Journal of Neuroendocrinology, *Vol. 2, No. 5*

Sex Differences and Steroid Control of Testosterone-Metabolizing Enzyme Activity in the Quail Brain

J. Balthazart, M. Schumacher[1] and L. Evrard
Laboratory of General and Comparative Biochemistry, University of Liège, Belgium.

Key words: aromatase, 5α-reductase, 5β-reductase, radioenzyme assay, reproduction.

Abstract

The activity of three testosterone-metabolizing enzymes (aromatase, 5α-reductase and 5β-reductase) was determined in the quail brain using the Palkovits punch technique combined with a very sensitive radioenzyme assay. Sex differences and the effects of gonadectomy and testosterone treatment on the activity of the three enzymes were quantified in eight brain nuclei which are implicated in the control of various aspects of reproductive behavior and physiology. The aromatase was only present in a few brain areas in which its activity was strongly controlled by testosterone. In two brain regions (medial preoptic nucleus and preoptic area in general) the activity of the enzyme was higher in males than in females. These sex differences disappeared in gonadectomized birds and in gonadectomized birds treated with testosterone, suggesting that they might only result from different circulating steroids in both sexes. However, in the posterior part of the medial preoptic nucleus, there was a strong tendency for the induction of aromatase by testosterone to be larger in males than in females. This supports our earlier finding that in the preoptic area, the aromatase activity is sexually differentiated. This difference probably has a restricted neuroanatomical localization and could only be demonstrated by more precise anatomical methods such as immunocytochemistry. The two testosterone reductases (5α and 5β) showed a more homogeneous distribution in the brain. They were not affected by the hormonal treatments or the sex of the birds except for the 5β-reductase which was significantly more active in three brain nuclei of the females (ventromedial nucleus of the hypothalamus, area hypothalamica lateralis and tuber) by comparison with the males. These sex differences were maintained irrespective of the hormonal status of the birds suggesting that they might be organizational in nature. The relation of these enzymes and their regulation to the control of reproduction is discussed and the usefulness of this approach combining punch technique and radioenzyme assay is evaluated.

The metabolism of testosterone (T) in the brain plays a critical role in the control of many aspects of reproduction including male sexual behavior and gonadotrophin secretion (1, 2). In the quail hypothalamus, T can be transformed into estradiol (E_2; aromatization) and 5α-dihydrotestosterone (5α-DHT; 5α-reduction), two metabolites which alone or in combination mimic most effects of T on reproduction (1, 3–5). In addition, the brain of all avian species studied so far contains a very active 5β-reductase which converts T into 5β-dihydrotestosterone (5β-DHT) (6–8) which appears to be devoid of any behavioral or physiological effects, at least as far as reproduction is concerned (1, 6, 9). The 5β-reductase therefore appears as a metabolic pathway which inactivates part of the T in the brain. By producing different amounts of active and inactive metabolites, the metabolism of T is one of the factors which determines the sensitivity of the brain to the steroid.

Previous studies on quail from our laboratory have identified changes in the activity of T-metabolizing enzymes that were related to the sex of the birds, their age or their endocrine condition (1, 5, 8, 10–15). These changes are limited to specific enzymes and brain areas. In each case, however, enzyme assays had been performed on relatively large brain samples (a few milligrams) so that the exact neuroanatomical localization of these changes could not be ascertained. By combining the Palkovits punch technique (16) with an extremely sensitive radioenzyme assay, we recently demonstrated that T-metabolizing enzymes have a very discrete anatomical localization, even within the preoptic-hypothalamic region (17). In the case of aromatase, this anatomical specificity could recently be confirmed by immunocytochemistry: aromatase-immunoreactive neurons are only found in specific nuclei or parts of nuclei in the brain (18).

The aim of the present work was to identify more precisely the brain sites in which the activity of T-metabolizing enzymes is sexually differentiated and/or modulated by T. The activities of

[1] Present address: Rockefeller University, Neuroendocrinology Laboratory, 1230 York Avenue, New York, New York 10021, USA.
Correspondence to: J. Balthazart, Université de Liège, Laboratoire de Biochimie Générale et Comparée (Bat. L1), 17 place Delcour, B-4020 Liège, Belgique.

three major enzymes (aromatase, 5α- and 5β-reductase) were measured in eight brain nuclei of male and female quail which were either gonadectomized (GNX) or GNX and treated with T or kept intact with their sexually mature gonads as control. The eight brain regions which were selected are implicated in the central control of various aspects of reproduction.

Results

All birds used for the measure of the activity of T-metabolizing enzymes were confirmed to be in the characteristic physiological and behavioral state that was expected based on their sex and endocrine condition. In particular, all males in the intact and GNX T-treated (GNX + T) groups showed copulatory behavior when tested with a receptive female while this behavior was absent in the four other groups, including the ovariectomized females treated with T. The few intact and GNX + T males which did not show sexual behavior during the tests were excluded from the experiment. The intact females were all regularly laying eggs before they were killed. The cloacal gland area which was measured at the end of the experiment also confirmed the effectiveness of gonadectomies and T treatments in both sexes (intact males: 274.5 ± 11.2 mm²; GNX males: 41.4 ± 3.0 mm²; GNX + T males: 236.8 ± 7.1 mm²; intact females: 140.7 ± 6.0 mm²; GNX females: 39.3 ± 18.8 mm²; GNX + T females: 159.6 ± 27.9 mm²; all means ± standard errors).

The measures of enzyme activity in intact males confirmed the neuroanatomical distribution which has been previously reported (17). The heterogeneity of the localization was especially noticeable in the case of the aromatase. The mean levels of enzymatic activities and the associated standard errors in the six experimental groups are shown in Figs. 1 to 3. The results of the statistical analysis of these data by the two-way analyses of variance (ANOVA) are summarized in Table 1.

Aromatase

Very high levels of aromatase activity were observed in the nucleus preopticus medialis (POM). The enzyme was also clearly present in the rest of the preoptic area (POA) as well as in the nucleus ventromedialis hypothalami (VMN), the area lateralis hypothalami (LHy) and the nucleus tuberis (TU). The enzyme activity was low to undetectable in all other nuclei. The two-way ANOVA revealed overall sex differences in the POM and POA and treatment effects in the POM, POA, VMN, LHy and TU (see Table 1). When a sex difference was observed it always resulted from a higher enzyme activity in males when compared to females (Fig. 1). Treatment effects always consisted of a decreased activity following castration or ovariectomy and in increases after exposure to T. A significant difference between males and females from corresponding treatment groups was only observed in the POM of intact birds. This actually resulted in a significant interaction between sex and treatment in the two-way ANOVA. No significant sex difference was observed in aromatase activity of GNX birds exposed to T.

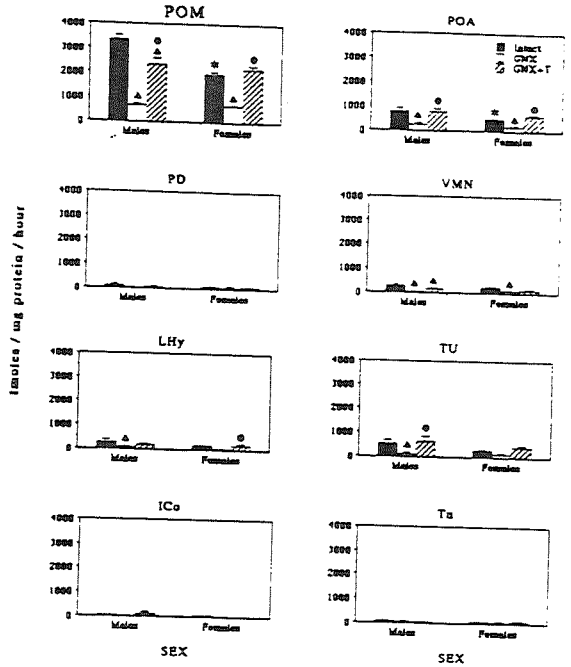

FIG. 1. Formation of estradiol (aromatization) by different nuclei of intact (black columns), gonadectomized (GNX; white columns) and testosterone-treated gonadectomized (GNX + T; hatched columns) adult male and female Japanese quail. Each column corresponds to the mean ± SEM of all experimental data. Individual means were compared by the Fisher protected least significant test and results are reported at the top of the columns: *P < 0.05 compared to males submitted to the same hormonal treatment; ▲P < 0.05 compared to intact birds of the same sex; ●P < 0.05 compared to castrated birds of the same sex. For abbreviations see legend to Fig. 4.

TABLE 1. Statistical Analyses of the Activity of Testosterone-Metabolizing Enzymes in the Brain of Intact, Gonadectomized and Testosterone-Treated Gonadectomized Male and Female Quail.

	Sex		Treatment		Interaction	
	F	P	F	P	F	P
Aromatase						
POM	16.66	0.001	93.06	0.001	13.15	0.001
POA	8.51	0.006	21.23	0.001	1.31	ns
PD	0.64	ns	1.86	0.17	0.70	ns
VMN	0.09	ns	10.92	0.001	0.63	ns
LHy	0.94	ns	5.52	0.008	0.96	ns
TU	2.75	0.106	6.13	0.005	0.92	ns
ICo	0.99	ns	1.06	ns	1.24	ns
Tn	0.31	ns	1.12	ns	1.34	ns
5α-Reductase						
POM	0.59	ns	1.21	ns	0.64	ns
POA	0.79	ns	1.37	ns	0.71	ns
PD	1.99	0.17	0.07	ns	2.32	0.114
VMN	0.79	ns	0.29	ns	0.02	ns
LHy	0.24	ns	2.44	0.101	0.29	ns
TU	2.65	0.113	0.74	ns	0.20	ns
ICo	0.01	ns	0.44	ns	0.12	ns
Tn	0.42	ns	0.34	ns	1.05	ns
5β-Reductase						
POM	0.82	ns	2.59	0.089	0.21	ns
POA	1.18	ns	1.14	ns	0.28	ns
PD	2.21	0.146	0.68	ns	0.77	ns
VMN	8.29	0.007	1.76	ns	1.08	ns
LHy	4.91	0.033	0.84	ns	1.55	ns
TU	6.66	0.014	0.19	ns	0.54	ns
ICo	1.65	ns	0.03	ns	0.09	ns
Tn	2.34	0.134	0.92	ns	0.60	ns

Data were analyzed by two-way ANOVA with the sex of the birds and their hormonal treatments as factors. Results presented are the F ratios and associated probabilities calculated for the effect of the sex of the birds, their hormonal treatment and the interaction between these two factors. ns = not significant (P > 0.20). See Fig. 4 for the abbreviations of the names of the different brain nuclei.

Since a sex difference in aromatase activity had been previously observed on several occasions in a microdissected brain region centered on the preoptic area (11, 12, 19, 20), an additional experiment was carried out to obtain more information on this problem. Aromatase activity was in this case measured separately in the rostral and caudal parts of the POM. Again, no statistically significant sex difference was seen in the aromatase activity of GNX birds submitted to the same replacement therapy with T. However, male values were numerically higher (25%) than those of the females in the posterior part of the nucleus (anterior POM: males (n = 11) 1385.84 ± 226.16, females (n = 10) 1358.20 ± 272.74 fmoles/mg protein/h, t = 0.079, ns; posterior POM: males (n = 10) 1216.35 ± 132.28, females (n = 6) 919.20 ± 127.87 fmoles/mg protein/h, t = 1.498, 2p = 0.156). It must be noted that absolute levels of aromatase were lower here than in the main experiment and this is very probably explained by the smaller diameter of the punch cannula which were used (800 versus 1,200 μm id). It has indeed recently been shown by immunocytochemistry that a large proportion of the aromatase-immunoreactive neurons are located at the periphery of the POM. Therefore these were presumably included in a POM punch collected with the 1,200 μm cannula but not with the 800 μm cannula.

5α-reductase

The neuroanatomical distribution of the 5α-reductase activity was much more homogeneous than for the aromatase. Enzyme activity was close to 1,000 fmoles/mg protein/h in all brain regions except in the LHy where higher levels of activity were observed. No overall effect of the sex of the birds, of their hormonal conditions or of the interaction between these factors could be detected (see Table 1). The *post-hoc* Fisher tests indicated, however, that 5α-reductase activity in the nucleus preopticus dorsolateralis (PD) was higher in ovariectomized females than in castrated males.

5β-reductase

A very active 5β-reductase was observed in all nuclei. Absolute levels of activity of this enzyme were usually 10 to 20 times higher than for the other two enzymes. The distribution of the 5β-reductase activity was also relatively homogeneous in the brain. The enzyme activity was sexually differentiated in three brain nuclei, VMN, LHy and TU (see Table 1). This sex difference had a small magnitude and resulted in only a few significant differences when groups of birds were compared two by two. These always concerned the T-treated animals. No significant effect of the hormonal manipulations was detected by the ANOVA although in two cases (VMN and LHy), the Fisher tests suggested an increase in enzyme activity following treatment of the females with T (P < 0.05).

Discussion

By increasing the sensitivity of a classical radioenzyme assay (11, 21) and combining it with the Palkovits punch technique (16), we were recently able to describe the distribution of T-metabolizing enzymes in the quail brain (17). Here, we demonstrate that this approach is suitable for studying sex differences and hormonal regulations of these enzymes. This combination of techniques provides a relatively good localization of the enzymes and, at the same time, allows a quantitative determination of their activity.

The exact localization of the punches is obviously critical in this approach. The low variance in the observed results demonstrates that a given brain area can be sampled in this way: the standard errors of the means on the figures, which reflect the sum of the assay errors, the individual variations and the dissection errors are still quite small. A more crucial question is whether this dissection procedure allows one to obtain homogeneous brain tissue or still confounds areas with and without enzyme under study. In the specific case of the aromatase, this question can now be answered

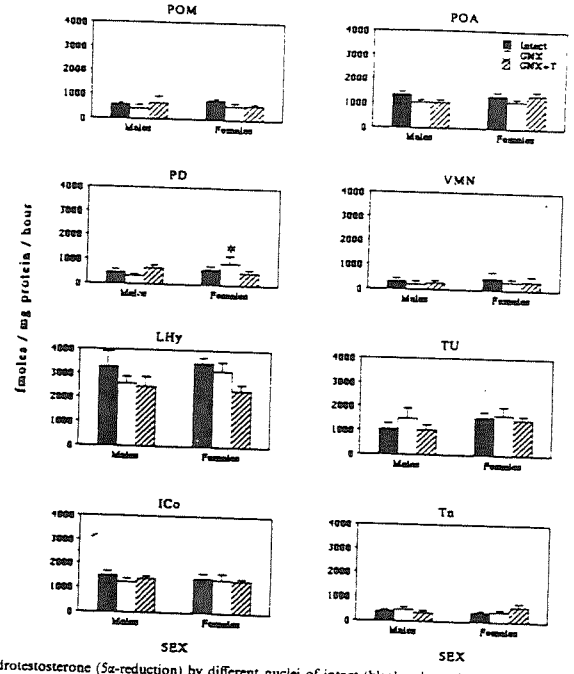

FIG. 2. Formation of 5α-dihydrotestosterone (5α-reduction) by different nuclei of intact (black columns), gonadectomized (GNX; white columns) and testosterone-treated gonadectomized (GNX + T; hatched columns) adult male and female quail. See Fig. 1 for additional details.

as an immunocytochemical procedure for the localization of this enzyme in the quail brain has recently become available (18, 22). This has demonstrated that aromatase-immunoreactive neurons are confined to only a few brain nuclei including the POM especially in its dorso-lateral borders, the septum in the region of the bed nucleus stria terminalis, the dorsal part of the VMN and the TU in the region of the nucleus inferioris hypothalami.

In the present study, as in another work on the rat brain (23), the areas that were dissected out were defined by the nuclei which are observed in Nissl-stained sections. Now that the immunocytochemical localization is known, it appears that this anatomical definition of the punches was adequate for some brain areas but not for others. In the preoptic area, for example, most if not all the aromatase-immunoreactive neurons are located within the POM, mainly at the periphery of the nucleus. The punch containing this nucleus was therefore adequately aimed at an homogeneous target zone. The aromatase activity that we found here in the area surrounding the POM should then be interpreted either as a contamination of this sample by the edges of the POM not removed with the punch or as a low activity that can be detected by the radioenzyme assay, but is not associated with a high enough concentration of the enzyme to allow its detection by immunocytochemistry. By contrast, punches aimed at the VMN defined in Nissl-stained material are more questionable as the aromatase-immunoreactive neurons are not really located within this nucleus but rather lie on its dorso-lateral borders (18, 24). A VMN punch should therefore only contain a fraction of the enzyme localized in this region and a part of it might have been picked up in the LHy punch. This type of analysis is possible at present only for the aromatase as this is the single enzyme that can be detected immunocytochemically. Now, that the distribution of the aromatase has been observed at a cellular level of anatomical resolution (18, 22, 24), it is important to mention that the amount of enzyme activity seems in every case to correspond closely to the intensity of the immunocytochemical signal. Immunoreactive neurons were found in all brain areas which contain a high enzyme activity (POM, POA, dorsal VMN, TU) but not in areas with low or no activity (PD, nucleus intercollicularis (ICo), nucleus taeniae (Tn)). Interestingly, the castration-induced decrease in aromatase activity and its increase following T-treatments are also reflected in immunocytochemistry: the POM of GNX + T males contains about five times more aromatase-immunoreactive cells than the POM of castrates (18). The existence of sex differences in the number of aromatase-immunoreactive neurons is currently under investigation. The absence of aromatase in the Tn, as observed by both immunocytochemistry and radioenzyme assay is in sharp contrast with the situation observed in the zebra finch in which high levels of activity were detected in this region (25, 26). This region is also thought to be homologous with parts of the mammalian amygdala which are known to contain an active aromatase (17, 23).

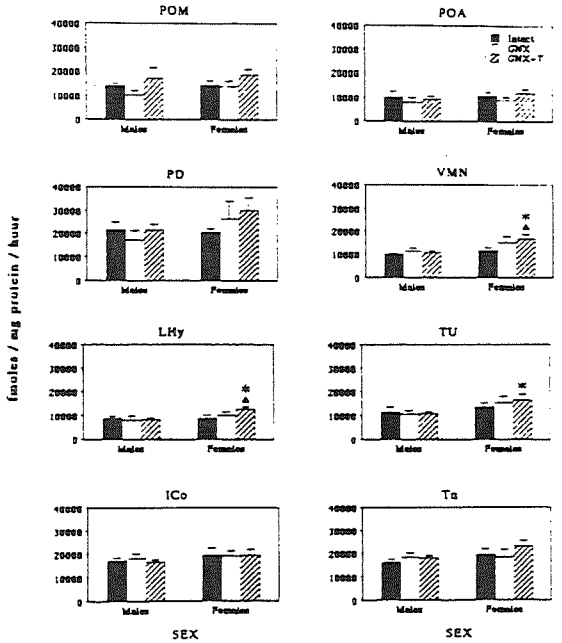

FIG. 3. Formation of 5β-dihydrotestosterone (5β-reduction) by different nuclei of intact (black columns), gonadectomized (GNX; white columns) and testosterone-treated gonadectomized (GNX + T; hatched columns) adult male and female quail. See Fig. 1 for additional details.

The neuroanatomical distributions of aromatase and 5α- and 5β-reductase observed in the present study were consistent with the results of our previous work and therefore do not need to be discussed here. The comparison of the localization of T-metabolizing enzymes and of steroid receptors suggests a number of questions concerning the biological role of the enzymes. Aromatase activity was only detected in nuclei (POM, POA, VMN, LHy, TU) which contain estrogen receptors, as demonstrated by autoradiography and immunocytochemistry (27, 28). The reverse relationship is however not true, and estrogen receptors are also found in areas which appear to be devoid of aromatase activity such as the ICo and Tn. These nuclei are therefore presumably sensitive to estrogens produced directly by the gonads. In addition, recent immunocytochemical studies using a double label technique for the aromatase and for estrogen receptors demonstrate that, at least within the POM, these two markers are not colocalized in most cells (Balthazart, Foidart, Surlemont and Harada, unpublished data). The mode of action of estrogens derived from central aromatization of T therefore remains difficult to appreciate. On the other hand, 5α-reductase activity was detected in all brain areas that were analyzed. Some of these are known to accumulate androgens in autoradiographic studies but others do not. In particular, the POM appears to be largely devoid of binding sites for androgens: radioactivity is retained in this nucleus following the injection of tritiated T but not of tritiated DHT (27). The bound radioactivity seen in POM after the injection of T is therefore likely to be present in the form of tritiated E_2, a fact also supported by the presence of an active aromatase and of binding sites for estrogens in this region (17, 18, 27, 28). The role of the 5α-DHT produced in this nucleus which is implicated in the control of male copulatory behavior (29) is therefore unclear. The synergism between E_2 and 5α-DHT, which is observed in the activation of male sexual behavior, might consequently result from an action of E_2 in the POM and of 5α-DHT at another brain site such as the septum, as suggested by a study on rats by Baum and collaborators (30). Detailed stereotaxic implant studies should be carried out to test this idea.

The present study confirms that the three main T-metabolizing enzymes have a completely different neuroanatomical distribution. It shows in addition that they are affected in a specific manner by the sex and hormonal condition of the birds. Sex

differences were observed for the aromatase and 5β-reductase activities, hormonal treatment only affected the aromatase, the 5α-reductase activity was never modified by either the sex or hormonal condition (see analysis in Table 1). This pattern of regulations appears to be very similar to the one recently observed in corresponding areas of the zebra finch brain using the same experimental approach (26). In this species, however, castration and treatment with T were very significantly affecting the activity of the 5α-reductase. The origins and significance of this apparent difference between species are unclear at present.

The activity of the 5α-reductase was not affected in any of the nuclei that were studied here by the sex of the birds or their gonadal state. In a previous study (11), we had observed a sex difference and a T induction of the activity of this enzyme in a part of the anterior hypothalamus labelled P2 which contained the LHy and a part of the VMN. Based on the present results, it appears that the brain area in which 5α-reductase activity is regulated is close to the LHy and VMN but does not include these nuclei. Additional studies on this region should be performed to identify the anatomical distribution of this T-inducible enzyme.

The presence of significant sex differences in the 5β-reductase activity (females higher than males) had been previously observed in the lobus paraolfactorius and preoptic area (11). Gonadectomy suppressed these differences and they were not restored by T so that it had been concluded that they were presumably caused by ovarian secretions. The situation observed here in three hypothalamic nuclei is substantially different as the sex differences were observed irrespective of the hormonal status of the birds. This suggests that the differences are organizational in nature and result from an irreversible action of neonatal steroids. This interpretation should now be experimentally tested.

The regulations of the aromatase appear to be substantially different from those of the 5β-reductase. As in our previous study based on a much coarser brain dissection (11), the activity of this enzyme was found to be sexually differentiated in the POM and POA of gonadally-intact birds (higher in males than in females). We also found that, in these and other (VMN, LHy, TU) brain regions, castration and T treatment, respectively, decreased and increased the aromatase activity. As male quail are known to have higher plasma levels of T than females (31), the sex difference in the activity of the aromatase might only reflect a differential activation of the enzyme by T in the two sexes. These regulations had been previously identified in the preoptic area obtained by a free-hand microdissection (11). We have also found in several studies using this free-hand dissection that induction by T of aromatase in the POA was significantly higher in males than in females (11, 12, 19, 20). The magnitude of this sex difference is however clearly affected by a number of presently unidentified factors which might include the duration of the exposure to T, the social experience of the birds or their hormonal environment during the pre- and early post-hatching life (20). The main experiment analyzing aromatase activity in the medial part of the POM did not detect this differential induction by T. In an additional experiment, evidence for a higher induction in the posterior part of POM in males was obtained but the difference with females did not reach statistical significance. A few interpretations can be offered to explain the failure to detect such a difference. It is first possible that we did not provide in the present experiment a T-treatment that was optimal for the induction of aromatase activity. This is suggested by the fact that the exogenous T did not restore cloacal gland size in GNX + T males to the level seen in intact males (see above). Similarly, the aromatase activity in the POM of GNX + T males, although it was significantly increased by T, did not reach the level seen in intacts. It is therefore possible that a longer treatment or a treatment with higher doses of steroid would produce a higher enzyme induction in males (to the level of the intact males) and make them significantly different from females submitted to the same manipulation (aromatase activity was significantly higher here in intact males than in GNX + T females). In addition, now that the exact distribution of the aromatase has been observed by immunocytochemistry, we know that the aromatase-positive neurons that were included in the POA as obtained by the free-hand dissection are essentially located in two clusters: the POM and a group of cells located just dorsal to the anterior commissure at the level of the lateral forebrain bundle in and under the nucleus accumbens and bed nucleus stria terminalis (18, 24). It is possible that the sex difference in T-inducibility of the aromatase concerns exclusively this dorsal population of neurons. Alternatively the dimorphism might be specific to a sub-region of the POM as suggested by the fact that in the second experiment presented here a numerical difference was found in the caudal but not in the rostral POM. It should then be assumed that in both experiments we never included the most dimorphic zone of the nucleus in the samples which were analyzed. Since most of the aromatase-immunoreactive neurons are located at the periphery of the POM this interpretation is possible. In this case, however, it remains to be explained why the sex difference was not observed in the rest of the preoptic area (POA). It is quite possible that many aromatase-positive cells at the POM periphery were destroyed during dissection by the borders of the punch needle and that this region contains a significant part of the dimorphic enzyme. It is likely that the dimorphism concerns a limited sub-region of the POA. The present approach is therefore not completely adequate to analyze such a neuroanatomically discrete difference. Immunocytochemical studies on this subject are now in progress in our laboratory.

In conclusion, the present studies demonstrate that T-metabolizing enzymes show specific sex differences and hormone-dependent changes in the quail brain. The aromatase is present in nuclei which control various aspects of reproductive behavior and physiology (POM, VMN, TU). It is strongly regulated by T in these areas and could therefore produce a chemical amplification of the response to the steroid. The 5β-reductase activity is present throughout the brain and more active in females than in males in some brain nuclei. This enzyme might therefore differentially inactivate T in males and females. The 5α-reductase is also present in fairly similar amounts in all brain nuclei but is apparently not influenced by the sex and the hormonal condition of the birds. No specific role for this enzyme in the control of reproduction is therefore suggested by the present data. However, independent studies, in which GNX animals were treated with either 5α-reduced androgens and inhibitors of the 5α-reduction, demonstrate that this metabolic pathway plays a key role in the activation of male reproductive behavior (3, 4, 32–35).

The present studies also show that the punch technique combined with a sensitive radioenzyme assay provide a useful method to analyze the distribution and regulation of T-metabolizing enzymes in the brain. Although this technique has its limitation in terms of the neuroanatomical resolution that can be obtained, it has the great advantage of providing quantitative data on enzymes activities. However, it is only by combining it with neurochemical methods of localization at the cellular level such as immunocytochemistry or *in situ* hybridization that a full understanding of the role of these enzymes in the control of reproduction will be obtained.

Materials and Methods

Subjects

Animals used in the main experiment were adult male (n = 57) and female (n = 72) Japanese quail (*Coturnix coturnix japonica*) which were bought from a local breeder (Fr. Lefevre, Boneffe, Belgium) at the age of 3 to 4 weeks. They were treated as described previously (11). From their arrival in the laboratory until the end of the experiment, they were exposed to a photoperiod of 16 h light/8 h dark with food and water available *ad libitum*. Until the beginning of the experiments, birds were kept in heterosexual groups; during the experiments they were isolated in individual cages. Birds were GNX or sham-operated under total anesthesia (Hypnodil, Janssen Pharmaceutica, Belgium; 15 mg/kg body wt) through a unilateral incision behind the ribs on the left side. Both testes were taken in males through this incision; in females, the left ovary only was removed as the right ovary is not developed and does not regenerate even after castration on the left side (36).

Two weeks after castration when birds had reached the age of sexual maturity (about 6 to 7 weeks), males and females were assigned at random to one of three experimental treatments: sham-operated birds with no hormone, GNX with no hormone or GNX with a T replacement therapy. In this way, six groups of birds were defined according to their sex and hormonal treatment; intact males (n = 21) and females (n = 24); sham-operated birds receiving empty implants, GNX males (n = 15) and females (n = 21); GNX birds receiving empty implants and GNX + T males (n = 21) and females (n = 27); GNX birds receiving Silastic implants filled with T. Birds which were treated with T received 40 mm (2 × 20 mm) of Silastic implants (Dow Corning tubing, number 602-252; id 1.57 mm, od 2.41 mm) filled with crystalline T (Sigma T-1500). Birds of the intact and GNX groups received empty implants of the same length. These capsules were implanted sc in the neck region.

Two to 3 weeks after these implantations, birds were tested twice for male copulatory behavior as previously described (3, 4). Their body weight was recorded and their cloacal gland area was measured (greatest length × width) to provide a measure of the effectiveness of the T treatment. Birds were then killed, brains were immediately removed from the skull and frozen on dry ice. They were stored at − 70 °C until used for the radioenzyme assays. The completeness of gonadectomy and the presence of Silastic implants were checked. Data relative to birds showing gonadal remnants or birds which had lost some of their implants were discarded before analysis and their brain was not used in the metabolism studies. Similarly, the brains of intact and GNX + T males which failed to display active sexual behavior during the tests were not used in the radioenzyme assays. Numbers mentioned in this paper are after the removal of these birds that were not appropriately treated.

In an additional experiment, 23 males and 22 females were submitted to exactly the same treatments as the GNX + T birds of the main experiment (gonadectomy at 4 weeks of age, implantation with 2 × 20 mm Silastic capsules filled with T, behavioral test, measures of cloacal gland size and body weight, sacrifice and dissection of the brain). The aromatase activity was then measured respectively in the rostral and caudal parts of the POM of these birds.

Brain dissection

The isolation of the different nuclei by the Palkovits punch technique (16) was performed according to methods which were previously described and have been fully validated for the quail brain (17). Briefly, the frozen brains were embedded in egg yolk and mounted on the specimen holder of a microtome with the forebrain up. Cryostat coronal sections were made until the tractus septomesencephalicus appeared completely as an inverted V-shape. During this process the plane of section was adjusted according to the stereotaxic atlas of the quail brain (37). Starting from the section in which the entire tractus septomesencephalicus first appeared, 200 μm-thick sections were collected until the level of the occulomotor nerves. Sections were thaw-mounted on glass microscope slides and stored on dry ice for not more than 1 day. Individual nuclei were isolated by punching them out of the sections with cannulae made from steel needles (id 800 or 1,200 μm).

Fig. 4 shows a schematic drawing of the punches that were taken in each bird during the main experiment. A total of eight brain areas were studied

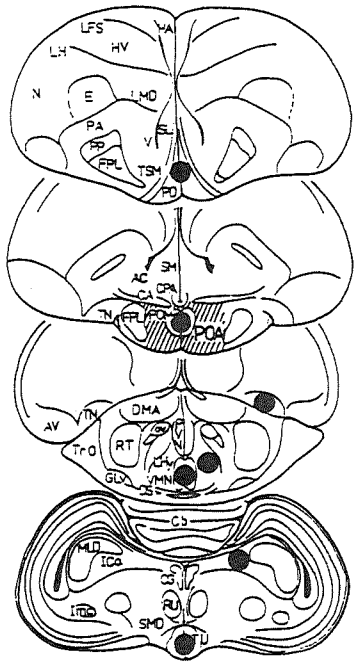

FIG. 4. Schematic drawing showing the localization of the nuclei dissected by the punch method in the adult quail brain. Sections from top to bottom are shown in a rostro-caudal order. Black dots represent the inner diameter of the cannula (800 μm). AC—nucleus accumbens; AV—archistriatum ventrale; CA—commissura anterior; Cb—cerebellum; CG—substantia grisea centralis; CPA—commissura pallialis; DMA—nucleus dorsomedialis anterior thalami; DS—decussatio supraoptica; E—ecostriatum; FPL—fasciculus prosencephali lateralis; GLv—nucleus geniculatus lateralis pars ventralis; HA—hyperstriatum accessorium; HV—hyperstriatum ventrale; ICo—nucleus intercollicularis; Imc—nucleus isthmi pars magnocellularis; LFS—lamina frontalis superior; LH—lamina hyperstriatica; LHy—area lateralis hypothalami; LMD—lamina medularis dorsalis; MLD—nucleus mesencephalicus lateralis pars dorsalis; N—neostriatum; OV—nucleus ovoidalis; PA—paleostriatum augmentatum; PD—nucleus preopticus dorsolateralis; POA—preoptic area; POM—nucleus preopticus medialis; PP—paleostriatum primitivum; PVN—nucleus periventricularis magnocellularis; RT—nucleus rotundus; RU—nucleus ruber; SL—nucleus septalis lateralis; SM—nucleus septalis medialis; SMD—supramamillary decussatio; TSM—tractus septomesencephalicus; TN—nucleus taeniae; TU—tuber; V—ventriculus; VMN—nucleus ventromedialis hypothalami. Nomenclature is based on previous publications on the quail and chicken brain (37–40, 43).

here, namely, the nucleus preopticus dorsolateralis (PD), the nucleus preopticus medialis (POM), the rest of the preoptic area (POA), the nucleus ventromedialis hypothalami (VMN), the area lateralis hypothalami (LHy), the nucleus taeniae (Tn), the nucleus intercollicularis (ICo) and the nucleus tuberis (TU) (37–40, 43). The POM was punched with a 1.200 μm cannula and the POA was later microdissected on the slide with a small scalpel blade. All other nuclei were dissected with a 800 μm cannula. The nuclei were punched from two consecutive sections in the medial parts of the nuclei. In addition, samples from three birds had to be pooled for the assay of enzymatic activities. The number of independent samples in each experimental group is consequently equal to one third of the number of animals that were used (seven intact males, eight intact females, five GNX males, seven GNX females, seven GNX+T males and nine GNX+T females). The punched areas were blown out of the needles in a glass tube and pooled samples were homogenized by ultrasonication in 120 μl of ice-cold STMM buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 at 20 °C, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol). Twenty μl of these homogenates were assayed for protein content by the method of Bradford (41). The remaining 100 μl were frozen in an acetone-dry ice-bath and stored at −70 °C until assayed for the activity of T-metabolizing enzymes.

During the additional experiment, POM aromatase activity only was studied. Therefore, due to the high enzyme activity in this nucleus, punches from two birds only had to be pooled to obtain detectable levels of activity. The aromatase activity was measured separately in the anterior and posterior part of the nucleus. In each case, the POM was punched out from two consecutive sections using a cannula of 800 μm id. All other aspects of this study were as described for the main experiment.

Measure of enzymatic activities

The activity of T-metabolizing enzymes was performed as described previously using procedures which have been fully validated for the quail brain (17). The brain homogenates were thawed on an ice-bath and 50 μl of STMM containing [^{3}H]testosterone (New England Nuclear, Brussels, Belgium; specific activity 168 Ci/mmol; final concentration: 25 nM) and $NADPH_2$ (final concentration: 1.2 mM = saturating level) were added. Tubes were incubated at 41 °C for 15 min and then the metabolites produced were extracted and purified by phenolic partition and thin layer chromatography as described in earlier publications from this laboratory (10, 11, 17, 42). Three metabolites were quantified: E_2, 5α-DHT and 5β-DHT. The identity of these metabolites has been confirmed for the quail hypothalamus by recrystallizations to specific activity and constant isotopic ratio (42). All results are finally expressed as femtomoles of metabolite produced per mg protein per hour.

Statistical analysis

The amounts of metabolites formed by the different brain nuclei were compared by two-way analyses of variance (ANOVA) with the sex of the birds and their hormonal treatment (intact, GNX with or without T-treatment) as factors. Individual means were compared when appropriate by the Fisher protected least significant difference test. Data are represented in the text and figures by their means and standard error of the mean.

Acknowledgements

We are indebted to Professor E. Schoffeniels for his continued interest in our research. This work was supported by grants from the National Institutes of Health, Bethesda, MD (HD 22064), the Belgian Fonds National de la Recherche Scientifique (Crédits aux Chercheurs), the Medical School of Liege; the University of Liege (Fonds Spécial pour la Recherche) and the EEC (SC1-0230-C/TT) to J. Balthazart and by a grant from the Belgian Fonds de la Recherche Fondamentale Collective (nbr. 2.4518.80) to Professor Schoffeniels.

Accepted 9 April 1990

References

1. Balthazart J. (1988). Steroid metabolism and the activation of social behavior. In: **Balthazart J.** ed. **Advances in comparative and environmental physiology**, vol 3: 105–159. Springer Verlag, Berlin.
2. McEwen BS. (1981). Neural gonadal steroid actions. **Science.** 211: 1303–1311.
3. Alexandre C. Balthazart J. (1986). Effects of metabolism inhibitors, antiestrogens and antiandrogens on the androgen and estrogen induced sexual behavior in Japanese quail. **Physiol Behav.** 38: 581–591.
4. Balthazart J. Schumacher M. Malacarne G. (1985). Interaction of androgens and estrogens in the control of sexual behavior in male Japanese quail. **Physiol Behav.** 35: 157–166.
5. Balthazart J. Schumacher M. (1984). Organization and activation of behavior in quail: role of testosterone metabolism. **J Exp Zool.** 232: 595–604.
6. Hutchison JB. Steimer TH. (1981). Brain 5β-reductase. A correlate of behavioral sensitivity to androgen. **Science.** 213: 244–246.
7. Balthazart J. Massa R. Negri-Cesi P. (1979). Photoperiodic control of testosterone metabolism, plasma gonadotrophins, cloacal gland growth, and reproductive behavior in the Japanese quail. **Gen Comp Endocrinol.** 39: 222–235.
8. Balthazart J. Ottinger MA. (1984). 5β-reductase activity in the brain and cloacal gland of male and female embryos of the Japanese quail (*Coturnix Coturnix japonica*). **J Endocrinol.** 102: 77–81.
9. Deviche P. Bottoni L. Balthazart J. (1982). 5β-dihydrotestosterone is weakly androgenic in the adult Japanese quail (*Coturnix coturnix japonica*). **Gen Comp Endocrinol.** 48: 421–424.
10. Schumacher M. Balthazart J. (1984). Sexual dimorphism of the hypothalamic metabolism of testosterone in the Japanese quail (*coturnix coturnix japonica*). **Prog Brain Res.** 61: 53–61.
11. Schumacher M. Balthazart J. (1986). Testosterone-induced brain aromatase is sexually dimorphic. **Brain Res.** 370: 285–293.
12. Balthazart J. Foidart A. Hendrick JC. (1990). The induction by testosterone of aromatase activity in the preoptic area and activation of copulatory behaviour. **Physiol Behav.** 47: 83–94.
13. Balthazart J. Schumacher M. (1984). Changes in testosterone metabolism by the brain and cloacal gland during sexual maturation in the Japanese quail. **J Endocrinol.** 100: 13–18.
14. Balthazart J. (1983). Hormonal correlates of behavior. In: **Farner DS. King JR and Parkes KC.** eds. **Avian biology**, 221–365. Academic Press, New York.
15. Balthazart J. Schumacher M. (1985). Role of testosterone metabolism in the activation of sexual behaviour in birds. In: **Gilles R and Balthazart J.** eds. **Neurobiology. Current comparative approaches.** 121–140. Springer-Verlag, Berlin.
16. Palkovits M. Brownstein MJ. (1983). Microdissection of brain areas by the punch technique. In: **Cuello AC.** ed. **Brain microdissection techniques.** 1–36. Wiley, New York.
17. Schumacher M. Balthazart J. (1987). Neuroanatomical distribution of testosterone metabolizing enzymes in the Japanese quail. **Brain Res.** 422: 137–148.
18. Balthazart J. Foidart A. Harada N. (1990). Immunocytochemical localization of aromatase in the brain. **Brain Res.** 514: 327–333.
19. Balthazart J. Devos F. Dohet A. Foidart A. Hugla JL. Radermaker F. Schumacher M. (1986). The induction of aromatase and sexual behavior by testosterone in male and female Japanese quail: a dose-response study. **IRCS Med Sci.** 14: 1188–1189.
20. Balthazart J. (1989). Correlation between the sexually dimorphic aromatase of the preoptic area and sexual behavior in quail: effects of neonatal manipulations of the hormonal milieu. **Arch Int Physiol Biochim.** 97: 465–481.
21. Hutchison JB. Steimer TH. (1986). Formation of behaviorally effective 17β-estradiol in the dove brain: steroid control of preoptic aromatase. **Endocrinology.** 118: 2180–2187.
22. Balthazart J. Foidart A. (1989). Immunocytochemical localization of aromatase in the quail brain. **Soc Neurosci Abstr.** 15: 430.18.
23. Roselli CE. Horton LE. Resko JA. (1985). Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. **Endocrinology.** 117: 2471–2477.
24. Balthazart J. Foidart A. Surlemont C. Harada N. The preoptic aromatase in quail: behavioral, biochemical and immunocytochemical studies. In: **Balthazart J.** ed. **Hormones, brain and behaviour in vertebrates.** Comparative Physiology, vol. 9. Karger, Basel. (In press).
25. Vockel A. Prôve E. Balthazart J. (1990). Sex- and age-related differences in the activity of testosterone-metabolizing enzymes in microdissected nuclei of the zebra finch brain. **Brain Res.** 511: 291–302.
26. Vockel A. Prôve E. Balthazart J. (1990). Effects of castration and testosterone treatment on the activity of testosterone metabolizing enzymes in the brain of male and female zebra finches. **J Neurobiol.** (In press).
27. Watson JT. Adkins-Regan E. (1989). Neuroanatomical localization of sex steroid-concentrating cells in the Japanese quail (*Coturnix japonica*): autoradiography with [^{3}H]-testosterone, [^{3}H]-estradiol, and [^{3}H]-dihydrotestosterone. **Neuroendocrinology.** 49: 51–64.
28. Balthazart J. Gahr M. Surlemont C. (1989). Distribution of estrogen receptors in the brain of the Japanese quail: an immunocytochemical study. **Brain Res.** 501: 205–214.
29. Balthazart J. Surlemont C. Copulatory behavior is controlled by the sexually dimorphic nucleus of the quail preoptic area. **Brain Res Bull.** (In press).
30. Baum MJ. Tobet SA. Starr MS. Bradshaw WG. (1982). Implantation of dihydrotestosterone propionate into the lateral septum or medial amygdala facilitates copulation in castrated male rats given estradiol systematically. **Horm Behav.** 16: 208–223.
31. Balthazart J. Delville Y. Sulon Y. Hendrick JC. (1987). Plasma levels of luteinizing hormone and of five steroids in photostimulated, castrated and testosterone-treated male and female Japanese quail (Coturnix coturnix japonica). **Gen Endocrinol (Life Sci Adv)** 5: 31–36.
32. Schumacher M. Balthazart J. (1983). The effects of testosterone and its metabolites on sexual behavior and morphology in male and female Japanese quail. **Physiol Behav.** 30: 335–339.
33. Balthazart J. Schumacher M. Malacarne G. (1984). Relative potencies of testosterone and 5α-dihydrotestosterone on crowing and cloacal gland growth in the Japanese quail (Coturnix coturnix japonica). **J Endocrinol.** 100: 19–23.
34. Södersten P. Eneroth P. Hansson T. (1988). Oestradiol synergizes with 5α-dihydrotestosterone or 3α but not 3β-androstanediol in inducing sexual behaviour in castrated rats. **J Endocrinol.** 119: 461–465.

35. Södersten P. Eneroth P. Hansson T. Mode A. Johansson D. Naslund B. Liang T. Gustafsson JA. (1986). Activation of sexual behaviour in castrated rats: the role of oestradiol. **J Endocrinol.** 111: 455–462.
36. Gibson WR. Follett BK. Gledhill B. (1975). Plasma levels of luteinizing hormone in gonadectomized Japanese quail exposed to short or to long daylengths. **J Endocrinol.** 64: 87–101.
37. Baylé JD. Ramade F. Oliver J. (1974). Stereotaxic topography of the brain of the quail. **J Physiol (Paris).** 68: 219–241.
38. Panzica GC. Viglietti-Panzica C. Calcagni M. Anselmetti GC. Schumacher M. Balthazart J. (1987). Sexual differentiation and hormonal control of the sexually dimorphic preoptic median nucleus in quail. **Brain Res.** 416: 59–68.
39. Panzica GC. Viglietti-Panzica C. Sanchez F. Sante Ph. Balthazart J. Effects of testosterone on a selected neuronal population within the preoptic sexually dimorphic nucleus of the Japanese quail. **J Comp Neurol.** (In press).
40. Kuenzel WJ. Masson M. (1988). **A stereotaxic atlas of the brain of the chick (Gallus domesticus).** The Johns Hopkins University Press, Baltimore.
41. Bradford MM. (1976). A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal Biochem.** 72: 248–254.
42. Schumacher M. Contenti E. Balthazart J. (1984). Partial characterization of testosterone-metabolizing enzymes in the quail brain. **Brain Res.** 305: 51–59.
43. Kuenzel WJ. VanTienhoven A. (1982). Nomenclature and location of avian hypothalamic nuclei and associated circumventricular organs. **J Comp Neurol.** 206: 293–313.