

## Sex- and age-related differences in the activity of testosterone-metabolizing enzymes in microdissected nuclei of the zebra finch brain

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Many effects of testosterone (T) in the zebra finch (*Taeniopygia guttata*) can be mimicked by T-metabolites, mainly estradiol and 5 $\alpha$ -dihydrotestosterone. We have therefore studied the neuroanatomical distribution of testosterone-metabolizing enzymes by means of the Palkovits punch technique combined with radioenzyme assay in the brain of adult and young male and female zebra finches. The activity of these enzymes was studied by a one-point assay in 5 nuclei of the song system (X, MAN, HVc, RA, ICo), 2 nuclei of the visual system (ectostriatum, nucleus rotundus) and in limbic and hypothalamic areas. Very noticeable was the presence of a very high aromatase activity in the hippocampal and parahippocampal region and in the nucleus taeniae and the absence of this enzyme in ICo. We found a higher aromatase activity in female than male HVc and RA and a higher 5 $\alpha$ -reductase activity in MAN, HVc, RA and ICo of males compared to females. The 5 $\alpha$ -reductase was more active in the preoptic area of females. A few sex-related differences in the activity of the 5 $\beta$ -reductase were also observed (higher activity in females than in males for area X and RA, but difference in the opposite direction for the ectostriatum). The statistical significance of these differences depended, to some extent, on the statistical technique used to demonstrate them, with the sex differences in RA being by far the most robust ones. Many age-related metabolic differences were also detected but these do not have a clear interpretation since the  $K_m$  of these enzymes also changes with age. Extremely low levels of 5 $\beta$ -reductase activity were found in the nuclei of the visual system in adult birds while this enzymatic activity was very high in young birds. The biological significance of this change with age remains obscure. Correlations are thus observed between the neuroanatomical distribution of T-metabolizing enzymes and of androgen and estrogen receptors with the important exception of ICo which has no aromatase but contains high concentrations of estrogen receptors. Testosterone-metabolizing enzymes are however also present in areas which are not known as steroid targets.

### INTRODUCTION

The song system of passerine birds has been used for many years as a favorite model system for studying the action of steroid hormones on the brain and behavior. It was first described by Nottebohm and his collaborators in the canary<sup>32</sup>. This system which is thought to control both song learning and song production consists of well-defined brain nuclei and fiber tracts. The major efferent components are the telencephalic hyperstriatum ventrale pars caudalis (HVc), which projects to the nucleus robustus archistriatalis (RA) and finally to the hypoglossal motoneurons. Other brain nuclei, such as the nucleus magnocellularis of the anterior neostriatum (MAN), the nucleus intercollicularis (ICo) and the area X of the lobus parolfactorius (X) are monosynaptically connected to either HVc or RA<sup>33</sup>.

Most nuclei of the song system are steroid-sensitive<sup>7</sup>

and sexually dimorphic in the zebra finch. The overall size of HVc, RA, and MAN is bigger in males than females and area X is not detectable at all by Nissl staining in females<sup>31</sup>. Moreover, the size of individual cells within these nuclei are different in males and females, males possessing larger cells<sup>17,19</sup>. Autoradiographic studies have shown that HVc, MAN, and RA take up more radioactivity following the injection of tritiated testosterone<sup>5,6</sup>.

Several of these features can be masculinized by treating young females with estradiol or testosterone during the first days of life<sup>17,19,25,29</sup>. In correlation with the masculinization of the neural elements, the behavior can also be altered by this neonatal exposure to steroids. Females treated with estradiol early in life can produce male-typical songs as adults when given testosterone<sup>19,36,37</sup>. It is assumed that sexual differentiation of the singing behavior in zebra finches results from a mascu-

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linization of males under the influence of estrogens during an early postnatal period. This hypothesis, based on the results of injection experiments, is supported by the recent finding that plasma levels of estradiol are actually higher in males than in females during the first 10 days after hatching<sup>23</sup>. However, this early sex difference in the circulating levels of estradiol was not found in a more recent study describing the hormonal milieu in young zebra finches and the difference was only found during a few days of the second week after hatching<sup>2</sup>. It is however clearly established that a treatment with exogenous estrogens can masculinize the brain of female zebra finches which are much older than 10 days post-hatch<sup>25,29</sup>, so that there is an apparent discrepancy between the ages at which exogenous estrogens are able to masculinize females and the age at which they are present in males. In addition, treatment of young female and male zebra finches with different antiestrogens have unexpectedly led to a masculinization or hypermasculinization of their behavior<sup>27,28</sup>.

A number of questions concerning the origin and the mode of action of the hormonal stimuli which differentiate the singing behavior in zebra finches thus remain unanswered. As we know from studies in mammals<sup>26</sup> and in birds<sup>4</sup>, the central aromatization of androgens within specific brain regions is a critical step in sexual differentiation although no evidence for this is, so far, available in zebra finches. It has however been shown that the neonatal treatment of young females with the aromatizable androgen, testosterone, can mimic the behavioral or morphological effects of estrogens<sup>17,49</sup>. For this reason we have investigated the ability of different brain areas of young and adult zebra finches to transform testosterone into estradiol. Testosterone is indeed present in substantial amounts in male and female zebra finches throughout their life<sup>23,38</sup> and could thus provide a differentiating stimulus if it was differentially aromatized in males and females.

There is also a growing body of evidence suggesting that the conversion of aromatizable androgens to estrogens plays an important role in the activation of adult behavior in zebra finches as shown in many other avian species<sup>1,9</sup>. In the castrated male zebra finch, aromatizable androgens are more effective than non-aromatizable steroids in restoring normal levels of courtship song. A treatment with a combination of dihydrotestosterone and estradiol is more effective than the administration of either steroid alone<sup>20</sup>. Moreover, recent studies indicate that androstenedione has to be converted to estrogenic metabolites in order to exert its effects. Castrates treated with androstenedione and the aromatase inhibitor ATD exhibit fewer courtship behaviors, less aggression and nest-building activity than males treated with androste-

nedione alone. Estradiol, when given concurrently with androstenedione and ATD, reverses the inhibitory effects of ATD<sup>55</sup>. All these studies independently confirm the role of aromatization in the activation of behavior.

The aim of the present study was to describe the neuroanatomical distribution of testosterone-(T-)metabolizing enzymes in the brain of male and female zebra finches at 20 days of age during the period of sexual differentiation and in adulthood when steroids are able to activate the full repertoire of reproductive behaviors. We also wanted to compare the neuroanatomical distribution of these enzymes with the distribution of receptors for testosterone and estradiol. We have studied 13 different nuclei which were isolated by means of the Palkovits microdissection technique described by Palkovits<sup>34,35</sup>. They include 5 nuclei of the song system, 3 nuclei of the preoptic area-hypothalamus and 3 limbic areas. Two areas of the tectofugal visual system, the ectostriatum and nucleus rotundus were studied as control non-steroid-sensitive nuclei.

## MATERIALS AND METHODS

### *Experimental animals*

All animals used in the experiments were obtained from the breeding colony of zebra finches (*Taeniopygia guttata castanotis*) established at the Department of Ethology, University of Bielefeld, F.R.G. Birds of both sexes were sacrificed when they were either 20 days (young) or over 100 days (adults) of age.

### *Microdissection of the brain*

The technique validated for the study of the quail brain<sup>43</sup> was used here with only minor modifications. After sacrifice, brains were quickly removed out of the skull and frozen on dry ice. This procedure does not affect the activity of T-metabolizing enzymes<sup>42</sup>. The frozen brains were embedded in egg yolk. They were mounted on the specimen holder of a microtome with the forebrain up. Coronal sections were made in a cryostat at -10 °C until the area X appeared. During this process, the plane of section was adjusted according to the stereotaxic atlas of the canary brain<sup>53</sup>. Starting from the first section where area X was visible, the brains were cut into 200- $\mu$ m serial sections until the RA disappeared. Sections were thaw-mounted on glass slides and stored on dry ice overnight. From these sections, individual nuclei were isolated by punching them out with a steel cannula from one to three consecutive sections (inner diameter 700  $\mu$ m). During the validation of the method, some sections were stained with Toluidine blue to verify the dissection. Fig. 1 shows a schematic drawing of the nuclei of the adult male brain which were dissected in this way.

Corresponding areas were dissected in adult females and in young of both sexes. Since a standard 700- $\mu$ m punch covers the entire cross-sectional area of many nuclei in the adult male, various amounts of extraneous tissue must have been included in the punches from females or young because some nuclei are smaller (e.g. RA, HVC or MAN) or not even visible (area X) in these cases. This strategy was adopted because it would have been extremely difficult or impossible to locate accurately in fresh frozen material some of the nuclei in females or in young. Reducing the size of the cannula in order to confine the punch within the nuclei would similarly be impossible. All comparisons presented in this paper thus refer to brain samples collected in homologous areas but do not necessarily imply that they were always located within a same nucleus. This aspect is further elaborated in the discussion of the

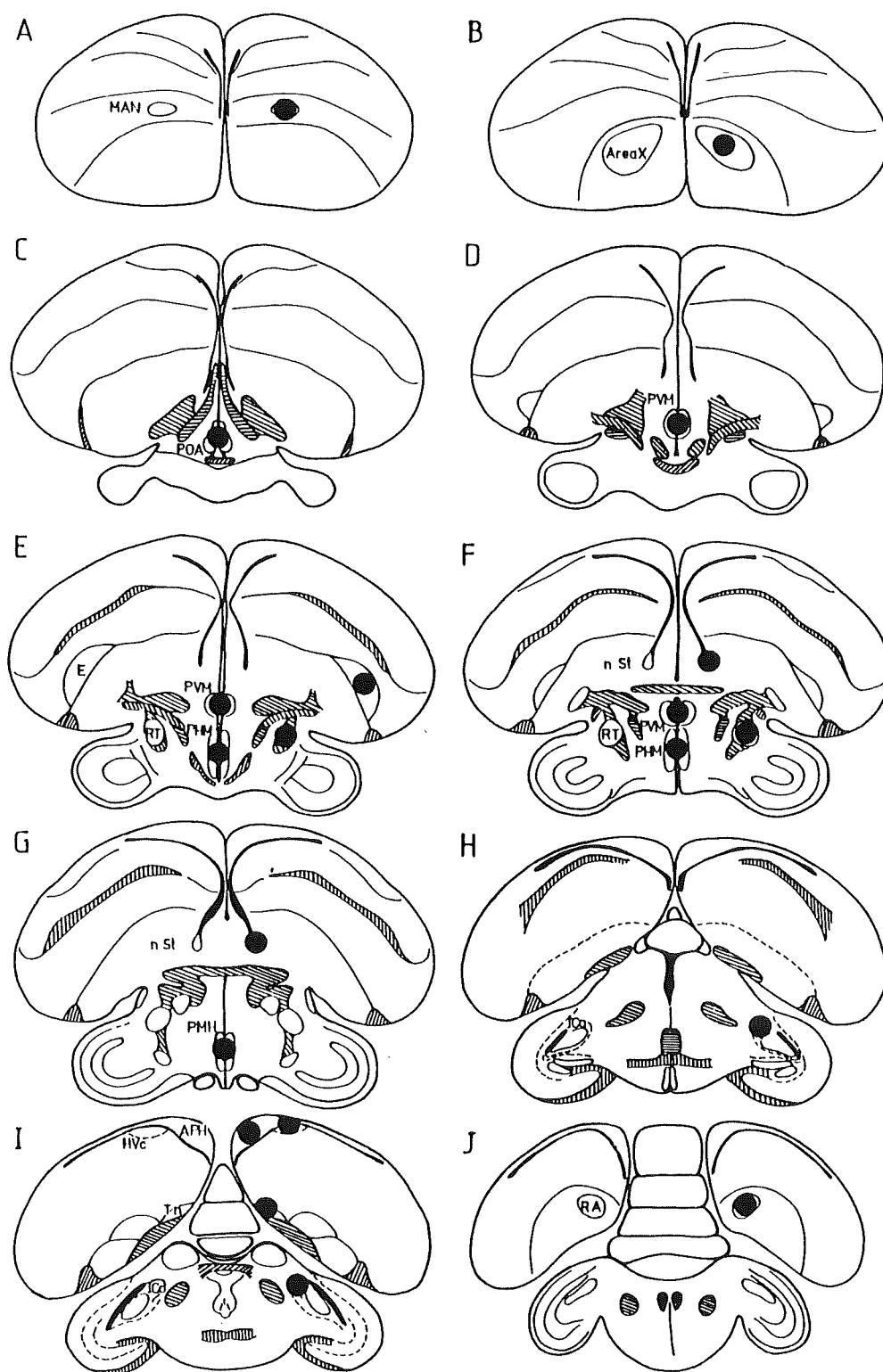


Fig. 1. Schematic drawing showing the localization of the nuclei dissected by the punch method in the adult male zebra finch brain. Corresponding brain areas were dissected in adult females and in young of both sexes. Sections A–J are ordered in rostral to caudal order. Black dots represent the inner diameter of the cannula. APH, area parahippocampalis; E, ectostriatum; HVC, hyperstriatum ventrale, pars caudalis; ICo, nucleus intercollicularis; MAN, nucleus magnocellularis of the anterior neostriatum; nSt, nucleus striaterminalis; PMH, nucleus medialis hypothalami posterioris; POA, nucleus preopticus anterioris; PVM, nucleus periventricularis magnocellularis; RA, nucleus robustus archistriatalis; Rt, nucleus rotundus; Tn, nucleus taeniae; X, area X of the lobus parolfactorius.

results and interpretations take this technical limitation into account.

Individual nuclei from 3 birds had to be pooled for the assay of enzymatic activity. The punched area was blown out of the needle into a glass tube and pooled samples were homogenized in 120  $\mu$ l of ice-cold STMM buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 at 20 °C, 5 mM MgCl<sub>2</sub>, 1 mM  $\beta$ -mercaptoethanol) by ultrasonication (50 W, 30 s). Twenty  $\mu$ l were removed for protein assay. The remaining 100  $\mu$ l were immediately frozen on dry ice and stored at -70 °C until future use.

#### Measure of enzymatic activities

The radioenzyme assay for testosterone-metabolizing enzymes has already been described<sup>44,54</sup>. For the assay, the 100- $\mu$ l homogenates were thawed in an ice-water bath. Immediately after thawing, 50  $\mu$ l of STMM buffer containing [<sup>3</sup>H]testosterone (New England Nuclear, spec. act. 168 Ci/mmol) at a final concentration of about 20 nM (23 nM in the experiment on young birds and in the first replicate of the experiment on adults which were run simultaneously and 21 nM in the second replicate of the experiment on adults) and NADPH<sub>2</sub> (final concentration 1 mg/ml or 1.2 mM = saturating level<sup>44</sup>) were added. Homogenates were then incubated at 41 °C for 5 min. Reactions were stopped by freezing samples at -20 °C. Steroids were extracted 3 times with diethylether after addition of 5  $\mu$ g of carrier steroids and about 3000 counts of [4-<sup>14</sup>C]estradiol (Amersham, spec. act. 56 mCi/mmol) and 3000 counts of 5 $\alpha$ -[4-<sup>14</sup>C]dihydrotestosterone (Amersham, spec. act. 57 mCi/mmol) for calculation of recovery of estrogens and androgens respectively. Radioactive steroids were always tested for purity by thin layer chromatography before use. The diethylether extracts were dried and after addition of 250  $\mu$ l of 1 M NaOH, androgens were extracted 3 times with a mixture of toluene:cyclohexane (1:1, v/v). After neutralization of the aqueous phase by 1 M HCl, estrogens were extracted 3 times with diethylether. Both androgens and estrogens were separated by thin layer chromatography on silica gel plates (estrogens: Macherey-Nagel 804023; androgens: Merck 5715) in a mixture of dichloromethane:ether (85:15, v/v). Androgens were revealed on the plates with Primulin (Sigma 7522) and estrogens with iodine vapors.

The different metabolites were eluted in 100  $\mu$ l of ethanol and 4 ml of Aqua-Luma and counted in a Beckman Liquid Scintillation Spectrometer using the preset <sup>3</sup>H/<sup>14</sup>C window. Counts were corrected for tracer recovery and the amounts of metabolites produced in control tubes (blanks) containing buffer, NADPH<sub>2</sub> and substrate but no brain homogenate.

Three metabolites were quantified: estradiol-17 $\beta$  (E<sub>2</sub>), 5 $\alpha$ -dihydrotestosterone (5 $\alpha$ -DHT) and 5 $\beta$ -dihydrotestosterone (5 $\beta$ -DHT). The identity of these 3 metabolites has been confirmed for the zebra finch hypothalamus by recrystallizations to specific activity and/or constant isotopic ratio<sup>54</sup>. Protein content of the homogenates was measured according to the method of Bradford<sup>12</sup>.

#### Experimental design

In a first experiment, we studied the neuroanatomical localization of the 3 testosterone-metabolizing enzymes in 12 different brain regions of male and female zebra finches at 20 days of age (see Fig. 1 for abbreviations and position of the nuclei). Thirty-six young birds (18 males and 18 females) were included in the study resulting in a number of independent samples equal to one-third of these figures (6 males, 6 females) since punches from 3 birds were pooled in each sample.

The second experiment was performed in two replicates on adult birds of both sexes (more than 100 days of age). A first replicate included 21 males and 15 females resulting in respectively 7 and 5 samples. The dissected brain regions were the same as in the study of young birds (see above and Figs. 1 and 2). Considering that this study had found many numerical differences related to sex which were not statistically significant, due presumably to the limited sample size, a second replicate was performed with 12 males and 12 females (4 final samples of each sex).

In addition to the nuclei previously sampled (see in Figs. 1 and 2), we punched out, in this replicate, the telencephalic nucleus MAN. In view of the high aromatase activity which had been detected in the area parahippocampalis (APH) in both young birds and adult birds of the first replicate, additional studies were carried out to analyze the distribution of this enzyme in the dorsal part of the caudal telencephalon. Eight different sub-areas were collected by a free-hand dissection with a scalpel blade from 200- $\mu$ m sections as described in Fig. 4.

In all cases, a small number of samples were lost during processing, resulting in slightly smaller sample sizes in the final results.

#### Statistical analysis

The amounts of metabolites formed by the different nuclei in both sexes were first compared in data from young birds and from adult birds in each replicate separately by two-way analyses of variance (ANOVA) with the sex of the birds and the neuroanatomical localization of the sample as factors. Sex differences in metabolism were then evaluated in each individual nucleus by the Bonferroni adaptation of the *t*-test for multiple comparisons (referred to below as the Bonferroni test or protected *t*-test). Results of these tests are indicated by asterisks on the figures. As it was expected that sex differences in T-metabolism would be localized in only a few steroid-sensitive nuclei, the comparison of male and female data was also performed by two-tailed Student's *t*-test. Although this procedure increases the chances of type I error, it is a defensible method from the biological point of view because it is the only one which allows for the detection of neuroanatomically discrete sex differences which are not detected by the global ANOVA. The ANOVA performed on data collected from a large number of areas in order to detect discrete sex differences increases the risk of type II error to such a degree that it is biologically inappropriate (see ref. 46 for further discussion). The reality of the differences obtained by the *t*-test can always be tested in independent replications of the experiments as was done here. Results from these *t*-tests are indicated on the figures by large dots in parentheses.

Many of the numerical differences between adult males and females observed separately in each replicate of the experiment were not statistically significant due to the limited number of samples available. The pooled data for both replicates were thus analyzed by three-way ANOVA with the sex of the birds, the anatomical location of the samples and the replication of the experiment as factors. Differences in enzyme activities between males and females were then evaluated by the two procedures described above. The *t*-tests were performed on data transformed, separately for each replicate, in percentages of the mean values observed in the corresponding male group to take into account differences which were observed between the absolute levels of activity in the two replicates of the experiment. All probabilities which are presented are two-tailed.

## RESULTS

### Young birds

In the first experiment, we studied the neuroanatomical localization of 3 testosterone-metabolizing enzymes in 12 different brain regions of male and female zebra finches at 20 days of age (see Fig. 1 for abbreviations). The mean levels of enzyme activities observed in 20-day-old birds are shown in Fig. 2.

The two-way ANOVA revealed that the production of testosterone metabolites was significantly different from one brain area to the other (aromatization:  $F_{11,97} = 13.6$ ,  $P < 0.0001$ ; 5 $\alpha$ -reduction:  $F_{11,106} = 8.49$ ,  $P < 0.0001$ ;

5 $\beta$ -reduction:  $F_{11,109} = 15.83$ ,  $P < 0.0001$ ). The overall activity of the 5 $\beta$ -reductase was significantly higher in males than in females ( $F_{1,109} = 7.64$ ,  $P = 0.0067$ ) but the activity of the other two enzymes was not sexually differentiated (aromatase:  $F_{1,97} = 2.31$ ,  $P = 0.132$ ; 5 $\alpha$ -reductase:  $F_{1,106} = 0.121$ ,  $P = 0.728$ ). Interactions between the effects of these two factors (sex of the birds and position of the nucleus) were significant in the case of the 5 $\alpha$ -reductase ( $F_{11,106} = 2.71$ ,  $P = 0.0041$ ) but not for the other two enzymes (aromatase:  $F_{11,97} = 1.27$ ,  $P = 0.249$ ; 5 $\beta$ -reductase:  $F_{11,109} = 1.77$ ,  $P = 0.067$ ).

The highest levels of aromatase activity were found in the limbic areas nucleus taeniae (Tn) and APH. Estradiol was also produced in substantial amounts by all hypothalamic nuclei and by 2 nuclei of the song system (HVc and RA). The activity of this enzyme was low or undetectable in the other nuclei. A few sex-related differences in aromatase activity were detected by the Bonferroni or by the *t*-test which are of questionable validity in this context as no overall sex difference was detected in the ANOVA. More estradiol was produced in two nuclei of females by comparison with the males: nucleus medialis hypothalami posterioris (PMH) ( $P < 0.05$  by Bonferroni,  $P < 0.01$  by *t*-test) and Tn ( $P < 0.05$  by Bonferroni). A difference in the opposite direction was seen in the ectostriatum (*t*-test only,  $P < 0.05$ ).

The 5 $\alpha$ -reductase was present and fairly active in all the nuclei that were studied. No sex-related difference in its activity could be detected except for the POA ( $P < 0.05$  by Bonferroni test).

The 5 $\beta$ -reductase was 2 to 3 orders of magnitude more active than the aromatase and the 5 $\alpha$ -reductase in all nuclei that were examined. Highest levels of enzyme activity were observed in the nuclei of the visual system, ectostriatum (E) and nucleus rotundus (Rt). In the *t*-test, the production of 5 $\beta$ -DHT was significantly higher in males than in females for 3 brain areas: the nucleus periventricularis magnocellularis (PVM), the Rt and the APH, but only one of these differences (Rt) was significant in the Bonferroni test.

#### Adult birds

The two replicates of this experiment provided very similar results. With only one exception (see 5 $\beta$ -reductase in nucleus stria terminalis [nSt]), the numerical differences observed in the first replicate of the experiment were reproduced in the second one. Only pooled data will thus be discussed here. Figure 3 presents the mean levels of aromatase, 5 $\alpha$ - and 5 $\beta$ -reductase activity observed in adult males and females (means of all data collected in the two replicates).

The three way ANOVA revealed that, as expected, the aromatase activity in adult birds varied significantly from

one brain area to the other ( $F_{10,150} = 22.92$ ,  $P < 0.0001$ ). Overall, females had a slightly higher aromatase activity than the males ( $F_{1,150} = 4.93$ ,  $P = 0.028$ ). There was also a significant effect of the experiment replicate ( $F_{1,131} = 11.14$ ,  $P = 0.001$ ) and two significant interactions between these factors (sex of birds by brain nucleus:  $F_{10,150} = 3.021$ ,  $P = 0.0017$  and brain nucleus by replicate:  $F_{10,131} = 3.354$ ,  $P < 0.0006$ ). The replicate effect originated from the fact that, as a mean, absolute levels of enzyme activity were slightly higher in the second than in the first set of data and this was more pronounced in some nuclei. The distribution pattern and the direction of the sex-related differences were however

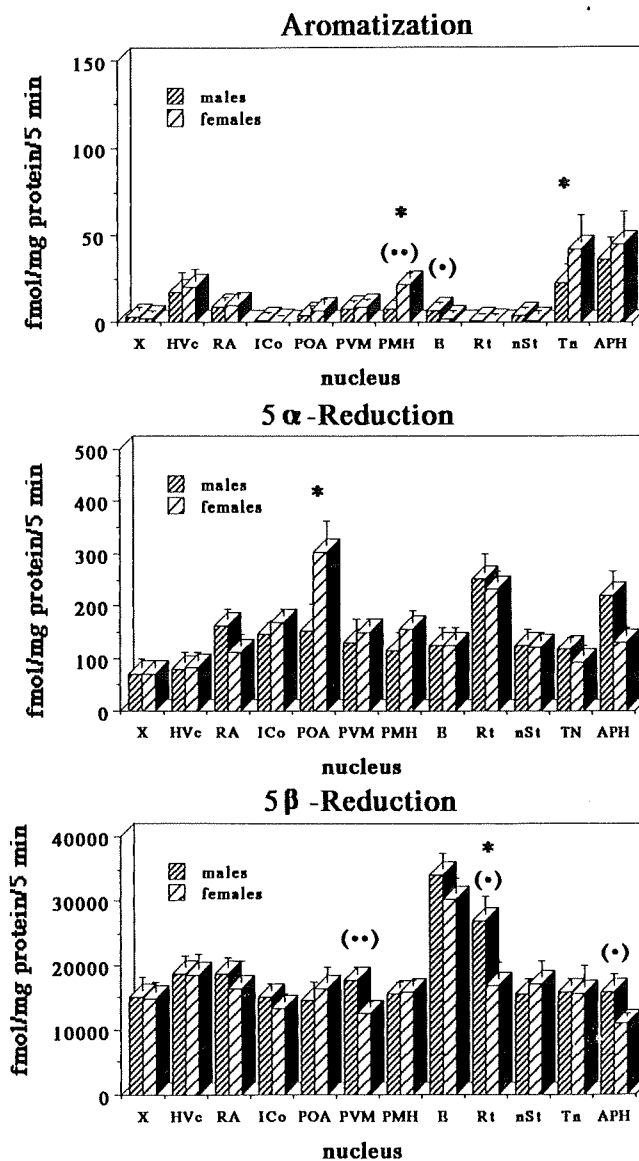


Fig. 2. Formation of estradiol (aromatization), 5 $\alpha$ -DHT (5 $\alpha$ -reduction) and 5 $\beta$ -DHT (5 $\beta$ -reduction) by different nuclei of 20-day-old male and female zebra finches. Each column corresponds to the mean  $\pm$  S.E.M. of 4–6 experimental points (punches from 3 individuals in each point). \* $P < 0.05$  for comparison between males and females within one nucleus by the Bonferroni test; • $P < 0.05$  and •• $P < 0.01$  by the *t*-test. For abbreviations, see legend of Fig. 1.

similar in both cases. They were analyzed in a 3-way ANOVA using replicates as one factor so that the differences between replicates do not affect their evaluation. All other interactions were not significant.

In adult animals, the distribution of the aromatase was similar to that seen in young birds although absolute levels of metabolite produced were higher in the adults. The aromatase was very active in the POA, the hypothalamic nuclei, in limbic areas (nSt, Tn and APH) and

in all nuclei of the song system (X, MAN, HVc and RA) with the exception of ICo. Little or no activity was detected in the nuclei of the visual system (E and Rt). Analysis by *t*-test of the data transformed in percentages revealed significant sex differences in a number of nuclei: in HVc, RA and APH, aromatase activity was significantly higher in females than in males. However the protected *t*-test only accepted two of these differences as significant (RA, APH). In one single nucleus (PVM), the aromatase activity was numerically higher in males than in females, but this difference was not significant even by the *t*-test ( $P < 0.10$ ).

In general, the production of  $5\alpha$ -DHT exceeded the formation of  $E_2$ . The activity of the  $5\alpha$ -reductase was different from one brain nucleus to the other (three-way ANOVA:  $F_{10,131} = 18.90$ ,  $P < 0.0001$ ) and was also, as a mean, higher in males than in females ( $F_{1,150} = 13.40$ ,  $P < 0.0004$ ). No systematic difference in the level of activity of this enzyme was found from one replicate of the experiment to the other ( $F_{1,150} = 3.09$ ,  $P = 0.081$ ) but significant effects of the interaction between brain nuclei and replicate ( $F_{10,150} = 3.88$ ,  $P < 0.0001$ ) and between sex of the birds and brain nuclei ( $F_{10,150} = 12.64$ ,  $P < 0.0001$ ) were present. Other interactions were far from being significant.

Highest levels of  $5\alpha$ -reductase were detected in the POA of both males and females and in the PVM and RA of males. Significant sex differences in enzyme activity were seen by *t*-test in MAN, HVc, RA and ICo where the males produced more and in some cases (e.g. RA) a lot more  $5\alpha$ -DHT than the females. A difference in the opposite direction (females higher than males) was observed in the nucleus preopticus anterioris (POA). The Bonferroni test only accepted the differences in RA and POA as significant at the 5% level. In all other nuclei studied, the enzyme was present but its activity was lower and not sexually differentiated. The activity of this enzyme was equal or even lower in adult than in young birds. A difference was particularly noticeable in Rt where the  $5\alpha$ -reductase activity was approximately double in young compared to adult birds.

The  $5\beta$ -reductase exhibited a very heterogeneous distribution in the adult bird brain. Extremely high levels of activity were seen in the nSt, whereas lowest levels (almost undetectable) were found in Rt. In general, the nuclei of the song system (with the exception of area X) tended to have a higher  $5\beta$ -reductase activity than the hypothalamic nuclei. This distribution of the enzyme activity was extremely different from the one which had been observed in the young birds (compare with Fig. 2). The absolute levels of activity were also markedly decreased compared to young animals. The three-way ANOVA demonstrated the presence of very significant

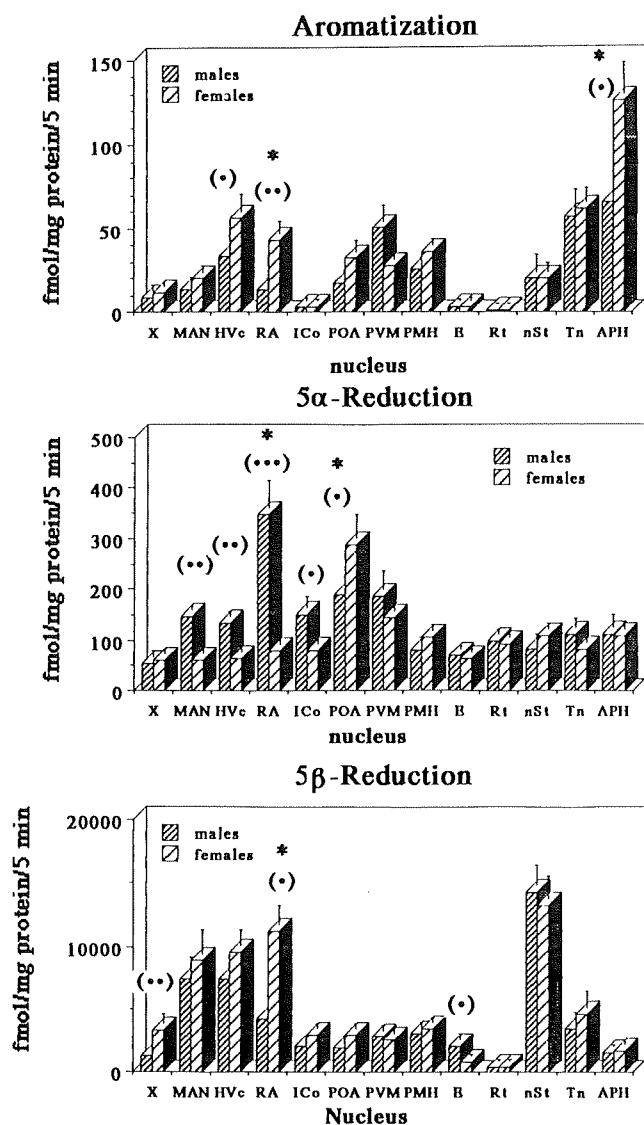


Fig. 3. Formation of estradiol (aromatization),  $5\alpha$ -DHT ( $5\alpha$ -reduction) and  $5\beta$ -DHT ( $5\beta$ -reduction) by different nuclei of adult male and female zebra finches. Each column corresponds to the mean  $\pm$  S.E.M. of 6–11 experimental points collected in two replicates of the experiment (punches from 3 individuals in each point). \* $P < 0.05$  for comparison between males and females within one nucleus by the Bonferroni test; (•)  $P < 0.05$ , (••)  $P < 0.01$  and (•••)  $P < 0.001$  by the *t*-test. The data submitted to the *t*-test were transformed within each replicate in percentages of the mean male results in order to compensate for the variations in the absolute level of enzyme activity (see text). For abbreviations, see legend of Fig. 1.

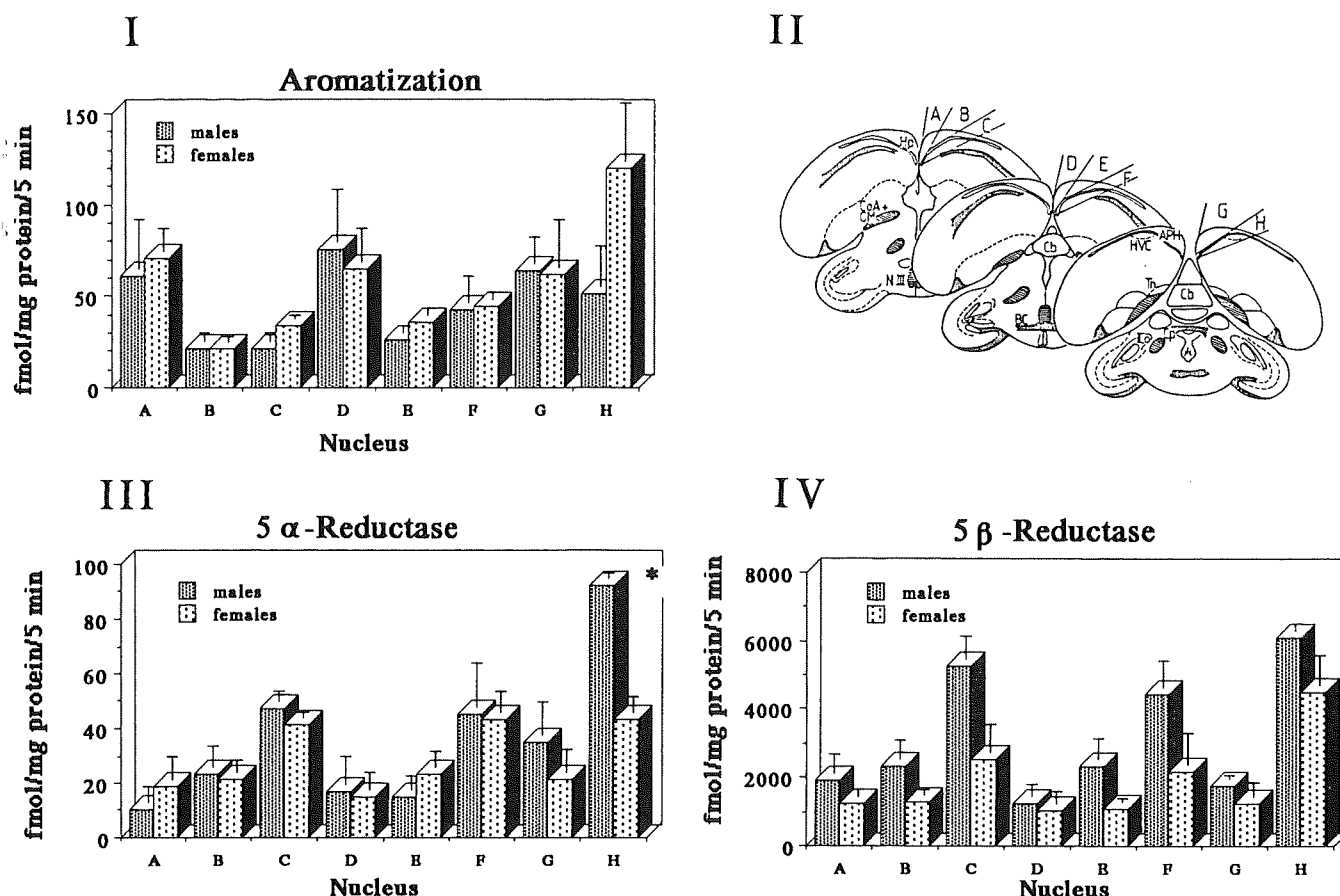


Fig. 4. Schematic drawing of the free-hand dissection of the dorsal telencephalon that was used to analyze the distribution of enzyme activities (panel II) and formation of estradiol (aromatization, panel I), 5 $\alpha$ -DHT (5 $\alpha$ -reduction, panel III) and 5 $\beta$ -DHT (5 $\beta$ -reduction, panel IV) by the 8 subregions (A–H) in adult male and female zebra finches. HVC had been punched out of the section before area H was dissected. Each column corresponds to the mean  $\pm$  S.E.M. of 3–4 experimental points. \* $P < 0.05$  for comparison between males and females within one nucleus by the Bonferroni and by the  $t$ -test. APH, area parahippocampalis; BC, brachium conjunctivum; Cb, cerebellum; CoA, commissura anterior; Cp, commissura posterior; Hp, hippocampus; HVC, hyperstriatum ventrale, pars caudalis; ICo, nucleus intercollicularis; OM, tractus occipitomesencephalicus; Tn, nucleus taeniae.

differences in enzyme activity according to the brain nucleus ( $F_{10,134} = 76.77$ ,  $P < 0.0001$ ) but also to the sex of the birds ( $F_{1,134} = 15.42$ ,  $P < 0.0001$ ) and the replicate of the experiment ( $F_{1,134} = 39.95$ ,  $P < 0.0001$ ). Significant interactions were also observed between sex and nucleus ( $F_{10,134} = 6.74$ ,  $P < 0.0001$ ), nucleus and replicate ( $F_{10,134} = 3.04$ ;  $P = 0.0017$ ) and between these 3 factors (sex  $\times$  nucleus  $\times$  replicate:  $F_{10,134} = 5.84$ ,  $P < 0.0001$ ).

The production of 5 $\beta$ -DHT was significantly higher in females than in males by  $t$ -test in the area X and the nucleus RA. A difference in the opposite direction (males higher than females) was observed in the ectostriatum. The more conservative (from a statistical point of view) Bonferroni test only accepted the difference in RA as significant. In the nSt, the mean values of 5 $\beta$ -reductase activity obtained after pooling the results of the entire experiment were numerically very similar. Contradictory results were however obtained for this nucleus in the two replicates of the experiment: in the

first replicate, males had a higher 5 $\beta$ -reductase activity than females ( $P < 0.05$  by  $t$ -test) and the opposite was true in the second replicate ( $P < 0.05$  by  $t$ -test). No explanation for this discrepancy can be proposed at present. Except for this single case (5 $\beta$ -DHT production in nSt), the results obtained in both replicates of this experiment for the 3 enzymes were in complete agreement. The significant effects of the replicate or of its interaction with other factors in the general ANOVAs were due to changes in the absolute levels of enzyme activity which sometimes were more pronounced in some nuclei than in others. The distribution pattern of the enzymes was however completely consistent from one part of the experiment to the other. In addition, every single sex difference in enzyme activity which was seen on the pooled data ( $t$ -tests or Bonferroni tests shown on the figures) corresponded to numerical differences in the same direction which were present in both subsets of results.

The dissection of the dorsal part of the caudal

telencephalon demonstrated the presence of high levels of aromatase activity in the entire region (Fig. 4).

The enzyme activity was however significantly different from one sub-area to the other (two-way ANOVA with sex of the birds and brain region as factors:  $F_{7,42} = 3.16$ ,  $P = 0.009$ ) but there was no overall effect of the sex of the birds ( $F_{1,42} = 2.62$ ,  $P = 0.11$ ) and no interaction between the two factors ( $F_{7,42} = 0.55$ ,  $P = 0.78$ ). The highest aromatase levels were seen in the hippocampus (A,D) and in the area under and medial to HVc (H). Lower levels of enzyme activity were found in the more lateral and rostral parts of the dorsal telencephalon (B,C,E,F).

The  $5\alpha$ - and  $5\beta$ -reductase activities also had a heterogeneous neuroanatomical distribution within the dorsal telencephalon ( $5\alpha$ -reductase:  $F_{7,40} = 10.02$ ,  $P < 0.0001$ ;  $5\beta$ -reductase:  $F_{7,33} = 14.88$ ,  $P < 0.0001$ ). An overall sex difference in the activity of these two enzymes was also detected ( $5\alpha$ -reductase:  $F_{1,40} = 4.22$ ,  $P = 0.046$ ;  $5\beta$ -reductase:  $F_{1,33} = 19.34$ ,  $P < 0.0001$ ). No interaction between these two factors (sex and brain area) was significant. The overall sex difference in  $5\alpha$ -reductase activity resulted essentially from a large difference present in the caudal and lateral sample (area H:  $P < 0.05$  by the *t*-test and by the Bonferroni test). Due to the small sample size (4 male and 4 females pools) no other sex-related difference within individual nuclei was significant.

## DISCUSSION

In this study, using a very sensitive radioenzyme assay which had previously been set up and validated for the zebra finch brain<sup>54</sup>, we could analyze the distribution of 3 testosterone-metabolizing enzymes in different brain nuclei which were microdissected by the Palkovits 'punch' method<sup>34,35</sup>. The distribution of the enzymes has been described and their activity has been quantified in adult and young zebra finches of both sexes. The results obtained provide many new insights concerning the biochemical mechanisms underlying both the sexual differentiation and the activation of reproductive behavior in this species.

In the zebra finch, high levels of aromatase activity are found in limbic structures, such as the nucleus taeniae, the hippocampus and area parahippocampalis and in the preoptic-hypothalamic areas. This corresponds to the situation described in mammals where highest levels of aromatase activity were found in the amygdala, in the preoptic area and in the hypothalamus<sup>39,45</sup>. Other studies in birds have only shown high aromatase activity in the diencephalon<sup>41,43,51</sup> but other brain regions were not systematically explored. It must however be noted that in

quail, an extremely low aromatase activity was observed in the nucleus taeniae (ref. 43 and Balthazart and Evrard, unpublished data), while high levels of activity were present in this nucleus of the zebra finch.

In addition, it is interesting that a high aromatase activity was also detected in 3 nuclei of the song system, MAN, HVc and RA, whose size and cell typology can be affected by estrogens acting during ontogeny<sup>17,19,25</sup>. These structures are thus able to produce estrogens locally by the intracellular conversion of androgens and this ability is already developed at 20 days of age.

A comparison of the sites where high aromatase activity is present with the distribution of estrogen receptors reveals some striking discrepancies. Some areas such as the preoptic-hypothalamic nuclei (POA, PVM and PMH) and limbic regions (Tn and lateral septum [nSt]) contain estrogen receptors as demonstrated by both autoradiography<sup>30</sup> and immunocytochemistry<sup>14,15</sup> and in addition have high aromatase activity. By contrast, in the song system, these two biochemical characteristics appear to be totally dissociated. Three nuclei of the song system, MAN, HVc and RA contain apparently no or very few estrogen receptors<sup>15,30,56</sup>, but have a high aromatase activity. On the other hand, the ICo, the only song control nucleus identified in all studies as a site with dense estrogen receptors (autoradiography<sup>30</sup>, immunocytochemistry<sup>15</sup>, binding assay on punched nuclei<sup>56</sup>) has no or barely detectable levels of enzyme. The same situation has in fact been described for the quail ICo which has a dense population of neurons containing estrogen receptors<sup>10</sup> but no detectable aromatase<sup>16</sup>.

Walters and collaborators<sup>56</sup> had suggested that estrogen receptors might be present in all nuclei of the song system but be detectable only in the absence of endogenous estrogens. There is evidence suggesting that in zebra finches castration does not totally suppress endogenous estrogens and therefore they treated castrated animals with ATD, an aromatase inhibitor in order to prevent formation of estrogens from the aromatization of androgens coming from an extragonadal source (adrenals?). Following this treatment with ATD, unoccupied estrogen receptors were significantly increased in some nuclei (MAN, ICo and area X) but not in others (POA, PVM, HVc and RA). The authors suggested that these differences between nuclei might be related to regional differences in aromatase activity. The results of the present experiments are not consistent with this interpretation. Our data show that aromatization is almost absent in ICo and low in area X where high increases in estrogen receptor concentrations were seen after treatment with ATD. In contrast, other nuclei, where no increase in estrogen receptors had been detected after ATD pretreatment, are shown here to contain an active

aromatase (e.g. POA, PVM, HVc and RA). The mechanism by which ATD increases the detectability of estrogen receptors in some brain nuclei but not in others thus remains unclear and might include regional differences in receptor regulation (see also ref. 13 for additional discussion).

As a consequence, the apparent discrepancy between the distribution of estrogen receptors and of aromatase activity is also unexplained. Recent publications<sup>41,50</sup> have shown that aromatase activity is not only found in the microsomal but also in synaptosomal subfractions of the cells. This local production of estrogens in the close vicinity of the synapses might provide steroid signals which could affect the brain activity by a non-genomic mechanism. It is indeed known that estrogens are able to modify the electrical activity of the neuronal membranes within seconds after their application which essentially rules out the possibility of a classical steroid effect mediated through a change in protein synthesis (for additional discussion see refs. 11,41). Whether the presence of aromatase activity in brain nuclei apparently devoid of intracellular estrogen receptors relates to these membrane effects of estrogens is unknown at present. In this context, the subcellular localization of the aromatase in the nuclei of the song system should certainly be investigated.

The very high levels of aromatase activity that were observed in a large portion of the dorsal telencephalon (essentially hippocampus and area parahippocampalis) are also intriguing. The aromatase in APH was at least twice as active as in any of the preoptic-hypothalamic nuclei. This brain region is not known as a classical target for steroids. However tract tracing studies have demonstrated that, in the canary, the APH is connected to a large number of steroid-sensitive areas such as the lateral septum, the nucleus taeniae, the preoptic area and parts of the hypothalamus<sup>24</sup>. The presence of estrogen receptors has also been demonstrated by immunocytochemistry in a large area under the lateral ventricle at the level of HVc in the zebra finch brain<sup>15</sup>. This brain region would correspond to the area named H in our free-hand dissection and which was shown to contain high levels of aromatase activity (see Fig. 4). Finally, preliminary experiments have shown that aromatase activity in the dorsal telencephalon of zebra finch can be increased by treating the birds with testosterone (Vockel and Balthazart, unpublished data). All these facts suggest that the high level of aromatase activity found in the dorsal telencephalon might be related directly or indirectly to the control of reproduction and additional studies should be performed to confirm this interpretation.

Substantial levels of  $5\alpha$ -reductase activity were found in all brain nuclei that were analyzed. It is interesting to

compare in the zebra finch song system the distribution of the  $5\alpha$ -reductase and of androgen receptors. In males, higher levels of enzyme activity were found in the nuclei which contain androgen receptors (MAN, HVc, RA and ICo) than in area X which is not androgen-sensitive<sup>5,21</sup>. The percentage or the number of cells labelled by androgen receptors is higher in males than in females in HVc, RA and MAN<sup>5,6</sup>. In parallel, the  $5\alpha$ -reductase activity was sexually differentiated in all these androgen-sensitive nuclei (see discussion below). The two biochemical sex dimorphisms affecting the  $5\alpha$ -reduction and the density of androgen receptors could thus contribute to explain the behavioral dimorphism affecting song in the zebra finch (T activates song in males but not in females<sup>3,18</sup>).

It is usually accepted that the  $5\beta$ -reduced androgens are biologically inactive compounds as far as activation of reproductive behavior is concerned and that the  $5\beta$ -reduction is an inactivation pathway for testosterone<sup>9,52</sup>. In the zebra finch, as in other birds<sup>44,54</sup>, the maximum velocity of the  $5\beta$ -reductase is much higher than that of the aromatase or  $5\alpha$ -reductase, thus making the enzyme able to metabolize large amounts of testosterone when it is present in excess. It must however be kept in mind that the enzymes which produce behaviorally active metabolites have a higher affinity ( $K_m$ ) for testosterone than the  $5\beta$ -reductase<sup>54</sup> so that in conditions where the substrate is present in limiting amount, as is usually the case in physiological conditions, estradiol and  $5\alpha$ -DHT will be formed preferentially. Many studies on several species of birds showed that  $5\beta$ -reductase activity was lower in steroid target areas than in non-target tissue (reviewed in refs. 8,9). The pattern of distribution of the  $5\beta$ -reductase observed here in the zebra finch to some extent contradicts this generalization. If, as expected, the enzyme activity was low in all hypothalamic nuclei as demonstrated previously in other species, it was very high in several nuclei of the song system which are known targets for androgens (MAN, HVc, RA). In addition, the lowest production of  $5\beta$ -DHT was observed in the two nuclei of the visual system, E and Rt, which are not steroid-sensitive. Even more intriguing is the fact that these two nuclei had a very active  $5\beta$ -reductase in young birds suggesting that the low levels seen in adults result from a specific regulation which took place during ontogeny. The mechanism underlying this physiological differentiation and its biological meaning is totally unclear at present.

The comparison of the data collected here in adult and in 20-day-old birds reveals interesting features. In general, the amounts of estradiol formed in adults were higher than in young and the apparent activity of the  $5\beta$ -reductase markedly decreased with age. Considering

that the size of several nuclei markedly changes with age in the zebra finch while the punched areas were similar in adults and young, it could be argued that the age-related changes in enzyme activity are merely a technical artifact (dilution of the nuclei in the surrounding tissue containing no enzyme). However, changes in the activity of the  $5\alpha$ -reductase were less obvious and the production of  $5\alpha$ -DHT appeared to be lower in adults than in young birds at least for some brain nuclei. The mechanism underlying these age-related changes in metabolite production are thus complex and are not just a consequence of the change in size of the nuclei. We showed previously that the maximum velocity of the aromatase and the  $5\beta$ -reductase changes with age in the zebra finch brain but at the same time the affinity ( $K_m$ ) of these enzymes for the substrate (testosterone) was drastically affected<sup>54</sup>. In the present study, the activity of the enzymes was measured at a single concentration of substrate since it would be technically impossible to perform saturation studies on a large number of areas dissected by the punch method. It is therefore impossible to know whether the changes in the amount of metabolites produced result from a difference in the affinity of the enzyme or a change in the maximum velocity and thus probably the concentration of the enzymes.

Even with these limitations, the present study clearly demonstrates that the 3 major T-metabolizing enzymes are present and active in 20-day-old zebra finches. The activity of these 3 enzymes is not sexually differentiated at that age, in contrast to what is seen in many nuclei of the adults. The intracellular metabolism of testosterone is consequently not the origin of the hormonal stimuli which are responsible for the sexual differentiation of the brain and behavior in this species. Whether these signals which affect differentially males and females originate in a sex-specific peripheral production of steroids or in a difference of steroid receptor concentration is not established at present.

Sex differences in the activity of the 3 testosterone-metabolizing enzymes were detected in several nuclei. Many of these differences were significant only when analyzed by the *t*-test, a procedure which might be criticized from a statistical point of view because it increases the chances of type I error. It must however be pointed out that, at the biological level, it is the only procedure which permits the evaluation of differences in a given nucleus independent of the experimental design<sup>46</sup> (it makes little sense to have the difference in nucleus Y0 significant in an experiment analyzing 3 nuclei but not in another concerned with 15). The statistically more conservative protected *t*-test (Bonferroni procedure) only accepted part of the differences shown to be significant by the *t*-test. In order to produce an objective and

complete report of the results, both types of statistics have been shown on the figures. To support the reality of the *t*-test results, we would like to point out, however, that many of the enzymatic differences that were detected by this procedure concern brain areas which are either steroid-sensitive or sexually dimorphic and where biochemical differences in steroid processing can be expected on an a priori basis. In addition, all differences which were identified by the *t*-test were numerically present in both replicates of the experiment even if they did not reach statistical significance due to the small sample size. We are thus inclined to believe that these differences are real, but a more conservative reader might consider them only as interesting suggestions for future studies.

It might be argued that the sex differences in metabolism which were observed here are just a consequence of the differences in nucleus size between males and females. The RA punches, for example, contained more extraneous (non-RA) tissue in females than in males. If we assume that the  $5\alpha$ -reductase is essentially localized in steroid-target tissues, while the  $5\beta$ -reductase has a high activity outside these targets, then the sex differences observed in the RA punches might indeed be a direct consequence from the smaller nucleus size in females. However this form of reasoning cannot explain the higher aromatase activity seen in females unless we postulate that this enzyme is more active outside than inside the nucleus, which makes little sense based on all available data. In the case of HVc, we can be even more confident about the reality of the metabolic differences between males and females because we actually measured enzymatic activities around the nucleus and they confirm the differences seen in or just around it. By a free-hand dissection of the dorsal telencephalon, we actually dissected the area around HVc (sample H in Fig. 4) after having punched out the nucleus itself. Enzyme assays showed that in both the punched HVc (including some extraneous tissue in females) and in the surrounding region (sample H) aromatase activity was higher in females but  $5\alpha$ -reductase was higher in males. This difference thus cannot be the result of a sex difference in the size of the nucleus. This specific case clearly indicates the existence of genuine enzymatic differences between the sexes. It also suggests that these are not necessarily confined to the nuclei of the song system and may also be found in the surrounding tissue. The punch technique is however inappropriate for obtaining detailed neuroanatomical localizations and it is only by the use of techniques such as immunocytochemistry or in situ hybridization that such a goal will be reached. It is, however, already clear at present that the distribution of neurochemical markers does not always correspond to

the brain nuclei as defined in Nissl-stained material: in zebra finches, for example, estrogen receptors are found around but not in HVC<sup>15,30</sup>.

Within the nuclei of the song system, aromatase activity was usually higher in females while the 5 $\alpha$ -reductase activity was higher in males (see Fig. 2 for the detail of statistics). The functional implications of these differences are not clear at present. The sex-related differences in 5 $\beta$ -reductase activity also do not have an obvious biological significance. It might be assumed that a low enzyme activity in RA of males permits the action of testosterone or its active metabolites while the higher activity in females contributes to make them less sensitive to the effects of testosterone. However, the same difference is seen in area X which is supposedly not androgen-sensitive and, in addition, a difference in the opposite direction occurs in the ectostriatum. A general theory of the biological role of testosterone metabolism in the zebra finch brain is thus impossible to propose. In this context, stereotaxic studies investigating the behavioral effects of androgenic and estrogenic metabolites of testosterone in the different song control nuclei of the zebra finch brain would be extremely desirable.

The nature of the sex differences affecting testosterone metabolism in adults is presently under investigation. Two questions are raised by these data. The first one is: are all these differences that were observed independent characteristics of the brain nuclei or are they causally related? It might be speculated that a higher enzyme activity in one nucleus could induce, by a transsynaptic or

hormonal mechanism, a higher activity in a distant but connected area. Alternatively, within the same nucleus, a difference in one enzyme could control the difference in another. Previous data in the literature do indeed demonstrate or, at least, strongly suggest that the 5 $\alpha$ - and 5 $\beta$ -DHT are able to control aromatase activity (see refs. 9,22 for discussion) and conversely that estradiol might affect the metabolism of androgens<sup>47,48</sup>. The second question concerns the activational versus organizational nature of these differences. Are they stable sex differences in metabolism which are still present when birds are exposed, as adults, to a same hormonal milieu (organizational differences) or differences which disappear in these conditions (activational differences). Based on previous research on quail using a similar experimental approach<sup>42</sup>, it can be expected that both types of results will be obtained. Additional studies will then be carried out to determine whether neonatal treatments with steroids are able to modify in an irreversible manner the activity of testosterone-metabolizing enzyme in some nuclei of the zebra finch brain.

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