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Effects of Corticosterone and DHEA on Doublecortin Immunoreactivity in the Song Control System and Hippocampus of Adult Song Sparrows

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ABSTRACT: **Adult neuroplasticity is strongly influenced by steroids. In particular, corticosterone (CORT) and dehydroepiandrosterone (DHEA) can have opposing effects, where CORT reduces while DHEA increases neurogenesis and neuron recruitment. It has been previously shown that in adult male song sparrows, DHEA treatment increases neuron recruitment throughout the telencephalon, including the lateral ventricular zone, while the effect of CORT treatment is restricted to HVC, one of the song control regions. These data suggest that the two steroids may differentially affect proliferation, migration, differentiation, and/or survival of new neurons. To determine if CORT or DHEA alters the migration and differentiation of young neurons, we examined an endogenous marker of migrating immature neurons, doublecortin (DCX), in HVC and hippocampus of adult male song sparrows that were treated with CORT and/or DHEA for 28 days. In HVC, DHEA increased the number of DCX-labeled round cells, while CORT had no main effect on the number of DCX-labeled cells. Furthermore, DHEA increased the area covered by DCX immunoreactivity in HVC, regardless of CORT treatment. In the hippocampus, neither DHEA nor CORT affected DCX immunoreactivity. These results suggest that DHEA enhances migration and differentiation of young neurons into HVC while CORT does not affect the process, whether in the presence of DHEA or not.** © 2013 Wiley Periodicals, Inc. Develop Neurobiol 74: 52–62, 2014

Keywords: **brain; dehydroepiandrosterone; HVC; songbird; stress**

INTRODUCTION

The structure of the adult brain shows dramatic changes in response to stress, aging, and hormones.

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The song control system in songbirds is the best-described example and undergoes pronounced structural and cellular changes throughout adulthood. The song control system in songbirds has two pathways: the motor pathway, which is involved in song production and the anterior forebrain pathway (AFP), which is involved in song learning. In the motor pathway, HVC (a proper name) projects onto robust nucleus of the arcopallium (RA), which in turn projects onto the respiratory motor neurons and syrinx (Kirn, 2010). The AFP is analogous to the cortico-basal ganglia pathway in mammals and begins at HVC, projecting onto Area X which synapses onto dorsal lateral nucleus of the medial thalamus (DLM). DLM then projects onto lateral magnocellular nucleus of the anterior nidopallium (LMAN), then projects onto either RA or Area X (Vates and Nottebohm, 1995).

The HVC, Area X, and RA show seasonal plasticity (Tramontin and Brenowitz, 2000). However, the increase in HVC volume is due to incorporation of new neurons while the increase in RA volume is due to greater neuron size and spacing (Smith et al., 1997, Tramontin et al., 1998, 2000). Among HVC neurons, only HVC-RA neurons in the motor pathway are replaced in adulthood whereas Area X projecting HVC neurons in AFP are not (Alvarez-Buylla et al., 1988, 1990, Kirn et al., 1991, Scharff et al., 2000). During neuroproliferation and neuron maturation, precursor cells proliferate in the lateral ventricular zone (Goldman and Nottebohm, 1983), and these neural progenitor cells then migrate to HVC and other regions of the forebrain where they differentiate into functional neurons. Not all new neurons survive (Nottebohm, 2002), and thus, the number of mature neurons and the size of associated brain regions result from differential rates of proliferation, migration, and survival of new neurons (Ming and Song, 2005).

The seasonal neuroplasticity is influenced by steroid hormones. The song control nuclei have androgen and estrogen receptors (Ball et al., 2002), as well as glucocorticoid (GC) receptors (Katz et al., 2008, Shahbazi et al., 2011), and administration of steroids affects nuclei volumes and neuron recruitment in the song control system and in the hippocampus (Hp). In male songbirds, testosterone or estradiol implants increase the volume of HVC, RA, and Area X (Nottebohm, 1981, Kirn et al., 1989, Rasika et al., 1994, Smith et al., 1997, Tramontin and Brenowitz, 2000, Tramontin et al., 2003). In the wild, the number of new neurons incorporated into HVC peaks in the fall when the plasma testosterone level is low (Kirn et al., 1994, Tramontin and Brenowitz, 1999, 2000). At the same time, the neuronal turnover is also higher in the fall, thus the total number of HVC neurons remains less than that of spring. Interestingly, testosterone and estradiol do not solely regulate seasonal behaviour and neuroplasticity. Male song sparrows maintain high aggression throughout the breeding and nonbreeding season. In this species, plasma levels of dehydroepiandrosterone (DHEA, an androgen precursor) remain high during the nonbreeding season compared with testosterone and estradiol (Soma and Wingfield, 2001). Administration of DHEA to male song sparrows increases HVC and RA volumes, as well as the number of bromodeoxyuridine-labeled neurons (BrdU, a marker for neuron recruitment) in HVC and Hp (Soma et al., 2002, Newman et al., 2010).

However, steroid hormones associated with stress have adverse effects on the brain, including the song control system and Hp. Among the song control nuclei, HVC appears to be most sensitive to chronic stress and GCs (Newman et al., 2010). Corticosterone (CORT), the predominant circulating GC in birds, reduces HVC volume, HVC neuron number, and the recruitment of new neurons in HVC (cells labeled with BrdU). In rats, chronic elevation of CORT *in vivo* and *in vitro* increases neuron death (Kimonides et al., 1999) and reduces neuron recruitment in Hp (Karishma and Herbert, 2002). Interestingly, this could be prevented by co-administration of DHEA in rats (Kimonides et al., 1999, Karishma and Herbert, 2002). Similar effects were observed in song sparrows where DHEA completely rescued the effects of CORT on volume of, neuronal recruitment into, and number of mature neurons in HVC (Newman et al., 2010). Taken together, these studies suggest that CORT has neurodegenerative effects, while DHEA rescues the detrimental effects of CORT.

The entire process ultimately leading to the recruitment of mature neurons is particularly complex with multiple steps at which these hormones could exert a regulatory effect. It is possible that CORT and DHEA affect different steps of this process, leading to the opposing phenomenon described above. Indeed, in song sparrows, DHEA administration increased the number of BrdU-labeled cells in general, including those throughout the telencephalon and Hp (Newman et al., 2010). In contrast, the reduction of BrdU-labeled cells by CORT was found only in HVC. Because CORT did not reduce BrdU-labeled cells along the ventricular zone, we hypothesize that CORT reduces neuron migration and survival in HVC, while DHEA promotes neuroproliferation as well as neuron migration and survival throughout the telencephalon. Here, we focused on migration and differentiation of immature neurons to HVC and Hp.

To determine how CORT and/or DHEA affect the migration and differentiation of immature neurons in the song control system and Hp, we treated male song sparrows with CORT and/or DHEA and then examined doublecortin (DCX) immunoreactivity. DCX is an endogenous protein expressed only in migrating and differentiating immature neurons (Francis et al., 1999; Gleeson et al., 1999) within 20–25 days of neuron's birth in the songbird telencephalon (Balthazart et al., 2008). If CORT and DHEA alter only survival of new neurons following migration, then we would expect to see no differences in DCX immunoreactivity in subjects exposed to CORT or DHEA. However, if both CORT and DHEA alter the migration and differentiation of new neurons, then we would expect a lower DCX immunoreactivity in CORT-implanted subjects and a higher DCX immunoreactivity in DHEA-implanted subjects.

MATERIALS AND METHODS

Animals

This study used the same subjects as in Newman et al. (2010). In June and July of 2007, a total of 36 adult male song sparrows (*Melospiza melodia*) were captured at Queens University Biological Station or around the University of Western Ontario. Birds were brought to the University of Western Ontario and housed individually in cages in four animal rooms. The photoperiod in all rooms was initially matched to ambient conditions and then was reduced gradually to a short-day photoperiod of 8L:16D. The birds were maintained on the short-day photoperiod for 1 month before the start of the experiment. The short-day photoperiod was used because, in songbirds, neuronal recruitment into HVC is highest during the nonbreeding season (Kirn et al., 1994, Tramontin and Brenowitz, 1999, 2000), and DHEA treatment during this season increases HVC size (Soma et al., 2002). Birds had *ad libitum* access to food (Mazuri small bird maintenance diet and millet) and water.

In November 2007, subjects were randomly assigned to one of the four treatments: control (two empty implants), CORT (one empty and one CORT implant), DHEA (one empty and one DHEA implant), and CORT + DHEA (one CORT and one DHEA implant). All four animal rooms had subjects from all four treatments. On the day of implantation (day 0 of experiment), birds were anesthetized with isoflurane (1.5%, 2 L/min O_2), and the Silastic implants (CORT: i.d. 1.47 mm, o.d. 1.96 mm, 12 mm in length; DHEA: i.d. 0.76 mm, o.d. 1.65 mm, 7 mm in length) were inserted subcutaneously through a small incision on the back (see Newman et al., 2010 for timelines). The opening of the skin was sealed with veterinary skin adhesive (Nexaband S/C, Medical Corporation, Raleigh, NC). The efficacy of steroid implants was validated previously in song sparrows (see Newman et al., 2010). Briefly, when silastic implants containing CORT were incubated in saline solution at ~40°C for 28 days, high levels of CORT (above 75 μg/mL) were maintained in the media over the full 28 days whether or not the implants had a hole. CORT implants *in vivo* elevated baseline plasma CORT levels for the first 3 days, then plasma levels became comparable to controls with empty implants for the remainder of the 28 days (plasma collected on day 4, 7, 21, and 28). This is likely because birds increased clearance and/or inhibited the hypothalamic-pituitary-adrenal axis, as shown by the suppressed adrenocortical response on day 7 and 21 (Newman et al., 2010). DHEA implants in vivo elevated plasma DHEA in both DHEA alone and DHEA and CORT group between days 7 and 28. Each bird also received three intramuscular injections of BrdU per day (cat no. B9285; Sigma, St. Louis, MO) on day 3 and on day 4 for the previous study.

On day 28 after implantation, birds were deeply anesthetized using ketamine and xylazine. They were perfused transcardially with heparinized saline, followed by buffered 4% paraformaldehyde. The brain was quickly removed from the skull and placed in 4% paraformaldehyde (~24 h) and then in 30% sucrose (~48 h) at 4°C. Brains were frozen on crushed dry ice and then stored at −80°C. The animal care and experimental procedures used in this experiment were approved by the University of Western Ontario Animal Care and Use Committee.

Immunohistochemistry

The immunohistochemistry used an antibody that was previously validated in songbirds, including loss of immunoreactivity after preabsorption and identification of the protein using Western blots (Boseret et al., 2007). Prior to the assay, we optimized the concentration of primary antibody for this species. We tested the primary antibody concentration of 1:200, 250, 500, and 1,000 and determined the optimal concentration to be 1:250. Brains were sliced into 30 μm coronal sections on a cryostat and then stored in a cryoprotectant solution of ethylene glycol and polyvinylpyrrolidone at -20°C. Every third section (i.e., 90 μm apart) was used to examine DCX immunoreactivity. Free-floating sections were thoroughly rinsed twice with 0.1 *M* phosphate-buffered saline (PBS; pH 7.5), and then incubated with 0.5% H_2O_2 for 15 min to eliminate endogenous peroxidase activity. Sections were rinsed three times with PBS, and then incubated with 10% Normal Horse Serum (cat no. S-2000; Vector Laboratories, Burlingame, CA) in PBS containing 0.3% Triton X-100 (PBS/T) for 30 min. Sections were incubated with primary antibody made in goat against DCX (polyclonal, 1:250 dilution, cat no. SC-8066; Santa Cruz Biotechnology, Santa Cruz, CA) in 0.3% PBS/T for ~24 h at 4°C. After rinsing with 0.1% PBS/T, sections were incubated with biotinylated horse anti-goat IgG secondary antibody (1:400 dilution) for 1 hr at room temperature, followed by rinses with 0.1% PBS/T. Sections were then incubated with avidin-biotin horseradish-peroxidase complex (VectaStain Elite ABC Kit, cat. no. PK 6100;

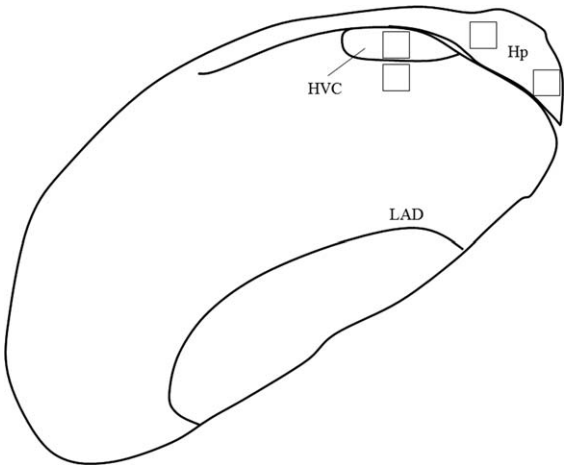

Figure 1 Schematic drawing of a coronal section of a song sparrow telencephalon hemisphere, with two sample squares in and just outside HVC and two sample squares in dorsal and medial hippocampus (Hp). Sample squares indicate the locations at which images were collected for further analysis. Abbreviation: LAD: lamina arcopallialis dorsalis.

Vector Laboratories) at dilution of 1:200 for 1 h, followed by rinses with 0.1% PBS/T. Immunoreactivity was visualized with 0.04% 3,3′-diaminobenzidine tetrahydrochloride (DAB, cat. no., D5637; Sigma). After rinsing with PBS, sections were mounted on microscope slides, dehydrated gradually with increasing concentrations of ethanol, and cleared of lipids with an organic solvent (NeoClear, cat no. 65038-71; EMD Chemicals, Mississauga, Ontario, Canada). Slides were covered with coverslips and mounting medium (Permount, cat no. SP15; Fisher Scientific).

Microscopy

Song control nuclei (HVC, Area X, and RA) and Hp of each brain were qualitatively and quantitatively analyzed for DCX-labeled (DCX+) cells using a Leica DM5500B microscope coupled with a Leica DFC 420C camera. The brain regions were first noted for presence or absence of DCX+ cells. In the regions with densely stained cells (HVC and Hp), we analyzed five coronal sections (90 μm apart) in both hemispheres. We measured (1) the percentage of a field of view (192 $\times$ 257 μm^2) covered by DCX+ cell bodies, dendrites, and axons and (2) the number of DCX+ cells. The five sections were selected such that the middle section contained the largest cross-section of HVC. For each of the five sections, two sampling fields were analyzed for each HVC and Hp. For HVC, one field was positioned in the center of HVC, and the other field was positioned just outside of HVC (in the adjacent nidopallium that is ventral to HVC; see Fig. 1). For Hp, one field was positioned dorsomedial to HVC (dorsal Hp), and the other field was positioned at the medial edge of Hp (medial Hp; Fig. 1). The experimenter who compiled and analyzed these images was blind to the treatment group of each subject.

For each field of interest, z-stack images of 0.63 μm steps through the focal planes were collected through the 40× objective lens and were then compiled using a montage mode in Leica Application Suite software. This created an image where all DCX+ cells and projections were in focus. Percent coverage by DCX+ cells and fibers (% cover) was determined using a threshold feature of the ImageJ program (NIH) after converting images into 32-bit gray scale. As seen before in canaries (Boseret et al., 2007, Balthazart et al., 2008, Yamamura et al., 2011), we observed two types of DCX+ cells: round and fusiform. Round and fusiform DCX+ cells typically represent fully differentiated neurons and migrating neurons, respectively (Balthazart et al., 2008). We counted these cell types separately. When fields of interest were missing due to tissue damage, the percentage cover or cell counts from the other hemisphere were substituted. We only included subjects in the statistical analysis that had 7 or more out of 10 sections (5 sections/hemisphere).

Statistical Analyses

Statistical analyses were carried out using PASW (previously SPSS) 18.0. We first tested for lateralization of all dependent variables using paired t-tests. Normality and homogeneity of variances were tested prior to each analysis and corrected via data transformation when appropriate. The effects of CORT and DHEA treatment on percentage DCX+ cover and total number of round and fusiform DCX+ cells in HVC were analyzed using a two-way ANCOVA with DHEA and CORT as fixed factors, respective values in the adjacent field in nidopallium as a covariate, and animal room number as a random factor. The effects of CORT and DHEA treatment on the total number of round and fusiform DCX+ cells in dorsal Hp and medial Hp were analyzed using two-way MANOVA. Results were considered significant at $\alpha \leq 0.05$ level. Data are presented as mean $\pm$ SEM.

RESULTS

HVC

DHEA treatment increased DCX staining in HVC. DHEA treatment significantly increased the percentage of the area covered by DCX immunoreactivity, which reflects the density of DCX+ cells and dendrites [Table 1; Fig. 2(A)]. CORT treatment, however, did not affect percentage cover. Nor was there an interaction between CORT and DHEA treatments, indicating that the presence of CORT did not influence the effects of DHEA on DCX+ in HVC.

We observed both round DCX+ cells and fusiform DCX+ cells in HVC. Similar to percentage cover, DHEA treatment significantly increased the total

Table 1 Effects of CORT and DHEA Treatments on DCX Immunoreactivity

Two-factor ANOVA	df	*F* ratio	*p* value
HVC % cover			
CORT	1,26	0.25	0.62
DHEA	1,26	7.90	**0.01**
CORT × DHEA	1,26	0.05	0.82
HVC fusiform cells			
CORT	1,26	4.41	0.21
DHEA	1,26	0.64	0.48
CORT × DHEA	1,26	0.23	0.66
HVC round cells			
CORT	1,26	0.02	0.90
DHEA	1,26	53.37	**0.006**
CORT × DHEA	1,26	0.25	0.65
HVC total no. of DCX+ cells			
CORT	1,26	0.06	0.83
DHEA	1,26	21.32	**0.023**
CORT × DHEA	1,26	0.34	0.60
Dorsal Hp fusiform cells			
CORT	1,20	1.04	0.32
DHEA	1,20	0.008	0.93
CORT × DHEA	1,20	1.04	0.32
Dorsal Hp round cells			
CORT	1,20	0.001	0.97
DHEA	1,20	2.98	0.10
CORT × DHEA	1,20	3.23	0.09
Dorsal Hp total no. of DCX+ cells			
CORT	1,20	0.09	0.77
DHEA	1,20	1.80	0.20
CORT × DHEA	1,20	1.80	0.20
Medial Hp fusiform cells			
CORT	1,20	0.03	0.88
DHEA	1,20	0.014	0.91
CORT × DHEA	1,20	0.24	0.63
Medial Hp round cells			
CORT	1,20	0.39	0.54
DHEA	1,20	0.01	0.92
CORT × DHEA	1,20	0.42	0.53
Medial Hp total no. of DCX+ cells			
CORT	1,20	0.28	0.60
DHEA	1,20	0.03	0.86
CORT × DHEA	1,20	0.24	0.63

CORT: corticosterone; DHEA: dehydroepiandrosterone; DCX: doublecortin.

number of DCX+ cells (round + fusiform combined) in HVC [Table 1; Fig. 2(B)]. When round and fusiform DCX+ cells were analyzed separately, it was evident that DHEA only increased the number of round DCX+ cells but had no effect on the number of fusiform DCX+ cells. CORT treatment did not affect the number of DCX+ cells in HVC. We found no interaction between CORT and DHEA on numbers of round or fusiform DCX+ cells.

Area X and RA

There were no or almost no DCX+ cells in Area X and RA in subjects from all treatment groups (Fig. 3). Area X was easily visualized by a lack of DCX staining, surrounded by moderate staining in the rest of the medial striatum. In contrast, the arcopallium intermedium, including RA, generally had no staining. Because of the near absence of staining, we did not compare DCX immunoreactivity across treatment groups for these areas.

Hippocampus

Dark staining of DCX+ cells was concentrated at the subventricular zone and near the medial tip of Hp

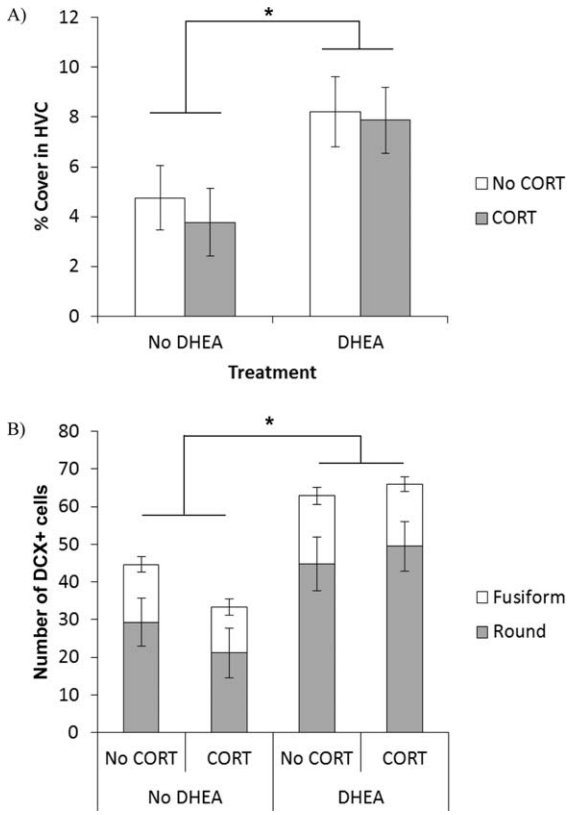

Figure 2 Effects of CORT and DHEA on (A) the percentage of a standardized area covered by DCX immunoreactivity and (B) the number of round and fusiform DCX+ cells in HVC of adult male song sparrows (*Melospiza melodia*) under short-day condition. Males were implanted with either (1) two empty silastic implants, (2) one empty implant and one CORT implant, (3) one empty implant and one DHEA implant, or (4) one CORT implant and one DHEA implant for 28 days. All bars represent untransformed, adjusted means that incorporate a covariate of respective values in the adjacent fields immediately ventral to HVC. Error bars depict SEM. *$p < 0.05$. Abbreviations: CORT: corticosterone; DHEA: dehydroepiandrosterone; DCX: doublecortin.

Figure 3 Photomicrographs illustrating little or no doublecortin (DCX) immunoreactivity in Area X (left) and RA (right). The perimeters of Area X and RA are shown with dashed lines. Abbreviation: RA: robust nucleus of the arcopallium. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Figs. 1 and 4). We also observed diffuse staining of round cells across the Hp. This diffuse staining could lead to overestimation of percentage cover, thus we omitted measuring percentage cover in Hp and only analyzed cell counts.

Neither steroid had significant effects on the number of DCX+ cells in Hp. The total number of DCX+ cells was always higher in medial Hp than in dorsal Hp (e.g., average of 25 vs. 2 cells in control birds), with large variation in dorsal Hp due to the small number of DCX+ neurons that were present. Neither CORT nor DHEA affected the total number of DCX+ cells (round cells + fusiform cells) in either field of the Hp [Table 1; Fig. 5(A,B)]. When we examined round cells and fusiform cells separately, the pattern remained the same. Neither CORT nor DHEA affected the number of round cells in either field of Hp. Similarly, neither steroid affected the number of fusiform cells in Hp.

DISCUSSION

Newman at al. (2010) showed that CORT decreased, while DHEA increased, HVC volume, mature neuron and neuron recruitment into HVC; however the mechanism was unknown. To determine if CORT and/or DHEA affect neuron recruitment and size of brain regions via altering migration and differentiation of young neurons, we examined DCX immunoreactivity in HVC and Hp of male song sparrows exposed to chronic elevation of CORT and/or DHEA. For HVC, we found that DHEA increased the number of round DCX+ cells as well as the percentage of the area covered by DCX+ migrating cells and their fibers, regardless of CORT treatment. CORT had no main effect on DCX immunoreactivity of young neurons in HVC. This suggests that the opposing effects of CORT and DHEA on HVC result from different mechanisms. We also found that DCX immunoreactivity in Hp was not affected by either CORT or DHEA.

We observed little or no DCX immunoreactivity in RA and Area X. RA has very low rates of neuronal incorporation after hatching and completely ceases to receive new neurons in adulthood (Alvarez-Buylla and Kirn, 1997, Wilbrecht and Kirn, 2004). The lack of DCX immunoreactivity in RA in our study is thus consistent with this and is in accordance with previous studies in canaries that reported no or almost no DCX staining in the arcopallium, especially in RA (Boseret et al., 2007, Balthazart et al., 2008). Newman et al. (2010) observed an increase in RA volume in response to DHEA without an increase in DCX+ cells (this study). This supports the earlier findings that the seasonal increase in RA volume is due to neurite growth (DeVoogd and Nottebohm, 1981, Maninger et al., 2009) rather than incorporation of new neurons. In contrast, Area X is one of the brain regions that continue to receive new neurons throughout adulthood (Alvarez-Buylla and Kirn, 1997, Wilbrecht and Kirn, 2004). In fact, Area X shows DCX mRNA expression in adult male zebra finches (Kim et al., 2006) and DCX immunoreactivity in adult canaries (Boseret et al., 2007, Balthazart et al., 2008). Yet, we observed very little DCX staining in Area X. Two previous studies showed that 14- or 28-day treatments of exogenous DHEA did not increase Area X volume in song sparrows under short-day conditions (Soma et al., 2002, Newman et al., 2010). These studies together suggest that during

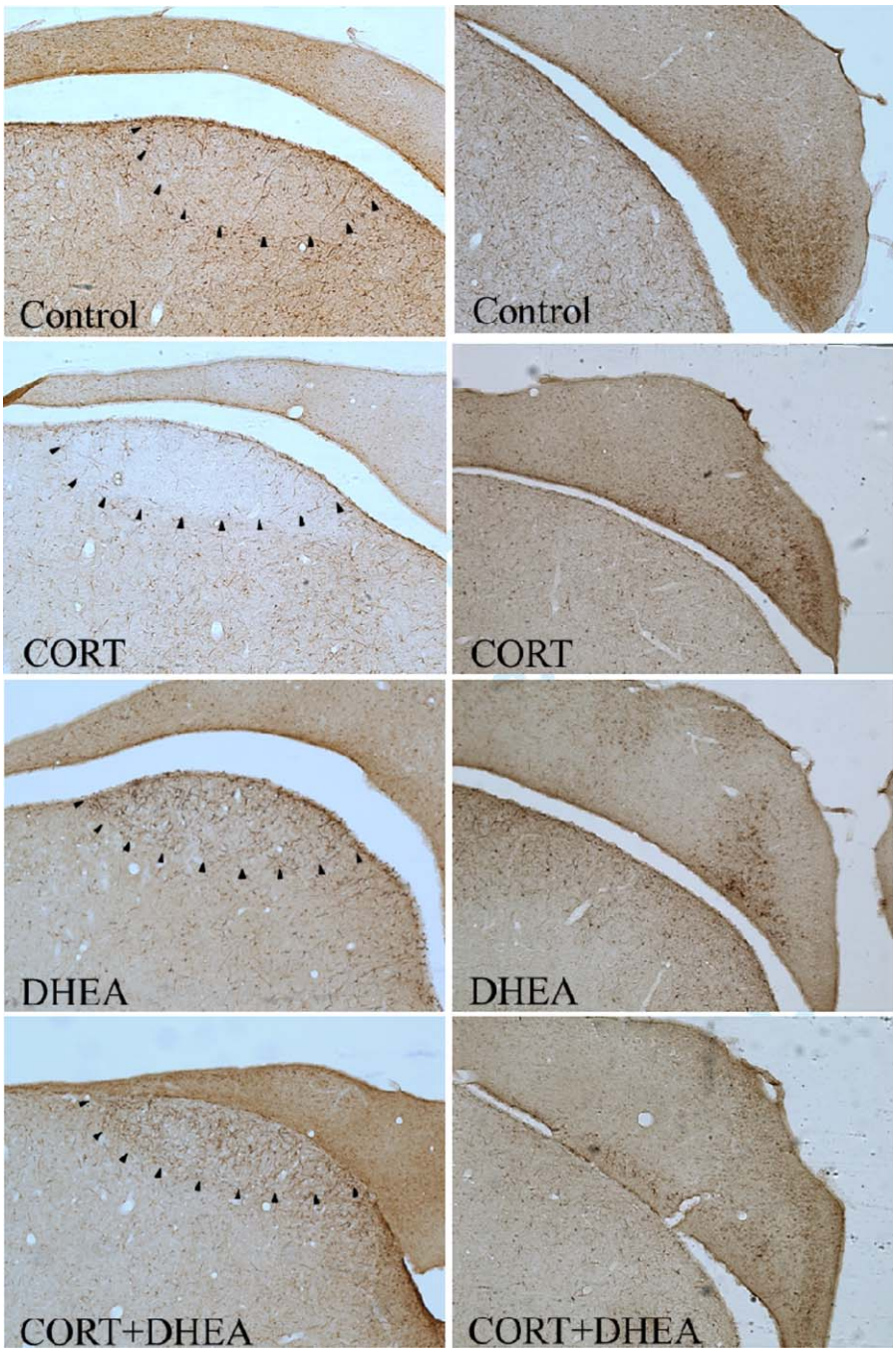

Figure 4 Photomicrographs of doublecortin (DCX) immunoreactivity in HVC (left column) and hippocampus (right column) from subjects given empty implants (control), CORT, DHEA, or CORT + DHEA for 28 days. The ventral edge of HVC is shown with arrows. Abbreviations: CORT: corticosterone; DHEA: dehydroepiandrosterone. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

nonbreeding season Area X is less sensitive to DHEA compared to HVC in this species.

In Hp of adult song sparrows, DCX immunoreactivity was present, but no effects of CORT or DHEA were observed. This is in contrast with mammalian studies. In rat Hp, CORT inhibits neuron recruitment, partly due to reduction of neuron migration, marked by a reduction in DCX+ cell number (Brummelte and Galea, 2010). Unlike rats, the Hp in birds appears to be less sensitive to CORT. Chronic elevation of

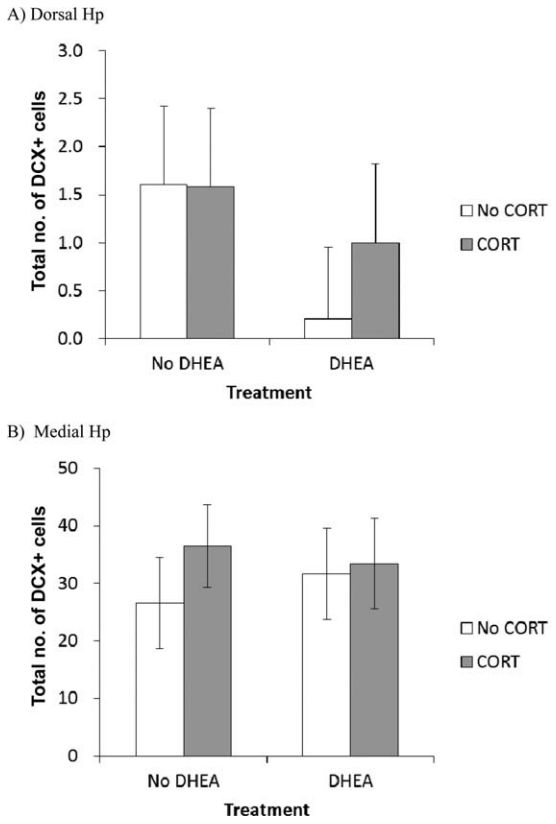

Figure 5 Effects of CORT and DHEA on the total number of round and fusiform DCX+ cells in (A) dorsal Hp and (B) medial Hp of adult male song sparrows (*Melospiza melodia*) under short-day condition. All bars represent raw mean values ± SEM. Abbreviations: CORT: corticosterone; DHEA: dehydroepiandrosterone; DCX: doublecortin; Hp: hippocampus.

CORT did not alter Hp volume (Pravosudov and Omanska, 2005, Newman et al., 2010), the total number of Hp neurons (Pravosudov and Omanska, 2005), or neuron recruitment in Hp as measured by BrdU incorporation (Newman et al., 2010). Our results are consistent with these previous studies in birds, since CORT did not modify DCX immunoreactivity in song sparrow Hp. DHEA also had no effect on DCX immunoreactivity in Hp of adult song sparrows, despite the fact that DHEA increased the number of BrdU-labeled cells (Newman et al., 2010). This indicates that DHEA increased neurogenesis in Hp through mechanisms other than promoting neuron migration (e.g., neuron survival).

Chronic elevation of DHEA increased DCX+ cells in HVC, suggesting that DHEA increases HVC volume and neuron recruitment in HVC in part by promoting migration and differentiation of young neurons. DHEA has been shown repeatedly to promote neuron survival in mammalian Hp (Karishma and Herbert, 2002, Charalampopoulos et al., 2008, Li et al., 2010, Shoae-Hassani et al., 2011). However, to our knowledge, this is the first study to suggest that DHEA stimulates neuron migration and differentiation into HVC, indicated by DCX immunoreactivity. Interestingly, DHEA only increased the number of round DCX+ cells and did not affect fusiform DCX+ cell number. Assuming that fusiform cells are migrating neurons whereas round cells are differentiating neurons (Balthazart et al., 2008; also see Herrick et al. 2006), this result suggests that DHEA treatment promoted not only migration, but also recruitment of young neurons to HVC. The fact that DHEA treatment increased both NeuN+ and round DCX+ cell numbers implies that DHEA also promoted differentiation and survival of those immature neurons in HVC. In fact, in embryonic rat forebrain, DHEA reduces apoptosis (Zhang et al., 2002). Similarly, in adult rat Hp, DHEA improves neuron survival (Karishma and Herbert, 2002). Newman et al. (2010) found that DHEA increased BrdU+ cells at the ventricular zone (Table 2). Taken together, these studies suggest DHEA promotes nearly all steps of neuroplasticity, from proliferation at the ventricular zone to survival of immature neurons. It is worth noting that the number of BrdU and the total number of DCX cells within HVC did not correlate significantly (linear regression, data not shown), highlighting the complex relationship between steroids and the multiple phases of neurogenesis. Further studies are needed to directly measure the effects of DHEA on neuron survival in songbird brains.

Neuronal recruitment into HVC is highest in the fall when plasma testosterone level is low (Kirn et al., 1994, Tramontin and Brenowitz, 1999, 2000). It has been hypothesized that decreasing testosterone levels causes apoptosis of HVC neurons creating space in HVC for new neurons to migrate into (Tramontin and Brenowitz, 1999, Thompson and Brenowitz, 2009). The birds used in this study were on the short-day photoperiod, thus photosensitive. The results of this study suggest that in male song sparrows, DHEA may contribute to the increased neuronal migration and differentiation of HVC neurons during nonbreeding seasons.

How DHEA may increase migration or survival of neurons in HVC is unknown. Intracellular receptors for DHEA have not been found (Widstrom and Dillon, 2004). Since abundant numbers of androgen and estrogen receptors are found in HVC (Balthazart et al., 1992, Gahr et al., 1993, Bernard et al., 1999, Soma et al., 1999) and DHEA is readily converted to androgens and estrogens in HVC (Soma et al., 2004a,2004b, Schlinger et al., 2008, Pradhan et al.,

Table 2 Summary of Effects of CORT and DHEA on Song Control System in Newman et al. (2010) and this Study

		CORT	DHEA	CORT × DHEA
HVC	HVC volume*	↓	↑	n.s.
	NeuN+ cell number*	↓	↑	n.s.
	BrdU+ cell number*	↓	↑	n.s.
	DCX+ % cover	n.s.	↑	n.s.
	DCX+ round cell number	n.s.	↑	n.s.
	DCX+ fusiform cell number	n.s.	n.s.	n.s.
RA	RA volume*	↓	↑	Significant; DHEA ↑ volume only in the absence of CORT
	DCX immunoreactivity	Very few DCX+ cells		
Area X	Area X volume*	n.s.	n.s.	n.s.
	DCX immunoreactivity	Very few DCX+ cells		

CORT: corticosterone; DHEA: dehydroepiandrosterone; DCX: doublecortin; RA: robust nucleus of the arcopallium; n.s.: not significant.

2010), it has been proposed that DHEA acts indirectly through androgen or estrogen receptors. In fact, 3-week treatment with testosterone, but not estradiol, increased fusiform and round DCX+ cells in HVC of female canaries (Yamamura et al., 2011). Interestingly, DHEA increased DCX immunoreactivity in HVC but not in Hp, despite the fact that DHEA increased BrdU+ cell numbers in both areas (Newman et al., 2010). This indicates that DHEA promotes different steps of neurogenesis depending on the brain region.

Chronic elevation of CORT did not alter DCX immunoreactivity in HVC, although CORT suppressed HVC volume and number of mature neurons in HVC in the previous study (Newman et al., 2010). This implies that CORT may be exerting its effects through triggering apoptosis of the neurons, rather than reducing neural migration or recruitment. In rodents, CORT regulates apoptosis in dentate gyrus (Gould et al., 1992; Cameron and Gould, 1994) through dampening brain-derived neurotrophic factor and other growth factors (Schaaf et al., 1997, Gubba et al., 2004, Jacobsen and Mørk, 2006). Surprisingly, we found CORT did not offset the positive effects of DHEA on DCX staining in HVC. Numerous studies demonstrate that DHEA and CORT have opposite effects, where DHEA rescues detrimental effects of CORT (Kalimi et al., 1994). In rats, DHEA rescued negative effects of CORT on neuron numbers (Kimonides et al., 1999), neurogenesis, and neuron survival in Hp (Karishma and Herbert, 2002). In song sparrows, DHEA prevented the reduction in number of mature neurons by CORT in HVC (Newman et al., 2010). In conclusion, this study demonstrates that DHEA increased DCX staining in HVC while CORT had no effect, despite the fact that DHEA and CORT had opposite effects on BrdU+, NeuN+ cell numbers in HVC as well as HVC volume. Taken together with previous studies, our study suggests that CORT and DHEA regulate brain anatomy and neurogenesis via distinct mechanisms, with CORT possibly reducing survival of neurons and DHEA enhancing proliferation, migration, recruitment, and survival of young neurons.

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