

Brain Aromatase, 5 α -Reductase, and 5 β -Reductase Change Seasonally in Wild Male Song Sparrows: Relationship to Aggressive and Sexual Behavior

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Received 4 October 2002; accepted 22 January 2003

ABSTRACT: In many species, territoriality is expressed only during the breeding season, when plasma testosterone (T) is elevated. In contrast, in song sparrows (*Melospiza melodia morphna*), males are highly territorial during the breeding (spring) and nonbreeding (autumn) seasons, but not during molt (late summer). In autumn, plasma sex steroids are basal, and castration has no effect on aggression. However, inhibition of aromatase reduces nonbreeding aggression, suggesting that neural steroid metabolism may regulate aggressive behavior. In wild male song sparrows, we examined the neural distribution of aromatase mRNA and seasonal changes in the activities of aromatase, 5 α -, and 5 β -reductase, enzymes that convert T to 17 β -estradiol, 5 α -dihydrotestosterone (5 α -DHT, a potent androgen), or 5 β -DHT (an inactive metabolite), respectively. Enzyme activities were measured in the diencephalon, ventromedial telencephalon (vmTEL, which includes avian amy-

dala), caudomedial neostriatum (NCM), and the hippocampus of birds captured during spring, molt, or autumn. Aromatase and 5 β -reductase changed seasonally in a region-specific manner. Aromatase in the diencephalon was higher in spring than in molt and autumn, similar to seasonal changes in male sexual behavior. Aromatase activity in the vmTEL was high in both spring and autumn but significantly reduced at molt, similar to seasonal changes in aggression. 5 β -Reductase was not elevated during molt, suggesting that low aggression during molt is not a result of increased inactivation of androgens. These data highlight the relevance of neural steroid metabolism to the expression of natural behaviors by free-living animals. © 2003 Wiley Periodicals, Inc. *J Neurobiol* 56: 209–221, 2003

Keywords: amygdala; DHEA; estradiol; estrogen; hypothalamus; limbic system; nucleus taeniae; reproduction; songbird; testosterone

Aggressive defense of an exclusive territory is critical for reproductive success and survival in many species.

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Contract grant sponsor: NIH NRSA; contract grant number: MH12978 (K.K.S.).

Contract grant sponsor: NIH; contract grant number: MH61994 (B.A.S.).

Contract grant sponsor: NSF; contract grant number: IBN 9631350 (J.C.W.).

Contract grant sponsor: J.D. French Alzheimer's Foundation (C.J.S.).

Contract grant sponsor: Alzheimer's Association (C.J.S.).

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DOI 10.1002/neu.10225

Territorial aggression has been clearly linked to plasma levels of testosterone (T) in numerous laboratory and field studies (e.g., Wingfield et al., 2001), and in most cases the effects of T are dependent on its metabolism to 17 β -estradiol (E₂) in the brain (Haug et al., 1986; Schlinger and Callard, 1990; Toda et al., 2001).

However, there are an increasing number of well-studied species in which aggression and plasma T are uncoupled. For example, seasonal changes in aggression do not parallel circulating T in several mammals and birds (Schwabl and Kriner, 1991; Soma and Wingfield, 1999; Jasnow et al., 2000; Canoine and Gwinner, 2002). Furthermore, castration does not de-

crease aggression in some species (e.g., Demas et al., 1999; Pinxten et al., 2000). Aggression may be dissociated from plasma T in some species or seasons because circulating T affects many tissues and has major costs, such as lower body condition, impaired immune function, and reduced survivorship (Wingfield et al., 2001a; Wingfield and Soma, 2002).

Male song sparrows in western Washington State, U.S.A. (*Melospiza melodia morphna*) are sedentary and aggressively defend territories against conspecifics year round, except during the prebasic molt in late summer (Wingfield and Hahn, 1994). In the nonbreeding season (autumn and early winter), males respond strongly to simulated territorial intrusions, even though the testes are completely regressed and plasma T, 5 α -dihydrotestosterone (5 α -DHT), androstenedione (AE), E₂, and estrone (E₁) levels are all nondetectable (≤ 0.15 ng/mL) (Wingfield and Hahn, 1994; Soma and Wingfield, 1999, 2001; K. Soma, M. Hau, and D. Painter, unpublished results). Furthermore, castration does not decrease nonbreeding aggression (Wingfield, 1994). In contrast, inhibition of aromatase, the enzyme that catalyzes the conversion of T to E₂, clearly decreases aggression in autumn, indicating a behavioral role for aromatase in the nonbreeding season (Soma et al., 1999c, 2000a,b).

As a first step towards identifying the location of behaviorally relevant aromatase, we examined aromatase mRNA distribution in the brain of song sparrows. In other songbirds, males have high levels of aromatase in the diencephalon and much of the telencephalon (Vockel et al., 1990; Balthazart et al., 1999; Metzdorf et al., 1999; Saldanha et al., 2000; Silverin et al., 2000). Aromatase expression in zebra finches (*Taeniopygia guttata*) is high in large portions of the caudal telencephalon, including nucleus taeniae (Tn), the hippocampus (HP), and the caudomedial neostriatum (NCM) (Shen et al., 1995; Saldanha et al., 2000).

As a second approach, we examined aromatase activity in brain regions thought to regulate aggression and looked for seasonal changes in aromatase that matched seasonal changes in aggression. Aromatase was examined during the breeding, molt, and nonbreeding seasons. Additionally, we examined 5 α - and 5 β -reductase in the same brain regions and seasons. 5 α -Reductase metabolizes T to 5 α -DHT, a potent androgen, and 5 β -reductase deactivates T to 5 β -DHT, a steroid that is considered behaviorally inactive (Hutchison and Steimer, 1981).

We examined enzyme activities in two regions that regulate motivated behaviors, such as sexual behavior and aggression: the diencephalon (Schlinger and Callard, 1989; Balthazart et al., 1999) and Tn (Thompson

et al., 1998; Cheng et al., 1999; Absil et al., 2002). Aromatase in the diencephalic medial preoptic nucleus is critical for the expression of male sexual behavior in Japanese quail (*C. coturnix*) (Balthazart et al., 1999). Diencephalic aromatase is also positively correlated with male aggressive behavior in Japanese quail (Schlinger and Callard, 1989) and male Lapland longspurs (*Calcarius lapponicus*) (Soma et al., 1999a). The neuroanatomy and functions of Tn have received less attention. However, Tn contains high levels of aromatase, androgen and estrogen receptors and projects to the hypothalamus and preoptic area (reviewed in Absil et al., 2002). Additionally, in wild male song sparrows, simulated territorial intrusions that elicit aggression also induce the expression of c-fos in Tn (Wingfield et al., 2001b and S. Meddle, unpublished results). Lesions of Tn affect male sexual behavior in a complex fashion (Thompson et al., 1998; Absil et al., 2002), but the effects on aggression have not been assessed. These studies suggest that Tn is homologous to the mammalian medial amygdala (Thompson et al., 1998; Cheng et al., 1999; Absil et al., 2002), a brain region that regulates social and motivated behavior in rodents (Newman, 1999). Thus, there is converging evidence that the diencephalon and Tn are good candidate regions in which to measure androgen-metabolizing enzymes with respect to the control of sexual and aggressive behaviors.

We also examined two regions that are not thought to regulate aggression, the HP and NCM. The HP is implicated in spatial memory, particularly in food-hoarding species that cache and retrieve food in autumn and winter (Saldanha et al., 1998). Song sparrows do not hoard food, and consistent with this observation, HP size does not vary seasonally in song sparrows (Lee et al., 2001). The NCM may function in song perception (Mello et al., 1992), which does not appear to vary seasonally in captive song sparrows (Reeves et al., 2001). Based on the above information, we hypothesized that androgen-metabolizing enzymes in the HP and NCM would not vary seasonally, making them useful "control" regions.

Data from song sparrows and other species raise the hypothesis that seasonal changes in aggression are positively correlated with aromatase in the diencephalon or Tn (e.g., Schlinger and Callard, 1989). A second hypothesis is that aggression is not correlated with aromatase but is negatively correlated with 5 β -reductase, which inactivates T (e.g., Delville et al., 1984). These hypotheses generate specific predictions. The first hypothesis predicts low aromatase activity during molt, when aggression is low, and high aromatase during the breeding and nonbreeding seasons, when aggression is high. The second hypothesis

predicts high 5 β -reductase activity during molt and low 5 β -reductase during the breeding and nonbreeding seasons.

MATERIALS AND METHODS

All animals were treated according to the NIH "Principles of Animal Care," and protocols were approved by the University of Washington and University of California Institutional Animal Care and Use Committees. Using *in situ* hybridization, we documented aromatase mRNA in the brain. Next, in two separate biochemistry experiments, we measured the activities of aromatase and other androgen-metabolizing enzymes in various brain regions and seasons.

Subjects

In Situ Hybridization. To examine the regional distribution of aromatase mRNA in the brain, we used *in situ* hybridization. Subjects were free-living male song sparrows caught during the spring breeding season ($n = 3$) or autumn nonbreeding season ($n = 1$).

Biochemistry Experiment 1: Regional Distribution of Androgen-Metabolizing Enzymes. Subjects were free-living male song sparrows caught at Montlake Reserve (King County, WA) in either late winter (late January–early February 1998, $n = 6$) or spring (early May 1998, $n = 6$).

Biochemistry Experiment 2: Seasonal Changes in Androgen-Metabolizing Enzymes. This experiment included subjects from all three major stages in the annual cycle of song sparrows: breeding, molt, and nonbreeding seasons (as in Soma and Wingfield, 2001). Subjects were free-living male song sparrows caught at Montlake Reserve or Pack Forest Research Station (Pierce County, WA) in autumn (early November 1999, $n = 3$), spring (early May 2000, $n = 3$), or molt (mid-August 2000, $n = 6$). Spring birds were in breeding condition, and autumn birds were all clearly in nonbreeding condition. Molting birds were replacing numerous feathers on the body, tail, head, and wings.

Field Protocol

Subjects were captured using mist nets and conspecific song playback (generally less than 3 min). Blood from the alar wing vein was collected (within 5–10 min of capture) into heparinized microhematocrit tubes and stored at 4°C until centrifugation (within 5 h). After centrifuging, plasma was collected and stored at –20°C. After collecting blood, we measured cloacal protuberance (CP) length. The CP is an androgen-sensitive organ used in sperm delivery.

Aromatase *In Situ* Hybridization

To examine the distribution of aromatase mRNA in song sparrow brain, we performed *in situ* hybridization. Subjects were captured in the field, taken to a laboratory, and sacrificed by rapid decapitation at 30 min after capture. Brains were collected, flash frozen on dry ice, and stored at –80°C. *In situ* hybridization was performed on 14 μ m coronal sections using well-established protocols (for details, see Freking et al., 2000). Briefly, the ³³P-labeled antisense riboprobe was generated from a 1.3 kb songbird aromatase cDNA (ZF1A-10). Hybridization was performed using standard procedures (Freking et al., 2000). To detect hybridization, slides were exposed to autoradiographic film for 3 days. Slides were then dipped in emulsion and stored at 4°C for 3 weeks before developing. Slides were lightly counterstained with thionin and examined with bright- and dark-field microscopy.

Tissue Collection for Biochemistry

Subjects were taken to a laboratory and killed by rapid decapitation at 30 min after capture. Androgen-metabolizing enzymes were measured in four brain regions: HP, ventromedial telencephalon (vmTEL) including Tn, caudomedial neostriatum (NCM), and diencephalon (DIEN) including the preoptic area. All regions were collected bilaterally.

The songbird HP is on the dorsal surface of the brain and was separated from the surrounding tissue by two parasagittal cuts (≈ 1 mm from the midline), starting just anterior to the cerebellum and going down ≈ 1 mm in depth (for details, see Saldanha et al., 1998, 1999; Soma et al., 1999a). The NCM lies just ventral to the HP and was dissected out as the dorsocaudal telencephalon that is within ≈ 1 mm of the midline, to a depth of ≈ 2 mm (Saldanha et al., 1999; Soma et al., 2000a). To collect the DIEN, the ventral surface was exposed, and a cut was made at the level of the mammillary bodies. The optic chiasm was removed, exposing the basal hypothalamus. The DIEN was removed to the depth of the anterior commissure, including the preoptic area (as in Soma et al., 1999a). The vmTEL was collected based on the position of Tn in other birds (Thompson et al., 1998; Soma et al., 1999a, b; Cheng et al., 1999) and aromatase *in situ* hybridization in song sparrows (see Results). Tn is a long, narrow nucleus that lies in the ventromedial portion of the caudal telencephalon, within 1 mm of the ventral edge of the telencephalon. Dissected tissues were immediately frozen on dry ice and stored at –80°C. Dissections were completed within 15 min of sacrifice. The length and width of the left testis were measured, and testis volume was calculated (Soma et al., 1999b).

Measurement of Androgen-Metabolizing Enzymes

Activities of aromatase, 5 α -, and 5 β -reductase were measured in tissue homogenates using the *in vitro* metabolism

of [^3H]androstenedione (AE) to [^3H]estrone (E_1), [^3H]estradiol (E_2), [^3H]5 α -androstenedione (5 α -A), and [^3H]5 β -androstenedione (5 β -A). This assay has been used in many species (for details, see Schlinger and Callard, 1989; Saldanha et al., 1998) and has been validated in song sparrows and closely related birds (Soma et al., 1999a, 2000a). For each experiment, all samples were analyzed in a single assay to avoid interassay variability.

Briefly, tissues were homogenized in 200 μL of ice-cold sucrose-phosphate buffer. Homogenates (180 μL) were incubated with 100 nM [^3H]AE (specific activity 74 Ci/mmol; NEN) for 5 min at 41°C in the presence of a NADH/NADPH-generating cofactor cocktail (20 μL) and 1 μg radioinert E_1 and E_2 (Steraloids, Newport, RI). Radioinert estrogens were included to protect formed tritiated estrogens from further metabolism (as in Soma et al., 2000a). Control tubes contained everything but tissue. Reactions were terminated by snap freezing. To correct for procedural losses, tubes containing a known amount of [^3H]AE or [^3H] E_1 were processed in parallel.

Steroids were extracted with diethyl ether (3 \times), and androgens were separated from estrogens by phenolic partition (2 \times) (as in Schlinger and Callard, 1989). Radioinert E_1 , E_2 , T, AE, 5 α -A, and 5 β -A (Steraloids) were added as markers, and steroids were separated using thin-layer chromatography (Schlinger and Callard, 1989). Steroids were visualized under ultraviolet light. After scraping the bands, tritiated steroids were eluted from the silica and measured in a scintillation counter. The cpm were corrected for background and procedural losses and are reported as femtomoles per milligram of protein. Protein content of the homogenates was measured by the method of Bradford (1976) using bovine serum albumin standards.

Measurement of Plasma Testosterone

Plasma T was measured using radioimmunoassay (see Wingfield and Farnar, 1975). Briefly, steroids were extracted with methylene chloride and separated using diatomaceous earth/glycol chromatography columns. For radioimmunoassay, we used a well-characterized T antibody (Wien Laboratories, NJ) (Wingfield and Hahn, 1994; Soma et al., 1999b). For each experiment, samples were analyzed in a single assay, to avoid interassay variation.

Statistics

Results are expressed as mean \pm S.E.M. Data were analyzed according to Zar (1984) using SuperANOVA for Macintosh. When appropriate, data were log-transformed prior to statistical analyses (Zar, 1984), and these are the results presented. Note that the results remain the same without log transformation; significant results remain significant when the raw data are used. Plasma samples with nondetectable hormone levels were assigned the assay detection limit for statistical tests.

To assess seasonal changes for each enzyme, enzyme activities were analyzed using a two-way ANOVA (season

\times brain region). Only if the interaction was significant did we examine the effect of season within each brain region (one-way ANOVA followed by *post hoc* Fisher's Protected LSD tests). This approach was specifically used to limit the number of statistical comparisons made. All tests were two-tailed, and α was set at 0.05.

RESULTS

Regional Distribution of Aromatase mRNA and Activity in Song Sparrow Brain

In Situ Hybridization. We examined the aromatase mRNA distribution in free-ranging male song sparrows in breeding condition. Alternate sections were Nissl-stained. The general pattern of aromatase mRNA (Figs. 1 and 2) was similar to that of other songbirds. In the diencephalon, hybridization was clear in the preoptic area (POA) and ventromedial nucleus (VMN) [Fig. 2(B,C)].

In the telencephalon, aromatase mRNA was detectable in the medial magnocellular nucleus of the anterior neostriatum (mMAN) and the surrounding area [Fig. 2(A)], as in other songbirds (Shen et al., 1995; Balthazart et al., 1996; Metzdorf et al., 1999). Aromatase mRNA was also detectable in the hyperstriatum (H) at the level of the rostral POA [Fig. 2(B)] and in the bed nucleus of the stria terminalis (BNST) at the level of the caudal POA [Fig. 2(C)]. More caudally, high levels of aromatase expression were present in NCM and Tn, and the HP also contained aromatase mRNA [Figs. 1 and 2(D)]. In developed slides but not autoradiographic film, a few labeled cells were visible in the caudomedial portion of HVC (as in Shen et al., 1995). In the most caudal portions of the telencephalon, aromatase was expressed in the neostriatum [Fig. 2(E)]. No labeled cells were visible in the robust nucleus of the archistriatum (RA) [Fig. 2(E)].

In addition, we performed *in situ* hybridization on the brain of a single wild nonbreeding male and the general distribution of aromatase mRNA was similar to that of breeding birds.

Regional Distribution of Enzyme Activities (Biochemistry Experiment 1)

Regional differences in aromatase, 5 β -, and 5 α -reductase activities were examined using biochemistry. Spring birds ($n = 6$) were in breeding condition with large testes and CPs and high plasma T levels (Table 1). Among winter birds ($n = 6$), no subjects were in breeding condition, and all winter subjects had smaller testes and CPs than breeding birds. However, upon closer inspection, two

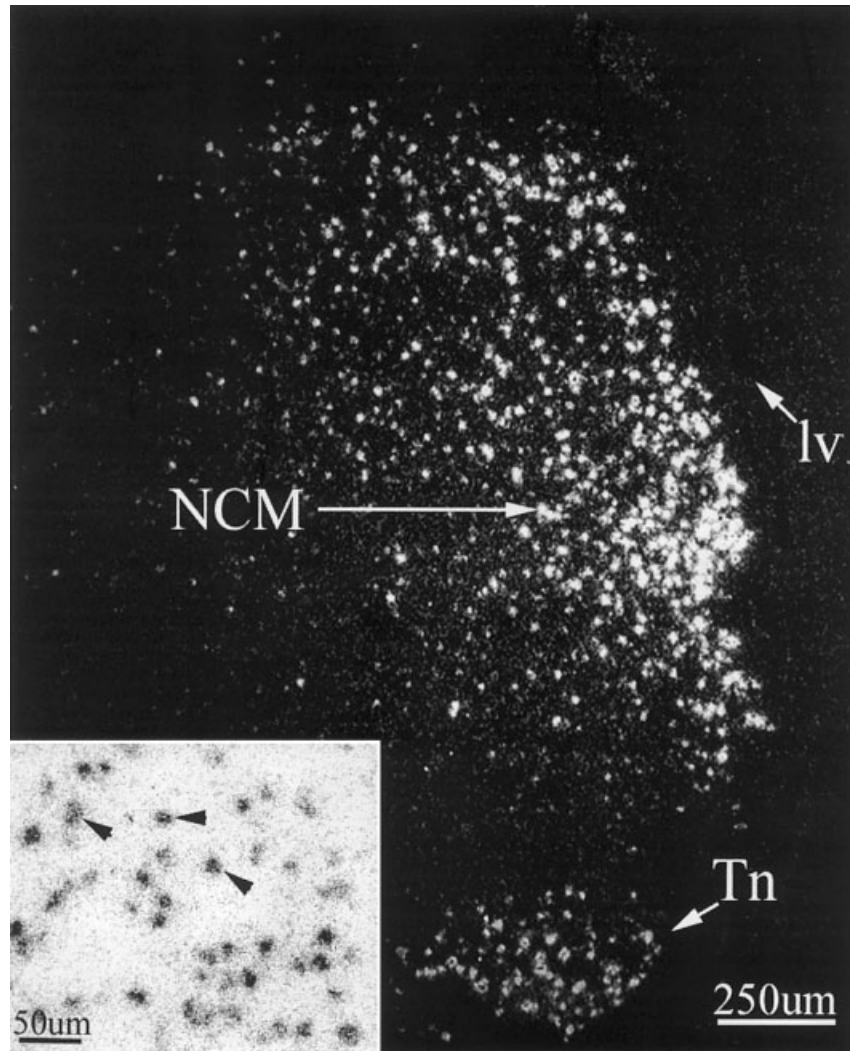


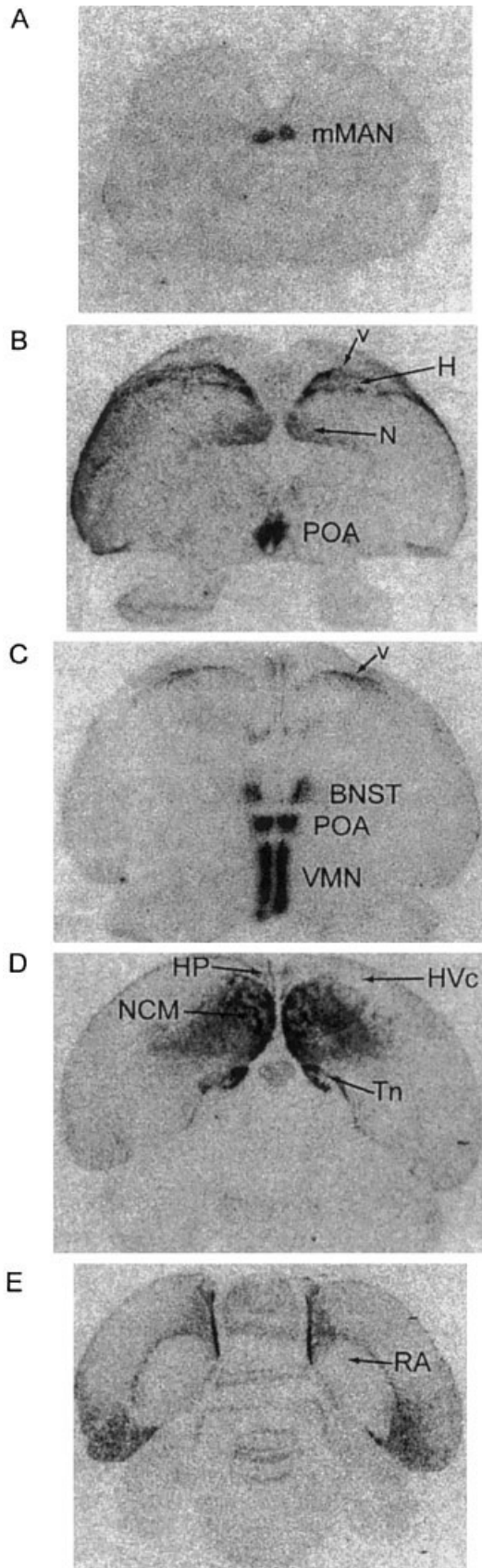
Figure 1 Dark- and bright-field photomicrographs of aromatase mRNA expression in song sparrow telencephalon. Subject was a free-ranging adult male song sparrow in spring. High levels of aromatase expression were detected adjacent to the lateral ventricle (lv) in the caudal telencephalon, including the caudomedial neostriatum (NCM) and nucleus taeniae (Tn). Inset: higher magnification showing silver grains over neurons in Tn, avian homologue to the mammalian medial amygdala.

winter subjects had completely regressed testes and basal plasma T (≈ 0.15 ng/mL) (nonbreeding birds), and four winter subjects had just started gonadal recrudescence and had slightly higher plasma T levels (prebreeding birds) (Table 1). Therefore, in an effort to be precise, winter birds were separated *post hoc* into nonbreeding and prebreeding subjects. The sample size is low for nonbreeding birds, so we did not analyze regional differences in these birds statistically.

For aromatase, we initially examined regional differences without regard to breeding condition ($n = 12$), and significant regional differences were present

($F = 18.36$, $p < 0.01$) (NCM > vmTEL > HP = DIEN). If the analysis is limited to breeding birds ($n = 6$) or prebreeding birds ($n = 4$), the patterns of results were similar to the analysis with all the subjects (Table 1).

For 5 β -reductase, analysis without regard to breeding condition ($n = 12$) indicated significant regional differences ($F = 8.71$, $p < 0.01$) (vmTEL = NCM > DIEN = HP). The pattern of results for breeding birds was the same (Table 1). The results for prebreeding birds were similar, except that the HP had significantly lower 5 β -reductase than all other regions (Table 1).



For 5α -reductase, analysis including all subjects ($n = 12$) indicated significant regional effects ($F = 4.92$, $p < 0.01$). The NCM had significantly lower 5α -reductase than the other regions, which were not different from each other. The same pattern of results was obtained if the analysis was restricted to breeding birds (Table 1). The results for prebreeding birds were also similar, except that the DIEN was not significantly different from NCM (Table 1).

Seasonal Changes in Androgen-Metabolizing Enzymes (Biochemistry Experiment 2)

Seasonal differences in enzymes were compared among the major stages of the annual cycle: breeding, molt, and nonbreeding. Breeding and nonbreeding birds are highly aggressive, while molting birds are less aggressive (Wingfield and Hahn, 1994).

Breeding Condition. Playback time to capture subjects did not differ among seasons ($F = 1.20$, $p = 0.35$). There were significant seasonal differences in plasma T, CP length, and testis volume (Table 2). Spring birds were in breeding condition. Molting birds had basal plasma T and their testes were almost fully regressed. Autumn birds all had basal plasma T levels and completely regressed testes and were all unequivocally in nonbreeding condition. These results are very similar to previous data from song sparrows (Wingfield and Hahn, 1994; Soma et al., 1999c, 2000a,b).

Androgen-Metabolizing Enzymes. For all regions, there was no seasonal change in milligrams of protein in homogenates ($F = 2.29$, $p = 0.12$), suggesting that dissections were consistent.

For aromatase (Fig. 3, top panel), there were significant effects of season ($F = 6.28$, $p < 0.01$) and season \times brain region ($F = 3.72$, $p < 0.01$). The effects of brain region have been described (see Ex-

Figure 2 Rostral (A) to caudal (E) distribution of brain aromatase mRNA from autoradiographic film. Subject was a wild male song sparrow in spring. (A) Expression in the medial magnocellular nucleus of the anterior neostriatum (mMAN). (B) Expression in the hyperstriatum (H) near the ventricles (v), the neostriatum (N), and preoptic area (POA). (C) Expression in the bed nucleus of the stria terminalis (BNST), POA, and ventromedial nucleus (VMN). (D) Expression in NCM, Tn, and HP. (E) Expression in the caudal neostriatum, but not in the robust nucleus of the archistriatum (RA).

Table 1 Regional Differences in Aromatase, 5 β -, and 5 α -Reductase Activities in Free-Ranging Male Song Sparrows (Experiment 1)

	Breeding	Prebreeding	Nonbreeding
Aromatase:			
HP	36.3 \pm 7.5 ^b	52.7 \pm 19.4 ^{bc}	28.1 \pm 8.5
vmTEL	140.5 \pm 14.4 ^a	100.5 \pm 18.6 ^b	81.5 \pm 15.6
NCM	224.5 \pm 58.4 ^a	267.2 \pm 69.7 ^a	79.0 \pm 20.6
DIEN	36.7 \pm 6.3 ^b	36.9 \pm 6.0 ^c	11.7 \pm 1.3
5 β -Reductase:			
HP	498.6 \pm 127.7 ^b	166.9 \pm 38.7 ^c	132.6 \pm 34.1
vmTEL	3591.5 \pm 734.8 ^a	2408.1 \pm 781.9 ^a	1363.2 \pm 622.7
NCM	3072.4 \pm 1406.3 ^a	1435.7 \pm 357.2 ^a	994.2 \pm 518.7
DIEN	480.8 \pm 135.4 ^b	545.7 \pm 133.9 ^b	263.1 \pm 20.7
5 α -Reductase:			
HP	155.1 \pm 28.2 ^a	193.0 \pm 66.3 ^a	110.5 \pm 3.7
vmTEL	144.1 \pm 40.1 ^a	196.3 \pm 56.7 ^a	24.3 \pm 21.0
NCM	70.3 \pm 32.5 ^b	9.5 \pm 5.6 ^b	66.3 \pm 66.3
DIEN	128.3 \pm 16.4 ^a	69.0 \pm 23.9 ^{ab}	69.7 \pm 28.8
Plasma T (ng/mL)	4.18 \pm 0.79	0.83 \pm 0.14	0.16 \pm 0.01
CP length (mm)	7.9 \pm 0.3	3.7 \pm 0.1	3.5 \pm 0.5
Testis volume (mm ³)	285.2 \pm 30.0	3.4 \pm 1.1	0.5 \pm 0.3

Within breeding and prebreeding groups, superscript letters denote regional differences (see Results). Regional differences were not analyzed in nonbreeding birds ($n = 2$). Seasonal differences were examined in a separate study (see Fig. 3).

periment 1) and were not included in Experiment 2 in order to limit the number of statistical comparisons. Across regions, aromatase was significantly higher in spring than in molt, and showed a trend to be higher in autumn than in molt ($p = 0.056$). Specifically, in the NCM and DIEN, aromatase was significantly higher in spring than in molt and autumn. Importantly, in the vmTEL, aromatase was similar in spring and autumn and significantly reduced during molt. Aromatase in vmTEL, which contains Tn, paralleled seasonal changes in aggression.

For 5 β -reductase (Fig. 3, middle panel), there were significant effects of season ($F = 8.45$, $p < 0.01$) and season \times brain region ($F = 2.70$, $p = 0.029$). Across regions, 5 β -reductase was significantly higher in spring than in molt and autumn. In the vmTEL and NCM, 5 β -reductase levels were significantly higher in spring than in molt. In the DIEN, 5 β -reductase was higher in autumn than in molt.

For 5 α -reductase (Fig. 3, bottom panel), there were

significant effects of season ($F = 6.32$, $p < 0.01$). Across regions, 5 α -reductase was significantly higher in spring than in molt and autumn.

DISCUSSION

We detected region-specific changes in brain aromatase, 5 α -, and 5 β -reductase activities over the annual cycle of free-living male song sparrows. The data highlight the importance of examining particular telencephalic regions at natural life history stages. Here, we examined three telencephalic regions that lie close to each other, and different patterns emerged for each one. In addition, few studies have examined the neural changes at molt that underlie the reduced singing and territorial behavior that is typical of a wide variety of molting birds. Song sparrows are well-suited for such studies because their behavioral and hormonal cycles in the field are known (e.g., Wingfield and Hahn,

Table 2 Breeding Conditions of Subjects in Experiment 2

	Spring	Molt	Autumn	p
Plasma T (ng/mL)	3.1 \pm 1.2 ^a	0.12 \pm 0.03 ^b	0.17 \pm 0.04 ^b	<0.01
CP length (mm)	8.6 \pm 0.4 ^a	5.2 \pm 0.5 ^b	4.0 \pm 0.1 ^b	<0.01
Testis volume (mm ³)	296.7 \pm 43.3 ^a	4.3 \pm 1.7 ^b	0.8 \pm 0.2 ^c	<0.01

p values are from ANOVA and denote seasonal differences. Seasons that are significantly different ($p < 0.05$) are denoted by superscript letters.

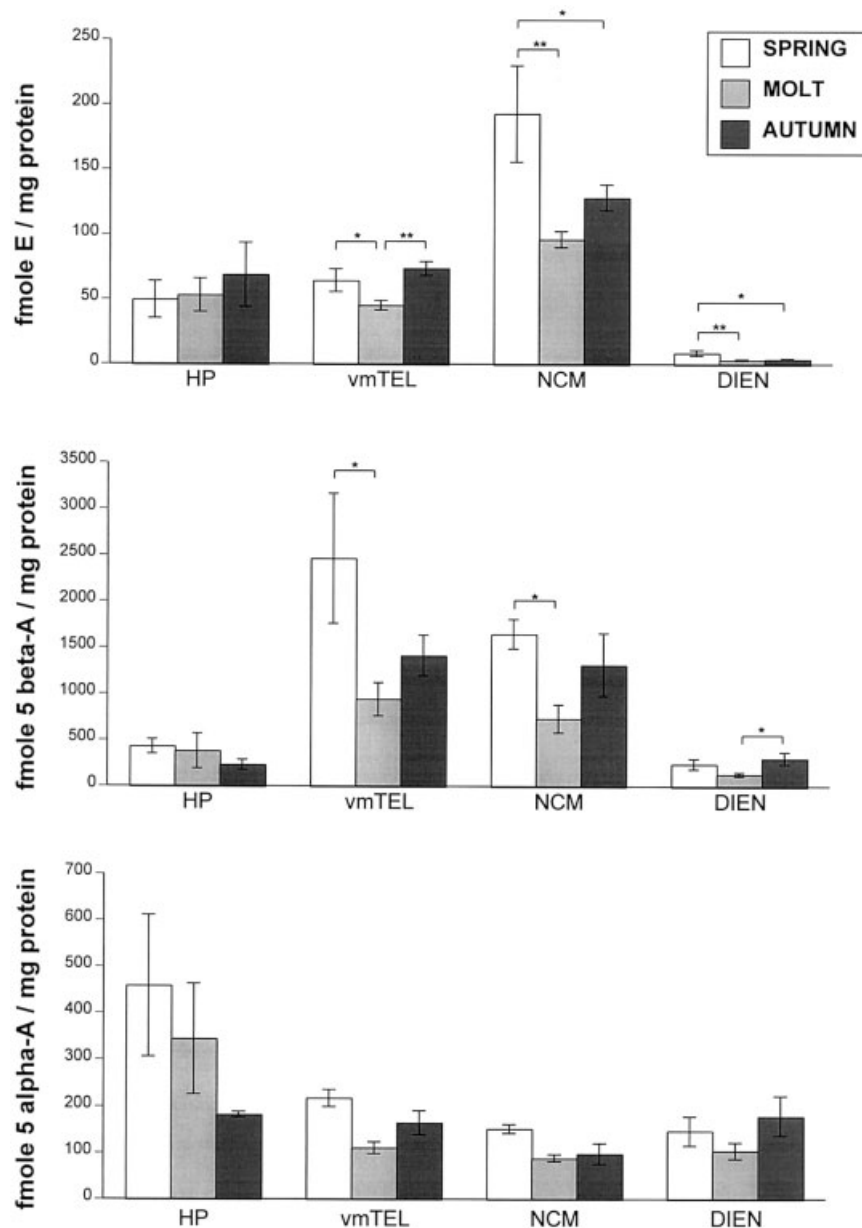


Figure 3 Seasonal changes in aromatase (top panel), 5β -reductase (middle panel), and 5α -reductase (bottom panel) in wild male song sparrow brain (Biochemistry Experiment 2). Enzyme products are estrone + estradiol (E) for aromatase, 5β -androstenedione (5β -A) for 5β -reductase, and 5α -androstenedione (5α -A) for 5α -reductase. Enzyme activities were measured in hippocampus (HP), ventromedial telencephalon (vmTEL) including nucleus taeniae, caudomedial neostriatum (NCM), and diencephalon (DIEN). Free-ranging subjects were captured in spring (breeding), molt, and autumn (nonbreeding). * $p < 0.05$, ** $p < 0.01$.

1994), and experimental manipulations have been done in the field (e.g., Soma et al., 2000b). Moreover, seasonal changes in natural behaviors, hormones, neuroanatomy, and neurochemistry are in many cases more pronounced in wild than in captive animals (Tramontin and Brenowitz, 2000; Wingfield et al., 2001), indicating the value of field work for under-

standing adult neuroplasticity and its relationship to natural behaviors.

Regional Differences

There are large regional differences in androgen-metabolizing enzyme activities in the song sparrow

brain. With regard to aromatase, activity is lowest in the DIEN, intermediate in the HP and vmTEL, and highest in the NCM. The *in situ* hybridization results also show high aromatase expression in NCM and Tn. Overall, the present data are consistent with previous studies of aromatase activity, mRNA, and immunoreactivity in other songbirds (Shen et al., 1995; Balthazart et al., 1996; Metzdorf et al., 1999; Saldanha et al., 2000). In song sparrows, aromatase mRNA in the song nucleus mMAN seems especially prominent, and mMAN aromatase might regulate song behavior. There is also robust aromatase expression in the rostral hyperstriatum near the lateral ventricles, and this aromatase may be involved in the production or migration of new neurons. In songbirds, high levels of neurogenesis continue in adulthood, and new neurons are derived from the division of radial cells in the ventricular zone (Alvarez-Buylla et al., 1990). In non-songbirds (e.g., Japanese quail, *C. coturnix*) and mammals, telencephalic aromatase is generally less abundant and more spatially restricted than in songbirds (for details, see Saldanha et al., 1998; Metzdorf et al., 1999; Silverin et al., 2000; Naftolin et al., 2001).

5 β -Reductase is abundant in song sparrow brain, as in other birds (Vockel et al., 1990; Saldanha et al., 1999). 5 β -Reductase is higher in the vmTEL and NCM than in the HP and DIEN. In other songbirds, 5 β -reductase activity is also highest in the telencephalon (Vockel et al., 1990; Soma et al., 1999a; but see Riters et al., 2001). The distribution of 5 β -reductase remains unclear because few studies have examined specific regions within the telencephalon (Vockel et al., 1990; Saldanha et al., 1999; Soma et al., 1999a; present study). No studies have examined the distribution of 5 β -reductase mRNA or immunoreactivity in the songbird brain. In general, telencephalic 5 β -reductase levels appear similar in songbirds and non-songbirds (Schlinger and Brenowitz, 2002). In contrast to birds, 5 β -reductase may not be highly expressed in the brains of most mammals (Schlinger and Brenowitz, 2002).

5 α -Reductase is highest in the HP and vmTEL, intermediate in the DIEN, and lowest in the NCM. Similarly, in other songbirds and in nonsongbirds, high 5 α -reductase activity can be found in both diencephalic and telencephalic regions (Vockel et al., 1990; Soma et al., 1999a; Riters et al., 2001). The distribution of 5 α -reductase in the songbird brain has not yet been examined using *in situ* hybridization or immunocytochemistry. In rodents, 5 α -reductase is expressed in glia and neurons of many regions in a complex age-dependent manner (Compagnone and Mellon, 2000).

Seasonal Changes

The three enzymes change across spring, molt, and autumn in a region-specific manner, indicating complex and dynamic steroid metabolism in the brain that occurs along with changes in hormones. Different seasonal patterns are found in regions very close to each other, suggesting brain microenvironments that differ in steroidal milieu. The sample sizes in the present study were limited by the difficulty of conducting seasonal studies on wild animals, as well as by constraints on the size of these biochemical assays. Nonetheless, the seasonal differences were often large and the within-group variabilities were generally low, which allowed us to detect significant seasonal changes. Seasonal changes in behavior, hormones, and the brain are often more pronounced in wild animals (e.g., Wingfield et al., 2001). Also, patterns were similar across experiments (compare spring birds in Biochemistry Experiments 1 and 2).

In the HP, a region involved in spatial memory, we did not detect much seasonal variation in androgen-metabolizing enzymes. These data are consistent with the observations that these song sparrows are not migratory, do not store food in winter, and show no seasonal change in HP volume (Lee et al., 2001).

In the NCM, a brain region involved in song audition and/or perception (Mello et al., 1992), all three enzymes are highest in spring, when the birds hear the most song. Although birds in autumn sing in response to a simulated territorial intrusion, basal singing in the absence of a simulated intrusion is low (Smith et al., 1997; Wingfield, 1994; Soma et al., 2002b). It is possible that hearing song up-regulates enzymes in NCM or, alternatively, that seasonal changes of enzymes in NCM produce changes in song perceptual abilities (Fusani et al., 2000). Song perception in captive male song sparrows does not appear to vary seasonally (Reeves et al., 2001), but neither wild nor molting birds have been examined. Local implants of aromatase inhibitors and/or estradiol to NCM would be an informative next step.

In the DIEN, aromatase is higher in spring than in molt and autumn. Thus, seasonal changes in DIEN aromatase are not correlated with male aggressive behavior. DIEN aromatase is, however, positively correlated with seasonal changes in plasma T and male sexual behavior, as in studies of other birds (e.g., Foidart et al., 1998; Silverin et al., 2000). DIEN aromatase, therefore, may closely match plasma T but not regulate nonbreeding aggression. Some studies have found a correlation between DIEN aromatase and aggression, but these studies were largely conducted on breeding birds with elevated plasma T

(Schlinger and Callard, 1989; Foidart et al., 1998; Soma et al., 1999a). There is one study in which DIEN aromatase has been correlated with aggression outside of the breeding season (Silverin and Deviche, 1991), but the songbird in this study (*Parus major*) has an autumnal peak in plasma T, unlike song sparrows. Taken together, these studies suggest that DIEN aromatase is correlated with aggression only when plasma T is elevated. The present data do, however, support the positive correlation between male sexual behavior and DIEN aromatase seen in other species (Balthazart et al., 1999).

With regard to 5β -reductase in the DIEN, activity is not higher at molt, but rather lower at molt. Thus, there is no support for the hypothesis that DIEN 5β -reductase is negatively correlated with seasonal changes in aggressive or sexual behavior in song sparrows. In contrast, in doves (*Streptopelia risoria*), DIEN 5β -reductase increases in long-term castrated birds that show no courtship behavior in response to exogenous testosterone (Hutchison and Steimer, 1981). Thus, in some species, increased DIEN 5β -reductase is associated with decreased male sexual behavior.

vmTEL and Seasonal Changes in Aggression

The vmTEL dissection was designed to include Tn, a distinct brain region in the archistriatum, and included adjacent areas as well. Tn expresses aromatase and estrogen receptors (Bernard et al., 1999). Lesions to Tn in quail affect male sexual behavior (Thompson et al., 1998; Absil et al., 2002). In wild male song sparrows, simulated territorial intrusions induce c-fos expression in Tn (Wingfield et al., 2001 and S. Meddle, unpublished results). Current experiments are examining the endocrine regulation of neuronal morphology in Tn.

Tn appears homologous to the mammalian medial amygdala, based on connectivity, steroid receptors, and lesion studies (Thompson et al., 1998; Cheng et al., 1999; Absil et al., 2002; see also Naftolin et al., 2001). In mammals, the medial amygdala is part of the steroid-dependent limbic system, a network that regulates social and motivated behavior, such as aggression and reproduction (Kling and Brothers, 1992; Newman, 1999). In rats, the medial amygdala has high aromatase levels that are reduced by castration and increased by T (Roselli et al., 1985), but there may be strain differences (e.g., Mathias et al., 1999; Naftolin et al., 2001). Aggressive encounters increase c-fos expression in the medial amygdala of hamsters (Kollack-Walker and Newman, 1995), and lesions of

the medial amygdala reduce male aggressive behavior in rats (e.g., Vochteloo and Koolhaas, 1987). The medial amygdala is not the only brain region involved in the regulation of a complex behavior such as aggression, but several lines of evidence indicate that it plays an important role.

In the song sparrow vmTEL (which contains Tn), aromatase activity is significantly reduced at molt and is similar in spring and autumn. Consistent with the present data, preliminary experiments using [3 H]DHEA as the substrate also indicate that similar amounts of [3 H]estrogen are formed in the vmTEL of spring and autumn song sparrows (Soma et al., 2002b). The seasonal pattern of aromatase in vmTEL is interesting because it resembles seasonal changes in territorial aggression (Wingfield and Hahn, 1994). During molt, adult male song sparrows do not respond strongly to simulated territorial intrusions, but after molt is completed, there is a resurgence of aggression in autumn. Aggression may be low during molt because aromatase activity is low in vmTEL. Similarly, in Lapland longspurs, there is a trend for aromatase in the vmTEL to match aggression within the breeding season (Soma et al., 1999a). In developing mice, amygdala aromatase is higher in aggressive mice than in nonaggressive mice (Compaan et al., 1994). Future work in song sparrows will examine aromatase in Tn using immunocytochemistry to understand the cellular basis of this seasonal plasticity. Experimental manipulations will also be useful, for example measuring behavioral effects of small E_2 or aromatase inhibitor implants near Tn.

Reduced aggression at molt is not the result of high 5β -reductase activity in vmTEL. On the contrary, 5β -reductase in vmTEL is reduced during molt. 5β -Reductase in vmTEL was not significantly lower in autumn than in spring, but 5β -reductase across all brain regions was significantly lower in autumn than in spring. Such a seasonal change could increase sensitivity of the brain to androgens during autumn and support the expression of nonbreeding aggression.

5α -Reductase activity in vmTEL is increased in spring but not significantly different between molt and autumn. It is unclear whether 5α -reductase regulates aggression in birds. In quail, neither a 5α -reductase inhibitor (4-MA) nor an androgen receptor antagonist (flutamide) decreases aggression (Schlinger and Callard, 1990).

The present data suggest that seasonal changes in aromatase activity in the vmTEL contribute to seasonal changes in aggression, but other mechanisms may also be important. For example, there are seasonal changes in androgen and estrogen receptor expression

in the brain (Gahr and Metzdorf, 1997; Soma et al., 1999b; Fusani et al., 2000). Also, plasma levels of thyroid hormones in birds tend to be increased at molt (Wingfield and Farner, 1993). In rodents, thyroid hormones inhibit the effects of estrogen on behavior (Dellovade et al., 1996). High thyroid hormones in molting song sparrows might reduce estrogen-dependent aggression. Additionally, neuropeptides in the septum are robust modulators of aggression in birds (Goodson, 1998) and might change seasonally.

Moreover, in song sparrows, plasma levels of dehydroepiandrosterone (DHEA), a precursor to aromatizable androgens, are reduced during molt and elevated during spring and autumn (Soma and Wingfield, 2001). DHEA treatment increases territorial singing and the volume of a forebrain song control nucleus (HVC) in autumn song sparrows (Soma et al., 2002c). DHEA might be secreted by the songbird adrenals (Soma and Wingfield, 2001) and then metabolized to AE by 3 β -hydroxysteroid dehydrogenase (3 β -HSD) in the songbird brain (Vanson et al., 1996; Soma et al., 2002a, b). We have detected 3 β -HSD activity in the song sparrow vmTEL, and DHEA is metabolized to AE and then to estrogen (Soma et al., 2002b). Preliminary data suggest that DHEA metabolism to AE and estrogen in the vmTEL is similar in spring and autumn (Soma et al., 2002b), consistent with the present results on vmTEL aromatase. Future studies will continue to examine the neuroendocrine regulation of territorial behavior in wild song sparrows, which has provided novel insights into the biology of naturally occurring aggression.

A. Tramontin, T. Sperry, and I. Moore kindly assisted with field work. S. Meddle generously shared unpublished results on c-fos expression.

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