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Activities of 3 β -HSD and Aromatase in Slices of Developing and Adult Zebra Finch Brain

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Abstract

Sex steroids influence the development and function of the songbird brain. Developmentally, the neural circuitry underlying song undergoes masculine differentiation under the influence of estradiol. In adults, estradiol stimulates song behavior and the seasonal growth of song control circuits. There is good reason to believe that these neuroactive estrogens are synthesized in the brain. At all ages, estrogens could act at the lateral ventricle, during migration, or where song nuclei exist or will form. We investigated the activity of two critical steroidogenic enzymes, 3 β -hydroxysteroid dehydrogenase/isomerase (3 β HSD) and aromatase, using a slice culture system. Sagittal brain slices were collected from juvenile (posthatch day 20) and adult zebra finches containing either the lateral ventricle, where neurons are born, or the telencephalic song nuclei HVC and RA. The slices were incubated with ³H dehydroepiandrosterone or ³H-androstenedione. Activity was determined by isolating certain products of 3 β HSD (5 α -androstenedione, 5 β -androstenedione, estrone, and estradiol) and aromatase (estrone and estradiol). Activities of both 3 β HSD and aromatase were detected in all slices and were confirmed using specific enzyme inhibitors. We found no significant difference in activity between adult males and females in either region for either enzyme. Juvenile female slices containing the lateral ventricle, however, showed greater levels of 3 β HSD activity than did similar slices from age-matched males. Determination of the activity of these critical steroidogenic enzymes in slice culture has implications for the role of neurosteroids in brain development.

Keywords

Songbird; neurosteroid; neurosteroidogenesis; estrogen; DHEA

INTRODUCTION

Estrogens are implicated in the masculine development of neural structures controlling song and in the seasonal growth and activation of song circuits in adults (Schlinger, 1998; Schlinger and Brenowitz, 2002). There is considerable evidence suggesting that these estrogens are formed in the brain (Holloway and Clayton, 2001; Schlinger and Arnold, 1991; 1992a). Aromatase catalyzes the conversion of androgens into estrogens. This enzyme is expressed in large amounts in the zebra finch telencephalon according to activity studies of brain

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homogenates (Schlinger and Arnold, 1991; 1992a; Vockel et al., 1990), cellular subfractions (Schlinger and Arnold, 1992b), and dissociated primary cell cultures (Schlinger et al., 1994).

The source of the androgenic substrate for telencephalic aromatization in songbirds remains unknown although the gonads, the adrenals and the brain are all possible sites of androgen synthesis (Freking et al., 2000; Holloway and Clayton, 2001; London et al., 2003; Schlinger et al., 1999; Soma et al., 2002). Testosterone is the dominant androgen secreted from the avian gonads (Wingfield and Silverin, 2002), but there is mounting evidence that DHEA is a significant avian hormone with potent neural actions (Goodson et al., 2005). The most likely mechanism for DHEA action on the brain is via conversion into more potent sex steroids by the action of the enzyme 3β -hydroxysteroid dehydrogenase/isomerase (3β HSD). This enzyme is responsible for the oxidation and isomerization of the inactive Δ^5 - 3β -hydroxy steroids [including pregnenolone and dehydroepiandrosterone (DHEA)] into active Δ^4 -keto steroids [progesterone and androstenedione (AE), respectively] (Fig 1), and we have evidence for its expression in the adult songbird brain. 3β -HSD activity has been detected in primary cell cultures of the zebra finch telencephalon (Vanson et al., 1996) and in brain homogenates of the adult zebra finch (Soma et al., 2004).

The synthesis of estrogens *de novo* was reported in the developing zebra finch brain from slices containing song nuclei HVC and RA (Holloway and Clayton, 2001). Not only were estrogens formed, but masculine connectivity between nucleus HVC and RA was established in male slices indicating that appropriate neural growth occurs under these *in vitro* conditions. Consequently, this system offers several advantages for the examination of steroid synthesis and metabolism. As compared to dissociated cell cultures, homogenates, and cellular subfractions, these largely undisrupted slices more closely resemble the *in vivo* condition. For example, because cells are not disrupted, requisite cofactors must already be co-localized with enzymes to drive reactions. In addition, enzymes catalyzing steroid-metabolism must be spatially co-localized for sequential reactions to occur. For these reasons, the slice culture system was used in this study to measure the activity of 3β -HSD and aromatase in both adult and juvenile (P20) zebra finch brains. Slices were prepared from two regions we speculate to be greatly impacted by steroids for the development of the avian neural song system: 1) a region housing song nuclei HVC and RA, and 2) a region containing the lateral ventricle, where new neurons are born throughout the life of the songbird (Alvarez-Buylla, 1990; Dewulf and Bottjer, 2005; Goldman and Nottebohm, 1983) and where the presence of steroidogenic enzymes have been detected (London et al., 2003).

METHODS

Animals

All zebra finches were bred in a colony under the care of the UCLA Life Sciences vivarium. Birds were maintained in a 12-h light/dark cycle with food and water available *ad libitum*. Protocols were approved by the UC Chancellor's Committee on Animal Care and Use and follow the National Institutes of Health Principles of Animal Care. Each breeding aviary housed 16-20 birds maintained at a 1:1 male to female ration. Nest boxes in each aviary were monitored daily to determine date of hatch (post hatch day 1 = P1). Juvenile birds were taken from their nests at P20. Adult birds (P > 90) were taken from non-breeding aviaries that housed approximately 30 individuals of the same sex.

Mapping

The distances of RA and HVC from the midline were mapped using 50 μ m sagittally sectioned adult and P20 thionin stained slices. The resulting maps were used in subsequent experiments

to mark the region containing RA and HVC using calipers and dye (1mL glycerol, 1mL ddH₂O 50:50, plus xylene cyanol).

Slice Culture

Tissue was collected immediately upon decapitation. Brains were removed and placed into aerated (99% O₂, 30min) ice cold artificial cerebral spinal fluid (aCSF) [199mM NaCl, 26.2mM NaHCO₃, 2.5mM KCl, 1mM NaH₂PO₄, 1.3mM MgSO₄, 2.5mM CaCl₂, 11mM glucose] (Holloway and Clayton, 2001). The brains were then split along the midline and the lateral ends of each hemisphere were cleaved, leaving 4 mm of sliceable tissue. A NVSL vibroslice (World Precision Instruments, FL) was used to collect the tissue slices. Brains were mounted on the flat lateral edges using Instant Super Glue™ (ND Industries). Each slice was placed into individual wells in a sterile 24-well flat-bottom polystyrene plates (Corning Inc, NY) containing cold, bubbled aCSF until ready for incubation with steroid precursors. The first, medial-most slice contained the lateral ventricle and was 500 µm thick. All slices thereafter, including those that housed the song nuclei RA and HVC, were 300 µm in thickness (see Fig 2). Each hemisphere contributed 1 slice containing the lateral ventricle and 3 slices containing song nuclei RA and HVC. Results represent steroid production from the single lateral ventricle slice and the average steroid production across the 3 song system slices. The time between decapitation and incubation with steroid precursors did not exceed 1 hr.

Measurement of Steroid Precursor metabolism

Assays were all conducted using age-matched animals of the opposite sex, though not always in a 1:1 ratio. Each animal per assay contributed a single lateral ventricle slice and three song system slices from the same hemisphere. All slices per assay were incubated in individual wells within the same 24-well polystyrene plate. Either tritiated sex steroid precursors [1,2,6,7-³H] androst-4-ene-3,17-dione (³H-AE) or [1,2,6,7-³H] dehydroepiandrosterone (³H-DHEA) (NEN Life Science Products) were used to measure 3β-HSD and aromatase activities, respectively. Both the incubating media as well as the tissue slices themselves were analyzed for steroid products throughout the study. Individual slices were incubated with 200nM of either ³H-AE or ³H-DHEA (Soma et al., 2004; Vanson et al., 1996) in 500µL warmed 1:1 DMEM/F-12 (Gibco) for 30 min (aromatase assay) or 3 hrs (3β-HSD assay), respectively (see Figs 3 & 4). The media was collected following incubation. A cold media wash (500µL) of the tissue in the well, used to remove any residual radioactive precursor from the tissue, was combined with the media samples. Tissue slices were homogenized using the Pellet Pestle® (Kontes, NJ) in 500µL sucrose-phosphate buffer (pH 7.4). A cold sucrose phosphate buffer wash (500µL), used to remove any residual radioactivity, was combined with the homogenized tissue samples. ³H androgen and estrogen products were isolated from the incubating media and tissue slices separately. Results reported represent the combined products of these separate isolations.

Biochemical and chromatographic isolation of steroid products were carried out as previously described (Soma et al., 2004; Vanson et al., 1996). Briefly, 5 mL ethyl ether was added to the samples, vortexed for 30 sec, and centrifuged at 2100 RPM, 4°C for 5 min. The aqueous portion was flash frozen in methanol-dry ice. The ether layer was poured into a separate tube and dried down. This procedure was repeated twice more before adding 1mL of CCl₄ and NaOH (1N) to the non-aqueous extract for the phenolic partition of androgens and estrogens. Seven hundred µL of the estrogen containing NaOH top layer was collected after the steroid samples were vortexed and centrifuged as above. An equal volume of CCl₄ was added to the extraction. Four hundred µL of the NaOH layer was extracted after vortexing and centrifuging as per above. An equal volume of ddH₂O and 2 mL ethyl acetate was added to the NaOH. Samples are vortexed for 20 sec and the NaOH is transferred to a new tube with a Pasteur pipette. This procedure is repeated twice more to ensure the complete transfer of estrogens from NaOH to ethyl acetate. The ethyl acetate tubes corresponding to the same sample were combined and

dried down. One mL ddH₂O was added to the tubes containing the remaining androgens and centrifuged as per the settings above. One and a half mL of the CCl₄ containing androgen layer was extracted and dried down. Both estrogens and androgens were stored in 1:1 MeOH:CH₂Cl₂. All procedures up to this point were conducted at 4°C. Resulting samples were spotted onto thin layer chromatography plates for separation. Cold steroids of interest were added to the samples to enhance ultraviolet visualization. Estrogens were run twice for 23 minutes in 3:1 ether:hexane while androgens were run twice for 28 minutes in 3:1 chloroform:ethyl acetate tanks.

Estrone (E1) and estradiol (E2) were collected from samples incubated with ³H-AE or ³H-DHEA. Since 5 α - and 5 β -reduced steroid products are major metabolites of AE, the androgens 5 α -androstenedione (5 α A) and 5 β -androstenedione (5 β A) were also collected from samples incubated with ³H-DHEA to more accurately quantify 3 β -HSD activity in the slice. Measurements of testosterone and AE production from DHEA were not included in this study due to difficulties in obtaining pure isolations. Our previous studies have shown that 5 β A and 5 α A separate cleanly from other potential steroid products using the described chromatographic procedures (Soma et al., 2004; Vanson et al., 1996). Primulin was used to visualize 5 α A and 5 β A under long wave (365nm) ultraviolet light illumination. E1 and E2 were placed for 5 min in tanks equilibrated with iodine for visualization. The appropriate bands were scraped from the plates and 400 μ L ddH₂O was added to the silica. Estrogen products were eluted from the silica twice with 2mL methanol whereas androgen products were eluted once. The amount of radioactivity within each individual sample (50% estrogens, 25% androgens) was counted in 5mL ScintiSafe™ 30% scintillation fluid for 2 minutes per sample. We have previously confirmed the specificity of these assays in songbirds using primary brain cell cultures (Soma et al., 2004; Vanson et al., 1996) and in adult brain homogenates (Soma et al., 2004; Vanson et al., 1996). To confirm reaction specificity in slices, several experiments were conducted including adding an equal concentration of fadrozole (aromatase inhibitor) and a 20x concentration of trilostane (3 β -HSD inhibitor) with the ³H-AE or ³H-DHEA substrate.

Statistical Analysis

Steroid production in age-matched animals was compared across sex and area (lateral ventricle vs. song system). Efforts were made in each experiment to incubate lateral ventricle and song system slices from at least one male and one female. Juvenile zebra finches cannot be sexed until they are sacrificed, so in some experiments more than 2 animals were sacrificed to produce at least one male and one female. We chose to incubate brain slices from all the animals creating an unequal sex ratio in these assays. Additionally, as described previously, the slices from each region were of different thickness and number. Finally, the data were generally not normally distributed. Therefore 2-tailed independent sample nonparametric Mann-Whitney U tests (SPSS v13.0) were used to evaluate steroid production between male and female slices as well as between lateral ventricle and song system slices (statistical significance set to $p < 0.05$). In addition, log transformed data was analyzed at each age by 2-way ANOVA followed by post-hoc comparisons. These latter analyses reached the same conclusions as did the nonparametric tests, with no additional interaction effects detected. Due to the fact that we believe the non-parametric tests are most appropriate, they are the only statistics presented.

RESULTS

Time course experiments in the presence or absence of specific 3 β HSD and aromatase inhibitors were conducted to determine optimal incubation times. Coronal adult and juvenile slices were incubated with ³H-AE in the presence or absence of fadrozole for 10, 30 and 90 mins. Aromatase, 5 β -reductase, and 5 α -reductase activities were measured by eluting E1, 5 β A and 5 α A, respectively. In Figure 3, the amount of estrogens, 5 β A and 5 α A eluted from

the incubating media and tissue samples depict a steady increase that plateaued prior to the 90 min time point. Inclusion of fadrozole to the incubation media dramatically reduced estrogen production, but 5β A and 5α A production proceeded unhindered. The 30 min time point on the upslope of the curve was selected for future experiments as it was the earliest time point that generated significant amounts of estrogens. The same steroid products were eluted from slices incubated with ^3H -DHEA in the presence or absence of trilostane (Fig 4). Trial incubation times included 60, 180 and 300 mins. Trilostane effectively prevented the production of all the aforementioned steroids and the 180 min time point was chosen for future experiments. In a separate experiment examining brain slices (2/group) incubated with ^3H -DHEA ($E1=178.4$ fmol/mg protein, $5\beta A=1411.41$ fmol/mg protein, $5\alpha A=0.0$ fmol/mg protein), fadrozole was shown to abolish estrogen production ($E1=0.0$ fmol/mg protein); conversely, there appeared to be a slight increase in the production of $5\beta A$ (2323.18 fmol/mg protein) and $5\alpha A$ (88.88 fmol/mg protein).

Aromatase activity

Adult sagittal brain slices of tissue were incubated with ^3H -AE for 30 min. ^3H -E1 and ^3H -E2 were subsequently extracted out of both the incubating media as well as the tissue slices themselves. Medial slices that contained the region of the lateral ventricle showed no significant difference between males ($n = 5$) and females ($n = 5$) in regards to aromatase activity as measured by the amount of total estrogens eluted. Slices containing the nuclei RA and HVC also showed no significant difference in aromatase activity between males ($n = 5$) and females ($n = 5$). In addition, the amount of estrogens produced in the lateral ventricular region was not significantly different from that produced in the song system region in either sex (Fig 5a).

Juvenile P20 sagittal brain slices were treated identically as above. Combined extracted E1 and E2 levels showed no significant difference for the medial slices that contained the region of the lateral ventricle between males ($n = 5$) and females ($n = 5$). Slices containing the nuclei RA and HVC also showed no significant difference in aromatase activity between males ($n = 5$) and females ($n = 5$). No detectable difference in estrogen production was measured between the lateral ventricle and song system regions for either sex (Fig 5b).

3β HSD activity

Adult slices were incubated with ^3H -DHEA to evaluate 3β HSD in conjunction with aromatase, 5α -reductase, and 5β -reductase. The following products were eluted: ^3H -E1, ^3H -E2, ^3H - 5α A, ^3H - 5β A. Data collected ($n = 4$ male, 4 female) indicated no significant difference between sexes, in either region, for any of the eluted steroid products (Fig 6a-c). However, a significantly higher amount of 5α A ($U=0.00$, $p=0.021$) and 5β A ($U=0.00$, $p=0.021$) were produced in the male song system slices as compared to the male lateral ventricular slices. Such significance was not achieved in females.

Juvenile P20 slices incubated with ^3H -DHEA also showed no sex differences between males ($n = 5$) and females ($n = 5$) for the production of estrogens or 5α A in the lateral ventricle region. However, 3β HSD in conjunction with 5β -reductase produced a significantly higher amount of 5β A in females as compared to males ($U=3.00$, $p=0.047$) (Fig 7c). In the slices containing song system nuclei, no significant difference between sexes was detected for any of the eluted steroid products. When comparing activity between the lateral ventricle and song system regions, significantly higher amounts of estrogens ($U=2.00$, $p=0.028$), 5α A ($U=2.00$, $p=0.028$), and 5β A ($U=2.00$, $p=0.028$) were produced in the song system region of males but not females (Fig 7a-c).

DISCUSSION

Data presented here confirms the presence and activity of 3β HSD and aromatase in intact, live slices from the developing and adult zebra finch telencephalon. Under these semi-natural conditions, ^3H -DHEA and ^3H -AE were converted into ^3H -estrogens as well as ^3H - 5α and 5β -reduced androgens. The activity of traditional 3β -HSD isoforms are dependent on the presence of nicotinamide adenine dinucleotide (NAD⁺) as a cofactor to catalyze the forward reaction of ^3H -DHEA into ^3H -AE (Payne and Hales, 2004). Our results indicate that the hydroxysteroid dehydrogenase/isomerase activity of 3β -HSD does indeed occur in the brain and that requisite levels of NAD⁺ are also present in these same brain cells. Aromatase utilizes NADPH as an electron donor to catalyze the formation of an aromatic ring on their androgenic substrate (Payne and Hales, 2004). Since tritiated substrates are acted upon by both 3β -HSD and aromatase in these slices, it falls to reason that either the same cells express both the enzymes and co-factors or that the enzymes are present in separate cells that are spatially co-localized within the slice. Whereas the distribution of aromatase-expressing cells is well characterized in the zebra finch (Saldanha et al., 2000) additional experiments are required to ascertain the precise cellular localization of 3β -HSD in the songbird brain. Importantly, these conversions occurred in two regions with implications for the development and function of neural structures controlling song: the lateral ventricle with its associated proliferative zone and the areas containing both RA and HVC. Although the song system nuclei and/or the lateral ventricle were concentrated in our slices, there exists a possibility that the surrounding tissue also included in the slice could have impacted our results and conclusions. Future experiments using enhanced techniques allowing for the isolation of the lateral ventricle and/or the song system nuclei in and of themselves would help to reduce this potentially confounding effect.

We report significant levels of both 3β HSD and aromatase activity in 500 μm thick midline slices containing the lateral ventricle, the birthplace of all new telencephalic neurons (Alvarez-Buylla, 1990; Dewulf and Bottjer, 2005; Goldman and Nottebohm, 1983). It is clear that in both P20 and adult slices, DHEA can be metabolized fully to estrogens and 5α -reduced androgens in this region of the songbird brain. In other vertebrates, DHEA has been shown to influence neurite outgrowth, neuron survival, and adult neurogenesis (Cardounel et al., 1999; Fiore et al., 2004; Karishma and Herbert, 2002; Kimonides et al., 1998; Li et al., 2001; Marx et al., 2000; Suzuki et al., 2004). DHEA has now been detected circulating in the blood of several avian species (Goodson et al., 2005). As with other species, the source of this DHEA is likely to be from the adrenals (Odell and Parker, 1984), but it is possible that DHEA is synthesized in or near the ventricular region of the songbird brain (London et al., 2003). Interestingly, 3β -HSD activity appeared to be lower along the lateral ventricle than that observed in slices containing HVC and RA. It is possible that high levels of endogenous steroidogenic activity occurring in this region (London et al., 2003) locally increases the concentration of radioinert substrate, thereby decreasing the conversion of radiolabeled substrate into detectable product. Future experiments examining the activity of the remaining steroidogenic enzymes, such as CYP17 and CYP11A1, are required to construct a complete picture of the neurosteroids produced during development in this highly prolific region of the forebrain.

It is also possible that the metabolism of androgens (DHEA and AE) occurs to a greater extent in the areas surrounding HVC and RA than at the lateral ventricle, especially in males. In this way, more estrogens and/or 5α -reduced androgens would be available to influence these song nuclei. Both nuclei express androgen receptor (Kim et al., 2004), so a greater synthesis of the potent androgen 5α -dihydrotestosterone might serve to activate these circuits more effectively. Estrogens clearly promote formation of the HVC/RA circuit just after P20 (Holloway and Clayton, 2001). In adults, exogenous DHEA stimulates growth of HVC, likely as a result of its local conversion into active estrogens (Soma et al., 2002).

There is no evidence here to support the view that the male brain synthesizes more active sex steroids via the combined actions of 3 β -HSD and aromatase. These results stand in contrast to expectations that the male brain synthesizes and responds to greater levels of estrogens than females (Holloway and Clayton, 2001; Schlinger, 1998). Two possibilities most likely account for this discrepancy. It is possible that enzymes or transporters upstream in the steroidogenic pathway may be expressed at higher levels in males, such as the steroidogenic acute regulatory protein (StAR), CYP11A or CYP17. Alternately, we could have detected a male bias in the activity of 3 β -HSD or aromatase had we examined slices held *in vitro* for a longer duration as did Holloway and Clayton (2001). Indeed, there is some evidence that 3 β -HSD in combination with 5 β -reductase was overall greater in females than in males. This latter result confirms our previous results pointing to greater telencephalic 3 β -HSD in adult female zebra finches compared to males (Soma et al., 2004). Efforts were made here to minimize stress of the birds before sacrifice to minimize potential confounds (Soma et al., 2004). We do not know why females have higher levels of 3 β -HSD in brain, or how this might play a role in neurosteroidogenesis that might impact masculine song system development. Nevertheless, the capacity to simultaneously examine steroid synthesis/metabolism, neurogenesis and neural development within the same brain slices promises to yield valuable information about significant neural steroid actions.

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References

- Alvarez-Buylla A. Mechanism of neurogenesis in adult avian brain. *Experientia* 1990;46:948–955. [PubMed: 2209804]
- Cardounel A, Regelson W, Kalimi M. Dehydroepiandrosterone protects hippocampal neurons against neurotoxin-induced cell death: mechanism of action. *Proc Soc Exp Biol Med* 1999;222:145–149. [PubMed: 10564538]
- Dewulf V, Bottjer SW. Neurogenesis within the juvenile zebra finch telencephalic ventricular zone: a map of proliferative activity. *J Comp Neurol* 2005;481:70–83. [PubMed: 15558733]
- Fiore C, Inman DM, Hirose S, Noble LJ, Igarashi T, Compagnone NA. Treatment with the neurosteroid dehydroepiandrosterone promotes recovery of motor behavior after moderate contusive spinal cord injury in the mouse. *J Neurosci Res* 2004;75:391–400. [PubMed: 14743452]
- Freking F, Nazairians T, Schlinger BA. The expression of the sex steroid-synthesizing enzymes CYP11A1, 3 β -HSD, CYP17, and CYP19 in gonads and adrenals of adult and developing zebra finches. *Gen Comp Endocrinol* 2000;119:140–151. [PubMed: 10936034]
- Goldman SA, Nottebohm F. Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. *Proc Natl Acad Sci U S A* 1983;80:2390–2394. [PubMed: 6572982]
- Goodson JL, Saldanha CJ, Hahn TP, Soma KK. Recent advances in behavioral neuroendocrinology: insights from studies on birds. *Horm Behav* 2005;48:461–473. [PubMed: 15896792]
- Holloway CC, Clayton DF. Estrogen synthesis in the male brain triggers development of the avian song control pathway *in vitro*. *Nat Neurosci* 2001;4:170–175. [PubMed: 11175878]
- Karishma KK, Herbert J. Dehydroepiandrosterone (DHEA) stimulates neurogenesis in the hippocampus of the rat, promotes survival of newly formed neurons and prevents corticosterone-induced suppression. *Eur J Neurosci* 2002;16:445–453. [PubMed: 12193187]
- Kim YH, Perlman WR, Arnold AP. Expression of androgen receptor mRNA in zebra finch song system: developmental regulation by estrogen. *J Comp Neurol* 2004;469:535–547. [PubMed: 14755534]
- Kimonides VG, Khatibi NH, Svendsen CN, Sofroniew MV, Herbert J. Dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) protect hippocampal neurons against excitatory amino acid-induced neurotoxicity. *Proc Natl Acad Sci U S A* 1998;95:1852–1857. [PubMed: 9465106]

- Li H, Klein G, Sun P, Buchan AM. Dehydroepiandrosterone (DHEA) reduces neuronal injury in a rat model of global cerebral ischemia. *Brain Res* 2001;888:263–266. [PubMed: 11150483]
- London SE, Boulter J, Schlinger BA. Cloning of the zebra finch androgen synthetic enzyme CYP17: a study of its neural expression throughout posthatch development. *J Comp Neurol* 2003;467:496–508. [PubMed: 14624484]
- Marx CE, Jarskog LF, Lauder JM, Gilmore JH, Lieberman JA, Morrow AL. Neurosteroid modulation of embryonic neuronal survival in vitro following anoxia. *Brain Res* 2000;871:104–112. [PubMed: 10882789]
- Odell WD, Parker LN. Control of adrenal androgen production. *Endocr Res* 1984;10:617–630. [PubMed: 6100259]
- Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev* 2004;25:947–970. [PubMed: 15583024]
- Saldanha CJ, Tuerk MJ, Kim YH, Fernandes AO, Arnold AP, Schlinger BA. Distribution and regulation of telencephalic aromatase expression in the zebra finch revealed with a specific antibody. *J Comp Neurol* 2000;423:619–630. [PubMed: 10880992]
- Schlinger BA. Sexual differentiation of avian brain and behavior: current views on gonadal hormone-dependent and independent mechanisms. *Annu Rev Physiol* 1998;60:407–429. [PubMed: 9558471]
- Schlinger BA, Amur-Umarjee S, Shen P, Campagnoni AT, Arnold AP. Neuronal and non-neuronal aromatase in primary cultures of developing zebra finch telencephalon. *J Neurosci* 1994;14:7541–7552. [PubMed: 7996194]
- Schlinger BA, Arnold AP. Brain is the major site of estrogen synthesis in a male songbird. *Proc Natl Acad Sci U S A* 1991;88:4191–4194. [PubMed: 2034664]
- Schlinger BA, Arnold AP. Circulating estrogens in a male songbird originate in the brain. *Proc Natl Acad Sci U S A* 1992a;89:7650–7653. [PubMed: 1502177]
- Schlinger BA, Arnold AP. Plasma sex steroids and tissue aromatization in hatchling zebra finches: implications for the sexual differentiation of singing behavior. *Endocrinology* 1992b;130:289–299. [PubMed: 1727704]
- Schlinger, BA.; Brenowitz, EA. Neural and Hormonal Control of Birdsong. In: Pfaff, DW., editor. *Hormones, Brain and Behavior*. Academic Press; 2002. p. 799-839.
- Schlinger BA, Lane NI, Grisham W, Thompson L. Androgen synthesis in a songbird: a study of cyp17 (17alpha-hydroxylase/C17,20-lyase) activity in the zebra finch. *Gen Comp Endocrinol* 1999;113:46–58. [PubMed: 9882543]
- Soma KK, Alday NA, Hau M, Schlinger BA. Dehydroepiandrosterone metabolism by 3beta-hydroxysteroid dehydrogenase/Delta5-Delta4 isomerase in adult zebra finch brain: sex difference and rapid effect of stress. *Endocrinology* 2004;145:1668–1677. [PubMed: 14670998]
- Soma KK, Wissman AM, Brenowitz EA, Wingfield JC. Dehydroepiandrosterone (DHEA) increases territorial song and the size of an associated brain region in a male songbird. *Horm Behav* 2002;41:203–212. [PubMed: 11855905]
- Suzuki M, Wright LS, Marwah P, Lardy HA, Svendsen CN. Mitotic and neurogenic effects of dehydroepiandrosterone (DHEA) on human neural stem cell cultures derived from the fetal cortex. *Proc Natl Acad Sci U S A* 2004;101:3202–3207. [PubMed: 14973190]
- Vanson A, Arnold AP, Schlinger BA. 3 beta-hydroxysteroid dehydrogenase/isomerase and aromatase activity in primary cultures of developing zebra finch telencephalon: dehydroepiandrosterone as substrate for synthesis of androstenedione and estrogens. *Gen Comp Endocrinol* 1996;102:342–350. [PubMed: 8804564]
- Vockel A, Prove E, Balthazart J. Sex- and age-related differences in the activity of testosterone-metabolizing enzymes in microdissected nuclei of the zebra finch brain. *Brain Res* 1990;511:291–302. [PubMed: 2334847]
- Wingfield, J.; Silverin, B. Ecophysiological Studies of Hormone-Behavior Relations in Birds. In: Pfaff, DW., editor. *Hormones, Brain and Behavior*. Academic Press; 2002. p. 587-647.

Fig 1. Steroidogenic Pathway

Simplified diagram of steroidogenic pathway. Steroids in bold. *Steroids*: DHEA, dehydroepiandrosterone; AE, androstenedione; 5αA, 5α-androstenedione; 5βA, 5β-androstenedione; E1, estrone; E2, estradiol; 5βDHT, 5β-dihydrotestosterone; 5αDHT, 5α-dihydrotestosterone. *Enzymes*: CYP11A1, cytochrome P450 side chain cleavage; CYP17, cytochrome P450 17α-hydroxylase/C17-20 lyase; 3βHSD, 3β-hydroxysteroid dehydrogenase/isomerase; 17βHSD, 17β-hydroxysteroid dehydrogenase. *Cholesterol transporter*: StAR, Steroid Acute Regulatory protein.

Fig 2. Caudal view of a sagittal adult telencephalon slice
 300 μ m paraformaldehyde fixed sagittal slice of adult male zebra finch telencephalon containing two song system motor nuclei: robust nucleus of the archipallium (thick arrow) and HVC (thin arrow). Note the intact tract that runs from HVC to RA.

Fig 3. Time course of incubation with ^3H -AE

Coronal juvenile slices incubated with 200nM ^3H -AE were incubated for 10, 30, and 90 mins in the presence or absence of the aromatase inhibitor fadrozole. Estrogenic and androgenic products were isolated and quantified. (A) ▲, estrogens; △, estrogens + fad; ■, 5αA; □, 5αA + fad. (B) ●, 5βA; ○, 5βA + fad.

Fig 4. Time course of incubation with ^3H -DHEA

Coronal adult slices incubated with 200nM ^3H -DHEA were incubated for 10, 30, and 90 mins in the presence or absence of the $3\beta\text{HSD}$ inhibitor trilostane. Estrogenic and androgenic products were isolated and quantified. (A) $\blacktriangle$, estrogens; $\triangle$, estrogens + trilo; $\blacksquare$, $5\alpha\text{A}$; $\square$, $5\alpha\text{A}$ + trilo. (B) $\bullet$, $5\beta\text{A}$; $\circ$, $5\beta\text{A}$ + trilo.

Fig 5. Adult and P20 aromatase activity in the lateral ventricle and song motor nuclei regions Adult (A) and juvenile (B) sagittal slices containing either the lateral ventricle or RA and HVC were cultured with 200nM ^3H -AE for 30 mins. Incubating media and tissue slices were processed for ^3H -E1 and ^3H -E2 steroid products to measure for total aromatase activity reported in fmol/mg protein/slice. No significant differences between sexes or between brain regions were detected for either age group. Data represents the means $\pm$ SEM for 5 males and 5 females in each age group.

Fig 6. Adult 3βHSD activity in slices containing the lateral ventricle or HVC and RA

Adult zebra finch sagittal telencephalon tissue slices containing the lateral ventricle or RA & HVC were incubated with 200nM ^3H -DHEA for 180 mins. Tritiated steroid products – E1 & E2 (A), 5αA (B), and 5βA (C)- were eluted from the incubating media and tissue slice to measure 3βHSD activity in conjunction with aromatase, 5α-reductase, and 5β-reductase, respectively. Results are reported in fmol/mg protein/slice. No significant differences in 3βHSD activity were detected between sexes in either brain region for any of the measured steroid products. Male song system slices produced significantly higher levels of 5αA and 5βA than male lateral ventricle slices. Data represents the means $\pm$ SEM for 4 males and 4 females. * $p < 0.05$.

Fig 7. P20 3βHSD activity in the lateral ventricle and song system regions

Juvenile zebra finch sagittal telencephalon tissue slices containing the lateral ventricle or RA & HVC were incubated with 200nM ^3H -DHEA for 180 mins. Tritiated steroid products – E1 & E2 (A), 5αA (B), and 5βA (C)- were eluted from the incubating media and tissue slice to measure 3βHSD activity in conjunction with aromatase, 5α-reductase, and 5β-reductase, respectively. Results are reported in fmol/mg protein/slice. Significant differences in joint 3βHSD and 5β-reductase activity were detected between sexes in lateral ventricle containing slices. Male song system slices manufactured significantly higher levels of all eluted steroid products than male lateral ventricle slices. Data represents the means $\pm$ SEM for 5 males and 5 females. * $p < 0.05$.