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Brain is the major site of estrogen synthesis in a male songbird

(aromatase/telencephalon/adrenal/testis/zebra finch)

BARNEY A. SCHLINGER AND ARTHUR P. ARNOLD

Department of Psychology and Laboratory of Neuroendocrinology of the Brain Research Institute, University of California, Los Angeles, CA 90024-1563

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ABSTRACT **The neural system controlling song in passerine birds can undergo striking morphological and functional changes during both development and adulthood, and many of these changes are regulated by estrogenic hormones. Estrogens circulate at high levels in blood of male songbirds and persist after castration. We measured the activity of aromatase, the enzyme that converts androgens to estrogens, in various tissues from adult male and female zebra finches. As expected, aromatase activity was present in male hypothalamus/preoptic area and pituitary and female ovary, but aromatase was unusually active in whole telencephalon of males and females. By contrast, activity was undetected in testes, adrenals, or other tissues of males. These results suggest that brain is the source of circulating estrogens in the male zebra finch and that estrogen actions on the song system result from local rather than peripheral aromatization.**

Behavioral and physiological studies indicate that conversion (aromatization) of androgens to estrogens takes place in the vertebrate brain and mediates many of the organizational and activational effects of testosterone (T) on male reproduction and sexual behavior (1–4). In the zebra finch (*Poephila guttata*), neonatal treatment with the aromatizable androgen T or 17β-estradiol (E_2) has permanent masculinizing effects on singing behavior and on the brain regions that control song (5, 6). As with mammalian studies, it was originally postulated that circulating T might be aromatized in brain to masculinize song in males (6). Subsequent studies in zebra finches and other songbirds showed that estrogens circulate at appreciable levels in both young and adult males. Presumably, this circulating estrogen is involved in the ontogeny and activation of singing behavior (7–12). However, castration of males does not prevent the development of song (10, 13–15), does not eliminate estrogens in the circulation of developing males (10), and can increase levels of estrogens in adult males (8). Accordingly, some investigators have speculated that these estrogens in males are derived from the adrenals (8, 15). We have measured the activity of aromatase, the enzyme that converts androgens to estrogens, in gonads, adrenals, and other tissues of adult zebra finches. In males, aromatase activity was detected only in brain and pituitary, and activity was extremely high in telencephalic microsomes. These findings suggest that brain is the source of circulating estrogens and that estrogen actions on the brain result from local rather than peripheral aromatization.

MATERIALS AND METHODS

Veinous or trunk blood was collected from intact nonbreeding adult males and females (held in single-sex aviaries) or adult males castrated for 3, 28, 60, or 140 days. Birds were sacrificed by decapitation, and tissues were quickly removed to iced aluminum foil. Aromatase activity was measured in whole-tissue homogenates or in microsomal preparations by quantifying the conversion of [7-^{3}H]- or [1,2,6,7-^{3}H]androstenedione to [^{3}H]estrone (E_1) and [^{3}H]E_2 by procedures validated previously for avian brain (16–18). Whole homogenates were prepared in 250 mM phosphate/50 mM sucrose buffer, pH 7.4, after 10 strokes in teflon/glass homogenizers (final volume, 250 or 500 μl). Vitellogenic ovarian follicles were pierced and crushed, and excess yolk was removed prior to homogenization. To prepare microsomes, whole homogenates were centrifuged at 1000 × *g* (15 min), producing a supernatant that was centrifuged at 10,000 × *g* (30 min) to produce another supernatant that was centrifuged at 100,000 × *g* (60 min) to produce the final microsomal pellet. To maximize our ability to detect estrogenic products in these experiments, we used a wide variety of incubation conditions. Tissues were incubated for 5, 10, 30, 60, or 90 min with 100 or 250 nM radiolabeled substrate having a specific activity of 11.0, 23.4, or 86.4 Ci/mmol (1 Ci = 37 GBq). In addition, adrenal and testicular tissues were usually pooled so that homogenates contained as much as or more tissue than ovarian homogenates. In some cases, 1 μg of E_1 was added to incubation tubes as a "trap" of the end product. For tissues in which aromatase activity was detected, conditions were optimized for the time of incubation and substrate concentration to quantify specific activity.

After ether extraction, estrogenic products were purified by phenolic partition (twice) and ethyl acetate extraction. Final residues were chromatographed on thin-layer silica gel plates, and radioactivity comigrating with radioinert E_1 and E_2 was determined as described (17). Results were expressed as femtomole of estrogen (E_1 + E_2) per whole tissue, or per milligram of protein as measured by the method of Bradford (19). Control tubes contained substrate and cofactors only. Procedural losses were estimated in tubes that contained 100,000 cpm of [6,7-^{3}H]E_1 (60 Ci/mmol), which were processed in parallel (17). Assay sensitivity (limit of detectable formed estrogen) was taken as twice the background cpm eluted from the E_1 region of TLC plates of control samples; the background cpm were corrected for assay losses, converted to femtomoles, and divided by incubation time and protein content per tissue. To verify authenticity, radiolabeled products from telencephalon, hypothalamus/preoptic area (HPOA), and ovary were recrystallized three times to constant specific activity.

E_2 and E_1 were measured in plasma by radioimmunoassay after purification by Celite chromatography at the University of California, Los Angeles, Population Research Center Hormone Assay Facility. These assays are used routinely on human and rodent plasma and have been validated for use in zebra finch (unpublished data). All assays included human and rat plasma standards for control. Assay variation was maintained within a 6% coefficient of variation for duplicate samples, with a lower limit of detection of approximately 28 and 42 pg/ml for E_2 and E_1, respectively.

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Abbreviations: T, testosterone; E_2, 17β-estradiol; E_1, estrone; HPOA, hypothalamus/preoptic area.

RESULTS

Plasma levels of estrogens were not significantly different in breeding (mixed-sex aviaries) or nonbreeding males and females (n = eight per group). Plasma E_2 levels (mean ± SEM) were similar for nonbreeding males (92 ± 13 pg/ml) and breeding males and females (87 ± 8 and 101 ± 16 pg/ml, respectively). Mean E_2 levels of nonbreeding females were 2-fold greater than those of other groups (195 ± 74 pg/ml), but these differences were not statistically significant. E_1 circulated at approximately 25–50% of E_2 levels in all birds. At various times after castration, estrogen levels in males either were unchanged from intact levels or were increased as much as 5-fold (unpublished data). Moreover, estrogen levels were unrelated to body weight or body fat content, suggesting that these are unimportant determinants of circulating estrogens (20). Thus, we have confirmed previous reports that estrogens circulate at comparable levels in adult males and females (7, 8) and are not reduced following castration of adult males (8).

Despite extensive efforts to optimize the assay, aromatase activity was undetected in intact or castrated male adrenals in five separate experiments with whole homogenates (average assay sensitivity, <0.679 fmol/min per mg of protein) (Fig. 1). Aromatase activity was also undetected in homogenates of right, left, or paired testes (<0.267 fmol/min per mg of protein). We also examined nonglandular tissues that can be a site of aromatase (20, 21) but found no activity in muscle, heart, lung, skin, kidney, adipose tissue, or uropygial glands of intact or castrated males. By contrast, in all experiments, ovary produced considerable estrogen (213–1160 fmol/min per mg of protein). This variability in ovarian aromatase activity probably reflects various stages of ovarian development in our samples. We quantified activity in HPOA, the region that generally has the highest aromatase activity in brain (1, 3, 4, 17, 18, 22, 23). We also measured activity in homogenates of whole brain because of reports that nonlimbic regions of the zebra finch brain also express high levels of aromatase (22, 24). In noncastrated (intact) males compared with males castrated for 30–140 days before removal of tissue, aromatase activity was greater in both the HPOA (9.56 ± 3.2 and 7.75 ± 2.13 fmol/min per mg of protein, respectively) and in whole brain (Fig. 1), but these differences were not significant. Aromatase was also detected in male pituitary as has been reported for other species (17, 18).

We considered the possibility that endogenous androgenic substrate or estrogen catabolizing enzymes interfered with our measures of aromatase in whole homogenates. Therefore, in several tissues, we measured aromatase in microsomal preparations, the purified subcellular fraction in which aromatase is generally enriched (16, 25). Our initial studies indicated that microsomal aromatase was saturated at 250 nM [^{3}H]androstenedione and that estrogen formation was linear for a 5-min incubation of ovary and telencephalon and a 30-min incubation of male HPOA. Therefore, we adopted these conditions to quantify microsomal aromatase in these tissues. However, aromatase activity was undetected in microsomal preparations of male adrenals or testes incubated for 10, 30, or 60 min (Fig. 2) (average assay sensitivity, <8.7 and 0.3 fmol/min per mg of protein for adrenals and testes, respectively). By contrast, activity in ovarian microsomes was high (760.8 ± 372 fmol/min per mg of protein). Interestingly, microsomes from telencephalon pooled from males or females had exceptionally high activity (male = 478.3 ± 65, female = 389.3 ± 29 fmol/min per mg of protein)—5-fold greater than male zebra finch HPOA and 3- to 16-fold greater than activity previously reported for microsomes from quail HPOA (16).

DISCUSSION

Under the conditions of our assays, brain and pituitary were the only sites of aromatization in males, suggesting that estrogens in plasma are synthesized in brain. We do not dismiss the possibility that aromatase is present in other tissues at levels below the sensitivity of these assays. How-

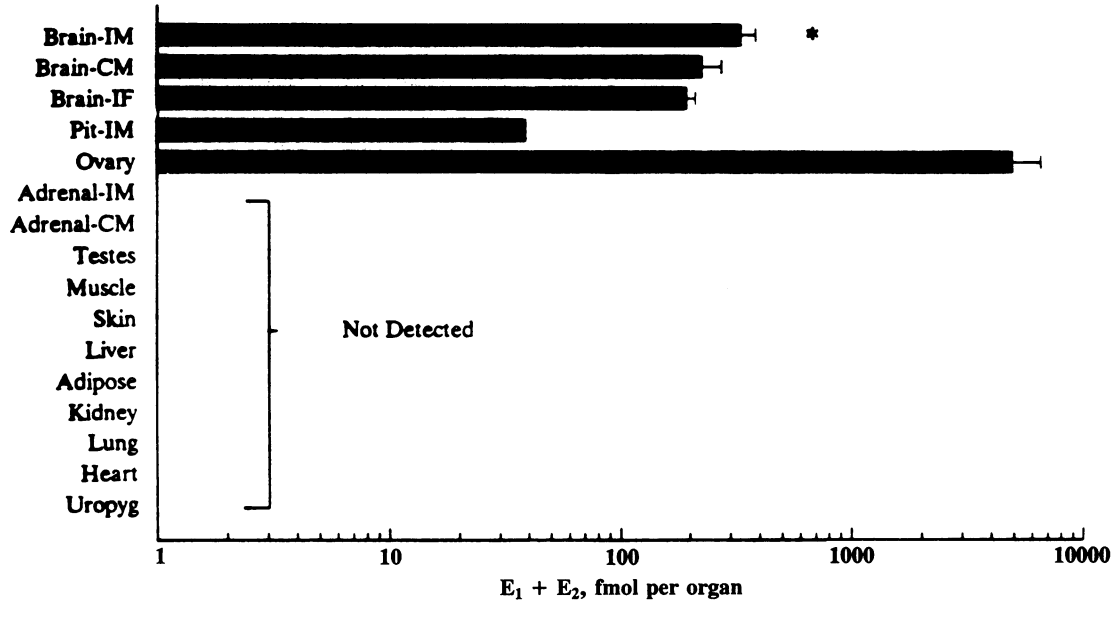

FIG. 1. Aromatase activity (mean ± SEM) in whole homogenates of tissues from adult intact (I) or castrated (C) male (M) or female (F) zebra finches. Aromatase was not detected in nine individual testes (right or left) or 10 pairs of testes nor in adrenals from 24 intact or 24 castrated males (adrenals were combined into six, three, three, or two pools containing two, six, five, or nine adrenals, respectively). Activities for whole-brain and ovary represent nine determinations, each from a separate animal. Values for male pituitary represent duplicate determinations on a pool of eight glands. Values for other tissues represent three determinations using intact and castrated males. Tissues from castrates were removed 30–140 days after castration. Values for brain were compared by one-way analysis of variance and Duncan's multiple range test (* = $P < 0.05$ compared to intact female but not castrated male). Muscle and skin were dissected from breast. Pit, pituitary; Uropyg, uropygial gland.

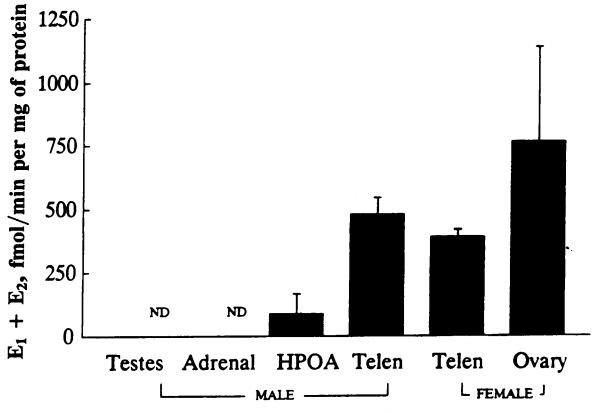

FIG. 2. Aromatase activity (mean ± SEM) in microsomes prepared from male and female zebra finches. Values represent pools of 8 or 30 testes and 9 or 28 adrenals (three determinations per tissue pool). Aromatase activity was also measured but undetected in the mitochondrial (10,000 × *g*) pellet from adrenals and testes. Other values are from three separate experiments (pools of four to eight tissues per experiment). The HPOA was dissected as described (17). Telencephalon (Telen) was taken as the tissue remaining after removal of HPOA, hindbrain, and optic lobes. ND, not detected.

ever, considering the abundance of aromatase in brain, the contribution of these tissues to the pool of circulating estrogen is likely to be low. Of greatest interest would be testicular or adrenal aromatase in close proximity to androgen-synthesizing enzymes. Several considerations suggest that the present assay conditions were sufficiently sensitive to detect even low levels of aromatase in these tissues. For example, we anticipated that it would be difficult to detect aromatase in adrenals because they are small and because competing or catabolizing metabolic pathways might remove substrate or estrogenic products. However, there is evidence that these factors did not significantly interfere with our measures of aromatase. First, when [^{3}H]E_1 (200,000 cpm) was used as the substrate in a 1-hr incubation of six adrenals, 100% of the radioactivity was recovered in ether extracts as E_1 or E_2, suggesting that there is little or no catabolism of estrogenic products in these homogenates. Nevertheless, the presence of an estrogen trap in some assays guarded against estrogen removal. Second, after incubating homogenates of six adrenals with 100 nM [^{3}H]androstenedione for 90 min, on average 70% of the initial androstenedione was recovered, indicating that availability of substrate was not limiting the rates of aromatization. Third, despite the absence of aromatase in this latter experiment, radioactivity was eluted from regions of TLC plates corresponding to testosterone, 5α-androstanedione, and 5β-androstanedione. This result suggests that 17β-hydroxysteroid dehydrogenase and 5α- and 5β-reductase remained active under these conditions despite the small amount of protein per adrenal. Moreover, when 6–12 adrenals or testes were incubated for 1–3 hr with [^{3}H]pregnenolone in the presence of 1 μg of radioinert E_1, formation of estrogens remained undetected, but authentic [^{3}H]testosterone, [^{3}H]androstenedione, and [^{3}H]progesterone were formed and verified in both adrenals and testes by recrystallization to constant specific activity (unpublished data). These results indicate that sufficient protein was present in our assays of adrenals to detect other steroidogenic enzymes and argue against the presence of nonspecific proteases that eliminated enzymatic activity. Finally, assuming that assay sensitivity represents maximum possible levels of undetected aromatization, aromatase activity in ovary must have been at least 314- and 33-fold greater than adrenal in homogenates and microsomes, respectively, and 787- and 798-fold greater than testicular homogenates and microsomes. At best, aromatization is a minor pathway in male adrenal and testes, with levels far below values for ovary or brain of males and females. Nevertheless, we assume that the synthesis of estrogens in brain of intact and castrated males utilizes androgens produced in the testes and adrenals, respectively.

The female telencephalon also possesses considerable aromatase, which may contribute estrogen to the circulation, in addition to that synthesized in ovary. However, because aromatizable androgens generally circulate at lower levels in female than in male songbirds (26), the contribution of brain to circulating estrogens in females may be lower than in males. Also, because peripheral catabolism of estrogens can be an important determinant of circulating estrogen levels, it is possible that a diminished removal of estrogens from the circulation in part contributes to the relatively high plasma levels of estrogens found in males and may account for the increased circulating estrogens seen in castrates (8).

The telencephalon of the adult male zebra finch possesses surprisingly high levels of aromatase (21, 24). Because other tissues lack this enzyme, we conclude that the telencephalon is also the most likely source of estrogens that are found to circulate at high levels in blood of male songbirds (7–11). This idea is supported further by the observation that levels of both telencephalic aromatase and plasma estradiol are low in nonpasserine birds (17, 21, 23, 27, 28). The evolution of high levels of telencephalic aromatase may be related in part to the presence in songbirds of the neural system controlling song. This system undergoes striking morphological and functional changes during sexual differentiation and song learning, two processes that are regulated by estrogen (29, 30). Estrogen has clear neurotrophic actions (31), and brain synthesis of estrogen is spatially and temporally correlated with neural plasticity in diverse vertebrate species (4, 32). Our data also suggest that rather than relying on estrogens from the periphery, the adult songbird brain itself provides the estrogens required for estrogenic regulation of neural plasticity in the song system. Although the present experiments bear only on adult levels of aromatase, it will be interesting to determine whether the brain is similarly the main site of estrogen synthesis during periods of sexual differentiation and song learning.

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