interaction $F(1,22) = 26.5, P < 0.01$; for bmal: time of day $F(1,22) = 12.9, P < 0.01$, interaction $F(1,22) = 121.2, P < 0.01$; Fig. 6, H and I].

Finally, in the adrenocortex, the clear rhythms of the three clock genes in AL rats were significantly reversed by RF [for per1: interaction $F(1,23) = 44.5, P < 0.01$; for per2: feeding condition $F(1,23) = 34.1, P < 0.01$, time of day $F(1,23) = 72.9, P < 0.01$, interaction $F(1,23) = 285.2, P < 0.01$; for bmal: feeding condition $F(1,23) = 6.15, P < 0.05$, time of day $F(1,23) = 35.3, P < 0.01$, interaction $F(1,23) = 377.4, P < 0.01$; Fig. 6, J-L]. RF also reversed the rhythms of clock gene expression in the medulla [for per1: interaction $F(1,23) = 47.2, P < 0.0001$; for per2: feeding condition $F(1,22) = 8.4, P < 0.01$, time of day $F(1,22) = 7.4, P < 0.05$, interaction $F(1,22) = 66.8, P < 0.0001$; for bmal: interaction $F(1,23) = 165.4, P < 0.0001$; data not shown].

DISCUSSION

Several genes representative of the mammalian endogenous molecular circadian clock displayed strong circadian expression patterns in each component of the HPA axis. Moreover, as in the SCN, per1 and/or per2 gene expression within each HPA axis element was ~180° antiphase with bmal gene expression, demonstrating a functional oscillatory clock gene expression pattern within these tissues. It is noteworthy, however, that
there was a different phase angle relationship between clock gene expression in the SCN and each component of the HPA axis. Interestingly, within the HPA axis, there was a very similar phase angle relationship between clock gene expression in the anterior pituitary and the adrenal cortex; however, the timing of these rhythms differed significantly from those in the PVN. Nevertheless, clock gene diurnal expression within each compartment of the HPA axis was significantly altered in rats exposed to daytime RF, whereas clock gene expression in the SCN was largely unaffected by this manipulation.

Circadian Expression of HPA Axis Functional Genes and Clock Genes

The regulatory influences of corticosterone on virtually all physiological systems may vary over the course of the day, since corticosterone secretion levels fluctuate with a large amplitude circadian rhythm. We found that the corticosterone secretion rhythm was in phase with the rhythm of adrenocortical expression of star, a steroidogenic factor encoding gene, and the clock genes per1 and per2. Adrenocortical expression of c-fos was also in phase with corticosterone secretion, which corresponds well to previous reports of adrenal Fos protein expression patterns (52). The c-fos gene is an immediate early gene that is generally induced following changes in cellular activation states in response to extracellular signals (11). Studies in mice show a strong circadian rhythm of genes that encode adrenal enzymes involved in steroidogenesis and catecholamine synthesis (39); a report published while this manuscript was in review presented evidence that adrenal star expression is under the direct control of Bmal-Clock (46).

In the PVN, crh hnRNA showed a clear circadian change, as previously reported (19, 54); moreover, we confirmed our earlier observations that c-fos expression is in antiphase with crh hnRNA rhythm (19). We have previously shown that glucocorticoids regulate crh hnRNA levels by suppressing transcription in the early evening (19), possibly through a direct glucocorticoid receptor (GR)-mediated repression of the crh promoter (57). In addition, replacement of adrenalectomized rats with tonic-release corticosterone pellets shifts the diurnal rhythm of crh hnRNA (54). One possibility is that the timing of crh transcription and/or translation is controlled by an interaction between glucocorticoids and the PVN clock genes in a manner similar to what has been described for the circadian regulation of the liver transcriptome (43). Functional and putative glucocorticoid response elements are present in the per1 (58) and bmal1 promoters (43). Consequently, activated GR may regulate clock gene expression, which in turn may time-dependently regulate a transcription factor that acts on the crh promoter.

Phase Relation of PVN and SCN c-fos and Clock Genes

We found clear oscillations in per1, per2, bmal1, and c-fos expression in the SCN, in agreement with previously reported data in the rat (4, 25). It is noteworthy that the peak c-fos mRNA levels observed at the end of the dark period in the SCN preceded the onset of the light period, suggestive of an anticipatory response. Interestingly, PVN c-fos, per1, per2, and bmal rhythms were in antiphase with the respective SCN genes. Extra-SCN neuronal clocks are often expressed in antiphase from the SCN (52), but not always (2, 52). It is not known what dictates the regional specificities of central nervous system clock gene expression. The PVN receives photoperiod cues indirectly through the SCN and DMH (45). In addition, DMH and arcuate nucleus relay temporal signals related to feeding and body temperature changes to the SCN (56). Therefore, it is plausible that the PVN intrinsic clock settings reflect the integration of photoperiod with nonphotic information related to arousal, feeding, and the general metabolic condition. The extent to which this integrated information is relayed to downstream components of the HPA axis remains to be determined. However, as noted above, if this is the case, then it is not reflected in similar phase angles for the respective clock gene expression in each tissue.

Effects of RF on HPA Axis Hormone Levels and Functional Gene Expression

Previous work that investigated the effects of RF on HPA axis activity focused on hormonal measures (30, 32). In this study, we examined the effects of RF on hormonal secretion as well as on functional gene and clock gene expression at each level of the axis.

We observed the expected anticipatory peak in corticosterone secretion (but not in ACTH secretion) 1 h before daytime feeding in RF rats. Shifts in diurnal peak corticosterone secretion do not necessarily require detectable shifts in ACTH peak secretion (32, 55). On the other hand, a shift in the adrenal sensitivity to exogenous ACTH that is entrained by time of feeding has been documented (32, 55). As discussed above, it is likely that slight changes in ACTH levels activate the adrenocortex to a different extent, depending on the time of

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day. RF also affected star gene expression within the adrenocortex, suggesting that RF resets the timing of peak steroidogenic activity of the adrenal gland. Finally, RF profoundly affected the adrenal oscillator, reversing the expression profile of each clock gene examined. Thus the RF-dependent shift in corticosterone peak secretion may result from both an altered response to changes in an extrinsic signal and the resetting of the adrenocortex functional state, probably as a result of altered clock gene expression [for example, by altering bmal expression and consequently affecting star expression (46)].

In addition to changes within the adrenocortex, substantial changes in the upstream components of the axis, particularly the PVN, are also observed in RF animals. Thus, crh hnRNA expression profile was reversed by RF. In another study, hypothalamic CRH peptide content was not affected by RF (32). It is possible that more sensitive assays may be needed to detect diurnal CRH peptide changes in the hypothalamus. PVN c-fos expression was also reversed, probably reflecting the altered neural activity derived from the extensive neuronal connection of the PVN with food-regulatory centers. Finally, clock gene expression was reversed by RF in both the anterior pituitary and PVN, indicating that feeding time is a strong zeitgeber in these regions. It would be interesting to determine in future studies whether the changes in PVN gene expression imposed by RF occur in parallel or contribute to the adrenal gland temporal and functional changes.

It is important to note that we conducted RF under normal LD conditions. Thus, if activity of the anatomical components of the HPA axis is strongly under the influence of the SCN, we may then expect to see only a minor effect of RF on gene expression throughout the HPA axis. To the contrary, we observed substantial effects of RF on these tissues. This supports the prospect that each HPA axis component has a strong link not only with the SCN but also with the FEO.

**Effects of RF on SCN c-fos and Clock Expression**

RF did not change the expression pattern of per1 and per2 in the SCN, in agreement with previous reports (15). However, we observed an effect of RF on bmal expression, which needs further confirmation. Hypocaloric but not normocaloric RF has been shown to change the oscillatory pattern of the master clock (35). Because our RF animals were gaining weight at a reduced rate compared with AL controls, they may have been under a mild hypocaloric state, which may have partially affected clock expression in the SCN. Interestingly, c-fos gene expression in the SCN was also affected by RF. This was unexpected given that RF did not alter Fos protein levels in the SCN in other studies (3), although it is worth mentioning that measures of SCN neuropeptide release can be altered by signals from feeding centers (29, 31). Thus it seems possible that the SCN responds to metabolic challenge in a graded manner: mild metabolic challenge (RF) may affect some SCN outputs and gene expression (c-fos) but not the master oscillator, and it is only with a more severe metabolic challenge (caloric restriction) that central clock function becomes altered (10).

In conclusion, we demonstrate in this study that each component of the HPA axis has pronounced circadian clock gene expression suggestive of intrinsic clock function. In addition, feeding cues are strong zeitgebers of the HPA axis functional state. This is manifest not only at the level of corticosterone secretion patterns but is also evident in the expression of genes related to hormone production and in clock gene expression. In contrast, RF had a limited effect on the SCN pacemaker, indicating the ability of feeding cues to override photoperiod influences on HPA axis intrinsic clock gene activity. This sensitivity of the HPA axis and glucocorticoid secretion to feeding signals may be crucial to the regulation of peripheral metabolic functions in response to food availability. Moreover, the apparent shift of HPA axis clock entrainment to time of meal presentation under LD conditions suggests that this system may be involved in the coordination of nocturnal vs. diurnal feeding patterns.

**ACKNOWLEDGMENTS**

We thank Michael VanElzakker for helping with the collection of tissue samples and Dr. Gregory Carey for help with the statistical analysis of the sinusoid curve fit of the data.

**GRANTS**

This work was supported by National Institute of Mental Health Grants MH-75968 and MH-065977 to R. L. Spencer.

**REFERENCES**


FOOD RESTRICTION AND HPA AXIS CLOCK GENES


