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Preoptic Aromatase in Quail: Behavioral, Biochemical and Immunocytochemical Studies

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The aromatase was identified in the brain of mammals more than 15 years ago [1, 2]. It has since been demonstrated that this enzyme is present and active in the brain of all vertebrates and plays a critical role in the control of a number of reproductive processes especially in the sexual differentiation and activation of male copulatory behavior [3-6]. This conclusion also applies to the Japanese quail (*Coturnix coturnix japonica*) which has been our model animal for the last 10 years. Studies by our and other laboratories have demonstrated that the conversion of testosterone (T) to estradiol (E₂) takes place in the brain of this species, that the activity of the enzyme catalyzing the reaction is strongly influenced by the sex and physiological condition of the birds and finally that the aromatase plays a key role in the control of several reproductive processes and in particular the sexual differentiation and the activation of copulatory behavior in the adult male [7-9]. We shall briefly review here the data concerning the distribution of aromatase in the quail brain and the regulation of its activity. Recently we were able to identify an immunocytochemical procedure which permits to localize the enzyme in the brain cells. Results collected so far with this technique are also summarized here. In order to provide adequate background on the functional importance of the brain aromatase, the evidence supporting the notion that this enzyme is a key control in the activation of male copulatory behavior will first be described.

Aromatization of T as a Limiting Factor in Behavior Activation

In male quail as in most other vertebrates [see however 10, 11 for remarkable exceptions in reptiles], T secreted by the testis is the major hormone which activates copulatory behavior in the male [12]. This is easily demonstrated by the facts that castration suppresses most reproductive behaviors including copulation while they will be rapidly restored by a treatment with exogenous T [13, 14]. There is however an array of arguments that support the idea that T does not act directly as a hormone in the brain but rather must be metabolized before it exerts some of its behavioral effects. In particular, the transformation of T into E_2 seems to be critical and it is firmly established that at least part of the androgen must be aromatized in order to activate behavior. The experimental arguments supporting this conclusion can be classified in four groups:

- (1) Estrogens per se are able to restore copulatory behavior in castrated male quail provided they are administered in fairly large quantities. This is true for E_2 and estradiol benzoate which are known to cross-react to some extent with the androgen receptors [15-17] but also for the synthetic estrogen, diethylstilbestrol (DES) which has a more specific affinity for estrogen receptors [18].
- (2) Aromatizable androgens (androgens which can be converted into estrogens such as T, androstenedione or androstenediol) activate copulation in castrates [13, 16] while nonaromatizable androgens such as 5 α -dihydrotestosterone (DHT) and the derived diols are either inactive or only show a very weak activity at very high doses [13, 16, 17].
- (3) Antiestrogens such as tamoxifen or nitromifene citrate (CI-628) block T-induced copulatory behavior which demonstrates that at least part of the T effects are mediated through interaction of estrogenic metabolites with estrogen receptors [18, 19].
- (4) Aromatase inhibitors such as androstatrienedione (ATD) inhibit T-induced sexual behavior in castrated male quail. This behavioral suppression was complete in a number of experiments in which the androgenic stimulation was given in the form of daily injections of testosterone propionate (TP) [16] or of stereotaxic implants of TP in the preoptic area (POA) [20] but we only obtained very weak effects of this compound when T was continuously administered in the form of subcutaneous silastic implants [18]. More recently, by increasing the dose of ATD which was administered, we were able to obtain a transitory suppression of the T-induced copulation in castrated quail. In parallel, the preoptic aromatase activity was strongly

depressed by the inhibitor but these effects only lasted for about a week after which a complete recovery was observed [21]. The use of ATD in very high doses raises however a question about the specificity of its action. It was indeed recently demonstrated that if present in high concentrations, ATD is able to decrease the binding of androgens to their receptor [22]. In addition, the aromatase inhibitor also partly suppresses the activity of the 5 α - and 5 β -reductases in quail when present in micromolar concentrations [23]. These nonspecific effects might contribute to explain the behavioral action of the compound.

A new aromatase inhibitor which is a nonsteroidal compound and apparently shows a high specificity was recently synthesized by Janssen Pharmaceutica (Beerse, Belgium). R76713 (6-[(4-chlorophenyl)(1*H*-1,2,4-triazol-1-yl)methyl]-1-methyl-1*H*-benzotriazole) is a triazole derivative which is a potent inhibitor of aromatase activity ($K_i = 0.72$ nM). It is also very specific as it does not affect the plasma levels of T, progesterone, 17 α -hydroxyprogesterone, corticosterone and 11-deoxycorticosterone when given in vivo and does not interact with the binding of androgens, estrogens, progestagens and corticosteroids to their respective receptors during in vitro assays [24]. We have used this compound to confirm the critical role of T aromatization in the activation of copulation in quail. A first experiment established that R76713 decreases the T-activated copulatory behavior in a dose-dependent manner. The brain aromatase was decreased in parallel and was in fact totally suppressed for the higher dose of inhibitor (1 mg/kg). Even at this high dose, no effect on the activity of other T-metabolizing enzymes could be detected. In addition, the growth of the cloacal gland, an androgen-dependent structure, was not affected. These facts suggested that the behavioral inhibition specifically resulted from the inhibition of estrogen production. This could be confirmed in a second experiment during which we successfully reversed the R76713-induced inhibition by giving to the birds a low dose of estrogen which was behaviorally ineffective by itself (fig. 1).

In this experiment, the activation of behavior which had been produced by one 20-mm silastic implant filled with T could be completely suppressed by the injection twice daily of 1 mg/kg of R76713. The dose of E_2 which was used here (20-mm silastic implant) had been shown previously to produce little or no behavioral effects [17]. This was confirmed here but interestingly this dose was able to restore copulation in the T-treated birds inhibited by R76713. This demonstrates that the androgens (T itself or its androgenic metabolites) synergize with estrogens (produced by local aromatization or

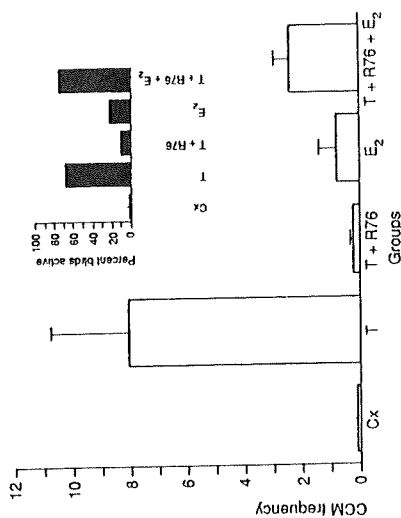


Fig. 1. Effects of a new specific aromatase inhibitor, R76713 (R76), on the T-induced sexual behavior in castrated (Cx) male quail. Castrated quail were treated with either T (20-mm silastic implants) or E₂ (20-mm silastic implants) alone or in combination with the aromatase inhibitor R76713 (R76; two injections daily at the dose of 1 mg/kg). Sexual behavior was quantified during 3 tests in standardized conditions. The total frequency and percentage of birds (insert) showing cloacal contact movements (CCM) are shown. Frequency data are mean ± SE.

exogenous in the present case) to produce their behavioral effects. This had previously been demonstrated by treating birds with exogenous 5 α -DHT and E₂ [17, 25]. It is interesting to note that the treatment with E₂ of birds inhibited by R76713 restored copulatory behavior in the majority of the birds (same percentage as in T-treated animals) but that the behavior frequencies remained significantly lower in the T + R76 + E₂ than in the T group. This is reminiscent of a situation we had observed previously by treating birds with 5 α -DHT and E₂. By varying the amounts of androgen and estrogen that were injected, we were able to obtain combinations which restored sexual behavior in the majority of the animals but the behavioral frequencies in birds treated with 5 α -DHT + E₂ were always lower than those in T-treated birds. This suggests that although exogenous E₂ given systemically can restore the behavior, this form of treatment cannot completely mimic the effects of estrogen produced in the brain by aromatization of T. The basis of this difference is not understood at present.

The Site of Action of T and Its Metabolites

For many years, it has been known that the POA is a major site of steroid action on reproductive behavior and this holds true for all the vertebrate groups that have been studied [26]. This has generated a lot of neuroanatomical and neuroendocrine research focused on this area and during the last decade, sexually dimorphic structures which were in some cases androgen-sensitive have been identified in the POA of a number of mammalian species [27-34]. Similarly in quail, we have identified a group of neurons in the medial preoptic region which is sexually dimorphic and T-sensitive. The volume of the medial preoptic nucleus (POM) when measured in Nissl-stained material is significantly larger in males than in females [35]. It is also reduced by castration and increased by a treatment with T [36, see also 37]. This nucleus was thus likely to be an important site of T action on behavior and actually it was shown that implants of TP or estradiol benzoate located in the medial POA in the vicinity of the dimorphic nucleus were able to activate copulation in castrated male quail [38]. We recently reexamined this question by using very small unilateral T implants in order to provide a better neuroanatomical localization of the effects and could demonstrate in this way that only implants located within the POM were able to activate behavior. The implication of the POM in the control of reproductive behavior was confirmed by electrolytic lesions aimed at the POA: only those lesions which were located within the nucleus disrupted the behavior [39].

Biochemical Studies on the Preoptic Aromatase

Our original studies demonstrated that the quail preoptic area-hypothalamus contains an active aromatase (maximum velocity: 500-1,000 fmol/mg protein/h) which has a very high affinity for T as a substrate ($K_m = \pm 20$ nM) [40, 41]. It was also quickly found that the levels of enzyme activity were higher in the POA than in the hypothalamus and than in any other brain region [40, 42]. Interestingly the preoptic aromatase activity is significantly higher in male than in female quail [40, 42]. This difference disappears in gonadectomized animals as levels of enzyme activity are decreased to baseline values but after induction of the enzyme activity by a same treatment with T in both sexes, aromatase activity was again found to be higher in males [40] (fig. 2).

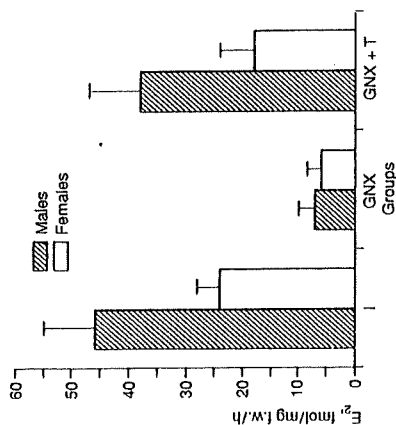


Fig. 2. Aromatase activity in the POA of male and female quail which were either gonadally intact (I) or gonadectomized (GNX) or gonadectomized and treated with silastic implants filled with testosterone (GNX + T). Productions of E_2 during the radioenzymatic assays are expressed as fmol/mg fresh weight/h (mean $\pm$ SE).

This sex difference in aromatase activity which persists even when birds are placed in similar hormonal conditions has been confirmed in several experiments although its magnitude appears to be lower (males about 25% higher than females) than originally thought [43, 44]. As the transformation of T into E_2 is a limiting factor in the activation of copulatory behavior, the sex difference in enzyme activity might be responsible, at least in part, for the behavioral sex dimorphism which has been previously described. It is indeed established that males only show sexual behavior in response to T. Copulatory behavior will very seldom be observed in females [14, 45] and this is the result of their demasculinization by early exposure to the ovarian steroids [45–47]. The limited production of E_2 , the active metabolite of T by the POA of females could therefore contribute to make them less sensitive to the behavioral effects of T. It must however be noticed that this cannot be the only limiting factor as females treated directly with estrogens still fail to show an active copulatory behavior [48].

The precise neuroanatomical localization of this sexually dimorphic aromatase was subsequently researched by combining sensitive radioenzyme assays with the Palkovits punch technique [49]. It was found that the highest levels of aromatase activity were in fact located within the sexually di-

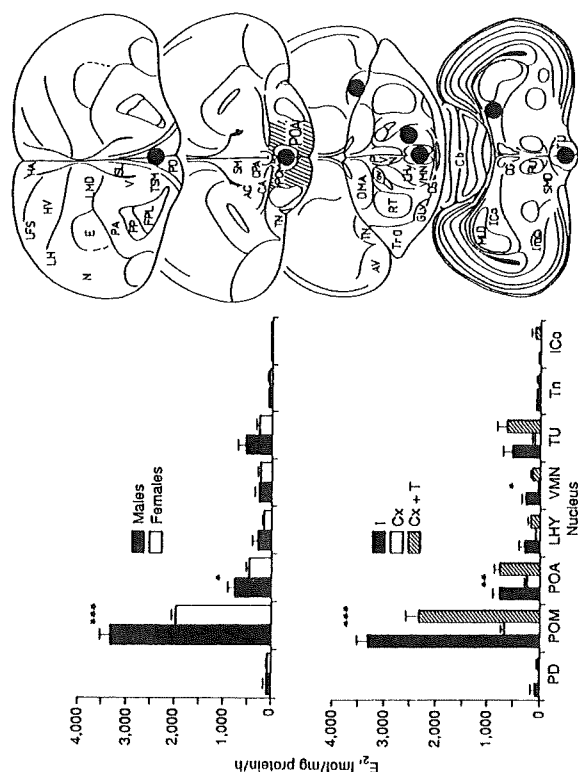


Fig. 3. Sex differences (top left) and effects of castration (Cx) and treatment with T (bottom left) on the aromatase activity in several nuclei of the quail brain dissected by the Palkovits punch technique. The right part of the figure illustrates in a schematic way the brain nuclei which were dissected. Productions of E_2 during the radioenzymatic assays are expressed as fmol/mg protein/h (mean $\pm$ SE). * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. AA: Nucleus archistriatus; AC: nucleus accumbens; AV: archistriatum; pars ventralis; CA: commissura anterior; CB: cerebellum; CG: central grey; CO: chiasma opticum; CPA: commissura pallii; DMA: nucleus dorsomedialis anterior thalami; DS: decussatio supraoptica; E: ectostriatum; FPL: fasciculus prosencephali lateralis; GLV: nucleus geniculatus lateralis, pars ventralis; HA: hyperstriatum accessorium; HV: hyperstriatum ventrale; ICa: nucleus intercollicularis; IH: nucleus inferior hypothalami; Imc: nucleus isthmi, pars magnocellularis; IN: nucleus infundibuli hypothalami; LFS: lamina frontalis superior; LH: lamina hypstriatica; LH: nucleus lateralis hypothalami; LMD: lamina medullaris dorsalis; MLD: nucleus mesencephalicus lateralis, pars dorsalis; MM: nucleus mamillaris medialis; NC: neostriatum caudale; Ov: nucleus ovoidalis; PA: paleostriatum augmentatum; PD: nucleus preopticus dorsolateralis; POA: preoptic area; POM: nucleus preopticus medialis; PP: paleostriatum primitivum; PVN: nucleus paraventricularis; RT: nucleus rotundus; RU: nucleus ruber; SL: nucleus septalis lateralis; SM: nucleus septalis medialis; SMD: supramammillary decussatio; SO: nucleus supraopticus; TN: nucleus taeniae; TSM: tractus septomesencephalicus; TU: nucleus tuberis; V: ventriculus; VMN: nucleus ventromedialis hypothalami.

morphic POM and that this enzymatic activity was dimorphic (fig. 3) [37]. In addition, the aromatase activity in the POM as well as in the rest of the POA was higher in males than in females but no sex difference in the activity of this enzyme could be detected in any of the other nuclei that were studied. The enzyme activity appeared to be controlled by T in these same areas (POM, POA) and also in the ventromedial nucleus (VMN) of the hypothalamus (fig. 3).

Immunocytochemical Localization of Aromatase in the Quail Brain

The study of aromatase activity in brain nuclei dissected by the punch technique provided a description of the distribution of the enzyme in the brain that allowed a detailed analysis of the changes in enzyme activity as a function of the sex of the birds or their physiological conditions. However, several questions could not find their answer by this method. The exact cellular and subcellular localization of the enzyme remained unknown. The nuclei that were dissected by the punch method were defined on the basis of Nissl-stained sections but there was no a priori reason to believe that the groups of aromatase-positive cells did match the cells groups recognized in classical histology. In addition, it was not possible by the product-formation assays to identify the mechanisms underlying the changes in enzyme activity observed for example after a treatment with T. It had been demonstrated by detailed kinetic experiments that the enzymatic differences observed in several physiological conditions were not due to changes in the affinity of the enzyme for its substrate (K_m) which suggested that the changes in enzyme activity were due to changes in enzyme concentration [40, 50] but alternative explanations were still possible. A mechanism based on the regulation of a noncompetitive inhibitor would also result in modifications of the enzyme velocity (V_{max}) with a constant K_m . Even if it was accepted that the enzyme concentration was changing as a function of the physiological state of the birds, several modalities were still possible: increase in the number of cells containing the enzyme, in the enzyme concentration within a constant number of cells or modification of the size of the brain area containing the enzyme. These questions could not find their answer in experiments based on the product-formation assay and other techniques such as immunocytochemistry were required. Purification of the aromatase in a variety of peripheral structures had been reported and the purified antigens had been used to produce both polyclonal and monoclonal antibodies [51–53]. If these

antibodies could be used successfully to identify the aromatase by immunocytochemistry in peripheral structures [54, 55] and to develop an immunological assay (ELISA) of the enzyme in the placenta [56], these techniques had not so far been applied to the brain of any species.

Taking advantage of the high activity and probably of the high concentration of aromatase in the quail POA, we were recently able to successfully identify by immunocytochemistry the enzyme in the brain of this species [57, 58]. A standard peroxidase-antiperoxidase procedure using diaminobenzidine as the chromagen was used in this study. The primary antibody which has been used is polyclonal and has been raised in rabbit against human placental aromatase. The antiserum was subsequently purified by ammonium sulfate fractionation and affinity chromatography with antigen-conjugated Sepharose 4B [52].

Immunoreactive cells were in this way identified in the POA, the hypothalamus and the limbic system at the exclusion of all other brain regions (fig. 4). In the POA, aromatase-immunoreactive cells were confined to the sexually dimorphic POM. These cells are actually a neurochemical marker for this nucleus and permit to outline its boundaries exactly as in the Nissl-stained sections (fig. 5, 6).

This correspondence of the immunoreactive material with the Nissl pattern can be verified throughout the rostrocaudal extent of the nucleus. Two groups of aromatase-containing cells are detected in hypothalamus. One is located at the dorsolateral edge of the VMN and clearly extends in the nucleus lateralis hypothalami. At a more caudal level, diagonal bands of cells are observed in the lateral posterior hypothalamus at the level of the nucleus inferior hypothalami. These cells also clearly mark the medial mamillary nucleus. Finally in the septal region, a group of cells was found below the lateral ventricles dorsal to the lateral forebrain bundle and to the anterior commissure. These cells are found in a fairly large area in the rostrocaudal plane and overlap with, but are not limited to, the nucleus accumbens and the bed nucleus of the stria terminalis. No labelled cells are detected in other parts of the brain. These localizations coincide very well with the distribution of aromatase activity which had been observed in the biochemical assays (fig. 3) [37]. Positive cells are found only in areas which demonstrated a high enzyme activity. With the exception of the POM, the distribution of aromatase-immunoreactive cells however does not correspond to nuclei as defined by classical histology techniques such as Nissl stains.

The distribution of estrogen receptors or estrophilins had been investigated previously in the quail brain by immunocytochemical techniques [59]

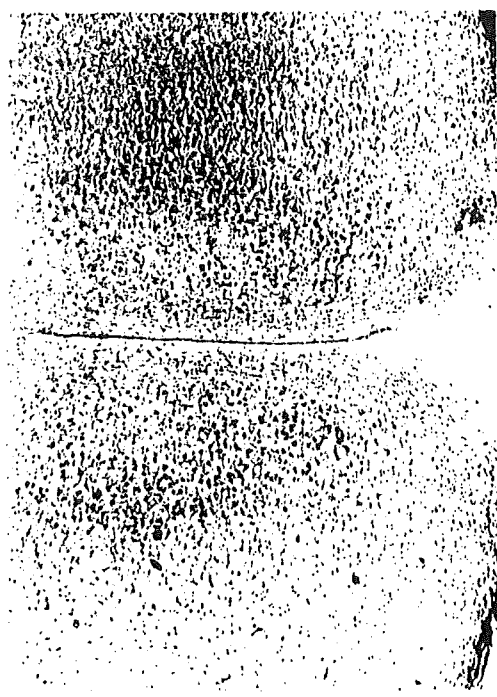


Fig. 5. Microphotograph illustrating the localization of the aromatase-immunoreactive neurons within the POM. Cryostat sections were stained with a polyclonal antibody raised against human placental aromatase using the peroxidase-antiperoxidase technique with diaminobenzidine as chromagen. Labelled cells outline the borders of the POM.

periphery. The POM represents an interesting case in that this nucleus contains high densities of both estrogen receptors and aromatase-containing cells (fig. 6).

The distribution of these two neurochemical markers within the nucleus is however quite different. Estrogen receptors are not limited to the POM. The highest density of immunoreactive cells is located in the medial portion of the nucleus. This density progressively decreases as one moves laterally but labelled cells are still found lateral to the nucleus. Aromatase-immunoreactive cells rather outline the lateral borders of the POM and in fact correspond to the POM region in which it was recently shown that neuronal size increases following T treatment [37]. Approximately 90% of the POM neurons are labelled by estrogen receptors [59] while preliminary data indicate that only 10-15% of them are aromatase-positive. At present, it is therefore impossible to ascertain that these two neurochemical markers are colocalized. Double-label immunocytochemistry experiments should however be able to answer this question. Preliminary results recently obtained

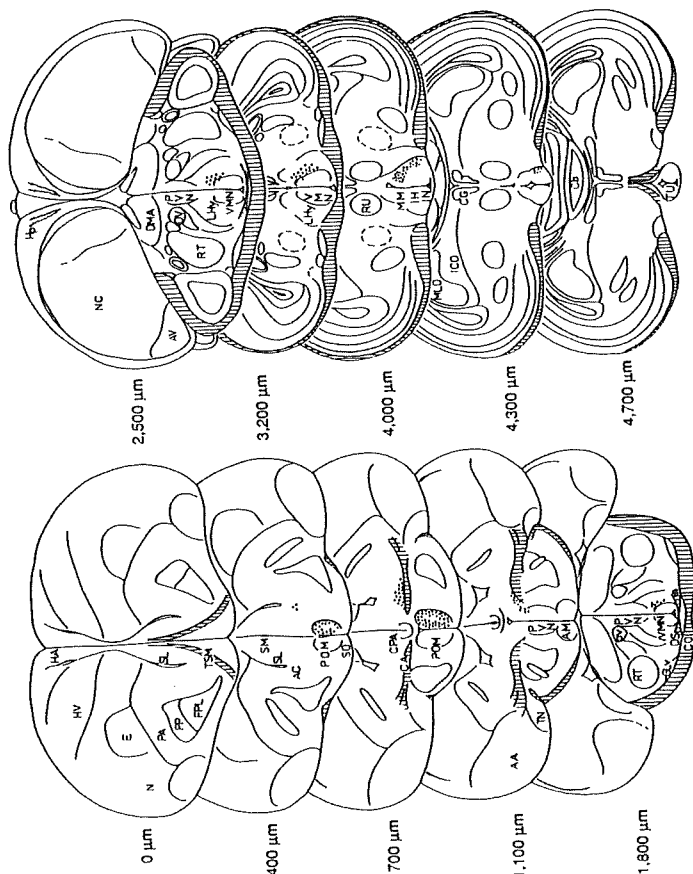


Fig. 4. Distribution of aromatase-immunoreactive cells (dots) in the brain of the quail. Schematic representations of coronal sections in the brain are presented in the rostral to caudal order from top to bottom and from left to right. Numbers at the left side of the sections represent distances in micrometers from the level of the tractus septo-mesencephalicus. See figure 3 for abbreviations.

using a monoclonal antibody directed against estrogen receptors purified from human mammary tumor [60; see 37 for a detailed presentation of these results]. The comparison of these two sets of immunocytochemical data clearly shows that all brain areas containing aromatase-immunoreactive cells also contain estrogen receptors. The reverse is however not true and several nuclei or brain regions such as the lateral septum, the nucleus taeniae and the nucleus intercollicularis are heavily labelled by antibodies directed against estrophilins while they contain no aromatase-immunoreactive cells. Presumably these structures are only affected by estrogens produced in the

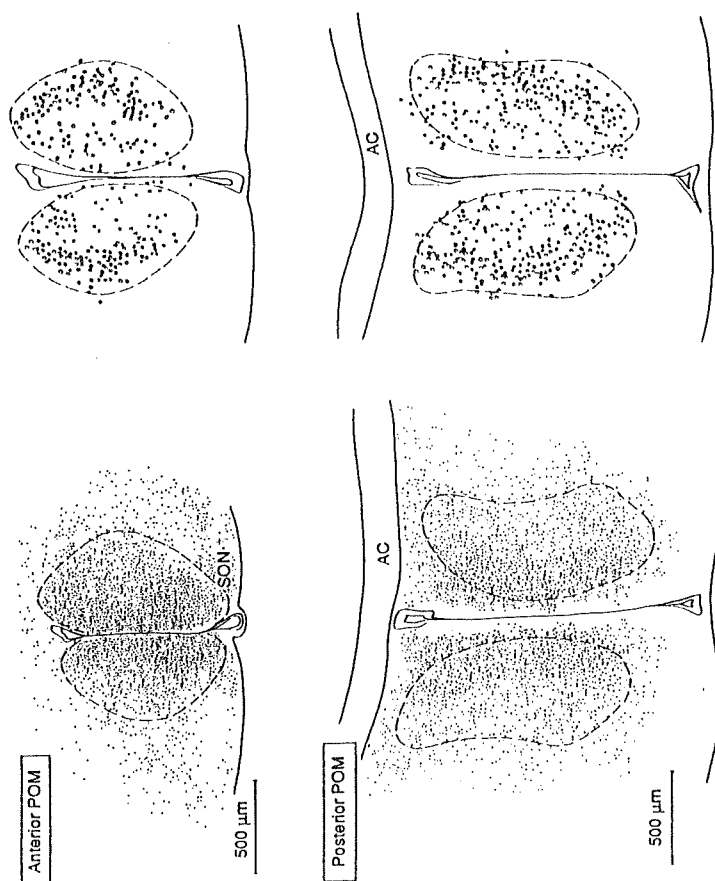


Fig. 6. Camera lucida drawings of sections in the POM at the anterior and posterior levels which were stained by immunocytochemistry for estrogen receptors (left) and for aromatase (right). Each dot represents one labelled cell. The boundaries of the nucleus have been drawn from adjacent sections which were stained for the Nissl material. Estrogen receptor-immunoreactive cells are present within the POM but also at lower density in the rest of the POA. Aromatase-immunoreactive cells are almost exclusively located in the POM especially in its lateral part. AC: Anterior commissure; SON: supraoptic nucleus.

already show that at least some of the aromatase-immunoreactive neurons are also positive for estrogen receptors.

The aromatase immunocytochemistry has also provided new information concerning mechanisms which mediate the induction by T of aromatase activity in the POA. The brain of castrated quail and of castrates which had

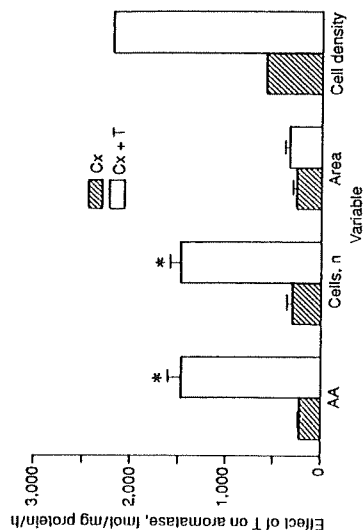


Fig. 7. Effects of a treatment with T in castrated (Cx) quail on the preoptic aromatase activity (AA) measured by radioenzyme assay and on the number of aromatase-immunoreactive cells in the POM. AA: Aromatase activity (fmol/mg protein/h); Cells, n: number of aromatase-immunoreactive cells in 4 consecutive median sections of the POM; Area: total surface of POM in the 4 sections which were counted (in $\text{mm}^2 \times 100$); Cell density: number of aromatase-immunoreactive cells/ $\text{mm}^3 \times 0.1$. [Drawn from data in 58.]

been treated for 5 days with T silastic implants were studied in parallel either for aromatase activity by radioenzyme assays or for aromatase-positive cells by immunocytochemistry. The enzyme activity in the POA had been increased by from 226 to 1,464 fmol/mg protein/h (a 645% increase; fig. 7).

The number of aromatase-immunoreactive cells was also counted for each bird in 4 consecutive sections located in the medial part of the POM in the rostrocaudal axis. Very few immunoreactive cells were found in castrates but these were approximately 5 times more numerous in the T-treated birds. The area of the POM which had been quantified was however slightly larger in T-treated birds than in castrates as it is known that T increases the POM volume. However, even after taking this factor into account, the estimated density of aromatase-immunoreactive cells was still much higher in T-treated than in castrated males.

These observations demonstrate two important facts. They first show that the increase in aromatase activity which is induced by T does not only reflect the modulation of the enzyme activity by a noncompetitive inhibitor. T directly affects the enzyme and it is extremely likely that, in fact, it increases its concentration. However, the change in immunoreactivity might also reflect a conformational change of a constant amount of enzyme and the

present data do not permit to differentiate between these two interpretations. On the other hand, the present immunocytochemical data provide detailed information on the neuroanatomical localization of these effects. They demonstrate that the increase in preoptic aromatase activity is the result of an enzymatic modification which is confined to the POM. Many cells which were not immunoreactive in castrated birds are positive following a treatment with T. The preferential localization of these neurons in the dorsolateral part of the POM fits in very well with the recent observation that T increases the neuronal size only in this subregion of the nucleus and not in its medial part [37].

Conclusions

The data which were reviewed above clearly demonstrate that the aromatization of T within the POA represents a critical neurochemical event in the chain of processes that lead to the activation of copulatory behavior in male quail. The enzyme which catalyzes the transformation of T into E₂ is preferentially, and almost exclusively, located in the POM, a sexually dimorphic structure. This nucleus is the critical target of T in the activation of behavior and the presence of aromatase at this level therefore fits in very well with the postulated role of the enzyme. The regulations of the enzymatic activity by T and its sexual dimorphism also support this notion.

We have recently achieved a successful localization of the aromatase in the quail by means of an immunocytochemical technique using a purified polyclonal antibody raised against the human placental enzyme. This detection is based on a heterologous procedure but we are nevertheless very confident in its specificity. This conclusion is based on a wide array of arguments: the antibody specifically inhibits aromatase activity in the quail brain, it binds to brain areas which are known to contain aromatase activity and this binding is suppressed by a preabsorption with the purified antigen. In addition, the subcellular distribution of the aromatase as seen by immunocytochemistry corresponds to the results of biochemical studies performed on subcellular fractions obtained by differential centrifugation and T modulate the immunoreactivity in parallel with the enzyme activity. This immunocytochemical procedure has already allowed us to describe in detail the neuroanatomical localization of the enzyme and to understand better the mechanisms which regulate its activity. The availability of this technique opens a new avenue which will allow us to study the role of aromatase in the

control of reproductive processes at a new level of anatomical regulation. The quail, in which the enzyme is confined within a sexually dimorphic nucleus which is strongly implicated in the activation of copulatory behavior, represents a unique model for these studies.

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Observations on Neuroendocrine Control of Sexual Behavior in the Male Common Marmoset (*Callithrix jacchus*)

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Declines in sexual activity after castration in male mammals may reflect widespread central actions of testicular hormones as well as peripheral effects upon the genitalia and secondary sexual adornments. The glans penis of some mammals is clothed in androgen-dependent epidermal 'spines' which overlie dermal tactile receptors. This fact led Beach and Levinson [1] to postulate that atrophy of penile spines after castration impairs sensitivity and hence affects sexual behaviour. Their hypothesis has never been adequately tested, however, since it has become clear that androgens exert their most powerful effects upon behaviour by acting upon the brain whereas their peripheral actions do not, by themselves, maintain sexual activity. Thus in the rat, peripheral structures and penile reflexes may be maintained after castration by treatment with a non-aromatizable androgen (dihydrotestosterone) but sexual behaviour is not activated by this procedure [2]. Conversely, oestradiol will restore most aspects of copulatory behaviour in castrated rats in the absence of peripheral effects upon the glans penis and other genital structures [3, 4]. The present report attempts to establish the role played by penile 'spines' in genital sensory feedback and copulatory behaviour in a New World primate species, the common marmoset. Firstly, the role played by sensory feedback from the penis for the attainment of intromission and ejaculation is described. Then effects of castration upon penile morphology and copulatory behaviour are reported. Finally a simple method for removing penile spines is employed, which avoids the usual complications of castration and hormonal manipulation. Evidence is presented that this procedure can influence both intromission latencies and duration of intravaginal thrusting in sexually experienced male marmosets.