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Hippocampal Neurogenesis Is Not Enhanced by Lifelong Reduction of Glucocorticoid Levels

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Abstract

Neurogenesis of dentate gyrus granule cells is generally considered to be negatively regulated by glucocorticoids. We tested the hypothesis that exposure to low plasma corticosteroid levels starting in the early postnatal period enhances granule cell proliferation rate during adulthood. Rat pups were adrenalectomized (ADX) on postnatal day 10 and were then "clamped" throughout life at low corticosterone levels via oral supplementation. Neurogenesis was determined using BrdU immunochemistry at 3 and 12 months in clamped rats as compared with age-matched, sham-operated controls. Rate of neurogenesis did not differ between the groups at either 3 or 12 months. It was significantly lower in 12-month-old compared with 3-month-old rats, despite the presence of an age-dependent increase of plasma corticosterone only in the sham-ADX rats. Granule cell layer volume, granule cell density, and granule cell degeneration (determined using apoptotic markers) were indistinguishable in the two groups, further supporting the comparable rate of neurogenesis under differing chronic glucocorticoid levels. In addition, whereas acute deprivation of plasma glucocorticoids (adrenalectomy) in adult rats evoked a burst of granule cell neurogenesis, complete elimination of these hormones (by stopping hormone supplementation) in adult, early-life ADX/clamped rats did not. These data do not support a simple inverse relationship between chronic plasma glucocorticoid levels and granule cell neurogenesis. Specifically, chronic modulation of glucocorticoid levels commencing early in life evokes additional, adaptive, and compensatory mechanisms that contribute to the regulation of granule cell proliferation.

Keywords

BrdU; granule cells; mineralocorticoid receptor; dentate gyrus; adrenalectomy

INTRODUCTION

In mammalian brain, the dentate gyrus (DG) is among the few areas where production of significant numbers of new neurons continues throughout life (Altman and Das, 1965; Bayer et al., 1982; Kuhn et al., 1996; Eriksson et al., 1998; Kornack and Rakic, 1999). This ongoing neurogenesis provides a continuous supply of newly formed, undifferentiated granule cells (Cameron and McKay, 2001; Bender et al., 2001; van Praag et al., 2002; Kempermann et al., 2003), which may play a role in certain memory processes of the aging hippocampus (van Praag et al., 1999; Lemaire et al., 2000; Shors et al., 2001; Drapeau et al., 2003). In rat DG, neurogenesis reaches a peak during the second postnatal week (Schlessinger et al., 1975), then declines progressively during adulthood (Seki and Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998; Nichols et al., 2001; Bizon and

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Gallagher, 2003; Heine et al., 2004). The loss of neurogenic potential of the aged DG has been proposed as a contributor to age-related deficits in cognitive functions involving the hippocampus (Cameron and McKay, 1999; Drapeau et al., 2003; but see Bizon and Gallagher, 2003; Merrill et al., 2003; Heine et al., 2004). Therefore, understanding the mechanisms that are responsible for age-related loss of neurogenic potential in the DG may be helpful for eventual strategies for prevention of aging-associated cognitive decline.

Among factors that influence granule cell neurogenesis, the role of adrenal steroids has been extensively studied. Glucocorticoids inhibit (Gould et al., 1991; Cameron and Gould, 1994), and adrenalectomy during adulthood increases granule cell neurogenesis (Gould et al., 1992; Rodriguez et al., 1998; Cameron and McKay, 1999; Nichols et al., 2001). In general, the rate of granule cell proliferation in the rat is inversely related to basal glucocorticoid levels. For example, adrenal steroid levels in the neonatal rat are relatively low for the first 2 postnatal weeks (Levine, 1970; Walker et al., 1991; Yi and Baram, 1994; Gould and Tanapat, 1999), coinciding with the period of maximal granule cell proliferation (Schlessinger et al., 1975). Basal plasma corticosterone (CORT; the major glucocorticoid in the rat) levels rise gradually after this period (Walker et al., 1991), concurrent with a decline in granule cell neurogenesis (Kuhn et al., 1996; Nichols et al., 2001; Bizon and Gallagher, 2003; Heine et al., 2004). During aging (> 12 months), plasma CORT reaches its highest levels (e.g., Sapolsky, 1992; but see Heine et al., 2004), whereas granule cell production decelerates further (Kuhn et al., 1996; Nichols et al., 2001; Bizon and Gallagher, 2003; but see Heine et al., 2004). This intriguing correlation of plasma glucocorticoids and granule cell neurogenesis has led to the hypothesis that elevated glucocorticoid levels are responsible for decreased neurogenesis in the aging hippocampus (Cameron and McKay, 1999; Drapeau et al., 2003). Here we tested this hypothesis by performing adrenalectomy in early postnatal rats and then keeping these rats on chronically low CORT levels (clamping) throughout life. If indeed glucocorticoid levels determine the rate of neurogenesis in the aging hippocampus, then granule cell production should be significantly increased in chronically clamped rats when compared with age-matched controls.

MATERIALS AND METHODS

Experimental Design

To evaluate neurogenesis, rats were injected with 5-bromo-2′-deoxyuridine (BrdU; Roche, Indianapolis, IN; 150 mg/kg body weight i.p.) and sacrificed 24 h (Experiments 1–4) or 28 days (Experiment 5) later:

- *Experiment 1*: Neurogenesis was evaluated at 12 months of age in rats that were adrenalectomized (ADX) on postnatal day (P) 10 and maintained on CORT supplement (ADX/clamped; $n = 7$), and compared with sham-ADX controls ($n = 6$).
- *Experiment 2*: Neurogenesis was evaluated at 3 months of age in rats that were ADX on P10 and maintained on CORT supplement ($n = 5$) compared with sham-ADX controls ($n = 5$).
- *Experiment 3*: To examine the neurogenic potential following the lifelong differing CORT levels, rats that had been ADX/clamped on P10 ($n = 4$) were deprived of their source of CORT 6 days before sacrifice (at 3 months of age), by stopping their CORT supplementation. Neurogenesis rate was compared with that of sham-ADX controls ($n = 3$).
- *Experiment 4*: To compare the acute effects of steroid withdrawal on neurogenesis in our experiments to those published, rats that were ADX as adults (3 months of age; $n = 5$) were injected with BrdU 6 days after adrenalectomy (with no CORT

supplement), and their neurogenesis rate was compared with that of sham-ADX controls ($n = 4$).

- *Experiment 5*: To determine the fate of BrdU-labeled cells, 3-month-old rats ($n = 3$) were injected with BrdU and sacrificed 28 days after injection.
- *Experiment 6*: To consider the potential role of adrenal steroid receptors in the adaptation to early- life onset chronic low plasma CORT levels, the expression of glucocorticoid and mineralocorticoid receptors (GR and MR, respectively) was studied. Rats were ADX and "clamped" on P10 and sacrificed on P19, P30, 3 or 12 months ($n = 5$ per group).

Animals

Sprague-Dawley-derived male rats (Zivic-Miller, Zelienople, PA) were born in our vivarium and maintained in this National Institutes of Health (NIH)-approved animal facility on a 12-h light/dark cycle with access to unlimited lab chow and water. Delivery was verified at 12-h intervals, and the date of birth was considered day 0. Litters were culled to 12 pups if needed and mixed among experimental groups: thus, effects of experimental manipulations were compared among littermates. For technical reasons, the experiments were performed in several batches, but each batch included both control and experimental groups. Following weaning, all rats were housed 2–3 per cage.

Surgical Procedures

Adrenalectomy or sham-adrenalectomy was performed under halothane anesthesia (~5 min/animal) on 10-day or 3-month-old rats via small bilateral dorsal incisions (Brunson et al., 2001a,b). The completeness of the adrenalectomy was verified by visual inspection at the time of sacrifice, as well as by assessment of plasma CORT. To permit normal mineralocorticoid function and based on pilot experiments, ADX animals were supplemented with aldosterone (subcutaneously, 2 μg/100 g body weight/day) during P10–21 (Walker et al., 1990; Brunson et al., 2001b). After weaning (P21), CORT (10 mg/L) was applied to the drinking solution (0.9% saline) (Walker et al., 1990; Akana and Dallman, 1997). This supplementation (clamping) leads to chronic "basal" glucocorticoid levels (Walker et al., 1990; Akana and Dallman, 1997), saturating MR but not GR (Reul and de Kloet, 1985; Herman, 1993). At the time of sacrifice, blood samples were collected via cardiac puncture and CORT levels were determined using a commercial radioimmunoassay (RIA) kit (ICN, Irvine, CA) as previously described (Eghbal-Ahmadi et al., 1999). Assay sensitivity was 0.5 μg/dl and intra-assay variability was approximately 5–7%.

All experiments were initiated at 8–10 a.m. to minimize diurnal variability of stress hormones (Watts et al., 2004). The experiments were also designed to minimize pain and discomfort, were carried out according to NIH guidelines and were approved by the Institutional Animal Care Committee.

Tissue Processing

Rats used for the neurogenesis study were sacrificed via injection of a lethal dose of pentobarbital and perfused transcardially with 0.9% saline followed by cold 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.3). Before starting perfusion, blood samples were collected from the left ventricle. Perfused brains were removed from the skull, postfixed (4 h in 4% paraformaldehyde/0.1 M phosphate buffer), cryoprotected (48 h in 25% sucrose/0.1 M phosphate buffer) and frozen in −50°C cold isopentane. Serial coronal sections (50 μm) were cut throughout the entire hippocampus on a cryostat. Slides were either processed immediately (BrdU analysis, co-localization study) or mounted on glass

slides and stored for future use. For in situ hybridization analyses, animals were sacrificed by rapid decapitation, and brains were immediately frozen on powdered dry ice.

BrdU Detection and Analysis

Every sixth section of a hippocampal series was processed for BrdU detection, as described previously (Bender et al., 2001). Free-floating sections were transferred to 2× SSC, then immersed in 50% formamide/2× SSC (2 h, 65°C), followed by 2 M hydrochloric acid (30 min at 37°C) to denature DNA. After neutralization with 0.1 M sodium borate (pH 8.5) and pre-incubation with phosphate-buffered saline (PBS) containing 0.3% Triton-X and 3% normal goat serum (NGS), sections were incubated with anti-BrdU (rat monoclonal IgG, Accurate Chemicals, Westbury, NY; 1:1,000) at room temperature (RT) for 24 h, followed by a biotinylated secondary antibody (anti-rat IgG, 1:600; Chemicon, Temecula, CA) and the avidin-biotin-peroxidase reaction components (Vector Laboratories, Burlingame, CA). BrdU-labeled nuclei were visualized by incubating sections in 0.04% 3, 3′ diaminobenzidine-solution containing 0.002% H_2O_2, 0.01% $NiCl_2$, and 0.01% $CoCl_2$. Eight anatomically matched sections were chosen from each series for BrdU analysis. In these sections, BrdU-immunoreactive nuclei were counted in the dorsal left and right hippocampus. Only nuclei that were located in granule cell layer or subgranular zone (defined as a two-cell body wide zone along the inner border of the granule cell layer) were included in the analysis. Data were correlated to granule cell layer surface area determined in adjacent cresyl violet-stained sections (see below) and presented as “BrdU-positive cells/mm^2 granule cell layer.” Data presentation as cell densities was chosen over a stereological analysis calculating total numbers, because the latter is more likely to incur sampling errors that could obscure minor differences among experimental groups. In these, as in the following experiments, all counts were performed by an observer blinded to the experimental group status of the sections.

Immunohistochemistry

Co-localization of BrdU with mature granule cell (calbindin D28k), glial fibrillary acidic protein (GFAP), or epithelial (rat endothelial cell antigen, RECA1) markers was examined using immunofluorescence. Free-floating sections were pre-treated as described above for BrdU, then incubated with rat anti-BrdU together with one of the following primary antibodies: polyclonal rabbit anti-calbindin D28k (1:800; Chemicon), monoclonal mouse anti-GFAP (1:1,000; Chemicon) or monoclonal mouse anti-RECA1 (1:20; SeroTec, Raleigh, NC), for 48 h at 4°C. First antibodies were subsequently detected with Alexa Fluor 488-conjugated secondary antibodies (Calbindin D28k, GFAP, RECA1) and biotinylated anti-rat IgG antibody followed by streptavidin Alexa Fluor 568-conjugate (BrdU; all fluorescent products were purchased from Molecular Probes, Eugene, OR). For each marker, two sections per brain were analyzed using a Zeiss LSM510 Meta confocal microscope (Argon Laser 488 mm and He–Ne Laser 543 mm). The number of double-labeled cells was determined and correlated to the total number of BrdU-labeled cells in these sections. Data are presented as “percentage of BrdU-labeled cells.”

For GFAP-immunohistochemistry, slide-mounted sections were incubated in 10 mM Na-citrate, pH 9.0 (30 min at 80°C), for antigen retrieval (Jiao et al., 1999). After cooling, sections were quenched with 0.3% H_2O_2 and immersed in 0.01 M PBS containing 0.3% Triton X-100, 0.2% sodium dodecyl sulfate (SDS) 1% bovine serum albumin (BSA), and 2% NGS for 1 h to block unspecific binding sites. GFAP antiserum (see above) was added and sections were incubated for >48 h at 4°C. Antigen binding was visualized using the avidin-biotin-peroxidase technique as described above. GFAP-immunopositive glial cells located in granule cell layer or subgranular zone were counted in two sections per brain (from dorsomedial hippocampus). Only cells with clearly visible somata and associated

radial processes were included in the analysis. Numbers were correlated to granule cell layer surface area and are presented as "GFAP-positive cells/mm^2 granule cell layer."

Analysis of Granule Cell Layer Volume and Granule Cell Density

Sections adjacent to those chosen for BrdU detection were stained with 1% cresyl violet acetate and analyzed for the following parameters. First, granule cell layer surface area was measured in each section using a counting grid; granule cell layer volume was then determined by multiplying the average value (total surface area/number of analyzed sections) with the distance from the septal to the temporal pole as calculated from the total number of sections in the series. Second, granule cell densities were determined by systematically counting granule cell nuclei included in a counting grid positioned above the midpoint of the granule cell suprapyramidal layer. Counting was performed in 10 units (1 U = 25 μm × 25 μm) of the grid by focusing through the entire vertical depth of the section at each position (revealing 3–4 layers of granule cells). Samples were taken from the eight sections (left and right hippocampus = 16 values per rat), which were adjacent to the eight sections used for BrdU analysis. Granule cell densities were computed as: N (neurons/mm^3) = $A[M/(L+M)]/V$, where A is the number of counted nuclei, L is the average length of nuclei, M is the section thickness, and V is the volume of the sampling area (Bender et al., 2003). All measurements and cell counts were performed under 400× magnification.

Analysis of Granule Cell Degeneration

Two separate methods were used to determine granule cell degeneration. First, granule cells were considered to be undergoing apoptosis based on morphological characteristics, including pyknotic, shrunken nuclei and an eosinophilic cytoplasm (Gerth et al., 1998). Thus, cells exhibiting dense chromatin forming crescent or ring-like structures or discrete clumps were counted in the cresyl violet-stained sections (see above). Single pyknotic nuclear clumps were excluded. Cells were counted in the same counting grid (and at the same positions) used to determine granule cell densities, and data are presented as "pyknotic cells/mm^2 granule cell layer." Second, in addition, terminal-transferase-mediated dUTP nick-end-labeling (TUNEL) was performed to detect cells undergoing apoptosis (modified from Heine et al., 2004). Slide-mounted sections (a series adjacent to those chosen for BrdU detection) were pre-treated in 0.1 M sodium citrate buffer (pH 6.0) in a microwave oven set at full power for 2.5 min. After cooling the jars, sections were pre-incubated with Proteinase K buffer (10 mM Tris-HCl, 2.6 mM $CaCl_2$, pH 7.6) for 10 min, followed by 20 μg/ml Proteinase K (Promega, Madison, WI) for 15 min at RT. After a brief rinse in double-distilled water and wash in PBS, sections were pre-incubated with terminal transferase (TdT) buffer (0.2 M sodium cacodylate/0.025 M Tris-HCl/0.25 mg/ml BSA, pH 6.6) for 10 min and incubated for 60 min at 37°C with 1 μl TdT and 1 μl biotin-16-UTP (Promega) per 100 μl reaction mixture. TdT reaction was stopped in 2× SSC (5 min), endogenous peroxidase activity was blocked with 0.3% H_2O_2 in PBS (15 min) and sections were transferred to streptavidin-peroxidase (Promega) for 1 h. Labeling was visualized by incubating sections in 3,3′ diaminobenzidine-solution containing H_2O_2, $NiCl_2$, and $CoCl_2$ (see above). TUNEL-positive cells were counted in the entire granule cell layer of at least four sections per brain (labeled cells in the two innermost layers were not included to avoid potential false-positives due to BrdU-induced DNA-breaks). Data are presented as "TUNEL-positive cells/mm^2 granule cell layer."

In situ hybridization histochemistry (ISH) for GR and MR mRNA: ISH and riboprobe labeling were performed as described previously (Eghbal-Ahmadi et al., 1999). Coronal sections (20 μm) of dorsal hippocampus were cut using a cryostat. Before hybridization, sections were brought to room temperature, air dried and fixed in fresh 4% buffered paraformaldehyde for 20 min, followed by dehydration and rehydration through graded

ethanols. Sections were exposed to 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 8 min and were dehydrated through graded ethanols. Prehybridization and hybridization steps were performed in a humidified chamber at 55°C in a solution of 50% formamide, 5× SET, 0.2% SDS, 5× Denhardt's, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast tRNA, 100 mM dithiothreitol and 10% dextran sulfate. After 1-h prehybridization, sections were hybridized overnight with antisense riboprobes radioactively labeled with ^{35}S-CTP (GR probe, a kind gift from Dr. J.N. Masters) or ^{35}S-UTP (MR probe, a kind gift from Dr. S. Rivest). The specificity of the probes has previously been established. After hybridization (with 1×10^6 cpm of riboprobe per section), sections were washed in 2× SSC for 5 min at room temperature and were exposed to RNase A (200 μg/ml) in 10 mM Tris-HCl (pH 8)/ NaCl for 30 min at 37°C. Sections underwent serial washes of increasing stringency at 55°C, the most stringent being at 0.03 × SSC for 1 h. Hybridized and washed sections were apposed to film (Hyperfilm β-Max, Amersham, IL) for 10–14 days. Semiquantitative analysis of the ISH signal was performed on digitized films using the ImageTool software program (UTHSC, San Antonio, TX) as previously described. For analysis, three matched dorsal hippocampal sections/animal were sampled, using unbiased methods (Eghbal-Ahmadi et al., 1999).

Statistical Analysis

The results are depicted as means with standard errors (SEM). Statistical significance was set at $P < 0.05$. Since most analyses compared two experimental groups, differences were evaluated using unpaired Student's *t*-test. For Figure 1, plasma corticosterone levels were analyzed with a two-way analysis of variance (ANOVA) with treatment (ADX) as a between subject variable and age as the within subject variable.

RESULTS

Adrenalectomy/Clamping Paradigm Results in Lifelong Reduction of Plasma Glucocorticoids

Adrenalectomy on P10, with CORT supplementation, influenced CORT plasma levels for life (effect of treatment: $F = 25.5$, $P < 0.0001$; Fig. 1). As shown in Figure 1, the adrenalectomy followed by "clamping" of CORT led to relatively constant low plasma levels (2.3–2.5 μg/dl) that are typical of basal CORT throughout the majority of morning hours (Dallman et al., 1987;Watts et al., 2004). Statistical divergence from sham-ADX levels emerged at 3 months, and was maintained to 12 months. Note that controls were sacrificed immediately upon removal from the cage at 8–10 a.m. However, it is reasonable to consider that in animals with intact adrenals, stressors (e.g., cage changes, other human contact) evoked occasional bursts of stress-level plasma glucocorticoids, that saturate GRs (Reul and de Kloet, 1985;Spencer et al., 1990). Note also that in both groups plasma CORT was increased at 12 months.

Hippocampal Neurogenesis Rate Does Not Differ Between ADX/Clamped and Sham-ADX Controls

While plasma CORT levels clearly distinguished the ADX/clamped from the intact group at both 3 months and 12 months, the number of BrdU-positive cells were similar in these two groups. As shown in Figure 2A, the rate of cell proliferation in 12-month-old rats was significantly lower than that of 3-month-old animals ($P < 0.0001$) for both the intact and ADX/clamped groups. However, proliferation rates did not differ between these groups at either age ($P = 0.73$ and 0.84 at 3 months and 12 months, respectively).

Figure 2 illustrates a typical DG after BrdU labeling in a 3-month (Fig. 2B) and a 12-month-old rat (Fig. 2C). Reduction in the number of BrdU-incorporating nuclei in the latter is

apparent. This reduction was similar in magnitude in both groups of rats (75% in intact; 76% in ADX/clamped rats). In contrast, the plasma CORT level was higher in the intact group (34 ± 6 μg/dl) versus the ADX/clamped group (10 ± 3 μg/dl; Fig. 1).

Mitotically active cells in subgranular zone do not only produce granule cells but also include progenitors of other cell types, e.g., epithelial cells and glial cells (Palmer et al., 2000) (Fig. 2D). Glial cells, in particular, have previously been shown to be sensitive to alterations in CORT levels (Gould et al. 1992; Nichols et al., 2001). To estimate the relative contribution of glial cells to the population of BrdU-incorporating cells, we injected BrdU into 3-month-old rats and determined the phenotype of BrdU-labeled cells 28 days later. At that time point, the majority of labeled cells (48%) had migrated into the granule cell layer and co-localized Calbindin D28k, suggesting differentiation into mature granule cells (Fig. 2D,E). Only 6% expressed the glial cell marker GFAP, whereas 7% had an epithelial cell phenotype; 39% of BrdU-labeled cells, mainly those still residing in or close to the subgranular zone, did not co-localize any of the cell-type markers studied. Because these cells probably represent slowly maturing granule cells (Heine et al., 2004), it can be estimated that the majority (>50%) of the BrdU-labeled cells in the current study differentiates into granule cells.

While only 6% of BrdU positive cells co-expressed GFAP at a given time during adulthood, this fact cannot exclude a subtle shift in the granule cell/glial cell production ratio after chronically low CORT, leading to a dissociation of the number of BrdU-labeled cells from the true granule cell production rates in the clamped rats. This possibility should result in altered glial cell density in DG. Therefore, the density of GFAP-expressing glial cells was compared between 12-month-old rats that had been clamped on low CORT throughout life and controls. As shown in Figure 2F, glial cell densities did not differ in clamped and sham-ADX controls, rendering a significant effect of lifelong low CORT on glial cell production rate unlikely.

Granule Cell Number and Viability Do Not Differ in Early-Life ADX/Clamped Rats and Controls

A modest but enduring increase of granule cell production in rats with constant low levels of CORT might be missed by the single sampling time-point using BrdU. However, such increased production (assuming equal cell death) should result in increased total numbers of granule cells. This can be measured as increased granule cell layer volume, higher granule cell packing density or both. As shown in Figure 3A,B, the volume and packing density of the granule cell layer were virtually identical in 12-month-old animals subjected to differing CORT levels throughout life. Similarly, no significant difference was found in 3-month-old rats (volume: 2.75 ± 0.12 mm^3 in clamped vs. 2.93 ± 0.17 mm^3 in control rats; density: 3.48 ± 0.1 × 10^5 cells/mm^3 in clamped vs. 3.63 ± 0.2 × 10^5 cells/mm^3 in control rats).

It could be argued that the lack of increased numbers of granule cells in the ADX/clamped group was attributable to an increased death rate, as found after adrenalectomy in adult rats (Sloviter et al., 1993). Therefore, we analyzed granule cell degeneration in 12-month-old intact and ADX/clamped animals (Fig. 3C) using two methods: (1) density of pyknotic nuclei; and (2) density of TUNEL-positive (apoptotic) nuclei. In both analyses, rates of granule cell degeneration were not significantly different between the groups (the lower number of TUNEL-positive compared with pyknotic nuclei is probably the result of a narrower time window for TUNEL detection of apoptotic nuclei). Taken together, these data support the notion that the rate of granule cell neurogenesis is not significantly affected by the chronically low plasma glucocorticoid levels.

Rate of Neurogenesis is Increased After Adrenalectomy of Adult Rats

The failure to detect increased neurogenesis rates in rats exposed to enduring low levels of plasma glucocorticoids might derive from technical issues with the BrdU method (Cameron and McKay, 2001; Rakic, 2002; Hayes and Nowakowski, 2002). Therefore, granule cell production was examined in 3-month-old rats that were ADX as adults and kept without CORT supplementation for 6 days (serum CORT levels of these rats were undetectable, i.e., <0.5 μg/dl in four of five animals, and 1.1 μg/dl in one). As expected from previous reports (e.g., Cameron and Gould, 1994; Rodriguez et al., 1998; Cameron and McKay, 1999; Nichols et al., 2001), adrenalectomy in adult rats provoked an increase of granule cell proliferation in subgranular zone (Fig. 4A). The lack of enhanced granule cell production in the ADX/clamped group in response to chronic low plasma CORT is therefore not explained by a lack of sensitivity of the BrdU method in our hands and requires other explanations.

Granule Cells From Rats With “Lifelong” Low CORT Die Upon Corticoid Withdrawal, but Their Proliferative Response is Impaired

Adrenalectomy early in life, with clamping of plasma CORT at levels saturating MR but not GR (de Kloet et al., 1990), might lead to adaptation of the granule cells to these hormone levels. Therefore, we investigated the responsiveness of granule cell neurogenesis to total elimination of plasma CORT, by withdrawing the hormone from the drinking solution of 3-month-old rats that were ADX/clamped on P10. In comparison with total withdrawal of CORT from intact 3-month-old rats (via adrenalectomy at this age, see above), a 6-day withdrawal of CORT from rats raised with chronic low CORT levels did not provoke enhanced neurogenesis (cf. Fig. 4B with Fig. 4A).

However, the effective elimination of plasma CORT induced apoptosis in the early-life ADX/clamped group, as demonstrated with both pyknotic nuclei and TUNEL analysis (Fig. 4D,F). This effect, likely attributable to loss of MR activation (Reul and de Kloet, 1985; Hornsby et al., 1996; Gerth et al., 1998), was similar in magnitude to the apoptosis evoked by a 6 day adrenalectomy in previously intact 3-month-old rats (Fig. 4C,E). Taken together, these data suggest that early-life onset of chronically low CORT levels influenced differentially the proliferative and apoptotic responses of granule cells to corticosteroid deprivation.

Transient Increase in Expression of Mineralocorticoid Receptor mRNA in Granule Cells of Chronically Clamped Rats

Granule cell neurogenesis and neurodegeneration are linked to the level of activation of the two corticosteroid receptors (Reul and de Kloet, 1985), the low-affinity GR (Woolley et al., 1991; Gass et al., 2000) and the high-affinity MR (Hornsby et al., 1996), respectively. The expression levels of these receptors vary inversely with CORT levels in both adult (Herman et al., 1989; Kalman and Spencer, 2002) and developing (Avishai-Eliner et al., 1999, 2001) rat DG. In turn, receptor levels help govern glucocorticoid secretion and plasma levels. Thus, higher expression of these receptors is associated with more efficient transduction of corticoid-evoked signals, including negative feedback of the hypothalamic-pituitary adrenal axis. Therefore, we studied the expression of MR and GR mRNAs in granule cells 9 days, 20 days, 3 months, and 12 months after ADX of neonatal (P10) rats, that were clamped with low CORT levels.

Unexpectedly, GR mRNA expression in granule cells of chronic low-CORT rats was not augmented at any time point when compared with sham-operated controls (Fig. 5A). This suggests that invariant low CORT levels during the critical period of granule cell proliferation lead to escape of GR expression levels from the effects of plasma glucocorticoids. For MR mRNA, its expression was strongly upregulated immediately after

ADX (ADX: 0.13 ± 0.05 relative optical density [ROD] units vs. control: 0.04 ± 0.03 ROD units; $P = 0.049$, *t*-test), and remained significantly higher than the control level for at least 20 days (ADX: 0.05 ± 0.01 ROD units vs. control: 0.02 ± 0.01 ROD units; $P = 0.043$, *t*-test; Fig. 5B). This increased MR expression level could amplify the effects of a given level of circulating CORT on hippocampal granule cells.

DISCUSSION

The major findings of these experiments are: (1) lifelong exposure to low plasma glucocorticoids, when commencing early in life, does not lead to chronic increase of granule cell neurogenesis; (2) hippocampal granule cell production correlates inversely with age in both intact and ADX/clamped animals, suggesting that aging-related reduction of neurogenesis is not governed exclusively by glucocorticoids; (3) the neurogenic potential (but not death-mechanisms) of granule cells in response to total deprivation of corticosteroids is lost when low plasma steroid levels are imposed starting early in life; and (4) increased MR levels and loss of GR regulation by plasma glucocorticoids may contribute to the adaptation of granule cell neurogenesis to lifelong low plasma levels of these hormones.

High levels of glucocorticoids inhibit production of new hippocampal granule cells (Gould et al., 1991, 1998; Cameron and Gould, 1994; Ambrogini et al., 2002). In addition, production of new granule cells declines with age (Seki and Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998; Nichols et al., 2001; Bizon and Gallagher, 2003; Heine et al., 2004), when glucocorticoid levels are higher (Sapolsky, 1992). Therefore, it has been hypothesized that chronic plasma glucocorticoid levels are a key regulator of the rate of granule cell neurogenesis (Gould et al., 1992; Cameron and McKay, 1999; Drapeau et al., 2003).

To consider the effects of lifelong low plasma corticosteroid on rates of granule cell neurogenesis, we adrenalectomized neonatal rats and clamped them at low CORT throughout life. This procedure eliminated daily circadian peaks and the stress-associated plasma glucocorticoid bursts. Surprisingly, granule cell production rate was similar in the two groups at 3 months of age. Further, although the procedure blunted the age-dependent increase of plasma CORT (the remaining increase is likely due to reduced clearance of CORT and/or contribution of ectopic adrenal hormone-secreting tissue in an occasional animal), the age-dependent decline of neurogenesis persisted in 12-month-old ADX/clamped rats.

Several paradigms of BrdU labeling have been used by a number of investigators to examine granule cell neurogenesis in adult rats (Cameron and McKay 1999, 2001; Hayes and Nowakowski, 2002; Heine et al., 2004), each providing advantages and disadvantages. We used a single, high dose pulse of BrdU followed by detection of BrdU incorporation after a short survival period (24 h). While this paradigm detects only a subset of the cells in S-phase, it provides an accurate estimate of the proliferative activity of hippocampal progenitor cells at a given time and under a given condition, whereas multiple injections of BrdU and/or longer survival incur the risk of disproportional representation of multiply-dividing cells in the analysis. In addition, with extended survival periods, cell death may occur, leading to an underestimate of the number of proliferating cells (Cameron and McKay, 2001). The disadvantage of this approach is a result of the relatively low number of cells in adult DG that are labeled, potentially affecting the sensitivity of the analysis. However, using this method, we detected enhanced granule cell proliferation after total CORT withdrawal in adult-ADX rats, but not in rats that had been clamped on low CORT throughout life (Fig. 4). These results suggest that the paradigm is sufficiently sensitive to

detect real differences in proliferative capacity of granule cell progenitors. Thus, the similar proliferation rates in the experimental groups suggest that early-life onset reduction of plasma CORT results in adaptive mechanisms that overcome the inverse relationship of plasma CORT and neurogenesis—mechanisms that might contribute to altered neurogenesis during aging. This conclusion is further supported by the observation that other parameters (e.g., granule cell volume and density) were also not different in chronically clamped compared with age-matched control rats.

Logical candidates for mediating granule cell adaptation to chronic changes in circulating corticosteroids are compensatory or adaptive changes in the expression of their receptors (Herman and Spencer, 1998). Such changes of receptor expression would facilitate a stable corticosteroid environment, which is particularly important in the neonatal rat to ensure proper brain development (Bohn, 1980; de Kloet et al., 1988). Indeed, we found a transient but robust enhancement of MR mRNA expression that lasted throughout the developmental period, when MR expression is dynamically regulated (Vazquez et al., 1998). Thus, by upregulating MR expression, granule cells may attempt to compensate for abnormally low CORT levels and maintain MR signaling at a level necessary for both control of neurogenesis and protection against apoptosis (Woolley et al., 1991; Hornsby et al., 1996; Gerth et al., 1998; Montaron et al., 2003). Indeed, our data, together with previous studies, suggest that this adaptive mechanism might be successful: chronically ADX/clamped rats, when tested as adults, performed without impairment in hippocampus mediated learning and memory tasks, even at age 12 months (Brunson et al., 2001b). As found here, increased MR expression did not persist into adulthood. In addition, when deprived entirely from adrenal steroids, granule cells from early-life clamped animals underwent apoptosis to a degree similar to that of animals ADX as adults, suggesting that compensatory mechanisms to protect from MR- dependent cell death are limited.

The apoptosis after total steroid withdrawal for 6 days in early-life ADX/clamped animals also excluded the possibility that granule cell neurogenesis was not enhanced because of altered clearance of CORT and/or long-lasting storage of the hormone in their tissues. In addition, the apparent dissociation between steroid-withdrawal-induced apoptosis and neurogenesis in these animals supports previous notions that neurogenesis and degeneration are independently regulated processes (Montaron et al., 2003). Finally, the similar rates of cell death observed in early-life ADX/clamped and intact rats throughout their lives (see Fig. 3C) does not support the notion that persistent, rapid turnover of granule cells, i.e., rapid cycling of proliferation and death evoked by low CORT, led to depletion of the precursor pool of the ADX/clamped rats.

An additional potential adaptive mechanism to changes in plasma CORT levels involves modulation of GR expression. In intact aging rats, increased GR in the granule cell layer was found (Fig. 5A), despite increased circulating CORT. Interestingly, this increase in GR expression was found also in early-life clamped rats, where age-related increased CORT was modest. This finding has two implications. First, it suggests a lifelong escape of the regulation of GR expression from plasma glucocorticoid levels in the early-life ADX group. Second, for both groups of aging rats, increased levels of GR have been considered a successful, compensatory mechanism for facilitating more effective negative glucocorticoid feedback, shutting off further activation of CORT secretion during stress (Liu et al., 1997). Thus, enhanced GR expression in aging rats is generally considered neuroprotective (Sapolsky, 1992;Stein-Behrens and Sapolsky, 1992).

While altered regulation of GR and MR expression following lifelong exposure to low plasma CORT was found here, the reduced neurogenic rate with age persisted, as found in intact animals. Thus, granule cell production rates were significantly lower in aging rats

regardless of whether their plasma CORT increased with age. These observations suggest that diminished granule cell production is largely regulated by additional, age-dependent factors that are not necessarily glucocorticoid-dependent. This conclusion is supported by the recent findings of Heine et al. (2004). These authors carried out a thorough analysis of lifetime granule cell production in Wistar rats, but found no correlation between basal corticosteroid levels and neurogenesis in middle-aged (12 months) and aged (24 months) rats.

Candidate mechanisms include growth factors (see Fabel et al., 2003, for a recent review). True, the production and release of some growth factors is influenced by glucocorticoids, probably via their binding to GR on cells neighboring the granule cell precursors (Cameron et al., 1993; Fabel et al., 2003). However, the findings here suggest that age-related effects might overcome the influence of glucocorticoid levels on release or action of neurogenesis-promoting factors. For example, the expression of insulin-like growth factor 1 (IGF1), a mediator of increased hippocampal neurogenesis in response to physical exercise (van Praag et al., 1999; Trejo et al., 2001), decreases upon aging (Anlar et al., 1999; Sonntag et al., 1999), potentially compromising neurogenesis (Åberg et al., 2000; Lichtenwalner et al., 2001). In addition, neuronal precursors in DG may be subject to replicative senescence, permitting only a defined number of proliferation cycles throughout life (Nichols et al., 2001; Olariu et al., 2003). Of note, the mechanisms for this effect may also involve glucocorticoids (e.g., via their actions on cellular oxidative stress, Roy and Sapolsky, 2003).

In summary, the regulation of granule cell neurogenesis throughout the life cycle is a complex and dynamic process. Both acute and chronic levels of plasma corticosteroids play a critical role in modulating granule cell production (and death). By manipulating glucocorticoid levels throughout life, the current studies illustrate an interesting divergence of neurogenesis and plasma corticosteroid levels, as well as adaptive mechanisms. In particular, these studies suggest that aging-related reduction of granule cell production rates is not a simple function of plasma glucocorticoids. Understanding the additional mechanisms involved should provide important information for prevention of aging-related cognitive dysfunction.

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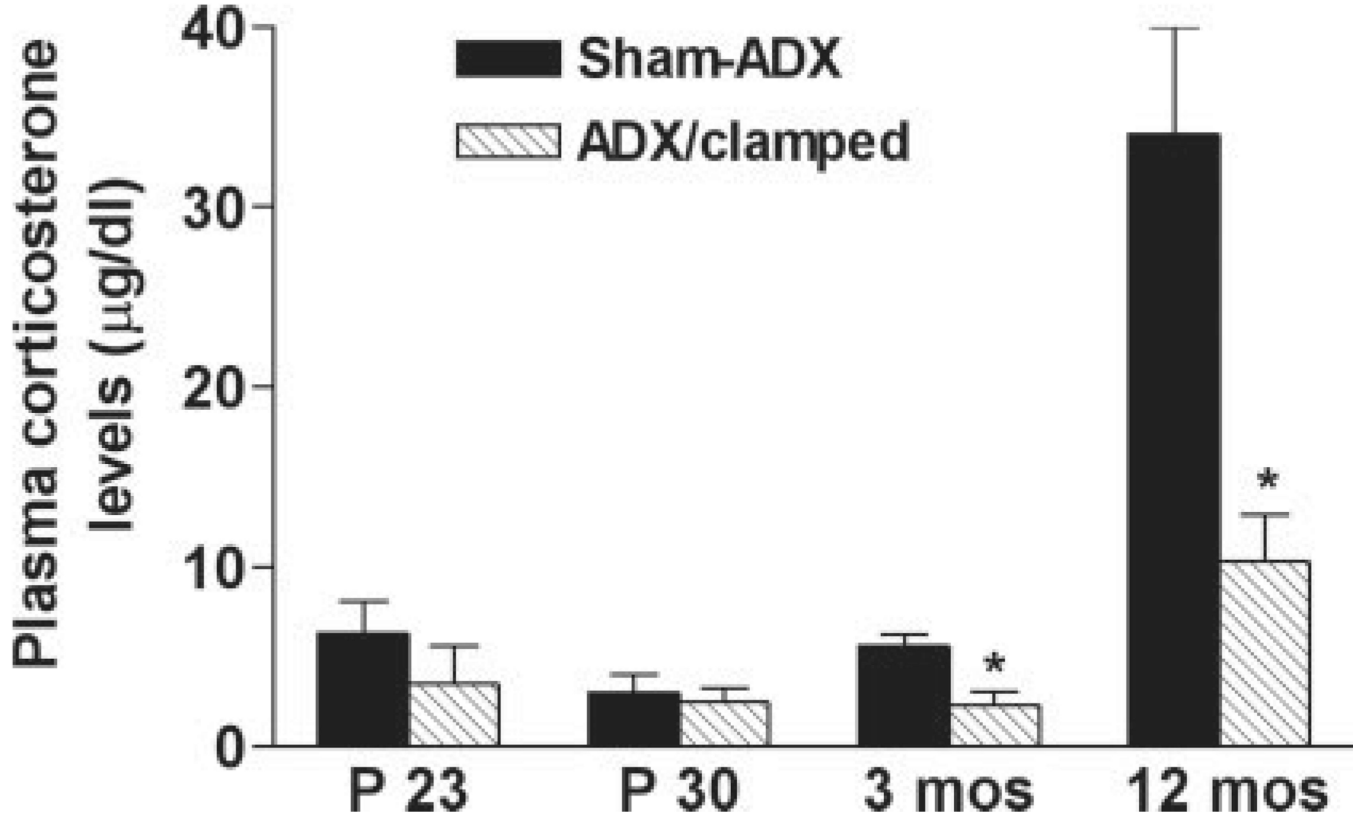

FIGURE 1.

Morning plasma corticosterone (CORT) levels of sham-adrenalectomized (Sham-ADX) or ADX/clamped rat groups. Starting after weaning (P23) plasma CORT levels of ADX/clamped animals averaged ~2.5 μg/dl, and remained at this range during adulthood. By middle age (12 months) increased plasma levels were found (see text), but these remained much lower than those in intact rats. In the latter group, an effect of age on plasma CORT was apparent (two-way ANOVA; $F = 25.5$, $P < 0.0001$).

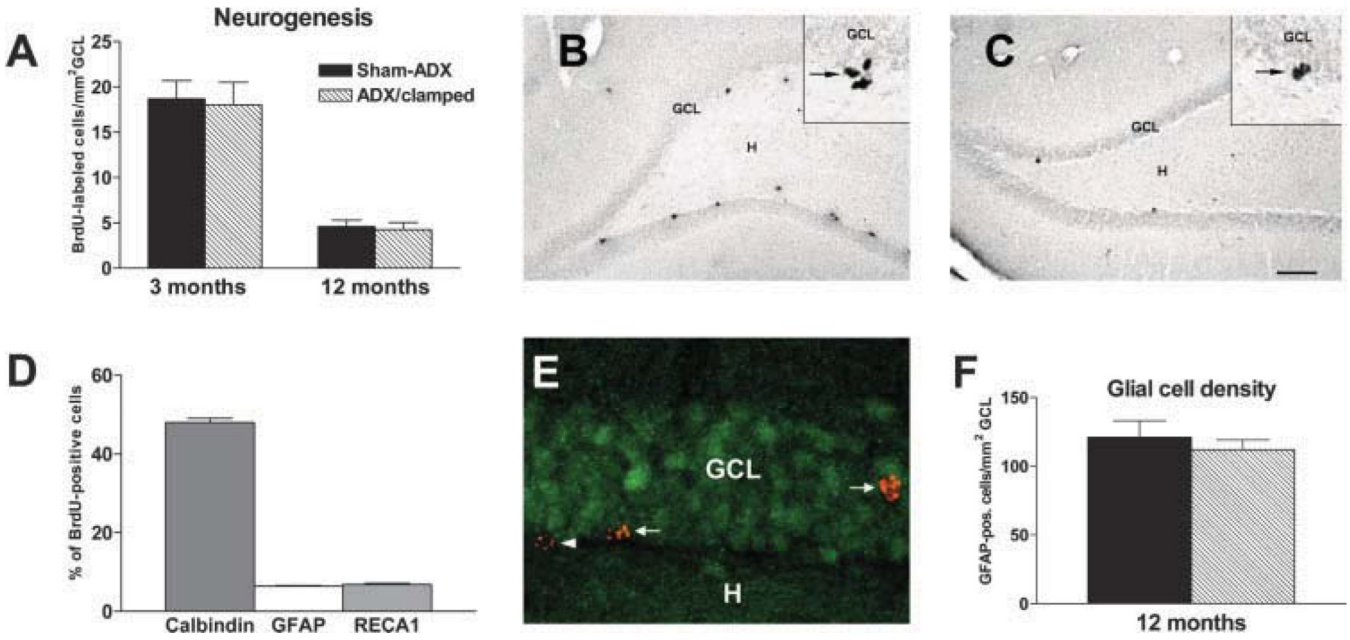

FIGURE 2.

Early postnatal adrenalectomy and persistent low-level plasma CORT do not influence neurogenesis. A: Rates of neurogenesis are similar in 3-month-old intact rats sham-adrenalectomized (Sham-ADX) compared with those reared at chronic low CORT levels (ADX/clamped). By 12 months, neurogenesis is diminished in both groups. B,C: Low magnification of the dentate gyrus in these two ages, highlighting the reduced abundance of BrdU-labeled cells at the hilar border of 12-month-old rats. An intact 3-month-old rat (B) and an intact 12-month-old (C) rat. Insets: Representative high-magnification photographs of BrdU-labeled nuclei (arrows) at the base of the granule cell layer (GCL). D: Twenty-eight days after BrdU injection (into 3-month-old rats), most BrdU-labeled cells (~48%) expressed calbindin D28k, indicating differentiation into mature granule cells. Minor populations were identified as glial (GFAP-expressing, ~6%) or epithelial (RECA1-expressing, ~7%) cells. E: Confocal image showing BrdU-labeled cells (red) that have migrated into GCL and express calbindin D28k (green; arrows). BrdU-labeled cells in subgranular zone, which do not express calbindin D28k (arrowhead), most likely represent glial, epithelial or slowly differentiating, immature granule cells. F: Density of glial cells in GCL (+subgranular zone) is not different in ADX/clamped compared with age-matched Sham-ADX control rats, indicating that lifelong exposure to low CORT does not alter glial cell production significantly. *$P < 0.05$, Student's *t*-test). H, hilus. Scale bars = 200 μm in B,C; 20 μm in insets; 15 μm E. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

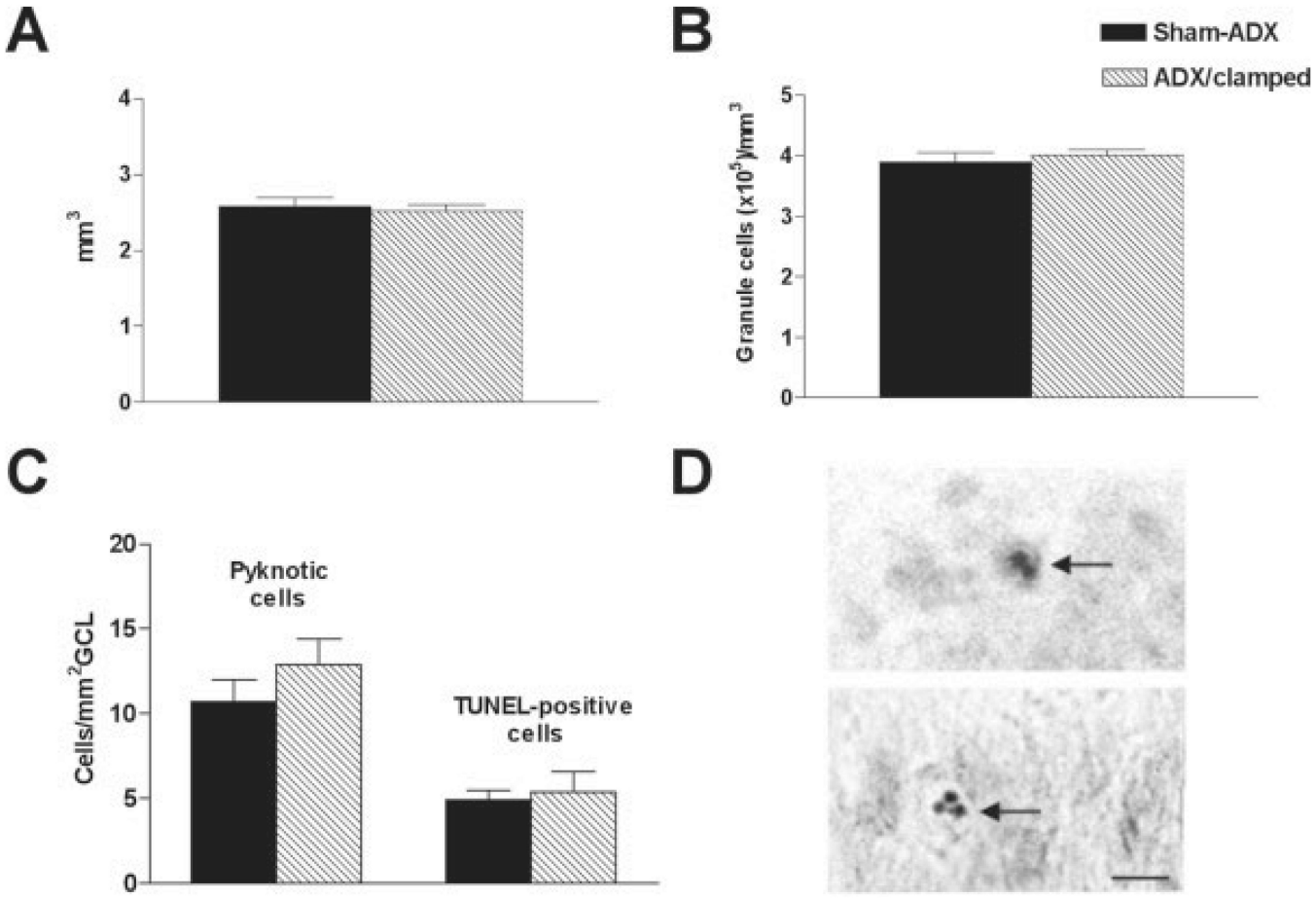

FIGURE 3.

Total numbers of granule cells are not higher in adrenalectomized (ADX)/clamped rats, and this fact is not due to increased cell death. Granule cell layer (GCL) volume (A), packing density (B), or apoptosis rate (C) were not different in 12-month-old ADX/clamped rats, when compared with age-matched sham-operated (Sham-ADX) rats. D: Examples of TUNEL-positive (upper panel) and pyknotic (lower panel) granule cell nuclei in GCL of a 12-month-old rat. Scale bar = 10 μm.

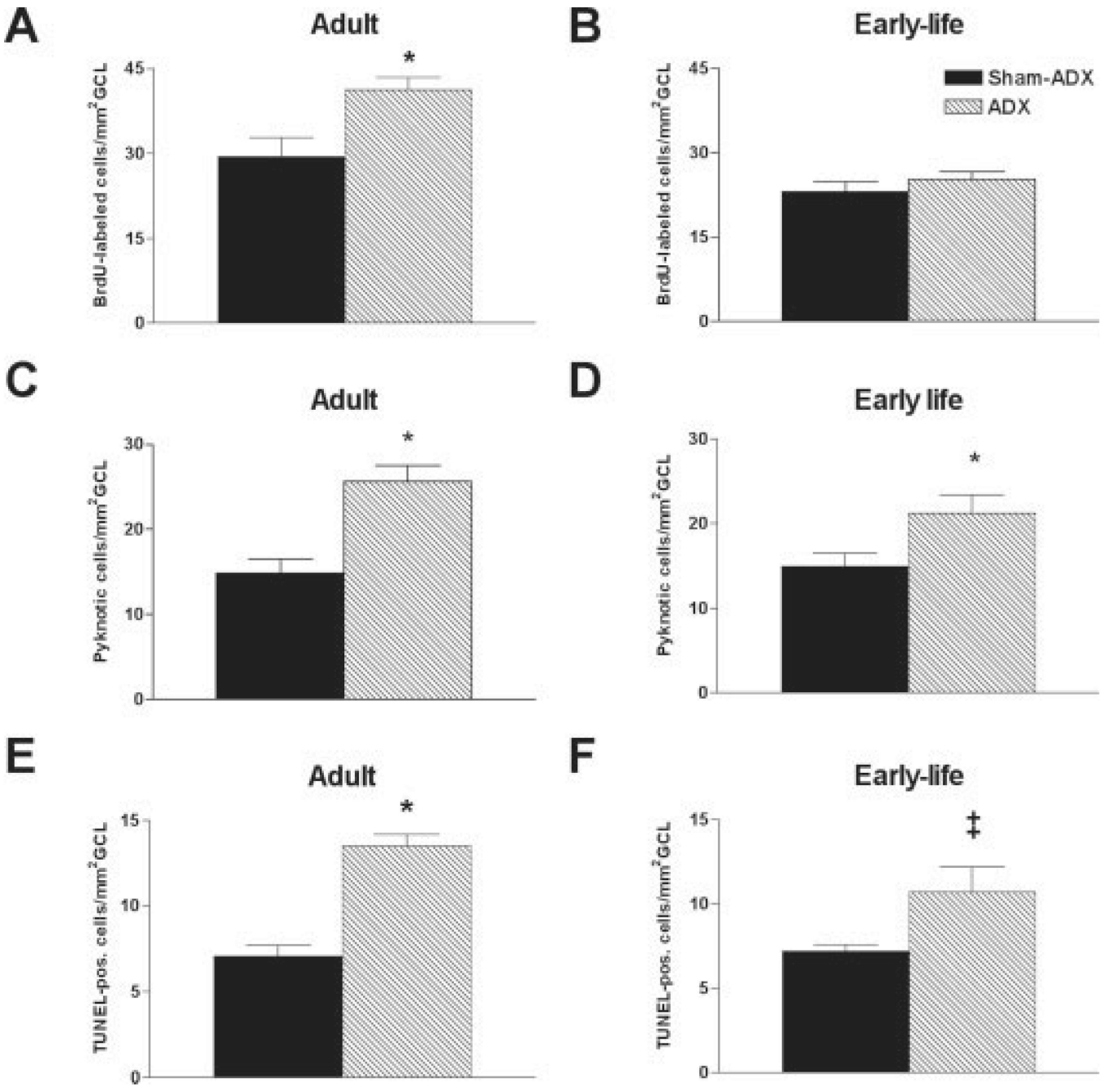

FIGURE 4.

Differential response of intact and adrenalectomized (ADX)/clamped rats to complete corticosterone (CORT) withdrawal. A: Adrenalectomy of 3-month-old rats resulted in increased granule cell production. B: Withdrawal of CORT from 3-month-old ADX/clamped rats did not enhance neurogenesis. However, apoptosis rate was significantly increased in both the adult-ADX (C,E) and the early-life ADX/clamped rats (D,F) after CORT withdrawal. (*$P < 0.05$; ‡$P = 0.06$; Student's *t*-test). GCL, granule cell layer.

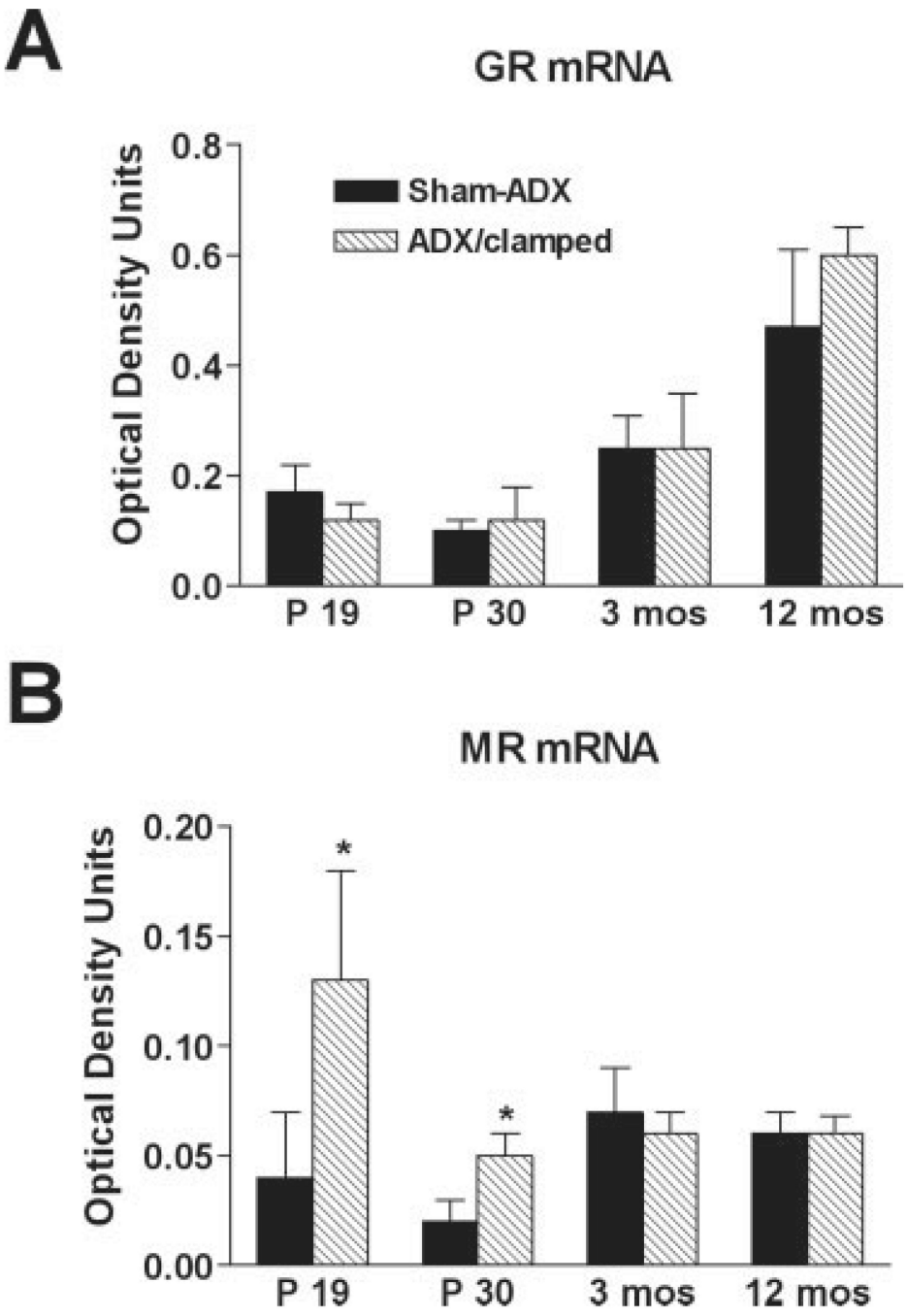

FIGURE 5.

Chronic low glucocorticoid levels selectively alter expression of hippocampal corticosteroid receptors. A: No difference was detected in GR mRNA levels in dentate gyrus of ADX/clamped rats at any time point compared with sham-adrenalectomized (ADX) controls. B: Levels of MR mRNA were transiently enhanced in granule cells of ADX/clamped animals. Upregulation of MR mRNA was pronounced during the first weeks after adrenalectomy and returned to control levels by 3 months of age. *$P < 0.05$, Student's *t*-test).