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The Role of the Hippocampal Mineralocorticoid and Glucocorticoid Receptors in the Hypothalamo–Pituitary–Adrenal Axis of the Aged Fisher Rat

MARÍA INÉS MORANO,[1] DELIA M. VÁZQUEZ, AND HUDA AKIL

Mental Health Research Institute, University of Michigan, Ann Arbor, Michigan 48109-0720

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The aging process has been frequently associated with hippocampal neurodegeneration, loss of corticosteroid receptors, and, at the same time, dysfunction of the hypothalamo–pituitary–adrenal (HPA) axis. We were interested in characterizing simultaneously the activity of the HPA axis and status of both corticosteroid receptors (mineralocorticoid or MR and glucocorticoid or GR) in the hippocampus of aged male Fisher-344 rats. We compared intact, adrenalectomized (ADX), and corticosterone-replaced ADX young (5–6 months) and old (26–27 months) rats, examining all the parameters in the same animals. Aged rats exhibited an unaltered basal rhythm and initial corticosterone response to restraint stress. However, the same old animals showed a delayed turn-off of the stress response and did so at different points of the corticosterone circadian cycle. The aged hippocampus showed a 40–50% lower MR and GR binding under all the conditions studied. This aging effect was not attributable to changes in the kinetics, affinity, or nuclear translocation of MR or GR. Intact aged rats exhibited also a 30–40% reduction of hippocampal MR and GR steady-state mRNA levels. Interestingly, after 36 h ADX only the aged hippocampus showed upregulation of MR and GR mRNA content to levels comparable to those in young rats. However, this increase in MR and GR mRNA content was not accompanied by a proportional increase in the B_{max} of these receptors, suggesting age-related translational or post-translational alterations. Moreover, corticosterone replacement was able to reverse the ADX-induced increase of MR and GR B_{max} in young and old hippocampi but it only reversed the upregulated mRNA levels of MR (and not GR) in the older group. The fact that corticosterone was able to modulate the biosynthetic rate of MR and GR strongly suggests that the decrease of receptors is functional and not simply due to cell death in the aged hippocampus. We propose that in the aged Fisher rat the loss of hippocampal corticosteroid receptors is previous to any change in the circadian rhythm of circulating corticosterone. Furthermore, the altered turn-off of the corticosterone stress response observed in the same animals may be related to the reduction of functional MR and GR but it is not due to high basal levels of corticosterone. © 1994 Academic Press, Inc.

INTRODUCTION

The hippocampus has been implicated in the neuronal regulation of the hipothalamic–pituitary–adrenal (HPA) axis activity (1–6). In the rat, the available evidence suggests that the hippocampal formation inhibits the adrenocortical system via an influence on the hypophysiotropic neurons of the hypothalamus. Thus, our laboratory has shown that dorsal or complete hippocampectomy produces increased levels of corticotropin releasing factor (CRF) and vasopressin (AVP) mRNAs in the paraventricular nucleus and hypersecretion of ACTH and corticosterone (4). In agreement, fornix transection results in elevation of hypophysial portal concentration of CRF, AVP, and oxytocin (6). Implication of the hippocampus as a suprahypothalamic site mediating glucocorticoid feedback inhibition of the HPA axis was first suggested by the demonstration that the hippocampus exhibits the highest brain levels of specific, high-affinity uptake and retention of corticosterone (7). Two corticosterone receptor systems have been found in rat brain, type I (K_d = 0.5–1 nM) and type II (K_d = 5–10 nM) (8). Both receptors have been cloned, identifying the type I as the mineralocorticoid receptor (MR) and the type II as the classic glucocorticoid receptor (GR) (9, 10). Studies using receptor autoradiography (8), immunohistochemistry (11), and *in situ* hybridization (12, 13) show that both MR and GR are highly expressed in hippocampal pyramidal neurons, although differentially distributed across the pyramidal cell layer. In contrast to MR, GR is also widely expressed in glial cells (8, 11–13). Both hippocampal receptors have been found to affect the HPA activity (14, 15). While MR was originally implicated in the regulation of the basal levels of ACTH during the nadir of the rhythm (14, 16, 17), more recently it has been suggested that MR is also

[1] To whom correspondence should be addressed.

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involved in the inhibition of the circadian ACTH peak (18). On the other hand, hippocampal GR has been proposed to mediate the negative feedback of the stress response (8, 14, 17) as well as the circadian ACTH peak (18).

In the rat, the aging process is frequently associated with dysfunction of the HPA axis (19–36) and, at the same time, with hippocampal neurodegeneration (37–40). One of the dominant hypotheses in the field links these two phenomena, suggesting that elevation of resting glucocorticoid levels at the nadir is typically observed in aged animals and further proposing that this is part of a cascade of events in which elevated circulating levels can lead to hippocampal cell death of the pyramidal layer, resulting loss of corticosteroid receptors and a further inability to keep the HPA axis in proper check, thereby leading to further damage (41). However, not all aging studies agree. While a number of studies have shown higher circulating levels of corticosterone in aged male rats under resting conditions (19–25), other investigations have failed to observe any age-related difference under the same conditions (26–36). On the other hand, the majority of the available evidence indicates that although young and old male rats exhibit a similar corticosterone stress response, the recovery to basal corticosterone levels after the stress is delayed in the older group (20, 29, 30, 33, but also see 36). These effects have been attributed to the loss of corticosterone receptors in the brain regions that mediate the feedback inhibition of the HPA activity (22, 38, 41–43). However, recent reports provide conflicting data. One group of researchers found no age-related changes in hippocampal MR or GR binding parameters in male Long Evans and Fisher-344 rats (44). Another group observed an increase in the affinity for corticosterone of GR, but not MR, in old Fisher-344 rats (45) and an impaired upregulation of GR binding capacity after adrenalectomy (ADX) in the hippocampus of the same animals (46). Moreover, in the Brown Norway rat, the hippocampus displayed a decrease of MR but not GR (36). Interestingly, although both MR and GR were decreased in the hippocampus of aged Wistar rats, only the MR loss could be reversed with an ACTH analog (42). In summary, all these conflicting data may be due to differences in the strain, sex (30), age of the animals at the time of the experiments, etc. Individual differences should be also taken into account according to Issa *et al.* (47).

Another important factor to be considered is that all the binding measurements of MR and GR were typically done after short-term ADX because of methodological constraints (once the corticosterone receptors are "activated" they are unable to exchange ligand in the *in vitro* binding assays; 48, 55). This was based on the assumption that this period of time is sufficient for the clearance of endogenous corticosterone but not for upregulation of the receptors in young rats (49). Consequently, the age effect on GR mRNA levels has been evaluated in the hippocampus of intact Long Evans rats but correlated with GR binding in ADX rats (50). vanEekelen *et al.* (36) studied both MR and GR under the same conditions; they used the less common Brown Norway rat. Therefore, it is rare in the literature to see all relevant parameters studied in the same animal. However, it is critical to get a full endocrine profile for a given animal, especially in view of the individual variability and the apparent effects of sex, strain, etc. Only a complete profile would allow a real characterization of the HPA axis of the animals and may begin to shed light on possible mechanistic changes leading to aging-induced dysregulation.

In order to clarify some of the current controversy regarding corticosterone receptors and HPA activity during aging, we designed a series of studies in which we compared intact, ADX, or corticosterone-replaced ADX young and old Fisher-344 rats and in which the plasma corticosterone circadian rhythm and stress response, plasma corticosterone binding activity, hippocampal MR and GR binding parameters, and mRNAs levels were measured in the *same* rat. Our results show that although the circadian rhythm of plasma corticosterone is similar in young and old animals, the aged rats exhibit an aberrant stress response during both the light phase and the onset of the dark period of the cycle. In addition, we find that although there is a significant reduction in the number of both corticosteroid receptors, the hippocampus capacity for modulation in response to changes in circulating corticosterone is more vigorous in the aged animal than that in the young animal.

MATERIALS AND METHODS

Animals

A total of 150 male Fisher-344 rats were obtained from the National Institute of Aging's specific pathogen-free colony at either 5–6 months (young controls) or 26–27 months (old) of age. Animals were group-housed, 2–3 per cage, and acclimated for 14 days to the new environment (12-h light–dark cycle, 6 AM lights-on) prior to experimentation. Food and water were given *ad libitum.* The health of the subjects was monitored frequently, and any rats exhibiting overt signs of pathology before or at the time of the sacrifice were excluded from the study.

Steroids

[1,2,6,7-^{3}H]corticosterone (75–105 Ci/mmol), [1,2,6,7-^{3}H]aldosterone (75 Ci/mmol), and [1,2,3,4-^{3}H]dexamethasone (70–110 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Unlabeled corticosterone and dexamethasone were obtained from Sigma (St. Louis, MO). The selective GR receptor agonists, RU28362 and RU26988, were a gift from Roussel-Uclaf (Romainville, France).

Adrenalectomy

Bilateral adrenalectomy and laparatomy (sham operation) were performed via a dorsal approach on animals

fully anesthetized with the inhalant metoxyflurane (Metofane; Pitman-Moore, Mundelein, IL). ADX rats received 0.9% saline as drinking water while the sham-operated (SHAM) rats continued to receive tap water.

Sequential Blood Sampling

Blood samples (~30 μl) were taken under resting conditions from the tail vein (within 30 s following the removal of the animals from their cages) at various times over the diurnal cycle. A safe-light was used during the dark phase to avoid exposure to light that might alter the circadian rhythm.

In the restraint stress studies, rats were placed at 8 AM (2 h after lights-on) or 6 PM (lights-off) into big wood restrainers for 60 min and then returned to their home cages. Unstressed control animals without stress were bled simultaneously. Blood samples (~30 μl) were taken from the tail vein at the following time points after the beginning of the restraint: 0, 1, 2, 3, 4, 6 h. Blood was collected in heparinized microhematocrit capillary tubes. Plasma obtained after centrifugation was frozen in dry ice and stored at −80°C until assayed.

In addition, trunk blood was obtained under resting conditions. Animals were sacrificed between 8 and 9 AM (less than 20 s following removal from their cages). Samples were collected in cold tubes containing EDTA, centrifuged, and stored at −80°C until assayed.

Corticosteroid Receptor Binding Assays

Adrenal steroid binding to intracellular receptors was measured *in vitro* in four different experiments. Intact rats were used in the first experiment. In the other experiments, ADX and SHAM rats were killed 36 h after the surgery. In the last experiment, ADX rats were also injected sc with 5 mg corticosterone in sesame oil (51) on the following two evenings before the sacrifice. Following decapitation, the brain of each animal was quickly removed and chilled on ice and both hippocampi were dissected. One hippocampus from each rat was frozen on dry ice and stored at −80°C for subsequent isolation of total nucleic acids (see below). The other hippocampus from each rat was placed in ice-cold TEDMG (10 m*M* Tris, 1 m*M* EDTA, 5 m*M* dithiothreitol, 20 m*M* sodium molybdate, and 10% (v/v) glycerol, pH 7.6) and rapidly homogenized. Homogenates were ultracentrifuged at 105,000*g* for 60 min at 0–2°C and the resulting supernatants (cytosols: 2–4 mg protein/ml) were added to incubation tubes containing the radiolabeled steroids with or without competitors.

A microassay technique (46) was adapted for these experiments. After overnight incubation (16–20 h, 4°C), bound and free hormones were separated by addition of an ice-cold dextran/charcoal suspension in TEDMG (0.05/0.5%). The tubes were incubated 10 min at 0–2°C and centrifuged. The supernatants were removed and counted in a Packard scintillation counter at 45% efficiency.

For single receptor binding capacity determinations, aliquots (50 μl) of cytosol from a single hippocampus were incubated with a saturating concentration (20 n*M*) of [^{3}H]dexamethasone either alone or with 2 m*M* RU28362 or 10 m*M* dexamethasone as competitors (each condition in triplicate). For saturation binding assays, aliquots of pooled cytosols from ADX rats were incubated in triplicate, with six different concentrations of [^{3}H]dexamethasone (0.5–20 n*M*). Parallel dilutions were also incubated with 100-fold excess of RU28362 and 500-fold excess of dexamethasone. Type II binding was derived from the RU28362 displacement of the [^{3}H]dexamethasone binding. Type I binding was determined from the difference between total and type II [^{3}H]dexamethasone binding. Binding in the presence of 10 μ*M* dexamethasone was used for determining nonspecific binding (~10% of the total binding). Protein content was measured by the method of Bradford (52) using BSA as standard, and the receptor densities were expressed as fmol/mg protein. The apparent maximal binding capacity (B_{max}) and dissociation constant (K_d) of type I and type II receptors were calculated by Scatchard analysis (53).

It has been found that [^{3}H]dexamethasone in the presence of RU26988 is an effective ligand for measuring type I adrenal steroid receptors *in vitro* (54, 55). In order to validate our technique, in a separate study we compared type I binding of young and old cytosols using parallel incubations with 20 n*M* [^{3}H]dexamethasone and 6 n*M* [^{3}H]aldosterone (both in the presence of 2 μ*M* RU28362). Nonspecific binding was determined in each case in the presence of an excess of dexamethasone (10 μ*M*). Similar values of B_{max} were obtained with both radioligands for each group of animals.

Finally, an attempt was made to compare the degree of endogenous occupation, without activation, of both adrenal steroid receptors in young and old rats. Using an adaptation of the Meaney *et al.* (56) method, in the fourth experiment parallel incubations of the hippocampal cytosols were performed during 4 and 24 h as described above. According to these authors and our own experience (not shown), after 4 h of incubation the radiolabeled steroid has not had time to exchange with the endogenous steroid, while after 20 h of incubation the total number of nonactivated corticosteroid receptors (occupied and unoccupied) could be determined. Therefore, the occupied, but not activated, type I and type II receptors were defined as the difference in specific binding from a sample incubated for 4 h compared to a sample from the same cytosolic fraction incubated for 24 h. Four groups of rats (young ADX, old ADX, young SHAM, and old SHAM), rapidly killed at 8 AM, were used in this study.

Corticosteroid Receptor mRNA Determinations

One hippocampus from each rat was frozen on dry ice and stored at −80°C until GR and MR mRNA levels were measured by RNase A protection assays. Total nucleic acids were extracted from each hippocampus by homog-

enization in 500 μl of LET buffer (10 m*M* Tris, 10 m*M* EDTA, 1% lithium dodecyl sulfate, pH 7.5) containing 200 μg/ml proteinase K (Boehringer–Mannheim, Indianapolis, IN) and incubation for 60 min at 42°C. After protein digestion the homogenates were phenol, phenol/chloroform, and chloroform extracted, and the nucleic acids were precipitated with ethanol and stored at −20°C.

The GR and MR RNase A protection assays were performed as reported previously (57). The RNA probes were synthetized as [^{32}P]UTP-labeled cRNAs from appropriate cDNA constructs in pGEM, using T7 and SP6 RNA polymerases. A GR clone (a gift from Dr. K. Yamamoto, UCSF) (9) spanning 450-bp of the 3′-translated region and untranslated flanking sequences and a 550-bp MR cDNA (10) fragment of the 3′-translated and untranslated regions of the mRNA were used as templates for GR and MR cRNA synthesis, respectively.

For each assay, 4 μl of the sample (1:20 dilution) was added to 8 μl of hybridization mix containing 2 to 5 fmol of each of the GR and MR riboprobes (40 m*M* PIPES, pH 6.4; 400 m*M* NaCl; 1 m*M* Na_2 EDTA; 50% freshly deionized formamide). Hybridization took place at 50°C during 14–16 h. RNase digestion was performed by incubation with 100 μl RNase buffer (10 m*M* Tris, pH 7.5; 5 m*M* Na_2 EDTA; 200 m*M* NaCl; 100 m*M* LiCl) containing 50 μg/ml DNase-free RNase A (Boehringer–Mannheim) for 30 min at room temperature. RNase activity was neutralized by adding 2 μl of 1% SDS and 2 μl proteinase K (10 mg/ml) at 37°C for 30 min. Samples were then ethanol precipitated, resuspended, and analyzed on 4% nondenaturing polyacrylamide gels. Gels were exposed to Kodak XAR films with intensifying screens at −70°C, and care was taken to avoid saturating the X-ray films. Protected bands were quantified by densitometry using Image software.

Determination of Hippocampal and Plasma Corticosterone

For corticosterone measurement in hippocampal subcellular fractions of intact rats, the method described in detail elsewhere (58) was used. Briefly, both hippocampi from each rat were rapidly dissected after decapitation and pooled (3 rats/determination). The tissues were homogenized in 0.32 *M* sucrose at 0–2°C and centrifuged at 850*g*. The crude nuclear pellets were washed twice and homogenized in water. The supernatants from the first centrifugation were spun at 105,000g to obtain the cytosolic fractions. Both fractions were extracted first with petroleum ether and then with dichloromethane. The extracts were evaporated to dryness and the corticosterone was resuspended in RIA buffer (50 m*M* sodium phosphate buffer, pH 7.5, containing 2.5% bovine serum albumin (BSA)).

For plasma corticosterone measurement, aliquoted samples (5 μl) from each animal were extracted with dichloromethane to eliminate endogenous transcortin. After evaporation of the solvent, the corticosterone was resuspended in RIA buffer. Recoveries of [^{3}H]corticosterone through the whole procedure were higher than 90%.

Corticosterone was assayed by RIA using a rabbit antiserum (B3a) raised against B-21-hemisuccinate:BSA. This antiserum, used at a final titer of 1:4000, cross-reacts 2% with cortisol and deoxycorticosterone and less than 0.3% with progesterone, estradiol, testosterone, or aldosterone. [^{3}H]corticosterone was used as tracer. The detection limit of the RIA was 1 pg of corticosterone, and the intra- and interassay coefficients of variation were 2 and 3%, respectively.

Plasma Corticosterone Binding Activity

Corticosterone binding activity in plasma was measured according to a modification of the Hammond and Lähteenmäki method (59). Briefly, each plasma sample was stripped of endogenous steroids by treatment during 30 min with a dextran/charcoal (0.25/2.5%) suspension in phosphate-buffered saline containing 0.1% gelatin, pH 7.4. After centrifugation, aliquots of the supernatants (1:400 plasma dilution) were incubated during 75 min with 1 pmol of [^{3}H]corticosterone in the presence or absence of 100-fold excess of corticosterone. A new treatment with the dextan/charcoal suspension was performed and the supernatants were removed. The concentration of [^{3}H]corticosterone specifically bound to plasma proteins was calculated after subtracting the nonspecific binding.

Statistical Analysis

Data are expressed as means ± SEM. Analysis of variance was used for testing overall differences between groups (two-factor ANOVA for the analysis of age and treatment or age and time of the day and three-factor ANOVA for the analysis of age, treatment, and time of the day). Post hoc comparisons were made by using the Fisher protected least-significant difference (probability value of less than 0.05).

RESULTS

Basal Corticosterone Levels in Young and Old Animals

In order to evaluate the hormonal status of our young and old Fisher-344 rats, we first studied their basal corticosterone levels over the diurnal cycle (Fig. 1). Both groups of animals showed the characteristic corticosterone circadian profile with a significant peak ($P < 0.0001$) at the beginning of the dark period. However, there were no significant differences between groups at any time point, indicating that young and old Fisher rats exhibit a comparable circadian pattern of circulating corticosterone. Interestingly, the plasma corticosterone levels of these Fisher rats were higher in the peak than those in young Sprague Dawley rats assayed at the same time ($F = 35 \pm 3$ μg/dl vs S–D = 20 ± 3 μg/dl, $n = 8$).

The capacity of plasma proteins to bind [^{3}H]corticosterone, with the major fraction represented by transcor-

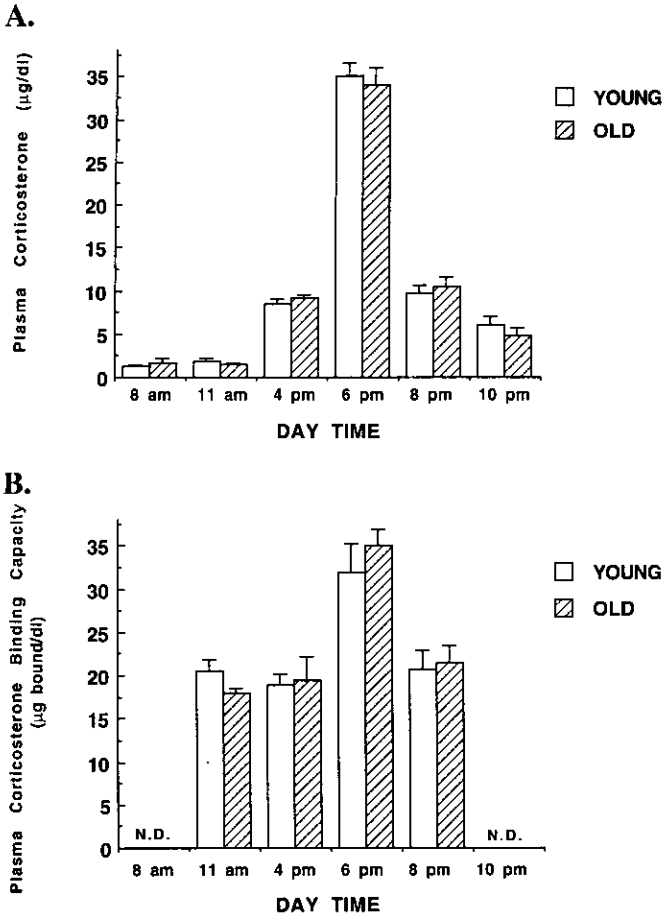

FIG. 1. Diurnal rhythm of plasma corticosterone levels (A, μg/dl) and corticosterone binding capacity (B, mg bound/dl) in young and old Fisher rats. Each bar represents the mean ± SEM of five animals. Lights-off, 6 PM. N.D., not detected. (A) time effect $P < 0.0001$; (B) time effect $P < 0.0001$.

tin or CBG, was also determined in the same samples. A circadian CBG rhythm was observed in the plasma of both groups, with higher levels in the interface light/dark of the cycle ($P < 0.0001$). Nevertheless, there were no group differences, suggesting that plasma free corticosterone is comparable in young and old Fisher rats.

Table 1 shows plasma determinations obtained when intact animals were quickly killed (less than 20 s following the removal from the home cages) between 8 and 9 AM. No group differences were observed either.

Stress-Induced Corticosterone Levels in Young and Old Animals

Stress-induced changes in circulating corticosterone were examined by applying 1 h restraint stress to young and old rats (Fig. 2). Stress was begun 2 h after lights-on (Fig. 2A) and at the beginning of the dark period (Fig. 2B). Nonrestraint (control) rats were bled simultaneously. There was a significant effect of time of day ($P < 0.0001$) and of treatment (restraint stress; $P < 0.0001$). Both groups achieved comparable concentrations of corticosterone at the end of the stress period (no age effect: $P < 0.6$ in AM and $P < 0.8$ in PM). However, old restrained animals showed a delayed recovery to basal corticosterone levels in comparison with young animals (age effect: $P < 0.002$ in AM, Fig. 2A and $P < 0.01$ in PM, Fig. 2B). Post hoc test demonstrated significant differences between the two groups of restrained rats 1 and 2 h after the termination of the stressor ($P < 0.05$).

Adrenal Weight of Young and Old Animals

Adrenal wet weight of aged rats was significantly increased over that of young animals ($P < 0.005$). However, when the adrenal weight was expressed per 100 g body weight, no group difference was found (Table 1).

Type I (MR) and Type II (GR) Receptors in Hippocampus of Intact Rats

Analysis of the results of cytosolic binding assays shows a significant decrease of both corticosteroid receptors, MR (−44%) and GR (−51%), in the hippocampus of aged intact animals (Fig. 3A; $P < 0.05$), suggesting a lower number of nonactivated binding sites in the older group. Anterior pituitaries and hypothalami from the same rats were examined in parallel for MR and GR binding and showed no age-related differences (result not shown).

While one hippocampus from each animal was used for the binding assay, the other one was employed for determination of MR and GR mRNA levels (see Materials and Methods). Each sample was hybridized with both probes and the RNase-protected mRNAs were analyzed on polyacrylamide gels as shown in Fig. 4. Single bands can be observed in the positions corresponding to MR and GR sizes. Using this technique, intact old rats exhibited 40% less MR and GR mRNA levels than the young ones (Fig. 3B, $P < 0.05$).

TABLE 1

Mean ± SEM of Multiple Variables in Young and Old Fisher Rats

	Young	Old
Plasma corticosterone (μg/dl)	1.6 ± 0.2 (30)	1.5 ± 0.1 (34)
Corticosterone binding capacity (μg bound/dl)	18.5 ± 0.8 (16)	17.2 ± 1.3 (14)
Body weight (g)	343 ± 7 (8)	405 ± 12 (8)*
Adrenal weight (mg)	52.4 ± 1.4 (21)	64.8 ± 2.6 (24)*
Adrenal weight (mg/100 g body weight)	15.6 ± 0.4 (8)	16.1 ± 0.9 (8)

Note. Basal corticosterone levels and corticosterone binding capacity were measured in animals killed between 8 and 9 AM. Group differences significant at *$P < 0.005$.

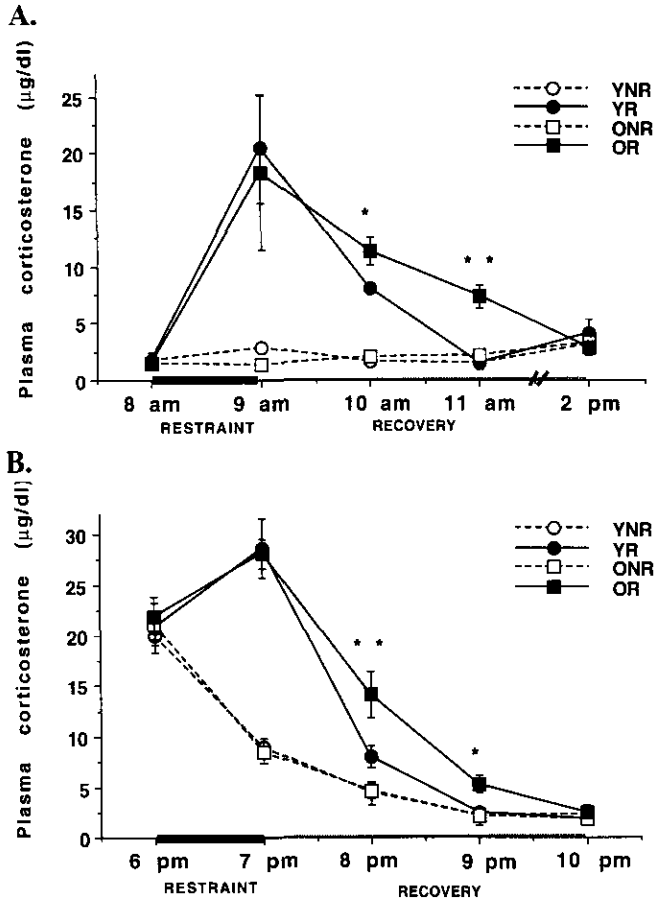

FIG. 2. Plasma corticosterone (μg/dl) levels in young (Y) and old (O) restraint (R) and nonrestraint (NR) rats. Restraint stress was performed 2 h after lights-on (A) and at the time of lights-off (B). Each point represents the mean ± SEM of four to five animals. Restraint group differences significant at $^*P < 0.05$ and $^{**}P < 0.01$.

Corticosterone Contents in Hippocampal Nuclei and Cytosols of Intact Rats

Aged rats show cytosolic MR and GR availability in the hippocampus lower than that in young animals in the presence of similar circulating levels of corticosterone (Fig. 3A, Table 1). Because it is not possible to detect nuclear MR and GR by *in vitro* exchange assays (55), we attempted to estimate the status of nuclear corticosterone receptors by measuring the amount of corticosterone sequestered in hippocampal nuclei and cytosol of both groups of animals. The results indicate that the intracellular content of corticosterone was decreased not only in the cytosol of the older hippocampal cells but also in its nuclear compartments (Fig. 5, $P < 0.05$).

Effect of Adrenalectomy on the Hippocampal Cytosolic Binding Capacity and Expression of the mRNAs of MR and GR

Adrenalectomized vs sham-operated rats. In order to compare the total number of MR and GR binding sites with the corresponding mRNA levels in the hippocampi of young and old rats, we decided to perform these measurements using ADX and sham-operated animals. Moreover, kinetic studies were also performed with hippocampi of young and old ADX rats. No significant differences between young and old ADX rats were observed in the association and dissociation rates of [^{3}H]dexamethasone or [^{3}H]aldosterone (results not shown).

Single receptor binding capacity determinations revealed a decrease in the apparent maximal binding (B_{max}) of MR and GR in the hippocampi at older age (SHAM or ADX) when compared to the corresponding young groups (age effect: $P < 0.002$; Fig. 6A). Both groups of animals, young and old, exhibited an approximately 100% increase in the B_{max} of MR and GR binding after 36 h of ADX ($P < 0.001$ SHAM vs ADX, Fig. 6A). In the young

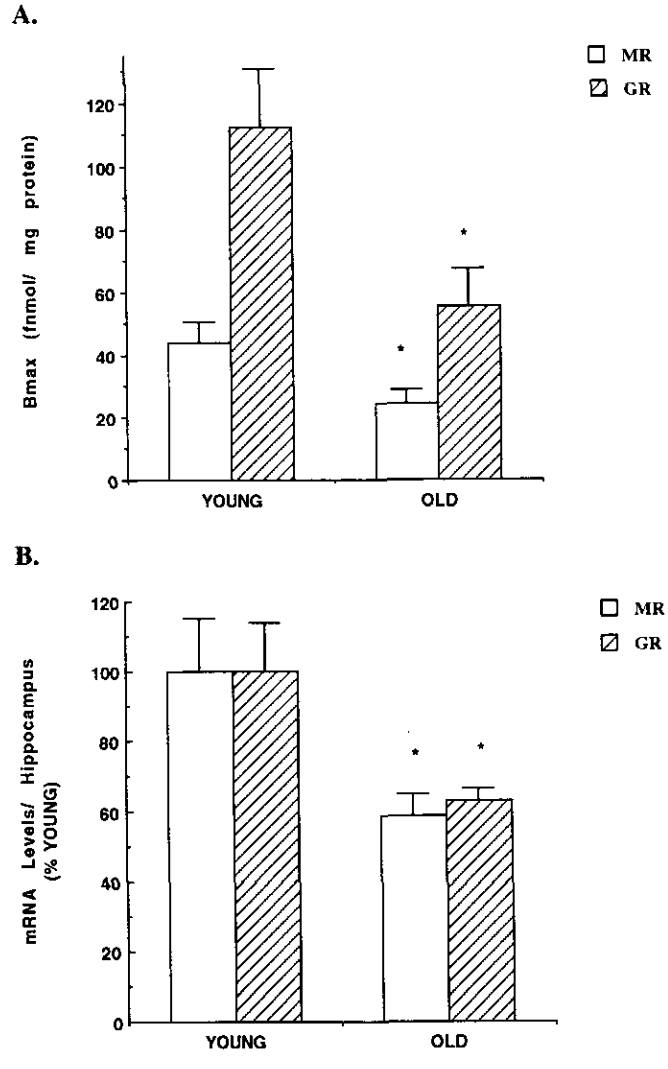

FIG. 3. Mineralocorticoid (type I) and glucocorticoid (type II) receptor (MR and GR) binding capacities (A, fmol/mg protein)) and mRNA levels (B) in the hippocampus of intact young and old rats. Each bar represents the mean ± SEM of five animals. Group difference significant at $^*P < 0.05$.

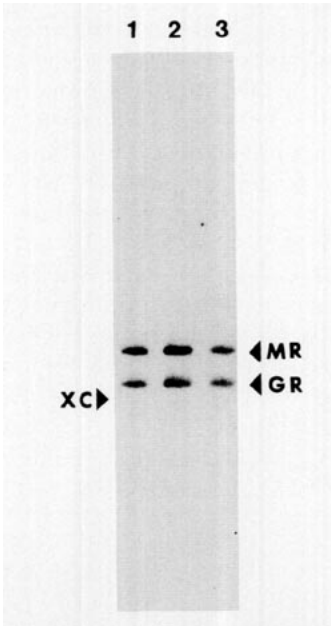

FIG. 4. MR and GR mRNA protection analysis. Each lane (labeled 1, 2, 3) contains a 1/20 dilution of a hippocampus protected using MR and GR cRNA probes as described under Materials and Methods. The resulting bands were digitized and quantified by optical densitometry. XC, xylene cyanol dye marker migrating typically at ~450 bp on a 4% *p*-acrylamide gel.

group this may be simply a consequence of the clearance of endogenous corticosterone since no change in MR or GR mRNAs was observed. However, the old ADX group showed significant upregulation of these mRNAs to levels similar to those of young groups ($P < 0.05$, Fig. 6B). Yet, this increase in MR and GR mRNA levels in the old ADX group was not accompanied by a proportional increase in the corresponding B_{max} of both receptors. Thus, the B_{max} of MR and GR in old ADX rats remained significantly lower than that of their young counterparts (Fig. 6A, $P < 0.05$ between old ADX and young ADX).

As shown in Fig. 7 by Scatchard analysis, the binding capacity of each receptor type was significantly reduced in the old ADX group. However, the apparent dissociation constants (K_d) were unchanged with age, indicating that the ligand affinities of MR and GR were similar in young and old rats.

MR and GR endogenous occupation. In one of the experiments, the difference in binding obtained between 4 and 20 h of *in vitro* incubation was used as an estimate of the degree of endogenous occupation, without activation, of these receptors (Fig. 8). Both MR and GR theoretical B_{max} were determined. More than 90% of both hippocampal receptors were unoccupied in ADX young and old rats. In the SHAM groups, with low circulating levels of corticosterone (<2 μg/dl), near 80% of GRs were unoccupied in young and old hippocampi. With respect to MR, the proportion of unoccupied receptors in old SHAM rats seems to be higher than that in young SHAM animals (53.1 ± 10.8% vs 31.3 ± 9.8%) although there is no significant difference between the groups.

Corticosterone replacement study. Finally, the effect of corticosterone treatment on the ADX young and old groups was studied. Table 2 shows that the ADX-induced increase of both MR and GR B_{max} was reversed in young and old hippocampi by sc injection of corticosterone (5 mg corticosterone in sesame oil each evening). The B_{max}s of hippocampal MR and GR in the ADX rats treated with corticosterone were not significantly different from those in SHAM animals. At the level of hippocampal mRNA contents, all three groups of young rats (SHAM, ADX, and ADX + corticosterone) had comparable values of MR and GR (Table 2). Interestingly, in the hippocampus of aged rats, the corticosterone treatment was able to reverse only the upregulation of MR mRNA levels produced by ADX ($P < 0.05$ between old ADX and old ADX + corticosterone). However, the hippocampal GR mRNA levels of old ADX and old ADX + corticosterone group were not significantly different, suggesting a different sensitivity of MR and GR mRNA to the dose of steroid in the replacement. In order to correlate B_{max} and mRNA levels of both receptors in each animal, a ratio was calculated between these two parameters (normalized with respect to young sham group). As shown in Table 2, in young rats, adrenalectomy increases the MR and GR ratios 2.6-fold over SHAM while treatment of the ADX rats with corticosterone return the ratio back to the SHAM level. Interestingly, the same treatment in older animals increases the ratio by only 1.6-fold for MR and 1.3-fold for GR ($P < 0.05$ between young and old ADX groups). While the MR ratio in old ADX rats was significantly different from the other groups ($P < 0.05$), no significant change was detected for the GR ratio in the same animals.

DISCUSSION

Many studies had previously related hippocampal neurodegeneration and the associated loss of corticosteroid

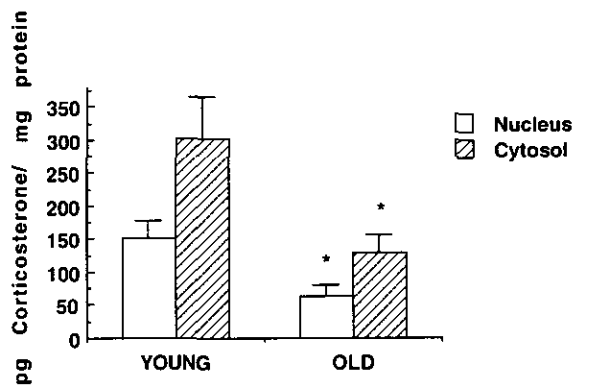

FIG. 5. Corticosterone contents in hippocampal nuclei and cytosols of intact rats. Each bar represents the mean ± SEM of four determinations. Group differences significant at $^*P < 0.05$.

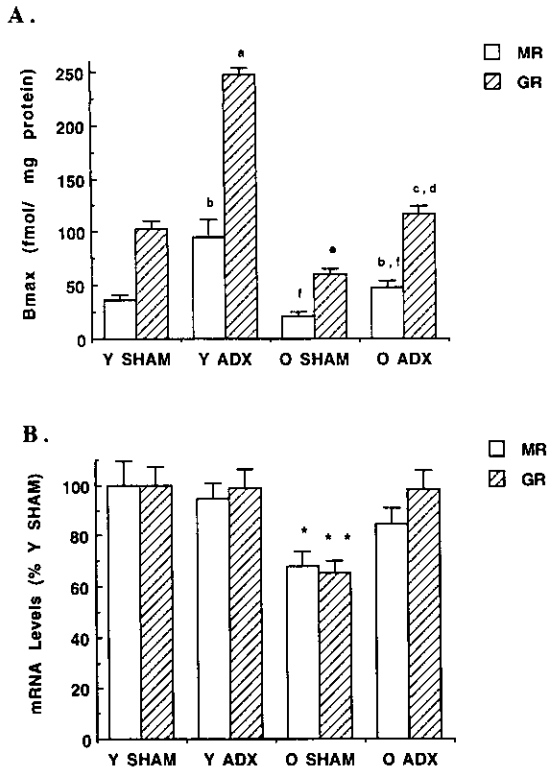

FIG. 6. Mineralocorticoid and glucocorticoid receptor (MR and GR) binding capacities (A, fmol/mg protein) and mRNA levels (B) in the hippocampus of SHAM or ADX young and old rats 36 h after the operation. Each bar represents the mean ± SEM of 9–11 animals. (A) Age and treatment effects show $P < 0.002$ for both receptors. Significant differences: $^{a}P < 0.001$, $^{b}P < 0.01$, and $^{c}P < 0.05$ from the corresponding SHAM group; $^{d}P < 0.001$, $^{e}P < 0.01$, and $^{f}P < 0.05$ from the corresponding young group. (B) $^{*}P < 0.05$ and $^{**}P < 0.01$ with respect to the other groups.

receptors with hyperactivity of the HPA axis (37, 39, 41), concluding that the hippocampal loss may be the consequence of high circulating levels of corticosterone. Since the corticosterone concentration seems to be critical in this context, we undertook the present study to simultaneously characterize HPA activity and hippocampal status of corticosteroid receptors in the *same* animal and obtained full endocrine profiles in the three groups of Fisher-344 rats: intact, adrenalectomized, and corticosterone-replaced adrenalectomized. Our study demonstrates that aged rats exhibit an unaltered basal rhythm and a normal initial glucocorticoid response to stress. Yet, the same rats show an age-related defect in the termination of the stress response. Indeed, this aberrant turn-off in aged rats is observed at different points of the diurnal cycle of corticosterone. At the level of corticosteroid receptors, the hippocampus was the only area implicated in the feedback mechanism of the HPA axis where age-related changes could be detected. The aged hippocampus shows a significant reduction in MR and GR binding capacity. This effect of aging on MR and GR binding was not attributable to changes in the kinetics, affinity, or nuclear translocation of the receptors. Moreover, our data demonstrate an age-related reduction of hippocampal MR and GR steady-state mRNA levels. Interestingly, only the aged hippocampus shows an upregulation of mRNA levels for both of these receptors 36 h after adrenalectomy, whereas receptor mRNA levels are unaltered following 36 h of ADX in younger rats. However, this increase in MR and GR mRNA content to levels comparable to those in young rats is not accompanied by a proportional increase in the binding capacity of these receptors in the aged rat. Thus, previous work correlating postadrenalectomy binding measurements of MR and GR with the corresponding mRNA levels in intact rats may ignore the fact that the aged hippocampus is more responsive at the transcriptional level to the removal of corticosterone than the young one. Finally, our corticosterone replacement study demonstrates that although the adrenalectomy-induced increase in MR and GR binding capacity is completely reversed by the treatment, only the upregulated MR mRNA levels were reversed in old rats, suggesting that transcription of the MR gene is more responsive than that of GR to this concentration of corticosterone.

Plasma Corticosterone Levels

The present study demonstrates that young and old male Fisher-344 rats did not differ in basal levels of cor-

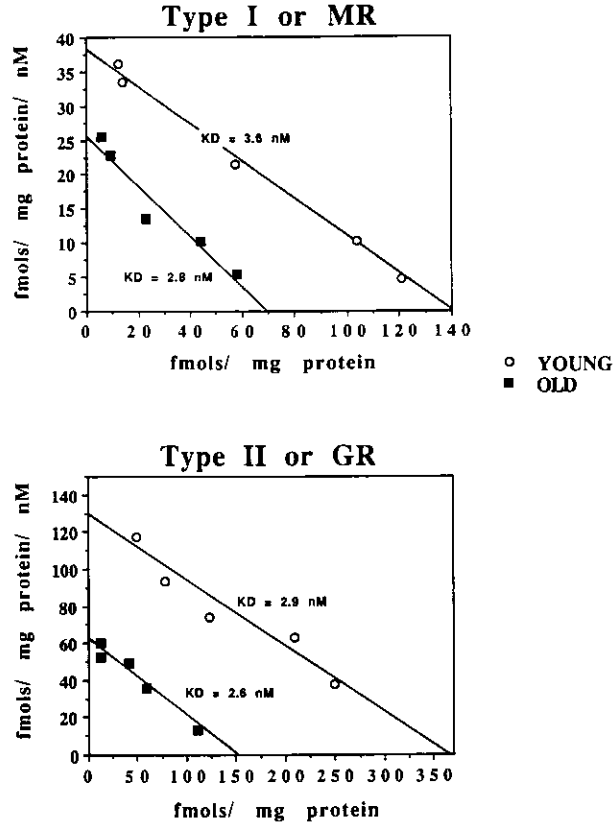

FIG. 7. Representative Scatchard plots of [^{3}H]dexamethasone binding (0.5–20 n*M*) in hippocampal cytosols of young and old ADX rats. Hippocampi were pool from three to five rats adrenalectomized 36 h prior to analysis. All R values >0.95.

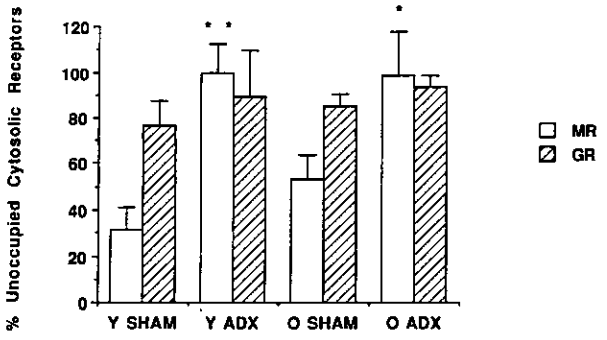

FIG. 8. Percentage of unoccupied mineralocorticoid and glucocorticoid receptors (MR and GR) in SHAM or ADX young and old rats 36 h after the operation. Each bar represents the mean ± SEM of five to eight rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ with respect to the corresponding SHAM groups.

ticosterone over the diurnal cycle. Although this result agrees with some reports (26–36), many other studies have shown a significant increase in basal corticosterone concentrations with age, leading to certain models of neuroendocrine aging and the role of elevated corticosterone in the process (19–25, 37, 41, 45). In a recent review of 17 published reports (60), Sapolsky concluded that there is an age-related elevation of circulating corticosterone levels throughout the circadian cycle if rats are studied under truly nonstressed circumstances (defined as <10 μg B/dl plasma during the circadian trough). Our experiments were designed to ensure valid basal nonstressed conditions. Indeed, our data (Fig. 1A, Table 1) showed very low corticosterone levels in young animals. Yet, we saw no difference in the aged animals, suggesting that it is possible for aged rats to exhibit completely normal basal patterns of circulating glucocorticoids even in nonstressed conditions. This issue is important as it shows that the loss of hippocampal corticosteroid receptors is not secondary to elevated circulating corticosterone. Rather, it is evident that hippocampal loss can take place even when basal hormonal levels are normal and may possibly anticipate the dysregulation of the circadian rhythm seen in some strains of rats.

Plasma corticosterone binding activity, determined in the same samples, showed no age-related change at any time-point (Fig. 1B). This observation suggests comparable plasma levels of free corticosterone in young and old rats. It also indicates an interesting strain difference because a 65% reduction of [^{3}H]corticosterone binding to CBG was described in the aged Norway Brown rat (36) and, also, higher titers of free corticosterone were detected during the PM phase of the cycle in the old Long Evans rat (25).

In contrast with the basal levels, there is an age-related defect in the corticosterone stress response of our rats. We performed 1 h restraint stress during the circadian trough and peak of circulating corticosterone cycle. Although no significant difference was observed in the corticosterone stress peak, the aged rat showed significantly elevated corticosterone levels during the recovery period. This phenomenon was apparent at both the peak and the nadir of the circadian rhythm (Fig. 2). When immobilization stress was performed in smaller restrainers, an even greater delay in the turn-off was observed (result not shown). Prolonged increase of plasma corticosterone secretion was also found in old rats after cold stress (20, 29). However, other studies, using different stressors, did not find a difference (28, 30, 26) or even reported a reduction (26) in the stress-induced corticosterone response of aged male rats. Moreover, it was described that aged female Fisher rats exhibit a reduced corticosterone response (30). Once again, therefore, difference in strain, sex, and kind of stressor should be considered in evaluating the effect of age on the HPA system. It should be pointed out that both circadian and stress-induced activities of the HPA axis are known to be inhibited by corticosterone feedback, though probably via separate

TABLE 2

Effect of Adrenalectomy and Corticosterone Treatment on the Binding Capacities and mRNA Content of MR and GR in Young and Old Fisher Rats

Treatment	Binding capacity (percentage of young sham)		mRNA content (percentage of young sham)		Ratio B_{max}/mRNA content		Corticosterone plasma level (μg/dl)
	MR	GR	MR	GR	MR	GR	
Young sham	100.0 ± 19.4*	100.0 ± 5.6*	100.0 ± 6.2	100.0 ± 7.5	1.04 ± 0.25**	1.01 ± 0.04**	1.6 ± 0.3
Young ADX	255.6 ± 42.4	254.5 ± 33.9	97.2 ± 4.5	105.0 ± 12.4	2.68 ± 0.30	2.59 ± 0.48	<0.1
Young ADX + B[a]	87.8 ± 7.4*	109.6 ± 4.6*	93.0 ± 18.5	101.7 ± 11.6	0.98 ± 0.13**	1.05 ± 0.07**	0.9 ± 0.2
Old sham	68.8 ± 10.7*	64.7 ± 15.6**	67.8 ± 8.2**	71.8 ± 7.2**	1.06 ± 0.18**	0.93 ± 0.22	1.8 ± 0.2
Old ADX	156.3 ± 17.1	140.1 ± 29.2	100.0 ± 10.8	105.2 ± 11.2	1.61 ± 0.18***	1.32 ± 0.24***	<0.1
Old ADX + B[a]	66.0 ± 8.6*	77.8 ± 5.0**	78.0 ± 2.9**	94.7 ± 3.2	0.89 ± 0.11**	0.86 ± 0.05	1.2 ± 0.2

Note. All values are means ± SEM of five animals. Significant differences: $^{*}P < 0.01$ and $^{**}P < 0.05$ from ADX groups; $^{***}P < 0.05$ from young ADX group.

[a] B, corticosterone treatment.

pathways (14). Taken together, the results described in this paper show that the aging process in the male Fisher rat results in a dissociation of these two activities of the HPA axis, i.e., these aged animals exhibit unaltered rhythm but altered turn-off of the stress response.

Mineralocorticoid and Glucocorticoid Receptor Binding Studies in the Aged Hippocampus

As pointed out in the Introduction, an age-related alteration of hippocampal corticosterone receptors is generally accepted (38, 41, 43). All the studies on aging agree regarding a reduction of the concentration of MR binding sites (23, 25, 36, 42, 47), with one exception (44). In contrast, depending on the rat strain, the hippocampal GR binding capacity was detected as reduced (25, 42, 47), unchanged (36, 44), or impaired in its ability to upregulate after long-term ADX (45, 46). Our results demonstrate that the aged Fisher rat exhibits a significant decrease of both hippocampal receptors, MR and GR. No change in the apparent K_d was obtained for either receptor (Fig. 6) in contrast with the report of Landfield and Eldridge (45) of higher GR affinity in older hippocampi. A 40–50% reduction of the apparent maximal binding capacities (B_{max}) of MR and GR was observed in the aged group under all the conditions studied: intact, SHAM, or 36 h ADX (Figs. 3A and 7A). However, estimates of the MR and GR B_{max} in intact and SHAM animals should be interpreted with caution. In the presence of circulating corticosterone, both of these receptors bind the ligand and become "activated" into forms which bind specific DNA response elements, but, at the same time, are unable to further exchange ligand (54). Therefore, only the nonactivated forms of MR and GR can be detected in hippocampal cytosols of intact and SHAM rats. Typically, removal of endogenous corticosterone by short-term ADX is used to calculate the total number of these receptors in cytosolic preparations, with the assumption that no significant upregulation occurs. While this assumption has received some support in young rats (55, 61), it had not been validated in older animals. Thus, the use of other techniques was required. As a first approach, we evaluated the amount of corticosterone in cellular fractions obtained from young and old intact rats. In older hippocampi, the corticosterone content was significantly lower in both fractions, nuclear and cytosolic (Fig. 5). This result indicates that the decreased number of nonactivated cytosolic receptors in intact aged rats cannot be attributed to an increase of nuclear translocation of these receptors. Indeed, it is clear that the aged animal has fewer receptors likely to modulate various target genes. In order to estimate the degree of occupation by endogenous corticosterone of the nonactivated cytosolic MR and GR we used the approach of Meaney *et al.* (56). The values we obtained of MR and GR occupancy in young rats agree with previous reports (61). During the corticosterone circadian trough, less than 20% of GR was occupied in young and old hippocampi. In contrast, MR occupation tends to be higher in the younger group although a significant age difference could not be demonstrated (Fig. 8).

Different possibilities need to be considered to explain a lower number of corticosterone receptors in aged hippocampi. A decreased rate of biosynthesis or an increased rate of degradation of MR and GR proteins are the first considerations. Indeed, an immunohistochemical demonstration of decreased GR protein has been reported in aged hippocampi of Sprague–Dawley rats (63). However, as Chang and Roth (62) pointed out, another possibility is that these receptors may become nonfunctional in the aged cells. The described assays depend on the ability of MR and GR to bind the radiolabeled ligand. Therefore, nonfunctional receptors could not be detected. In this respect, the association of MR and GR with a protein complex which involves several heat–shock proteins including hsp-90 and hsp-70 is thought to be critical to their ability to bind steroids (64). An age-related decrease of some of the associated proteins or a change in the cellular milieu which modify the stability of the receptor complex could also result in reduced MR and GR binding capacities.

In sum, this series of studies on MR and GR in the hippocampus supports the idea of a profound decrease in functional receptors in the aged animals and shows that, in our animals, the phenomenon exists with or without adrenalectomy and cannot be attributed to altered affinities or to differential translocation of the receptors into the nuclear compartment.

Mineralocorticoid and Glucocorticoid Receptor mRNA Levels in the Aged Hippocampus

To investigate whether the loss of MR and GR binding in the hippocampus of our aged rats was associated with lower levels of mRNA expression of these receptors, we simultaneously evaluated both parameters within each rat. A 30–40% decrease of MR and GR mRNA levels was detected in intact and SHAM old hippocampi (Figs. 3B and 7B, Table 2). These changes could be due to alterations in the MR and GR transcription process or in the stability of the corresponding mRNAs. This result is in agreement with the decrease of GR mRNA reported in intact aged Long Evans (50) and Brown Norway (36) rats. However, in this latter strain there was no significant difference in MR mRNA levels between young and old intact rats (36). We also studied the effect of 36 h ADX and corticosterone replacement over the hippocampal corticosterone receptors. At this time, only the aged ADX group was able to upregulate the mRNA levels of both receptors. Interestingly, although the old ADX hippocampi reached MR and GR mRNA levels comparable to those of the young ones, the corresponding binding capacities of both receptors were not increased to the same extent (Fig. 7, Table 2). While time course studies will be necessary, the data at this post-ADX time are consistent with the possibility that MR and GR may exhibit trans-

lational or post-translational alterations in the aged hippocampi. Moreover, these data indicate that while the post-ADX elevation of MR and GR cytosolic binding could be attributed exclusively to the clearance of endogenous corticosterone in the young rats, upregulation of both MR and GR mRNA levels complicates any interpretation of the results in the aged group. At the protein level, treatment with corticosterone was able to reverse the ADX-induced increase of MR and GR binding capacities in young and old hippocampi, presumably because of the activation of the receptors by the exogenous corticosterone (Table 2). However, this corticosterone treatment only reversed the upregulated mRNA levels of hippocampal MR but not GR in the older group (Table 2). Thus, at the gene expression level MR seems to be more responsive than GR to low doses of corticosterone. It should be noted that this is the only case described in the paper in which a change in MR did not parallel a similar change in GR.

The mRNA studies support the idea that the decreased level of receptor binding in the aged rat is, at least in part, due to a decrease in the biosynthetic capacity of these proteins, as revealed by a decrease in the size of the message pool. These lower mRNA levels observed in the aged hippocampal cells may reflect a decrease in neurons associated with the natural aging process. However, that this decrement is selectively reversed by adrenalectomy and modulated by corticosterone strongly suggests that this is not simply due to cell death, but to a reversible lowering of the biosynthetic rate of the corticosteroid receptors in the aged hippocampus.

While the high responsiveness of MR and GR to the glucocorticoid environment argues against an explanation purely in terms of cell death, it does not preclude the real possibility that there is significant neuronal loss in the aged rat. Indeed, Sapolsky *et al.* (41) have demonstrated that the hippocampus of aged Fisher rats exhibits loss of neuronal cells and shows a lower number of corticosterone binding sites per neuron. Quantitative *in situ* hybridization experiments are in progress to describe the anatomical localization of the MR and GR mRNA changes in our animals.

The Role of the Hippocampus in the HPA Axis of Aged Rats

Earlier findings of corticosterone hypersecretion in aged rats under both basal and poststress conditions led to models of age-related neurodegeneration. Lanfield *et al.* first proposed a correlation between hippocampal degeneration and HPA abnormalities (37, 39). They also reported a protective effect of ADX over hippocampal pathology in aged rats when the operation is performed in middle-age (37). Later, Sapolsky *et al.* (41) proposed that increased circulating corticosterone levels produce downregulation of corticosterone receptors and loss of neurons in a "feed forward cascade" of the aging process. More recently, deKloet (15) proposed that a change in balance of MR- and GR-mediated effects alters the ability to maintain the homeostasis, which progressively would create a condition of age-related disturbances, in neuroendocrine regulation and behavioral adaptation. Evidently, the view of the role of the hippocampus as a simple checkpoint of the stress termination is an incomplete one. As Issa and co-workers (47) and DeKloet (15) have pointed out, this structure is a complex interface among stress biology on the one hand and learning and memory on the other. We have recently proposed a view of the hippocampus as an integrator of information, a structure which assigns "salience" or priority to various stimuli (65). In that context, the hippocampus would integrate information about the state of stress of the organism (by monitoring glucocorticoids) along with retrieving recent information from memory as a component of its information processing.

Issa *et al.* (47) examined the relationship between HPA dysfunction and cognitive impairment in aged Long Evans rats. These authors have demonstrated that cognitively impaired animals which show higher corticosterone levels in the dark phase of the cycle and delayed recovery to basal corticosterone levels after restraint-stress also exhibit the lowest number of MR and GR receptors. Although we did not screen our Fisher rats behaviorally, individual differences appeared very small in all the parameters evaluated within the aged group. The aging process in these rats does not affect the basal corticosterone circulating levels nor the corticosterone stress peak, although we cannot discard that changes throughout multiple levels of the aged HPA axis may compensate the effects of a decrease in hippocampal corticosteroid receptors. Studies on the different levels of the HPA axis in these aged rats are currently in progress in our laboratory.

The present work carried out in male Fisher rats demonstrates another interesting aspect of the relationship between the hippocampus and the HPA axis during aging. A dissociation between the circadian and stress-induced activities of the HPA axis in the older animals is observed in the presence of similar hippocampal decrease of both corticosterone receptors. It appears that at this stage of aging (26–27 months), the hippocampal loss of glucocorticoid receptors does not necessarily lead to a disruption of the corticosterone circadian rhythm. In addition, the altered termination of the corticosterone stress response and the loss of corticosteroid receptors in the hippocampus are not related to high circulating levels of corticosterone under basal conditions. Therefore, it is not likely that the reduction of functional MR and GR in the hippocampus is a consequence of elevated corticosterone circulating levels. The sequence of events may be different from what others propose. Early in the aging process the hippocampal loss of steroid receptors is accompanied with a normal circadian rhythm of corticosterone and with good sensitivity to corticosterone under conditions of sustained feedback (corticosterone replacement study) but with inefficient poststress turn-off following certain stressors

(i.e., restraint). Later on, most aspects of HPA activity may become aberrat and a continuous dysregulation of even basal corticosterone levels may appear in more "advanced" cases.

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