Localization and Regulation of Glucocorticoid and Mineralocorticoid Receptor Messenger RNAs in the Hippocampal Formation of the Rat

James P. Herman, Paresh D. Patel, Huda Akil, and Stanley J. Watson

Mental Health Research Institute
Ann Arbor, Michigan 48109

Messenger RNAs coding for glucocorticoid (GR) and mineralocorticoid (MR) receptor proteins were localized to discrete subfields of the hippocampal formation by *in situ* hybridization histochemistry, using cRNA probes of approximately equivalent specific activity. Both GR and MR mRNAs were present in all subfields examined; GR mRNA was of greatest abundance in CA1, while MR mRNA was most densely labeled in CA3. In all subfields examined, MR mRNA was considerably more abundant than GR mRNA. Removal of circulating glucocorticoids by adrenalectomy precipitated an up-regulation of GR mRNA in subfields CA1–2 and the dentate gyrus, which was reversed by dexamethasone replacement. High doses of dexamethasone significantly down-regulated GR mRNA in CA3. In contrast, adenalectomy produced significant up-regulation of MR mRNA only in subfield CA1–2. The data indicate that steroid receptor mRNAs are differentially distributed in hippocampus, and that sensitivity to steroids occurs within defined structural domains of the hippocampal formation. (Molecular Endocrinology 3: 1886–1894, 1989)

INTRODUCTION

The hippocampus has long been considered to play a role in feedback regulation of the hypothalamo-pituitary-adrenocortical (HPA) axis (1–6). Recent data from our laboratory has revealed that hippocampal removal elicits a pronounced up-regulation of CRF and, to some extent, vasopressin mRNAs in hypophysiotropic neurons of the hypothalamic paraventricular nucleus, confirming an inhibitory role of the hippocampus on HPA function (6). The notion that the hippocampus may be a site of steroid feedback interactions is supported by findings demonstrating that this structure contains the highest number of glucocorticoid-concentrating neurons in the rat brain (7, 8). Recent studies have defined at least two glucocorticoid receptor (GR) subtypes which differ in affinity for corticosterone (B) (9–11). The classical (or type 2) GR binds both B and dexamethasone, while the type 1 receptor binds B but not dexamethasone and strongly resembles the kidney mineralocorticoid receptor (MR) in its physicochemical properties (9–11). Evidence from binding studies indicate that hippocampal type 1 (or mineralocorticoid-like) receptors bind B with a higher affinity than type 2 receptors and are more extensively occupied at physiological levels of B (11). These data have lead some to postulate different functions for the two hippocampal receptors in HPA regulation, with the type 1 receptors putatively involved in circadian rhythmicity, and the GR involved in inhibition of the stress response (11, 12).

Receptor autoradiographic (10) studies have shown that type 1 and type 2 binding is differentially distributed within the rat hippocampal formation. Immunohistochemical data further demonstrate a gradient of GR protein across hippocampal subregions, which agrees well with the binding data (13). Heterogeneity of type 1 and type 2 binding and GR localization is paralleled by binding data indicating a greater sensitivity of receptors localized to subfield CA3 to high levels of glucocorticoids and aging (14, 15), suggesting a functional as well as anatomical differentiation of type 1 and type 2 receptors in hippocampus. However, it remains to be determined whether regional differences in receptor localization are intimately associated with subfield-specific glucocorticoid negative feedback regulation of the HPA axis.

While glucocorticoid receptor binding and immunohistochemistry provide valuable clues regarding function and regulation of type 1 and type 2 GR proteins in hippocampus, both procedures have methodological limits that somewhat compromise their ability to accurately assess these processes. Receptor binding studies require removal of endogenous steroids (via adrenalectomy) before quantitation, which changes the basal state of the HPA axis and may influence type 1 and type 2 regulatory processes. Immunohistochemical analysis does not require adrenalectomy, but is not

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readily amenable to quantitation. The ability to study hippocampal steroid receptors by immunohistochemistry is also limited by the unavailability of quality antibodies to the type 1 receptor. Recently, it has become possible to use *in situ* hybridization methods to assess mRNA levels at the cellular level. Levels of mRNA coding for a given protein can be inferred to represent the state of activation of the cellular machinery synthesizing this protein. In that this technique allows receptor mRNAs to be detected in intact (*i.e.* nonadrenalectomized) animals in an anatomical context and allows results to be meaningfully quantified, *in situ* hybridization histochemistry represents a valuable new method for addressing issues of type 1 and type 2 GR localization and regulation in the brain.

Recently, our laboratory has isolated a cDNA clone for the MR from a rat hippocampal library (16). This clone has over 90% nucleotide homology with the MR isolated from kidney by Arriza and colleagues (17), leading us to postulate that the species encoded by the hippocampal MR mRNA accounts for most, if not all, type 1 binding in this structure. We have used this clone and a GR cDNA clone, generously provided by K. Yamamoto, to synthesize high specific activity cRNA probes for detailed *in situ* hybridization analysis of MR and GR mRNA expression in hippocampus. The efficacy of the *in situ* hybridization method for selective localization of MR and GR mRNAs in rat brain has recently been demonstrated (18–20). Our results further characterize regional distribution of MR and GR mRNA within the hippocampus, compare relative amounts of MR and GR mRNAs within defined hippocampal subfields, and characterize glucocorticoid regulation of these two receptor mRNAs.

RESULTS AND DISCUSSION

Controls and Verification of Adrenalectomy

The characteristics of MR and GR cRNA probes used in our semiquantitative analyses of localization and regulation of respective GR mRNAs are summarized in Table 1. It is important to note that the probes were closely matched for specific activity and C-G content and contained no regions of significant nucleotide homology. In addition, both probes were directed against similar regions of the MR and GR mRNA molecules (3′ untranslated region, located 5′ to the first potential polyadenylation site). The specific hybridization of MR and GR cRNA probes to complementary mRNAs in tissue sections was confirmed by utilization of sense-strand RNA probes and analysis of thermal dissociation characteristics of cRNA-mRNA hybrids in the presence of triethylammonium chloride (which destabilizes RNA-RNA hybrids, lowering melting temperature (Tm) by approximately 30 C) (21). No positive hybridization signal was observed in sections hybridized with sense-strand GR and MR RNA probes (Fig. 1). Complete loss of signal was observed at high temperature (60 C) in the presence of triethylammonium chloride. The empirical melting temperatures of MR and GR cRNA:mRNA hybrids (MR, 52 C; GR, 45 C) are consistent with theoretical Tm values calculated for hybrids of corresponding C-G content, length, and percent basepair mismatch (22) (in this case assumed to be 0%) in the presence of triethylammonium chloride.

Table 1. Design of Glucocorticoid and Mineralocorticoid cRNA Probes

	MR probe	GR probe
Length (nt)	347	456
No. of UTPs	132	137
C-G Content (%)	38.9	39.9
SA ($\times 10^4$ Ci/mmol)	4.13	4.28

GR cRNA probe was directed against hormone-binding domain, 3′ untranslated region. MR cRNA probe was directed against 3′ untranslated region. Homology between probes was less than 43%; no stretch had more than 10 successive nucleotides with 100% homology.

Glucocorticoid regulation of hippocampal MR and GR mRNA was assessed by removal of the adrenal glands (ADX), ADX and subsequent replacement with the type 2 GR ligand dexamethasone (ADX/DEX), and administration of large doses of dexamethasone to normal rats (DEX). To verify the efficacy of adrenalectomy, plasma B levels were determined for each experimental group. No detectable levels of B were present in plasma from ADX or ADX/DEX rats. B levels were severely depressed in DEX rats (0.17 ± 0.13 μg/dl), consistent with down-regulation of B release by the high dose of dexamethasone. The mean plasma B level for sham-operated rats was 2.15 ± 0.84 μg/dl.

Localization and Relative Abundance of GR and MR mRNAs in Hippocampal Subfields

The present results demonstrate that MR and GR mRNAs can reliably be localized to individual subfields of the hippocampus by *in situ* hybridization histochemistry. GR mRNA was localized to all subfields of the hippocampal formation examined and in the dentate gyrus (DG). Hippocampal subfields were defined in accordance with the sterotoxic atlas of Paxinos and Watson (23). Signal intensity was greatest over subfield CA1–2, of intermediate intensity over the DG, and sparsest over subfields CA3-CA4 (Fig. 2A). Quantitation of signal revealed a significant effect of subfield on hybridization density across the hippocampal formation by one-way analysis of variance [ANOVA; $F(2,114) = 17.32$; $P = 0.001$], highlighted by significant differences in disintegrations detected per mm^2 between subfield CA1–2 and subfield CA3 and the DG, and between DG and subfield CA3 (all comparisons by Newman-Keuls test, $P < 0.05$; Fig. 2B). Precise definition of subfield CA4 was not permitted by our autoradiographs due to scatter of cells in the hilus of the dentate gyrus.

MR mRNA was also detected in all subfields examined (Fig. 3A). In contrast to GR mRNA, MR mRNA

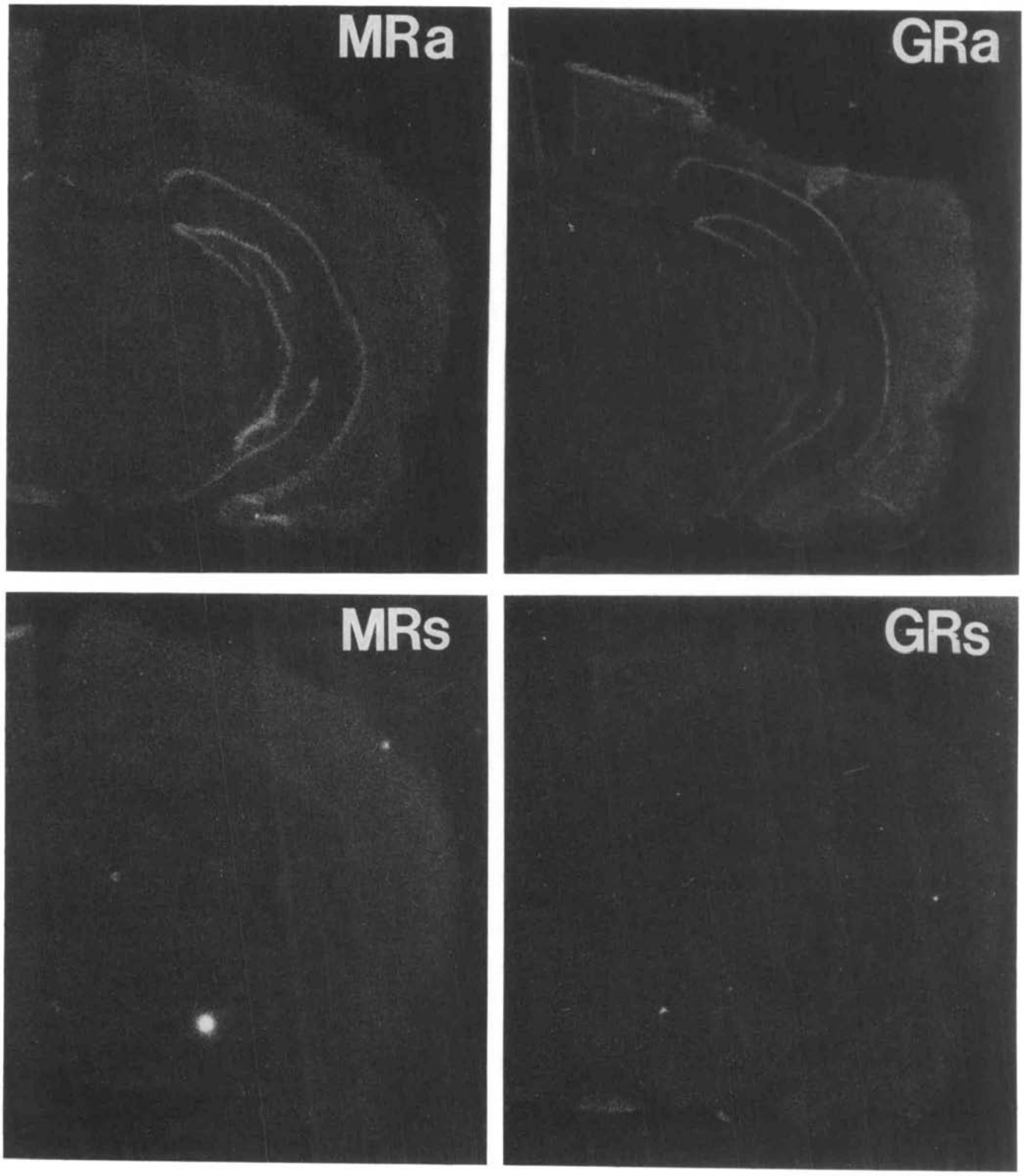
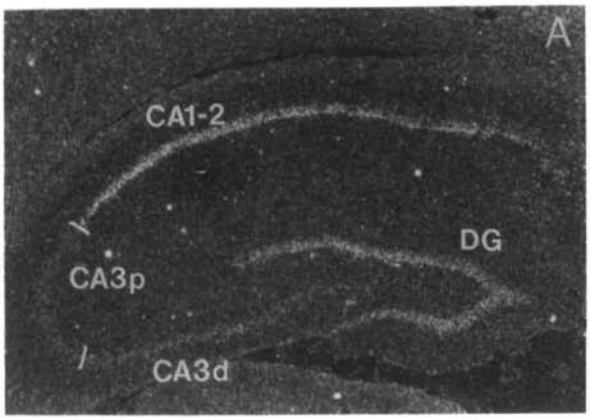


Fig. 1. Sections through the Caudal Hippocampus of a Normal Rat, Incubated with Antisense cRNA Probes Complimentary to MR (MRa) and GR (GRa) mRNAs, and Message-Sense RNA Probes for MR (MRs) and GR (GRs) mRNAs

Positive signal can be observed in the hippocampal formation and cortex using MRa and GRa probes. No signal is observed in either region for the MRs or GRs (sense-strand) probes, illustrating that radiolabeled probes of the same length and C-G content as the antisense probes do not result in positive signal.



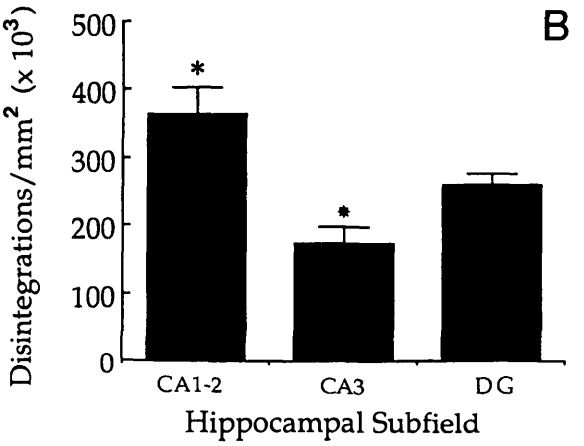
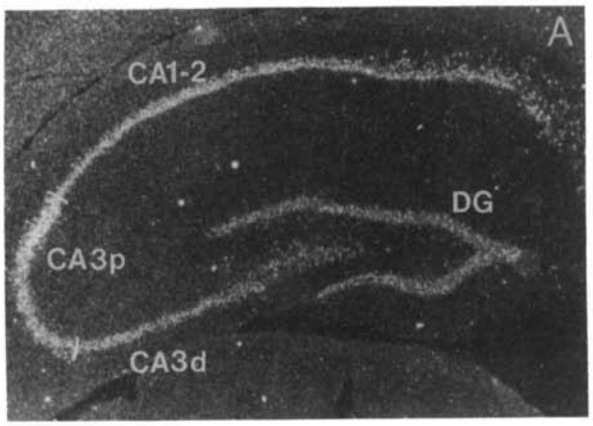


Fig. 2. Localization and Abundance of GR mRNA in Hippocampal Subfields

A, Photomicrograph illustrating distribution of GR mRNA in the dorsal hippocampus. The densest hybridization signal is observed in CA1–2, with considerably lesser signal in CA3prox(p), CA3dis(d) (see below and text for differentiation of these regions), and CA4. The dentate gyrus (DG) signal is intermediate in intensity. B, Semiquantitative analysis of digitized images through the hippocampus of normal rats, illustrating the distribution of GR mRNA in different hippocampal regions. There was a significant effect of subfield on GR mRNA content by one-way ANOVA (see text). Values labeled on the *ordinate* axis should be multiplied by 1000 to yield true disintegrations per mm^2. Subfield CA1–2 contains significantly higher levels of GR mRNA than did either CA3 or the DG ($P <$ 0.05, by Newman-Keuls test). Hybridization signal in CA2–3 was significantly less than that in the dentate gyrus (*, $P <$ 0.05).



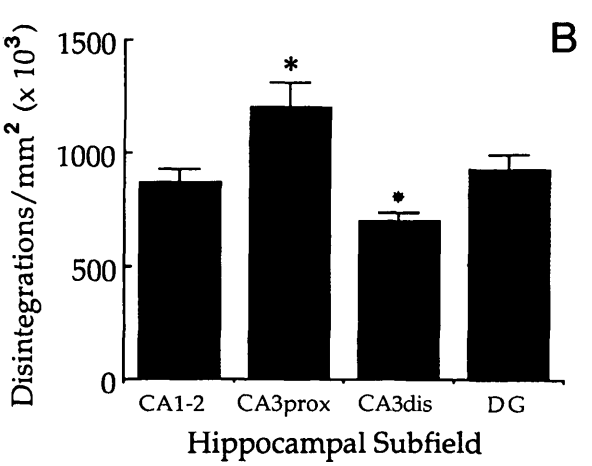


Fig. 3. Localization and Abundance of MR mRNA in Hippocampal Subfields

A, Photomicrograph illustrating distribution of MR mRNA in the dorsal hippocampus. The region of CA3 proximal to the pyramidal cells of CA1–2 (CA3prox) shows the densest hybridization, followed by CA1–2 and DG, which are approximately equal in intensity. Positive signal is weakest in the region of CA3 (CA3dis; see text) distal to CA1–2 (CA3d). This section is adjacent to that shown in Fig. 2A; comparison between the two shows marked differences in GR and MR mRNA localization in subfield CA3prox (CA3p), and more intense labeling of MR mRNA in all subfields. See Table 2 for comparison. B, Semiquantitative analysis of digitized images through the hippocampal formation of normal rats, illustrating the distribution of MR rnRNA in different hippocampal regions. There was a significant effect of subfield on MR mRNA content by one-way ANOVA (see text). In contrast to GR mRNA, MR mRNA was densest in CA3prox, differing significantly from all other groups (*, $P < 0.05$, Newman-Keuls test). Subfields CA1–2 and the DG exhibited similar density, while the signal subfield CA3dis was the weakest, being significantly lower that that of CA3prox and DG ($P < 0.05$).

exhibited heaviest hybridization over hippocampal subfield CA3, particularly in that portion of CA3 immediately proximal to the CA2-CA3 junction [CA3prox]. Hybridization density decreases abruptly at the point where subfield CA3 is oriented parallel to the proximal dorsal and ventral blades of the DG [herein referred to as distal CA3 (CA3dis)]. The localization of intense and relatively weak hybridization signal within subfield CA3 roughly obeys the division of CA3 into CA3a-b and CA3c described by Lorente de No (24). In contrast, CA1-2 and the DG exhibited intermediate levels of positive signal which were consistent across the subfields. Quantitation of signal resulted in a significant effect of subfield on hybridization density (disintegrations detected per mm^2) by one-way ANOVA [$F(3,174) = 8.32$; $P < 0.001$]. Subfield CA3prox differed significantly from all subfields, and subfield (CA3dis) from the DG (all comparisons by Newman-Keuls test, $P < 0.05$; Fig. 3B).

Localization of the two receptor mRNAs differs sub-

stantially across hippocampal subfields, with MR mRNA distributed according to the gradient CA3prox > CA1–2 = DG > CA3dis, and GR mRNA distributed according to the gradient CA1–2 > DG > CA3. In general, the hippocampal MR and GR mRNA gradients across subfield agree well with those described previously by others (18–20). Our quantitative data further indicate a marked prevalence of MR mRNA in that region of CA3 (CA3prox) immediately proximal to the CA2-CA3 border, representing an apparent heterogeneity of MR rnRNA production within CA3 proper. The subfield differences we and others have observed in hippocampal MR and GR mRNA levels suggest that the two receptors are regulated independently in the hippocampus and lend further support to the hypothesis that they are functionally heterogeneous receptor species.

With some notable exceptions, the distribution of MR and GR mRNAs roughly parallel results from receptor binding experiments using *in vitro* labeling of brain sections with type 1- and type 2-selective ligands, respectively, and with immunohistochemistry using monoclonal antibodies directed against the GR. Receptor autoradiographic data agree quite well with the mRNA data described above regarding type 1 receptor distribution, finding a gradient suggesting CA1 > DG > CA3. Unfortunately, CA3prox was not resolved in the receptor binding studies (10). In agreement with previous protein localization by immunohistochemistry (13), GR mRNA is most abundant in CA1, intermediately abundant in DG, and least prevalent in CA3. However, results do not completely agree with the receptor autoradiography data of Reul and de Kloet (10), which find lowest type 2 receptor binding in CA1. This discrepancy may represent a real subfield-specific difference in message translatability, a posttranslation modification of the receptor protein, or perhaps more rapid turnover of receptor protein in this particular subfield. However, the possibility that methodological factors associated with the respective protocols contribute to observed differences cannot be presently ruled out.

MR/GR mRNA Ratios in Hippocampus

Comparison of adjacent sections through the hippocampus hybridized with GR and MR cRNA probes (for example, compare Figs. 2A and 3A) suggests that hippocampal MR mRNA is of higher abundance than GR message. Since probes were labeled to roughly the same specific activity, it was possible to make semiquantitative comparisons between the relative amounts of GR *vs.* MR mRNA in individual subfields of the hippocampus. Results of quantitative analysis of MR- and GR mRNA-generated signals from digitized autoradiographs are shown in Table 2. In all subfields, detected levels of MR mRNA were more abundant than detected levels of GR mRNA, with differences most pronounced in subfield CA3 and least pronounced in CA1–2.

Although matched for specific activity, the shorter length of the MR cRNA probe raised the possibility that the high MR/GR mRNA ratio may be due in part to superior tissue penetration of the shorter MR cRNA probe. Therefore, additional experiments were performed using a MR cRNA probe, also directed against the 3′ untranslated region, which was 94 nucleotides longer [550 nucleotides (nt)] than the GR cRNA probe (456 nt). Again, adjacent sections were hybridized with MR and GR cRNA probes. For calculation of MR/GR mRNA ratios, the MR signal intensity was corrected for the difference in specific activity of the two probes. As seen in Table 2, it is evident that both the 550-nt MR cRNA probe and the 347-nt MR cRNA probe yielded similar MR/GR ratios. The observed MR/GR ratio, thus, appears to be independent of the MR cRNA probe lengths employed in this study.

Table 2. MR/GR Ratios in Hippocampus

Region	MR Probe Characteristics	MR/GR ratio
CA1-2	347 nt, 3′UT	1.5:1
	550 nt, 3′UT, coding	1.7:1
CA3prox	347 nt, 3′UT	5.0:1
	550 nt, 3′UT, coding	2.5:1
CA3dis	347 nt, 3′UT	2.5:1
	550 nt, 3′UT, coding	2.7:1
DG	347 nt, 3′UT	2.2:1
	550 nt, 3′UT, coding	1.7:1

All MR/GR ratios were calculated relative to the 456-nt cRNA probe used for GR *in situ* hybridization. Ratios were based on optical density measures over relevant hippocampal subfields from digitized images of *in situ* hybridization autoradiographs. MR and GR data were corrected for differences in specific activity before calculation of MR/GR ratios. UT, Untranslated region.

Higher levels of MR than GR mRNA in hippocampus are further confirmed by RNAse protection experiments performed in our laboratory, using RNA extracted from whole hippocampal homogenates (Patel, P. D., and S. J. Watson, unpublished observations). The 2.2:1 MR/GR mRNA ratio agrees well with those calculated for individual subfields by *in situ* hybridization histochemical analysis (see Table 2).

The observed MR/GR ratios imply a greater rate of synthesis of MR than GR message in the rat hippocampus. Data from binding studies seems to indicate the converse, *i.e.* a greater concentration of type 2 (*i.e.* glucocorticoid) than type 1 (*i.e.* mineralocorticoid-like) receptors in hippocampus (12). Coupled with the observed higher affinity of MR than GR for the endogenous glucocorticoid B (12), higher MR mRNA and lower type 1 binding relative to GR may reflect a faster turnover rate of hippocampal MRs. This hypothesis is derived from the assumption that in order for the hippocampal MR species to have greater amounts of mRNA, a lesser amount of detectable receptors, and a higher affinity than the GR, the cycle of synthesis/expression/binding of this receptor subtype must be accelerated relative to that of the GR. Such an accel-

eration would imply that the biological signal carried by the MR is reported more frequently than GR-mediated messages to relevant cellular sectors.

Regulation of Hippocampal GA and MR mRNAs

Hippocampal GR mRNA appears to be regulated by steroids in a site-specific manner. Effects of steroid manipulations on detected message in individual subfields were analyzed by one-way ANOVA, followed by Newman-Keuls *post-hoc* analysis. There was a significant effect of treatment on mRNA detected in subfields CA1–2 [$F(3,141) = 16.14$; $P < 0.001$] and CA3 [$F(3,118) = 4.38$; $P < 0.01$] and in the DG [$F(3,132) = 5.73$; $P = 0.001$; Fig. 4]. In response to ADX, GR mRNA was significantly increased in subfield CA1–2 and in the DG, but not in subfield CA3 ($P < 05$, by Newman-Keuls test). In both cases administration of exogenous DEX to adrenalectomized animals (ADX/DEX) returned GR mRNA to within the range of sham-operated animals (Fig. 5). DEX administration alone (DEX group), on the other hand, reliably decreased GR message levels only in subfield CA3 ($P < 0.05$, by Newman-Keuls test; Fig. 5).

The change in hippocampal MR mRNA after ADX was not as widespread as that in GR message. Significant effects of treatment on MR message content were observed only in subfield CA1–2 [$F(3,144) = 4.87$; $P < 0.01$], with the ADX group showing increased MR mRNA relative to the sham and DEX groups ($P < 0.05$, by Newman-Keuls test). Replacement of ADX animals with DEX did not return MR message to normal levels (Fig. 4). The overall effect of treatment on MR message in subfields CA3prox and CA3dis did not achieve statistical reliability ($P = 0.12$ and 0.10, respectively). No effect of treatment on MR mRNA was seen in the DG.

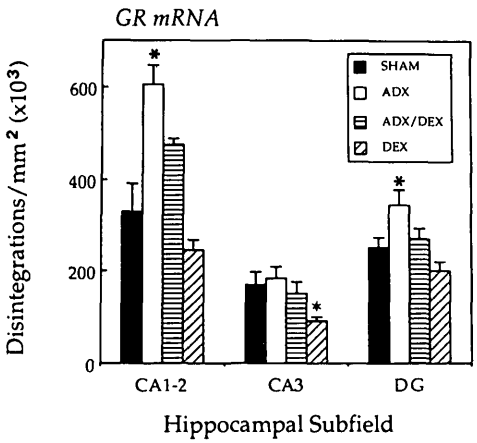


Fig. 4. Semiquantitative *in Situ* Hybridization Analysis of Glucocorticoid Receptor (Type 2) mRNA (GR mRNA) from Sections through the Hippocampus of Sham-ADX (SHAM; n = 6), ADX (n = 5), ADX/DEX (n = 4), and Chronic DEX-Treated (DEX; n = 5) Rats

Overall one-way ANOVAs revealed significant effects of treatment of GR mRNA in all hippocampal subfields. *Post-hoc* analysis using Newman-Keuls test revealed that GR mRNA is significantly increased in subfields CA1–2 and the DG in the ADX group relative to that in SHAM animals (*, $P < 0.05$), whereas ADX/DEX resulted in no change from SHAM levels. DEX treatment alone (DEX) caused a decrease in GR mRNA in subfield CA3 relative to that in SHAM rats.

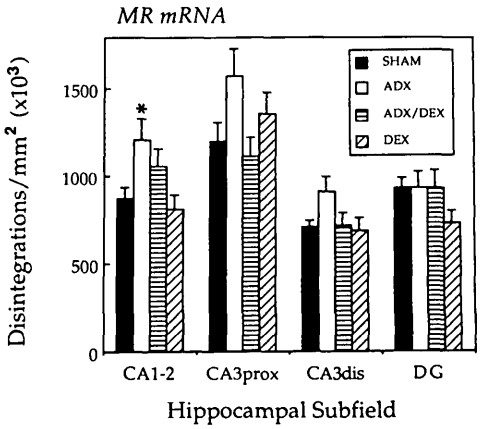


Fig. 5. Semiquantitative *in Situ* Hybridization Analysis of MR mRNA from Sections through the Hippocampus of Sham-ADX (SHAM; n = 6), ADX (n = 5), ADX/DEX (n = 4), and Chronic DEX-Treated (DEX; n = 4) Rats

One-way ANOVA revealed a significant effect of treatment on MR mRNA only in subfield CA1–2. Subsequent *post-hoc* analysis revealed significant differences between the ADX group and SHAM animals (*, $P < 0.05$) in subfield CA1–2; ADX/DEX animals did not differ from either the ADX or SHAM group.

Our results indicate that hippocampal GR mRNA levels are regulated by glucocorticoids and are responsive to replacement with the (type 2) glucocorticoid receptor agonist DEX. These data parallel results of previous receptor binding studies, which have demonstrated an up-regulation of hippocampal GRs after ADX. *In situ* hybridization further revealed that GR synthesis, as inferred from mRNA levels, was regulated by DEX (and, hence, type 2 GRs) in a site-specific manner. ADX-induced up-regulation was most pronounced in CA1–2 and DG and did not occur in CA3. The increase in GR mRNA synthesis in CA1–2 and DG was type 2 receptor-specific, as it was normalized by the GR agonist DEX. On the other hand, CA3 GR mRNA was significantly down-regulated by large doses of DEX. While the significance of the high dose steroid responsivity/ADX insensitivity of CA3 is obscure, it is of interest to note that CA3 is particularly sensitive to damage by high levels of glucocorticoids and aging (14, 15), suggesting that steroid binding in this region may be more integrated with the metabolic machinery of this neuronal population than of CA1–2 or DG neurons.

MR mRNA does not exhibit the same pattern of response to adrenalectomy as GR message, at least at the time point examined (8 days). Significant changes were limited to CA1–2 and were of a lower relative magnitude than those seen under the same conditions for GR mRNA [*e.g.* a 33% (MR) *vs.* an 80% (GR) change in CA1–2]. However, it should be noted that the abso-

lute change in disintegrations detected per mm^2 is greater for MR than for GR mRNA, indicating by inference that on a molar basis more new MR than GR mRNA molecules are being synthesized in CA1-2 of ADX rats. Which type of change is most important from a cellular biological standpoint, *e.g.* absolute level *vs.* change from baseline, depends on the translational/posttranslational mechanisms of the cell type in question.

The differences in response of MR and GR mRNAs to ADX indicate that receptor genes have a differential sensitivity to endogenous steroids. The pattern of changes in MR and GR mRNA level 8 days postadrenalectomy parallels findings from receptor binding studies, in that type 2 receptor number is some 2-fold higher than type 1 receptor number at this time point (11). Whether the difference between levels of hippocampal type 1 and type 2 binding and corresponding MR and GR mRNA levels reflect differences in the ability of the activated steroid-receptor complex to suppress basal GR and MR synthesis remains to be determined.

Analysis of MR mRNA in the ADX/DEX group indicates that DEX replacement is unable to return ADX-induced increases in CA1 to normal levels. The relative ineffectiveness of DEX replacement may be due to the limited ability of DEX to bind to the type 1 receptor *in vivo* (25), leaving a substantial number of hippocampal type 1 receptors unbound. In a similar vein, high doses of DEX given to nonadrenalectomized rats are unable to down-regulate MR mRNA in any hippocampal subfield (DEX group), suggesting a relative independence of MR mRNA synthesis from the effects of type 2 ligands. The MR specificity of ADX-induced MR mRNA increases in the hippocampus is presently being determined using specific MR and GR ligands.

The data included in this study demonstrate GR and MR mRNA localization and steroid regulation in the rat hippocampus. Regulation of both GR and MR mRNA content with adrenalectomy is commensurate with their putative roles as feedback modulators of HPA function; the differences in magnitude of response to steroid loss clearly suggest that the two receptors may perform quite different functions in tuning the HPA axis. Overall, the relatively small and restricted effects of steroids on MR and GR mRNA levels in hippocampus imply that MR and GR gene regulation is not tightly coupled to steroid negative feedback. It is probable that steroid binding represents only a portion of cellular input contributing to modulation of steroid receptor proteins in the brain.

Parallels between binding studies describing localization and regulation of type 1 glucocorticoid receptors (9–11) and MR mRNA data (Refs. 18 and 20 and this study) lead us to believe that the hippocampal MR mRNA species encodes a receptor species accounting for type 1 binding in the hippocampus. While it is clear that the hippocampal MR mRNA encodes a MR protein, its primary function in the hippocampus appears to be associated with B binding. Unlike the kidney, hippocampus has no detectable levels of the B-inactivating enzyme 11β-hydroxysteroid dehydrogenase, thus rendering circulating B predominant over mineralocorticoids in its ability to bind type 1 receptors (26). This effective unveiling of high affinity hippocampal type 1 receptors to B clearly suggests a major role for B in regulating functional properties of this structure at multiple levels (*i.e.* high and low affinity B receptors) and suggests that the MR gene encodes a receptor protein capable of multiple functions critically dependent on the tissue-specific environment.

MATERIALS AND METHODS

Regulation and Localization of Hippocampal MR and GR mRNAs

Subjects Subjects were male Sprague-Dawley rats, weighing between 200–250 g at the beginning of the experiments. All animals were housed in hanging wire cages in a temperature- and humidity-controlled vivarium quarters, maintained on a 12-h light, 12-h dark cycle.

Treatment Protocols For regulation studies rats were either ADX or sham-ADX via a dorsal approach. The animals were subsequently divided into four groups: one group received adrenalectomy with twice daily injections of 25 μg DEX (ADX/DEX; 1000 and 1600 h), one group was ADX and injected twice daily with DEX vehicle (ADX), one group was sham-ADX and injected twice daily with DEX vehicle (sham), and one group was sham-ADX and given twice daily injections of 100 μg DEX (DEX). DEX was dissolved in a small amount of ethanol and diluted to appropriate concentrations with bacteriostatic saline solution. ADX animals received 0.9% saline as drinking water.

All animals received injections for a period of 8 postsurgical days, at the end of which time they were overdosed with pentobarbitol. The thoracic cavity was subsequently exposed, and a 5-ml blood sample was rapidly removed directly from the left ventricle, after which the animal was perfused transcardially with 0.9% heparinized saline solution, followed by 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Brains were removed and postfixed for 1–2 h at 4 C, followed by cryoprotection in a 30% sucrose-20 mM sodium phosphate buffer solution (pH 7.4) overnight at 40 C. Brains were blocked in the coronal plane, frozen in liquid nitrogen, and stored at −70 C. Sections were cut in the coronal plane at 10 μm on a Bright-Hacker cryostat (Hacker Instruments, Fairfield, NJ), thaw-mounted onto polylysine-coated slides, and stored at −70 C until processed for *in situ* hybridization.

Blood samples were introduced into evacuated tubes containing EDTA and spun at 1500 rpm for 10 min, and plasma was collected in tubes containing 0.5 ml 1 N HCl. B was assayed by a competitive protein binding assay (27).

Probe Preparation GR and MR probes were designed to be as closely matched as possible in number of UTPs and C-G content, thus allowing approximately equivalent specific activity and probe "stickiness," respectively. Probe design is summarized in Table 1. The GR probe was a 456-nt fragment of a cDNA clone provided by Keith Yamamoto, directed against the final 50 nucleotides of the protein-coding region and the 3′ untranslated region of the GR mRNA. The GR insert was subcloned into the *Xba*I-*Eco*RI site of pGEM 4, and was transcribed from the T7 promoter with T7 RNA polymerase. The MR probe was a 347-nt *Pst*I-*Eco*RI fragment of our MR clone, directed against the 3′ untranslated region of the MR mRNA. The MR insert was subcloned into pGEM 3, and was transcribed from the SP6 promoter using SP6 polyrnerase. The nt sequences of the two probes did not contain any regions of high homology, obviating potential cross-hybridiza-

tion. Probes were synthesized using a SP6 transcription system, with [^{35}S]UTP, and unlabeled UTP was added to yield specific activities of 4.13 and 4.28 × 10^4 Ci/mmol for the MR and GR probes, respectively.

***In Situ* Hybridization** Sections were removed from the −70 C freezer and incubated with a 1 μg/ml solution of proteinase-K to permeabilize the tissue and inactivate endogenous RNase. After proteinase-K treatment, sections were incubated successively in water (1 min), 0.1 M triethanolamine (pH 8.0; 1 min), and 0.25% acetic anhydride in 0.1 M triethanolamine (10 min), after which tissue was washed in 2 × SSC[1] (5 min) and dehydrated through graded ethanols. Sections were then hybridized with 1.5 × 10^6 dpm [^{35}S]UTP-labeled GR or MR cRNA probes in a hybridization buffer containing 75% formamide, 10% dextran sulfate, 3 × SSC, 50 mM sodium phosphate buffer (pH 7.4), 1 × Denhardt's solution, 0.1 mg/ml yeast tRNA, and 0.1 mg/ml sheared salmon sperm DNA in a total volume of 20 μl. Tissue sections were covered with coverslips, the coverslips were sealed to the slides with rubber cement, and the slides were incubated overnight at 50 C. On the following day the rubber cement was removed, the coverslips were soaked off in 2 × SSC, and tissue was washed in fresh 2 × SSC for 10 min. To remove single stranded probe not hybridized with endogenous mRNAs, sections were incubated for 30 min in a 200 μg/ml solution of RNase-A at 37 C. Tissue was then washed in increasingly stringent SSC solutions (2, 1, and 0.5 ×; 10 min each), followed by a 1-h wash in 0.5 × SSC at the hybridization temperature. After the final wash, tissue sections were dehydrated through graded ethanols and prepared for detection by x-ray and emulsion autoradiography.

Hybridization Controls Several control procedures were employed to verify specificity of GR and MR hybridization. First, sense-strand RNA probes of the same length and specific activity as the GR (456 nt) and MR (347 nt) cRNA probes were synthesized from the same plasmid inserts by transcription using SP6 and T7 RNA polymerases, respectively. Sense-strand hybridization protocols were carried out in parallel with cRNA hybridizations. This control procedure assures that probes of the same approximate size and C-G content will not produce positive signal in tissue sections. Second, cRNA-rnRNA hybrids were melted by incubation of hybridized tissue sections at increasingly stringent conditions (low salt, high temperature). True hybrids are known to show predictable dissociation with increases in stringency. Wash conditions were as follows: 2.4 M triethylammonium chloride, 2 mM EDTA, and 0.1% sodium dodecyl sulfate in 50 mM Tris buffer, pH 8.0 (28); wash temperatures of between 22–90 C were evaluated. Triethylammonium chloride reliably reduces temperatures required to melt hybrids, allowing thermal stability to be assessed at temperatures that do not cause tissue damage (21, 28).

Detection and Analysis Effectiveness of the *in situ* hybridization procedure was verified by positive x-ray signal after a 48-h exposure. Sections were then dipped, along with L-[^{35}S] methionine-labeled brain paste standards, in Kodak NTB2 nuclear emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with distilled water. Sections were exposed for 21 days, at the end of which time all autoradiographs were developed in undiluted Kodak D-19 developer (2 min) and fixed with Kodak rapid fix (3 min). Brain paste standards had been prepared with different amounts of radioactivity, cut at 10 μm, and vapor fixed before dipping. Three images, corresponding to 1) subfield CA1–2 and CA3prox, 2) subfield CA3dis, and 3) dentate gyrus, were digitized from a given hippocampal section (×100 magnification) and analyzed using a Loats Associates *in situ*/Grain image analysis program. Briefly, analysis consisted of establishing a standard curve relating the optical density of standards with the radioactivity of the appropriate brain paste standard, obtained by directly counting adjacent standard sections in a Beckman βcounter (Palo Alto, CA). The standard curve of best fit was selected (in this instance, a second order fit). Areas of interest in digitized images of hippocampus were sampled, and the obtained optical density value was converted to disintegration values according to the standard curve of best fit. Backgrounds selected over the molecular layer of the hippocampus were subtracted from each value obtained. Selection of the molecular layer as background was based on both the presence of definable portions of this layer in each image and the consistency of optical density measures from this region across the three regions sampled from each section.

[1] 2 × SSC = 0.30 mM NaCl, 0.03 mM Na citrate, pH 7.2.

Relative Abundance of MR and GR mRNAs in Different Subregions of the Hippocampal Formation

Treatment Protocol Normal rats were used for assessment of the relative abundance of hippocampal MR and GR mRNAs. Regional differences in MR/GR mRNA ratios were established by the following series of experiments. 1) Alternate sections through hippocampus of normal rats were incubated with the MR- and GR-specific cRNA probes listed in Table 1, using the *in situ* hybridization conditions described above. 2) Alternate sections through the hippocampus of normal rats were hybridized *in situ* with the GR cRNA probe described above or a MR cRNA probe of substantially longer length than that employed in Exp 1 above. This probe was 550 nt in length, contained 149 UTPs, and had a C-G content of 46%; it was directed against the 3′ untranslated region of the MR mRNA. Due to the larger number of UTP residues, the MR cRNA probe was of higher specific activity than the GR probe (5.31 × 10^4 Ci/mmol). Signal intensity was corrected for specific activity before determination of MR/GR ratios. Hybridization conditions were identical to those described above.

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Address requests for reprints to: James P. Herman, Mental Health Research Institute, 205 Washtenaw Place, Ann Arbor, Michigan 48109–0720.

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