J. Steroid Biochem. Molec. Biol. Vol. 40, No. 4–6, pp. 557–570, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

TESTOSTERONE METABOLISM IN THE AVIAN HYPOTHALAMUS

J. BALTHAZART

Laboratory of General and Comparative Biochemistry, University of Liège (Bat L1), 17 place Delcour, 4020 Liège, Belgium

Summary—Many central actions of testosterone (T) require the transformation of T into several metabolites including 5α-dihydrotestosterone (5α-DHT) and estradiol (E_2). In birds as in mammals, 5α-DHT and E_2, alone or in combination, mimic most behavioral effects of T. The avian brain is, in addition, able to transform T into 5β-DHT, a metabolite which seems to be devoid of any behavioral or physiological effects, at least in the context of reproduction. By *in vitro* product-formation assays, we have analyzed the distribution, sex differences and regulation by steroids of the 3 main T metabolizing enzymes (aromatase, 5α- and 5β-reductases) in the brain of the Japanese quail (*Coturnix c. japonica*) and the zebra finch (*Taeniopygia guttata castanotis*). In the hypothalamus of quail and finches, aromatase activity is higher in males than in females. It is also decreased by castration and increased by T. The activity of the 5α-reductase is not sexually differentiated nor controlled by T. The 5β-reductase activity is often higher in females than in males but this difference disappears in gonadectomized birds and no clear effect of T can be observed at this level. The zebra finch brain also contains a number of steroid-sensitive telencephalic nuclei [e.g. hyperstriatum ventrale, pars caudale (HVc) and robustus archistriatalis (RA)] which play a key role in the control of vocalizations. These nuclei also contain T-metabolizing enzymes but the regulation of their activity is substantially different from what has been observed in the hypothalamus. Aromatase activity is for example higher in females than in males in HVc and RA and the enzyme in these nuclei is not affected by castration nor T treatment. In these nuclei, the 5α-reductase activity is higher in males than in females and the reverse is true for the 5β-reductase. These sex differences in activity are not sensitive to gonadectomy and T treatment and might therefore be organized by neonatal steroids. We have been recently able to localize aromatase-immunoreactive (AR-ir) neurons by ICC in the brain of the quail and zebra finch. Positive cells are found in the preoptic area, ventromedial and tuberal hypothalamus. AR-ir material is found in the perikarya of cells and fills the entire cellular processes including axons. At the electron microscope level, immunoreactive material can clearly be observed in the synaptic boutons. This observation raises questions concerning the mode of action of estrogens produced by central aromatization of T.

INTRODUCTION

In males of most vertebrate species, testosterone (T) is one of the major steroids which control the physiological and behavioral components of reproduction. In particular, the activation of copulatory behavior in the male critically depends on the action of T in the preoptic area (POA). In the brain, as in many other androgen-target structures, the activity of T can be modulated by its conversion into active vs inactive metabolites. In the brain of both birds and mammals, T can be transformed into several metabolites including estradiol-17β (E_2; aromatization) and 5α-dihydrotestosterone (5α-DHT; 5α-reduction). Depending on the species, E_2 and 5α-DHT, alone or in combination, are able to mimic most if not all behavioral effects of T [1–3]. The avian brain, in addition, is able to transform T into 5β-dihydrotestosterone (5β-DHT; 5β-reduction), a metabolite which seems devoid of any biological activity at least in the context of reproduction. The 5β-reduction of T is usually considered as an inactivation shunt for the hormone [1, 4]. The brain metabolism of T is therefore of special interest in birds as the modulation of enzyme activities leading to the production of active or inactive metabolites can in principle regulate the neural responsiveness to the androgen.

During the last 10 years, research in my laboratory has analyzed the contribution of the intracellular T metabolism to the regulation of

Proceedings of the VIIIth International Congress on Hormonal Steroids, The Hague, The Netherlands, 16–21 September 1990.

reproductive behavior in birds. Two avian species which are common models in behavioral endocrinology have been used in these studies: the Japanese quail (*Coturnix coturnix japonica*) and the zebra finch (*Taeniopygia guttata castanotis*). Two experimental approaches have been used to investigate this question. On one hand, *in vivo* experiments using T metabolites, antihormones and metabolism inhibitors have been performed to establish the behavioral action of androgenic and estrogenic steroids. These compounds were injected systemically or directly implanted in the POA of castrated quail and the behavioral responses that were elicited were quantified in standardized situations. On the other hand, the activity of the major T metabolizing enzymes (aromatase, 5α- and 5β-reductase) has been quantified by *in vitro* radioenzyme assays (product-formation assays) in the brain of birds and correlated with their behavior. These assays were done in birds of different ages or sex as well as in castrated animals submitted to various replacement therapies with steroid hormones so that precise relationships could be established between the activity of brain enzymes and the reproductive behavior of the subjects.

I shall briefly describe here the major results demonstrating that androgen metabolism in the brain of quail and zebra finches plays a critical role in the control of male reproductive behavior in these species. The changes in the activity of brain enzymes as a function of age, sex or hormonal condition will then be reviewed and the physiological implications of these enzymatic adaptations will be discussed. During the last year, we were also able to localize by immunocytochemistry the aromatase in the avian brain. This new approach has opened a new field of investigation in the area of steroid metabolism research and the first results gathered so far will be considered at the end.

T METABOLISM AND ACTIVATION OF MALE REPRODUCTIVE BEHAVIOR IN QUAIL

In quail, male copulatory is activated by T produced by the gonads. Birds functionally castrated by exposure to short days or birds surgically castrated are inactive [5, 6] and treatment with T restores sexual activity within one week [6, 7]. In the specific case of copulatory behavior, aromatization of T into E_2 is absolutely required for the activation of the behavior. This notion is supported by the following facts: aromatizable androgens such as T or androstenedione activate the behavior while nonaromatizable androgens such as 5α-DHT are not or less active [5]; aromatase inhibitors such as androstratrienedione (ATD) or R76713 inhibit T-induced copulatory behavior in functionally or surgically castrated males [5, 7, 8]; antiestrogens such as tamoxifen or nitromifene citrate (CI-628) block T-induced sexual behavior [9, 10]. These compounds are active after systemic injection but also when implanted directly in minute amounts within the POA thereby demonstrating that T aromatization needs to take place within the brain in order to activate the behavior [8, 11]. Consistent with this implication of aromatization are also the facts that the behavior can be activated directly by systemic injections or stereotaxic implantation in the POA of estrogens such as E_2 or diethylstilbestrol [5, 10–13].

The fact that copulatory behavior can be activated in castrated quail by estrogens alone does, however, not rule out a possible contribution of androgens in this process. Treatment with 5α-DHT alone or with the nonaromatizable androgen R1881 indeed weakly stimulates male sexual behavior in castrated quail [14–16] (although negative results obtained with 5α-DHT have also been reported [17, 18]). In addition, a clear synergism between 5α-DHT (or R1881) and estrogens can be experimentally demonstrated in the activation of this behavior [14, 16, 18] (see [1] for review).

IMPLICATION OF T METABOLISM IN THE SEXUAL DIFFERENTIATION OF BEHAVIOR IN QUAIL

Masculine reproductive behavior is sexually differentiated in quail. If treated with doses of T which activate sexual behavior in castrated males, females almost never show copulatory behavior when presented to a stimulus bird [6, 19]. It is currently accepted that the sexual differentiation of copulatory behavior in quail follows a pattern in which the male is the neutral sex (i.e. which develops in the absence of gonadal steroids) and the female phenotype results from the early exposure to estrogens [20]. This notion is essentially supported by the finding that injection of estrogen into male eggs during early incubation suppresses the capacity of the male to respond when adult to a treatment with T by showing copulatory

behavior [21, 22]. This "demasculinization" would spontaneously take place in females following exposure to the ovarian estrogens and indeed we showed recently that plasma levels of E_2 are significantly higher in female than in male embryos [23].

Surprisingly both T and E_2 are able to demasculinize the behavior of male embryos if injected in the egg during the early incubation [21]. In an extensive series of experiments, it could however be demonstrated that the demasculinization of copulation by exogenous T is mediated by its conversion to estrogen. The differentiating effects of T are indeed blocked by the simultaneous administration of antiestrogens or of aromatase inhibitors and they are mimicked by aromatizable but not by nonaromatizable androgens [24]. It was confirmed recently that an active aromatase is indeed present in the POA-hypothalamus of quail embryos as early as day 10 of incubation [25]. At that early age, the enzyme is however not inducible by T and it is only after day 14 of incubation that this control mechanism of the aromatase activity becomes established [25, 26].

The fact that T is present in substantial amounts in the plasma of developing embryos [23] and that the aromatase in the POA is already active on day 10 of incubation suggested that in physiological conditions, males could actually be partially demasculinized by their endogenous androgens acting through aromatization. This possibility was tested by treating male embryos with the aromatase inhibitor ATD and quantifying their sexual behavior as adults. It could be demonstrated that embryonic exposure to ATD slightly increases the level of male-typical behavior suggesting that a weak demasculinization takes place in normal males [27]. This effect is however of a very small magnitude probably because embryonic males are protected from the demasculinizing effects of their androgens by an extremely active 5β-reductase which is present in their hypothalamus [28] and actively transforms T into 5β-DHT, a compound which is not aromatizable and has no effect on the differentiation of sexual behavior [22].

T METABOLISM IN THE ACTIVATION AND DIFFERENTIATION OF MALE REPRODUCTIVE BEHAVIOR IN ZEBRA FINCHES

In zebra finches as in quail, the activation of several reproductive behaviors depends on the central aromatization of T. In castrated finches, aromatizable androgens are more effective than nonaromatizable ones in restoring normal levels of courtship song [29]. Recent studies also show that androstenedione has to be converted into estrogenic metabolites in order to exert its behavioral effects. If androstenedione-treated birds are concurrently exposed to the aromatase inhibitor, ATD they exhibit fewer courtship behaviors, less aggression and nest building activity than males treated with androstenedione alone [30]. Song production and song learning is controlled in this species by a discrete network of telencephalic and mesencephalic nuclei which are steroid-sensitive and sexually dimorphic [31, 32]. The sexual differentiation of singing (adult males but not adult females sing in response to T in this species) and of the brain nuclei controlling its production can be modified by exposure to exogenous steroids during the first days of life. Estrogens and aromatizable androgens masculinize these characteristics in females [33, 34]. Although sex differences in the plasma levels of estrogens have been reported in young zebra finches (higher levels in males than in females: [35, 36]), these are limited in duration and do not completely cover the period during which exogenous estrogens are able to masculinize the brain and behavior of the birds (see [37] for discussion). In addition, castration does not lead to a decrease in circulating E_2 in this species so that the origin of the estrogens (adrenal secretion, brain aromatization?) remains unclear [36]. A role for the central aromatization of androgens in the process of sexual differentiation might therefore be considered.

THE ASSAY OF T-METABOLIZING ENZYMES IN THE AVIAN BRAIN

The data reviewed above has established the importance of T metabolism in the activation and differentiation of reproductive behavior in quail and zebra finches. Until recently, little or no evidence was however available concerning the distribution of T metabolizing enzymes in the brain of these species. Similarly the regulation of these enzymatic activities as a function of the sex, age or endocrine condition of the animals had never been analyzed. Since the original demonstration of aromatase in the brain by Naftolin *et al.* [38], this enzyme and the steroid reductases have classically been studied by product-formation assays. This approach has also been used in our studies.

The activity of aromatase, 5α- and 5β-reductase was measured in brain samples by *in vitro* radioenzyme assays which quantified the transformation of radioactive T into the corresponding metabolites (E_2, 5α-DHT, 5β-DHT). These procedures were first adapted and validated for the avian brain [39, 40]. Briefly, the procedure used in all studies is based on the incubation at 41°C of the brain homogenates with tritiated T in the presence of a suitable concentration of co-factor ($NADPH_2$). The metabolites are then extracted by organic solvents, purified by phenolic partition and thin layer chromatography, and quantified by scintillation counting. Initial validations demonstrated that the amounts of metabolites which are produced in the selected conditions are linear as a function of time of incubation, and amount of tissue present in the sample. The co-factor is also present in saturating conditions.

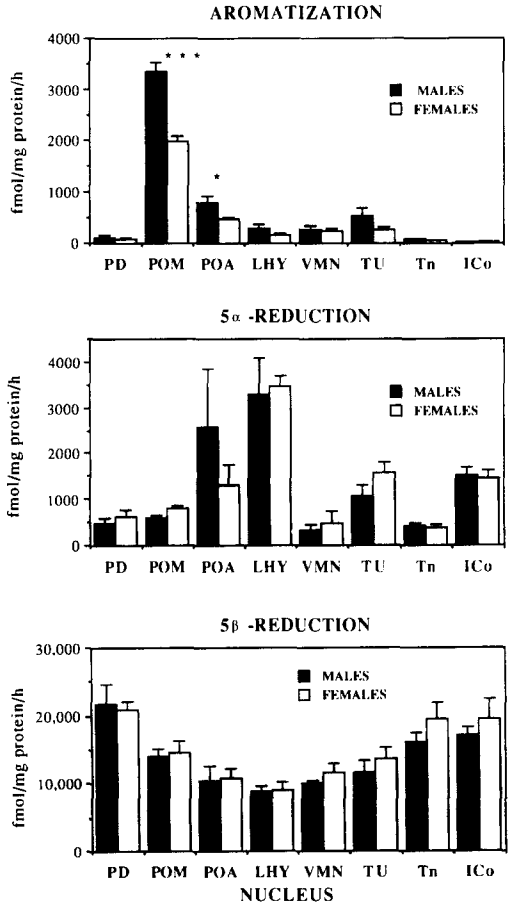

Fig. 1. T metabolism in the brain of intact sexually mature male and female quail as studied by the Palkovits punch technique combined with radioenzyme assay. Data are means ± SEM. * = $P < 0.05$; *** = $P < 0.001$ compared to corresponding male data by the Student *t*-test. PD: pars dorsalis of POA; Tn: nucleus taeniae; (redrawn from data in [46]).

The original studies were performed on brain samples weighing a few milligrams which were obtained by a free-hand dissection and corresponded to more or less defined brain regions (e.g. POA vs posterior hypothalamus [40–42]). More recently, the radioenzyme assay procedure was modified and its sensitivity increased in order to permit quantification of enzyme activities in smaller samples. The Palkovits "punch technique" [43, 44] was then adapted for the quail and zebra finch brain so that the activity of T-metabolizing enzymes could be measured on punched nuclei [37, 45–47]. We shall concentrate here on these most recent anatomically well-defined results.

ACTIVITY OF T-METABOLIZING ENZYMES IN QUAIL

The distribution of aromatase, 5α- and 5β-reductase was first studied in the brain of sexually mature intact male and female quail [45, 46].

Major results obtained in this study are shown in Fig. 1. Both aromatase and 5α-reductase are heterogeneously distributed in the quail brain. The activity of the 5β-reductase is more constant from one nucleus to the other although the enzyme seems to be less active in the POA-hypothalamus [nuclei POM, lateral hypothalamus (LHY), ventro-medial (VMN) and tuberal hypothalamus (TU)]. The highest levels of aromatase activity (AA) are observed in the medial preoptic nucleus (POM), a sexually dimorphic [48] T-sensitive [49] structure located around the third ventricle in the POA. AA in this nucleus and in the POA in general is higher in males than in females. No other significant sex-related difference in enzymatic activities could be observed.

The hormonal-dependence of T-metabolizing enzymes was subsequently investigated in males and females by comparing enzyme activities in sexually mature intact and gonadectomized quail as well as in gonadectomized birds submitted to a replacement therapy with T. Results of these manipulations in males are shown in Fig. 2.

AA in males was significantly decreased by castration and increased by T in the POM, the entire POA and in the VMN. Similar trends were seen in the TU but they did not reach statistical significance. The activity of two reductases was not significantly changed by these hormonal treatments.

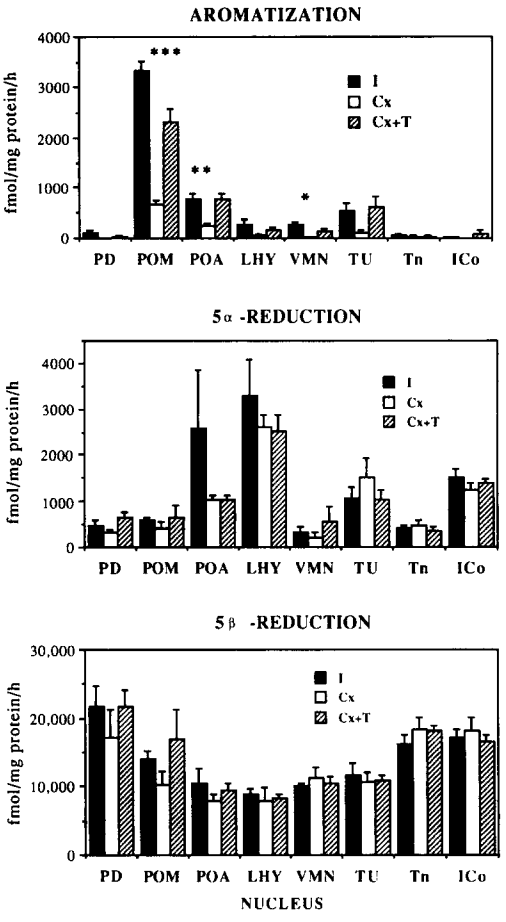

Fig. 2. Effect of castration and testosterone treatment on the T metabolism in the brain of male quail. Data are means ± SEM. Significant effects of treatments (as calculated by ANOVA) are indicated by asterisks above the columns. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. I = Intact sexually mature birds; Cx = castrated males; Cx + T = castrated males treated with silastic implants filled with T; (redrawn from data in [46]).

In previous studies [41, 42] based on a free-hand dissection of the hypothalamus, a few additional modulations of the reductases had been reported. It had been shown that the activity of the 5α-reductase was higher in males than in females in the rostral part of the hypothalamus while the 5β-reductase was more active in the POA of females. These differences were however only observed in gonadally intact birds and disappeared following gonadectomy. They were probably the result of a differential activation of the enzymatic activities by the testicular or ovarian steroids. These sex differences were not found in the present studies based on the Palkovits "punch technique" (overall analysis of variance demonstrate however higher 5β-reductase activity in several nuclei of the female brain; see [46] for details) presumably because here we studied discrete brain nuclei which are only a small fraction of the larger samples previously analyzed. The exact anatomical localization of the sex differences in reductase activities therefore remains to be established.

The sex difference and steroid regulation of AA in the POA and more specifically in the POM are especially important in the context of the control of sexual behavior. In our original study [42], a higher AA had been detected in the POA of males compared to females. Gonadectomy decreased the enzyme activity to baseline levels in both sexes but T replacement resulted in a differential induction of the enzyme in males and females so that the preoptic AA was higher in T-treated castrated males than in T-treated ovariectomized females. Such a sex difference in aromatase induction by T has now been replicated in several independent experiments [7, 50, 51] although the magnitude of this difference appears to be lower than originally thought (males being about 10–25% higher than females).

The sex difference and induction by T of AA was shown in the punch studies [46] to be essentially confined to the POM (see Figs 1–2). This observation is of special relevance for the control of reproduction because we showed recently that this nucleus is a critical site of action for T in the restoration of copulatory behavior in castrated male quail. Stereotaxic implantation of 27 g needles filled with crystalline T within POM activated copulation in castrated quail but implants positioned elsewhere in the POA even a few hundred microns lateral to POM were ineffective [52]. In addition, electrolytic lesions in the POA suppress copulatory behavior in quail only if they destroy a significant part of the POM. Lesions of similar size in other parts of the POA are ineffective [52].

The activity of aromatase in the POM varies in parallel with copulatory behavior (both are decreased by castration and induced by T). The sex difference in POM-AA might therefore contribute to explain the relative insensitivity of females to the activating effects of T on sexual behavior (see above): they would not produce enough of the active metabolite, E_2. It is clear, however, that this is not the only mechanism that mediates the behavioral sex difference because we know that systemic treatment of ovariectomized females with doses of estrogens which are sufficient to activate copulation in males is still ineffective [12]. If the lower

aromatase of females was the only limiting factor, it should be by-passed by the direct administration of the reaction product of the enzyme.

It must also be noted that, in the punch study, we did not find a significant difference in POM-AA between gonadectomized T-treated males and females although means indicated a strong tendency in this direction especially in the caudal part of the nucleus. It seems therefore that AA might be truly differentiated in subregions of the POA but the radioassay of punched nuclei might not provide enough anatomical resolution to permit a meaningful analysis of these differences. This problem is currently analyzed by immunocytochemical techniques (see below) which appear much more appropriate here.

In additional experiments, we also analyzed the quantitative relations between the induction of AA in the POA and the activation of copulatory behavior by T in quail [7, 50]. These showed that the induction of AA is dose- and time-dependent. AA levels normally seen in sexually mature males are restored in castrated birds by treatments with 20–40 mm long silastic capsules filled with T which produce physiological levels of the steroid. The minimal dose of T which reliably restores copulatory behavior approximately doubles the AA in POA.

A significant increase in preoptic AA is already observed within 16 h after the implantation of the T-filled capsules and the induction is maximal after 48 h. Activation of the behavior follows a similar time-course but with a delay of about 24–48 h. These results are thus all consistent with the idea that the preoptic AA is a limiting factor for the activation of copulatory behavior. When the activity of the enzyme is blocked by an aromatase inhibitor such as ATD or R76713, the restoration of sexual behavior is suppressed or strongly delayed [7, 8] (see Fig. 3).

These data clearly implicate in a causal way the preoptic aromatase in the control of male reproductive behavior in quail.

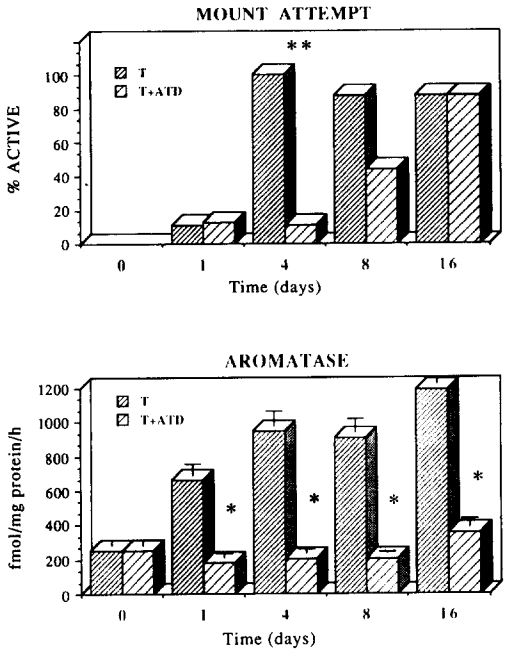

Fig. 3. Effects of chronic treatment with T (20 mm silastic capsules) alone or in combination with the aromatase inhibitor, ATD on the activation of sexual behavior (% of birds showing mount attempts) and on the AA in the POA-hypothalamus. Birds received the hormonal treatments for various durations (0–16 days). They were then tested for behavior, sacrificed and the metabolism of T was quantified in their brain by *in vitro* radioenzyme assays (redrawn from data in [7]).

ACTIVITY OF T-METABOLIZING ENZYMES IN ZEBRA FINCHES

Similar studies of T-metabolizing enzymes were recently carried out on the zebra finch brain. In song birds, steroid-sensitive structures are found in the telencephalon in addition to the limbic structures (for review see [53]). A network of interconnected nuclei implicated in the acquisition and expression of vocal behavior are clearly visible in Nissl-stained sections [32, 54, 55]. They have been shown by autoradiography to accumulate T or its metabolites [31, 56]. It was therefore of interest to research whether these so-called song control nuclei were also able to metabolize T.

Medium to high levels of aromatase, 5α- and 5β-reductase were found in all these nuclei with the possible exception of the area X (X) which is interestingly the only nucleus of song system that does not appear to be steroid-sensitive [53]. In zebra finches like in quail, these enzymes were heterogeneously distributed in the brain (see Fig. 4).

The activity of the 5β-reductase was again low in the hypothalamic nuclei compared to most parts of the telencephalon. Surprisingly, the highest levels of AA were not found in the limbic structures but appeared in a dorsal region of the telencephalon including the area parahippocampalis (APH) which had originally been selected as a control structure.

Statistical analysis of the data revealed significant metabolic sex differences in a number of brain nuclei. Aromatase was higher in

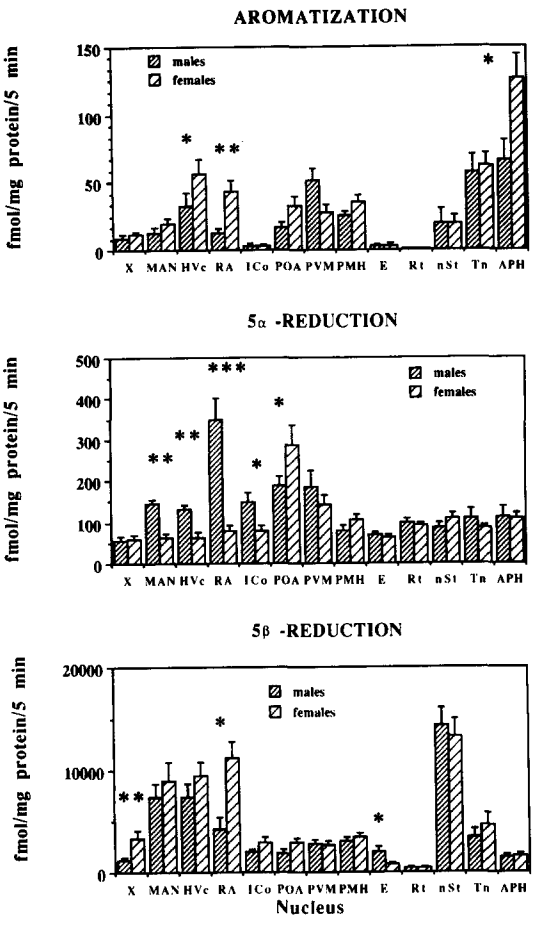

Fig. 4. T metabolism in the brain of intact sexually mature male and female zebra finches as studied by the Palkovits punch technique combined with radioenzyme assay. Data are means ± SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ compared to corresponding male data by the Student t-test. E: ectostriatum; Rt: nucleus rotundus; nSt: nucleus striaterminalis; Tn: nucleus taeniae; (redrawn from data in [57]).

females than in males in the hyperstriatum ventrale, pars caudale (HVc), robustus archistriatalis (RA) and APH. In one single nucleus the periventricularis magnocellularis (PVM), the AA was numerically higher in males than in females but this difference did not reach statistical significance ($P < 0.10$). The 5α-reductase was significantly more active in the nucleus magnocellularis of the anterior neostriatum (MAN), HVc, RA and nucleus intercollicularis (ICo) of males compared to females. A difference in the opposite direction was seen in the POA. Finally, the production of 5β-DHT was significantly higher in females than in males in the area X and in the nucleus RA while in the ectostriatum (E) a difference in the opposite direction was observed.

This pattern of T metabolism therefore appears to be quite different from the results obtained in quail. High levels of aromatase are found in the POA-hypothalamus and in the limbic system in general (e.g. nucleus taeniae which is the avian homologue of the amygdala in mammals) but in addition the telencephalic nuclei of the song system also show an active enzyme. Sex differences in AA are observed in parts of the telencephalon but it is usually the females which have the more active enzyme contrary to what had been observed in the POA-hypothalamus of quail.

The activity of the two reductases was also sexually differentiated in a number of nuclei, the magnitude of these differences being especially large in RA. It might of course be argued that these differences are only a consequence of the difference in nucleus size between males and females. The punches of RA contained for example more extraneous tissue in females than in males as the punch cannula was the same in both sexes and the RA of males is significantly larger. If we assume then that the 5α-reductase is only present in the steroid-target tissues while the 5β-reductase is located outside these structures, the sex difference in reductase activity in RA might be due only to the smaller size of the nucleus in females. However, this reasoning would then not explain the higher female aromatase in this nucleus unless we assume that this enzyme is also located outside the structure which makes little sense based on all available data. The detailed comparison of all the observed sex differences therefore strongly suggests that these are real and that they are not simply an artifact of the dissection procedure (see [57] for a more detailed discussion). The punch technique is however not really appropriate to analyze this type of difference with a high level of anatomical resolution and other techniques such as immunocytochemistry should be used for this purpose (see below).

The presence of high levels of AA in the APH was an unexpected finding in the previous experiment and additional assays were then carried out to map more precisely the distribution of this telencephalic aromatase (see Fig. 5).

Serial 200 μm thick sections were cut on a cryostat and the dorsal parts of these sections were then dissected with a scalpel blade as indicated in Fig. 5 in order to obtain 8 independent brain samples in the hippocampal and APH region (labeled A–H in the figure). This demonstrated the presence of high levels of AA in the entire region. The enzyme activity was however significantly different from one area to

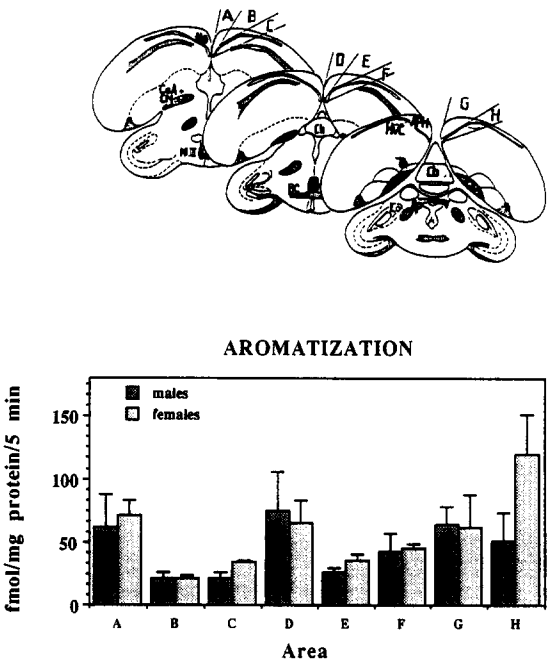

Fig. 5. Schematic drawing of the free-hand dissection of the dorsal telencephalon that was used to analyze the distribution of T-metabolizing enzymes in adult male and female zebra finches (top) and results of the aromatase assays in these fractions (bottom). Each column corresponds to the mean ± SEM of 3–4 experimental points. BC: brachium conjectivum; Cb: cerebellum; CoA: commissura anterior; Cp: commisura posterior; Hp: hippocampus; OM: tractus occipitomesencephalicus; Tn: nucleus taeniae; (redrawn from data in [57]).

the other and the highest levels were observed in the hippocampus (fractions A and D) and in the area ventro-medial to HVc (fraction H; HVc had been punched out of these sections before they were dissected). There was no statistical difference between AA levels in males and females in this region although data strongly suggested the presence of higher enzyme activity in females for some of the fractions (not statistically significant due to the small number of samples that were analyzed.

Like in quail, the control by steroids of the activity of the aromatase and of the two reductases was subsequently investigated by repeating these assays in gonadectomized birds of both sexes which received or not a replacement therapy with testosterone. This confirmed a number of sex differences in enzyme activity and revealed that some but not all these differences were controlled by the circulating steroids in the adult birds [47]. Selected examples of these studies are shown in Fig. 6.

The analysis of these data by two way analyses of variance confirmed the presence of overall metabolic sex differences (for aromatase in RA and APH; for the 5α-reductase in HVc and RA) and demonstrated significant interactions between the sex of the birds and their hormonal condition (for the 5α-reductase in POA and PVM). In addition, the higher level of AA in males compared to females was seen again in PVM even if it did not reach significance. The enzymatic activities reacted very differently to the gonadectomy and T replacement. In some cases, they were decreased in castrated birds and induced to normal male levels by treatment with T (e.g. aromatase and 5α-reductase in POA and PVM) suggesting that the sex differences observed in intact birds were the result of a differential activation by testicular or ovarian steroids. In the telencephalic nuclei, however, a totally different type of result was obtained and in general the hormonal treatment had no effect on the activity of these enzymes (see [47] for a more detailed presentation of these results). This suggests that the enzymatic sex differences which had been obtained (e.g. higher aromatase in the RA of females, higher 5α-reductase in the RA of males) might be organizational in nature: they could be the consequence of irreversible modifications in brain morphology and physiology induced by neonatal steroids. Experiments manipulating the hormonal environment of young zebra finches should now be performed to directly test this possibility.

In conclusion, these experiments have revealed a number of major differences between the quail and the zebra finch as far as brain T-metabolizing enzymes are concerned. Their anatomical distribution is substantially different and in particular, the presence of high levels of AA in the zebra finch telencephalon raises questions regarding the role of estrogens produced at this level. Sex differences in opposite directions are seen in the study of AA in the hypothalamus of both species and in the telencephalic nuclei of the zebra finch. The regulation by steroids of AA is very different in the POA-hypothalamus (increased enzyme activity in the presence of T) and in the telencephalon of the zebra finch (apparent absence of control). This might suggest that different forms of the enzyme are present in the diencephalon and in the telencephalon and biochemical experiments should be carried out to directly test this interesting possibility. The presence of major sex differences in the activity of the 5α- and 5β-reductase in several nuclei of the song system also suggests that this differential T metabolism might be responsible, at least in part, for the sex

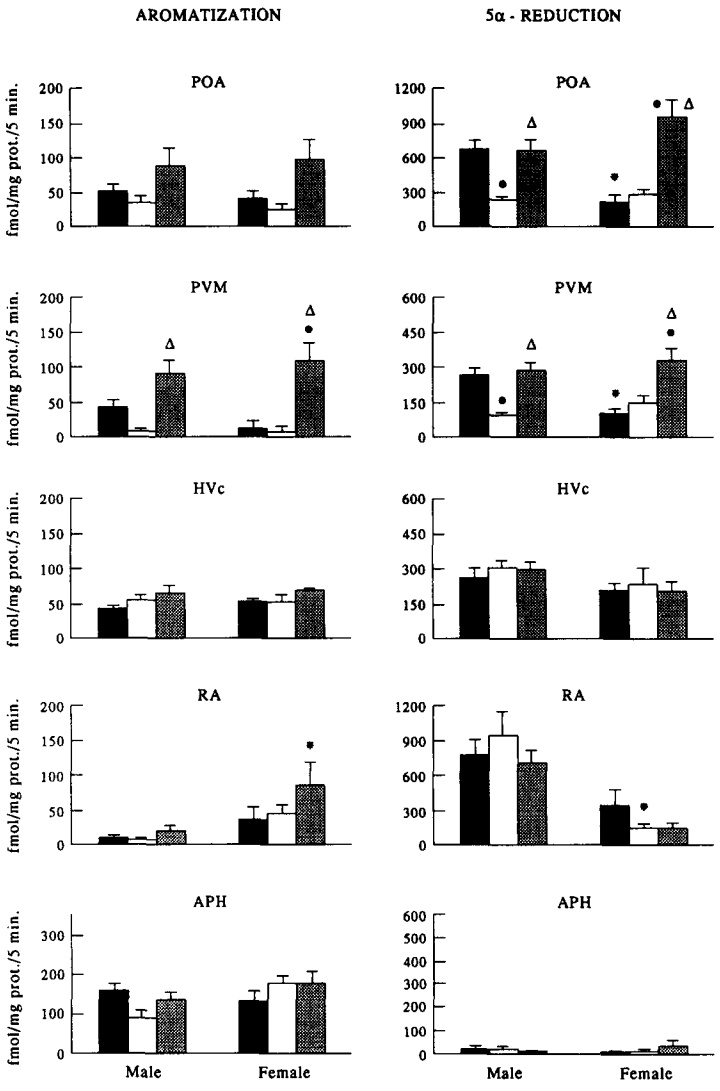

Fig. 6. Formation of E_2 (aromatization) and of 5α-DHT (5α-reduction) by different nuclei of intact (■), gonadectomized (□) and T-treated gonadectomized (▩) adult male and female zebra finches. Each column corresponds to the mean ± SEM of 8 data points in males and 5 data points in females. Individual means were compared by the Newman–Keuls tests following calculation of significant F ratios in the analyses of variance. △ = $P < 0.05$ compared to gonadectomized birds of the same sex, ● = $P < 0.05$ compared to intact birds of the same sex and * = $P < 0.05$ compared to males submitted to the same hormonal treatment; (redrawn from data in [47]).

difference in the vocal behavior of these birds (male but not female zebra finches sing in response to T). The fact that these differences are not controlled by the adult T levels and are therefore presumably organizational in nature certainly supports this interpretation.

Finally, it might be interesting to recall here a striking point in the zebra finch endocrinology: the source of estrogens in males might not be the testes as no decrease and even increases in the plasma levels of this steroid have been reported following castration [36] (see also [58] for similar results in young sparrows). It might therefore be significant that in this species a large part of the dorsal telencephalon contains an active aromatase. The estrogens produced at this level might therefore influence the behavior both in the context of differentiation and activation. Whether this brain aromatase is capable of maintaining high levels of circulating estrogens in the absence of gonads (by aromatization of adrenal steroids?) remains however to be investigated.

MECHANISMS UNDERLYING THE CHANGES IN AROMATASE ACTIVITY

The aromatase in the POA-hypothalamus of quail and zebra finches and in some

telencephalic nuclei of the zebra finch appears to be directly related to the activation of sexual behavior. The activity of this enzyme is, in addition, modulated by many factors. In quail, the preoptic AA changes according to the age of the birds (higher in sexually mature males than in chicks [59]), their sex (higher in males than in females [41, 42]) or their endocrine condition (higher in sexually mature or T-treated males than in castrates [7, 42]). In zebra finches, changes as a function of age and endocrine status have also been described [37, 40].

In quail, detailed kinetic experiments have demonstrated that these changes in activity never implicate modifications of the affinity of the enzyme for its substrate (K_m). In each case where the maximum velocity (V_{max}) of the aromatase is increased, its K_m remains constant [42, 59] (Balthazart, unpublished data). This suggests that it is the enzyme concentration which changes from one physiological condition to the other. These data are however also consistent with a change of enzyme activity that would be caused by the presence of a noncompetitive inhibitor in birds with a low enzymatic activity; this would also decrease the V_{max} without affecting the K_m. In order to discriminate between these alternative explanations (changes in enzyme concentration vs enzyme inhibitor), pools of adult male and female POA homogenates were incubated either separately or in the same tube in the presence of increasing amounts of radioactive T. It was predicted that if aromatase was simply more concentrated in males than in females, then the enzymatic velocity in an incubation of a mixture of male and female POA would be the mean of the velocities observed in separate incubations. On the contrary, if the brain of females contained an enzymatic inhibitor, then the reaction rate in the co-incubation should be less than the mean. It could actually be equal to the velocity observed in females provided that the inhibitor was not present in limiting amounts. Results of such an experiment are shown in Fig. 7.

The affinity of the aromatase for its substrate was similar in males, in females and in the pooled homogenate (approx. 20 nM; see Fig. 7). As expected the maximum velocity of the reaction was higher in males (310.90 ± 17.41 fmol/mg protein/15 min) than in females (125.18 ± 5.06 fmol/mg protein/15 min). The velocity measured in the co-incubation was intermediate (202.64 ± 12.03 fmol/mg protein/15 min) and actually very close to the arithmetic mean of the male and female data (218). This type of experiment therefore argues against the presence of an endogenous aromatase inhibitor in the POA of female quail and strongly suggests that the higher V_{max} in males is due to a higher concentration of the enzyme. An independent confirmation of this conclusion has recently been obtained by immunocytochemistry.

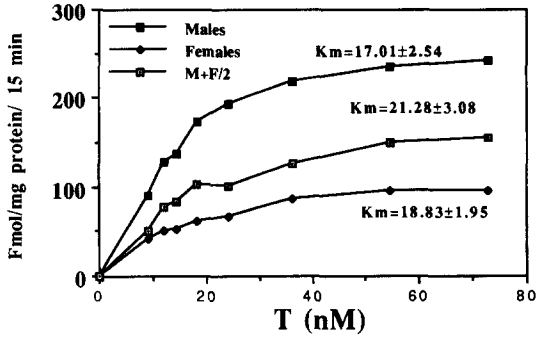

Fig. 7. Saturation analysis of aromatase activity in the POA of sexually mature adult male and female quail. Male and female homogenates were incubated with increasing concentrations of tritiated T either alone or after equal amounts of both homogenates had been pooled in the same tube (M + F/2). The affinity of the enzyme for its substrate was similar in the 3 sets of incubations and the maximum velocity in the co-incubations was approximately equal to the mean of the values observed in males and females (see the text for additional information).

By contrast, kinetic analyses of the preoptic aromatase in zebra finches of different ages between hatching and sexual maturity reveal significant changes in the enzyme characteristics (see Fig. 8).

It was observed that during the first 80 days of postnatal life, the V_{max} of the aromatase decreases in male and female zebra finches while the enzyme affinity increases (K_m decreases [40]). The major changes in enzyme affinity actually occur between 40 and 80 days of age, that is approximately when the birds become sexually mature. It might therefore be speculated that these changes are caused by the rising levels of gonadal steroids although direct experimental evidence for this interpretation is not available at present. In zebra finches the mechanisms underlying the modification of the aromatase apparent affinity during ontogeny are still unclear. The observed enzyme kinetics are very complex and difficult to interpret. They could reveal the presence of inhibitors of the enzyme or alternatively suggest that during the maturation there is a change in its nature. Additional biochemical experiments only could discriminate between these alternatives.

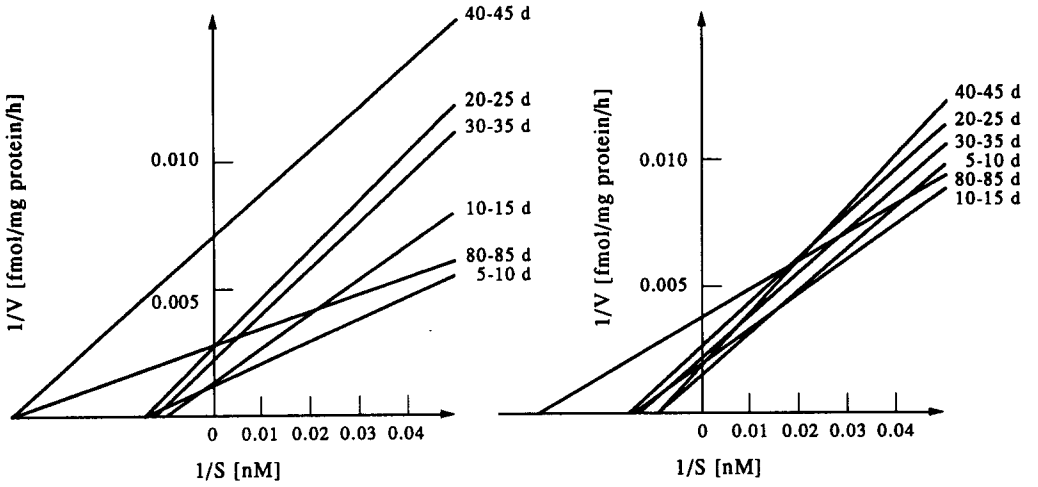

Fig. 8. Double reciprocal plots (Lineweaver–Burke plots) of the production of E_2 (aromatization) vs substrate concentration in the POA-hypothalamus of male and female zebra finches of different ages between 5 and 85 days post-hatch. Data demonstrate variations in both the K_m (intercept with the X axis) and the V_{max} (intercept with the Y axis) of the enzyme.

THE IMMUNOCYTOCHEMICAL LOCALIZATION OF AROMATASE

The product formation assays were adequate for quantifying the activity of T-metabolizing enzymes but they did not permit an accurate mapping of their distribution. Recently, we have been able to obtain a polyclonal antibody which specifically recognizes the aromatase of the avian brain. It has been used to study by immunocytochemistry the distribution and regulation of aromatase at a cellular level of resolution.

Aromatase-immunoreactive cells have in this way been localized on fresh frozen sections or on sections obtained in fixed brains by a classical peroxidase–antiperoxidase (PAP) immunocytochemical procedure using a polyclonal antibody raised in rabbits against human placental aromatase and purified by affinity chromatography [60]. Although the final proof of specificity cannot be obtained in this heterologous technique because quail brain aromatase is not available for preabsorption controls, it is clear, based on immunocytochemical controls and on the comparison of immunocytochemistry with radioenzyme assay results that the procedure identifies the molecule which is responsible for the enzymatic activity (see [61] for detail).

Although the immunocytochemical identification of aromatase in the brain has only been achieved very recently, several important facts have already been established by this technique. At the cellular level, it could be seen that the immunoreactive product fills the entire cytoplasm of the cells, including long cell processes but always leaving a clear nucleus [61, 62]. At the electron microscope level, immunoreactive aromatase was found in the rough endoplasmic reticulum throughout the cytoplasm, including the full length of neural processes [63]. There were also many synaptic boutons which contained aromatase-immunoreactive (AR-ir) clear synaptic vesicles. These AR-ir positive boutons were found forming synapses with AR-ir positive and negative neurons. These synapses were both axo-somatic and axo-dendritic. This subcellular localization of aromatase is compatible with previous biochemical studies demonstrating that this is a microsomal enzyme. In addition, the presence of immunoreactive material in presynaptic boutons is totally consistent with the recent finding that significant AA is located in synaptosomal fractions after differential centrifugation of quail brain homogenates [64]. The presence of immunoreactive aromatase in synaptic vesicles certainly indicates an unorthodox role for this enzyme or its estrogenic products in the brain. This could include a role in the synaptic zone for locally formed E_2 or its metabolites including the catechol estrogens which are potent inhibitors of the catechol-*o*-methyl transferase and could in this way alter the metabolism of catecholamines.

AR-ir perikarya were found in all preoptic, hypothalamic and septal areas which had previously been shown to contain AA by *in vitro* product formation assays [45, 46, 61]. In the POA, the immunoreactive neurons were confined to the POM which is T-sensitive and

sexually dimorphic in quail [48, 49]. AR-ir cells therefore represent a neurochemical marker for this sexually dimorphic nucleus. Their presence fits in perfectly well with the previous demonstration of high levels of AA in the nucleus (see Figs 1 and 2).

The aromatase immunocytochemistry has also provided new information about the mechanisms which mediate the induction by T of AA in the POA. The brain of castrated quail and of castrates which had been treated with T were studied in parallel either for AA (measured by radioenzyme assays on the entire POA) or for AR-ir neurons [61, 65]. After 5 days of exposure to physiological levels of T (induced by 40 mm long silastic implants), the enzyme activity had increased by about 645%. The number of immunoreactive cells was then counted in 4 consecutive sections located in the medial part of the POM. These cells were very scarce in castrates but their number was about 5 times higher in the T-treated birds. This strongly suggests that the increase in AA which is observed following treatment with T results from an increased concentration of the enzyme. This represents an independent confirmation of a conclusion which had been reached before based on kinetic data (see above).

The number of AR-ir perykarya has also been recently quantified in the brain of male and female quail. As shown in Fig. 9, positive cells are more numerous in the POM of males compared to females. However, this difference only reached statistical significance in the more caudal part of the nucleus around the level of the anterior commissure (Balthazart and De Clerck, unpublished data).

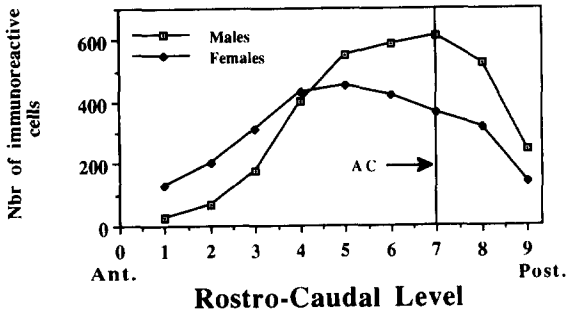

Fig. 9. Distribution of AR-ir cells in the POM of adult male and female Japanese quail. The number of immunoreactive cells has been counted in one section every 100 μm. The level of the different sections (1–9) has been standardized by reference to the anterior commissure (AC). Ant.: anterior part, Post.: posterior part of the POM.

The difference in enzyme activity which had been detected by the product formation assays (see Fig. 1) therefore only concerns a subregion of the POM. Techniques such as immunocytochemistry appear to be adequate for the analysis of differences which have such a discrete neuroanatomical localization. As mentioned above, a sex difference in the inducibility of the AA has also been observed in the POA of quail but its exact localization has eluded us so far. Based on these recent immunocytochemical data, it seems that the differential inducibility might also be localized in the caudal part of the POM and future studies should quantify the AR-ir perikarya in this region in gonadectomized males and females submitted to a same replacement therapy with T.

CONCLUSION

Product formation assays carried out on microdissected brain regions or more recently on nuclei dissected by the Palkovits "punch technique" have demonstrated that T-metabolizing enzymes have a discrete distribution in the brain of the Japanese quail and the zebra finch. This has been confirmed recently by immunocytochemistry in the case of aromatase. The activity of these enzymes is affected by the age, sex and endocrine condition of the birds in a manner which is specific for the species, the brain region and the enzyme considered. This metabolism leads to the production of compounds which are either implicated in the differentiation or activation of reproductive behavior or are devoid of behavioral effects. The modulation of the enzyme activities therefore permits an amplification or inactivation of the effects of T in the brain. In a number of cases, it has been shown that the changes in AA parallel the changes in sexual behavior and the enzyme activity probably represents a limiting factor in the activation of behavior by T. It has always been assumed that T and its metabolites activate sexual behavior by an interaction with the classical intracellular steroid receptors. A number of recent reports [66] including our finding of aromatase immunoreactivity at the synapse level suggest, however, that steroids might also exert biological effects by an action at the membrane or even at the synapse level. This possibility should be seriously considered for future work in behavioral neuroendocrinology.

Acknowledgements—I am indebted to Professor E. Schoffeniels for his continued interest in my research. The work from my laboratory which is described in this paper 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) and the EEC (SC1-0230-C/TT) to J. Balthazart and by a grant from the Belgian Fonds de la Recherche Fondamentale Collective (No. 2.4518.80) to Professor Schoffeniels. I would like to thank here all my recent collaborators and especially Michael Schumacher, Agnes Foidart, Chantal Surlemont, Linda Evrard, Anne De Clerck and Angela Vockel who have been of invaluable help in collecting all the data described in this review.

REFERENCES

1. Balthazart J.: Steroid metabolism and the activation of social behavior. In *Advances in Comparative and Environmental Physiology* (Edited by J. Balthazart). Springer Verlag, Berlin, Vol. 3 (1989) pp. 105–159.
2. Martini L.: The 5α-reduction of testosterone in the neuroendocrine structures. Biochemical and physiological implications. *Endocrine Rev.* **3** (1982) 1–25.
3. McEwen B. S.: Neural gonadal steroid actions. *Science* **211** (1981) 1303–1311.
4. Hutchison J. B. and Steimer Th.: Brain 5β-reductase. A correlate of behavioral sensitivity to androgen. *Science* **213** (1981) 244–246.
5. Adkins E. K., Boop J. J., Koutnik D. L., Morris J. B. and Pniewski E. E.: Further evidence that androgen aromatization is essential for the activation of copulation in male quail. *Physiol. Behav.* **24** (1980) 441–446.
6. Balthazart J., Schumacher M. and Ottinger M. A.: Sexual differences in the Japanese quail: behavior, morphology and intracellular metabolism of testosterone. *Gen. Comp. Endocr.* **51** (1983) 191–207.
7. Balthazart J., Foidart A. and Hendrick J. C.: The induction by testosterone of aromatase activity in the preoptic area and activation of copulatory behavior. *Physiol. Behav.* **47** (1990) 83–94.
8. Balthazart J., Evrard L. and Surlemont C.: Effects of the non-steroidal aromatase inhibitor, R76713 on testosterone-induced sexual behavior in the Japanese quail (*Coturnix coturnix japonica*). *Horm. Behav.* **24** (1990) 510–531.
9. Adkins E. K. and Nock B. L.: The effects of the antiestrogen CI-628 on sexual behavior activated by androgen and estrogen in quail. *Horm. Behav.* **7** (1976) 417–429.
10. Alexandre C. and Balthazart J.: Effects of metabolism inhibitors, antiestrogens and antiandrogens on the androgen and estrogen induced sexual behavior in Japanese quail. *Physiol. Behav.* **38** (1986) 581–591.
11. Balthazart J. and Surlemont C.: Androgen and estrogen action in the preoptic area and activation of copulatory behavior in quail. *Physiol. Behav.* **48** (1990) 599–609.
12. Schumacher M. and Balthazart J.: The effects of testosterone and its metabolites on sexual behavior and morphology in male and female Japanese quail. *Physiol. Behav.* **30** (1983) 335–339.
13. Watson J. T. and Adkins-Regan E.: Activation of sexual behavior by implantation of testosterone propionate and estradiol benzoate into the preoptic area of the male Japanese quail (*Coturnix japonica*). *Horm. Behav.* **23** (1989) 251–268.
14. Balthazart J., Schumacher M. and Malacarne G.: Interaction of androgens and estrogens in the control of sexual behavior in male Japanese quail. *Physiol. Behav.* **35** (1985) 157–166.
15. Deviche P. and Schumacher M.: Behavioural and morphological dose-responses to testosterone and to 5a-dihydrotestosterone in the castrated male Japanese quail. *Behav. Proc.* **7** (1982) 107–121.
16. Schumacher M., Alexandre C. and Balthazart J.: Interactions des androgènes et des oestrogènes dans le contrôle de la reproduction. *C. R. Acad. Sci. Paris, Serie III* **305** (1987) 569–574.
17. Adkins E. K.: Effects of diverse androgens on the sexual behavior and morphology of castrated male quail. *Horm. Behav.* **8** (1977) 201–207.
18. Adkins E. K. and Pniewski E. E.: Control of reproductive behavior by sex steroids in male quail. *J. Comp. Physiol. Psychol.* **92** (1978) 1169–1178.
19. Adkins E. K. and Adler N. T.: Hormonal control of behavior in the Japanese quail. *J. Comp. Physiol. Psychol.* **81** (1972) 27–36.
20. Adkins-Regan E.: Sex steroids and the differentiation and activation of avian reproductive behaviour. In *Hormones and Behaviour in Higher Vertebrates* (Edited by J. Balthazart, E. Pröve and R. Gilles). Springer-Verlag, Berlin (1983) pp. 219–228.
21. Adkins E. K.: Effect of embryonic treatment with estradiol or testosterone on sexual differentiation of the quail brain. *Neuroendocrinology* **29** (1979) 178–185.
22. Schumacher M., Hendrick J. C. and Balthazart J.: Sexual differentiation in quail: critical period and hormonal specificity. *Horm. Behav.* **23** (1989) 130–149.
23. Schumacher M., Sulon J. and Balthazart J.: Changes in serum concentrations of steroids during embryonic and post-hatching development of male and female Japanese quail (*Coturnix coturnix japonica*). *J. Endocr.* **118** (1988) 127–134.
24. Adkins-Regan E., Pickett P. and Koutnik D.: Sexual differentiation in quail: conversion of androgen to estrogen mediates testosterone-induced demasculinization of copulation but not other male characteristics. *Horm. Behav.* **16** (1982) 259–278.
25. Schumacher M., Hutchison R. E. and Hutchison J. B.: Ontogeny of testosterone-inducible brain aromatase activity. *Brain Res.* **441** (1988) 98–110.
26. Schumacher M. and Hutchison J. B.: Testosterone induces hypothalamic aromatase activity during early development in quail. *Brain Res.* **377** (1986) 63–72.
27. Adkins-Regan E.: Exposure of embryos to an aromatization inhibitor increases copulatory behaviour of male quail. *Behav. Proc.* **11** (1985) 153–158.
28. Balthazart J. and Ottinger M. A.: 5β-reductase activity in the brain and cloacal gland of male and female embryos of the Japanese quail (*Coturnix coturnix japonica*). *J. Endocr.* **102** (1984) 77–81.
29. Harding C. F., Sheridan K. and Walters M. J.: Hormonal specificity and activation of sexual behavior in male zebra finches. *Horm. Behav.* **17** (1983) 111–133.
30. Walters M. J. and Harding C. F.: The effects of an aromatization inhibitor on the reproductive behavior of male zebra finches. *Horm. Behav.* **22** (1988) 207–218.
31. Arnold A. P., Nottebohm F. and Pfaff D. W.: Hormone concentrating cells in vocal control areas of the brain of the zebra finch (*Poephila guttata*). *J. Comp. Neurol.* **165** (1976) 487–512.
32. Nottebohm F. and Arnold A. P.: Sexual dimorphism in the vocal control areas in the song bird brain. *Science* **194** (1976) 211–213.
33. Gurney M. E.: Hormonal control of cell form and number in the zebra finch song system. *J. Neurosci.* **1** (1981) 658–673.
34. Gurney M. E. and Konishi M.: Hormone-induced sexual differentiation of brain and behavior in zebra finches. *Science* **208** (1980) 1380–1383.
35. Hutchison J. B., Wingfield J. C. and Hutchison R. E.: Sex differences in plasma concentrations of steroids during the sensitive period for brain differentiation in the zebra finch. *J. Endocr.* **103** (1984) 363–369.
36. Adkins-Regan E., Abdelnabi M., Mobarak M. and

Ottinger M. A.: Sex steroid levels in developing and adult male and female zebra finches (*Poephila guttata*). *Gen. Comp. Endocr.* **78** (1990) 93–109.
37. Vockel A., Pröve E. and Balthazart J.: Sex- and age-related differences in the activity of testosterone-metabolizing enzymes in microdissected nuclei of the zebra finch brain. *Brain Res.* **511** (1990) 291–302.
38. Naftolin F., Ryan K. J., Davies I. J., Reddy V. V., Flores F., Petro Z., Kuhn M., White R. J., Takaoka Y. and Wolin L.: The formation of estrogens by central neuroendocrine tissues. *Rec. Prog. Horm. Res.* **31** (1975) 295–319.
39. Schumacher M., Contenti E. and Balthazart J.: Partial characterization of testosterone-metabolizing enzymes in the quail brain. *Brain Res.* **305** (1984) 51–59.
40. Vockel A., Pröve E. and Balthazart J.: Changes in the activity of testosterone-metabolizing enzymes in the brain of male and female zebra finches during the post-hatching period. *Brain Res.* **463** (1988) 330–340.
41. Schumacher M. and Balthazart J.: Sexual dimorphism of the hypothalamic metabolism of testosterone in the Japanese quail (*Coturnix coturnix japonica*). *Prog. Brain Res.* **61** (1984) 53–61.
42. Schumacher M. and Balthazart J.: Testosterone-induced brain aromatase is sexually dimorphic. *Brain Res.* **370** (1986) 285–293.
43. Palkovits M.: Isolated removal of hypothalamic or other brain nuclei of the rat. *Brain Res.* **59** (1973) 449–450.
44. Palkovits M. and Brownstein M. J.: Microdissection of brain areas by the punch technique. In *Brain Microdissection Techniques* (Edited by A. C. Cuello) Wiley, New York (1983) pp. 1–36.
45. Schumacher M. and Balthazart J.: Neuroanatomical distribution of testosterone metabolizing enzymes in the Japanese quail. *Brain Res.* **422** (1987) 137–148.
46. Balthazart J., Schumacher M. and Evrard L.: Sex differences and steroid control of testosterone-metabolizing enzymes activity in the quail brain. *J. Neuroendocr.* **2** (1990) 675–683.
47. Vockel A., Pröve E. and Balthazart J.: Effects of castration and testosterone treatment on the activity of testosterone metabolizing enzymes in the brain of male and female zebra finches. *J. Neurobiol.* **21** (1990) 808–825.
48. Viglietti-Panzica C., Panzica G. C., Fiori M. G., Calcagni M., Anselmetti G. C. and Balthazart J.: A sexually dimorphic nucleus in the quail preoptic area. *Neurosci. Lett.* **64** (1986) 129–134.
49. Panzica G. C., Viglietti-Panzica C., Calcagni M., Anselmetti G. C., Schumacher M. and Balthazart J.: Sexual differentiation and hormonal control of the sexually dimorphic preoptic medial nucleus in quail. *Brain Res.* **416** (1987) 59–68.
50. Balthazart J., Devos F., Dohet A., Foidart A., Hugla J. L., Radermaker F. and Schumacher M.: The induction of aromatase and sexual behavior by testosterone in male and female Japanese quail: a dose-response study. *I. R. C. S. Med. Sci.* **14** (1986) 1188–1189.
51. Balthazart J.: Correlation between the sexually dimorphic aromatase of the preoptic area and sexual behavior in quail: effects of neonatal manipulations of the hormonal milieu. *Archs Int. Physiol. Biochem.* **97** (1989) 465–481.
52. Balthazart J. and Surlemont C.: Copulatory behavior is controlled by the sexually dimorphic nucleus of the quail preoptic area. *Brain Res. Bull.* **25** (1990) 7–14.
53. Ball G. F.: Chemical neuroanatomical studies of the steroid-sensitive songbird vocal control system: a comparative approach. In *Hormones, Brain and Behaviour in Vertebrates. 1. Sexual Differentiation, Neuroanatomical Aspects, Neurotransmitters and Neuropeptides. Comparitive Physiology, Vol. 8* (Edited by J. Balthazart). Karger, Basel (1990) pp. 148–167.
54. Nottebohm F., Kelley D. B. and Paton J. A.: Connections of vocal control nuclei in the canary telencephalon. *J. Comp. Neurol.* **207** (1982) 344–357.
55. Stokes T. M., Leonard C. M. and Nottebohm F.: The telencephalon, diencephalon, and mesencephalon of the canary, *Serinus canaria*, in stereotaxic coordinates. *J. Comp. Neurol.* **156** (1974) 337–374.
56. Nordeen K. W., Nordeen E. J. and Arnold A. P.: Estrogen accumulation in zebra finch song control nuclei: implications for sexual differentiation and adult activation of song behavior. *J. Neurobiol.* **18** (1987) 569–582.
57. Vockel A., Pröve E. and Balthazart J.: Sex- and age-related differences in the activity of testosterone-metabolizing enzymes in microdissected nuclei of the zebra finch brain. *Brain Res.* **511** (1990) 291–302.
58. Marler P., Peters S., Ball G. F., Dufty A. M. and Wingfield J. C.: The role of sex steroids in the acquisition and production of birdsong. *Nature* **336** (1988) 770–772.
59. Hutchison J. B. and Schumacher M.: Development of testosterone-metabolizing pathways in the avian brain: enzyme localization and characteristics. *Dev. Brain Res.* **25** (1986) 33–42.
60. Harada N.: Novel properties of human placental aromatase as cytochrome *P*-450: purification and characterization of a unique form of aromatase. *J. Biochem.* **103** (1988) 106–113.
61. Balthazart J., Foidart A. and Harada N.: Immunocytochemical localization of aromatase in the brain. *Brain Res.* **514** (1990) 327–333.
62. Balthazart J., Foidart A., Surlemont C., Vockel A. and Harada N.: Distribution of aromatase in the brain of the Japanese quail, ring dove and zebra finch: an immunocytochemical study. *J. Comp. Neurol.* **301** (1990) 276–288.
63. Naftolin F., Leranth C. and Balthazart J.: Ultrastructural localization of aromatase immunoreactivity in hypothalamic neurons. *Endocrine Soc.* (Abstr. 669) (1990).
64. Schlinger B. A. and Callard G. V.: Localization of aromatase in synaptosomal and microsomal subfractions of quail (*Coturnix coturnix japonica*) brain. *Neuroendocrinology* **49** (1989) 434–441.
65. Balthazart J., Foidart A., Surlemont C. and Harada N.: Preoptic aromatase in quail: behavioral, biochemical and immunocytochemical studies. In *Hormones, Brain and Behavior in Vertebrates. Comparitive Physiology Vol. 9* (Edited by J. Balthazart). Karger, Basel (1990) pp. 45–62.
66. Schumacher M.: Rapid membrane effects of steroid hormones: an emerging concept in neuroendocrinology. *TINS* **13** (1990) 359–362.