

Defense of Adrenocorticosteroid Receptor Expression in Rat Hippocampus: Effects of Stress and Strain*

JAMES P. HERMAN, STANLEY J. WATSON, AND ROBERT L. SPENCER

Department of Anatomy and Neurobiology (J.P.H.), University of Kentucky Medical Center, Lexington, Kentucky 40536-0084; Mental Health Research Institute (S.J.W.), University of Michigan, Ann Arbor, Michigan 48109; and Department of Psychology (R.L.S.), University of Colorado, Boulder, Colorado 80309

ABSTRACT

Neuronal mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) proteins are glucocorticoid-activated transcription factors that bind identical DNA response elements yet transduce distinct physiological/transcriptional actions. The present study assessed regulation of adrenocorticosteroid receptor RNA and protein following intermittent stress exposure, using Sprague Dawley (S-D) and stress-hyperresponsive Fischer 344 (F344) rat strains. The F344 (but not S-D) strain showed enhanced acute stress responsivity and enhanced corticosterone secretion following prolonged stress. F344 rats also showed reduced responsiveness to a novel stressor after prolonged stress exposure, suggestive of enhanced glucocorticoid negative feedback. Upon prolonged stress, F344 rats down-regulated MR hnRNA

in CA1, CA3, and dentate gyrus. Transcriptional changes were accompanied by decreased expression of the α 5' messenger RNA (mRNA) form, consistent with altered promoter utilization. In contrast, β 5' splice variant, full-length mRNA, and MR protein expression were not affected by stress in either strain, implying that transcriptional changes do not affect overall mRNA or protein expression. GR protein was increased in pyramidal and granule cell somata/nuclei of F344 rats despite lack of a change in mRNA expression. These data suggest that prolonged stress elicits restricted changes in MR and GR expression in the F344 strain only. Overall, stable expression of adrenocorticosteroid receptors is rigorously defended in hippocampal neurons, apparently through transcriptional and post-transcriptional mechanisms. (*Endocrinology* 140: 3981–3991, 1999)

NEURONAL mineralocorticoid (MR) and glucocorticoid (GR) receptors integrate information on circulating glucocorticoids into changes in gene transcription. Actions of both receptor species are mediated by homo- or heterodimer binding to glucocorticoid response elements (GRES) present in regulatory regions of responsive genes (1, 2). Binding studies indicate that the neuronal MR is extensively occupied at low circulating levels of glucocorticoids (3), suggesting that the MR is having a constant transcriptional action on responsive genes. The MR appears to have trophic actions on hippocampal neurons, protecting dentate gyrus granule cells from adrenalectomy-induced cell death (4) and maintaining expression of the antiapoptotic gene Bcl-2 in pyramidal cell populations (5). By contrast, the neuronal GR is occupied only during periods of high glucocorticoid secretion (e.g. stress) (3, 6). Glucocorticoids acting through the GR to increase Ca^{2+} influx (7), disrupt LTP (8–10) and impair memory (11), suggestive of hippocampal dyshomeostasis. This hypothesis is supported by known deleterious effects of high glucocorticoid levels on pyramidal cell dendritic complexity (12) and viability (13).

Given the diametrically opposing effects of MR and GR on hippocampal cell integrity and function, it is important to understand mechanisms regulating expression of these molecular species. Neuronal MR expression is regulated by glu-

corticoids. Adrenalectomy increases MR binding in hippocampal cell extracts and MR messenger RNA (mRNA) expression in CA1 and CA2 pyramidal cells (14, 15). The MR has three known splice-site variants in the 5' untranslated region of the mRNA, immediately adjacent to the translation start site (15–17). Variant RNAs are likely generated by alternative promoter utilization (18). Adrenalectomy selectively increases expression of the α splice-site variant mRNA (15), suggesting that the α -associated promoter may be preferentially involved in MR up-regulation following steroid removal. Expression of MR hnRNA is increased following ADX and decreased by acute stress in a glucocorticoid-dependent fashion (19, 20), consistent with glucocorticoid negative regulation of the MR gene at the level of transcription.

Glucocorticoids also play a major role in control of GR biosynthesis. Both GR mRNA and protein are markedly increased in all hippocampal subfields following adrenalectomy (14, 21–23), with the most substantial elevations seen in CA1 (14). These changes are reversed by low doses of glucocorticoids or aldosterone and can be mimicked by systemic administration of MR antagonists (24), implying MR regulation (25, 26). Expression of GR mRNA and GR binding are decreased by very high levels of glucocorticoids *in vivo* and *in vitro* (24, 27, 28), consistent with negative regulation through the GR. However, chronic stress has inconsistent effects on GR binding, protein expression, and mRNA levels (29–35), suggesting that physiological increases in glucocorticoid secretion may not be sufficient to down-regulate the GR.

Given the intimate relationship between glucocorticoid levels and GR and MR expression, stress-induced changes in corticosteroid receptor balance are likely keyed to the net

Received December 23, 1998.

Address all correspondence and requests for reprints to: James P. Herman, Ph.D., Department of Anatomy and Neurobiology, University of Kentucky Medical Center, 800 Rose Street, Lexington, Kentucky 40536-0084. E-mail: jpher00@pop.uky.edu.

* This work was sponsored by Grants AG-12962 (to J.P.H. and R.S.) and MH-42251 (to S.J.W.).

amount of glucocorticoid released. Glucocorticoid stress responses are subject to substantial individual or strain differences that mitigate the impact of stress on the organism. For instance, the F344 strain consistently shows exaggerated stress-induced corticosterone secretion relative to other rat strains (36), suggesting genetically programmed HPA hyperresponsiveness.

While considerable work has been directed toward delineating regulation of adrenocorticosteroid receptor expression by manipulations of steroid environment, the manner in which endogenous, stress-induced changes in glucocorticoids regulate MR and GR transcription, mRNA expression, and protein levels has yet to be definitively addressed. In the present study, we investigate mechanisms underlying stress regulation of adrenocorticosteroid receptors in hippocampus. Sprague Dawley and F344 strains will be examined to test the hypothesis that adrenocorticosteroid receptor down-regulation will be intensified in populations predisposed to enhanced HPA stress responsiveness.

Materials and Methods

Subjects

Subjects were male Sprague Dawley and F344 rats (Harlan Sprague Dawley, Inc., Indianapolis, IN), weighing between 250–300 g at the beginning of the stress experiments. All rats were maintained on a 12-h light, 12-h dark cycle in a constant temperature/humidity environment and had access to food and water *ad libitum*. Animal procedures were approved by the University of Kentucky IACUC.

Chronic intermittent stress regimen

Groups of male F344 and S-D rats were exposed to a chronic intermittent stress paradigm previously characterized by our laboratory (27). In this paradigm, rats are exposed to a randomized selection of different stressors twice daily over a period of 15 days. Stressors used in the experiment included: restraint (2 h in plastic restraint cages (Plas Labs, Lansing, MI), cold exposure (2 h in a 4 C cold room), cold water swim (20 min in 16–18 C water), warm water swim (40 min in 26–30 C water), vibration [6 animals per cage placed on a shaker for 2 h (1 cycle/sec)], crowding (6 rats per cage, overnight) and isolation (1 animal per cage, overnight). Handled (removed from their home cages and handled twice daily) and unhandled groups of rats were included as controls. Nine to ten rats were included in each group.

During the treatment protocols, all rats in the unhandled, handled, and stress groups received two acute stress tests. The first stress test was the first stress administered to rats comprising the stress group and was thus equally novel for all groups. In this test, animals were placed in restraint cages for 30 min. Blood was sampled by tail-nick immediately upon placement in restrainers and immediately before release. Sixty minute and 120 min samples were subsequently collected under light restraint to obtain stress time-course data. The second acute stressor was administered 10 days after the initial restraint exposure, and represented a novel stress for all groups. In this test, animals were placed in an open field for 5 min, and blood collected at 30, 60, and 120 min time points following stress initiation (under light manual restraint). Thus, all animals were exposed to at least two stressors; unhandled and handled rats had discrete exposures 15 and 5 days before kill, whereas stressed rats received bidaily stress over the entire period.

Rats were killed by rapid decapitation between 0900 and 1100 h on the morning after administration of the last stressor (16 h after the final stressor). Brains were removed and frozen in isopentane (–40–50 C), and trunk blood collected for analysis of plasma stress hormones.

To characterize the MR antibody used in these studies, four normal, unstressed S-D rats were overdosed with Pentobarbital and perfused transcardially with 100 ml of 0.9% saline followed by 350 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. Brains were removed and postfixed overnight in a 4% paraformaldehyde so-

lution, then sunk in a 30% sucrose solution. Brains were frozen on dry ice.

Tissue processing

Fresh frozen brains were sectioned at 15 μ m on a Bright-Hacker cryostat and thaw-mounted onto Superfrost Plus (Fisher, Chicago, IL) slides. Slides were maintained at –20 C until processing for *in situ* hybridization or immunautoradiography. Fixed tissues were sectioned at 30 μ m sections on a sliding microtome in the coronal plane. Sections were stored in a cryoprotectant solution at –20 C until further processing.

Hybridization probes

Assessment of MR RNA regulation was accomplished using probes that specifically recognized: 1) all mature MR mRNA forms (550 bp, complementary to the coding region and 3' untranslated region of rat MR mRNA); 2) the α 5' splice variant (180 bp, complementary to exon 1 α); 3) the β 5' splice variant (310 bp, complementary to exon 1 β); and 4) a coding region intron spanning exons 2 and 3 (approximately 300 bp). Assessment of GR RNA regulation was performed using: 1) a probe complementary to a sequence within the intron spanning exons 7 and 8 of the GR gene (approximately 800 bp) (courtesy of Keith Yamamoto, UCSF); and 2) a probe recognizing all mature GR mRNA forms (456 bp, complementary to the coding region and 3' untranslated region of rat GR mRNA). Labeling reactions included 60 μ Ci 33 P-UTP (specific activity 2900 Ci/mmol), 1 \times SP6 transcription buffer (Roche Molecular Biochemicals), 15 mM dithiothreitol, 200 μ M GTP, CTP and ATP, 10 μ M UTP, 40 U placental RNase inhibitor (40 U/ μ l) (Roche Molecular Biochemicals), 1 μ g linearized plasmid DNA, and 20 U of appropriate RNA polymerase (SP6 or T7, Roche Molecular Biochemicals). Reactions were incubated at 37 C for 90 min. Subsequently, 12 U of RNase-free DNase I (Roche Molecular Biochemicals) was added to digest the DNA template and after 5 min at 37 C, the reaction mix was diluted to 100 μ l with diethylpyrocarbonate (DEPC)-treated water and ethanol precipitated with 7.5 M ammonium acetate.

In situ hybridization

Tissue sections were removed from the –20 C freezer and fixed for 10 min in 4% buffered paraformaldehyde. Slides were then rinsed twice in 5 mM DEPC-treated potassium PBS (pH 7.5) (KPBS) (5 min), twice in KPBS/0.2% glycine, and twice in KPBS. Slides were then acetylated by a 10 min treatment with 0.25% acetic anhydride and 0.1 M triethanolamine (pH 8.0), rinsed twice in 0.2 \times SSC (5 min) and dehydrated in graded alcohols. Labeled probes were added to a hybridization buffer containing 50% formamide, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 335 mM NaCl, 1 \times Denhardt's, 200 μ g/ml salmon sperm DNA, 150 μ g/ml yeast transfer RNA, 20 mM dithiothreitol, and 10% dextran sulfate. Probes were denatured for 5 min at 70 C and 50 μ l (1 \times 10⁶ cpm) of diluted probe applied to each slide. Slides were coverslipped, placed in moistened chambers, and incubated overnight at 55 C. Following hybridization, coverslips were removed in 0.2 \times SSC and rinsed in fresh 0.2 \times SSC for 10 min. Slides were treated with RNase A (50 μ g/ml) for 30 min at 37 C and transferred to fresh 2 \times SSC, then rinsed three times in 0.2 \times SSC 10 min per wash, followed by a 1 h wash in 0.2 \times SSC at 65 C. Slides were dehydrated in graded alcohols, dried at room temperature and exposed for 14–21 days to Kodak BioMAX film (Eastman Kodak Co., Rochester, NY).

Immunautoradiography

Alternate series of tissue sections from rats processed for *in situ* hybridization were removed from the –20 C freezer, warmed to room temperature, and ringed with a PAP pen (RBI). Slides were then immersed in a blocking buffer containing a 1:50 dilution of normal donkey serum in 10 mM potassium PBS (KPBS), pH 7.2, for 30 min at room temperature. The normal serum corresponded to the species in which the secondary antibody was made (see below). Slides were then placed in humidified chambers and sections covered with 500 μ l of primary antiserum [rabbit anti-MR (MR214), 1:5000 dilution or mouse anti-GR (BuGR2) (BioAffinity Reagents, Golden, CO), 1:4000] in blocking buffer

(normal serum diluted 1:50 in KPBS), and incubated overnight at 4 C. After primary antiserum incubation, slides were rinsed three times in 10 mM KPBS and incubated in a secondary antibody solution containing either ^{35}S -labeled donkey antirabbit IgG (MR214) or ^{35}S -labeled donkey antimouse IgG (BuGR2), diluted 1:500 in KPBS. After 1 h of incubation, the secondary antibody was removed and the sections washed three times in KPBS and dried under a stream of cold air. Slides were then exposed to x-ray film for 3 to 5 days. Immunohistochemical controls included 1) incubation of tissue with preabsorbed MR214; 2) omission of primary antibody; and 3) omission of secondary antibody. No specific signal was seen following any control procedure. To verify linearity of signal detection, sequential dilutions of ^{35}S -labeled donkey antirabbit IgG and ^{35}S -labeled donkey antimouse IgG were blotted onto nitrocellulose and processed for immunoradiography, as above (34). All intensity readings derived from tissues fell within the linear range of the standard curve relating gray level to radioactivity.

Brain sections from normal perfused rats were used to verify appropriate distribution of MR immunoreactivity by the MR214 antiserum. Sections were removed from cryoprotectant and washed extensively in 50 mM KPBS, pH 7.2. Sections were then blocked in KPBS containing 4% normal goat serum (Vector Laboratories, Inc.) for 30 min, followed by incubation overnight with rabbit polyclonal MR antibody, diluted 1:5,000 in 50 mM KPBS in the presence of 0.4% Triton X-100 (TX) (Sigma Chemical Co.), 1% BSA, and 2% normal goat serum (Vector Laboratories, Inc.). On day two, sections were rinsed extensively in 50 mM KPBS and subsequently incubated for 1 h in biotinylated goat antirabbit antiserum, diluted at 1:1,000 in KPBS with 0.4% TX. Sections were then rinsed three times in 50 mM KPBS and subsequently incubated for 1 h in a 1:200 dilution of Elite ABC complex solution (Vector Laboratories, Inc.). Staining was visualized using nickel-enhanced diaminobenzidine (DAB) chromagen. Sections were rinsed twice in 50 mM KPBS, followed by two washes of 0.1 M sodium acetate (pH 6.0). The chromagen solution contained 250 mg/ml nickel ammonium sulfate, 3.5 mg/ml DAB and 0.01% hydrogen peroxide. The reaction was allowed to proceed for 3–5 min, at which point sections were rinsed several times in 50 mM KPBS to stop the reaction. Sections were mounted onto Superfrost slides, dehydrated, and coverslipped with DPX mountant.

Western blot analysis

Western blot analysis was conducted as described previously (24). Briefly, frozen hippocampi were homogenized in a 50 mM Tris buffer (pH 7.2, 4 C) containing 6 mM MgCl_2 , 1 mM EDTA, 10% (wt/vol) sucrose, 1% SDS, and a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 3 mM benzamide, 1 mM leupeptin, 1 $\mu\text{g}/\text{ml}$ of pepstatin, 1 $\mu\text{g}/\text{ml}$ antipain, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ of soybean trypsin inhibitor). Homogenates were ultracentrifuged (105,000 \times g) and the supernatants adjusted to a final protein concentration of 4 mg/ml (DC protein assay, Bio-Rad Laboratories, Inc., Hercules, CA). Supernatants were mixed with Laemmli's sample buffer and boiled for 5 min. Samples (50 μg) were separated by SDS-PAGE, using 7.5% bis-acrylamide gels, and separated proteins electrophoretically transferred onto nitrocellulose. GR protein was detected on nitrocellulose blots by the monoclonal antibody, BUGR2 (Affinity BioReagents, Inc.), using enhanced chemiluminescent labeling (ECL, Amersham Pharmacia Biotech, Ar-

lington Heights, IL). The optical density of GR reactive bands (approximately 97 K_d) visible on x-ray film were determined densitometrically (see below). Parallel blots revealed that ECL detection produces a linear relationship between amount of blotted protein and chemiluminescent signal, validating its use in the present experiments.

Hormone assays

Plasma samples were collected and stored at -20°C . Plasma CORT was assessed by RIA, using a double-antibody kit from ICN (with ^{125}I -labeled CORT used as tracer). All plasma samples for each assay were processed at the same time.

Plasma corticosteroid binding globin was measured using a competitive binding procedure adapted from Westphal (37). Plasma was diluted (1:450 final dilution) in 10 mM Tris buffer containing 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerin, pH 8.0. Diluted samples were incubated (4 C) in triplicate overnight with a saturating concentration of ^3H corticosterone (15 nM) \pm an excess of unlabeled corticosterone (10 μM). Macromolecular-bound steroid was separated from unbound steroid by mixing with dextran-coated activated charcoal followed immediately by centrifugation. The bound fraction (supernatant) was mixed with scintillation cocktail and the relative amount of radioactivity determined by a liquid scintillation counter. CBG was expressed as nmol specific ^3H corticosterone binding/liter plasma.

Data analysis

In situ hybridization, immunoradiography, and Western blot data were analyzed using NIH Image 1.55 software for Macintosh. Hippocampal subfields were defined according to the rat brain atlas of Paxinos and Watson (35); measurements were taken from subfield CA1, CA3, dentate gyrus and frontal cortex. Background signal was sampled from the molecular layer of the hippocampus and was subtracted from all regions to obtain corrected gray level measures. Gray level measurements of Western blot ECL data and secondary spotted on nitrocellulose were taken in like fashion, with background determined over negative regions of the blots.

Results

Verification of the efficacy of the chronic stress procedure is presented in Table 1. Plasma corticosterone (CORT) was elevated in F344 rats relative to the S-D strain across all groups [$F(1,43) = 5.94$, $P < 0.05$]. There was a significant effect of chronic stress on plasma CORT [$F(2,43) = 3.40$, $P < 0.05$], highlighted by a significant increase in stressed F344 rats relative to handled and unhandled controls. Differences between stressed S-D rats and unhandled and handled controls did not reach statistical significance. There was a significant effect of both stress [$F(2,47) = 3.18$, $P = 0.05$] and strain [$F(1,47) = 5.43$, $P < 0.05$] on plasma ACTH. Overall, the F344 strain showed higher ACTH levels than S-D rats and

TABLE 1. Effects of chronic stress on F344 and Sprague Dawley rats

		Unhandled	Handled	Stress
CORT (ng/ml)	Sprague Dawley	53.2 \pm 11.4	52.9 \pm 14.1	95.5 \pm 15.6
	F344	63.9 \pm 28.3	60.3 \pm 22.5	171.3 \pm 46.9 ^{a,b}
ACTH (pg/ml)	Sprague Dawley	152.3 \pm 17.4	91.2 \pm 14.4	139.5 \pm 36.2 ^a
	F344	179.2 \pm 33.9	142.5 \pm 31.3	250.7 \pm 61.0 ^{b,c}
CBG (nM)	Sprague Dawley	371.1 \pm 49.8	289.5 \pm 29.5	466.6 \pm 58.0
	F344	717.4 \pm 93.6 ^b	739.3 \pm 115.0 ^b	741.4 \pm 32.8 ^b
Adrenal weight (mg/100 g)	Sprague Dawley	13.2 \pm 0.4	13.5 \pm 0.5	16.9 \pm 0.7 ^a
	F344	15.9 \pm 0.6 ^b	16.1 \pm 0.8 ^b	20.4 \pm 0.9 ^{a,b}
Thymus weight (mg/100 g)	Sprague Dawley	148.9 \pm 6.2	158.6 \pm 8.3	126.4 \pm 7.6 ^a
	F344	112.7 \pm 3.6 ^b	105.1 \pm 5.3 ^b	86.9 \pm 4.0 ^{a,b}

^a Significant difference between stressed and handled and unhandled groups, $P < 0.05$.

^b Significant differences between strains, $P < 0.05$.

^c Significant difference between stressed and handled groups, $P < 0.05$.

significant elevations in ACTH secretion following stress. Plasma corticosteroid binding globin (CBG) was higher in F344 rats relative to the S-D strain [$F(1,25) = 52.6, P < 0.05$]. However, there was no effect of stress on plasma CBG levels. Significant adrenal hypertrophy [$F(2,51) = 23.8, P < 0.05$] and thymic atrophy [$F(2,51) = 10.1, P < 0.05$] were observed following stress in both strains. The F344 strain showed significantly lower thymus weight [$F(1,51) = 72.6, P < 0.05$] and elevated adrenal weight [$F(1,51) = 29.9, P < 0.05$] relative to S-D across all treatment groups. In both strains tested, stress groups showed significant increases in adrenal weight and decreases in thymus weight relative to unhandled and handled controls ($P < 0.05$, Newman-Keuls test).

Acute stress responses were tested in all rats at the commencement of testing and 4 days before they were killed. Initial presentation of restraint stress revealed a marked elevation in both basal and stress-induced glucocorticoid secretion in the F344 strain relative to S-D rats. Repeated-measures ANOVA revealed a significant effect of strain [$F(1,68) = 43.58, P < 0.05$] and time [$F(2,68) = 41.48, P < 0.05$] on corticosterone secretion, marked by significant differences between the F344 and S-D groups at all time points ($P < 0.05$, Newman-Keuls test). This difference was supported by an elevated integrated CORT secretion in the F344 group [$t(34) = 41.37, P < 0.05$] (Fig. 1A). Analysis of responsiveness to a novel stressor was tested 10 days following initiation of the stress/handling procedure, with data analyzed by three-way ANOVA, using strain and treatment as factors with time as a repeated measure. As was the case for the initial stress exposure, the novel stressor resulted in substantial increases in CORT release over time [$F(2,60) = 25.75, P < 0.05$] and a significant effect of strain [$F(1,60) = 55.28, P < 0.05$]. Analysis of integrated CORT secretion (Fig. 1B) revealed a significant effect of strain on magnitude of the CORT response to a novel stressor [$F(1,29) = 17.19, P < 0.05$], and a significant stress by strain interaction [$F(2,29) = 3.34, P < 0.05$]. Posthoc analysis

indicated a reduced integrated CORT response in the stressed F344 group relative to unhandled controls ($P < 0.05$, Fisher's LSD test).

Regulation of MR gene and mRNA expression was assessed by *in situ* hybridization analysis. Localization of the MRin, MR α , MR β , and MR-3'UT in hippocampus are presented in Fig. 2. As noted, all four species were localized in pyramidal and granule cell layers of the hippocampus. Some minor subfield-specific intensity variations were noted; in support of previous data (15, 19), MRin signal was enriched in DG, MR α and MR-3'UT signals were most intense in CA2, and MR β was distributed evenly across all subfields.

Regulation of MR gene and mRNA expression by chronic stress is noted in Figs. 3 and 4. Following chronic stress exposure, overall expression of MR hnRNA (Fig. 3A) was decreased in CA1 [$F(2,47) = 5.32, P < 0.05$], CA3 [$F(2,47) = 4.32, P < 0.05$] and DG [$F(2,47) = 4.17, P < 0.05$]. Significant strain by stress interaction effects were observed in CA1 [$F(2,47) = 3.84, P < 0.05$] and DG [$F(2,47) = 3.52, P < 0.05$], with a trend toward an interaction effect seen in CA3 [$F(2,47) = 2.40, P = 0.10$]; these effects appear to reflect the fact that MR hnRNA was decreased by stress in F344 rats but was not affected in the S-D strain. In support of this hypothesis, posthoc analysis revealed that MR hnRNA was reduced in CA1 and CA3 of F344 rats relative to unhandled and handled controls ($P < 0.05$); however, there was no effect of stress on MR hnRNA expression in S-D rats.

Analysis of MR α variant mRNA expression (Fig. 4A) revealed a significant effect of stress in CA3 only [$F(2,51) = 4.98, P < 0.05$]. However, stress by strain interaction effects were again observed in CA1 [$F(2,51) = 3.46, P < 0.05$] and DG [$F(2,51) = 4.79, P < 0.05$], reflecting differential depletion of MR α mRNA in stressed F344 rats. Expression of MR β mRNA, representing the other major MR 5' splice variant, was not affected by stress or strain (Fig. 4B).

Importantly, there was no effect of stress or strain on

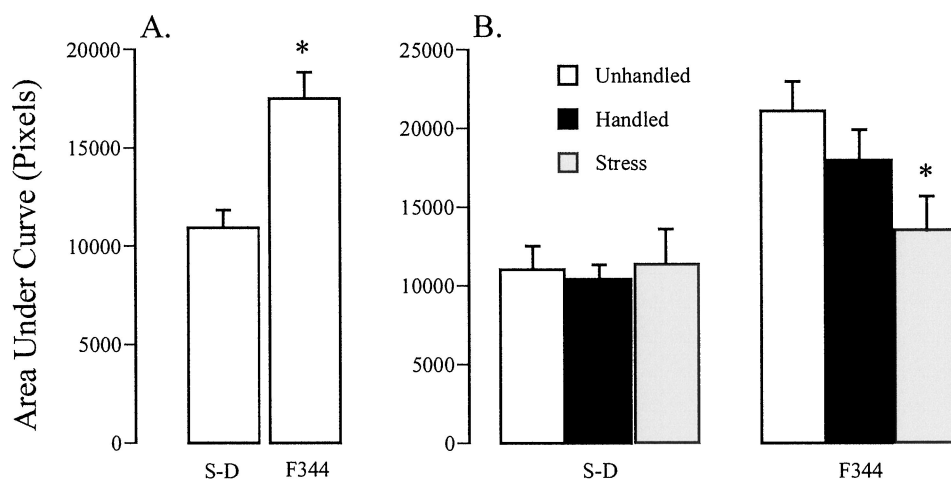


FIG. 1. Integrated plasma CORT responses of S-D and F344 rats to stress. Integrated CORT responses were determined by measurement of area under the curves relating plasma CORT levels with time poststress. A, Response to initial presentation of restraint stress. F344 rats show substantially larger integrated CORT responses than S-D animals. B, Response to a novel stressor (open field exposure) in groups of rats tested 10 days after a single exposure to restraint (unhandled), 10 days of twice-daily handling (handled) and 10 days of chronic intermittent stress exposure. S-D rats show no change in magnitude of the CORT response following exposure to prolonged stress. In contrast, F344 rats show diminished responsiveness to novelty following chronic stress exposure, reflected as a significant difference between the stress group and unhandled controls ($P < 0.05$).

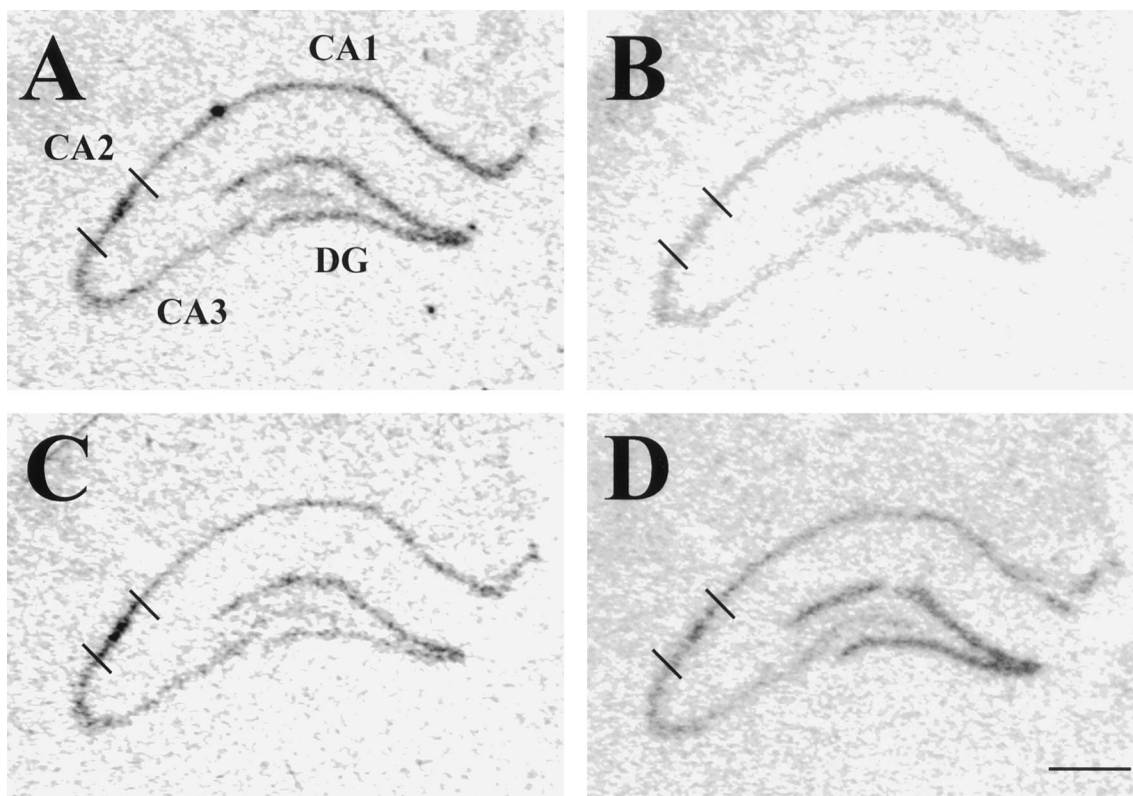


FIG. 2. Localization of MR mRNA and hnRNA species in rat hippocampus. MR mRNA (A) is localized throughout the hippocampus, including cornu ammonis fields CA1-CA3 and the dentate gyrus. Expression is particularly enriched in CA2. MR β splice variant mRNA (B) is expressed at similar levels throughout the hippocampus. MR α variant expression (C) corresponds more closely with that of MR mRNA, with an enrichment of expression in CA2. MR hnRNA localization (D) is distributed throughout hippocampus also, and appears to highest in DG. Magnification bar, 100 μ m.

full-length MR mRNA, as detected by a probe recognizing the 3' coding and proximal 3' untranslated region of the message. MR-3'UT mRNA expression was not affected by either stress or strain (Fig. 3A). Thus, the changes in MR hnRNA and MR α mRNA did not appear to impact the mature message pool at the poststress time point examined.

Localization of MR protein in hippocampus is illustrated in Fig. 5. Immunautoradiographic localization of MR-immunoreactivity (ir) indicates expression in all pyramidal cell layers and in dentate gyrus granule cells (Fig. 5A). The distribution of radiolabeled signal corresponded with that observed by standard immunohistochemical staining (Fig. 5B), serving to verify that the immunautoradiographic technique detects MR antigen in appropriate tissues. No autoradiographic signal was observed in sections reacted in the absence of primary antibody. Immunostaining was blocked by preincubation with GST-MR fusion protein, verifying antibody specificity. The distribution of MR immunoreactivity observed using both detection methods corresponded well with the localization of MR mRNA (Fig. 5C); indeed, the intensity of MR staining was greatest in regions showing the highest levels of MR message, such as subfield CA2.

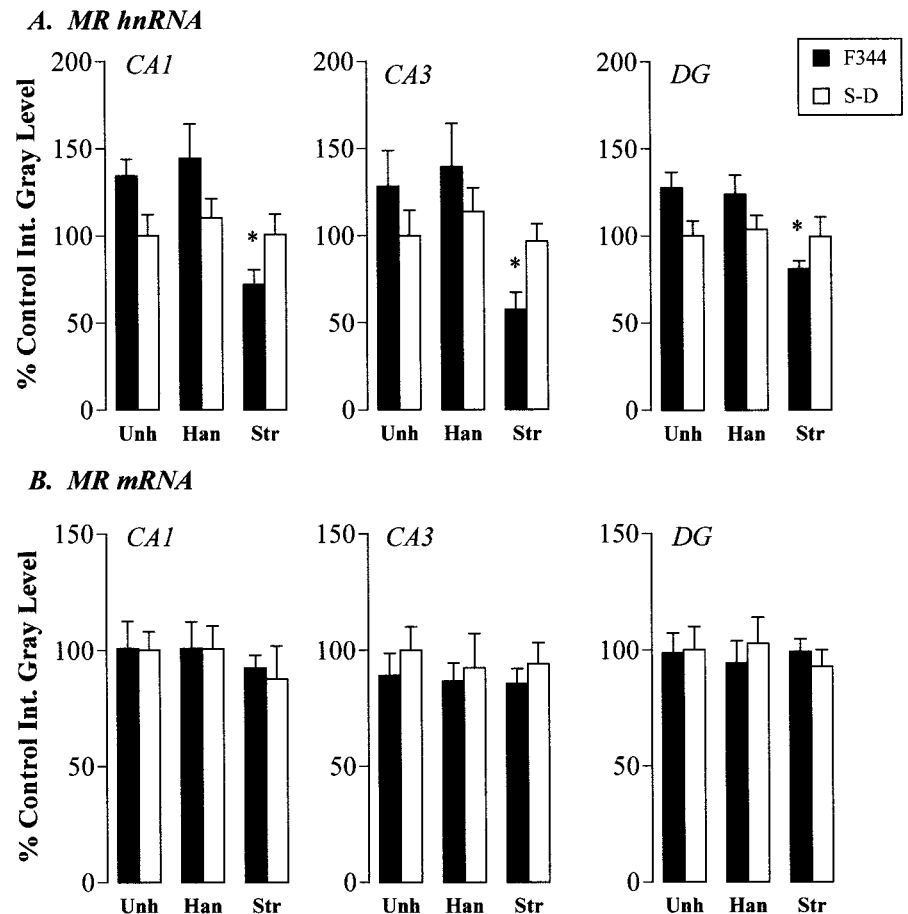
Immunautoradiography was employed to assess MR protein regulation, using sections neighboring those used for *in situ* hybridization analyses. This method was characterized in our laboratory and others as an anatomical method for semiquantitative analysis of protein expression (34, 36–

38). In agreement with the full-length MR mRNA data, densitometric analysis did not indicate any significant effect of chronic stress or strain on MR protein expression in any hippocampal subfield (Fig. 6). Note that Western blot analysis did not prove sufficiently sensitive to allow quantitative assessment of MR protein in hippocampal extracts (data not shown).

To assess stress modulation of GR levels, additional series were processed for assessment of GR hnRNA and mRNA (by *in situ* hybridization) (Fig. 7). No changes in GR hnRNA (Fig. 7A) or mRNA (Fig. 7B) were seen following chronic intermittent stress in any hippocampal region. In addition, GR hnRNA and mRNA did not differ among the two strains examined.

In contrast, stress-induced alterations in GR protein expression (Fig. 8A), as assessed by immunautoradiography, were observed in CA3 [$F(2,29) = 4.24, P < 0.05$], and there was an interaction between stress and strain in this subfield [$F(2,29) = 3.90, P < 0.05$]. Posthoc analysis revealed that stress increased GR immunoreactivity in stressed F344 rats relative to both handled and unhandled controls ($P < 0.05$, Newman-Keuls test); however, GR immunoreactivity was not affected in S-D rats. Significant effects of stress on GR immunoreactivity were also seen in DG [$F(2,29) = 3.62, P < 0.05$]; again, GR immunoreactivity was altered in stressed F344 rats relative to unhandled animals, whereas no changes were observed within the S-D strain. In contrast with CA3

FIG. 3. Semiquantitative analysis of MR heteronuclear RNA (hnRNA) (A) and mRNA (B) in CA1, CA3, and dentate gyrus of unhandled (Unh), handled (Han) and stressed (Str) rats [F344 and Sprague Dawley (S-D) strains]. In the F344 strain, MR hnRNA was significantly decreased by chronic stress (relative to both handled and unhandled controls) in all hippocampal regions examined (*, $P < 0.05$, Newman-Keuls test). No changes were observed in the S-D strain. In contrast, there were no strain or stress-induced changes in MR mRNA expression in any region examined.



and DG subfields, GR immunoreactivity was not affected by stress in CA1.

To further assess stress-induced changes in adrenocorticosteroid receptor protein, Western blot analysis was performed on homogenates taken from the contralateral hippocampus of animals used for immunohistochemical analysis (Fig. 8B). In contrast with the immunohistochemical data, no changes in GR protein expression were detected by this method.

Discussion

Strain effects on stress responsiveness

The chronic variable stress regimen increased basal CORT and ACTH secretion in the present study. Importantly, CORT and ACTH increases were significantly increased only in the F344 group, confirming the previously observed stress hyperreactivity characteristic of this strain (38). The relatively circumscribed changes in CORT levels in the stressed S-D strain should not be interpreted as an ineffective stress regimen, as both strains showed substantial adrenal hypertrophy and thymic atrophy (consistent with prolonged elevations in HPA activity). In addition, basal CORT levels in the unhandled and handled groups in this study were slightly elevated, perhaps due to the use of the trunk blood collection procedure. These elevations may add variability to the dataset and thereby prohibit changes in the S-D chronic stress group from achieving statistical reliability. Finally, to

isolate effects of chronic stress exposure from the influence of episodic changes evoked by individual stressors, rats were killed 16 h after the last stress (during the period of the circadian trough). Thus, basal levels cannot be assumed reflect the cumulative effect of repeated episodic increases in glucocorticoids seen by the animal. It is important to note that even quite small increases in CORT may be sufficient to alter MR-mediated feedback of basal HPA activity and permit glucocorticoids to impair ongoing physiologic homeostasis (39).

In accordance with previous reports (38), F344 rats show enhanced CORT secretion upon initial presentation of an acute stressor. Strain-related hypersecretion of CORT is also seen upon presentation of a novel stressor (open field) to handled or unhandled F344 rats 10 days into the chronic stress regimen. However, stressed F344 rats show reduced CORT responses to novelty following prolonged stress exposure. These data stand in contrast to stressed S-D rats, which show responses of similar magnitude to handled or unhandled controls. The decreased response to the novel stressor in the F344 strain may be due to differential elevations in basal CORT levels by prolonged stress, suggesting enhanced feedback inhibition of the HPA system.

Despite the large strain differences in basal and stress-induced HPA activation, hippocampal adrenocorticosteroid receptor mRNA and protein are remarkably similar across the two strains. These data suggest that the HPA-related

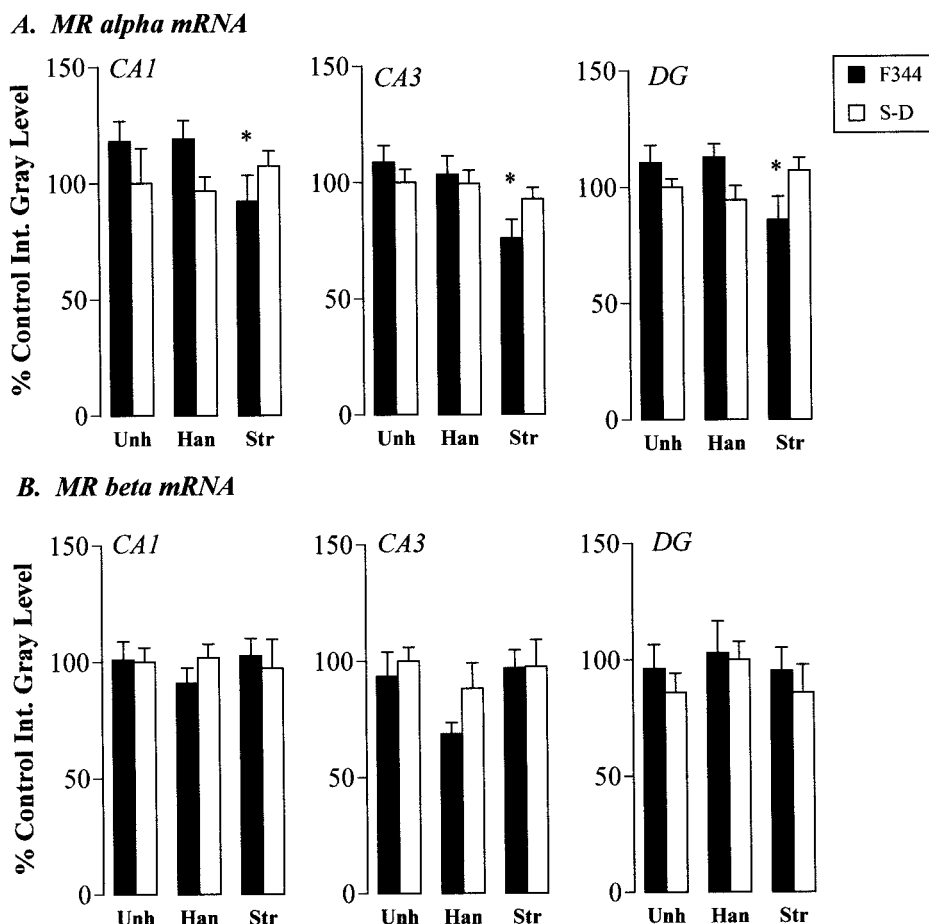


FIG. 4. Semiquantitative analysis of MR 5' splicing variant RNAs in CA1, CA3, and dentate gyrus of unhandled (Unh), handled (Han), and stressed (Str) rats [F344 and Sprague Dawley (S-D) strains]. As was the case for MR hnRNA, expression of the α 5' splice-site variant (A) was decreased by chronic stress exposure (relative to both handled and unhandled controls) in the F344, but not S-D strain (*, $P < 0.05$, Newman-Keuls test). No strain or stress changes were seen in expression of the β 5' splice-site variant (B).

strain differences are likely independent of hippocampal MR and GR expression in the regions of hippocampus examined in this study.

Stress, strain, and regulation of MR biosynthesis

Chronic stress results in down-regulation of MR hnRNA expression in F344 but not S-D rats, consistent with a strain-specific responsiveness of MR gene transcription to the effects of chronic stress. Stress down-regulation of MR hnRNA expression is accompanied by reduced expression of the MR α 5' splice variant in the F344 strain, consistent with decreased synthesis of this mRNA species. Notably, expression of the MR β splice variant is not affected by stress in either rat strain, suggesting that stress may preferentially affect specific MR promoter regions. The lack of a substantial loss of MR β , together with the lack of a stress effect on full-length MR mRNA or protein, indicates that hippocampal neurons maintain stable levels of MR in the face of changes in transcriptional activity, perhaps through modulation of mRNA stability and/or translational efficacy.

The data suggest that the MR gene is susceptible to down-regulation by long-term exposure to elevated glucocorticoids. Previous studies from our group and others provide evidence supporting this hypothesis. For example, acute restraint elicits marked reduction of MR hnRNA in all hippocampal subfields (19). Down-regulation of MR hnRNA is due to stress-induced CORT secretion (20). Expression of MR

mRNA shows a marked circadian rhythm, with decreased expression occurring shortly after the time of peak CORT secretion (21, 40). Adrenalectomy, which removes circulating glucocorticoids, elevates MR mRNA (15, 16, 22), consistent with release of glucocorticoid inhibition. Moreover, *in situ* hybridization studies indicate that ADX effects are most pronounced in hippocampal subfields CA1 and CA2 (14, 15), regions corresponding to those showing highest expression of GR mRNA and protein (14, 41, 42).

The lack of a significant effect of chronic stress on MR mRNA, as measured using the MR-3'UT probe, is somewhat at odds with previous studies. For example, a previous study in our lab demonstrated a significant decrease in MR mRNA following an analogous chronic stress regimen using S-D rats (31), despite similar CORT hypersecretion. It is possible that the discrepancy between the present report and our earlier study may be due to differences in susceptibility of the two shipments of S-D rats to stress or order of presentation of the random stressors in the two studies. Whatever the reason for the observed interstudy variance, it is clear that elevated HPA activity induced by the chronic intermittent stress paradigm is not sufficient to reduce steady-state MR message, despite effects on expression of MR hnRNA or the MR α mRNA splice variant. The lack of a significant change in MR mRNA generally supports data from previous studies, which show no changes in expression following repeated immobilization stress (32). Interestingly, chronic social stress de-

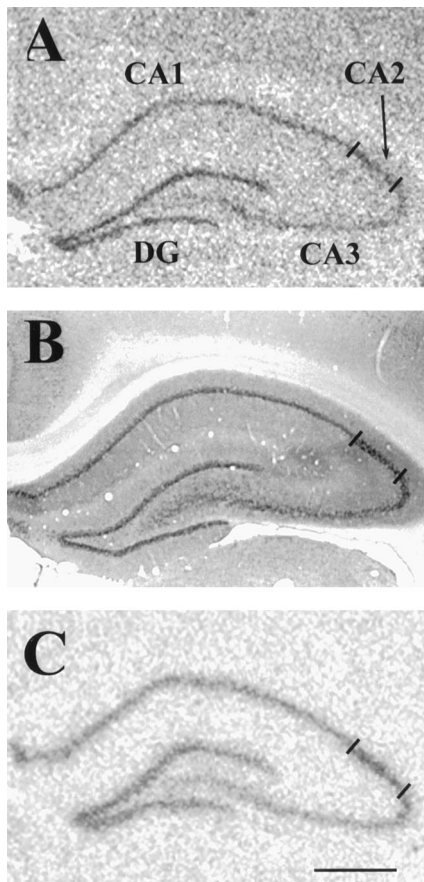


FIG. 5. Localization of MR immunoreactivity in hippocampus. A, Immunohistochemical detection of MR protein in hippocampus results in specific signal in all hippocampal subfields, including CA1–3 and the dentate gyrus (DG). Distribution of immunoreactivity agrees with that seen by standard immunohistochemical detection using chromagen staining (B). In both cases, specific immunostaining was revealed by omission of primary or secondary antibody or preabsorption with MR fusion protein. Expression of MR protein agrees with the distribution of MR mRNA as visualized by *in situ* hybridization (C). Magnification bar, 100 μ m.

creases CA1 MR (and GR) mRNA levels in a subset of subordinate animals (30), suggesting that long-term MR mRNA regulation may be mitigated by perceived stress intensity.

The length of exposure to stress may play a role in determining how MR (as well as GR) levels may be regulated. For example, adrenalectomy-induced MR and GR mRNA up-regulation appears to attenuate rapidly over time (43); similar observations have also been noted for MR protein. However, recent studies in our laboratory indicate that the expression of MR and GR mRNA does not change substantially over a wide range of stress exposure times (3–28 days) (McCreary, B. J., M. Paskitti, and J. P. Herman, unpublished observation), suggesting that any adaptation of MR and GR expression occurs quite rapidly.

The observed stability of MR mRNA expression following stress may reflect compensatory regulation of MR transcription or stability by neuronal circuitry influenced by stress exposure. The effects of glucocorticoid secretion on MR mRNA pools may be countered by enhanced activation of MR expression through the influence of stress-activated neu-

ronal pathways. Consistent with this possibility, it is known that MR mRNA levels are decreased by removal of noradrenergic or serotonergic innervations of the hippocampus (44, 45), and increased following treatment with norepinephrine/serotonin reuptake blockers (antidepressants) (46). Chronic stress is known to up-regulate both noradrenergic activity (47) and serotonin binding (48) in the hippocampus, suggesting that neurotransmitter-evoked increases in MR transcription/stability may compete with glucocorticoids for control of steady-state MR mRNA levels.

Stress regulation of MR α and MR β mRNA expression agree in principal with data derived from ADX rats. Adrenalectomy elicited approximately 83–120% increases in MR α mRNA in subfields CA1–3 of the hippocampus, without concomitant increases in MR β message (15). Increased MR α expression was accompanied by a somewhat attenuated change in full-length MR mRNA (39–47%) (15), suggesting that the effects of MR α up-regulation on the mature message pool was diluted by steady-state levels of MR β expression. The opposite effect may be occurring in the present situation, where significant decreases in the apparently glucocorticoid-sensitive MR α pool are attenuated by unaffected expression of MR β splice variant.

The strain-specific effect of chronic stress on MR gene and α splice variant regulation may be related to glucocorticoid secretion. The F344 strain shows clear elevation of CORT secretion relative to the S-D group following stress (36, 49). Given the responsiveness of the MR gene to glucocorticoids *in vivo*, it is likely that the strain difference in MR down-regulation may be related to enhanced cumulative and/or episodic secretion of glucocorticoids in the F344 strain.

Stress, strain, and regulation of GR biosynthesis

As was the case with the MR, effects of stress on GR regulation were strain dependent. Neither F344 nor S-D rats showed changes in mature GR mRNA expression, indicating that message production is not affected by prolonged stress. These results agree with previous studies using S-D rats in this stress paradigm (24). Similar results are seen using probes recognizing GR intronic sequences, suggesting a lack of long-term transcriptional activation. Glucocorticoid receptor protein expression, as measured by Western blot, was also unaffected by stress exposure, indicating no substantial change in the overall hippocampal pool of GR protein. However, immunohistochemical analysis revealed subtle subfield-specific increases in GR immunoreactivity in CA3 and DG of stressed F344 rats. Given the ability of the latter technique to resolve protein expression at the subfield level, it is possible that this increase was missed by analysis of protein expression across the entire hippocampus. Subfield-specific increases may represent regional enhancement of GR protein, perhaps occurring at the translational level. Alternatively, increased GR levels may represent enhanced translocation of GR immunoreactivity from cytoplasm to nucleus, where it is more readily detected by densitometric analysis. The latter possibility is consistent with the observed increases in glucocorticoid levels seen in F344 rats, and suggest that strain differences may be a product of altered GR occupation rather than biosynthesis.

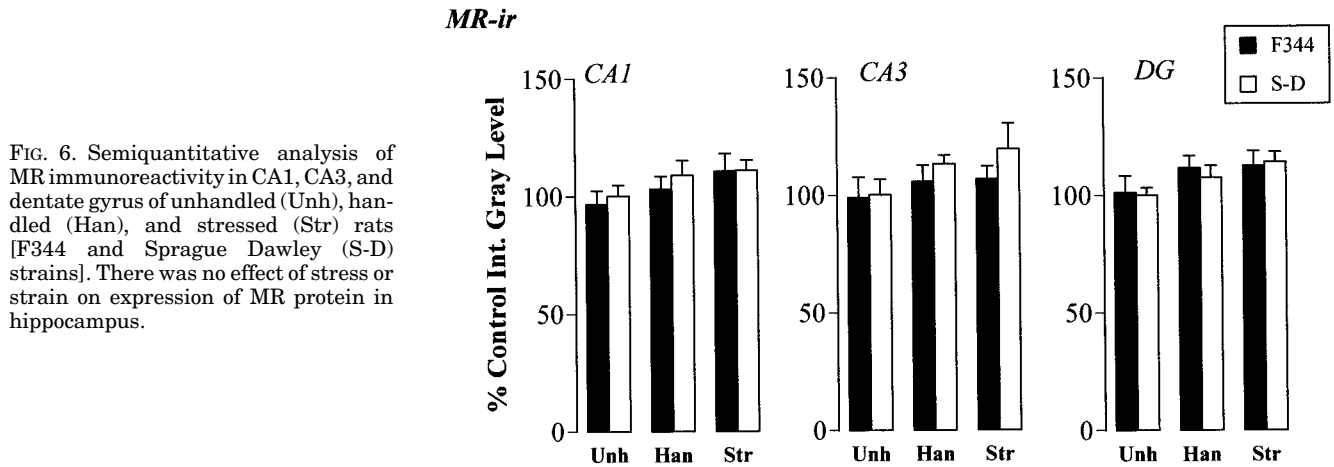


FIG. 6. Semiquantitative analysis of MR immunoreactivity in CA1, CA3, and dentate gyrus of unhandled (Unh), handled (Han), and stressed (Str) rats [F344 and Sprague Dawley (S-D) strains]. There was no effect of stress or strain on expression of MR protein in hippocampus.

A. GR hnRNA

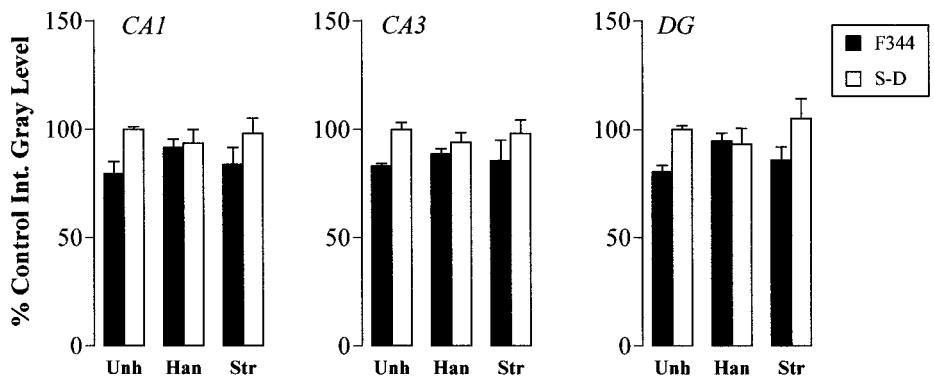
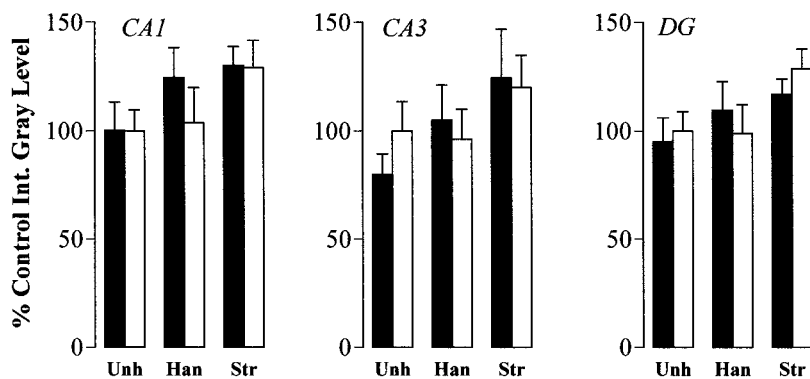


FIG. 7. Semiquantitative analysis of GR hnRNA and mRNA in CA1, CA3, and dentate gyrus of unhandled (Unh), handled (Han), and stressed (Str) rats [F344 and Sprague Dawley (S-D) strains]. There was no effect of strain or stress on GR gene transcription or mRNA expression in any hippocampal subfield.

B. GR mRNA



It is notable that the largest enhancement in GR immunoreactivity is seen in CA3. This region is known to exhibit dendritic atrophy following stress or high-dose glucocorticoid treatment (12), despite the fact that cellular GR mRNA and protein levels are lowest in this hippocampal subfield (50). Thus, it is possible that increases in nuclear GR translocation may participate in the dendritic rearrangements characteristic of this cell population.

It is notable that the increase in GR protein in CA3 occurs in the same group showing attenuated responsiveness to heterotypic stressors during chronic stress exposure. However, CA3 GR immunoreactivity is not correlated with the

magnitude of the stress response, suggesting that enhanced GR protein levels do not predict altered responsiveness. In addition, lesions of large regions of CA3 do not alter stress responsivity (51), indicating that the GR changes seen in this region are unlikely to be responsible for strain-specific differences in negative feedback. Subsequent analysis is necessary to determine whether strain differences are due to altered GR levels in other stress-relevant structure (such as the PVN and anterior pituitary).

Previous studies document varying degrees of GR mRNA down-regulation following stress or glucocorticoids. The present paradigm usually produces minimal decreases in GR

A. GRir (immunoautoradiography)

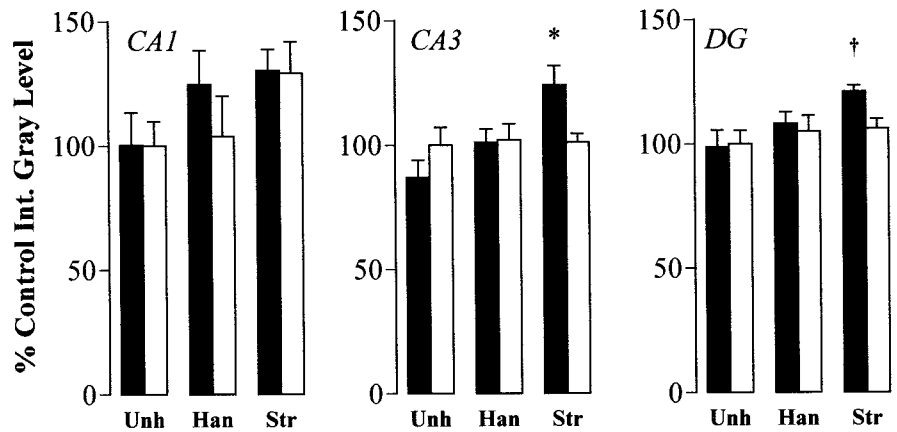
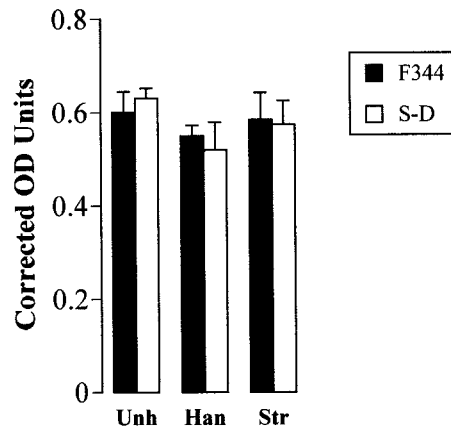


FIG. 8. Semiquantitative analysis of GR protein in the hippocampus of unhandled (Unh), handled (Han), and stressed (Str) rats [F344 and Sprague Dawley (S-D) strains]. Immunoautoradiographic analysis (A) revealed increases in GR immunoreactivity in CA3 of F344 (but not S-D) rats relative to unhandled and handled controls (*, $P < 0.05$, Newman-Keuls test). GR immunoreactivity was also elevated in the dentate gyrus of stressed F344 rats relative to the unhandled (but not handled) control group (†, $P < 0.05$, Newman-Keuls test). In contrast, Western analysis (B) performed on the contralateral hippocampus revealed no stress induced changes in immunoreactivity obtained from cell extracts.

B. GR-ir (Western)



mRNA levels (24, 31), consistent with previous studies using other stress paradigms (30, 32). In contrast, chronic immobilization can produce larger (40–50%) decrements in hippocampal GR expression in multiple rat strains, suggesting that the stability of GR expression may be modulated by stressor intensity or duration (29, 34). Nonetheless, the present report indicates that stress regimens sufficient to elicit episodic or chronic glucocorticoid hypersecretion need not result in substantial loss of hippocampal GR expression.

Overall, the present study suggests the existence of mechanisms defending stable levels of adrenocorticosteroid expression in the face of prolonged periods of elevated glucocorticoid secretion. Thus, chronic stress appears to have minimal impact on mature MR and GR mRNA pools and protein expression, despite the ability to decrease MR hnRNA and reduce the expression of one of the two major splice variants. Maintenance of stable levels of MR and GR biosynthesis appears to be a priority for hippocampal neurons, and perhaps reinforces the potential importance of the MR and GR in neuronal homeostasis and development (4, 5, 52). These data highlight the physiologic importance of both receptors in ongoing neuronal function and suggest that alteration in expression of one or both receptors by disease or aging may play a role in predisposing the hippocampus to dysfunction or degeneration.

Acknowledgments

We would like to thank Dr. Ines Morano, Dr. Claudio Caamano, and Sharon Burke for generation and characterization of the MR antibody used in this study; Dr. Keith Yamamoto for supplying GR intron and exon clones; and Mark Dolgas, Dreauna Rucker, Garrett Bowers, Xiaohang Wang, David Morrison, and Crystal Cotter for expert technical assistance.

References

- Liu W, Wang J, Sauter NK, Pearce D 1995 Steroid receptor heterodimerization demonstrated *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* 92:12480–12484
- Trapp T, Rupprecht R, Castern M, Reul JHM, Holsboer F 1994 Heterodimerization between mineralocorticoid and glucocorticoid receptor: a new principle of glucocorticoid action in the CNS. *Neuron* 13:1457–1462
- Reul JM, deKloet ER 1985 Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology* 117:2505–2511
- Woolley CS, Gould E, Sakai RR, Spencer RL, McEwen BS 1991 Effects of aldosterone or RU28362 treatment on adrenalectomy-induced cell death in the dentate gyrus of the adult rat. *Brain Res* 554:312–315
- McCullers DL, Herman JP 1998 Mineralocorticoid receptors regulate Bcl2 and p53 mRNA expression in rat hippocampus. *NeuroReport* 9:3085–3089
- Spencer RL, Young EA, Choo PH, McEwen BS 1990 Adrenal steroid type I and type II receptor binding: estimates of *in vivo* receptor number, occupancy, and activation with varying level of steroid. *Brain Res* 514:37–48
- Kerr DS, Campbell LW, Thibault O, Landfield PW 1992 Hippocampal glucocorticoid receptor activation enhances voltage-dependent calcium conductances: relevance to brain aging. *Proc Natl Acad Sci USA* 89:8527–8531
- Foy MR, Stanton ME, Levine S, Thompson RF 1987 Behavioral stress impairs long-term potentiation in rodent hippocampus. *Behav Neural Biol* 48:138–149
- Pavlidis C, Watanabe Y, McEwen BS 1993 Effects of glucocorticoids on hippocampal long-term potentiation. *Hippocampus* 3:183–192

10. Shors TJ, Seib TB, Levine S, Thompson RF 1989 Inescapable versus escapable shock modulates long-term potentiation in the rat hippocampus. *Science* 244:224–226
11. Bodnoff SR, Humphreys AG, Lehman JC, Diamond DM, Rose GM, Meaney MJ 1995 Enduring effects of chronic corticosterone treatment on spatial learning, synaptic plasticity, and hippocampal neuropathology in young and mid-aged rats. *J Neurosci* 15:61–69
12. Magarinos AM, McEwen BS 1995 Stress-induced atrophy of apical dendrites of hippocampal CA3 neurons: comparison of stressors. *Neuroscience* 69:83–88
13. Sapolsky RM 1994 The physiological relevance of glucocorticoid endangerment of the hippocampus. *Ann NY Acad Sci* 746:294–304; discussion 304–307
14. Herman JP, Patel PD, Akil H, Watson SJ 1989 Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger RNAs in the hippocampal formation of the rat. *Mol Endocrinol* 3:1886–1894
15. Kwak SP, Patel PD, Thompson RC, Akil H, Watson SJ 1993 5'-heterogeneity of the mineralocorticoid receptor messenger ribonucleic acid: differential expression and regulation of splice variants within the rat hippocampus. *Endocrinology* 133:2344–2350
16. Patel PD, Kwak SP, Herman JP, Young EA, Akil H, Watson SJ 1992 Functional heterogeneity of type I and type II corticosteroid receptor expression in rat hippocampus. In: Sheppard KE, Boublik JH, Funder JW (eds) *Stress and Reproduction*. Raven Press, New York, pp 1–17
17. Castrén M, Damm K 1993 A functional promoter directing expression of a novel type of rat mineralocorticoid receptor mRNA in brain. *J Neuroendocrinol* 5:461–466
18. Zennaro MC, Le Menuet D, Lombes M 1996 Characterization of the human mineralocorticoid receptor gene 5'-regulatory region: evidence for differential hormonal regulation of two alternative promoters via nonclassical mechanisms. *Mol Endocrinol* 10:1549–1560
19. Herman JP, Watson SJ 1995 Stress regulation of mineralocorticoid receptor heteronuclear RNA in rat hippocampus. *Brain Res* 677:243–249
20. Herman JP, Watson SJ 1994 Glucocorticoid regulation of stress-induced mineralocorticoid receptor gene transcription *in vivo*. *Ann NY Acad Sci* 746:485–488
21. Herman JP, Watson SJ, Chao HM, Coirini HM, McEwen BS 1993 Diurnal regulation of glucocorticoid receptor and mineralocorticoid receptor mRNAs in the rat hippocampus. *Mol Cell Neurosci* 4:181–190
22. Reul JM, Pearce PT, Funder JW, Krozowski ZS 1989 Type I and type II corticosteroid receptor gene expression in the rat: effect of adrenalectomy and dexamethasone administration. *Mol Endocrinol* 3:1674–1680
23. Tornello S, Orti E, DeNicola AF, Rainbow TC, McEwen BS 1982 Regulation of glucocorticoid receptors in brain by corticosterone treatment of adrenalectomized rats. *Neuroendocrinology* 35:411–417
24. Herman JP, Spencer RL 1998 Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression *in vivo*. *J Neurosci* 18:7462–7473
25. Chao HM, Ma LY, McEwen BS, Sakai RR 1998 Regulation of glucocorticoid receptor and mineralocorticoid receptor messenger ribonucleic acids by selective agonists in the rat hippocampus. *Endocrinology* 139:1810–1814
26. Miller AH, Spencer RL, Husain A, Rhee R, McEwen BS, Stein M 1993 Differential expression of type I adrenal steroid receptors in immune tissues is associated with tissue-specific regulation of type II receptors by aldosterone. *Endocrinology* 133:2133–2140
27. Oakley RH, Cidlowski JA 1993 Homologous down-regulation of the glucocorticoid receptor: the molecular machinery. *Crit Rev Eukaryot Gene Expr* 3:63–88
28. Sapolsky RM, McEwen BS 1985 Down-regulation of neural corticosterone receptors by corticosterone and dexamethasone. *Brain Res* 339:161–165
29. Gomez F, Lahmame A, de Kloet ER, Armario A 1996 Hypothalamic-pituitary-adrenal response to chronic stress in five inbred rat strains: differential responses are mainly located at the adrenocortical level. *Neuroendocrinology* 63:327–337
30. Chao HM, Blanchard DC, Blanchard RJ, McEwen BS, Sakai RR 1993 The effect of social stress on hippocampal gene expression. *Mol Cell Neurosci* 4:543–548
31. Herman JP, Adams D, Prewitt CM 1995 Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. *Neuroendocrinology* 61:180–190
32. Mamalaki E, Kvetnansky R, Brady LS, Gold PW, Herkenham M 1993 Repeated immobilization stress alters tyrosine hydroxylase, corticotropin-releasing hormone and corticosteroid receptor ribonucleic acid levels in rat brain. *J Neuroendocrinol* 4:689–699
33. Eldridge JC, Brodish A, Kute TE, Landfield PW 1989 Apparent age-related resistance of type 2 hippocampal corticosteroid receptors to down-regulation during chronic escape training. *J Neurosci* 9:3237–3242
34. Makino S, Smith MA, Gold PW 1995 Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. *Endocrinology* 136:3299–3309
35. Sapolsky RM, Krey LC, McEwen BS 1984 Stress down-regulates corticosterone receptors in a site-specific manner in the brain. *Endocrinology* 114:287–292
36. Dhabhar FS, Miller AH, McEwen BS, Spencer RL 1995 Differential activation of adrenal steroid receptors in neural and immune tissues of Sprague Dawley, Fischer 344, and Lewis rats. *J Neuroimmunol* 56:77–90
37. Westphal U 1971 *Steroid-Protein Interactions*. Springer, New York
38. Dhabhar FS, McEwen BS, Spencer RL 1997 Adaptation to prolonged or repeated stress—comparison between rat strains showing intrinsic differences in reactivity to acute stress. *Neuroendocrinology* 65:360–368
39. Dallman MF, Akana SF, Bhatnagar S, Bell ME, Strack AM, Bottomed out: metabolic significance of the circadian trough in glucocorticoid concentrations. *Int J Obes*, in press
40. Holmes MC, French KL, Seckl JR 1997 Dysregulation of diurnal rhythms of serotonin 5-HT_{2C} and corticosteroid receptor gene expression in the hippocampus with food restriction and glucocorticocoids. *J Neurosci* 17:4056–4065
41. Fuxe K, Wikstrom AC, Okret S, Agnati LF, Harfstrand A, Yu ZY, Granholm L, Zoli M, Vale W, Gustafsson JA 1985 Mapping of glucocorticoid receptor immunoreactive neurons in the rat tel- and diencephalon using a monoclonal antibody against rat liver glucocorticoid receptor. *Endocrinology* 117:1803–1812
42. Ahima RS, Harlan RE 1990 Charting of type II glucocorticoid receptor-like immunoreactivity in the rat central nervous system. *Neuroscience* 39:579–604
43. Holmes MC, Yau JL, French KL, Seckl JR 1995 The effect of adrenalectomy on 5-hydroxytryptamine and corticosteroid receptor subtype messenger RNA expression in rat hippocampus. *Neuroscience* 64:327–337
44. Yau JLW, Seckl JR 1992 Central 6-hydroxydopamine lesions decrease mineralocorticoid, but not glucocorticoid receptor gene expression in the rat hippocampus. *Neurosci Lett* 142:159–162
45. Seckl JR, Dickson K, Fink G 1990 Central 5,7-dihydroxytryptamine lesions decrease hippocampal glucocorticoid and mineralocorticoid receptor messenger ribonucleic acid expression. *J Neuroendocrinol* 2:911–916
46. Seckl JR, Fink G 1992 Antidepressants increase glucocorticoid and mineralocorticoid receptor mRNA expression in rat hippocampus *in vivo*. *Neuroendocrinology* 55:621–626
47. Nisenbaum LK, Abercrombie ED 1993 Presynaptic alterations associated with enhancement of evoked release and synthesis of norepinephrine in hippocampus of chronically cold-stressed rats. *Brain Res* 608:280–287
48. McKittrick CR, Blanchard DC, Blanchard RJ, McEwen BS, Sakai RR 1995 Serotonin receptor binding in a colony model of chronic social stress. *Biol Psychiatry* 37:383–393
49. Dhabhar FS, McEwen BS, Spencer RL 1993 Stress response, adrenal steroid receptor levels and corticosterone-binding globin—a comparison between Sprague-Dawley, Fischer 344 and Lewis rats. *Brain Res* 616:89–98
50. Herman JP 1993 Regulation of adrenocorticosteroid receptor mRNA expression in the central nervous system. *Cell Mol Neurobiol* 13:349–372
51. Herman JP, Dolgas CM, Carlson SC 1998 Ventral subiculum co-ordinates situation-specific neuroendocrine and behavioral stress responses. *Neuroscience* 86:449–459
52. Vazquez DM, Lopez JF, Morano MI, Kwak SP, Watson SJ, Akil H 1998 Alpha, beta, and gamma mineralocorticoid receptor messenger ribonucleic acid splice variants: differential expression and rapid regulation in the developing hippocampus. *Endocrinology* 139:3165–3177