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Circulating estrogens in a male songbird originate in the brain

(aromatase/telencephalon/zebra finch/gonads/adrenals)

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ABSTRACT **Gonadal steroids act on brain to regulate the development and expression of vertebrate reproductive behavior. In addition, steroid targets in brain are an integral part of the feedback regulation of gonadal steroidogenesis. The actions of androgens in brain are often mediated by enzymatic activation or inactivation of circulating hormone, including local conversion of androgen to estrogen. We now report that in zebra finches, the brain synthesizes large quantities of estrogen from androgen and releases this estrogen into blood. Since there appears to be no other significant source of estrogen synthesis in adult male zebra finches, it is likely that the brain directly controls plasma levels of this steroid by contributing most or all of the high levels of estrogen found in the circulation.**

The actions of estrogens (Es) on brain are essential for the development and expression of song in male passerine (oscine) birds. In the zebra finch (*Poephila guttata*), males sing a courtship song that females lack. Correspondingly, the telencephalic neural system controlling song is much larger in males than females (1). However, if young females are treated with E, they develop a masculinized neural song system and the capacity to sing as adults (2–6). These E effects on the song system are extensive; they influence neuron survival, size, dendritic arbor, or hormone sensitivity within several brain structures (7–11). Thus, Es are thought to be the normal masculinizing hormone in developing males. Such E effects on the male brain are not unusual, but in the vast majority of vertebrates, Es in males circulate at low or undetectable levels. However, the enzyme that catalyzes the conversion of androgen to E, aromatase cytochrome P450, can be present near E targets in the brain. By controlling local concentrations of E, aromatization controls occupancy of nearby E receptors and, hence, regulates those actions of testicular androgens that are mediated by E receptors (12–16).

By contrast, in both developing and adult songbirds, males have high estradiol (E_2) levels in blood, often equal to or greater than those seen in adult breeding females (17–19). Because high E levels in blood may be involved with brain sexual differentiation (20) and with the capacity of birds to learn song (21), there has been great interest in the tissue origin of the E in blood and the regulation of its synthesis. This search has become more challenging since traditional experimental approaches to reduce circulating sex steroids, castration or photoperiod manipulation, do not generally eliminate circulating E in males, and perhaps also females (22–24). Using an *in vitro* assay that quantifies the conversion of [^{3}H]androstenedione (AE) to [^{3}H]estrone (E_1) and [^{3}H]E_2, we (25, 26) have found aromatase to be abundant in ovary of females at all ages (from 3 days to adulthood) but undetectable in testes, adrenals, or a variety of other peripheral tissues of intact or castrated males at any age. By contrast, aromatase was abundant in microsomes prepared from telencephalon (TEL) of both males and females (refs. 25 and 26 and see also refs. 27 and 28). Because aromatase activity in male TEL was high, similar to ovary, and absent elsewhere, we have postulated that the brain is the origin of E seen in the circulation of male songbirds (25).

To test this hypothesis, we developed methods to measure aromatase activity in zebra finches *in vivo* and have used these experimental approaches to determine (*i*) whether E made from androgen in brain can be found in the circulation; (*ii*) whether physiological blood levels of aromatizable androgen (AA) can be converted by brain to E and released into the circulation; and (*iii*) whether *in vivo* measures of E synthesis could reveal sites of aromatase activity undetected by *in vitro* assays, especially in tissues outside of brain.

MATERIALS, METHODS, AND RESULTS

Experiment 1: E Synthesis After Intracerebral Injection of [^{3}H]AA. We asked whether [^{3}H]AE injected directly into the medio-dorsal TEL (n = 5) near the higher vocal center, a known aromatase-rich brain region (27, 28), could cause [^{3}H]E to appear in blood emanating from the brain (i.e., collected from the jugular vein). To control for the possibility that [^{3}H]E detected in the jugular might reflect E synthesized outside of brain that had recirculated through the brain, we also made similar injections into an adjacent aromatase-poor region, the cerebellum (CB, n = 5), of separate animals. [1,2,6,7-^{3}H]AE (1 ng; 86.4 Ci/mmol; 1 Ci = 37 GBq) in ethanol (EtOH) was mixed with 10 μl of EtOH/propylene glycol (9:1, vol/vol), and the EtOH was dried under N_2 gas. Birds were anesthetized with Equithesin (0.05 ml/15 g), placed in a stereotaxic apparatus, and injected with 1 μl by using a 10-μl Hamilton syringe and a blunt-end 26-gauge needle. After 5 min, the needle was withdrawn from the brain, and birds were bled to death by inserting a 27-gauge needle on a heparinized syringe into the jugular ≈0.5 cm from the skull. The concentrations of [^{3}H]androgens and [^{3}H]Es were measured in plasma (final volume, 150–300 μl) as described (29). Briefly, plasma (or tissue homogenized in 250 mM sucrose/50 mM potassium phosphate, pH 7.4) was extracted (three times) with a 10-fold excess ethyl ether and Es were purified by phenolic partition (twice) and ethyl acetate extraction (three times). Es and androgens (which remain after phenolic partition) were then separately chromatographed twice on thin-layer silica plates in ether/hexane, 3:1 (vol/vol), after adding radioinert carriers. Es were visualized by iodine vapors; androgens were detected under UV irradiation after spraying plates with primulin. Regions of chromatoplates were scraped and material was eluted in methanol/H_2O 6:1 (vol/vol), and then mixed with Biofluor scintillation solution (New England Nuclear) before radioactivity was measured in a scintillation counter. The

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Abbreviations: E_2, estradiol; E, estrogen; E_1, estrone; T, testosterone; AE, androstenedione; TEL, telencephalon; CB, cerebellum; AA, aromatizable androgen; df, degree of freedom.

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amounts of [^{3}H]E_1 and [^{3}H]E_2 were quantified separately and then summed as a measure of total E synthesis.

Five minutes after injections, there was no significant difference between the TEL and CB injections in jugular plasma levels of radiolabeled AA {[^{3}H]AE plus [^{3}H]testosterone (T)}, 115.8 ± 20.4 vs. 91.8 ± 22.7 fmol/ml [mean ± SEM; Student's t test = 0.78, degrees of freedom (df) = 8; P = 0.453; Fig. 1] or 5β-androstanedione, 70.3 ± 27.9 vs. 37.1 ± 12.8 fmol/ml (t = 1.08; df = 8; P = 0.310), a product of the major androgen inactivating pathway (5β-reduction) in avian brain (29–31). These results indicate that injected androgen did not differentially leak from these brain regions and was not catabolized unequally. Nevertheless, after TEL androgen injections, [^{3}H]E was present in jugular plasma, 6.17 ± 0.79 fmol/ml, but only small amounts were found in plasma after CB injections, 0.64 ± 0.29 fmol of E_1/ml (t = 6.59; df = 8; $P < 0.0002$). [^{3}H]E_1 in jugular blood was verified by recrystallization (three times) to a constant specific activity, 111.2 dpm crystals (C_{dpm})/112.1 mother liquor dpm (ML_{dpm}) [1.96% error; 100% of initial dpm (I_{dpm} recovered)]. Moreover, [^{3}H]E levels in jugular blood were greatest 5–10 min after telencephalic androgen injections, were detected after injecting as little as 165 pg of [^{3}H]AE, and were similar after 1 or 6 ng of [^{3}H]AE. Presumably, diffusion and metabolism delay the jugular E peak by 5–10 min.

Immediately after bleeding, whole brains of injected birds were removed, homogenized, and extracted to determine [^{3}H]steroid content. Significantly greater levels of [^{3}H]E were present in brain after TEL than CB androgen injection, 26.14 ± 7.94 vs. 2.28 ± 1.60 fmol per brain [t = 2.95; df = 8; $P < 0.02$)] (Fig. 1), and these amounts were significantly correlated with the [^{3}H]E content in blood (Spearman rank correlation; r = 0.738, n = 10, $P < 0.02$). These data indicate that androgens can be converted in brain to Es that can reach the circulation. Group similarities in blood androgen content and the limited presence of E in blood after CB injections suggest that little, if any, of the E in jugular plasma after injections into TEL was synthesized outside of brain and recirculated through the brain. In addition, in a separate group of birds, the spread of radioactivity after 1 ng of [^{3}H]AE injection into brain was examined by thaw–mount autoradiography. Radioactivity was concentrated at the site of injection, though some was present within adjacent ventricles after TEL injection or over the dorsal TEL after CB injection. The limited presence of radioactivity over aromatase-positive TEL presumably accounted for the small amount of [^{3}H]E in jugular plasma after CB injection.

Experiment 2: E Synthesis After Systemic Injections of [^{3}H]AA. Although E may be found in blood after direct injections of androgen in brain, we assume that the brain derives its aromatizable androgenic substrate from the circulation, after secretion by the testes (in intact males) or adrenals (in castrates) (25). Moreover, although aromatase may be poorly expressed outside of brain, low levels of activity [below detection by *in vitro* assays (25, 26)] in numerous tissues might lead to detectable Es in blood. Therefore, to test whether the male brain or peripheral site(s) in males can remove androgens from blood, convert them to Es, and release E back into the circulation, we injected [^{3}H]AE or [^{3}H]T {[1,2,6,7-^{3}H]T (87.1 Ci/mmol)} directly into the vascular system and measured radioactive products in blood entering or leaving the head of males. For these studies, [^{3}H]androgen in EtOH was mixed with 50 μl of avian saline (0.75% NaCl) and EtOH was evaporated under N_2. Anesthetized birds were injected directly into the jugular vein as it enters the thorax (≈1.3 cm from the skull) via a 50-μl air-tight Hamilton syringe with a 30- to 33-gauge needle. Injections in which the clear saline was seen to pass down the jugular into the body cavity were deemed successful; others were discarded. Blood was then collected from either the carotid artery (as an estimate of peripheral aromatization) or from the jugular vein near the base of the skull (as a measure of brain aromatization). Radioactivity within the carotid artery was taken as an estimate of [^{3}H]steroid entering the brain and of [^{3}H]steroid exiting the body.

Five minutes after injection of 6 or 36 ng of [^{3}H]AE or [^{3}H]T into the jugular, AA was present in blood within the carotid and jugular at near physiological levels (175–4400 pg/ml; 612–15,372 fmol/ml). [^{3}H]E was present in carotid plasma of 4 of 11 females after injection of 6 ng of [^{3}H]AE or T (3.28 ± 1.49 fmol/ml, n = 11 or 3 of 3 females after 36 ng of [^{3}H]AE (39.03 ± 8.83 fmol/ml, n = 3) (Fig. 2) but not in males injected with 6 ng of [^{3}H]AE or T (n = 7) or 36 ng of [^{3}H]AE (n = 3). [^{3}H]E_2 in jugular plasma was verified by recrystallization (96.3 C_{dpm}/101.7 ML_{dpm}; 5.0% error; 79% I_{dpm} recovered). Although carotid blood does not fully represent the peripheral circulation, the sex differences in Es found in the carotid suggest that peripheral (nonbrain) aromatization can be detected by measuring carotid E levels. Thus, females have Es in the carotid using these procedures, but males do not, presumably because only females have a significant peripheral source of E, the ovary. Despite the absence of [^{3}H]E in arterial blood entering the male brain, [^{3}H]E was detected in venous blood leaving the male brain (in jugular) (11 of 13 males after 6 ng of [^{3}H]AE or T, 7.76 ± 2.64 fmol/ml, n = 13; 3 of 3 males after 36 ng of [^{3}H]AE, 44.17 ± 4.17 fmol/ml, n = 3) (Fig. 2). These data imply that androgens were taken up by brain, converted to E, and released back into the bloodstream. Moreover, 5 min after similar 6-ng [^{3}H]AE injections into jugular of separate males, [^{3}H]E was extracted from TEL

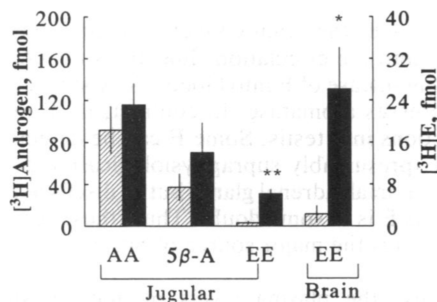

FIG. 1. Jugular plasma levels (mean ± SEM; fmol/ml of plasma) of radiolabeled AAs (T and AE), 5β-androstanedione (5β-A), or total Es (EE = E_1 plus E_2) and whole-brain E content (fmol of EE per brain) 5 min after 1 ng of [^{3}H]AE injection into adult male TEL (solid bars) or CB (hatched bars). Left axis shows androgen values in plasma; right axis shows E values in plasma or brain. *, $P < 0.02$; **, $P < 0.0002$ (when compared with CB-injected birds).

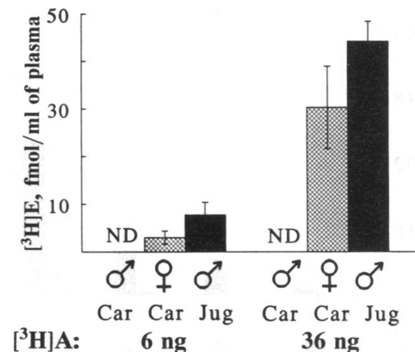

FIG. 2. Carotid (Car) or jugular (Jug) plasma levels of radiolabeled E (E_1 plus E_2) 5 min after 6 or 36 ng of [^{3}H]AE or [^{3}H]T ([^{3}H]A) was injected into male or female jugular. ND, not detected. Data from three experiments were combined to calculate the mean ± SEM.

homogenates (33.0 ± 10.7 fmol/TEL, n = 4) but not from homogenates of CB (n = 4) (not illustrated). The absence of [³H]E in CB is additional evidence that the brain did not uniformly receive E from the circulation. Moreover, these data suggest that the contribution of Es to the circulation by brain is regionally specific and correlated with the distribution of aromatase activity.

Experiment 3: E Synthesis After Injections of [³H]AA into Gonad or Adrenal. We presume that Es present in the female carotid were synthesized in the ovary and that the lack of carotid E in males implies little testicular or adrenal synthesis of E. Unfortunately, it is not technically feasible to measure arterial or venous blood of the small gonads and adrenals of zebra finches. However, to test whether testes or adrenals, which synthesize androgens, might synthesize E when androgens are present in high concentrations, we injected [³H]androgen directly into ovaries, left testes, or left male adrenals and measured radiolabeled products in the carotid plasma 5 min later. Under Equithesin anesthesia, [³H]AE or [³H]T was injected through a small incision in the animal's left side into gonads or adrenals (in 1 or 4 μl of propylene glycol) as described for brain. Sufficient carotid blood was collected into heparinized capillary tubes to yield 100–200 μl of plasma from each bird. Plasma was pooled from at least two birds for each determination.

[³H]E was detected in 12 of 17 plasma pools (n = 33 birds) after 1, 3, 6, or 36 ng of androgen ([³H]AE or [³H]T) was injected directly into ovary (Fig. 3). [³H]E_1 found in carotid after ovarian [³H]AE injections was verified by recrystallization as described above (84.3 C_{dpm}/84.7 ML_{dpm}; 0.1% error; 100% I_{dpm} recovered). In addition, [³H]E was detected in 3 of 18 plasma pools (n = 41 birds) after 3, 6, or 36 ng of androgen was injected into male adrenals. By contrast, [³H]E was undetected in 9 plasma pools (n = 18 birds) after injection of 1, 3, or 6 ng of androgen into left testis. In cases in which 36 ng of [³H]AA was injected, the injected ovary, injected left (plus the right) male adrenal, and brains of injected animals were collected, frozen immediately (−80°C), and later extracted to determine [³H]steroid content. [³H]AAs were present in large amounts in both ovaries and male adrenals (2052 ± 543 vs. 680 ± 518 fmol per organ; t = 1.83, df = 10, P = 0.10). Nevertheless, [³H]E was very low (n = 2) or undetected (n = 4) in adrenal homogenates (0.56 ± 0.37 fmol per organ, n = 6) but was present in ovarian homogenates (229.5 ± 90.1 fmol per organ, n = 6; Fig. 4). In addition, [³H]E was extracted from the TEL of adrenal-injected males (44.93 ± 7.68 fmol per TEL; n = 4) and ovarian injected females (55.0 ± 2.6; n = 2; Fig. 4) but was absent from the CB. These data indicate that E can be detected in carotid plasma after synthesis in a peripheral tissue (ovary), and they confirm our *in vitro* measures that indicate little or no aromatase activity

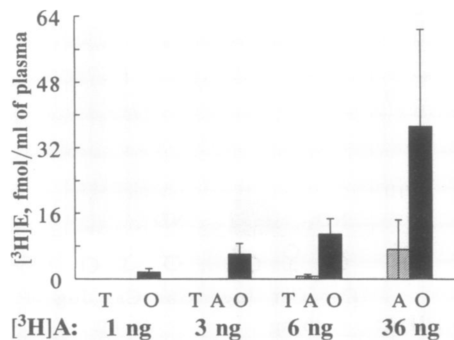

FIG. 3. Carotid plasma levels of radiolabeled E (E_1 plus E_2) 5 min after 1, 3, 6, or 36 ng of [³H]AE or [³H]T was injected into the left testis (bars T) or left adrenal (bars A) of adult males or ovary (bars O) of adult females. Adrenal was not injected with 1 ng; testis was not injected with 36 ng.

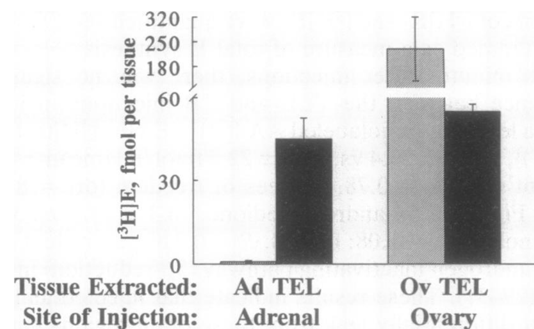

FIG. 4. Radiolabeled E content (E_1 plus E_2) extracted from homogenates of adrenals (left plus right; Ad) or TEL of males or ovary (Ov) or TEL of females 5 min after 36 ng of [³H]AE was injected into the left male adrenal or female ovary. Note break in left axis.

in testes (25). The data may also indicate that some aromatization occurs in the male adrenal. However, [³H]E in blood after adrenal injection was not reliably detected and was present in only small amounts after injection of large quantities of [³H]AA. Moreover, even when [³H]E was present in plasma after adrenal injection, [³H]E was either low or undetectable in extracts from the adrenal itself. This contrasts with both ovary and male brain, which contain large amounts of E relative to blood after direct androgen injections. Therefore, it is possible that Es present in blood after adrenal injections were synthesized elsewhere, for example, in brain where the TEL on average contained 80 times the adrenal content of [³H]E after adrenal [³H]androgen injection. Although these data point to a nonadrenal site of E synthesis, we cannot dismiss the possibility that small amounts of E in blood of castrated adult or developing males are derived from the adrenals. Nevertheless, we conclude that most of the E present in the circulation of males comes from the brain for the following reasons: (*i*) [³H]E was consistently identified in blood leaving the brain after direct telencephalic androgen injections, (*ii*) much greater quantities of [³H]E were found in venous than in arterial blood of the brain after vascular [³H]AA injections, (*iii*) [³H]E was consistently extracted in large amounts from aromatase-containing brain regions after brain, adrenal, or vascular [³H]AA injections, and (*iv*) aromatase activity is high in homogenates of TEL and ovary but not in testes or adrenals (25).

GENERAL DISCUSSION

The present data establish that when the zebra finch brain is exposed to AA, either via direct injection or through the vascular system, the brain converts the androgen to E and releases E into the circulation. Injections of AA into ovary also result in release of E into blood, as expected because the ovary expresses aromatase. In contrast, no E enters blood after injections into testis. Some E can be detected in blood after large (presumably supraphysiological) androgen injections into the small adrenal gland, but as discussed above, the origin of this E is in some doubt. Thus, these results suggest that the brain is the major source of plasma E in male zebra finches.

Classically, the plasma levels of gonadal steroids are thought to be regulated by gonadotrophins that act on the gonads to stimulate steroidogenesis. In turn, the gonadotrophins are regulated by negative feedback by the circulating steroids. In zebra finch males, we assume the plasma levels of AA are regulated by such a classical negative feedback loop between the gonads (or the adrenals) and the hypothalamo-hypophyseal system. If E synthesis by brain is

constitutive and depends solely on the levels of androgenic substrate available from the blood, then E levels would be coregulated by androgenic negative feedback mechanisms and would parallel the circulating level of AAs. However, since plasma levels of E have been reported to vary independently of levels of AAs in zebra finches (18, 20, 22), it is intriguing to speculate that cerebral synthesis of E might be regulated independently, by unknown factors. If so, then the regulation of plasma E in male zebra finches might not fit classical concepts of regulation of plasma levels of gonadal steroids. It will be exciting to discover the factors that regulate E synthesis in this species.

Since females also possess high levels of aromatase in TEL and AA in blood, the brain of females may also contribute significantly to Es found in blood, especially after ovariectomy (22). Importantly, this tissue distribution of aromatase in adults is similar to that in hatchling zebra finches at ages when the brain is sensitive to the sexually differentiating effects of E (26). Between 1 and 13 days of age, aromatase is undetectable in testes, adrenals, or other tissues of males but is present in high amounts in ovary and TEL of both males and females. Because AAs circulate at high levels (20, 22, 26), it is likely that the brain may also be the source of circulating E in males at these ages. This implies that the estrogenic masculinization of song relies on E synthesized in brain.

The observation that the brain is the source of circulating E changes our concept of E action in the central nervous system of this species. We no longer conceive of the brain as simply a target for Es in blood but rather view the brain as an active modulator of circulating E levels. With this view, we wonder whether there are E targets in males both inside and outside of the brain that are regulated by E formed in brain. Although it appears that the neuroendocrine control of E levels in songbirds is unique, our results raise the question whether regulation of sex steroids follows a similar pattern in other species such as teleosts, in which brain-derived Es may also contribute to circulating hormone levels in adults (32) or during ontogeny in mammals when neural aromatase can be expressed transiently at higher levels (33).

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