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# Diurnal Regulation of Glucocorticoid Receptor and Mineralocorticoid Receptor mRNAs in Rat Hippocampus

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**The present studies were undertaken to determine whether hippocampal glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) biosynthesis, as inferred from mRNA expression, exhibit diurnal patterns of activation which may reflect or predict changes in plasma adrenocorticosteroid levels. Animals received either adrenalectomy (ADX) or sham adrenalectomy and were sacrificed at 4-h intervals throughout the diurnal cycle. Hippocampal and control brain areas were assayed for regional GR and MR mRNA changes via semiquantitative *in situ* hybridization histochemical analysis. The results indicate subfield-specific significant circadian rhythms in both GR and MR mRNAs. A significant diurnal rhythm in GR mRNA expression was seen in the dentate gyrus (DG), which took the shape of a monotonic curve with a marked trough at 1500 h after lights on (1 h after lights off). A similar pattern was evident in subfield CA1, although the effect fell short of statistical significance. No rhythm was seen in CA3. In response to ADX, GR mRNA was markedly increased in both CA1 and CA3; these increases appeared to be independent of circadian influences. In contrast, ADX effects in DG were quite limited and appeared to eliminate the circadian GR mRNA trough. Bimodal diurnal rhythms in MR mRNA expression were observed in all subfields and commonly exhibited troughs at 1500 h after lights on and 0300 h after lights on. These rhythms appeared to be related to circulating steroids, as ADX eliminated both the 1500 and 0300 h troughs, resulting in flat levels of MR mRNA expression corresponding roughly to the circadian peak. Notably, no diurnal GR or MR mRNA rhythms were observed in frontoparietal cortex, nor were any GR mRNA changes seen in the dorsomedial thalamus or hypothalamic paraventricular nucleus. Indeed, ADX was ineffective in altering adrenocorticosteroid receptor mRNA expression in any extrahippocampal region examined. These results indicate that GR and MR mRNAs exhibit hippocampus-specific diurnal rhythms in expression which are controlled to a greater (MR) or lesser (GR) extent by circulating steroids. The apparent steroid sensitivity of hippocampal adrenocorticosteroid receptor populations may be involved in the expression of rhythmic changes in hippocampal function associated with HPA regulation and information processing.** 

## INTRODUCTION

One of the outstanding characteristics of the hypothalamo–pituitary–adrenocortical (HPA) axis is a marked circadian rhythm in adrenocortical steroid secretion. This rhythmicity exhibits a diurnal peak coinciding with the active phase of the organism's sleep–wakefulness cycle (light phase in human, dark phase in rat). In recent years, it has been proposed that the hippocampal formation may play a role in generation of diurnal hypothalamo–pituitary–adrenocortical rhythms, perhaps via type 1 and/or type 2 glucocorticoid receptors indiginous to hippocampal neuronal populations (1–3). Support for this hypothesis comes from several sources: first, it has long been thought that the hippocampus plays an inhibitory role in activation of the HPA axis. Notably, hippocampal ablation or fornix section have been shown to desynchronize daily patterns of corticosterone (CORT) and ACTH release, rendering secretory patterns relatively constant across the circadian cycle at levels midway between known peak and nadir values (4–6). In addition, steroid implants, particularly into the ventral hippocampus, appear to desynchronize diurnal CORT secretions (7). Finally, intracerebroventricular injection of the type 1 receptor antagonist RU28318 appears to eliminate AM–PM differences in circulating CORT levels, suggesting a specific interaction between type 1 receptor binding and endogenous CORT rhythms (2). Together with the preferential localization of type 1 receptors and mRNA to the hippocampal formation (8–10) and the high-affinity–low-capacity characteristics of type 1 binding in hippocampal cytosol (11), these data have led some to hypothesize a role for this receptor subtype in regulation of circadian HPA rhythms (1, 12).



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In addition to an apparent role in HPA regulation, the hippocampus also exhibits inherent diurnal rhythms. In general, measures of cellular activation (responsivity to perforant path stimulation (13), blood flow (14), and cFOS staining (15)) suggest that hippocampal activation is maximal during the PM (in rats and mice), concurrent with the animal's waking cycle and with elevated CORT levels. Hippocampal long-term potentiation (LTP) has also been shown to be enhanced in the waking period; interestingly, adrenalectomy appears to desynchronize this day–night differential, suggesting steroid involvement in generation of diurnal LTP rhythms and perhaps efficacy of information processing (16). Neurotransmitter second messenger metabolism likewise shows diurnal variance; hippocampal norepinephrine and serotonin appear increased in the AM (17), whereas hippocampal (but not cortical) calcium–calmodulin-dependent adenylate cyclase activity increases in the PM (18). In line with its putative role in circadian HPA entrainment, type 1 glucocorticoid receptor binding also exhibits day–night differences, with PM levels showing a 65% increase over AM in adrenalectomized rats; interestingly, type 2 binding shows no apparent AM–PM differential (12). In all, it is clear that the hippocampus has numerous avenues of interaction with circadian information, which can either be integrated into appropriate modulation of ongoing hippocampal function or used as a reference for regulation of downstream neuronal systems, such as the HPA axis.

The above lines of evidence support the notion that the hippocampus can serve as an integrator for circadian rhythmicity of the HPA axis. Vital to further assessment of hippocampal–HPA interactions is an understanding of mechanisms underlying regulation of hippocampal rhythmicity and rhythmic modulation of hippocampal outflow. In that the hippocampus is in a prime position to integrate diurnal glucocorticoid fluctuations, it is clearly essential to determine whether endogenous adrenal corticosteroid receptors are in a position to directly affect hippocampal function. In the present studies, we focus on diurnal regulation of hippocampal type 1 (or mineralocorticoid (MR)) and type 2 (or glucocorticoid receptor (GR)) mRNAs. As it is generally accepted that mRNA levels reflect the capacity of the cell for production of the respective molecular species, establishing the nature of daily GR and/or MR mRNA rhythms yields valuable clues regarding how the hippocampus can modulate steroid receptivity with respect to time of day.

## METHODS

*Experimental subjects.* Subjects were male Sprague–Dawley rats (150–200 g, Charles River, Kingston, NY). Rats received either adrenalectomy (ADX) or sham adrenalectomy (SHAM) under ketamine/acepromazine anesthesia. ADX rats were maintained on 0.9% saline. Half the rats were housed under standard light conditions (lights on 0500 h to 1900 h) and half on a reverse light–dark cycle (lights on 1900 h to 0900 h). Seven days after ADX or SHAM operation, animals were sacrificed by rapid decapitation at times corresponding to 4-h intervals throughout the day–night cycle (0300, 0700, 1100, 1500, 1900, and 2300 relative to lights on). Trunk blood was collected from all rats for determination of plasma CORT levels and for verification of adrenalectomy; brains were rapidly removed and frozen on powdered dry ice. Brains were stored at −70°C until histological processing.

*In situ hybridization.* Hippocampi and hypothalami were sectioned at 16 μm on a IEC cryostat, thaw mounted onto gelatin-coated slides, and the sections stored at −70°C until processing. Prior to hybridization, all slides were fixed in cold 4% paraformaldehyde (30 min), rinsed in several washes of 2× SSC (1× SSC = 150 m*M* NaCl, 15 m*M* NaCitrate), and incubated in 0.2 μg/ml Proteinase K (Boeringher–Mannheim) at 37°C for 15 min. Sections were then rinsed in $H_2O$ for 1 min, 0.1 *M* triethanolamine (pH 8.0) for 1 min, and acetylated with acetic anhydride (0.25%, in 0.1 *M* triethanolamine) for 10 min at room temperature. Following the acetylation step, sections were rinsed in 2× SSC (5 min) and dehydrated through graded ethanols.

Antisense $^{35}$S-labeled cRNA probes were constructed for: (1) rat GR (456-bp *Xba*I–*Eco*RI fragment, subcloned in pGem3, directed at the 3′ extreme of the protein coding region and the 3′ untranslated (UT) region (courtesy K. Yamamoto, UCSF)); and (2) rat MR (550 bp *Eco*RI–*Hin*dIII fragment subcloned in pGem4, directed at the 3′ extreme of the protein coding region and the 3′ UT region). All probes were produced by *in vitro* transcription. Plasmids containing subcloned cDNAs were linearized with the appropriate 5′-overhang producing restriction enzyme to yield probes of desired length and G:C composition. Standard labeling reactions included 1 μg linearized plasmid, 1X-SP6 transcription buffer (Bethesda Research Labs), 250 μCi α-$^{35}$S-UTP (>1000 Ci/mmol, dried; Amersham), 150 μ*M* ATP, 150 μ*M* CTP, 150 μ*M* GTP, 12.5 m*M* dithiothreitol, 3.0 units/μl RNAsin (Promega) and 0.5 units/μl SP6 or T7 RNA polymerase (Boehringer-Mannheim). The reaction was incubated for 90 min at 37°C, and the labeled probe separated from free nucleotide over a Sephadex G50-50 column equilibrated in 0.1 *M* Tris–HCl, pH 7.5, 12.5 m*M* EDTA, 0.15 *M* NaCl, 0.2% SDS, and 10 m*M* DTT. $^{35}$S-labeled α-UTP was added to the transcription reaction in amounts calculated to yield specific activities estimated at $1.71 \times 10^5$ Ci/mmol for the GR probe, and $1.80 \times 10^5$ Ci/mmol for the MR probe. Note that specificity of these probes has been documented previously, using sense-strand and RNase controls and melting point analysis (9).

$^{35}$S-labeled cRNAs probes were diluted in hybridization buffer (75% formamide, 10% dextran sulfate, 3× SSC, 50 m*M* sodium phosphate buffer, pH 7.4, 1× Denhardt's, 0.1

mg/ml yeast tRNA, and 0.1 mg/ml sheared salmon sperm DNA) to yield 1,000,000 dpm/30 μl. Aliquots of 30 μl were applied to each section, the sections were coverslipped, and the coverslips were sealed with rubber cement. Adjacent hippocampal sections were hybridized with probes complementary to rat MR or rat GR mRNAs; hypothalamic sections (through the PVN) were hybridized with probes complementary to GR mRNA. All slides were incubated overnight at 55°C in sealed plastic boxes containing moistened foam. Coverslips were then removed, the slides rinsed in 2× SSC and immersed in fresh 2× SSC for 20 min. The tissue was treated with RNase A (200 μg/ml) at 37°C for 30 min to degrade any remaining single-stranded cRNA. Sections were then washed successively in 2×, 1×, and 0.2× SSC for 10 min each, followed by a 60-min wash in 0.2× SSC at 65°C. Sections were dehydrated through alcohols and exposed to Kodak XAR X-ray film.

*Image analysis.* Semiquantitative analyses of *in situ* hybridization autoradiographs were conducted utilizing Macintosh-based Image software (courtesy Wayne Rasband, NIH). Sections from experimental and control animals were matched for rostrocaudal level. GR and MR determinations in hippocampus were made from digitized X-ray autoradiographs, with individual subfields delineated on the basis of cytoarchitectonics and known labeling patterns (9). Hypothalamic measurements (GR mRNA) were made from emulsion-dipped autoradiographs to permit clear delineation of medial parvocellular and posterior magnocellular cell groups. Brain-paste standards were processed in parallel to ensure that all signal was in the linear response range of the detection system used (film or emulsion). For hippocampal sections, optical density (OD) measures were corrected for background (via subtraction) and expressed as OD units/mm$^2$. Integrated OD measures were precluded by variability in the overall size of the hippocampal subfields quantified from animal to animal. For hypothalamic sections, OD measures were corrected for background and multiplied by the area sampled, yielding integrated OD units. Mean values for all animals were determined from three to eight sections through the hippocampus and/or hypothalamus and used in the subsequent analysis of group effects. Within-group analyses were performed by one-way ANOVA, followed by post hoc analysis (Duncan's multiple range test). Between-groups analysis was performed by two-way ANOVA, with groups again distinguished by post hoc analysis.

## RESULTS

*Hippocampal MR and GR mRNA distribution.* Figure 1 shows the characteristic pattern of hippocampal MR and GR mRNA distribution. Within the hippocampus proper, MR mRNA exhibits densest hybridization in subfields CA2 and proximal subfield CA3a (CA2–3a) and

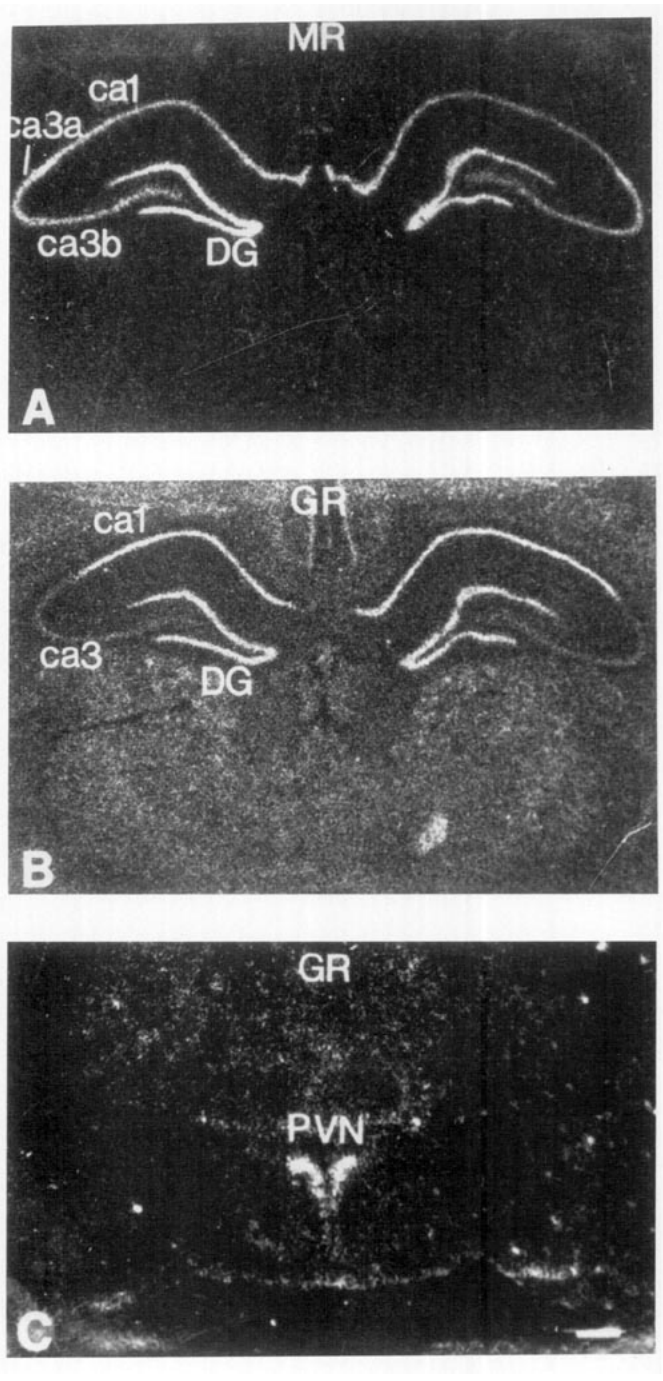


**FIG. 1.** Localization of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNA in hippocampus (A, B) and paraventricular nucleus (PVN) (C). Note the dense hybridization of MR mRNA throughout the hippocampus, particularly enriched in subfield CA2-CA3a and dentate gyrus (DG); substantially lower levels of hybridization are seen in the frontoparietal cortex. In contrast, GR mRNA is present in greatest abundance in CA1 and DG, with CA2-CA4 showing modest hybridization density. GR mRNA can also be appreciated in numerous extrahippocampal areas, including frontoparietal cortex and dorsomedial thalamus. GR mRNA signal can be appreciated in the PVN, as seen in C; note that numerous thalamic nuclei also express GR mRNA at this anatomical level. Magnification bar = 500 μm.

dentate gyrus. CA1, distal CA3 (CA3b), and CA4 also show substantial MR-generated signal. Note that the frontoparietal and cingulate cortex exhibit low levels of MR signal, while thalamic regions are generally negative. GR mRNA is preferentially localized to subfield CA1 and dentate gyrus, with substantially lower levels present in CA2–3a and CA3b and CA4. In contrast with MR mRNA, substantial hybridization signal can be observed for GR mRNA in frontoparietal and cingulate cortices and dorsal thalamic nuclei.

*Diurnal MR rhythms.* Semiquantitative analysis of MR mRNA expression in the hippocampus revealed a consistent picture of fluctuation across the day–night cycle. Statistical analysis of the SHAM data by one-way ANOVA revealed significant effects of time of day on MR mRNA expression in all subfields examined (CA1 (Fig. 2A): $F(5,23) = 3.94$; $P < 0.01$, CA2–CA3a (Fig. 2B): $F(5,23) = 2.81$, $P < 0.05$; CA3b (not shown): $F(5,23) = 3.52$, $P < 0.05$; DG (Fig. 2C): $F(5,23) = 3.61$, $P < 0.05$). The daily rhythms appeared in phase with one another and consisted of bimodal curves, with conspicuous troughs seen at 0300 and 1500 (corresponding to the first points following lights on and lights off, respectively). The MR mRNA rhythm was specific for hippocampus, as no significant diurnal variation was observed in overlying cortex ($F(5,23) = .76$, $P = 0.6$) (Fig. 2D).

Removal of endogenous glucocorticoids by ADX induces a small but significant increase in MR mRNA levels in all subfields examined (significant effects of treatment on MR mRNA levels by two-way ANOVA: CA1 (Fig. 2A): $F(1,51) = 15.00$, $P < 0.01$; CA2-CA3a (Fig. 2B): $F(1,51) = 19.15$, $P < 0.01$; CA3b (not shown: $F(1,51) = 15.87$, $P < 0.01$; DG (Fig. 2C): $F(1,51) = 7.36$, $P < 0.01$). The overall magnitude of increase was greatest in CA2–CA3a and CA3b (36 and 37%, respectively) and least in dentate gyrus (18%). ADX effects were again specific to the hippocampus, as there was no effect of ADX on cortical MR mRNA expression (Fig. 2E) ($F(1,51) = 0.73$, $P = 0.4$). Interestingly, the effects of ADX appeared to vary with time of day: post hoc analysis using Duncan's multiple range test indicate that steroid removal significantly increased MR signal intensity at time points associated with trough periods in the diurnal MR rhythms. When analyzed separately (one-way ANOVA), ADX groups showed no significant diurnal MR mRNA expression (CA1: $F(5,28) = 1.90$, $P < 0.25$; CA2–CA3a: $F(5,28) = 2.09$, $P < 0.25$; DG: $F(5,28) = 2.12$, $P < 0.25$; cortex, $F(5,28) = 1.00$, $P < 0.25$), suggesting that the effects of ADX eliminate the daily rhythm.

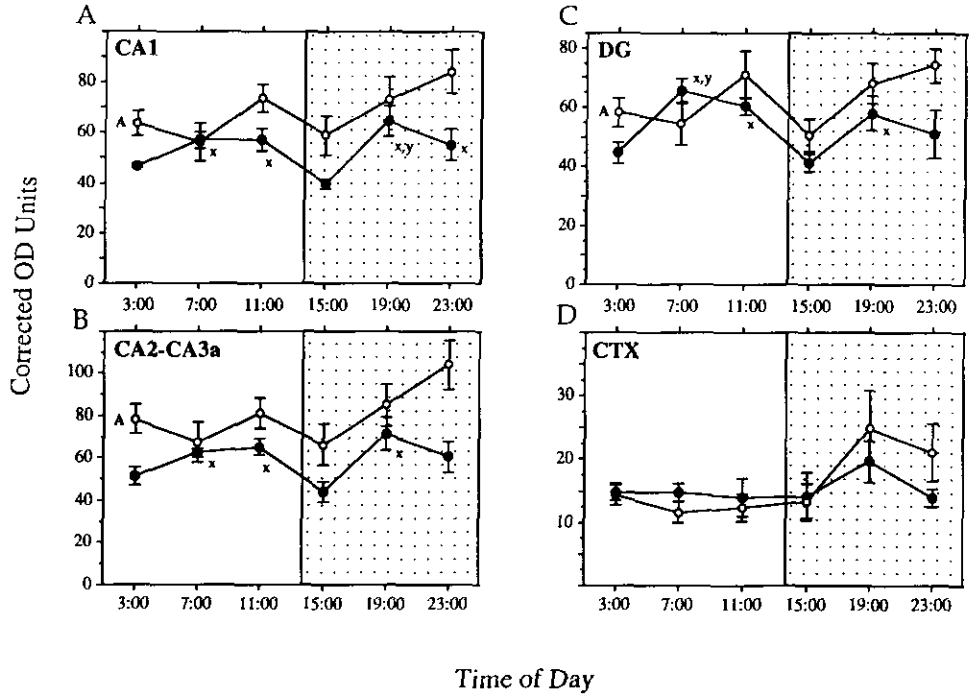


**FIG. 2.** Diurnal regulation of MR mRNA expression in hippocampal subfields and cortex. SHAM data are represented by filled circles, ADX by open circles. Statistical analysis by two-way ANOVA revealed that ADX significantly increased MR mRNA expression in CA1, CA2–CA3a, CA3b (not shown), and DG (denoted by A) across the circadian cycle. No ADX-induced changes were present in frontoparietal cortex. Analysis of the SHAM data by one-way ANOVA demonstrated that circadian MR mRNA rhythms were present in all hippocampal subfields; the rhythms appeared bimodal, with definable troughs at 03:00 and 15:00 h after lights on. Post hoc analysis revealed significant differences between individual points in the rhythm; x's next to individual data points reflect significant increases over the 15:00 time point, y's significant differences from the 03:00 h time point. In contrast to the SHAM data, no rhythm could be observed in the ADX group across the diurnal cycle.

*Diurnal GR rhythms.* Endogenous GR mRNA rhythms displayed a markedly different pattern than those observed for MR mRNA. Analysis of the SHAM data by one-way ANOVA revealed significant effects of time of day on GR mRNA levels only in the DG (Fig. 3C) ($F(5,23) = 2.75$, $P < 0.05$); the main effect of time of day approached significance in CA1 (Fig. 3A) ($F(5,23) = 2.16$, $P = 0.1$). No effects of time of day were seen in CA3 (Fig. 3B) or frontoparietal cortex (Fig. 3D). The diurnal change seen in GR mRNA in DG (as well as the trend seen in CA1) were different in character from those seen for MR mRNA, in that the curves were monotonic, with a single trough occurring at 1500 h (1 h following lights off). In the case of the DG, 1500 h values differed significantly from the 0300, 0700, and 2300 h time points (Duncan's multiple range test, $P < 0.05$), comprising a 40% decrease from peak levels seen at 0300 h. The decrease in CA1 GR mRNA levels from 0300 to 1500 h was more modest (25% reduction).

The effects of ADX on GR mRNA were quite heterogeneous across hippocampal subfields. ADX induced significant increases in GR mRNA levels in all hippocampal subfields (significant effects of treatment on GR mRNA levels by two-way ANOVA: CA1 (Fig. 3A): $F(1,52) = 104.2$, $P < 0.01$; CA3 (Fig. 3B): $F(1,52) = 48.98$, $P < 0.01$; DG (Fig. 3C): $F(1,52) = 18.75$, $P < 0.01$); however, the magnitude of these effects varied from subfield to subfield. In DG, for instance, ADX had the effect of flattening the diurnal rhythm, such that the circadian trough was essentially eliminated (ADX differed significantly from SHAM only at the 1100, 1500, and 1900 h time points, Newman–Keuls test, $P < 0.05$). In the DG, ADX produced, on average, a 30% increase over SHAM levels. In contrast, ADX significantly increased GR mRNA at all time points in CA1 and CA3, increasing GR expression 88 and 81%, respectively. No ADX effects were observed in frontoparietal cortex ($F(1,52) = 0.16$, $P = 0.7$).

Interestingly, analysis of the ADX data taken alone revealed a significant effect of time of day on GR mRNA levels only in CA1 ($F(5,29) = 3.89$, $P < 0.01$); here, the 0300 h was significantly greater than the 0700, 1100, 1900, and 2300 h time points, and the 1500 h time point differed from the 1100 and 1900 h time points. CA3 ($F(5,29 = 1.67$, $P < 0.25$) and DG ($F(5,29) = 1.05$, $P > 0.25$) showed no significant diurnal effects. Again, effects of ADX were specific for hippocampus, as no ADX-induced changes were seen in frontoparietal cortex (Fig. 3D).

Semiquantitative analysis of GR mRNA signal in the PVN and dorsomedial thalamus is illustrated in Fig. 4. In contrast to hippocampus, the parvocellular PVN and dorsomedial thalamus showed no endogenous diurnal rhythm and did not appear to be modulated by ADX.

*Comparison of GR, MR mRNA, and plasma CORT rhythms.* In Fig. 5, the daily diurnal DG MR and GR mRNA rhythms derived from the SHAM groups are superimposed on curves reflecting plasma CORT levels taken from the same animals. Note that the 1500 h trough values in both MR and GR mRNA coincide with the rising phase of the diurnal CORT rhythm. In contrast, the 2300–0300 h trough in MR mRNA corresponds to a period of low circulating CORT levels.

## DISCUSSION

The results of the present study present a complicated picture of GR and MR mRNA regulation in brain. Among all regions examined in the present studies, the hippocampus alone shows pronounced changes in MR and GR mRNAs across the diurnal cycle, consistent with the hypothesis that the hippocampus acts as an integrator of diurnal HPA signals. However, the hippocampus does not act in unitary fashion, but shows significant heterogeneity of GR and to a lesser extent, MR mRNA expression in response to time of day and steroid removal across its component subfields. The data suggest that an interaction between neural drive and steroid regulation of steroid receptor synthesis occurs at the level of the individual hippocampal subfield.

The hippocampal MR (type 1 receptor) represents a high affinity, low capacity receptor subtype which is believed to be extensively bound by steroids even at low circulating levels. The exact extent of occupation has been argued in the literature (3, 19, 20), but it is generally believed to be at least 70% bound even at nadir CORT levels (e.g., <1.0 μg/dl) (19). For this reason, the MR in particular has been hypothesized to be involved in circadian regulation of HPA function and behavioral states, in that binding to this receptor is meaningful only at low CORT levels. Therefore, understanding of circadian MR regulation is particularly important to current hypotheses of HPA regulation. The data presented here present an interesting pattern. Notably, MR mRNA exhibits a significant diurnal rhythm in SHAM animals, whose shape indicates troughs in MR mRNA production at 2300, 0300, and 1500 h relative to lights on. This pattern is generally consistent across all hippocampal subfields. In response to ADX, hippocampal MR mRNA levels show a general increase, with the most pronounced increases seen at times corresponding to the SHAM diurnal troughs (e.g., 0300, 1500, and 2300 h). The magnitude of ADX changes varied with hippocampal subfield, with pyramidal cell layers (CA1, CA2–CA3a, CA3b) showing ADX-induced increases (33–37%) greater than those of granule cell layers of the DG (18%). The net effect of ADX in all subfields appears to be either (1) a flattening of the normal MR rhythm, since no effect of time was seen on MR expression when the ADX groups were analyzed alone, or (2) specific augmentation of MR expression at the 0300 and 2300 h time points, since all subfields showed a trend toward decreased MR mRNA expression at 1500

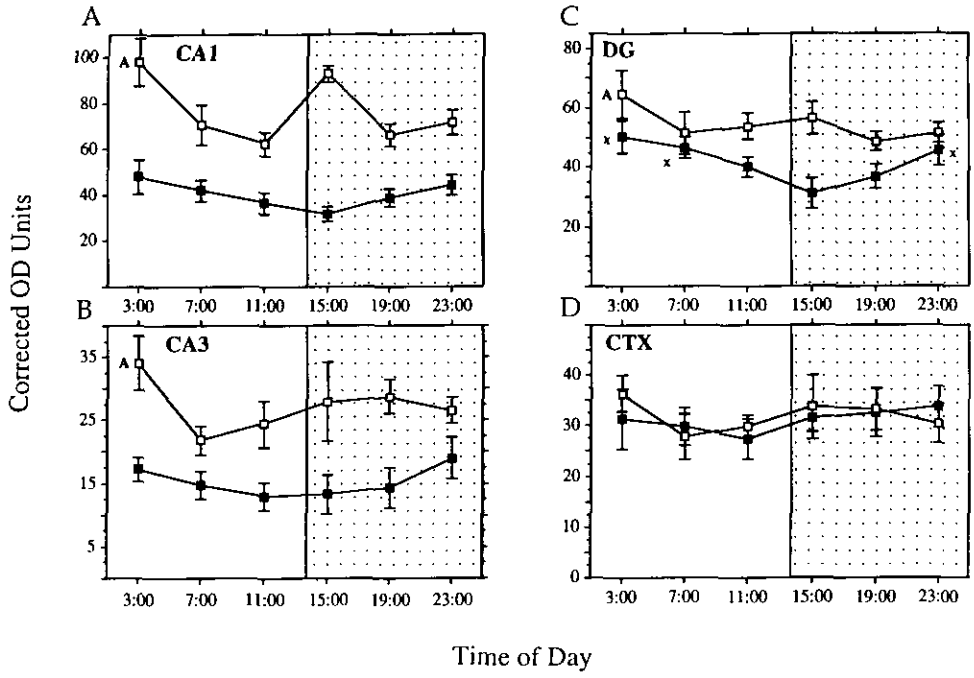


**FIG. 3.** Diurnal regulation of GR mRNA expression in hippocampal subfields and cortex. SHAM data are represented by filled squares, ADX by open squares. GR mRNA exhibits significant up-regulation in all hippocampal subfields in response to ADX, whereas no ADX changes were seen in cortex. The SHAM data analyzed alone revealed a significant effect of time of day on GR mRNA expression in the DG only, where GR message appears to exhibit a unimodal rhythm with a trough around the time of lights off (15:00 *h*) (x's denote points which differ reliably from the 15:00 h values). A similar pattern was seen in CA1; however, statistical analysis could not definitively verify this effect. Analysis of the ADX groups alone showed significant circadian fluctuations only in CA1; in this region, levels of GR mRNA expression were *increased* at the 15:00 and 03:00 h time points. Note in addition that the effects of ADX differed qualitatively in different regions; whereas ADX induced cycle-wide increases in GR mRNA levels in CA1 and CA3, in DG ADX only eliminated the circadian trough.

h. In all cases, effects of time of day and ADX on MR mRNA were only seen in the hippocampus (no parallel changes could be detected in frontoparietal cortex).

In all, the data appear to suggest that steroids are involved in entraining the diurnal rhythm in MR mRNA expression. In the absence of steroids, diurnal MR mRNA expression displays a flat profile, with the mean level of MR expression approximating that of peak daily values in SHAM rats (e.g., CA1, mean value in ADX = 69.2 vs daily high for SHAM = 64.7 (1900 h); CA2–CA3a, 82.0 vs 71.6 (1900 h); CA3b, 57.3 vs 53.7 (1900 h); DG, 63.8 vs 65.4 (0700 h)). The similarity between ADX and peak daily values indicates an interaction between circulating steroids and MR mRNA expression which is manifest as effective reductions in MR synthesis at two particular periods in the diurnal cycle, that around the time of lights off and that around the time of lights on. Comparison between MR mRNA levels and circulating CORT values (derived from the same animals) allow for some tentative interpretations of these data. It is notable that the trough in MR expression occurs at a point in the diurnal cycle (1500 h) when circulating steroids are rising. The ability of ADX to prevent this trough may indicate either that increased (or increasing) steroid levels act at the hippocampal MR gene to inhibit MR mRNA synthesis at these time points, or that steroids act at a distance to modulate activity of neurons synapsing on hippocampal neurons. The former explanation appears more attractive at present, since the hippocampal MR changes appear to be parallel across subfields receiving different classes of afferent input (CA1 vs CA3 vs DG).

The sharp drop in MR mRNA expression seen at 1500 h, corresponding to increased or increasing plasma CORT levels, renders it tempting to speculate that this MR trough represents type 2 receptor regulation of type 1 synthesis. This notion is derived from the fact that type 2 occupation will be increasing over this particular time period, whereas type 1 receptors should be extensively occupied. However, it should be noted that in the absence of knowledge of MR mRNA half-life, it is impossible to tell when transcription and/or RNA half-life is being influenced by steroid, i.e., it is unknown whether changes are being initiated at 0300 h (when type 1 binding is most relevant) or 1100 h (when type 2 binding has a predominant influence). The ability of MR mRNA to exhibit a precipitous drop and recovery over an 8-h period would suggest that the message half-life is indeed quite short (a maximum of 6.5 h, assuming a zero-order decay), and therefore that the observed decreases are occurring during a period in which type 2 binding is rapidly changing.

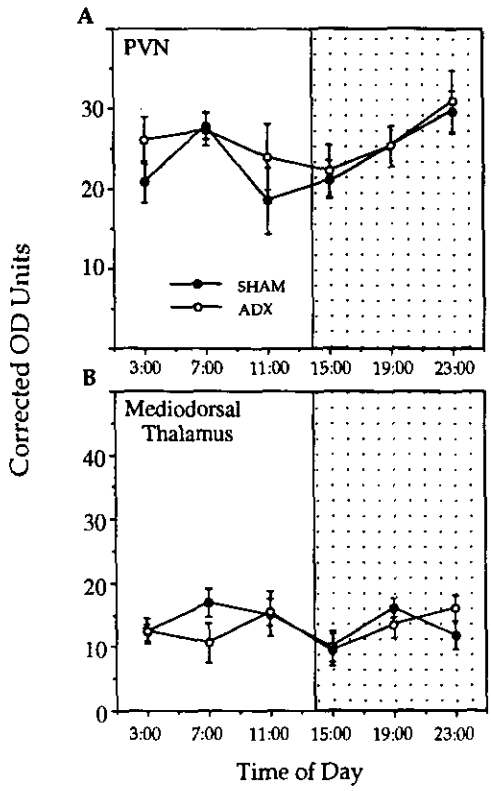


**FIG. 4.** Diurnal regulation of GR mRNA expression in paraventricular nucleus (PVN) and dorsomedial thalamus. SHAM data are represented by filled circles, ADX by open circles. In contrast to the hippocampal data, no effect of either ADX or time of day on GR mRNA expression could be observed in these extrahippocampal regions.

The second MR mRNA trough, occurring around the time of lights on (2300–0300 h), also deserves comment. This trough is consistent across subfields and is for the most part eliminated by ADX, suggesting steroid interactions with the rhythm at this point as well. What stands out about these particular time points is the relationship to the daily CORT cycle, in that this trough corresponds to periods of decreasing circulating CORT levels. Binding of CORT to the type 1 receptor may account for these observations, in that CORT levels may be low enough at these points to effectively see this receptor species. However, if this is the case, it is difficult to explain, in terms of steroid binding alone, how the diurnal "peaks" are generated. If type 1 binding were involved in generation of the trough, clearly the effectiveness of this regulation would have to be counteracted by synaptic input at the diurnal peaks. Alternatively, the 2300–0300 h trough may be neuronally generated, perhaps via steroid-sensitive circadian-entrained inputs to the hippocampus, to adjust the abundance of the type 1 receptor to biologically meaningful levels at these particular points in time.

In contrast with MR mRNA, regulation of GR mRNA expression is heterogeneous across hippocampal subfields, both with respect to diurnal rhythms and steroid depletion. GR mRNA exhibits a significant circadian rhythm only in DG, although there is a trend exhibited in CA1. Unlike the MR rhythm, the diurnal curve is monotonic: GR mRNA steadily decreases from peak levels at 0300 h to a trough at 1500 h, at which point levels begin rising and return to peak levels by 2300 h. In the DG, the development of this trough is completely prevented by ADX, suggesting that the steady decrease seen over the 0700–1500 h period is mediated directly or indirectly by circulating steroids. Indeed, in this respect GR mRNA regulation in the DG is quite similar to MR mRNA regulation, in that the effect of steroid removal is an elimination of rhythmicity, maintaining GR expression at peak diurnal levels.

There is substantial evidence to suggest that GR biosynthesis is subject to downregulation by interactions with the GR protein (21, 22), presumably at the genomic level (23, 24). The circadian data presented here suggest that

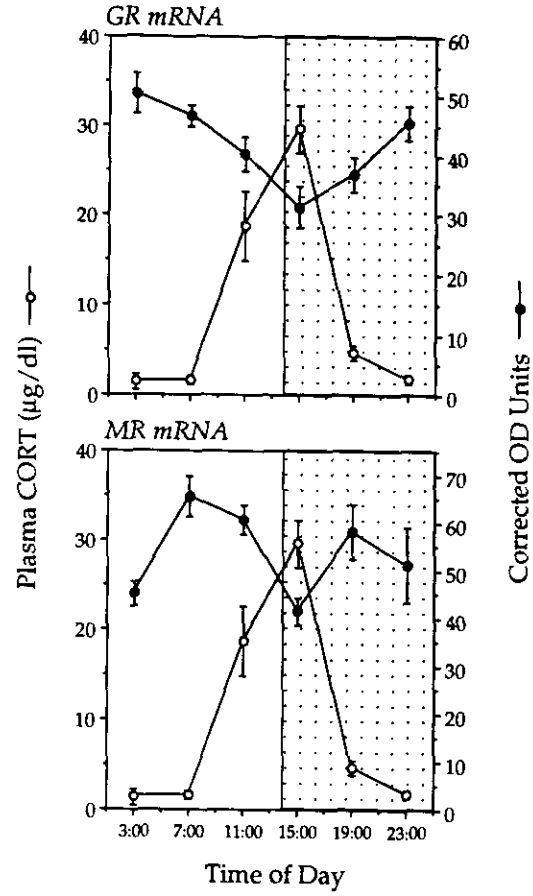


**FIG. 5.** Relationship of MR and GR mRNA rhythms in dentate gyrus to circadian CORT levels in the same experimental subjects. Plasma CORT data are represented by open circles, mRNA data by closed circles. Data are taken from SHAM rats only. Note that the trough seen at 15:00 h in both MR and GR mRNA rhythms appears to correspond roughly to the rising phase of plasma CORT, suggesting a possible regulation of these receptor mRNAs by CORT across the diurnal cycle.

at least in the DG, GR mRNA production may be subject to direct regulation by corticosterone. As was the case for MR mRNA, the lack of knowledge of *in vivo* half-life prohibits clear attribution of GR downregulation to type 1 or type 2 binding.

The results of DG GR mRNA regulation stand in stark contrast to changes seen in other hippocampal subfields. In CA1 and CA3, ADX induces a marked (81–88%) increase in GR mRNA production which is evident at all points in the circadian cycle. In CA3, this increase represents a "shift" of an arhythmic curve to a higher level of GR mRNA expression. In CA1, ADX effects are more complicated; indeed, not only does ADX increase GR mRNA levels at all points across the day–night cycle, but it also produces a diurnal rhythm distinct from that seen in SHAM animals. In particular, high levels of GR mRNA are seen at 0300 and 1500 h, both of which are statistically differentiable from surrounding time points. Thus, steroids are involved in determination of the amplitude of GR mRNA expression in CA1 and CA3, rather than merely entraining the diurnal rhythm. Moreover, in the case of CA1, ADX apparently unmasks a drive of GR production which is normally tightly regulated by steroids, and suggests that in this subfield GR regulation is subject to multifaceted influences from both humoral and neuronal sources.

The apparent heterogeneity seen in the responses of individual hippocampal subfields to circadian influences and steroids illustrates the complexity of hippocampal GR and MR regulation. However, it is striking to see such consistent trends toward lower GR and MR expression at the same time point. Therefore, the possibility that such decreases may be a technical artifact associated with differential tissue integrity/preservation/etc. must be discounted. First, it is notable that neither diurnal nor ADX-induced changes can be observed in cortex or thalamus (in the case of GR). These data both verify the confinement of effects to the hippocampus and render it unlikely that differences are associated with tissue artifact. Second, alternate series for the same animals were processed for $\beta$-adrenoreceptor autoradiography (25) and *in situ* hybridization analysis of GAP-43 mRNA expression (26). In neither case were marked decrements in binding/hybridization intensity observed at the 1500 h time point, indicating further that tissue artifact is unlikely to generate the pattern of results seen in these studies.

The observed changes in MR and GR mRNA in response to steroids agree with our previous report, in which we show significant ADX-induced changes in GR mRNA in CA1 and DG and MR mRNA in CA1 in animals sacrificed at 1000 AM–1200 PM (4–6 h after lights on) (9). Interestingly, the lack of significant changes in CA3 GR mRNA in our previous report (9) can be explained by the apparent low levels of GR message content in CA3 of ADX rats at a comparable time point (700 h). Similarly, the 700 h time point appears to be quite poor in distinguishing among ADX and control groups in the present study, again explaining to some degree the small MR mRNA changes observed previously (9).

Unlike the hippocampus, no effects of either time of day or steroid depetion were seen on MR mRNA levels in the frontoparietal cortex or GR mRNA levels in frontoparietal cortex, dorsomedial thalamus, arcuate nucleus, or PVN. These data suggest that hippocampal corticosteroid receptors are distinctly and specifically receptive to information concerning time of day and/or steroid levels, and imply that modulation of steroid receptors in hippocampus is an active process with probable physiological implications. Physiological consequences of diurnal changes in steroid receptor number may be related to anything from regulation of subsequent corticosteroid secretion to modulation of the efficiency of information processing; additional experiments are clearly required to fully characterize the significance of the present findings.

It should be noted that the present study analyzed only a few of the many regions containing MR and GR mRNA. It remains to be determined whether other brain regions show diurnal and ADX effects on adrenal corticosteroid receptor mRNAs, or if such effects are indeed confined to the hippocampus. In addition, the lack of effect of ADX on MR and GR mRNAs in extrahippocampal regions suggests that synthesis of these receptors in brain is subject to modulation at many levels, of which direct regulation by glucocorticoid hormones is but one.

Striking with respect to HPA regulation is the glaring lack of diurnal rhythms of GR mRNA in the PVN. These data replicate the findings of Kwak *et al.*, which show no diurnal variation of GR or MR mRNA in PVN microdissections by RNase protection analysis (27). The PVN is known to be central to integration of ACTH release (28), and the CRH/AVP containing neurons resident within it apparently react to local glucocorticoids to limit ACTH secretagogue release and synthesis. These neurons also show endogenous rhythmicity of CRH mRNA production and possibly release (27–31). The apparent lack of GR mRNA changes in the PVN, both across the diurnal cycle and following ADX, suggest that GR levels are maintained despite ongoing changes in circulating steroid levels. Given the stability of the GR and MR mRNAs in the face of changes in CRH mRNA synthesis, it is unlikely that the PVN directly integrates circadian HPA rhythms via endogenous receptors. Indeed, the marked diurnal fluctuations in hippocampus suggest that hippocampal circuits may be more central to basal HPA regulation, as suggested by several previous reports. The role of the stable population of PVN GRs may be more related to stress regulation. It may well be essential for the organism to maintain a full complement of PVN corticosteroid receptors to provide a mechanism for efficient negative feedback inhibition of stress responses at the level of the PVN at all points in the diurnal cycle.

It should be noted that while the mRNA data point toward meaningful changes in MR and GR expression across the circadian cycle, several points bear consideration. First, mRNA predicts, but does not reflect, changes in protein levels. For example, mRNA is subject to post-transcriptional regulation; changes in mRNA content may be exaggerated or diminished by factors influencing half-life and degradation. Second, mRNA does not address receptor binding and translocation of bound receptors to the cell nucleus, which are dependent on ligand concentrations. Finally, there are multiple forms of MR mRNA in hippocampus, differing in their 5′ untranslated domains, all of which are loaded on ribosomes (32). Thus, there is a potential for additional levels of MR regulation by translation of different 5′ sequences; the functional significance of these different MR variants is unknown at this time. In all, while mRNA provides an estimate of meaningful changes in GR and MR biosynthesis across the diurnal cycle, the physiological impact of these changes remains to be defined.

The implications of the observed diurnal data for regulation of the HPA axis are presently unclear. Both MR and GR mRNAs exhibit circadian patterns of expression which are at least partially under the control of circulating steroid levels. In this respect, it is likely that steroids play at least some role in modulating the effectiveness of steroid binding at the level of the hippocampal neuron, and are therefore involved in regulating any of a number of classes of hippocampal rhythmic activity, including neuronal excitability, learning, blood flow, and HPA activation, in tune to circulating CORT levels. The possibility of circadian HPA regulation is especially interesting with regard to the literature on hippocampal–HPA interactions, which generally indicate desynchronization of HPA rhythms upon hippocampal damage or disconnection (4–6). Further research will determine whether the hippocampus occupies a central position in modulation of the body's glucocorticoid milieu across the day–night cycle.

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