

Decrements in Nuclear Glucocorticoid Receptor (GR) Protein Levels and DNA Binding in Aged Rat Hippocampus

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Hippocampal glucocorticoid receptors (GRs) are believed to play a major role in age-related cognitive decline and cellular vulnerability. It has been proposed that these receptors mediate damaging effects of elevated glucocorticoid secretion on cellular function. In the present report we present evidence that intracellular trafficking of the GR is impaired with hippocampal aging, manifest as decreased nuclear translocation and deficient DNA binding. We also present evidence that chaperone proteins responsible for GR nuclear translo-

cation are decreased with hippocampal aging. Age-related nuclear GR decreases are not observed in hypothalamus, indicating regional specificity of trafficking deficits. Aging did not affect nuclear or cytosolic MR levels. These data suggest that GR signaling is diminished, rather than enhanced, during hippocampal aging. Diminished GR signaling capacity may attenuate the beneficial effects of glucocorticoids on hippocampal regulation of memory and stress integration. (*Endocrinology* 143: 1362–1370, 2002)

GLUCOCORTICIDS mediate a wide variety of systemic and brain functions, facilitating efficient adaptation to internal and external challenge. Aged rats and humans show progressive loss of control of the hypothalamo-pituitary-adrenocortical (HPA) axis, resulting in hypersecretion of glucocorticoids during times of stress. Inappropriate glucocorticoid secretion has been implicated as a mitigating factor in numerous diseases of aging, including depression, cognitive decline, and Alzheimer's disease (1–4). Thus, understanding and controlling the factors underlying glucocorticoid signaling are of considerable importance to the treatment and prevention of age-related disease.

Glucocorticoids signal by binding corticosteroid receptors. Two types of corticosteroid receptors have been characterized to date. The mineralocorticoid receptor (MR) is a high affinity, low capacity receptor that is heavily occupied even at low levels of circulating glucocorticoids (5). The glucocorticoid receptor (GR) binds glucocorticoids with lower affinity and is extensively bound under conditions of adrenocortical stimulation (circadian drive or stress) (5). Corticosteroid receptors are translocated to the cell nucleus through an active transport process involving a chaperone complex, consisting of heat shock proteins (hsp) 90 and 70 (both inducible and constitutive) and hsp40, heat shock organizing protein, and an immunophilin moiety (6). hsp90 and constitutively active hsp70 (hsc70) are the essential elements of the chaperone complex (7). hsp90 must be present to allow for high affinity steroid binding to the GR and for general function of the

complex as a whole. hsc70 is responsible for proper folding of the complex.

Once in the nucleus, receptor proteins dimerize and bind consensus recognition sequences on DNA, thereby activating or repressing transcription of a wide variety of genes. The virtual identities of DNA-binding domains of the two receptors are consistent with findings that both receptors bind the same DNA recognition element (8).

The hippocampus is a clear point of interaction between the aging process and glucocorticoid signaling. Lesion studies indicate that this region is intimately involved in stress inhibition and spatial memory (9, 10), both of which are impaired in aging animals. The hippocampus expresses very high levels of corticosteroid receptors (11, 12), making it a prime target for age-related changes in glucocorticoid secretion. Notably, intrahippocampal injection of the GR antagonist RU38486 or GR gene deletion can impair the performance of avoidance or spatial memory tasks (13–15), suggesting involvement of the GR in hippocampal processes targeted by the aging process. The similarity between the effects of lesion/GR knockout and aging are consistent with attenuated GR signaling, suggesting decreased receptor function.

The exact mechanism responsible for reduced GR action is presently unclear. Previous studies suggest that reduced signaling may be due to a loss of receptor-bearing neurons combined with glucocorticoid-mediated down-regulation of receptor expression (4). However, recent studies have cast doubt on the generality of hippocampal cell death in aging (16), and GR down-regulation is not observed in all aging studies (17). In the present study we present evidence indicating that age-related hippocampal GR deficits are associated with changes in GR nuclear transport, which reduce the

Abbreviations: GRE, Glucocorticoid response element; HPA, hypothalamo-pituitary-adrenocortical; hsc, constitutively active heat shock protein 70; hsp, heat shock protein; KPBS, potassium PBS; PLSD, protected least significant difference; RNase A, ribonuclease A; TBST, Tris-buffered saline with 1% Tween 20.

ability of the GR to transduce glucocorticoid signals in this brain region. The results suggest that age-related changes in hippocampal function may be associated with decreased glucocorticoid signaling capacity.

Materials and Methods

Subjects

Male Fischer 344/Brown-Norway F₁ hybrid rats, aged 3, 15, and 30 months at the time of arrival, were used in this study. All animals were housed three per cage at University of Kentucky or University of Cincinnati at a constant temperature-humidity vivarium on a 12-h light, 12-h dark cycle. All animal protocols were reviewed and approved by the respective institutional animal care and use committees.

Animals were killed by rapid decapitation. Brains were rapidly removed and bisected in the midsagittal plane. The left half was flash-frozen in isopentane cooled to -40 or -50 C on dry ice and stored at -80 C until processing for *in situ* hybridization and immunohistochemistry. The hippocampus was dissected free of the right hemisphere and immediately homogenized in ice-cold buffer (see below). Half-brains were sectioned at 15 μ m in a Bright-Hacker cryostat and thaw-mounted onto SuperFrost Plus slides (Gold Seal, Portsmouth, NH). Sections were collected through the hippocampus. All sectioned tissue was stored at -20 C.

In situ hybridization

Tissue series were fixed by immersion in 4% phosphate-buffered paraformaldehyde for 10 min. Slides were then transferred through a series of solutions starting with 5 mM potassium PBS (KPBS) for 10 min, followed by 5 mM KPBS with 2% glycine for 10 min, and back into 5 mM KPBS for 10 min. After this, the slides were treated in a solution of 0.1 M tetraethylammonium with 0.25% acetic anhydride. After pretreatment, slides were rinsed twice for 5 min each time in 0.2 \times SSC and dehydrated through graded ethanols and chloroform.

Antisense cRNAs were synthesized to specifically recognize all mature GR mRNA forms (456-bp coding region and 3'-untranslated region of rat GR mRNA; K. Yamamoto, University of California, San Francisco, CA) and all MR mRNA forms (550-bp coding region and 3'-untranslated region of rat MR mRNA; P. Patel and S. Watson, University of Michigan, Ann Arbor, MI). Probes were synthesized by *in vitro* transcription using SP6 or T7 RNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN) and [³³P]UTP (Amersham Pharmacia Biotech, Arlington Heights, IL). Labeling reactions included 60 μ Ci [³³P]UTP (SA, 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 ribonuclease (RNase) inhibitor (40 U/ μ l; Roche Molecular Biochemicals); 1 μ g linearized plasmid DNA; and 20 U of the appropriate RNA polymerase. Labeling reactions were incubated for 90 min at 37 C, the template DNA was digested with 12 U RNase-free deoxyribonuclease I (Roche Molecular Biochemicals), and labeled probe was purified by ammonium acetate precipitation.

The ³³P-labeled probe was diluted in hybridization buffer along with sheared salmon sperm DNA, Denhardt's solution, yeast tRNA, and dithiothreitol to yield 1,000,000 cpm/50 μ l. Each slide received 50 μ l hybridization mixture and was then coverslipped. All slides were incubated overnight at 55 C in sealed plastic boxes lined with filter paper moistened with 50% formamide. Coverslips were then removed, the slides were rinsed briefly in 2 \times SSC and then immersed in 2 \times SSC for 20 min. Sections were treated with RNase A (100 μ g/ml) at 37 C for 30 min and subsequently washed three times in 0.2 \times SSC for 10 min each time, followed by a 60-min wash at 65 C. Tissue was dehydrated through graded ethanols and exposed to Kodak BioMax x-ray film (Eastman Kodak Co., Rochester, NY).

Immunohistochemistry

Alternate series from brains processed for *in situ* hybridization were removed from -20 C and warmed to room temperature. To block nonspecific binding, tissue was then immersed in a solution containing 1:50 normal donkey serum, consistent with the origin of the secondary antibody, in 10 mM (KPBS) for 20 min. Residual fluid was blotted off, and

primary antibody was applied (GR: BuGR2, 1:4000 dilution, Affinity BioReagents, Inc., Golden, CO; MR: 1:4000 dilution, courtesy of S. J. Watson, University of Michigan). Slides were incubated overnight in humidified chambers at 4 C. Primary antibody was removed by washing the slides three times in KPBS for 5 min each time, and sections then incubated with ³⁵S-labeled donkey antimouse (GR) or donkey antirabbit (MR) secondary antibody (1:500 dilution in KPBS) for 1 h at room temperature. Slides were then washed three times in KPBS for 10 min, followed by a rinse in nanopure water. Sections were then dehydrated through graded ethanols and exposed to Kodak BioMax x-ray film. Previous studies from our laboratory have documented the applicability and specificity of immunohistochemical assessment of GR and MR (18, 19).

EMSA

Whole cell and nuclear protein extracts were used for gel-shift analyses. For whole cell extracts, hippocampi (\sim 200 mg) were homogenized in 20 mM Tris (pH 7.5) buffer containing 1.5 mM EDTA, 40 mM KCl, 5% glycerol, 0.5 mM dithiothreitol, and protease inhibitors [aprotinin (5 μ g/ml), leupeptin (5 μ g/ml), pepstatin A (5 μ g/ml), and Pefabloc (0.5 mM)] in a total volume of 1 ml. Homogenates were spun at 16,500 rpm for 1 h, and supernatant was harvested, snap-frozen, and stored at -80 C. Nuclear extracts were obtained using the procedure of Deryckere and Gannon (20). Briefly, tissue was homogenized in Dounce homogenizers (five strokes; Kontes Co., Vineland, NJ) in 10 mM HEPES (pH 7.9) containing 0.6% IGEPAL CA-630, 150 mM NaCl, 1 mM EDTA, and 0.5 mM Pefabloc on ice. Homogenates were then transferred to a fresh tube, debris was pelleted in a microcentrifuge for 30 sec, and the supernatant was transferred to a fresh tube and incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 4000 rpm (4 C) for 5 min. Pellets were then resuspended in 20 mM HEPES containing 25% glycerol, 420 mM NaCl, 1.2 mM MgCl₂, 0.2 mM EDTA, 2 mM benzamide, 0.5 mM dithiothreitol, 5 μ g/ml leupeptin, and 5 μ g/ml Pefabloc and incubated on ice for 20 min, and debris was pelleted for 15 sec in a microcentrifuge. Supernatants were snap-frozen on dry ice. For both whole cell and nuclear extracts, protein concentrations were determined by the method of Bradford (21).

The EMSA probe was an oligonucleotide corresponding to a consensus glucocorticoid response element (GRE; 5'-AGTAGCTAGAA-CATCCTGTACAGTCGA-3'; Genosys, The Woodlands, TX). Annealed oligonucleotides were 5'-end-labeled using T4 polynucleotide kinase and [α -³²P]ATP. Probe (50,000 cpm) was added to binding reactions containing binding buffer [10 mM Tris (pH 7.5) containing 50 mM NaCl, 1 mM EDTA, and 5% glycerol], 0.5 mg/ml BSA, 11 ng/ml poly(dI-dC), and 10 mg protein extract in a total volume of 20 μ l. Binding reactions were incubated on ice, at which point gel loading buffer was added. Samples were loaded onto 4% nondenaturing polyacrylamide gels, and electrophoresis was performed in 0.5 \times TBE (160 V, 1.5–2 h). Gels were fixed in 10% methanol and 5% glacial acetic acid for 10 min, rinsed three times, and dried under vacuum. Gels were exposed to Kodak XAR5 x-ray film overnight at -80 C. On all gels, controls included reactions run without protein samples and reactions run in the presence of a 100-fold excess of unlabeled oligonucleotide. The specificity of DNA binding was confirmed by supershift, involving preincubation of reactions with 1 μ l GR antibody.

Western blot

Proteins (10–15 μ g) were separated on a 7.5% SDS-polyacrylamide gel using the Laemmli method (22). Samples were normalized according to protein content, with equal amounts of each protein loaded per well. The proteins were transferred electrophoretically to nitrocellulose. After transfer, the nitrocellulose was stained with Ponceau S to verify equal protein loading and transfer. The membranes were blocked overnight at 4 C in a solution of 10% nonfat dry milk in Tris-buffered saline with 1% Tween 20 (TBST). Primary antibody incubation was carried out for 1 h at room temperature. Primary antibodies [GR: Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), GR M-20; MR: Santa Cruz Biotechnology, Inc., MCR (N-17); hsp90: Transduction Laboratories, Inc. (Lexington, KY); hsp70: Transduction Laboratories, Inc.; hsc70: Santa Cruz Biotechnology, Inc.; calcineurin: Transduction Laboratories, Inc.)] were diluted 1:2,000 (GR and MR), 1:1,500 (calcineurin), or 1:1,000 (hsp90, hsp70, and

hsc70) in TBST. The membranes were washed with TBST and incubated for 1 h in horseradish peroxidase-conjugated secondary antibody [antirabbit, Amersham Pharmacia Biotech; antigoat, Sigma (St. Louis, MO); antimouse, Amersham Pharmacia Biotech] diluted 1:10,000 in 2% nonfat dry milk in TBST. After washing in TBST, proteins were visualized using the Amersham Pharmacia Biotech ECL Plus chemiluminescent reagents. Exposure to ECL Hyperfilm was typically for 1–15 min.

Data analysis

In situ hybridization, immunohistochemistry, and Western blot data were analyzed using NIH Image 1.62 software. For anatomical analyses measurements were taken from subfields CA1 and CA3, dentate gyrus, and frontal cortex, as previously described (18, 19, 23). Briefly, subfields CA1 and CA3 and the dentate gyrus were defined cytologically and manually sampled from the dorsal hippocampus. Background signal was sampled over the corpus callosum and was subtracted from all regions to obtain corrected gray level measures. Six to 12 hippocampi were sampled per subject, with the mean value for each region from each animal used in subsequent statistical analysis. Gray level measurements of Western blot ECL and GRE binding were taken in like fashion, with background determined over negative regions of the blots. The data from all experiments were analyzed using one-way ANOVA, with Fisher's protected least significant difference (PLSD) *post hoc* test to distinguish differences among age groups.

Results

Initial experiments examined the expression of GR mRNA and protein in aged rat hippocampus (Fig. 1). There was a significant effect of age on GR mRNA levels in subfields CA1 and CA3 ($P < 0.05$; Fig. 1A), with no parallel changes seen in frontoparietal cortex (data not shown). In CA1, GR mRNA was decreased in middle-aged and aged animals relative to

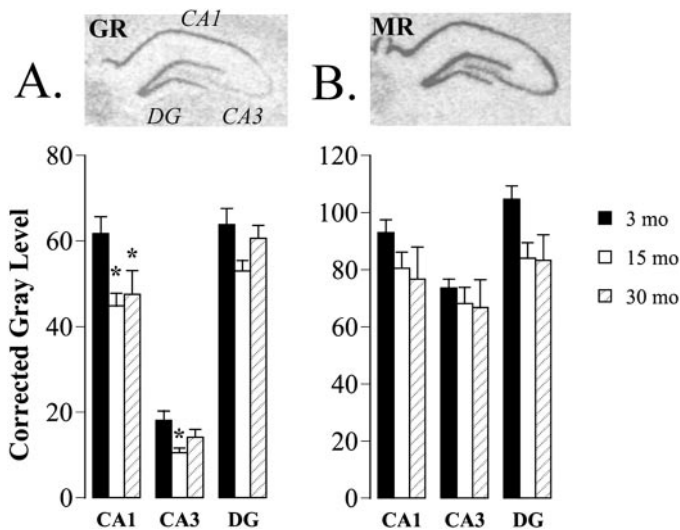


FIG. 1. Expression of GR and MR mRNA in hippocampus of young (3-month-old), middle aged (15-month-old), and aged (30-month-old) F344/BN F₁ rats. A, Distribution and regulation of GR and MR mRNA in the hippocampus. GR mRNA is expressed at highest levels in CA1 and dentate gyrus. One-way ANOVA revealed age-related decrements in GR mRNA levels in subfields CA1 ($P < 0.05$) and CA3 ($P < 0.05$), but not in dentate gyrus (DG). In CA1, GR mRNA was decreased in both 15- and 30-month-old handled groups relative to the 3-month-old group (*, $P < 0.05$, by Newman-Keuls test); in CA3, GR expression was decreased in the middle-aged group relative to young rats (\dagger , $P < 0.05$, by Newman-Keuls test). B, Distribution and regulation of GR and MR mRNA in hippocampus. MR mRNA is distributed throughout all hippocampal subfields. There were no effects of age on MR mRNA levels in any hippocampal subfield. In all cases, $n = 6$ /age group.

young animals; in CA3, differences were seen between young and middle-aged groups ($P < 0.05$, by Fisher's PLSD test). There was no effect of age on MR mRNA expression in any region (Fig. 1B). Overall, these data suggest that decreases in GR mRNA levels occur relatively early in the aging process and are not accompanied by changes in MR gene expression.

In contrast with the mRNA data, analysis of corticosteroid receptor protein levels by immunohistochemistry (Fig. 2) revealed no effect of age on GR or MR expression (Fig. 2, A–C), suggesting that GR mRNA changes seen in neighboring sections do not accurately predict resting protein levels. Western blot analysis performed on cytosolic extracts from the contralateral hippocampus of the same animals was in agreement with the results of immunohistochemical analysis (Fig. 2D), showing no significant effect of age on GR protein levels.

To examine intracellular distribution of GR protein, nuclear and cytosolic extracts were prepared from hippocampi of additional young, middle-aged, and aged rats. No age-related changes in cytosolic GR levels were observed (Fig. 3, A and C). In contrast, a clear decrease in nuclear GR protein

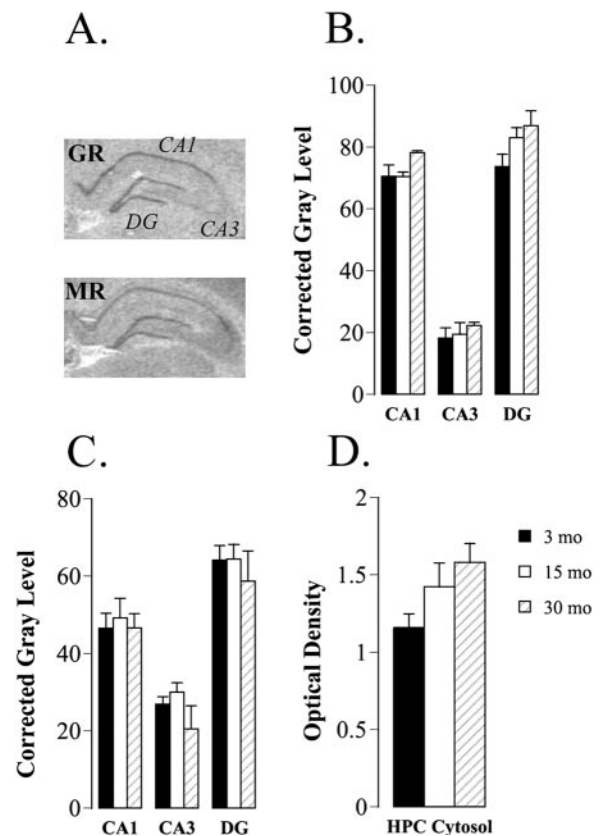


FIG. 2. Expression of GR and MR immunoreactivity (ir) in hippocampus of young (3-month-old), middle-aged (15-month-old), and aged (30-month-old) F344/BN F₁ rats. A, Distribution of GR and MR immunoreactivity in hippocampus, visualized by immunohistochemistry. The pattern of GR and MR protein localization agrees with distribution of mRNAs. B, Hippocampal GR immunoreactivity was not affected by age. C, There were no effects of age on MR protein levels in any hippocampal subfield. D, Western blot did not reveal changes in GR expression with age ($P = 0.07$). In all cases, $n = 6$ /group.

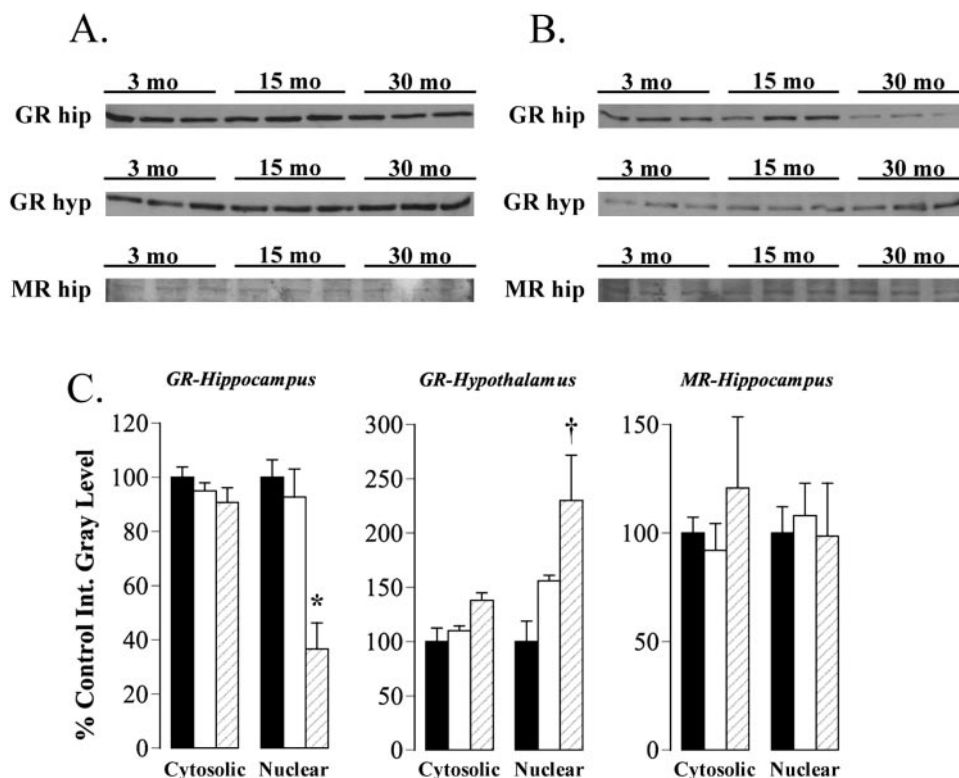


FIG. 3. Western blot analysis of hippocampal GR and MR immunoreactivity (ir) in cytosolic (A) and nuclear (B) samples of young (3-month-old), middle-aged (15-month-old), and aged (30-month-old) F344/BN F₁ rats. A, Cytosolic GR was not altered in aged hippocampus or in the hypothalamus. No changes in cytoplasmic MR levels were observed with age. B, Nuclear GR protein levels were markedly reduced in hippocampal (hip) extracts. Deficits were not observed in hypothalamic extracts (hyp), which, in fact, showed age-related increases in GR. Nuclear MR levels were not affected by age in the hippocampus. C, A marked age-related decrease in GR immunoreactivity was seen by one-way ANOVA in hippocampal nuclei ($P < 0.01$), with aged animals showing lower nuclear GR levels than either young or middle-aged groups ($P < 0.05$, by Fisher's PLSD test), with no change in cytosolic GR ($n = 9/\text{group}$). In aged hypothalamus, GR was increased in both nuclear ($P < 0.05$) and cytosolic ($P < 0.05$) compartments, with aged animals showing greater GR levels than young rats in both regions ($n = 3/\text{group}$; $P < 0.05$, by Fisher's PLSD test). MR immunoreactivity was not affected by age in either compartment ($n = 9/\text{group}$). *, Significantly different from 3- and 15-month-old groups, $P < 0.05$; †, significantly different from the 3-month-old group, $P < 0.05$.

was observed in aged animals, consistent with reduced nuclear GR transport or retention (Fig. 3, B and C; $P < 0.01$). No decrement in nuclear (or cytosolic) MR was observed in hippocampus (Fig. 3, A–C), indicating that the corticosteroid receptor translocation deficit was limited to the GR. The loss of nuclear GR was specific for hippocampus, as hypothalamic GR levels increased in aged animals ($P < 0.05$; Fig. 3, B and C). Immunoreactivity for the MR was not detectable in cytosolic or nuclear samples from the hypothalamus.

Decreased nuclear GR immunoreactivity predicts a decrement in nuclear GR DNA binding and consequent transcriptional efficacy. This possibility was examined using EMSA. Analysis of whole cell extract DNA binding was performed on protein samples obtained from the contralateral hippocampus of animals used for anatomical analyses, from the same extracts used for Western blot analysis. Nuclear extracts were obtained from an additional set of animals processed in parallel. Incubation of either whole cell or nuclear extracts with a consensus GRE binding sequence resulted in significant retardation in migration of labeled oligonucleotide (Fig. 4A). Retardation was not seen in the absence of protein and was blocked by the addition of excess unlabeled oligonucleotide to the binding mixture (Fig. 4A). Antibodies directed against the GR (polyclonal, courtesy of S. J. Watson, University of Michigan) were

used in supershift experiments. Preincubation of extracts with GR antibody resulted in supershifted bands, consistent with binding of oligonucleotides with antigen/antibody complexes (Fig. 4A). Overall, the data indicate detection of specific interactions between protein and GRE sequences employed in these studies.

EMSA revealed decreased GRE-binding activity in whole cell and nuclear hippocampal extracts of aged and, to a lesser extent, middle-aged animals (Fig. 4B). The linearity of the detection scheme was assessed by estimating relative change in integrated gray level with increases in quantities of protein loaded in the gel (Fig. 4C). The relationship between signal intensity and protein was linear over a substantial range of signal intensities, verifying the utility of the technique for detection of relative changes in DNA binding. Subsequent densitometric analysis of EMSA data revealed a significant effect of age on GRE binding in both whole cell ($P < 0.05$) and nuclear ($P < 0.05$) extracts (Fig. 4D). Young animals exhibited greater GRE binding capacity than either middle-aged or aged animals using either whole cell or nuclear extracts ($P < 0.05$, by Newman-Keuls test). These data are consistent with the Western blot analyses and suggest that reduced GR nuclear translocation may be the cause of reduced DNA binding.

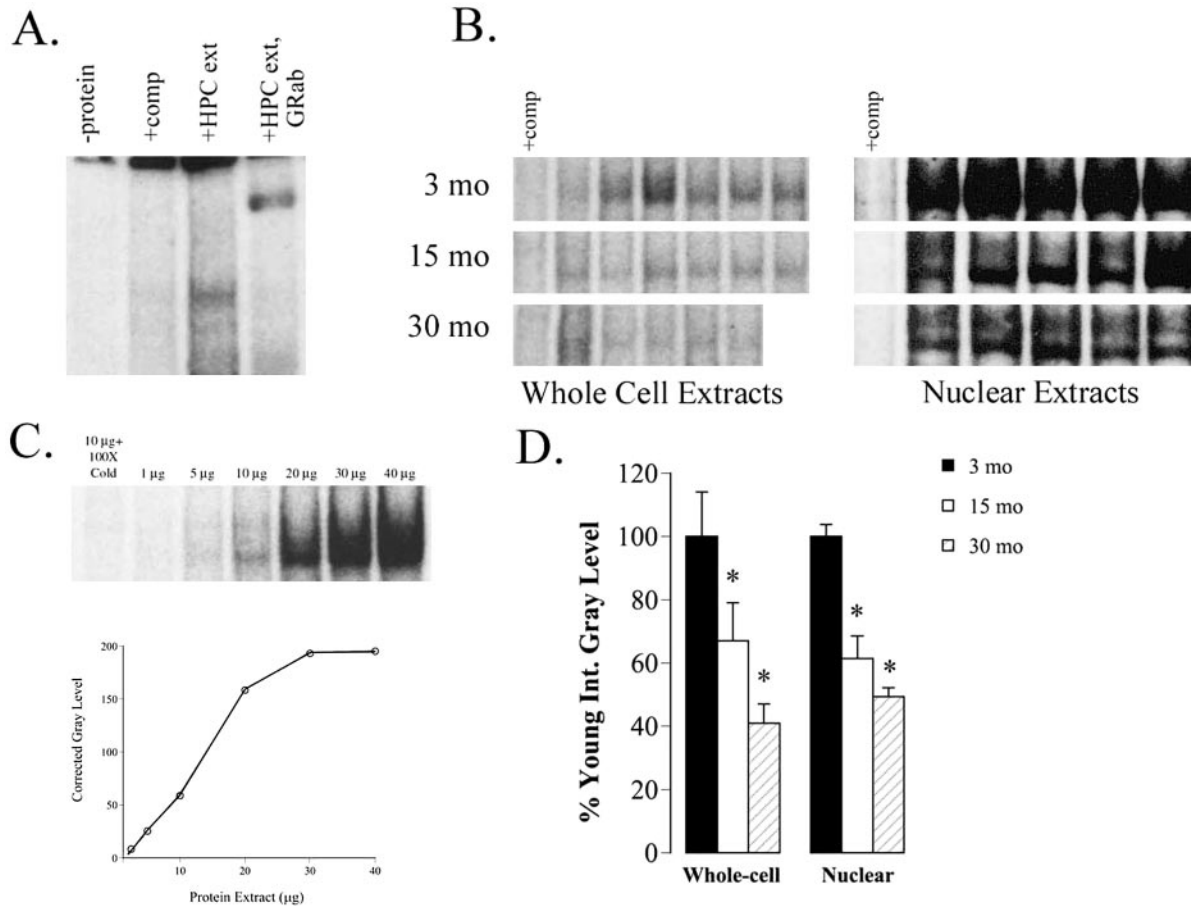


FIG. 4. GRE-binding activity in hippocampal protein extracts of young (3-month-old), middle-aged (15-month-old), and aged (30-month-old) F344/BN F₁ rats after handling or chronic stress exposure. A, Specificity of the EMSA protocol. No retardation of GRE oligonucleotide migration is seen in the absence of hippocampal protein or with 10 μ g hippocampal (HPC) protein extract preincubated with a 100-fold excess of unlabeled GRE nucleotide. Specific bands were observed in the presence of hippocampal extract, and this band could be supershifted by incubation of the binding reaction with 1 μ l GR antibody (ab). B, GRE binding in whole cell and nuclear hippocampal extracts of 3-month-old (*upper*), 15-month-old (*middle*), and 30-month-old (*lower*) animals. Age-related decreases in GRE binding could be observed in both whole cell and nuclear protein preparations. C, Relationship between the intensity of the EMSA bands and the amount of protein loaded in EMSA reactions, using protein from the same sample of hippocampal extract. Note the protein-dependent increase in corrected gray level, saturating at about 30 μ g protein. A linear relationship between amount of protein and signal was observed between 1–20 μ g, indicating an ability to use this analysis scheme for semiquantitative analysis between 10–160 gray level units. Semiquantitative analyses were conducted using signal intensities within the linear range of the signal-protein curve. D, Semiquantitative analysis of GRE binding revealed age-related decreases in GRE binding in both whole cell ($P < 0.05$) and nuclear ($P < 0.01$) protein preparations. Whole cell GRE binding was substantially decreased in the hippocampus of the 15- and 30-month-old groups relative to 3-month-old animals ($n = 5$ –6/group; *, significantly different from the 3-month-old group, $P < 0.05$, by Fisher's PLSD test). EMSA analysis performed on nuclear extracts from hippocampi of a second cohort of animals ($n = 6$ /group) revealed substantial decreases in nuclear GRE binding in 15- and 30-month-old rats relative to young animals (*, significantly different from 3-month-old group, $P < 0.05$, by Fisher's PLSD test).

Nuclear trafficking of the GR is regulated by the macromolecular hsp90 heterocomplex. Additional Western analyses were performed to determine whether deficits in GR translocation were associated with decreased chaperone expression or trafficking. The results are presented in Fig. 5. Aged animals showed pronounced decreases in hsp90 and hsc70 in both nuclear and cytoplasmic extracts. Expression of inducible hsp70 was not affected by age. To assess the specificity of the observed trafficking changes, we examined the nuclear and cytosolic expression of calcineurin in our aging samples. Calcineurin is a phosphatase molecule involved in immune function and calcium signaling and is distributed in both nucleus and cytoplasm (24, 25). No age-related changes in calcineurin were observed in either cellular compartment.

TABLE 1. Plasma corticosterone levels in F344/BN F₁ hybrid rats

	3 months	15 months	30 months
Plasma corticosterone (ng/ml)	54.6 \pm 19.2	37.5 \pm 8.5	100.6 \pm 17.1 ^a

^a Significantly different from young and middle-aged groups, $P < 0.05$.

Age-related decreases in nuclear GR are not due to reduced corticosterone secretion. As can be seen in Table 1, resting corticosterone levels are significantly increased in aged F344/BN F₁ rats in the current study.

Discussion

The hippocampal GR is implicated in numerous physiological and pathological processes associated with the aging

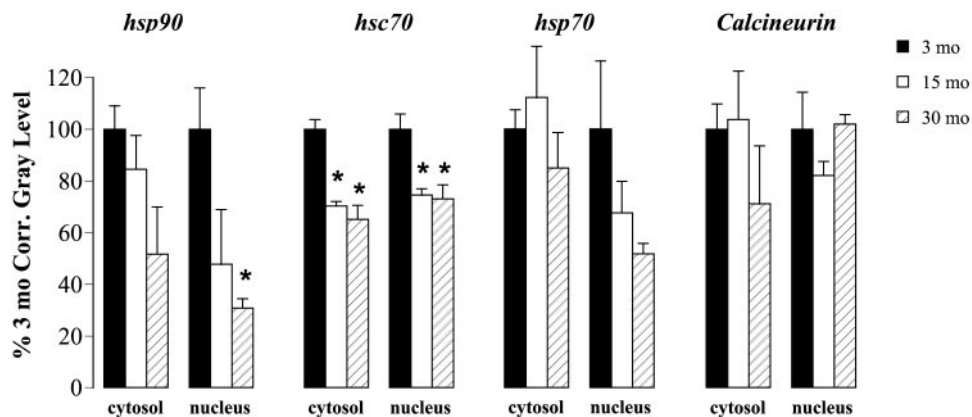


FIG. 5. Western blot analysis of hippocampal hsp90, hsc70, hsp70, and calcineurin immunoreactivity (ir) in nuclear (A) and cytosolic (B) samples of young (3-month-old), middle-aged (15-month-old), and aged (30-month-old) F344/BN F₁ rats. Analysis of hsp90 immunoreactivity revealed significant decrements in both cytosolic and nuclear extracts ($P < 0.05$) from aged hippocampus relative to young animals (*, significantly different from the 3-month-old group, $P < 0.05$, by Fisher's PLSD test). Decrements in hsc70 expression were seen in both middle-aged and aged groups ($P < 0.05$). Cytosolic and nuclear hsp70 and calcineurin immunoreactivity were not affected by age ($n = 3/\text{group}$).

process, including calcium buffering, learning and memory, and stress integration (4, 26). The current study suggests that age-related decrements in receptor translocation and availability of chaperone proteins reduce the physiological impact of the GR by attenuating entry into the nucleus and subsequent transcriptional regulation. The data are consistent with known decrements in GR-related hippocampal processes with age. For example, hippocampus-mediated inhibition of HPA stress responses is attenuated in aged rats (27), consistent with the loss of glucocorticoid feedback (4). Furthermore, age-related changes in spatial memory parallel those seen after GR deficiency or blockade (13, 15), consistent with removal of a positive GR action in cognition. The present study indicates that aging is associated with diminished, rather than enhanced, GR signaling in the cell nucleus, and that glucocorticoid-related deficits seen in aged rats are due to subtraction of beneficial actions of GR on hippocampal function.

The observed reduction in GR signaling capacity indicates that the net impact of glucocorticoids on neuronal function will be diminished in the aged hippocampus. As such, age-related glucocorticoid hypersecretion is less likely to impinge on hippocampal cell physiology or viability. However, the reduced GR signaling capacity seen in aged rats does not preclude increased glucocorticoid activation at an earlier age. Indeed, alterations in CRH expression and increased glucocorticoid secretion occur in middle-aged animals (27, 28), suggesting that altered HPA function may allow for cumulative effects of glucocorticoids on neurons. The negative effects of earlier glucocorticoid elevations on dendritic arborization (29) and granule cell division (30) may play a role in initiating processes resulting in reduced nuclear GR transport in aging.

Age-related decreases in nuclear GR are not due to reduced corticosterone secretion. Indeed, circulating corticosterone levels were slightly elevated in this aged cohort. In conjunction with our previous data indicating similar levels of plasma corticosteroid-binding globulin in aged F344/BN F₁ rats (27), these results indicate that free corticosterone

levels are probably increased in aged animals, which should enhance, rather than attenuate, GR translocation.

The lack of change in nuclear MR protein may be due to differential control of GR *vs.* MR transport. For example, *in vitro* studies indicate that MR is localized in the cell nucleus in the absence of hormone (31), suggesting a ligand-independent transport process. In addition, the low abundance and high affinity glucocorticoid binding of the MR may allow readier access to the nuclear translocation apparatus. Whatever the mechanism, the data suggest that aging produces a very different corticosteroid receptor complement in the nucleus of hippocampal neurons. The availability of a full complement of MR in the face of reduced GR may enhance transcriptional actions of MR-MR homodimers. The MR is involved in viability-promoting aspects of glucocorticoid signaling, serving to promote dentate gyrus granule cell survival (32), enhance pyramidal cell excitability (33), and positively regulate the expression of antiapoptotic genes in CA1 (34). As such, the reduction in nuclear GR may produce a transcriptional scenario favoring trophic effects of low levels of glucocorticoids. However, the trophic influences associated with reduced GR may not be compatible with optimal neuronal function. The complementary effects of MR and GR on cellular processes have led to the hypothesis that the intracellular ratio of MR and GR may dictate whether glucocorticoid signals will have positive or negative consequences (12). For example, selective antagonism of either MR or GR produces deficits in different aspects of spatial memory (35), implying that both are necessary for optimal glucocorticoid signaling. This interaction is further supported by evidence that the MR and GR heterodimerize (36), suggesting that the two receptors can influence gene expression in a coordinate manner. Thus, the selective reduction in GR relative to MR may have profound consequences for hippocampal cell physiology, serving to attenuate beneficial as well as deleterious actions of glucocorticoids.

Reduced GR mRNA with age is consistent with the findings of previous studies (28, 37–39). Importantly, this decrease occurs in middle age (28) and coincides with similar

decreases in CRH mRNA expression (27, 28). Thus, it is likely that the GR mRNA decrease is a product of maturation rather than old age *per se*. Despite the reduced mRNA levels, no age-related changes were observed in protein levels by immunautoradiography or Western blot using whole cell extracts. Thus, it is evident that decreased GR mRNA is not predictive of protein expression, suggesting enhanced protein translation processes, reduced protein degradation, or increased mRNA stability with age.

In contrast to the marked decrease in nuclear GR immunoreactivity seen in hippocampal nuclear extracts of aged rats, immunautoradiographic and whole cell Western blot analyses did not reveal decreased GR protein expression. In both cases the deleterious effects of aging on nuclear levels were probably diluted by normal expression of GR in the cytosol.

Age-related decreases in GR mRNA, nuclear protein, and DNA binding are unlikely to reflect reduced hippocampal neuron number. Although cell counts could not be performed in the animals employed for biochemical measures, the lack of change in MR mRNA/protein, total GR protein, and cytosolic GR and MR immunoreactivity makes it unlikely that the decreases in nuclear GR and DNA binding are due to cell loss. Further, the *in situ* hybridization and immunautoradiography data do not show differential age-related changes in pyramidal *vs.* granule cell populations, which would be expected if CA1 or CA3 cells were selectively lost in aging. Finally, reduced GR mRNA and GRE binding are seen in middle-aged rats, which do not show substantial neuron loss in any model examined to date. Indeed, the generality of hippocampal cell loss in aging is currently a topic of debate (16) and may or may not occur in this rat strain.

The GRE data probably reflect diminished GR DNA binding in the cell nucleus, given that nuclear MR levels are not affected by aging. However, it should be noted that the resolving power of the EMSA assay is not sufficient to definitively distinguish GR and MR homo- or heterodimers. Furthermore, it is not known whether the affinity of MR for the consensus GRE differs from that of GR *in vivo* or, indeed, whether the MR is capable of binding distinct DNA consensus sequences. Thus, whereas the results are consistent with reduced GR action, interpretation of the gel shift data *viz.* MR is limited.

Middle-aged animals show loss of GRE binding without concomitant decreases in nuclear GR levels. These data suggest that additional age-related factors may mitigate the ability of the GR to bind DNA. Importantly, the GR exhibits protein-protein interactions with numerous transcription factors, including c-Jun (40), nuclear factor- κ B (41), and gonadal steroid hormone receptors [*e.g.* AR (42)]. Thus, it is possible that modulation of other nuclear protein factors may interfere with GR signaling even in middle-aged animals. In support of this idea, it is known that homeostatic and synaptic regulators of transcription factor activation show gradual changes across the life span; for example, serum T levels decrease progressively with age in rats (43), hippocampal expression of 5-HT₇ receptor mRNA is decreased in middle-aged animals (44), and neuronal nitric oxide synthase is elevated in hippocampus by middle age (45). Thus, middle

age is marked by changes in numerous neuromodulatory signaling pathways that may indirectly impact on GR DNA binding capacity.

In the hypothalamus, nuclear GR levels were increased in aged animals. Analysis of the ratio of nuclear to cytosolic GR suggests enhanced, rather than reduced, transport. Elevated nuclear GR may be due to transient or cumulative increases in circulating corticosterone levels, which would serve to increase cytosolic binding and subsequent translocation (46, 47). Indeed, circulating corticosterone levels are significantly increased in the aged cohort used in this study, perhaps accounting for the elevated nuclear GR level. These data indicate that GR nuclear transport or retention is functionally intact in the hypothalamus, suggesting that age-related GR reductions are cell or region specific.

Preferential loss of nuclear GR in hippocampus *vs.* hypothalamus is consistent with the prominence of this structure as a target for age-related dysfunction. The hippocampal formation is among the regions most vulnerable to disease processes (*e.g.* Alzheimer's disease) (48) and is often among the earliest regions to show morphological abnormalities with age (49, 50). The hippocampus also shows selective accumulation of oxidized proteins in aging (51), which may increase competition for chaperone proteins.

Although correlative, the observed reduction in hsp90 in the nuclear and cytosolic fractions suggests an involvement in age-related nuclear GR protein reductions. As hsp90 is essential for ligand binding and transport of the GR into the nucleus (7, 52), decreased cytosolic hsp90 may reflect a reduced amount of GR able to bind ligand and thus render the GR less likely to translocate. Interestingly, the effects of age on hsc70 are evident at 15 as well as 30 months. As such, this change is unlikely to account for specific decreases in nuclear GR in the 30-month group. However, the data do not preclude involvement of hsc70 in progressive decrement in nuclear GR and GRE binding seen across the aging process.

hsc70 and hsp90 are involved in folding and transport of numerous cellular proteins. The hsps also bind misfolded proteins, perhaps as a mechanism to limit cellular damage (53). Notably, neuronal aging is associated with accumulation of modified (*e.g.* oxidized) proteins (54); as such, reduced GR translocation may be associated with increasing competition for cytoplasmic hsps. Thus, the combination of reduced hsp90/hsp70 protein levels and enhanced competition for available hsps may contribute to the observed impairment in nuclear GR protein levels and DNA binding.

The mechanism for age-related decreases in hippocampal hsp90 and hsc70 levels is currently undefined. Whereas altered glucocorticoid signaling represents a plausible mechanism for this down-regulation, *in vivo* data on glucocorticoid regulation of hsp90 are conflicting. Previous reports indicate that hsp90 mRNA expression is negatively regulated by glucocorticoids in the hippocampus (55); however, steady state protein levels are not affected by either glucocorticoid manipulations or stress (56). Alternative possibilities, including age-related changes in calcium buffering (57) or second messenger signaling (58), remain to be evaluated.

Notably, no concurrent decrement in calcineurin was observed in either nucleus or cytoplasm in the aged hippocampus. Calcineurin is a protein phosphatase that is involved in

calcium signal transduction and immune function (24, 25) and is not known to use the hsp90 chaperone complex for translocation. The equivalence of calcineurin levels across the aging spectrum indicates that the GR reduction is not due to general aging decrements in nuclear translocation efficiency or age-related artifacts in nuclear/cytosolic protein extraction.

Overall, the current study indicates that despite elevations in HPA activity, nuclear transport of GR is reduced in the aged hippocampus. Impaired nuclear GR transport or retention, whether due to modification of transcription factor proteins or competition for the nuclear trafficking machinery, may alter the transcriptional capacity of affected neurons. The GR is a critical regulator of cellular and organismic homeostasis, and attenuated nuclear signaling of this (and perhaps other) nuclear transcription factors will probably have profound implications for aging neurons.

Acknowledgments

The authors thank Mark Dolgas and Garrett Bowers for invaluable technical support, and Dana Zeigler, Deanna McCullers, and Dr. Helmer Figueiredo for helpful comments on the manuscript.

Received August 23, 2001. Accepted December 12, 2001.

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This work was supported by NIA Grants AG-12962 (to J.P.H. and R.L.S.), AG-10836 (to J.P.H.), and AG-00242 (to K.J.S.).

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