Corticosterone and Dehydroepiandrosterone Have Opposing Effects on Adult Neuroplasticity in the Avian Song Control System

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ABSTRACT
Chronic elevations in glucocorticoids can decrease the production and survival of new cells in the adult brain. In rat hippocampus, supraphysiological doses of dehydroepiandrosterone (DHEA; a sex steroid precursor synthesized in the gonads, adrenals, and brain) have antiglucocorticoid properties. With male song sparrows (Melospiza melodia), we examined the effects of physiological doses of corticosterone, the primary circulating glucocorticoid in birds, and DHEA on adult neuroplasticity. We treated four groups of nonbreeding sparrows for 28 days with empty (control), corticosterone, DHEA, or corticosterone + DHEA implants. Subjects were injected with BrdU on days 3 and 4. In HVC, a critical song control nucleus, corticosterone decreased, whereas DHEA increased, HVC volume, NeuN+ cell number, and BrdU+ cell number. Coadministration of DHEA completely reversed the neurodegenerative effects of chronic corticosterone treatment. In an efferent target of HVC, the robust nucleus of the arcopallium (RA), DHEA increased RA volume, but this effect was blocked by coadministration of corticosterone. There were similar antagonistic interactions between corticosterone and DHEA on BrdU+ cell number in the hippocampus and ventricular zone. This is the first report on the effects of corticosterone treatment on the adult song control circuit, and HVC was the most corticosterone-sensitive song nucleus examined. In HVC, DHEA is neuroprotective and counteracts several pronounced effects of corticosterone. Within brain regions that are particularly vulnerable to corticosterone, such as the songbird HVC and rat hippocampus, DHEA appears to be a potent native antiglucocorticoid.

INDEXING TERMS: adult neurogenesis; BrdU; cortisol; DHEA; hippocampus; NeuN; neurosteroid; songbird; stress

Neurogenesis continues during adulthood across vertebrates and is profoundly influenced by steroids. In mammals, cells proliferate in the subventricular zone and dentate gyrus and migrate to the olfactory bulb and granular layer of the hippocampus, respectively (Cameron et al., 1993; Alvarez-Buylla and Garcia-Verdugo, 2002). In birds, neuronal progenitor cells migrate from the lateral ventricular zone (VZ) to many areas in the telencephalon (Alvarez-Buylla and Kirn, 1997; Goldman, 1998; Nottebohm, 2002). Songbirds have high neuronal turnover in HVC and area X, two nuclei of the song control system (Nottebohm, 1981; Alvarez-Buylla and Kirn, 1997).

Glucocorticoids, which are secreted by the adrenal glands during stress and bind to receptors that act as transcription factors, are potent steroidal modulators of adult neurogenesis. In adult male rats, corticosterone

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MATERIALS AND METHODS

Animals

Wild adult male song sparrows (Melospiza melodia) were used for experiments 1B and 2. Subjects were captured using mist nets and conspecific playback and were then transferred to the University of Western Ontario (for specific capture locations, see descriptions of experiments 1B and 2 below). Subjects were housed individually with ad libitum access to water, food (Mazuri small bird maintenance diet + white millet seeds), and grit and kept on a natural photoperiod. Subjects were gradually shifted to a short photoperiod (8L:16D) and nonbreeding condition. Subjects were maintained on 8L:16D for at least 1 month prior to treatment. All song sparrows were used in accordance with the Canadian Council on Animal Care following procedures approved by the University of Western Ontario Animal Care and Use Committee.

Steroid implants

Implants were made from silastic tubing (for corticosterone: i.d. 1.47 mm, o.d. 1.96 mm; for DHEA: i.d. 0.76 mm, o.d. 1.65 mm). For corticosterone, silastic implants with effective lengths of 10–15 mm have been used previously in songbirds to elevate corticosterone moderately above baseline levels (Astheimer et al., 2000; Breuner and Hahn, 2003; Martin et al., 2005), so we used an effective length of 12 mm. Implants were packed with crystalline corticosterone (catalog No. C2505; Sigma-Aldrich, Oakville, Ontario, Canada) and sealed with liquid silastic. In previous studies, corticosterone release from silastic implants was facilitated by making a small hole in the implant (Silverin, 1998; Astheimer et al., 2000). We used a 22-gauge needle to make one hole in each corticosterone implant near the end of the effective length (i.e., not through the liquid silastic plug). For DHEA, we used an effective length of 7 mm, which clearly and consistently elevates plasma DHEA levels in songbirds within the physiological range (Soma et al., 2002; Goodson et al., 2005b). Implants were packed with crystalline DHEA (catalog No. A8500-000; Steraloids, Newport, RI) and sealed with liquid silastic.

Experiment 1: validation of corticosterone implants

In experiment 1A, the corticosterone implants were tested in vitro. To examine the release of corticosterone from silastic implants over 28 days, we incubated corticosterone implants with zero, one, three, or six needle holes per implant (n = 5 per group) in microcentrifuge tubes containing 550 µl assay buffer at ~40°C. Implants were incubated for 3 days, then transferred to fresh
medium and incubated for 4 days, then transferred to fresh medium and incubated for 7 days. Empty implants were incubated for 3 days (n = 5) or 28 days (n = 5). Samples were stored at −20°C. At day 28, all corticosterone implants still contained steroid.

In experiment 1B, the corticosterone implants were tested in vivo. Subjects (n = 12) were captured in August from around Guelph, Ontario, Canada (43°33’N, 80°15’W) and the University of Western Ontario (43°0.5’N, 81°16.8’W). On day 0, subjects received corticosterone implants (n = 6) or empty implants (control, n = 6; Fig. 1A). Three subjects in the control group and one subject in the corticosterone group either continued or began molting feathers during the experiment, and these molting subjects were excluded because molt dramatically alters stress physiology in song sparrows and other songbirds (Romero, 2006; Newman et al., 2008b; Newman and Soma, 2009).

Three days prior to implantation (day −3), a baseline blood sample (~50 μl) was collected from the brachial vein within 3 minutes of entering the room. Baseline blood samples were also collected on days 1, 2, 3, and 4 (~25 μl on each day). On days 7 and 21, a baseline blood sample (~50 μl) was collected, and another blood sample (~50 μl) was collected after 30 minutes of restraint in an opaque bag. On day 28, a final baseline blood sample was collected. Blood was kept on ice until centrifuged, and plasma was stored at −20°C. On day 28, all implants were present, and all corticosterone implants still contained steroid.

Subjects were treated for 4 weeks because 1) over 3 weeks is required for the migration, maturation, and integration of neurons in the adult song system (Nottebohm, 2002); 2) prior studies in rats on the effects of corticosterone treatment or chronic stress use a similar timeline (Wong and Herbert, 2004; Joels et al., 2007); and 3) removing the implants during the middle of the experiment would have introduced an additional stressor. Finally, as demonstrated in Results (Fig. 2A), plasma levels of corticosterone were elevated only for the first few days of treatment, suggesting that the corticosterone dose and treatment duration were physiological.

Experiment 2: effects of corticosterone and DHEA treatment on adult neuroplasticity

Subjects

Subjects were wild adult male song sparrows (n = 36 total) captured at the Queens University Biological Station...
(44°34’N, 76°19.5’W; n = 12) or near the University of Western Ontario (n = 24; June 4 to July 6, 2007). Subjects were housed in individual cages in four separate rooms (n = 9 per room) under conditions identical to those of experiment 1B. Subjects were gradually shifted to a short-day photoperiod (8L:16D). A short photoperiod was used in this experiment because 1) in the songbird brain, the rate of incorporation of new neurons into HVC is highest during the nonbreeding season (Tramontin and Brenowitz, 1999, 2000; Nottebohm, 2002); 2) individual variation in both circulating corticosterone and DHEA is lowest during the nonbreeding season (Newman et al., 2008b); 3) DHEA treatment has a large effect on song production and HVC volume in the nonbreeding season (Soma et al., 2002); and 4) neural DHEA metabolism is highest during the nonbreeding season (Pradhan et al., 2010). We randomly assigned subjects to one of four treatment groups: empty implant + empty implant (control group), corticosterone implant + empty implant (corticosterone group), DHEA implant + empty implant (DHEA group), or corticosterone implant + DHEA implant (corticosterone + DHEA group).

Steroid treatments started from 3 November to 7 December, 2007, depending on the room. There were birds from each treatment group in each room. On day 0 (Fig. 1B), we anesthetized subjects (1.5% isoflurane, 2 liters/minute O2) and inserted silastic implants (see above) s.c. on the back. Implants were inserted through a small incision in the skin, which was then sealed with veterinary skin adhesive (Nexaband S/C; Closure Medical Corporation, Raleigh, NC).

**Experimental timeline**

On day 3 and day 4 (Fig. 1B), all subjects received three intramuscular injections (at 0900, 1300, and 1700 hours) of the thymidine analogue bromodeoxyuridine (BrdU; catalog No. B9285; Sigma). BrdU is incorporated into cells that are synthesizing DNA (in the S-phase of mitosis) at the time of BrdU exposure (Thompson and Brenowitz, 2009). BrdU was dissolved in 0.1 M phosphate-buffered saline (PBS; pH 7.4, 100 μl volume, 0.015 g/ml), which resulted in a BrdU dose of 65 mg/kg, similar to that used in previous songbird studies (Wang et al., 2002; Hoshooley et al., 2007).

Baseline blood samples were collected from the brachial vein within 3 minutes of entering the room. Two days prior to implantation (day -2), a baseline blood sample (~100 μl) was collected. On days 7 and 21, a baseline blood sample (~100 μl) was collected, and subjects were restrained in an opaque paper bag for 30 minutes. After this restraint, another blood sample was collected (~100 μl). To minimize stress, blood was not collected on treatment days 1–4 as in experiment 1B. On day 28, a baseline blood sample (~100 μl) was collected prior to death. Blood was kept on ice until centrifuged, and plasma was stored at −20°C. Subjects were weighed, and fat score (on a scale of 0–5) was recorded on days −2, 7, and 21. Subjects were not weighed immediately prior to death (day 28).

After blood collection on day 28, subjects were deeply anaesthetized with ketamine and xylazine and transcardially perfused with cold heparinized saline followed by buffered 4% paraformaldehyde (pH 8.5, as in previous songbird studies; see, e.g., MacDonald et al., 2006). The brain was removed from the skull (completely pneumatized in all subjects, which is indicative of adulthood), postfixed in 4% paraformaldehyde for 24 hours, and then cryoprotected in 30% sucrose until saturated (~48 hours). Brains were then frozen on pulverized dry ice and stored at −80°C. We also collected the spleen and syrinx as positive controls because these are known targets for corticosterone and DHEA, respectively (Soma et al., 2002; Shini et al., 2008). These tissues were postfixed in 4% paraformaldehyde for 24 hours and then weighed, and tissue mass was corrected for day 21 body mass. Testes were regressed in all subjects (mean volume of one testis ± SEM: 0.83 ± 0.1 mm3), consistent with nonbreeding condition. Finally, we checked the silastic implants. All implants were present, and all corticosterone and DHEA implants contained steroid.

**Steroid measurement**

Corticosterone levels were measured in plasma and in medium without an extraction step (as in Newman et al., 2008b), with a sensitive and specific corticosterone radioimmunoassay (ImmuChem 07-120103; MP Biomedicals, Orangeburg, NY). The lowest point on the corticosterone standard curve was 3.12 pg corticosterone per tube. Intraassay variation was 5.9%, and interassay variation was 9.5% (low control) and 7.6% (high control; n = 7 assays).

DHEA levels were measured in plasma after extraction with dichloromethane (as in Newman et al., 2008b) with a sensitive and specific DHEA radioimmunoassay (DSL 8900; Diagnostic Systems Laboratories, Webster, TX). The lowest point on the DHEA standard curve was 2 pg DHEA per tube. Intraassay variation was 9.5%, and interassay variation was 5.2% (low control) and 11.7% (high control). Recovery of 50 pg exogenous DHEA from a pool of song sparrow plasma (n = 6 replicates) was 143%. Plasma concentrations were corrected for recovery. Both the corticosterone and the DHEA radioimmunoassays have been validated extensively for song sparrow plasma (Newman et al., 2008a,b; Newman and Soma, 2009).

**Immunocytochemistry**

Brains were sectioned in the coronal plane at 30 μm thickness on a cryostat. Two sets of sections were
collected into PBS to examine BrdU and NeuN immunoreactivity, and a third set was collected into cryoprotectant and stored at −20°C.

To detect BrdU+ cells (see Fig. 3A), free-floating sections were washed in PBS and then immersed in 2 N HCl for 40 minutes. Sections were washed in 0.1 M sodium borate (with 0.5% HCl) for 10 minutes and then washed in PBS. Sections were then incubated in 0.5% H2O2 for 30 minutes at room temperature and then washed in PBS. Sections were blocked in 10% normal goat serum (Vector) for 30 minutes and then exposed to an anti-BrdU/IdU monoclonal primary antibody raised in mouse (immunogen: 5-iodo-2′-0-deoxyuridine-5′-monophosphate conjugated through the phosphate group to albumin; clone IU-4; purified immunoglobulin; Caltag MD5000, Burlington, Ontario, Canada; Vanderlaan et al., 1986). Sections were exposed to this anti-BrdU antibody (diluted 1:500 in PBS with 0.3% Triton) for 20 hours at room temperature. This antibody has been used previously in chick brain at the same dilution (Carnahan et al., 1994), and we tested a range of dilutions on song sparrow brain sections. In pilot studies, we also tested various BrdU antibodies (from BD Pure and BD Pharmingen, San Diego, CA; Chemicon, Billerica, MA; Caltag) and chose the antibody that gave the highest signal to noise ratio, similarly to Leuner et al. (2009). Next, sections were washed in PBS with 0.1% Triton (3 × 5 minutes) and then exposed to the biotinylated secondary antibody (1:250 in PBS with 0.3% Triton; goat anti-mouse IgG; Vector, Burlingame, CA) for 1 hour. Sections were washed in PBS with 0.1% Triton (3 × 5 minutes), and then treated with avidin-biotin-peroxidase reagent (Vector Elite ABC kit) for 1 hour. BrdU labelling was visualized by exposing the sections to a diaminobenzidine solution (Sigma FastDAB). Sections were then washed thoroughly in PBS, mounted on glass slides, dehydrated through a series of graded ethanols, and cleared in solvent (Harleco Neo-Clear; EMD Chemicals, Mississauga, Ontario, Canada), and then coverslips were affixed with Neo-Mount (EMD Chemicals). As a negative control, 12 brain sections from one subject that had not been injected with BrdU were processed for BrdU staining; staining was absent in these sections. As an additional negative control, 20 brain sections from different subjects that had been injected with BrdU were processed for BrdU staining with the primary antibody omitted; staining was absent in these sections.

We also examined NeuN, a neuron-specific protein that is expressed in the large majority of neuronal cell types (Mullen et al., 1992). To detect NeuN+ cells (Fig. 3B–E), sections were processed similarly; however, sections were not exposed to HCl or sodium borate and were exposed to an anti-NeuN monoclonal primary antibody raised in mouse (immunogen: purified cell nuclei from mouse brain; clone A60; purified immunoglobulin; Chemicon MAB377; Mullen et al., 1992). Sections were exposed to this anti-NeuN antibody (diluted 1:2,000 in PBS with 0.3% Triton) for 20 hours at room temperature, as in Phillmore et al. (2006). NeuN immunoreactivity is present in cytoplasm and nucleus in birds in most neuronal cell types (Mullen et al., 1992; Hoshooley and Sherry, 2007). NeuN immunoreactivity is absent in a few neuronal cell types, such as cerebellar Purkinje cells (Mullen et al., 1992). Thus, as negative control, we examined NeuN immunoreactivity in Purkinje cells of song
sparrows. Purkinje cells were also examined using Nissl staining. As expected, cerebellar Purkinje neurons were detectable in the Nissl-stained sections but were absent in the NeuN-stained sections (Fig. 4). As a second negative control, six sections of song sparrow intestine were processed for NeuN, and staining was absent in these

**Figure 3.** Representative immunocytochemical staining for BrdU and NeuN. A: BrdU⁺ cells in the lateral ventricular zone (VZ). B: NeuN⁺ cells in HVC. C: NeuN⁺ cells in HVC; the dorsal edge of HVC is defined by the lateral ventricle and the ventral edge is indicated by arrows. Dorsal is upward, and medial is to the left. D: NeuN⁺ cells in area X; perimeter is indicated by arrows. E: NeuN⁺ cells in RA, perimeter is indicated by arrows. Scale bars = 25 μm in A, B; 250 μm in C–E.

**Figure 4.** Comparison of Nissl (thionin) staining (A) with NeuN immunoreactivity (B) in the cerebellum. Although NeuN is specific to neurons, NeuN is known to be absent in some neuron types, such as cerebellar Purkinje neurons (Mullen et al., 1992). The lack of NeuN immunoreactivity in song sparrow Purkinje neurons thus serves as a negative control and demonstrates the specificity of the NeuN antibody. Scale bars = 25 μm.
sections. As a third negative control, 20 brain sections from different subjects were processed for NeuN staining with the primary antibody omitted; staining was absent in these sections. Note that, because a few neuronal cell types are not NeuN immunoreactive, we refer to “NeuN+ cell number” rather than “neuron number.”

Microscopy

All brain measurements were made by an observer blind to the treatment group. To obtain estimates of total telencephalon volume, slides with NeuN-labelled sections were scanned at 2,400 dpi into a computer using a high-resolution (2,400 dpi) flatbed scanner. The left and right telencephalon area was measured from every twelfth section in NIH ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Telencephalon volume was calculated by multiplying the area of each section by the sampling interval (360 μm). We also used NeuN immunoreactivity to measure the volume of three nuclei in the song control system: HVC, RA and area X. HVC is critical for both song production and learning and projects to both RA and area X. RA is primarily involved in song production, and area X is involved in song learning and auditory feedback. Finally, NeuN immunoreactivity was used to measure hippocampus volume, as in Hoshooley and Sherry (2004). We captured images from at least 10 sections per subject per brain region, with the distance between sections dependent on the size of the regions of interest. We captured images from every NeuN-labelled section containing the HVC, RA, and area X (90 μm apart, ~12 sections per subject) and from every twelfth section containing hippocampus (360 μm apart, ~20 sections per subject) using a Leica Digital CCD camera mounted on a Leica DM5000 B light microscope (Leica Microsystems Inc., Richmond Hill, Ontario, Canada) through a 10 objective lens. Leica Application Suite software was used to make area measurements of HVC, RA, area X, and hippocampus in one hemisphere; volume was calculated by multiplying the area of each brain region in each section by the sampling interval (90 μm or 360 μm). Values were then multiplied 2 to obtain an estimate of total volume (both hemispheres). Song nuclei, hippocampus, and telencephalon volumes do not differ between the left and right hemispheres (Tramontin et al., 2000; Pravosudov et al., 2006).

To estimate the number of HVC neurons (i.e., NeuN+ cell number in HVC), we used the stereological optical fractionator method (Glaser et al., 2007) on sections labelled for NeuN. NeuN+ cells were counted in HVC, because HVC is known to be sensitive to both androgens and glucocorticoids (see, e.g., Soma et al., 2002; Buchanan et al., 2004). In one hemisphere, NeuN+ cells were counted in every ninth section that contained HVC (i.e., 270 μm apart; n = ~10 sections per subject). With a ×20 objective, we used the Leica Application Suite software driving a motorized stage to cover HVC with a 270 × 270-μm sampling grid. Then, under a ×63 oil objective, the section was moved to the first square in the sampling grid. At each point in the sampling grid (270 μm apart), the counting frame was 30 × 30 μm. Moreover, we measured the thickness of the tissue section by focusing on its top and bottom edges in three counting frames per section. We did not count cells in the top and bottom 1 μm of the section (guard zones) except in a few cases where the section was ≤7 μm thick, in which case we counted all of the cells. Because of shrinkage during processing, the section thickness was 7.7 ± 0.3 μm (range 6–11 μm). We focused through the section by 1-μm intervals and counted all of the cells that came into focus within the 5 μm × 30-μm² section. We confirmed that the NeuN stain completely penetrated the 30-μm tissue section. In three subjects from each treatment group, we counted the number of separate NeuN+ cells that came into focus at each 1-μm interval in 10 counting frames per subject (n = 120 frames of view). There was no statistically significant effect of section depth or treatment group on NeuN+ cell number (data not shown). The total population of cells in one-half of HVC was then estimated using the following formula:

\[ \text{Total population} = n \times (1/\text{ssf}) \times (1/\text{ssf}) \times (1/\text{ssf}) \]

where \( n \) = total number of cells counted, \( \text{ssf} \) = section sampling frame (i.e., 1/9, because we counted every ninth section), \( \text{ssf} \) = area sectioning frame (30 μm²/270 μm²), and \( \text{ssf} \) = optical dissector height (5 μm/tissue section thickness. The total was multiplied by 2 to obtain an estimate of total HVC NeuN+ cell number (both hemispheres).

To estimate the number of BrdU+ cells in HVC, in the hippocampus, along the VZ, and in the telencephalon (not including HVC and hippocampus), BrdU+ cells were counted exhaustively in one hemisphere of the brain. Exhaustive sampling was used rather than a stereological approach because BrdU+ cells were relatively infrequent. In such cases, exhaustive sampling is more accurate than sampling from a small subset of sections (Hoshooley and Sherry, 2004). For all four regions, BrdU+ cells were counted in all sections containing HVC (90 μm apart). In the rest of the brain (rostral and caudal to HVC), BrdU+ cells were counted in every twelfth section (360 μm apart). The number of counted BrdU+ cells was then multiplied by the section interval (i.e., 12 or 3) and subsequently multiplied by 2 to obtain an estimate for both hemispheres. Also, we checked for an effect of corticosterone and DHEA treatments on the size of BrdU+ cell nuclei for a subset of 400 BrdU+ cells along the VZ.
(n = 50/treatment group) and in the telencephalon (n = 50/treatment group). There was no effect of treatment on BrdU+ cell nucleus diameter along the VZ (control: 5.49 ± 0.11 μm; corticosterone: 5.84 ± 0.14 μm; DHEA: 5.85 ± 0.13 μm; corticosterone + DHEA: 5.89 ± 0.15 μm; one-factor ANOVA: F_{3,199} = 1.93, P = 0.13) or in the telencephalon (control: 5.53 ± 0.13 μm; corticosterone: 5.56 ± 0.11 μm; DHEA: 5.50 ± 0.11 μm; corticosterone + DHEA: 5.77 ± 0.13 μm; one-factor ANOVA: F_{3,199} = 1.01, P = 0.39). Thus, BrdU+ profile size did not differ systematically across groups. For both NeuN and BrdU photomicrographs, images were adjusted for contrast and brightness in Leica Application Suite software.

Statistical analysis

In experiment 1, in vitro results were analyzed using one-factor ANOVAs at each time (days 3, 7, 21, 28) to analyze the effect of the number of needle holes on corticosterone levels in media. As a result of small sample sizes for the in vivo validation, the effect of corticosterone implants on plasma corticosterone levels was analyzed using Welch’s t-tests. The effects of acute restraint stress on days 7 and 21 were calculated using difference scores (stressed — baseline). The effect of corticosterone treatment on the difference scores was analyzed on days 7 and 21 by using Welch’s t-tests. Area under the curve was calculated with the trapezoid rule (Breuner et al., 1999; Cockrem et al., 2008) to estimate the total integrated amount of corticosterone present in the plasma, and a Welch’s t-test was used to test the effect of corticosterone treatment on integrated corticosterone levels.

In experiment 2, the effects of corticosterone and DHEA treatments on telencephalon volume, telencephalon BrdU+ cell number, HVC volume, HVC NeuN+ cell number, HVC BrdU+ cell number, RA volume, area X volume, hippocampus volume, hippocampus BrdU+ cell number, VZ BrdU+ cell number, brain mass, syrinx mass, and spleen mass were assessed using two-factor ANOVAs. We tested for main effects of corticosterone treatment and DHEA treatment, as well as an interaction between corticosterone and DHEA treatments. Because each factor in the ANOVA had only two levels, significant main effects without a significant interaction were interpreted without further post hoc analysis. However, significant interactions were explored by comparing groups using Tukey’s honestly significant difference (HSD) post hoc tests. The effects of corticosterone and DHEA treatment on body mass and fat score were also evaluated using two-factor ANOVA on each measurement day (days 7 and 21). The effects of steroid treatments and treatment duration on plasma corticosterone and DHEA levels were analyzed by using two-factor mixed-design ANOVAs in experiment 2, where treatment day was a within-subjects factor and treatment group was a between-subjects factor. A two-factor mixed-design ANOVA was used to analyze the effect of acute restraint stress on plasma steroid levels, where stress was a within-subjects factor and treatment group was a between-subjects factor. The effects of 30 minutes of acute restraint stress were measured on day 7 and day 21.

For all experiments, data were log transformed if necessary to achieve normality and homogeneity of variance. All data were analyzed with JMP 7 (SAS, Cary, NC). Data are presented as mean ± SEM.

RESULTS

Steroid levels

In vitro corticosterone levels

In experiment 1A, corticosterone-filled silastic implants continued to release corticosterone over the course of 28 days in vitro. A serial dilution of media samples revealed that the samples required a 1:100,000 dilution (0.0005 μl per RIA tube) prior to analysis. Corticosterone levels in medium containing empty silastic implants for 3 days (n = 5) or 28 days (n = 5) were below detection (<3.12 pg/tube). Corticosterone levels in medium containing corticosterone implants were extremely high, even when the corticosterone implants had no needle holes (Supp. Info. Fig. 1). On days 3 and 7, there was no significant effect of number of needle holes (day 3: F_{3,19} = 2.18, P = 0.13; day 7: F_{3,19} = 0.29, P = 0.83). On day 21, when implants had been incubating for the longest period of time (14 days), there was a significant effect of needle hole number (F_{3,19} = 6.52, P = 0.005), and post hoc tests revealed that corticosterone levels were lowest in media containing corticosterone implants with zero holes. On day 28, there was no significant effect of needle hole number (F_{3,19} = 2.56, P = 0.10).

Baseline plasma corticosterone levels

To validate corticosterone implants in vivo in experiment 1B, we started collecting plasma on day 1 (Fig. 1A), and baseline plasma corticosterone levels were significantly higher in the corticosterone group than in the control group on days 1 and 2 and tended to be higher on day 3 (Fig. 2A). Baseline plasma corticosterone levels were not significantly different on subsequent days. Furthermore, the integrated amount of corticosterone was significantly higher in the corticosterone group (Welch’s t-test: t = 3.85, P = 0.02; Fig. 2B, Table 1). In experiment 2, the first measurement of baseline plasma corticosterone after implantation was on day 7 (Fig. 1B) to avoid stressing the subjects soon after BrdU administration.
Baseline plasma corticosterone levels (Table 2) were not significantly affected by treatment \( (F_{3,138} = 0.99, P = 0.40) \) or sampling day \( (F_{3,138} = 1.87, P = 0.14) \), and there was no interaction \( (F_{9,138} = 1.25, P = 0.27) \).

**TABLE 1.**
Effects of Corticosterone and DHEA Treatments on NeuN Measurements and BrdU+ Cell Number

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<tr>
<td>Corticosterone</td>
<td>1,31</td>
<td>6.14</td>
<td>0.02</td>
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<td>DHEA</td>
<td>1,31</td>
<td>15.69</td>
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<td>Corticosterone × DHEA</td>
<td>1,31</td>
<td>1.32</td>
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<tr>
<td>HVC BrdU+ cell number</td>
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<tr>
<td>Corticosterone</td>
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<tr>
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<td>RA volume</td>
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<tr>
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<td>Area X volume</td>
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<tr>
<td>Corticosterone</td>
<td>1,24</td>
<td>3.33</td>
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<td>1,24</td>
<td>2.35</td>
<td>0.14</td>
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<td>Corticosterone × DHEA</td>
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<td>0.59</td>
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<td>Hp volume</td>
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<tr>
<td>Corticosterone</td>
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<td>1.07</td>
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<tr>
<td>DHEA</td>
<td>1,28</td>
<td>0.32</td>
<td>0.58</td>
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<td>Corticosterone × DHEA</td>
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<td>0.81</td>
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<tr>
<td>Hp BrdU+ cell number</td>
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<td>Corticosterone</td>
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<td>0.04</td>
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<td>VZ BrdU+ cell number</td>
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<td></td>
<td></td>
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<tr>
<td>Corticosterone</td>
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<td>0.25</td>
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<tr>
<td>DHEA</td>
<td>1,26</td>
<td>7.99</td>
<td>0.01</td>
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<tr>
<td>Corticosterone × DHEA</td>
<td>1,26</td>
<td>18.05</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

**Baseline plasma DHEA levels**

DHEA treatment significantly increased plasma DHEA levels throughout the course of experiment 2 (DHEA was not measured in experiment 1). For baseline plasma DHEA levels (Table 3), there was a significant interaction between treatment and sampling day \( (F_{9,138} = 3.46, P = 0.0008) \). Post hoc tests revealed that plasma DHEA levels were higher in the DHEA and corticosterone + DHEA groups on days 7, 21, and 28, relative to control and corticosterone groups. In the DHEA-treated groups, plasma DHEA levels remained within the physiological range for this song sparrow population, insofar as baseline plasma DHEA levels before implantation ranged from 1.2 to 17 ng/ml.

**Stressed plasma corticosterone levels**

In experiments 1B and 2, the effect of acute restraint stress was measured on day 7 and day 21. In experiment 1B on day 7, the stress-induced increase in plasma corticosterone levels was significantly greater in controls than in the corticosterone group (stress – baseline corticosterone levels: control group = +171.1 ± 38.5 ng/ml; corticosterone group = −8.9 ± 4.6 ng/ml; \( t = 3.14, P = 0.04 \)). Similarly, on day 21, the stress-induced increase in plasma corticosterone levels was significantly greater in controls than in the corticosterone group (stress – baseline corticosterone levels: control group = +23.4 ± 6.1 ng/ml; corticosterone group = −10.0 ± 10.4 ng/ml; \( t = 2.59, P = 0.05 \)). In experiment 2 on day 7, there was a significant interaction between stress and treatment \( (F_{3,61} = 4.50, P = 0.007) \). Post hoc tests revealed that acute restraint stress increased plasma corticosterone levels in all groups except the corticosterone group. In experiment 2 on day 21, there was also significant interaction between stress and treatment \( (F_{3,67} = 2.96, P = 0.04) \). Post hoc tests revealed that acute restraint stress increased plasma corticosterone levels in all groups and that stressed plasma corticosterone levels were greater in the controls than in the corticosterone group.

**Stressed plasma DHEA levels**

There was no main effect of stress on DHEA levels on day 7 and day 21 and no interaction with treatment;
however, there was a main effect of treatment (day 7: $F_{3,67} = 51.29, P < 0.0001$; day 21: $F_{3,67} = 24.96, P < 0.0001$). Post hoc tests revealed that, on both days, plasma DHEA levels were greater in the DHEA and corticosterone + DHEA groups compared with the control and corticosterone groups.

### Neural attributes

**Telencephalon**

For the volume of the entire telencephalon, there were no main effects of corticosterone treatment or DHEA treatment and no interaction (Table 1, Fig. 5A). Furthermore, neither corticosterone nor DHEA treatment affected brain mass, and there was no interaction (data not shown). For telencephalon BrdU$^+$ cell number, there was no main effect of corticosterone, but there was a main effect of DHEA and a significant interaction effect. BrdU$^+$ cells were more abundant in the telencephalon of the DHEA group than in all other groups (Tukey’s HSD, $P < 0.05$; Fig. 5B). DHEA treatment increased recruitment of new cells into the telencephalon, but only in the absence of corticosterone treatment. Stated another way, corticosterone treatment had no effect on recruitment of new cells into the telencephalon, except in the presence of DHEA treatment.

**HVC**

Corticosterone and DHEA treatment had significant effects on HVC (Table 1). For HVC volume, HVC NeuN$^+$ cell number, and HVC BrdU$^+$ cell number, there were main effects of both corticosterone and DHEA treatment, but there was no interaction effect. Corticosterone significantly decreased, whereas DHEA significantly increased, HVC volume, HVC NeuN$^+$ cell number, and HVC BrdU$^+$ cell number (Fig. 6).

**RA**

For RA volume, there were main effects of both corticosterone and DHEA treatment but also a significant interaction effect between corticosterone and DHEA treatment (Table 1). RA volume was greatest in the DHEA treatment groups.

### Table 3.

Effects of Corticosterone and DHEA Treatments on Plasma DHEA Levels (ng/ml; Experiment 2)

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Day -2 (baseline)</th>
<th>Day 7 (baseline)</th>
<th>Day 7 (stressed)</th>
<th>Day 21 (baseline)</th>
<th>Day 21 (stressed)</th>
<th>Day 28 (baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.4 ± 1.3</td>
<td>1.4 ± 0.5</td>
<td>1.0 ± 0.5</td>
<td>2.6 ± 1.3</td>
<td>0.8 ± 0.3</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>2.2 ± 1.1</td>
<td>1.4 ± 0.3</td>
<td>0.7 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>2.6 ± 1.8</td>
</tr>
<tr>
<td>DHEA</td>
<td>2.1 ± 1.3</td>
<td>8.7 ± 1.8</td>
<td>12.6 ± 1.8</td>
<td>10.2 ± 1.9</td>
<td>11.0 ± 2.5</td>
<td>7.6 ± 1.5</td>
</tr>
<tr>
<td>Corticosterone + DHEA</td>
<td>3.8 ± 1.2</td>
<td>12.9 ± 1.9</td>
<td>13.5 ± 2.5</td>
<td>15.4 ± 3.7</td>
<td>20.6 ± 5.5</td>
<td>10.3 ± 3.1</td>
</tr>
</tbody>
</table>

**Figure 5.** Effects of corticosterone (Cort) and DHEA treatments on telencephalon volume (A) and BrdU$^+$ cell number in the telencephalon (excluding HVC, hippocampus and VZ; B). Numbers in bars indicate sample sizes, and the asterisk indicates a significant difference from all other treatment groups.
group compared with all other groups (Tukey’s HSD, $P < 0.05$; Fig. 7A). Thus, DHEA treatment increased RA volume, but only in the absence of corticosterone treatment.

**Area X**

For area X volume, there were no main effects of corticosterone or DHEA treatment and no interaction effect (Table 1, Fig. 7B).

**Hippocampus**

For hippocampus volume, there were no main effects of corticosterone or DHEA and no interaction effect (Table 1, Fig. 8A). For hippocampal BrdU$^+$ cell number, there was no main effect of corticosterone, but there was a main effect of DHEA and a significant interaction effect (Table 1, Fig. 8B). As in the telencephalon, BrdU$^+$ cells were more abundant in the hippocampus of the DHEA group than in all other groups (Tukey’s HSD, $P < 0.05$).

**Lateral VZ**

For BrdU$^+$ cell number along the VZ, there was no main effect of corticosterone treatment but a significant main effect of DHEA treatment and a significant interaction (Table 1). BrdU$^+$ cells were more abundant along the VZ in the DHEA group (Tukey’s HSD, $P < 0.05$; Fig. 9), as in the telencephalon and hippocampus.

**Peripheral measures**

To assess the physiological effects of corticosterone and DHEA treatment in experiment 2, we took several peripheral body measurements. For body mass on days 7 and 21, there were no main effects of corticosterone or DHEA treatments and no interaction (data not shown). For fat score on days 7 and 21, there was no main effect of corticosterone, but there was a main effect of DHEA, and there was no interaction. On both days 7 and 21, DHEA treatment decreased the fat score (as in Soma et al., 2002; data not shown). For spleen mass, there was a significant main effect of corticosterone treatment ($F_{1,35} = 4.63, P = 0.04$), where corticosterone significantly decreased spleen mass, but there was no main effect of DHEA ($F_{1,35} = 2.58, P = 0.12$) and no interaction ($F_{1,35} = 0.23, P = 0.64$). For syrinx mass, there was no significant effect of corticosterone treatment ($F_{1,35} = 1.10, P = 0.30$) and no interaction ($F_{1,35} = 0.01, P = 0.91$), but there was a significant main effect of DHEA ($F_{1,35} = 33.82, P < 0.0001$), where DHEA significantly increased syrinx mass (as in Soma et al., 2002).

**DISCUSSION**

This is the first study to report the effects of corticosterone on the adult song system and the opposing effects
Corticosterone and DHEA effects on songbird brain

We demonstrate for the first time that a physiological, rather than pharmacological, DHEA dose can counteract the effects of chronic corticosterone treatment in the brain. Specifically, corticosterone treatment decreased, whereas DHEA treatment increased, HVC volume, NeuN$^+$ cell number, and BrdU$^+$ cell number. Corticosterone and DHEA had additive effects in HVC, and DHEA coadministration completely blocked the neurodegenerative effects of chronic corticosterone treatment. These data add to a growing literature on antiglucocorticoid neuroprotection. For example, recent evidence suggests that the neurodegenerative effects of stress and corticosterone on the adult brain can also be attenuated by estradiol treatment (Takuma et al., 2007) or voluntary exercise (Chang et al., 2008).

Figure 7. Effects of corticosterone (Cort) and DHEA treatments on RA (A) and area X (B) volume. Numbers in bars indicate sample size, and the asterisk indicates a significant difference from all other treatment groups.

Figure 8. Effects of corticosterone (Cort) and DHEA on hippocampus volume (A) and hippocampal BrdU$^+$ cell number (B). Numbers in bars indicate sample sizes, and the asterisk indicates a significant difference from all other treatment groups.

of corticosterone and DHEA in the songbird brain. We demonstrate for the first time that a physiological, rather than pharmacological, DHEA dose can counteract the effects of chronic corticosterone treatment in the brain. Specifically, corticosterone treatment decreased, whereas DHEA treatment increased, HVC volume, NeuN$^+$ cell number, and BrdU$^+$ cell number. Corticosterone and DHEA had additive effects in HVC, and DHEA coadministration completely blocked the neurodegenerative effects of chronic corticosterone treatment. These data add to a growing literature on antiglucocorticoid neuroprotection. For example, recent evidence suggests that the neurodegenerative effects of stress and corticosterone on the adult brain can also be attenuated by estradiol treatment (Takuma et al., 2007) or voluntary exercise (Chang et al., 2008).

Plasma corticosterone levels

In recent avian studies, long-term corticosterone treatment via implants increased baseline plasma corticosterone levels only during treatment days 1–4 (Angelier et al.,
Thus, in experiment 1, we examined the effects of corticosterone implants during days 1–4 and then on days 7, 21, and 28. Baseline plasma corticosterone levels were elevated in the corticosterone group on days 1 and 2, but, by day 4, corticosterone levels were elevated in both treatment groups, likely because of daily handling and blood collection. On day 7, corticosterone levels in both groups had declined, and baseline corticosterone levels remained low throughout the rest of the experiment. Transient increases in circulating corticosterone in response to corticosterone treatment are similar to those reported for corticosterone-implanted mice (Herrmann et al., 2009) and rats (Bodnoff et al., 1995) and for young rats given corticosterone in water (Enkel and Koch, 2009). By day 7, the corticosterone implants may have 1) stopped releasing corticosterone, 2) suppressed endogenous corticosterone secretion, and/or 3) increased metabolism or clearance of circulating corticosterone. Consistent with the idea that endogenous corticosterone secretion was suppressed, acute restraint stress on days 7 and 21 had less or no effect on plasma corticosterone levels in the corticosterone group. Similarly, in kestrels (*Falco tinnunculus*), acute restraint stress did not increase plasma corticosterone levels after 8 days of corticosterone treatment (Müller et al., 2009). Furthermore, the results from experiment 1 suggest that, in vitro, the corticosterone implants did not stop releasing corticosterone during the 28 days of treatment. In fact, corticosterone levels in the media were extremely high, even during the last 7 days of incubation in fresh medium (equivalent to days 21–28 in vivo). Although it is possible that, in vivo, the corticosterone implants are encapsulated and isolated from blood vessels, the same would presumably be true for the DHEA implants, which were directly adjacent to, and in contact with, the corticosterone implants in the CORT + DHEA group; however, DHEA levels in this group were high throughout the treatment period.

**Corticosterone and DHEA effects on neural morphometry and NeuN**

Corticosterone treatment decreased HVC volume and HVC NeuN** cell number by ~30%. In contrast, compared with controls, corticosterone did not affect RA, area X, hippocampus, or overall telencephalon volumes. These results suggest that HVC is particularly sensitive to glucocorticoid exposure in adult songbirds. Zebra finches treated with corticosterone during early development have reduced HVC volume but unaffected RA volume in adulthood (Buchanan et al., 2004). Moreover, stressors during early development, such as parasite infection and food restriction, result in reduced HVC volume in adults (Spencer et al., 2005) and fledglings (MacDonald et al., 2006). How corticosterone affects HVC volume remains unclear. Glucocorticoid receptors are present in the songbird brain (Breuner and Orchinik, 2001; Hodgson et al., 2007; Katz et al., 2008), but whether they are expressed in HVC is unknown. These effects of corticosterone on HVC are similar to the effects of corticosterone on the rat dentate gyrus, where corticosterone decreases neuron survival (Sapolsky et al., 1985; Karishma and Herbert, 2002).

DHEA treatment increased HVC volume and NeuN** cell number by ~35%, similar to Soma et al. (2002). Although there is no known classical intracellular steroid receptor for DHEA (Widstrom and Dillon, 2004), DHEA is converted to androgens and estrogens in the songbird brain (Soma et al., 2004a,b; Schlinger et al., 2008; Pradhan et al., 2010), and these potent metabolites of DHEA can bind to receptors in HVC (Soma et al., 1999; Ball et al., 2003). Testosterone or estradiol treatment increases HVC volume in song sparrows and white-crowned sparrows (Tramontin et al., 2003; Soma et al., 2004a,b), and estradiol treatment increases HVC neuron number in canaries (*Serinus canaria*; Hidalgo et al., 1995). Also, HVC volume and neuron number show dramatic seasonal changes in adult song sparrows and are maximal during the breeding season, when plasma levels of testosterone and estradiol are also maximal (Smith et al., 1997; Soma et al., 1999). DHEA treatment also increased RA volume by ~77%, but did not affect area X or hippocampus volumes. The effect of DHEA on RA differs from that described by Soma et al. (2002), in which treatment for only 14 days did not significantly increase RA; however, there was a trend in the same direction.
Androgen receptors are highly expressed in HVC and RA, with lower levels of expression in area X and hippocampus (Soma et al., 1999; Kim et al., 2004). Therefore, DHEA might affect HVC and RA directly, or changes in HVC might have downstream effects on RA (Brenowitz, 2008). Furthermore, HVC also expresses high levels of estrogen receptors in some songbird species (Bernard et al., 1999). Thus, regional differences in DHEA metabolism and steroid receptors may govern the regional specificity of DHEA effects.

In HVC, corticosterone and DHEA treatments had independent additive effects, rather than interactive effects. Corticosterone and DHEA may act via separate mechanisms or converge on the same mechanism. For example, corticosterone and DHEA could regulate neurotrophic factors. Brain-derived neurotrophic factor (BDNF) increases neuron survival in HVC (Rasika et al., 1994). In rat hippocampus, BDNF is decreased by corticosterone (Jacobsen and Mørk, 2006) but is not affected by DHEA (Gubba et al., 2004; Pinnock et al., 2009). Alternatively, corticosterone and DHEA may converge on the same mechanism, such as the phosphatidylinositol-3 kinase/Akt pathway. In cultured rat neurons, corticosterone reduces phosphorylation of Akt and increases apoptosis (Nitta et al., 2004), whereas DHEA increases phosphorylation of Akt and decreases apoptosis (Zhang et al., 2002).

**Corticosterone and DHEA effects on BrdU⁺ cell number**

We also examined the number of new cells incorporated into the adult song control system and hippocampus using BrdU. In HVC, corticosterone decreased BrdU⁺ cell number by ~60%, whereas DHEA increased BrdU⁺ cell number by ~80%. These effects of corticosterone and DHEA are similar to, but more pronounced than, their effects on HVC volume and NeuN⁺ cell number. Again, the effects of corticosterone and DHEA on BrdU⁺ cell number in HVC were additive and could be mediated by mechanisms similar to those mentioned above.

In contrast to HVC, the effects of corticosterone and DHEA on BrdU⁺ cell number in the hippocampus, along the VZ, and in the rest of the telencephalon were interactive, rather than additive. In these three regions, corticosterone treatment alone did not affect BrdU⁺ cell number, but corticosterone coadministration completely prevented the DHEA-induced increase in BrdU⁺ cell number. The effects of corticosterone treatment on cell proliferation in the brain are not consistent across species. Our results are consistent with data from chickadees, in which corticosterone treatment for 51 days did not affect hippocampal volume or hippocampal BrdU⁺ cell number (Pravosudov and Omanska, 2005), even though corticosterone receptors are expressed in the songbird hippocampus (Hodgson et al., 2007). However, our results differ from those in zebra finch brain slices, in which, in vitro, corticosterone decreased BrdU⁺ cell number along the VZ (Katz et al., 2008). Furthermore, corticosterone treatment reduces BrdU⁺ cell number in the dentate gyrus of rats (Cameron and Gould, 1994; Wong and Herbort, 2004), although repeated acute restraint stress did not affect cell proliferation in the mouse dentate gyrus (Snyder et al., 2009). Thus, it is possible that there are species differences in the sensitivity to corticosterone in these brain areas. Alternatively, corticosterone treatment could alter the proportions of proliferating cells that mature into neurons, thus altering the phenotype of new cells in these brain areas without affecting the total number of new cells.

In the absence of exogenous corticosterone, DHEA might increase BrdU⁺ cell number by decreasing local levels of corticosterone via modulation of 11β-hydroxysteroid dehydrogenase (11β-HSD) isozymes 1 and 2. 11β-HSD1 regenerates active glucocorticoids from inactive metabolites, whereas 11β-HSD2 catalyzes the activation of glucocorticoids. Recent evidence suggests that 11β-HSD2 is expressed in the developing songbird brain (Katz et al., 2010). 11β-HSD1-mediated synthesis of local glucocorticoids is reduced by 7-hydroxy-DHEA derivatives, because these DHEA derivatives are the preferred substrates for 11β-HSD1 (Müller et al., 2006a,b). Additionally, in peripheral tissues, DHEA decreases expression of 11β-HSD1 (Apostolova et al., 2005) and increases the activity of 11β-HSD2 (Balazs et al., 2008). Alternatively, DHEA may act via the serotonergic system. In adult male rats, DHEA potentiates fluoxetine-induced cell proliferation in the dentate gyrus, and this effect is blocked by corticosterone treatment (Pinnock et al., 2009).

Corticosterone might block the effects of DHEA on BrdU⁺ cell number by reducing DHEA metabolism or by inactivating DHEA metabolites. First, corticosterone might decrease DHEA metabolism by 3β-hydroxysteroid dehydrogenase (3β-HSD). In the zebra finch brain, acute corticosterone effects on cell proliferation were not affected by 3β-HSD inhibition (Katz et al., 2008), but chronic corticosterone treatment might decrease cell survival over the long term. Second, in rodents, expression of hydroxysteroid sulfotransferase 2, which converts free hydroxysteroids (e.g., estradiol) to their inactive sulfated forms, is induced by a synthetic glucocorticoid (Runge-Morris et al., 1999; Gong et al., 2008). Future studies should determine whether chronic corticosterone treatment alters DHEA metabolism or estrogen inactivation in the songbird brain.
DHEA as a native antiglucocorticoid

In conclusion, this study demonstrates region-specific effects of corticosterone and DHEA in the adult song sparrow brain. In HVC, corticosterone decreased, whereas DHEA increased, HVC volume, NeuN⁺ cell number, and BrdU⁺ cell number. If corticosterone-induced neurodegeneration in HVC, a critical part of the song control system, is associated with behavioral changes within and across seasons, then periods of prolonged stress during adulthood could substantially affect song production and, subsequently, fitness. DHEA treatment blocked the neurodegenerative effects of chronic corticosterone treatment in HVC. These data suggest that DHEA may be useful in the treatment of glucocorticoid-related neurodegeneration and stress-related psychiatric illness. DHEA is an endogenous steroidal prohormone that can be easily administered orally or percutaneously (Labrie et al., 1996), readily crosses the blood–brain barrier, and is well tolerated in humans (Maninger et al., 2009). The present data provide crucial experimental evidence that DHEA, at physiological concentrations, can act as a native antiglucocorticoid in the adult brain.

ACKNOWLEDGMENTS

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LITERATURE CITED


