

# Dehydroepiandrosterone Metabolism by 3 $\beta$ -Hydroxysteroid Dehydrogenase/ $\Delta$ 5- $\Delta$ 4 Isomerase in Adult Zebra Finch Brain: Sex Difference and Rapid Effect of Stress

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Dehydroepiandrosterone (DHEA) is a precursor to sex steroids such as androstenedione (AE), testosterone (T), and estrogens. DHEA has potent effects on brain and behavior, although the mechanisms remain unclear. One possible mechanism of action is that DHEA is converted within the brain to sex steroids. 3 $\beta$ -Hydroxysteroid dehydrogenase/ $\Delta$ 5- $\Delta$ 4 isomerase (3 $\beta$ -HSD) catalyzes the conversion of DHEA to AE. AE can then be converted to T and estrogen within the brain. We test the hypothesis that 3 $\beta$ -HSD is expressed in the adult brain in a region- and sex-specific manner using the zebra finch (*Taeniopygia guttata*), a songbird with robust sex differences in song behavior and telencephalic song nuclei. In zebra finch brain, DHEA is converted by 3 $\beta$ -HSD to AE and subsequently to estrogens and 5 $\alpha$ - and 5 $\beta$ -reduced androgens.

3 $\beta$ -HSD activity is highest in the diencephalon and telencephalon. In animals killed within 2–3 min of disturbance, baseline 3 $\beta$ -HSD activity in portions of the telencephalon is higher in females than males. Acute restraint stress (10 min) decreases 3 $\beta$ -HSD activity in females but not in males, and in stressed animals, telencephalic 3 $\beta$ -HSD activity is greater in males than in females. Thus, the baseline sex difference is rapidly reversed by stress. To our knowledge, this is the first demonstration of 1) brain region differences in DHEA metabolism by 3 $\beta$ -HSD, 2) rapid modulation of 3 $\beta$ -HSD activity, and 3) sex differences in brain 3 $\beta$ -HSD and regulation by stress. Songbirds are good animal models for studying the regulation and functions of DHEA and neurosteroids in the nervous system. (*Endocrinology* 145: 1668–1677, 2004)

DEHYDROEPIANDROSTERONE (DHEA), A SEX steroid precursor (Fig. 1), has numerous effects on the developing and adult brain. Recent studies have shown that DHEA can affect neurite outgrowth, neuron survival, neurotransmitter signaling, adult neurogenesis, and adult neuroanatomy (1–4). Potent neurotrophic and neuroprotective effects of DHEA are seen in a variety of experimental systems (5–7). Behaviorally, DHEA can affect sexual behavior, aggression, learning, and mood (2, 8–11).

Songbirds provide excellent opportunities for studying the effects of DHEA on behavior and the brain. Singing is a natural and learned behavior that is critical for social interactions in both aggressive and reproductive contexts (12). Sex steroids such as testosterone (T) and estradiol (E<sub>2</sub>) can stimulate singing (13–15) via actions on a circuit of discrete and steroid-sensitive brain regions (16, 17). In some songbird species, this song control circuit is highly plastic in adult animals and shows large increases in the sizes of entire telencephalic regions in response to T and E<sub>2</sub> (15, 18, 19). T treatment can also increase neuron number, neuron size,

dendritic arborization, and synapses within song nuclei in several songbird species (15).

The effects of DHEA have been examined in song sparrows (*Melospiza melodia*). Interestingly, free-ranging male song sparrows sing and aggressively defend their territories in autumn and winter, when they are in nonbreeding condition and plasma T and E<sub>2</sub> levels are undetectable (20, 21). DHEA, however, is detectable in the circulation of song sparrows in autumn (21). Nonbreeding adult male song sparrows were treated with a physiological dose of DHEA. DHEA treatment increased territorial singing behavior (11). Moreover, DHEA increased the size of a telencephalic song control nucleus (HVC; see <http://www.avianbrain.org> for revised avian brain terminology) (11). Within only 2 wk, DHEA increased the volume of HVC by 50% (similar to maximal size in breeding song sparrows). This is one of the largest reported effects of DHEA on the adult brain. Taken together, these data indicate that songbirds are good animal models for studying the functions of DHEA in the nervous system.

Although several studies have described the effects of exogenous DHEA on the brain, the mechanism of action remains unclear in most cases. There is no known intracellular steroid receptor for DHEA (22). Recent reports, however, suggest that DHEA can directly bind to receptors on the plasma membrane (23, 24). Alternatively, DHEA might be converted within the brain to sex steroids, which then bind to androgen and estrogen receptors. Consistent with the

Abbreviations: 5 $\alpha$ -A, 5 $\alpha$ -Androstenedione; 5 $\beta$ -A, 5 $\beta$ -androstenedione; AE, androstenedione; DHEA dehydroepiandrosterone; E<sub>1</sub>, estrone; E<sub>2</sub>, 17 $\beta$ -estradiol; 3 $\beta$ -HSD, 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta$ 5- $\Delta$ 4 isomerase; NAD, nicotinamide adenine dinucleotide; POA, preoptic area; T, testosterone.

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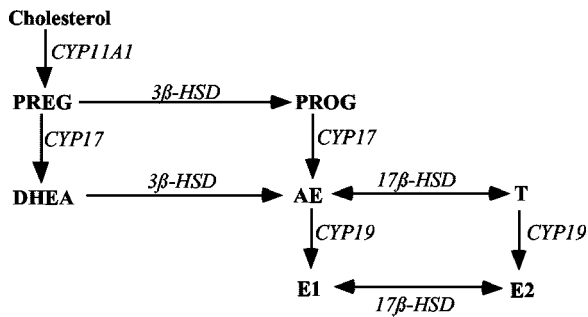


FIG. 1. Simplified diagram of sex steroid synthesis. Steroids are in **bold**, and enzymes are in *italics*. Steroids: PREG, pregnenolone; PROG, progesterone. Enzymes: CYP11A1, cytochrome P450 side-chain cleavage; CYP17, cytochrome P450 17 $\alpha$ -hydroxylase/C17,20 lyase; CYP19, P450 aromatase.

latter hypothesis, the behavioral and neural effects of DHEA in song sparrows are similar to those of T and E<sub>2</sub> (11, 19).

Here, we test the hypothesis that the adult songbird brain can metabolize DHEA via the enzyme 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta$ 5- $\Delta$ 4 isomerase (3 $\beta$ -HSD) (Fig. 1) to androstenedione (AE). AE can be subsequently converted to potent androgens and estrogens by the songbird brain (25). In songbirds, very little is known regarding 3 $\beta$ -HSD in the brain. 3 $\beta$ -HSD activity was detected in primary cell cultures from the telencephalon of developing zebra finches (*Taeniopygia guttata*) (26). Zebra finches show large sex differences in song behavior and song circuit neuroanatomy, and they exhibit high rates of brain steroid metabolism (27). Much is known about steroidogenesis in zebra finches, but it is still unclear whether brain 3 $\beta$ -HSD is expressed in adults or in tissue that has not been cultured. Therefore, we examined 3 $\beta$ -HSD activity in the brains of adult male and female zebra finches.

In addition, we examined the effects of acute restraint stress on DHEA metabolism by 3 $\beta$ -HSD. DHEA is involved in physiological responses to stress and can counteract some of the effects of stress and glucocorticoids on the brain (4, 10, 28, 29). Here, we tested the hypothesis that the neural metabolism of DHEA is regulated by stress.

## Materials and Methods

### Subjects

Protocols were approved by the University of California Chancellor's Committee on Animal Care and Use and followed the National Institutes of Health Principles of Animal Care. Subjects were adult zebra finches (90+ d old), based on morphology, skull ossification, and gonadal maturation. Animals were maintained in mixed-sex breeding aviaries. There was a 12-h light, 12-h dark light cycle, and food and water were available *ad libitum*.

### Tissue collection

Animals were captured and then killed by rapid decapitation. One group of animals was killed within 2–3 min of disturbance (*i.e.* entering the aviary room). This was the shortest amount of time in which it was possible to enter an aviary, catch an animal, bring it to the laboratory, and kill the subject. This first group is operationally defined as the "baseline," relative to the second group. In the second group, animals were restrained in a dark cloth bag for 10 min before they were killed. Such a restraint is a common paradigm for giving a standard stressor to songbirds in studies of corticosterone secretion (30, 31). This second group is operationally defined as "stressed." Subjects killed within 2–3 min of disturbance did experience some stress, although less than sub-

jects restrained for 10 min, and for this reason are labeled "baseline" rather than "unstressed." Many studies have shown that plasma corticosterone levels do not increase in songbirds until after 3 min of restraint (30).

The brain was rapidly dissected, and tissues were immediately frozen on dry ice. We collected the following tissues: 1) midbrain and hindbrain; 2) cerebellum; 3) optic lobes; 4) diencephalon (hypothalamus and thalamus) and the preoptic area (POA); 5) rostral telencephalon, including nucleus X and magnocellular nucleus of the anterior nidopallium; 6) medial central telencephalon, including the septum and bed nucleus of the stria terminalis; 7) lateral central telencephalon; and 8) caudal telencephalon, including nucleus taeniae and the song nuclei HVC and robust nucleus of the arcopallium. In addition, in some subjects we collected the syrinx, the muscular vocal organ in songbirds.

The dissection protocol closely followed previous studies (32–34). Briefly, the cerebellum was collected first. Next, a cut was made on the ventral surface at the level of the mammillary bodies, and the midbrain/hindbrain was collected. The optic lobes were dissected by cuts along the lateral margins of the hypothalamus. The POA-diencephalon was then removed to the depth of the anterior commissure. To isolate the rostral telencephalon, an incision was made at the anterior border of where the POA had been. To separate the caudal and central telencephalon, an incision was made at the anterior border of where the cerebellum had been. Finally, the central telencephalon was bisected into medial and lateral portions. Tissues were stored at  $-80$  C.

### Measurement of DHEA metabolism by 3 $\beta$ -HSD

To examine 3 $\beta$ -HSD activity, we measured the *in vitro* conversion of tritiated DHEA by brain homogenates. Tissue from different subjects was pooled only during validation studies. In some experiments, we included a cold trap of radioinert AE in the incubation medium to prevent metabolism of formed tritiated AE by aromatase, 5 $\alpha$ -reductase, and 5 $\beta$ -reductase (26). In other experiments, we did not include an AE cold trap, to examine the formation of estrogens, 5 $\alpha$ - and 5 $\beta$ -reduced androgens from DHEA. 5 $\alpha$ -Reductase produces active androgens, whereas 5 $\beta$ -reductase produces behaviorally inactive androgens (35). Validation studies (see *Validations of 3 $\beta$ -HSD assay*) showed that the two methods (with and without AE cold trap) produced data that were highly correlated, and both methods yielded similar patterns when examining sex differences.

*With AE cold trap.* Tissues were homogenized in 200  $\mu$ l of ice-cold sucrose-phosphate buffer (pH 7.4) with glass-Teflon homogenizers (10 strokes). Homogenates (180  $\mu$ l) were incubated with [1,2,6,7-<sup>3</sup>H]DHEA (specific activity = 74 Ci/mmol; NEN Life Science Products). [<sup>3</sup>H]DHEA was purified by thin-layer chromatography before use. The [<sup>3</sup>H]DHEA concentration was 200 nM, similar to previous studies (26). This substrate concentration was sub-saturating, but approximately 80% of [<sup>3</sup>H]DHEA remained at the end of incubations (our unpublished results). In two initial validation studies (see *Validations of 3 $\beta$ -HSD assay*), lower substrate concentrations were used. Radioinert AE (25  $\mu$ M; Steraloids, Newport, RI) was added to protect formed [<sup>3</sup>H]AE from further metabolism. Incubations also included 1.1 mM nicotinamide adenine dinucleotide (NAD) (20  $\mu$ l), a cofactor for 3 $\beta$ -HSD. Control tubes contained everything but tissue. Incubations were carried out at 41 C with shaking for 180 min. Reactions were terminated by snap-freezing in methanol/dry ice. To determine procedural losses, a tube containing a known amount of [<sup>3</sup>H]AE was processed in parallel.

Steroids were extracted with diethyl ether (three times) and then separated by thin layer chromatography. Thin-layer silica gel plates were run in a mixture of chloroform: ethyl acetate (4:1) for 18 min (two times). Steroids were visualized under UV light after spraying with primulin. The appropriate bands were scraped from the plates, tritiated steroids were eluted from the silica with methanol, and aliquots were counted in a scintillation counter. The counts per minute were adjusted for background values and procedural losses, and data are reported as femtomoles per milligram of protein. Protein content of the homogenates was measured by the Bradford method using BSA standards.

*Without AE cold trap.* In some experiments, we did not include a cold trap of radioinert AE, which permitted metabolism of formed tritiated AE. AE is converted to 5 $\beta$ -androstenedione (5 $\beta$ -A), 5 $\alpha$ -A, and estrone (E<sub>1</sub>) by the actions of 5 $\beta$ -reductase, 5 $\alpha$ -reductase, and aromatase, respectively.

AE is also converted to T by 17 $\beta$ -HSD, but T was difficult to measure in this system because of high background values.

In the absence of an AE cold trap, the procedures were similar to those described above, with the following modifications. First, exogenous NAD was not included in the incubation, because preliminary results suggested that exogenous NAD interferes with aromatase activity. We assume that the source of the cofactor in these experiments is endogenous NAD. Second, to determine procedural losses of androgens and estrogens, tubes containing known amounts of [<sup>3</sup>H]AE and [<sup>3</sup>H]E<sub>1</sub> were processed in parallel. Third, after the ether extraction, androgens and estrogens were separated by phenolic partition (two times). Fourth, androgens were chromatographed as described above, and estrogens were chromatographed in ether:hexane (3:1) for 23 min (two times). Estrogens on TLC plates were visualized by exposure to iodine vapors.

### Validations of 3 $\beta$ -HSD assay

We performed several types of validation studies. First, we confirmed that AE, 5 $\beta$ -A, 5 $\alpha$ -A, and E<sub>1</sub> do not comigrate with other metabolites of DHEA, such as 7-hydroxy-DHEA, androstenediol, and androstenediol. Second, a timecourse study determined an appropriate duration for incubations. Third, we determined whether specific pharmacological inhibitors of 3 $\beta$ -HSD and aromatase (trilostane and fadrozole, gifts of Micron Technologies and Novartis Pharma, respectively) reduced [<sup>3</sup>H]AE and [<sup>3</sup>H]estrogen production (36, 37). Fourth, we determined whether a cold trap of radioinert AE decreases the production of metabolites of [<sup>3</sup>H]AE, such as [<sup>3</sup>H]5 $\beta$ -A. Fifth, 3 $\beta$ -HSD activity was directly compared in samples measured with and without a cold trap of radioinert AE to assess whether the results were positively correlated. Sixth, [<sup>3</sup>H]AE and its metabolites were recrystallized to constant specific activity. Radioinert steroid (20 mg) was added to tritiated product (2000 cpm), and recrystallization (three times) was performed in methanol and distilled water, as described in detail previously (26, 38). After the third recrystallization, we compared the specific activity (cpm/mg) of the final crystals with the specific activity of the mother liquor. We also determined whether the specific activity of the final crystals was similar to the initial specific activity (percentage of recovery).

### Regional differences

Subjects were adult male zebra finches ( $n = 5$ ) that were killed within 3 min of disturbance. The brain was dissected as described above.

In addition, the rostral telencephalon, medial central telencephalon, and caudal telencephalon were bisected along the midline. The halves were included in two separate assays, one with and one without a cold trap of radioinert AE. This allowed us to also examine regional differences in [<sup>3</sup>H]DHEA metabolism to [<sup>3</sup>H]5 $\beta$ -A, [<sup>3</sup>H]5 $\alpha$ -A, and [<sup>3</sup>H]E<sub>1</sub>.

### Effects of sex and stress

First, we examined adult males ( $n = 6$ ) and females ( $n = 6$ ) that were collected within 3 min of disturbance (baseline). This experiment included the telencephalic regions, diencephalon, midbrain/hindbrain, and optic lobes.

Second, we examined adult males ( $n = 5$ ) and females ( $n = 5$ ) that were restrained for 10 min before they were killed (stressed). This experiment focused on the telencephalon (rostral, medial central, and caudal regions), which showed some sex differences in the first experiment (see preceding paragraph) and contains several sexually dimorphic song control nuclei.

The first and second experiments showed different patterns. To resolve this discrepancy and eliminate the possibility of interassay variation, we conducted another experiment. In the third experiment, we examined baseline and stressed males and females in the same assay ( $n = 6$  per group, 24 subjects total). This experiment focused on the central telencephalon, which showed sex differences in the other experiments.

### Hormone measurements

Baseline and stressed levels of plasma DHEA and corticosterone were measured in adult male and female zebra finches ( $n = 24$  subjects total) using techniques validated for birds (21, 39). Trunk blood was collected at the time they were killed into heparinized microhematocrit tubes and

centrifuged. Plasma was collected and stored at  $-20$  C. DHEA and corticosterone were measured in separate assays. For each assay, steroids were extracted with methylene chloride, and then steroids were measured in duplicate by RIA with specific antibodies (Endocrine Sciences, Calabasas, CA). Water blanks were included in each assay. These procedures have been described in detail (21, 39).

### Statistics

Data are shown as mean  $\pm$  SE of the mean. Data were analyzed using Systat for Windows. Data were log-transformed where appropriate before statistical analyses, as indicated in *Results*. In studies of regional differences, repeated measures ANOVA was used when comparing multiple brain regions taken from an individual subject. For two-way ANOVA analyses (sex  $\times$  stress), *post hoc* tests (Fisher's protected least significant difference test) were conducted only if the interaction was significant. All tests are two tailed, and  $\alpha$  was set at 0.05.

## Results

### Assay validations

**Timecourse.** A timecourse study was performed to determine an appropriate duration for incubations. For this experiment, we used 100 nM [<sup>3</sup>H]DHEA, based on previous studies (26, 38, 40). In samples without an AE cold trap, we detected the formation of [<sup>3</sup>H]AE, [<sup>3</sup>H]5 $\beta$ -A, and [<sup>3</sup>H]E<sub>1</sub> (Fig. 2). [<sup>3</sup>H]E<sub>2</sub> was detected only at 180 min, and levels were very low. [<sup>3</sup>H]5 $\alpha$ -A was not measured in this experiment but was produced in other studies (see below). In samples with an AE cold trap, very little [<sup>3</sup>H]5 $\beta$ -A was formed, and no [<sup>3</sup>H]estrogens were formed.

Total product formation was linear between 30 and 180 min, and products were sufficiently abundant to be reliably detected above background at 180 min (Fig. 2). In a second timecourse study, we determined that AE formation was linear between 180 and 240 min as well. Therefore, we chose an incubation duration of 180 min.

**Pharmacological inhibitors.** We determined whether trilostane, a competitive 3 $\beta$ -HSD inhibitor, decreased the formation of [<sup>3</sup>H]AE. In this assay, we used 75 nM [<sup>3</sup>H]DHEA and 1.5  $\mu$ M trilostane and included an AE cold trap. We used a lower substrate concentration because this facilitated detection of the effects of trilostane. Trilostane concentration (20 $\times$  substrate concentration) was based on previous studies (40–42). Trilostane abolished [<sup>3</sup>H]AE production in the telencephalon (Fig. 3), as well as in the POA-diencephalon, cerebellum, and syrinx.

We also determined whether fadrozole, an aromatase inhibitor, decreased [<sup>3</sup>H]estrogen formation by brain tissue. For this assay, we used 200 nM [<sup>3</sup>H]DHEA and 200 nM fadrozole (36) and did not include an AE cold trap. Fadrozole decreased [<sup>3</sup>H]E<sub>1</sub> production by 97% (Fig. 3). There was no effect on [<sup>3</sup>H]AE formed, indicating that fadrozole does not inhibit 3 $\beta$ -HSD.

**Effect of AE cold trap.** The inclusion of a cold trap of radioinert AE should protect formed [<sup>3</sup>H]AE from further metabolism (26). In this experiment, we examined the effects of an AE cold trap (25  $\mu$ M). The AE cold trap increased the amount of [<sup>3</sup>H]AE and decreased the amount of [<sup>3</sup>H]5 $\beta$ -A (Fig. 4). [<sup>3</sup>H]5 $\alpha$ -A and [<sup>3</sup>H]E<sub>1</sub> levels were very low, even in samples without an AE cold trap, perhaps because samples had low amounts of protein in this assay. Also, the AE cold trap

FIG. 2. Timecourse of DHEA metabolism by  $3\beta$ -HSD in adult zebra finch brain. DHEA was metabolized to AE, and formed AE was subsequently metabolized to  $5\beta$ -A and  $E_1$  by  $5\beta$ -reductase and aromatase, respectively.

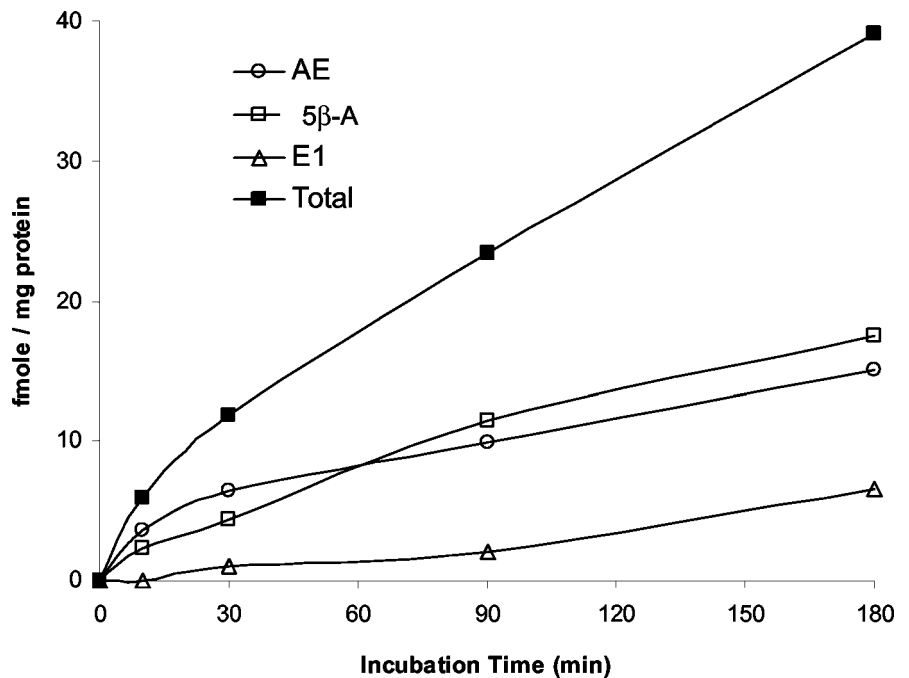
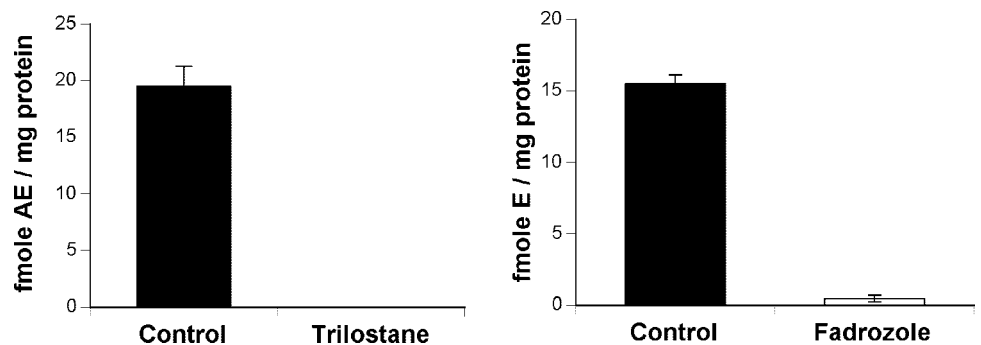


FIG. 3. Assay validation using specific pharmacological inhibitors of steroidogenic enzymes with telencephalic tissue. Trilostane is an inhibitor of  $3\beta$ -HSD, and fadrozole is an inhibitor of aromatase. Relative to controls (vehicle), trilostane abolished AE production ( $n = 4$  replicates per group). Relative to controls (vehicle), fadrozole decreased estrogen production by 97% ( $n = 5$  replicates per group).



reduced the overall activity of  $3\beta$ -HSD measured (femtomoles AE +  $5\beta$ -A), which is consistent with end-product inhibition (42–44).

In a separate experiment, we determined whether  $3\beta$ -HSD activity measurements using the two methods (with and without an AE cold trap) were positively correlated. In this experiment, we measured  $3\beta$ -HSD activity in telencephalic homogenates ( $n = 25$  animals). Homogenates were split and assayed in parallel using both methods, which allowed for a direct comparison. As above, the AE cold trap reduced  $3\beta$ -HSD activity, but  $3\beta$ -HSD activities were highly correlated between the two assays (Pearson correlation,  $r = 0.85$ ,  $P < 0.0001$ ).

**Product recrystallization.** Tritiated products were recrystallized (three times) to constant specific activity (Table 1). Recrystallizations confirmed the identity of metabolites.

#### Regional differences

In adult males ( $n = 5$ ), we examined regional differences in  $3\beta$ -HSD activity. In the first assay, we included an AE cold trap (Table 2). We detected a significant effect of region (repeated measures ANOVA:  $F_{6,24} = 3.13$ ,  $P = 0.021$ ). *Post hoc*

analyses with paired *t* tests revealed that the POA-diencephalon was significantly different from the cerebellum, optic lobes, midbrain/hindbrain, and medial central telencephalon ( $P < 0.05$ ). The midbrain/hindbrain was significantly different from the optic lobes and caudal telencephalon ( $P < 0.05$ ).

In the second assay, we focused on the telencephalic regions and did not include an AE cold trap, permitting us to analyze the androgenic and estrogenic metabolites of DHEA (Table 3). Regional differences were examined using repeated measures ANOVA, and if the overall ANOVA was significant, then *post hoc* tests were conducted using paired *t* tests. As above,  $3\beta$ -HSD activity did not differ among the telencephalic regions examined (Table 3).  $5\beta$ -A,  $5\alpha$ -A, and  $E_1$  were expressed as a percent of total  $3\beta$ -HSD metabolites, and these percentages are indices of  $5\beta$ -reductase,  $5\alpha$ -reductase, and aromatase, respectively. The percentage of  $5\beta$ -A was significantly lower in the caudal telencephalon (Table 3). The percentage of  $5\alpha$ -A was significantly higher in the rostral telencephalon (Table 3). Note that relatively little  $5\alpha$ -A was formed, suggesting that this is a minor pathway for DHEA metabolism in the brain. The percentage of  $E_1$  was signifi-

cantly higher in the caudal telencephalon (Table 3). In the caudal telencephalon, approximately 50% of [ $^3\text{H}$ ]DHEA metabolized by  $3\beta\text{-HSD}$  ended up as [ $^3\text{H}$ ]E $_1$ , consistent with the high aromatase expression in this region (45).

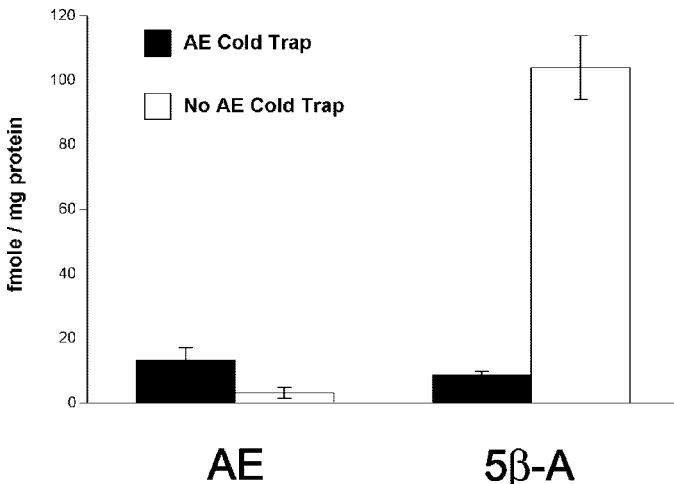


FIG. 4. Effect of a cold trap of radioinert AE on levels of [ $^3\text{H}$ ]AE and [ $^3\text{H}$ ]5 $\beta$ -A. The cold trap increased the amount of [ $^3\text{H}$ ]AE and decreased [ $^3\text{H}$ ]5 $\beta$ -A, indicating that the cold trap reduced metabolism of formed [ $^3\text{H}$ ]AE ( $n = 4$  replicates per group).

TABLE 1. Validation of the identities of DHEA metabolites by recrystallization to constant specific activity

Metabolite	Subject	Tissue	S.A. of crystals	S.A. of mother liquor	Error	% Recovery
AE	Male	Testis	155.4	145.4	0.03	100
AE	Female	Rostral telencephalon	166.7	171.5	0.01	100
AE	Male	Caudal telencephalon	143.6	143.2	0.002	96
E $_1$	Male	Telencephalon	63.7	70.6	0.05	73
5 $\beta$ -A	Female	Telencephalon	162.7	168.7	0.02	100
5 $\alpha$ -A	Male	Telencephalon	103.5	104.0	0.002	89

Note: Specific activity (S.A.) is expressed in cpm/mg.

TABLE 2. Regional differences in DHEA metabolism (mean  $\pm$  SEM) in adult male zebra finches ( $n = 5$ )

	fmol AE/mg protein	fmol 5 $\beta$ -A/mg protein	3 $\beta$ -HSD activity (fmol AE + 5 $\beta$ -A/mg protein)
POA-diencephalon	102.58 $\pm$ 18.73	0 $\pm$ 0	102.58 $\pm$ 18.73
Rostral telencephalon	96.42 $\pm$ 27.27	2.73 $\pm$ 1.92	99.16 $\pm$ 29.13
Medial central telencephalon	81.51 $\pm$ 18.4	2.54 $\pm$ 1.15	84.05 $\pm$ 19.35
Optic lobes	78.58 $\pm$ 16.99	0 $\pm$ 0	78.58 $\pm$ 16.99
Caudal telencephalon	65.99 $\pm$ 7.47	1.49 $\pm$ 0.86	67.48 $\pm$ 8.25
Cerebellum	57.23 $\pm$ 9.75	0.84 $\pm$ 0.29	58.07 $\pm$ 9.78
Midbrain/hindbrain	54.11 $\pm$ 8.14	0 $\pm$ 0	54.11 $\pm$ 8.14

Note: This assay included a cold trap of radioinert AE to reduce subsequent metabolism of formed tritiated AE. Therefore, no 5 $\alpha$ -A or estrogens were formed, and little 5 $\beta$ -A was formed. See *Results* for details of statistical tests.

TABLE 3. Regional differences in DHEA metabolism to AE and AE metabolites (mean  $\pm$  SEM) within the telencephalon of adult male zebra finches ( $n = 5$ )

	Total metabolites (fmol AE + 5 $\beta$ -A + 5 $\alpha$ -A + E $_1$ /mg protein)	5 $\beta$ -A/Total (%)	5 $\alpha$ -A/Total (%)	E $_1$ /Total (%)
Rostral telencephalon	643.39 $\pm$ 204.68	44.5 $\pm$ 5.9 <sup>a</sup>	2.9 $\pm$ 0.3 <sup>a</sup>	20.7 $\pm$ 3.8 <sup>a</sup>
Medial central telencephalon	564.66 $\pm$ 208.27	48.1 $\pm$ 6.9 <sup>a</sup>	1.8 $\pm$ 0.3 <sup>b</sup>	26.8 $\pm$ 5.4 <sup>a</sup>
Caudal telencephalon	415.30 $\pm$ 68.16	24.0 $\pm$ 5.9 <sup>b</sup>	1.2 $\pm$ 0.2 <sup>b</sup>	53.5 $\pm$ 5.5 <sup>b</sup>
F $_{2,8}$ ( $P$ )	1.78 (0.23)	17.25 (<0.01)	16.03 (<0.01)	40.09 (<0.01)

Note: This assay did not include a cold trap of radioinert AE, and formed tritiated AE was metabolized to 5 $\beta$ -A, 5 $\alpha$ -A, and E $_1$ . F and P values from repeated measures ANOVA. Within a column, letters denote regions that are significantly different.

### Sex differences and effect of acute stress

**Baseline animals.** We measured  $3\beta\text{-HSD}$  activity in adult males ( $n = 6$ ) and females ( $n = 6$ ) that were killed within 2–3 min of disturbance. Multiple brain regions were examined in four separate assays, under identical conditions. All assays included an AE cold trap to protect formed AE from further metabolism. In the medial central telencephalon,  $3\beta\text{-HSD}$  activity was significantly higher in females (Fig. 5A;  $P = 0.020$ ). Similarly, in the lateral central telencephalon and POA-diencephalon,  $3\beta\text{-HSD}$  activity was also significantly higher in females (Table 4). In the rostral telencephalon, there was a trend for higher activity in females, but this was not significant (Table 4;  $P = 0.092$ ). In the optic lobes, a region not known to be sexually dimorphic, there was no sex difference in  $3\beta\text{-HSD}$  ( $P = 0.998$ ). Overall, in baseline animals,  $3\beta\text{-HSD}$  activity in several forebrain regions was higher in females than males.

**Stressed animals.** Next, we examined  $3\beta\text{-HSD}$  activity in adult males ( $n = 5$ ) and females ( $n = 5$ ) that were stressed (restrained for 10 min before they were killed). In this experiment, we focused on the telencephalon, which showed sex differences in baseline animals (see above) and contains sexually dimorphic song control nuclei. First, the medial central telencephalon was examined in an assay with an AE cold trap. In contrast to the first experiment, males had signifi-

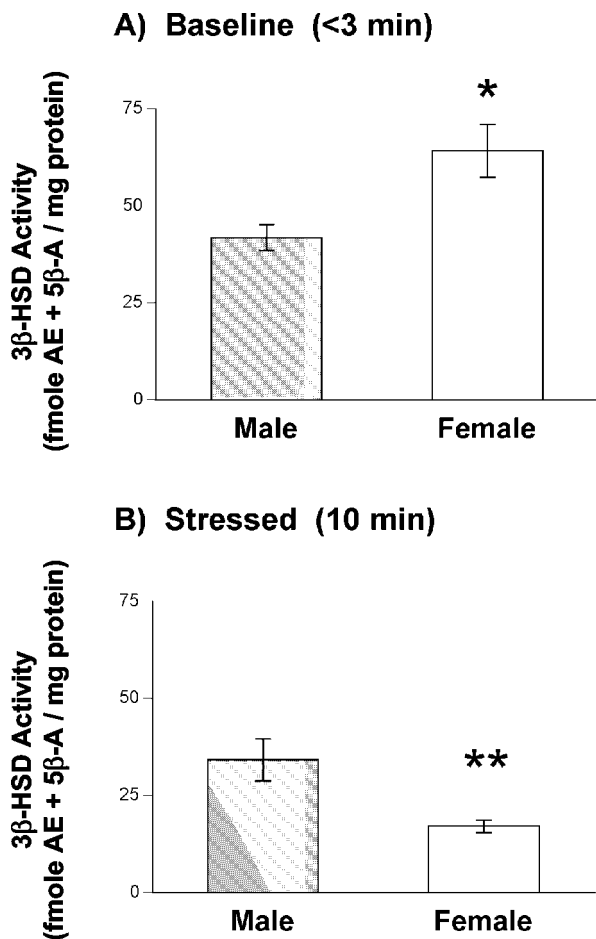


FIG. 5. DHEA metabolism by  $3\beta$ -HSD in the telencephalon of baseline and stressed animals in separate assays. A, In baseline animals (killed within 2–3 min of disturbance),  $3\beta$ -HSD activity in the medial central telencephalon was significantly higher in females ( $P = 0.020$ ).  $n = 6$  subjects per sex. B, In stressed animals (10 min restraint),  $3\beta$ -HSD activity in the medial central telencephalon was significantly higher in males ( $P = 0.003$ ).  $n = 5$  subjects per sex.

cantly higher  $3\beta$ -HSD activity in the medial central telencephalon than females (Fig. 5B; log-transformed data,  $t = 3.89$ ,  $P = 0.003$ ). Next, we examined the rostral telencephalon and caudal telencephalon, and this assay did not include an AE cold trap. The lack of an AE cold trap permitted us to examine the metabolism of DHEA to estrogens, especially in the caudal telencephalon where aromatase is high. Total  $3\beta$ -HSD activity in the rostral and caudal telencephalon was significantly higher in males than females (Table 5). Of the AE metabolites, only  $5\alpha$ -A production showed a slight sex difference in the rostral telencephalon (Table 5). Thus, in acutely stressed animals, males had higher  $3\beta$ -HSD activity in the forebrain than females.

**Baseline and stressed animals.** To resolve the different patterns seen above and to eliminate the possibility of interassay variation, we conducted a third experiment. We examined baseline and stressed males and females in the same assay ( $n = 6$  per group). We focused on the central telencephalon (medial and lateral portions combined), which showed sex differences in the previous experiments (see above). The

assay included an AE cold trap. Log-transformed data were analyzed by two-way ANOVA. In the central telencephalon (Fig. 6), there was no significant effect of sex ( $F_{1,20} = 0.001$ ,  $P = 0.973$ ), a significant effect of stress ( $F_{1,20} = 6.046$ ,  $P = 0.023$ ), and a significant interaction between sex and stress ( $F_{1,20} = 11.259$ ,  $P = 0.003$ ). *Post hoc* analyses (Fisher's protected least significant difference test) revealed a sex difference in baseline animals (female > male;  $P = 0.026$ ) and in stressed animals (male > female;  $P = 0.029$ ). *Post hoc* tests revealed that stress did not affect  $3\beta$ -HSD activity in males ( $P = 0.533$ ) but significantly decreased activity in females ( $P < 0.001$ ).

#### Plasma hormone levels

Plasma corticosterone levels (Fig. 7) were significantly increased in response to stress ( $F_{1,20} = 37.27$ ,  $P = 0.0001$ ) in males and females. There was no effect of sex ( $F_{1,20} = 0.35$ ,  $P = 0.56$ ) or the interaction between sex and stress ( $F_{1,20} = 0.03$ ,  $P = 0.87$ ) on circulating levels of corticosterone. Plasma DHEA levels (Fig. 7) were unaffected by sex ( $F_{1,20} = 0.14$ ,  $P = 0.72$ ), stress ( $F_{1,20} = 1.02$ ,  $P = 0.32$ ), or the interaction between these factors ( $F_{1,20} = 0.03$ ,  $P = 0.87$ ).

#### Discussion

The present data indicate that there are regional and sex differences in DHEA metabolism by  $3\beta$ -HSD in the adult brain. Moreover, sex differences in  $3\beta$ -HSD activity are sensitive to acute stress. To our knowledge, this is the first study to describe 1) brain region differences in DHEA metabolism by  $3\beta$ -HSD, 2) sex differences in  $3\beta$ -HSD in the brain, and 3) rapid modulation of  $3\beta$ -HSD activity in any tissue. Although numerous studies have examined  $3\beta$ -HSD in peripheral tissues (e.g. gonads, adrenals), far fewer studies have characterized  $3\beta$ -HSD in the brain. The present data are consistent with the hypothesis that some effects of DHEA on the brain are mediated by local neural conversion of DHEA to sex steroids. Neural DHEA metabolism may contribute to sex differences in neuroanatomy and behavior, particularly in response to stress.

#### The adult brain metabolizes DHEA to AE and estrogen

Studies of neural  $3\beta$ -HSD activity have generally used cell lines or primary cultures from developing animals (46–48), and there has been less work on uncultured tissue (44). Importantly, steroidogenic enzymes may be affected in unpredictable ways by the process of culturing cells. In this regard, data from tissue homogenates can complement cell culture experiments. Moreover, studies using tissue homogenates can more easily examine the adult/aging brain and the effects of stress. Such studies are of interest because of DHEA's effects in the elderly (2, 49) and antiglucocorticoid effects (28, 50).

Using tissue homogenates, we document the metabolism of DHEA by  $3\beta$ -HSD in the adult zebra finch brain. The activity of  $3\beta$ -HSD was validated in several ways. First, trilostane, a  $3\beta$ -HSD inhibitor, abolished AE production when added to the incubation medium. Trilostane, however, also inhibited brain  $5\beta$ -reductase (our unpublished results).

**TABLE 4.** Sex differences in baseline  $3\beta$ -HSD activity (fmol AE +  $5\beta$ -A/mg protein, mean  $\pm$  SEM) in adult zebra finches killed within 3 min of disturbance

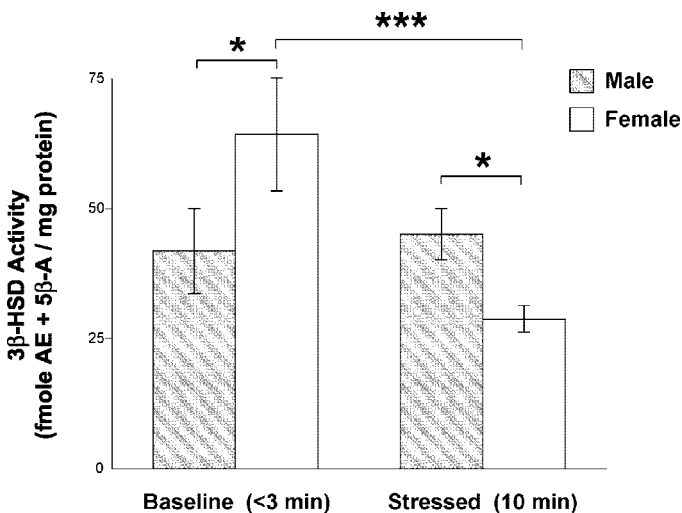
	Male (n = 6)	Female (n = 6)	P
Assay 1			
Rostral telencephalon	58.14 $\pm$ 6.74	81.43 $\pm$ 10.27	0.092
Caudal telencephalon	49.53 $\pm$ 7.38	59.37 $\pm$ 5.44	0.312
Assay 2			
Lateral central telencephalon	43.82 $\pm$ 3.00	59.29 $\pm$ 4.84	<b>0.025</b>
Assay 3			
POA-diencephalon	38.49 $\pm$ 5.23	58.44 $\pm$ 5.94	<b>0.036</b>
Assay 4			
Midbrain/hindbrain	37.03 $\pm$ 3.72	47.77 $\pm$ 5.02	0.119
Optic lobes	58.45 $\pm$ 7.34	58.47 $\pm$ 7.83	0.998

Note: These assays included a cold trap of radioinert AE to reduce metabolism of formed tritiated AE. *P* values from *t* tests (two tailed). Assay 2 also included the medial central telencephalon (see Fig. 5A). **Boldface** indicates  $P \leq 0.05$ .

**TABLE 5.** Sex differences in  $3\beta$ -HSD activity (mean  $\pm$  SEM) in stressed adult zebra finches (killed after a 10-min restraint stress)

	Male (n = 5)	Female (n = 5)	P
Rostral telencephalon			
Total metabolites (fmol AE + $5\beta$ -A + $5\alpha$ -A + E/mg protein)	823.50 $\pm$ 135.83	291.51 $\pm$ 54.69	<b>0.005</b>
$5\beta$ -A/Total (%)	60.8 $\pm$ 7.4	58.2 $\pm$ 4.3	0.755
$5\alpha$ -A/Total (%)	1.8 $\pm$ 0.4	0.3 $\pm$ 0.3	<b>0.020</b>
E/Total (%)	7.1 $\pm$ 2.5	8.8 $\pm$ 2.3	0.626
Caudal telencephalon			
Total metabolites (fmol AE + $5\beta$ -A + $5\alpha$ -A + E/mg protein)	507.99 $\pm$ 45.61	165.76 $\pm$ 17.11	<b>&lt;0.0001</b>
$5\beta$ -A/Total (%)	45.1 $\pm$ 16.2	51.5 $\pm$ 10.0	0.627
$5\alpha$ -A/Total (%)	0 $\pm$ 0	0 $\pm$ 0	
E/Total (%)	27.9 $\pm$ 6.9	25.1 $\pm$ 8.6	0.818

Note: This assay did not include a cold trap of radioinert AE. *P* values from *t* tests (two tailed). **Boldface** indicates  $P \leq 0.05$ .



**FIG. 6.** DHEA metabolism by  $3\beta$ -HSD in the central telencephalon of baseline and stressed animals in a single assay. Acute stress significantly decreased  $3\beta$ -HSD activity in females but not males (n = 6 subjects per group).

Second, fadrozole, an aromatase inhibitor, nearly abolished estrogen production without affecting AE production. Third, a cold trap of radioinert AE greatly reduced the production of  $5\beta$ -A, an AE metabolite. Fourth, AE and its metabolites were recrystallized to constant specific activity. Similar studies in another songbird, song sparrows, gave identical results (our unpublished results).

Studies of neural  $3\beta$ -HSD activity have largely focused on the conversion of pregnenolone to progesterone, and less is

known about the metabolism of DHEA to AE. In rat hippocampal and hypothalamic cultures, DHEA is metabolized to AE and estrogens (47, 48). Similar results have been obtained with zebra finch telencephalic cultures (26). In human fetal brain tissue, DHEA is also metabolized to AE (51). Such studies of DHEA metabolism have relevance for understanding the effects of DHEA on the brain. In some cases, the neurotrophic and neuroprotective effects of DHEA and estrogen are similar, perhaps because DHEA is converted to estrogen within the brain (11, 19, 52, 53). Although DHEA at pharmacological doses might bind directly to estrogen receptors, this mechanism is unlikely to be relevant under physiological conditions because DHEA has very low affinity for estrogen receptors (54). Future experimental work in songbirds will determine whether the behavioral and neural effects of DHEA can be blocked by an aromatase inhibitor or androgen receptor antagonist.

#### Regional differences

DHEA metabolism by  $3\beta$ -HSD was generally highest in the forebrain and lower in the midbrain and hindbrain. Similar results have been obtained in song sparrow brain (our unpublished results). The present study is the first to examine regional differences in DHEA metabolism by  $3\beta$ -HSD, although previous studies have investigated  $3\beta$ -HSD using pregnenolone as the substrate. For example, in adult male rats,  $3\beta$ -HSD activity is high in the amygdala and septum, intermediate in the hippocampus, low in the hypothalamus, and undetectable in the parietal cortex (55). In adult male Japanese quail (*Coturnix japonica*) and ring doves (*Streptopelia*

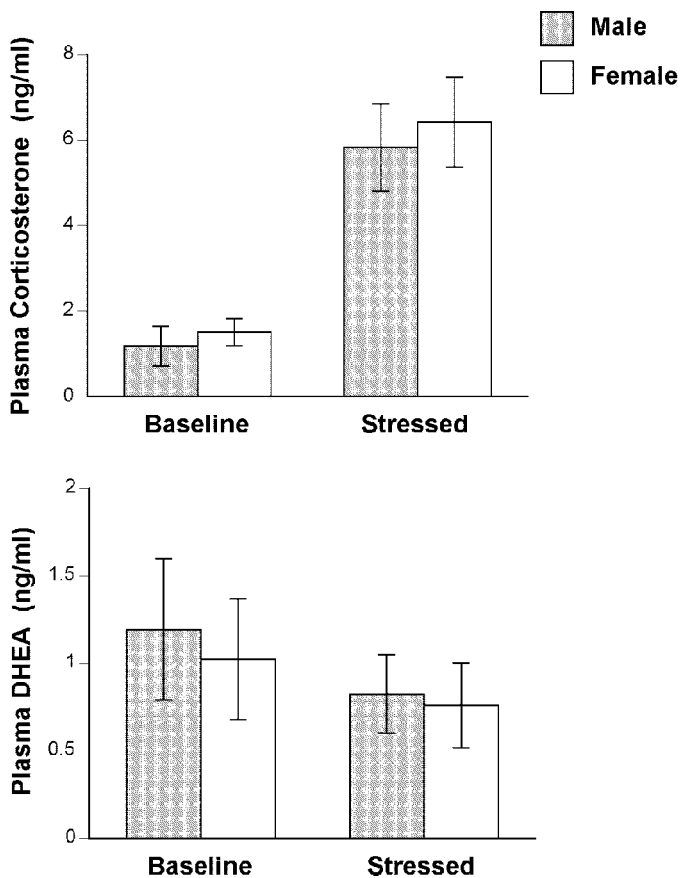


FIG. 7. Plasma corticosterone and DHEA levels in adult zebra finches. Acute stress increased plasma corticosterone levels similarly in males and females. Acute stress did not affect plasma DHEA concentrations.  $n = 6$  subjects per group.

*risoria*),  $3\beta$ -HSD activity is highest in the forebrain and lower in the midbrain and cerebellum, similar to the present results (56, 57).

The distributions of  $3\beta$ -HSD protein and mRNA have also been examined. In adult male rats,  $3\beta$ -HSD mRNA is expressed widely in the nervous system, including the cortex, hippocampus, hypothalamus, cerebellum, and spinal cord (58, 59). In quail, RT-PCR studies suggest that  $3\beta$ -HSD mRNA is highest in the cerebellum and lowest in the diencephalon (60), although enzyme activity is higher in the diencephalon than cerebellum (56). These data suggest that  $3\beta$ -HSD mRNA and activity show different regional patterns in quail. Results in adult zebra finches using *in situ* hybridization suggest that  $3\beta$ -HSD mRNA is high in the optic tectum, cerebellum, and hindbrain (61). Thus,  $3\beta$ -HSD mRNA and activity may be distributed differently in zebra finches as well. In a frog,  $3\beta$ -HSD immunoreactive cells are present in the hypothalamus, and immunoreactive fibers are visible in the diencephalon and telencephalon (62). In zebrafish (*Danio rerio*),  $3\beta$ -HSD immunoreactive cells are detected in the dorsal telencephalon, hypothalamus and cerebellum, and immunoreactive fibers are widely distributed (41). Thus, brain  $3\beta$ -HSD is seen in a variety of species and may be a general property of the vertebrate brain. The distribution of  $3\beta$ -HSD varies from species to species, suggest-

ing that brain  $3\beta$ -HSD serves different functions in fish, amphibians, birds, and mammals.

#### Sex differences and effect of stress

In baseline animals (killed within 2–3 min), females have higher  $3\beta$ -HSD activity than males in the POA-diencephalon and the medial and lateral portions of central telencephalon. These tissues contain steroid-sensitive regions known to be important in reproductive behavior, such as the POA, hypothalamus, septum, and bed nucleus of the stria terminalis.

In contrast, in stressed animals (killed after a 10-min restraint stress), males have higher levels of  $3\beta$ -HSD activity than females in the telencephalon. This sex difference is seen in all regions of the telencephalon examined and in assays with and without an AE cold trap. Interestingly, stress rapidly decreases telencephalic  $3\beta$ -HSD activity in females but not in males. In song sparrows, stress increases brain  $3\beta$ -HSD activity in males of this species (our unpublished results). Little is known about the factors that regulate  $3\beta$ -HSD activity in the nervous system (44, 63), although stress may affect  $3\beta$ -HSD activity via GABA or endozeptines (64, 65).

This is the first report of sex differences in  $3\beta$ -HSD in the brain. Previous studies in mice have reported sex differences in  $3\beta$ -HSD activity in the gonad and liver (66). Note that sex differences in brain  $3\beta$ -HSD activity could be missed if the tissue is not collected in a systematic manner that accounts for stress. In quail, there is no sex difference in brain  $3\beta$ -HSD mRNA using RT-PCR (56). It is possible that  $3\beta$ -HSD activity, but not mRNA, shows sex differences in the brain. In support of this hypothesis, the sex difference we observe in  $3\beta$ -HSD activity is rapidly affected by stress. Effects on this timescale are unlikely to be the result of changes in gene transcription.

In stressed animals, plasma corticosterone titers are similar in male and female zebra finches, but higher  $3\beta$ -HSD activity in the male telencephalon may contribute to sex differences in the effects of stress on the brain. For example, in rats, stress affects neurosteroid levels and learning differently in males and females (67, 68). Interestingly, stress facilitates classical conditioning in males but impairs conditioning in females (67). Decreased brain  $3\beta$ -HSD activity in stressed females might be a mechanism for making the female brain more sensitive to the effects of stress. There are reasons to suspect that DHEA metabolism is involved in the stress response. In rodents and humans, DHEA has several antiglucocorticoid actions. For example, DHEA ameliorates the damaging effects of corticosterone on the hippocampus (4, 28, 29). In addition, acute stress increases plasma DHEA in humans (69), although not in song sparrows (21) or zebra finches (present study). Songbirds may regulate DHEA action at the level of local metabolism rather than circulating hormone concentrations.

Stress has rapid effects on  $3\beta$ -HSD activity. Within 10 min, brain  $3\beta$ -HSD activity decreases significantly in females. The mechanisms underlying this change remain unclear. One possibility is posttranslational modification of the enzyme, such as phosphorylation. Recent evidence suggests that phosphorylation of brain aromatase rapidly decreases its activity (70, 71). A second possibility is changes in endogenous substrates or endogenous inhibitors of  $3\beta$ -HSD (42).

Future studies will address the rapid regulation of 3 $\beta$ -HSD. The rapid changes in 3 $\beta$ -HSD and aromatase activities in the brain suggest that these enzymes are important for the minute-by-minute control of brain steroid levels in response to environmental stimuli. Fast changes in adrenal and gonadal steroidogenesis have been attributed to the actions of steroidogenic acute regulatory protein (72), but in the brain there may be additional points of regulation.

### Conclusions

3 $\beta$ -HSD in the adult songbird brain can metabolize DHEA to AE, and AE can be subsequently converted to potent androgens and estrogens. In birds, DHEA might be synthesized by the gonads, adrenals, or perhaps the brain itself (21, 73). Thus, brain 3 $\beta$ -HSD can be viewed as part of a pathway to metabolize circulating DHEA from the periphery or as part of a neurosteroidogenic pathway. The present data are consistent with the hypothesis that some neural effects of DHEA are mediated by its conversion to sex steroids within the brain (11, 52). Not all neural effects of DHEA, however, are necessarily mediated via 3 $\beta$ -HSD (23). Given the diverse actions of DHEA on the nervous system and behavior in animal and human studies (2, 3, 22, 74), elucidating the underlying mechanisms of action is an important issue.

Also, future studies will determine whether there are sex differences in brain 3 $\beta$ -HSD during zebra finch development. If so, this could be an important mechanism for sexual differentiation of the brain in this species, which shows dramatic sexual dimorphism of song nuclei and song behavior. Studies of zebra finch brain slice cultures suggest that there may be sex differences in brain steroidogenic enzymes during development (75, 76). For these reasons, songbirds such as zebra finches provide excellent opportunities for studying the distribution, regulation, and functions of steroidogenic enzymes in the brain.

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