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Steroid Control and Sexual Differentiation of Brain Aromatase

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Brain aromatase (ARO) activity in the quail is markedly enhanced by testosterone (T). This effect only becomes detectable after several hours and reaches its maximum within a few days, which suggests enzymatic induction at the genomic level. This idea is reinforced by the fact that T also increases the ARO protein, as observed by immunocytochemistry (ICC) and the ARO mRNA, as measured by reverse transcriptase–polymerase chain reaction (RT–PCR). These changes can be mimicked by the administration of estrogens and therefore presumably require T aromatization. In our first test, injection of the non-steroidal ARO inhibitor, R76713 (racemic vorozole®), unexpectedly revealed an increase in ARO immunoreactivity in the preoptic area (POA) of treated birds. This property of R76713 was shared by another non-steroidal inhibitor, fadrozole, but not by two steroidal inhibitors, androstatrienedione (ATD) and 4-hydroxy-androstenedione (OHA). These last two compounds markedly decreased the concentration of brain ARO as estimated by ICC. In parallel, ATD and OHA decreased ARO mRNA concentration measured by RT–PCR but vorozole and fadrozole had no effect on these concentrations in the POA, and only caused them to decrease slightly in the posterior hypothalamus. Together, these data indicate that the removal of estrogens caused by steroidal inhibitors decreases the synthesis of ARO, presumably at the transcriptional level. Additional regulatory mechanisms apparently take place after the injection of non-steroidal inhibitors and probably include increased half-life of the protein. The induction of ARO activity by steroids appears to be greater in males than in females, but this difference has been difficult to localize and confirm by assay methods. We therefore analysed by ICC the tridimensional distribution of ARO-ir neurons in the POA of males and females that were sexually mature or gonadectomized and treated with T-filled or control empty implants. Localized sex differences and effects of T were detected in this way. In particular, males had more ARO-ir cells than females in the lateral POA but a difference in the opposite direction was evident in the medial part of this area. These sex differences are largely activational (i.e. caused by the higher T levels in males) but they may also reflect organizational effects of neonatal steroids. Castration decreased ARO-ir cell numbers in the lateral POA, but increased it in the periventricular region. This anatomically specialized control by T may be mediated by three potential mechanisms that are discussed and comparatively evaluated: a migration of ARO neurons towards the ventricle after castration; a differential colocalization of ARO with estrogen receptors or a differential modulation of ARO neurons by catecholaminergic inputs. © **1997 Elsevier Science Ltd**

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INTRODUCTION

The enzyme aromatase, that catabolizes the transformation of androgens into estrogens, has been identified in the brain of species belonging to all different classes of vertebrates with the possible exception of agnathans [1–3]. This enzyme appears to play a key role in the control of reproductive function, in particular male sexual behaviour. It has now been firmly demonstrated that the activation of male copulatory behaviour by testosterone (T) requires its aromatization into an estrogen, and that this enzymatic transformation is a limiting factor in behavioural induction (see [4, 5] for reviews). This critical role

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played by aromatase has been particularly well established in Japanese quail (*Coturnix japonica*). Behavioural studies have indeed revealed that the behavioural effects of T: (a) can be mimicked by the administration of natural (e.g. estradiol; E_2) and synthetic (e.g. diethylstylbestrol; DES) estrogens but not by non-aromatizable androgens; (b) are blocked by the concurrent administration of antiestrogens but not by antiandrogens; and finally (c) are almost completely suppressed by the concurrent administration of aromatase inhibitors (see [6] for review). Aromatase activity has also been measured in the brain of this species, and these studies have identified several correlations between the changes in aromatase activity in the preoptic area (POA) (a region directly implicated in the activation of copulation) and changes in behaviour [6, 7]. In particular, it could be demonstrated that treatments with T that activate copulatory behaviour also increase aromatase activity in the male POA, and that the enzymatic induction closely precedes in time the appearance of the behaviour (see Fig. 1). When an aromatase inhibitor was injected in parallel with T, the enzyme induction and the behavioural activation were both blocked in parallel [8].

During more than 20 years after the first identification of aromatase activity in the brain [9, 10], the cellular localization of the enzyme could not be identified because of the absence of suitable antibodies that would permit its visualization by immunocytochemistry (ICC). In the late 1980s and early 1990s, a few immunocytochemical studies presented a distribution of aromatase-immunoreactive (ARO-ir) material in the rat brain with the use of polyclonal antibodies raised against purified human placental aromatase (e.g. [11–14]). In all this work, important discrepancies were, however, identified between the distribution of immunoreactive cells and of aromatase activity.

A few years ago, we established an immunocytochemical procedure that permits the visualization of aromatase in the quail brain [15, 16], and localizes the immunoreactive cells exclusively in areas that were previously shown to contain aromatase activity [17, 18]. This allowed, for the first time, an analysis at the cellular level of the controls of aromatase. In this way it could be demonstrated that T, besides increasing the preoptic-hypothalamic aromatase activity [17, 19–21], also increases the number of cells expressing this enzyme, and therefore also presumably the enzyme concentration [15, 22, 23]. Because many effects of T in the brain are mediated at the cellular level by its locally produced metabolite, E_2, subsequent studies investigated the potential role played by estrogens in the control of aromatase activity and ARO-ir cells in the quail brain. This work showed that both natural (E_2) and synthetic (DES) estrogens are able, like T, to increase both aromatase activity and the number of ARO-ir cells [24–26]. This suggested that estrogens, presumably produced by local aromatization of androgens, are responsible for most, if not all, effects of T on the expression of the aromatase enzyme (positive feedback of the product on its enzyme). Experiments designed to test this conclusion led to apparently conflicting results.

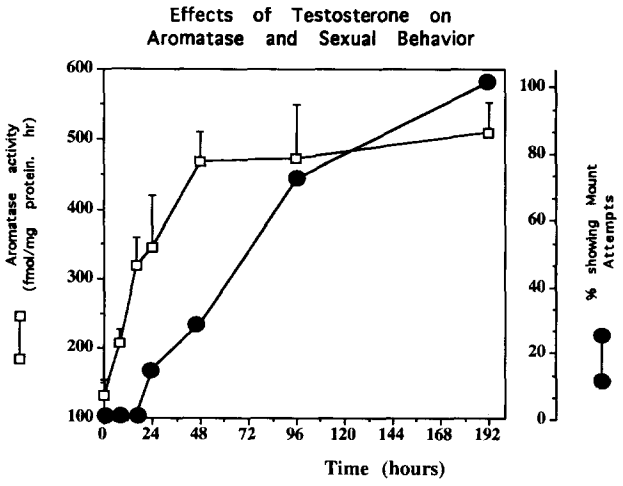

Fig. 1. Time course of the activation of sexual behaviour (mount attempts) and of the induction of aromatase activity in the POA-anterior hypothalamus after treatment with T (40 mm long silastic implants) of castrated male quail. The first statistically significant increase in enzyme activity is seen after 16 h. Activation of copulatory behaviour follows with a delay of 1–2 days (redrawn from data in [8]).

THE NON-STEROIDAL AROMATASE INHIBITOR, R76713 (RACEMIC VOROZOLE) INCREASES AROMATASE IMMUNOREACTIVITY

We attempted to disassociate the effects of androgens and estrogens in the control of aromatase synthesis by comparing ARO-ir cells in castrated male quails that were treated with T, associated or not with daily injections of aromatase inhibitors. This therefore produced two groups of subjects that were exposed to androgens and estrogens (treatment with T) or to androgens alone (T associated with an aromatase inhibitor) that could be compared to castrates that were essentially steroid-free. The first of these studies analysed the effects of a non-steroidal aromatase inhibitor, R76713 or racemic vorozole™ (VOR; a triazole derivative). It could be confirmed that at the doses that had been used, the inhibitor completely blocked the activation by T of copulatory behaviour (a well-known estrogen-dependent response) as well as the aromatase activity in the POA [27]. However, contrary to what had been expected, racemic VOR only partly inhibited the T-induced increase of ARO-ir cell numbers in the tuberal hypothalamus and did not

modify at all the effects of this aromatizable androgen on aromatase in the POA and anterior hypothalamus. Furthermore, VOR unexpectedly increased the apparent optical density of the aromatase-containing cells throughout the brain, which suggested that the inhibitor had somehow increased the concentration of the enzyme [22].

In another study, we also analysed the time-course of these effects of VOR on the density of aromatase immunostaining and demonstrated that this increase takes place gradually over the course of several days (maximum observed at the end of the experiment i.e. after 14 days of treatment). This suggested that the augmented immunostaining could not result from a change in enzyme conformation that should have been observed more rapidly, but rather reflected a gradual increase of the enzyme concentration [28].

DIFFERENTIAL EFFECTS OF STEROIDAL AND NON-STEROIDAL AROMATASE INHIBITORS ON AROMATASE

The preceding observations raised the question of whether the apparent increase in aromatase concentration was related to the inhibition of aromatase activity or reflected a specific property of VOR. It was also desirable to identify more precisely the mechanism underlying the increase of aromatase immunoreactivity induced by the treatment with this compound. In order to address these problems, a series of studies were performed in which the effects of VOR were compared to those of another non-steroidal inhibitor, fadrozole (CGS 16949A or FAD; an imidazole derivative) and of two steroidal inhibitors, androstatrienedione (ATD) and 4-hydroxy-androstenedione (OHA).

The effectiveness of the different inhibitors was first confirmed by assessing the biological effects of the blockade of estrogen production, specifically by quantifying the inhibition of the T-induced copulatory behaviour. As expected on the basis of previous work utilizing these compounds [27, 29–34], the four inhibitors decreased aromatase activity in the quail brain and, as a consequence, almost completely blocked the activation of copulatory behaviour by T in castrated males [28,35]. Their effects on aromatase immunoreactivity were, however, strikingly different.

Because they strongly inhibited the local production of estrogens, the two steroidal inhibitors (ATD and OHA) completely blocked the induction of ARO-ir cells that was induced by the treatment with T, and numbers of immunoreactive cells in these two groups were comparable to numbers observed in castrated birds not treated with T. In contrast, no significant decrease in ARO-ir cell numbers was associated with the treatments with the two non-steroidal aromatase inhibitors (VOR and FAD) and numerical increases were actually observed in FAD-treated subjects. Similar effects were essentially observed in the six brain areas that were studied, and in particular in the anterior part of the medial preoptic nucleus (POM) and of the tuberal hypothalamus, as illustrated in Fig. 2 (top panels).

Visual inspection strongly suggested that the density of the immunoreactive material was much higher in birds treated with the non-steroidal inhibitors than in the other four groups. The optical density of this material was therefore quantified by microdensitometry in selected fields belonging to the six brain regions in which ARO-ir cells had been counted. Sections were digitized with a video camera attached to a Macintosh IIci computer and these images were analysed with the IMAGE software (W. Rassband, NIH, Bethesda, MD, U.S.A.). For each brain region considered, the field containing the largest number of ARO-ir cells under a 20× objective was used for quantification. The entire field was digitized, transformed into a binary image and the area covered by immunoreactive cells was determined by an interactive thresholding method. The mean optical density of this area was recorded and the specific optical density of the ARO-ir material was calculated by subtracting from this value the optical density of a field from the same section that did not contain any stained cells (non-specific staining). This provided a measure of optical density for ARO-ir cells in the range 0 (white) to 255 (black).

These densitometric studies confirmed that the experimental treatments reliably affected the optical density, and therefore presumably the concentration, of ARO-ir material in the quail brain (significant effects by analyses of variance in all brain regions considered). Representative data for the anterior part of the POM and tuber are shown in Fig. 2 (middle panels). These significant overall effects mostly resulted from the increased optical density observed in the two groups that were treated with the non-steroidal inhibitors (VOR and FAD). This property was definitely not shared by the steroidal aromatase inhibitors, ATD and OHA: the optical density of ARO-ir cells in birds receiving these compounds was in fact lower than in the birds treated with T alone, and sometimes even lower than in the castrated birds exposed to no hormone (see [28,35] for details of the statistical analysis of these data).

Taken together, the measures of cell numbers and cell densities therefore strongly suggested the presence of an important difference between steroidal and non-steroidal aromatase inhibitors. Because these inhibitors block the endogenous production of estrogens and because it is well known that, in birds, estrogens increase aromatase activity, immunoreactivity and synthesis [19, 24–26], we had expected that injection of all inhibitors would decrease the number and/or optical density of the ARO-ir cells. This prediction

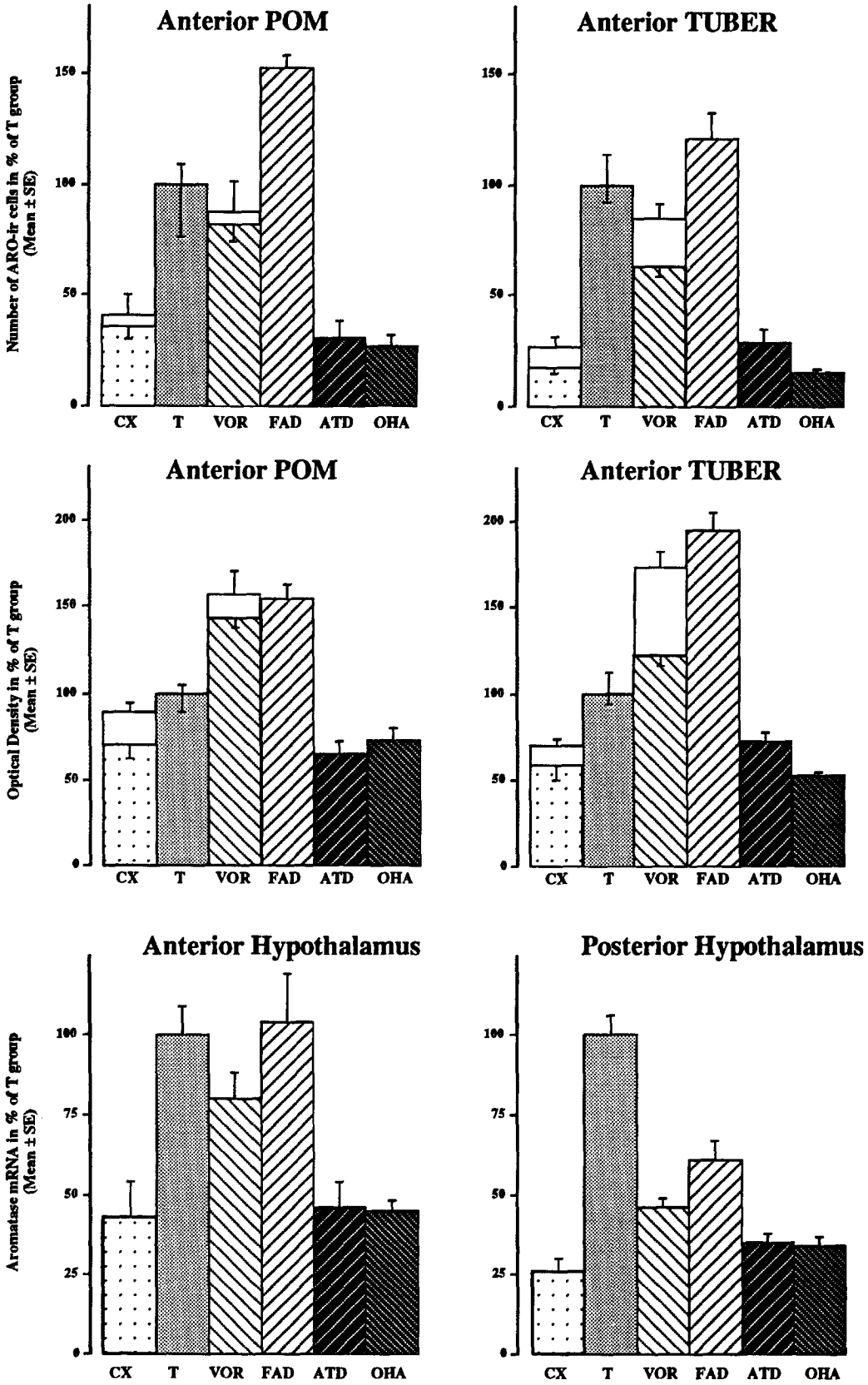

Fig. 2. Effects of testosterone (T) associated or not with non-steroidal (vorozole (VOR) and fadrozole (FAD)) or steroidal (androstatrienedione (ATD) and 4-hydroxy-androstenedione (OHA)) aromatase inhibitors on the number of ARO-ir cells (top) and their optical density (middle) in the anterior part of the POM or of the tuberal hypothalamus, and on the concentration of aromatase mRNA as measured by RT-PCR in the anterior or posterior hypothalamus (bottom). The effects of the four inhibitors on ARO-ir cells (number and density) were analysed in the course of two independent experiments, performed using slightly different methods. All data are therefore expressed as the percentage observed in the corresponding T group to facilitate direct comparisons. CX and VOR-treated birds were included in both experiments, and the two sets of data are presented to illustrate the good inter-experiment reproducibility of the effects. The standard errors relative to each experiment are also plotted independently above and below the bars (redrawn from data in [28] and [35]).

was fulfilled for ATD and OHA but not for VOR and FAD and actually, changes in the opposite direction were detected. This was so despite the fact that all compounds markedly decreased aromatase activity as indicated by direct enzyme activity assays [27, 29–34], and by the observation that in the subjects specifically used in this experiment a nearly complete suppression of copulatory behaviour was observed [28, 35]. The increased density of the immunoreactive signal in birds treated with the non-steroidal inhibitors (and of cell numbers in the FAD group) even suggested that they increase the enzyme concentration, and it was therefore decided to perform additional experiments to obtain more information on the potential mechanism underlying this effect.

We had previously shown that the T-induced increase in aromatase activity and in the number of ARO-ir cells reflects a pretranslational (presumably transcriptional) control of the enzyme concentration as revealed by the increase in aromatase mRNA that was measured by reverse transcriptase–polymerase chain reaction (RT–PCR) [36]. Therefore, in a subsequent experiment we investigated whether the differential effects of aromatase inhibitors on aromatase immunostaining were paralleled by different effects on the aromatase mRNA concentrations. Castrated subjects that were chronically treated with T silastic implants were injected twice a day for 4 days with the four aromatase inhibitors that had been used previously. Their brains were then dissected, and separate assays of the aromatase mRNA concentrations were performed in the anterior hypothalamus-POA and in the posterior hypothalamus by RT–PCR as previously described [36].

As expected on the basis of our previous work [36], treatment with T markedly increased the aromatase mRNA concentration in both the anterior and posterior hypothalamus. We had also shown before that the effects of T at this level are mainly caused by its metabolite, E_2 [25], and accordingly, treatments with the steroidal aromatase inhibitors (ATD, OHA) that blocked endogenous estrogen production completely blocked the increase in mRNA concentration in both parts of the brain (Fig. 2, bottom panels). Some anatomical specificity was, in contrast, observed in the response to VOR and FAD. In the posterior hypothalamus, both compounds significantly inhibited the T-induced increase of aromatase mRNA concentration, although this inhibition was not complete and a significant increase in comparison with castrates that did not receive T was still present [35]. In the POA–anterior hypothalamus, however, no effects on these two non-steroidal inhibitors could be observed and T increased the aromatase mRNA concentration in birds simultaneously treated with VOR or FAD as it did in birds receiving no inhibitor.

Taken together, the results of these experiments evaluating the aromatase protein and mRNA by ICC and RT–PCR, respectively, raise a number of intriguing questions. These questions are discussed below in parallel with a presentation of the mechanisms that potentially control aromatase synthesis and activity. It is well established that aromatase levels are controlled by T acting through its estrogenic metabolites [Fig. 3(A)]. However, this model of regulation only includes a positive feedback and the negative signals that presumably maintain the system in steady state have not been formally identified. For this discussion, we will assume that such mechanisms exist and that a negative feedback system links the enzyme activity to its concentration and to the concentration of its messenger RNA [Fig. 3(A')]. The mechanisms of action of the different aromatase inhibitors can then be considered in the light of these putative control systems, and three partly independent questions emerge from the studies described above.

1. Why is the T-induced increase in aromatase mRNA blocked by ATD and OHA but not VOR and FAD?

   A limited difference in the effects of steroidal and non-steroidal inhibitors on aromatase mRNA concentration was noted in the posterior hypothalamus, but this divergence appeared quite extreme in the POA-anterior hypothalamus, where no inhibition at all was found after treatment with VOR and FAD. The origin of this difference between steroidal and non-steroidal inhibitors remains unclear at present. Previous *in vitro* and *in vivo* experiments demonstrate that non-steroidal aromatase inhibitors are equally if not more potent than steroidal inhibitors [37, 38]. This was confirmed in the present studies by the fact that both types of compounds inhibited to the same extent male copulatory behaviour. Recent *in vitro* work in our laboratory also confirms that all four compounds are able to suppress by more than 90% aromatase activity when added to incubations of quail hypothalami at a concentration of 10^{-4}M (Baillien and Balthazart, unpublished data). There is therefore no reason to believe that significant amounts of estrogens were still produced in birds treated with VOR or FAD, but not in the other two groups treated with steroidal inhibitors.

   It may be postulated that the androgenic action of T explains the increase in aromatase mRNA concentration observed here in the anterior hypothalamus [Fig. 3(C')]. It has indeed been shown previously that even if they do not increase aromatase activity or aromatase mRNA concentrations in isolation, pure non-aromatizable androgens such as 5α-dihydrotestosterone (5α-DHT) or methyltrienolone synergize with estrogens to produce these increases [25, 26]. This indicates that they are not completely devoid of activity. However, if T or 5α-DHT is able to increase aromatase mRNA in the absence of estrogenic metabolites such as E_2,

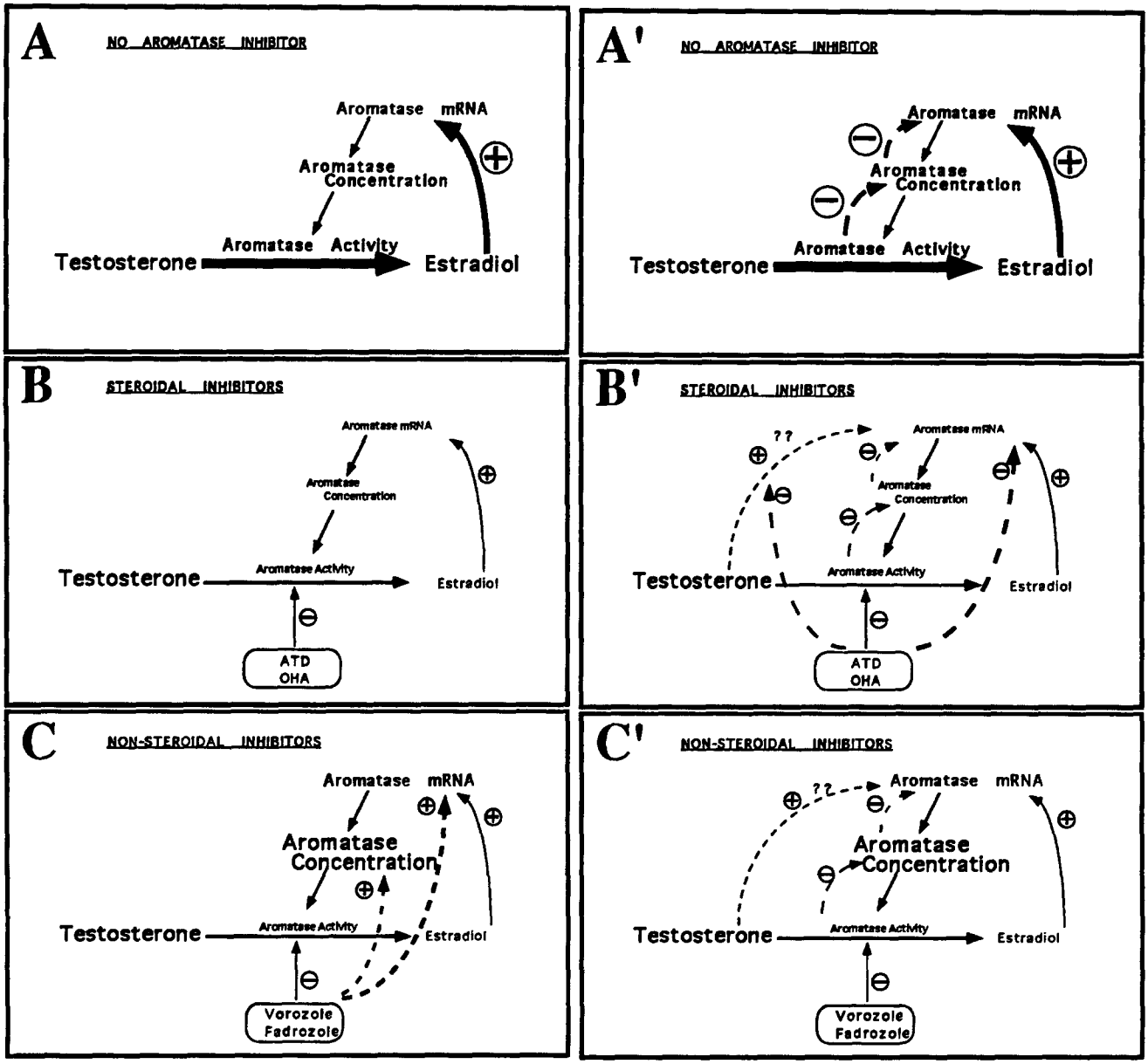

Fig. 3. Schematic models of the putative mechanisms implicated in the induction of aromatase synthesis and its control by steroidal and non-steroidal aromatase inhibitors. The width of arrows and size of characters used to name elements in the model have been adjusted to reflect higher or lower enzymatic activities or concentrations. Two sets of alternative models are presented. In the left columns, all regulations are based only on the well-established positive feedback of estrogens on aromatase activity (panels A, B and C). In the right columns, we have also included a putative, so far unidentified, negative feedback mechanism that helps maintain the steady-state levels of enzyme activity. The role of steroidal and non-steroidal aromatase inhibitors has then be reconsidered in this context (panels A′, B′ and C′). See text for additional comments.

additional hypotheses need to be formulated to explain why such an increase in mRNA concentration was not observed in the ATD and OHA groups. It could be argued that because ATD and OHA are steroid molecules they display some anti-androgenic activity [Fig. 3(B′)]. However, there is little evidence for this type of action in the literature [39]. At high doses, ATD blocks the 5α-reduction of T in the quail brain [40], but because 5α-reduced androgens are poorly or not effective in stimulating aromatase [25], the inhibition of 5α-reductase activity should not have a significant effect in the control of aromatase.

Therefore, we would like to suggest two possible ways in which such a differential action of steroidal and non-steroidal inhibitors can be taken into account. They are derived from the two models of aromatase control presented in Fig. 3 (panels A and A′). Either we assume that the model shown in panel A fully explains aromatase control. The drop in estrogen production is then sufficient to explain the decrease in aromatase mRNA concentration

and in aromatase protein (immunocytochemical signal) observed after treatment with ATD or OHA [Fig. 3(B)]. The lack of decrease in mRNA and the increase in the immunocytochemical signal detected after treatment with the aromatase inhibitor can then be explained only by assuming that these compounds increase the half-life of the protein, and/or directly stimulate aromatase transcription and translation by steroid-independent mechanisms that are so far unidentified [dotted arrows in Fig. 3(C)]. This notion is indirectly supported by the observation that treatment of castrated quail with VOR increases the density of aromatase immunoreactivity even in birds that are not treated with T [22], which indicates the possibility of a steroid-independent control.

Alternatively, one can assume that the aromatase activity in some way exerts a negative feedback control on the aromatase and aromatase MRNA concentrations [Fig. 3(A')]. The blockade of the enzyme activity by VOR or FAD injections would then remove these inhibitory inputs, and this would result in an increased concentration of aromatase and aromatase mRNA that would not require an action of these inhibitors in addition to the suppression of enzyme activity [Fig. 3(C'); see also point 2 below for a possible mechanism that could produce this effect]. However, if this scenario is correct, one additional hypothesis must then be formulated to explain why this control by enzyme activity was not detected in the ATD- and OHA-treated birds. Because these compounds are steroids, it might be suggested that they could act as antiestrogens and in this way directly interact with the steroid-sensitive sites at the DNA level that control aromatase transcription [Fig. 3(B')].

2. What causes the increased density of aromatase immunoreactivity in birds treated with VOR or FAD?

Treatment of T-treated male quail with the aromatase inhibitors VOR or FAD increases the density of aromatase immunostaining in the brain more than treatment with T alone [22,28,35]. This denser immunostaining only develops on a long-term basis (1–2 weeks), which suggests that it is caused by an increase in protein (aromatase) concentration rather than by a mere change in protein conformation that should be observed much more rapidly [28]. Aromatase mRNA concentration was assayed by RT–PCR to determine whether this putative increase in protein concentration reflects an increased transcription of the corresponding message, and the results of that experiment clearly invalidated this interpretation. In the anterior hypothalamus, the aromatase mRNA concentration was similar in birds treated with T alone or in combination with VOR or FAD. In the posterior hypothalamus, RNA concentration was even lower in birds that had been injected with the inhibitors. In contrast, the optical density of ARO-ir cells was higher in birds treated with the inhibitors in all brain regions, which suggests that the increase in optical density and presumably in protein concentration cannot be explained by an increased transcription of the RNA encoding for the enzyme. It could be postulated that the translation of the aromatase RNA molecules was increased by the two non-steroidal aromatase inhibitors, but the mechanism mediating a translational regulation of this type cannot easily be conceived.

Because the aromatase inhibitors directly interact with the enzyme and not with its messenger RNA, it is more likely that VOR and FAD increase the brain aromatase concentration by increasing the half-life of the protein. In the absence of detailed information on the catabolic processes that regulate aromatase concentration, it is difficult to evaluate this possibility experimentally. However, both biochemical and immunocytochemical experiments have identified, in the quail brain, the presence of aromatase throughout the full extent of axons including the presynaptic boutons [41,42]. ICC at the electron microscope level has recently demonstrated the presence of ARO-ir material at the surface of small vesicles packed at the presynaptic level [42]. There is currently no evidence that this material can be excreted in physiological conditions (e.g. after depolarization), but if this was the case, it could then be postulated that the inhibition of aromatase activity by VOR or FAD in some way blocks this extrusion of aromatase-containing vesicles from the presynaptic boutons. It is then easy to imagine that this process could lead to an accumulation of enzyme in the cells. This scenario is, however, largely speculative at the present time.

3. Why are effects of non-steroidal inhibitors on aromatase neuroanatomically differentiated?

It is interesting that the RT–PCR assays demonstrated that both non-steroidal inhibitors produced a significant inhibition of the T-induced increase in aromatase mRNA concentration in the posterior but not in the anterior hypothalamus. On the basis of our current understanding of aromatase control, this anatomical specificity could find its origin in three possible mechanisms.

It could first be suggested that the inhibition of estrogen production was more effective in the caudal than in the rostral parts of the hypothalamus, resulting in a greater depletion of estrogens and consequently in a more complete inhibition of transcription. Experimental data, however, give no support to this idea. It was previously established that VOR decreases the aromatase activity below castrate levels in the POA of male quail [27] and, in addition, the present study indirectly confirmed these data by showing that both VOR and FAD

strongly inhibit the T-induced male sexual behaviour, which is known to depend directly on the preoptic aromatase activity [28,35].

A second interpretation would assume that the steroid specificity of aromatase induction is different in the rostral and caudal parts of the hypothalamus with a bigger dependence of the enzyme on androgenic metabolites in the anterior hypothalamus and on estrogens in the caudal hypothalamus. Experiments performed to test this theory specifically by treating castrated birds with non-aromatizable androgens (5α-DHT, methyltrienolone) or with estrogens (E2 or DES) have, however, failed so far to provide evidence supporting this idea [19,24–26,43].

It is therefore tempting to relate this anatomically differentiated regulation to previous data demonstrating a differential colocalization between aromatase and estrogen receptors in different brain areas [44,45], and to assume that non-steroidal inhibitors differently affect aromatase in the anterior and posterior hypothalamus because controls of enzyme transcription are different in the two brain areas. In the tuberal hypothalamus, a large proportion of the ARO-ir cells (70–80%) contain detectable levels of immunoreactive estrogen receptors. It can therefore be assumed that, in this brain region, locally produced estrogens control aromatase transcription in an autocrine mode of action. The microsomal aromatase catalyses the transformation of T into E_2, and this steroid then diffuses to the nucleus of the cell where it was formed and exerts genomic effects including the increase of aromatase transcription. In the POA, estrogens would, in contrast, affect aromatase transcription in an indirect manner because, in this brain region, most ARO-ir cells do not appear to contain estrogen receptors [44]. It is possible that, in the preoptic region, E_2 formed by aromatization (in estrogen receptor negative neurons) diffuses to an adjacent cell that contains estrogen receptors to exert its effects. The synthesis of aromatase should then be controlled by this adjacent cell acting transynaptically. In addition, long-distance projecting neurons that are themselves estrogen-sensitive could play a similar role in the control of the enzyme. We have previously suggested that catecholaminergic neurons could be implicated in this process [5, 7, 46]. The control of preoptic aromatase would therefore be exerted by steroids indirectly in a way that is very reminiscent of the control of luteinizing hormone releasing hormone (LHRH) [47–49].

Indirect controls of this nature obviously make it easier to explain why a major decrease in estrogen production is not immediately reflected in a decrease in aromatase mRNA concentration. Indirect inputs that control the aromatase transcription could only vary progressively, they could temporarily compensate for the lack of estrogens, they could be sensitive to androgenic steroids or they could be directly affected in an unidentified way by these aromatase inhibitors. It will only become possible to test these ideas when the control mechanism(s) of aromatase are identified. It remains, however, that the differential colocalization of aromatase and estrogen receptors and the anatomically specified effects of non-steroidal aromatase inhibitors clearly suggest that different mechanisms control aromatase in the POA-anterior hypothalamus and in the tuberal hypothalamus.

SEX DIFFERENCES AND CONTROL BY T OF ARO-IR CELLS: THREE-DIMENSIONAL ANALYSIS REVEALS LOCALIZED EFFECTS

Studies measuring aromatase activity by a product-formation assay in the entire POA originally demonstrated that the activity of this enzyme is under the control of T and is sexually differentiated in several species of birds including the Japanese quail [17, 21, 50]. In this species, a high level of aromatase activity is normally observed in sexually mature males. The enzyme activity drastically decreases after castration and is restored to the level of intact birds by a treatment with exogenous T [8,17].

The preoptic aromatase activity is also significantly higher in males than in females and interestingly, this enzymatic sex difference is still present (although its magnitude has decreased) when birds of both sexes are gonadectomized and treated with the same dose of exogenous T [8, 17, 51]. These sex differences and T-induced changes in aromatase activity nicely parallel differences observed in the copulatory behaviour of corresponding subjects: castration suppresses and T treatment restores copulatory behaviour [52–54], and males, but not females, show male-typical copulatory responses [54–56].

In the Japanese quail, a sexually dimorphic nucleus has been identified in the POA: the medial POM is larger in males than in females [57]. This nucleus is also T-sensitive: its volume regresses after castration and is restored by a treatment with T [58, 59]. The POM plays a critical role in the activation of male sexual behaviour: electrolytic lesions and stereotaxic implantations of steroids or antihormones demonstrate that the POM is a necessary and sufficient site of steroid action for the activation of copulation [6]. Additional studies also demonstrate that T must be aromatized locally within the POM to activate the behaviour [27,60–62].

When this nucleus was discovered and its significance for the control of reproductive behaviour was recognized, aromatase activity was measured specifically in the nucleus microdissected by the "Palkovits" punch technique [63]. This demonstrated that high levels of enzyme activity are present in the dimorphic

POM [18], and that this enzyme is also controlled by the circulating levels of T and is significantly higher in sexually mature males than in females [64].

More recently, it became possible to visualize by ICC the aromatase-containing cells in the quail brain [15,16], and in agreement with biochemical data, immunocytochemical studies demonstrated that, in the POA, most if not all ARO-ir cells are located within the cytoarchitectonic boundaries of the POM [16,65]. Detailed studies were then performed to analyse by semi-quantitative anatomical methods the control of preoptic ARO-ir neurons. Very few ARO-ir elements were found in the POM of castrated males by comparison with sexually mature intact males, but their number dramatically increased after a 2-week treatment with T [15], especially in the caudal part of the nucleus [23,66] (Fig. 4).

A sex difference in the number of preoptic ARO-ir cells (males>females) was also identified in the posterior part of the POM at the level of the anterior commissure [23, 66]. These changes and sex differences in the number of aromatase-positive cells presumably reflect the differences in enzymatic activity that have been identified by product-formation assays (males>females; T-treated males>castrated males). They support the idea that changes in aromatase activity reflect changes in the amount of enzyme (see [5,67] for additional discussion).

Interestingly, these immunocytochemical studies identified no difference in the total number of ARO-ir cells in the entire POM between males and females that had been gonadectomized and treated with T [23, 66]. This somehow contradicted the biochemical studies that had demonstrated that a sex difference of aromatase activity exists in these endocrine conditions [8, 17, 51]. The POM is, however, a heterogeneous structure: neurons located in the lateral part, contrary to those located in the medial part of the nucleus, react differentially in males and females to the same treatment with T [59]. They are also permanently affected by embryonic treatments with estrogens [68]. Furthermore, the number of ARO-ir neurons located in the lateral part of the nucleus, which are highly sensitive to T, is markedly decreased by castration, whereas cells located more medially are less affected by steroid removal [69].

Quantitative studies of the distribution of ARO-ir neurons in the sexually dimorphic POM were therefore undertaken in order to determine the specific localization of ARO-ir cell populations that are different in sexually mature male and female quail and are responsible for the sex difference in aromatase activity. This work was also designed to research whether parts of these dimorphic populations remain sexually differentiated when birds are gonadectomized and treated with T.

Male (M) and female (F) quail were studied either as sexually mature birds (I) or after gonadectomy (CX; bilateral castration of males and unilateral ovariectomy of females) associated or not with a replacement therapy with T administered by subcutaneous implantation of silastic capsules filled with the steroid (CX + T). This combination of sex and endocrine treatments defined six experimental groups (MI, FI, MCX, FCX, MCX + T, FCX + T). Serial sections (30 μm thick) in the coronal plane were collected throughout the rostro-caudal extent of the POA and stained by ICC for aromatase with a primary antibody raised against quail recombinant enzyme (see [66] for details of procedures). This procedure and its specificity have previously been validated for use in the quail brain [15,70].

The distribution of ARO-ir cells was then studied in every third section in the entire rostro-caudal extent of the POM. All sections were first drawn under the microscope (objective 10×) with the help of a camera lucida and ARO-ir cells marked and counted on these drawings. Ten serial sections (90 μm apart) were quantified for each bird. The most caudal section was located at the rostral end of the commissura anterior (CA), whereas the most rostral section (CA-9) was located approximately 810 μm (9×90) more rostrally. This allowed us to reconstruct the distribution of the ARO-ir cells along the rostro-caudal axis of the POM (Fig. 5).

This study confirmed a number of previously established results (e.g. [23,65]). Larger numbers of ARO-ir cells were located in the caudal part of the POM than in its most rostral portion. Castration in both sexes markedly decreased the number of these cells, but again this effect was more prominent in the caudal part of the nucleus. Sexually mature gonadally intact males (MI) had more ARO-ir cells than females in the same hormonal state (FI), but this difference was no longer observed when subjects were placed in similar endocrine conditions (comparison MCX vs FCX or MCX + T vs FCX + T).

In order to obtain a view in space of this distribution, the location of ARO-ir cells within each section was also mapped quantitatively. A transparent grid was laid over the drawing of each section to permit the counting of positive cells in spatially identified sub-regions. This grid was composed of 14×10 squares in the dorso-ventral and medio-lateral directions, respectively, with each square corresponding to 116×116 μm on the actual brain section. The anatomical position of the grid was standardized by placing its top left corner at the tip of the third ventricle on each section, and the left side of the grid was aligned with the edge of the third ventricle (except at level CA where the top left of the grid was placed at the corner defined by the crossing of the third ventricle and the ventral edge of the anterior commissure). The number of ARO-ir cells was then counted in each square of the grid, and means were computed for each square/section in the rostro-caudal axis and

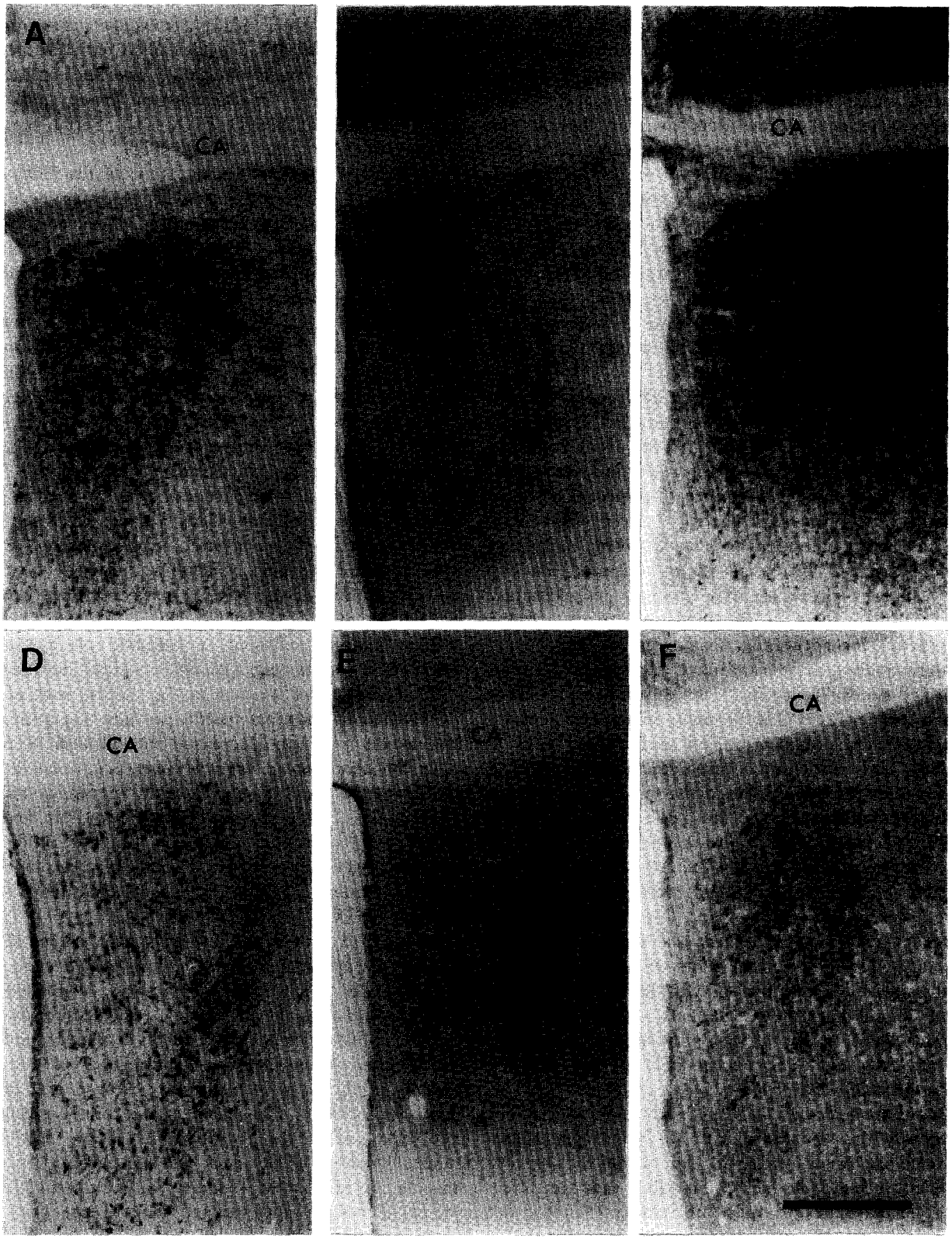

Fig. 4. Photomicrographs illustrating the distribution of ARO-ir cells in the POM (level of the anterior commissure; CA) of male (A–C) or female (D–F) quail that were either gonadally intact and sexually mature (A, D), or gonadectomized (B, E) or gonadectomized and treated with testosterone (C, F). The medial part of the brain and the third ventricle are on the left in each panel. Magnification bar = 400 μm.

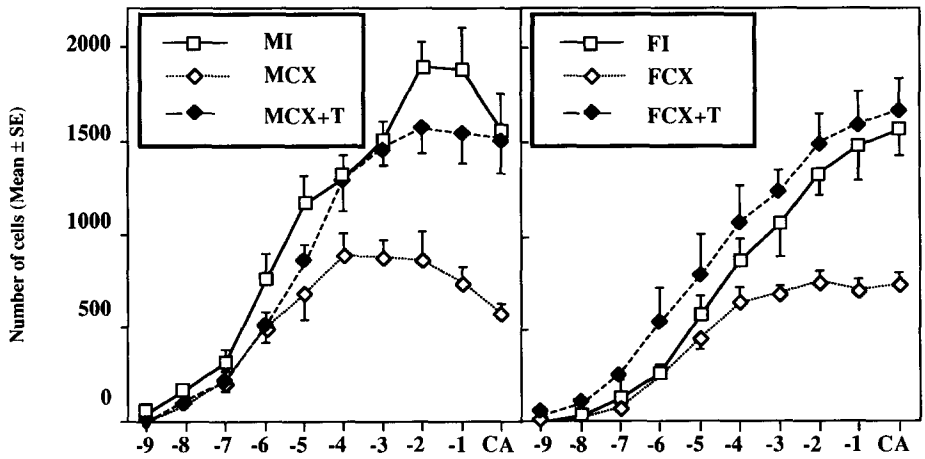

Fig. 5. Distribution of ARO-ir cells along the rostro-caudal axis of the POM of male and female quail that were either sexually mature and gonadally intact (MI, FI), or gonadectomized (MCX, FCX) or gonadectomized and treated with testosterone (MCX + T, FCX + T). Successive sections in the rostro-caudal axis were realigned for different birds using the anterior commissure (CA) as landmark. Every third section was stained and quantitatively analysed (CA-9 to CA). Data presented are the means (±SE) of the total numbers of ARO-ir cells counted in the POM in 30 μm-thick sections (redrawn from data in [66]).

experimental group. These data were plotted in three-dimensional graphs for each rostro-caudal level by experimental group combination, and representative data obtained for sections located in the caudal part of the POM at the level of the anterior commissure and are presented in Fig. 6. An interesting spatial heterogeneity in the distribution of ARO-ir cells and in their reaction as a function of the sex or endocrine condition was revealed in this way.

The distribution of positive cells appeared to be somewhat different in males and females, but these differences were difficult to assess in this kind of graphic presentation. In order to obtain a better view of the putative sex differences in ARO-ir cell distribution, additional graphs were prepared by subtracting the matrices of data (in the dorso-ventral and medio-lateral directions) relative to the two sexes, and representing the resulting matrix of differences in three dimensions again. Positive scores on the vertical axis indicated in this case that more cells were present at a specific location in the male than in the female group. Negative scores (below the horizontal plane) conversely were associated with brain areas where females have more ARO-ir cells than males. These differential graphs are presented in the third row of Fig. 6 for intact, CX and CX + T birds, respectively. With this analysis, it became clear that despite the fact that intact males had in general more ARO-ir cells than the corresponding females (see Fig. 5), a difference in the opposite direction could be observed at specific locations, for example in the ventro-medial part of the nucleus. It must also be stressed that the analyses presented here as an example correspond to sections obtained at the level of the anterior commissure, where the overall number of ARO-ir cells was identical in groups MI and FI. When a similar analysis was repeated at more rostral levels, where males have an overall number of cells higher than females, the dorso-lateral excess of cells in males was still observed but at the same time larger numbers of ARO-ir cells were still present in females compared to males when the ventro-medial part of the POM was considered (see [66]).

These sex differences, favouring either males or females as a function of the specific location in the POM, usually disappeared after gonadectomy so that the differential graphs similar to those of the third line in Fig. 6 were almost completely flat and centered on the zero point of the vertical axis. Treatment of both males and females with the same dose of T, however, re-established some significant sex differences. Even if these differences had a smaller magnitude than in gonadally intact subjects, their pattern was strikingly similar, with males having more cells than females in the dorso-lateral POM, whereas differences in the opposite direction were present in the ventro-medial part of the nucleus. These qualitative impressions were confirmed by detailed statistical analyses using two-way analyses of variance with repeated factors and *post-hoc* comparisons of data relative to specific locations, but the detailed presentation of these results goes beyond the scope of the present study (see [66] for detail).

CASTRATION DECREASES THE TOTAL NUMBER OF ARO-IR CELLS IN THE MALE POM BUT LOCAL INCREASES ARE ALSO OBSERVED BY A THREE-DIMENSIONAL ANALYSIS

The three dimensional study of the ARO-ir cell distribution also suggested the presence of experimental effects (due to castration and T treatment) that had

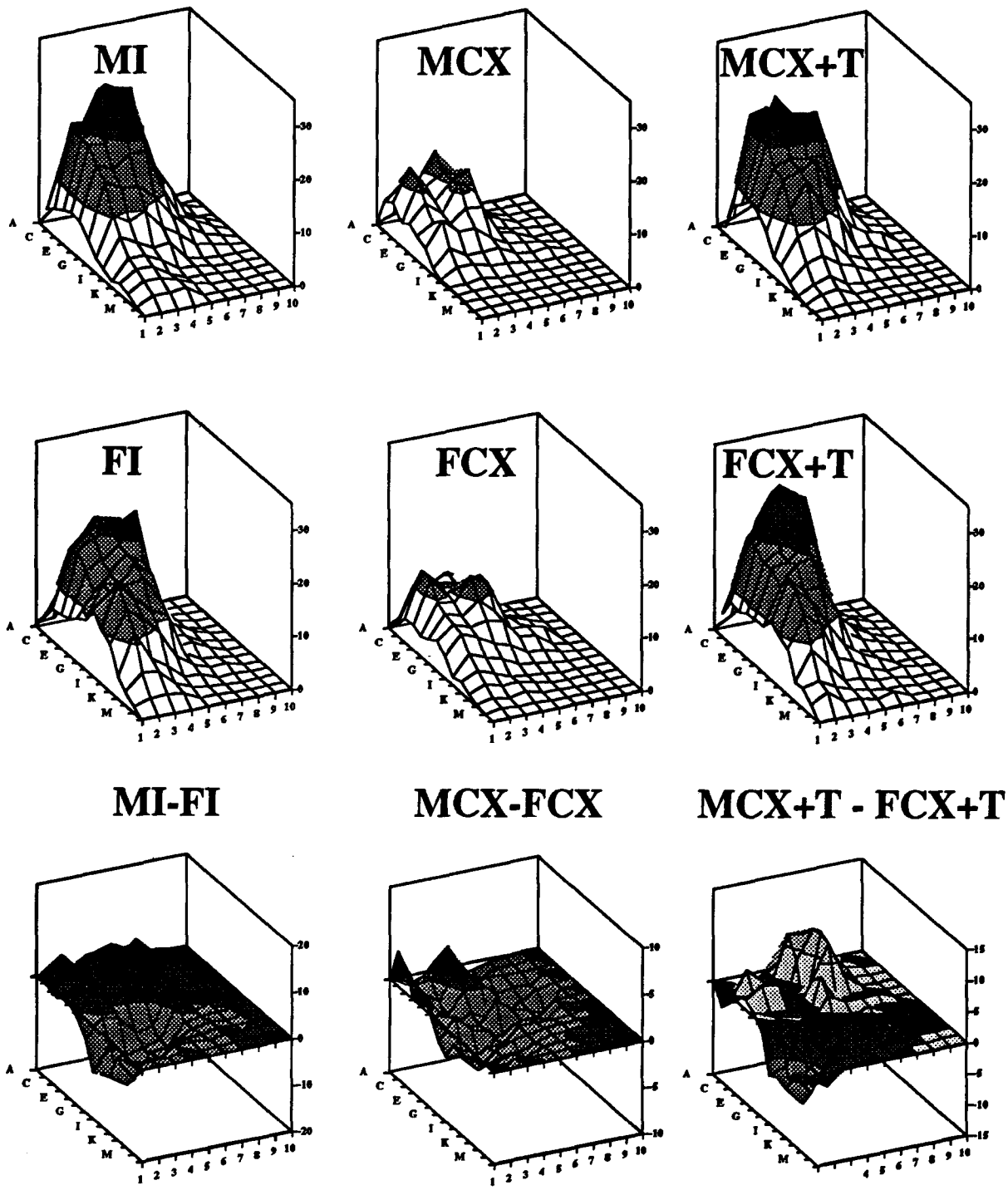

Fig. 6. Three-dimensional representation of the distribution of ARO-ir cells in sections collected at the level of the anterior commissure in the six groups of male or female quail that were either sexually mature and gonadally intact (MI, FI), or gonadectomized (MCX, FCX) or gonadectomized and treated with T (MCX + T, FCX + T). The distribution of cells in each experimental group is indicated by a three-dimensional graph in which the two horizontal coordinates, respectively, represent the medio-lateral and the dorso-ventral axis of the POM, whereas the vertical coordinate represents the mean number of ARO-ir cells present at each specific location. Column 1 in each graph corresponds to the edge of the third ventricle and square A1 corresponds to the top of the third ventricle (see text). The shading of the surface provides indications on the actual position in the vertical axis, and a different greytone is used for each interval between marks on this axis. The third line of graphs illustrates the sex difference in the distribution of ARO-ir cells in intact (MI–FI), gonadectomized (MCX–FCX) and T-treated birds (MCX + T–FCX + T) from left to right, respectively. In each panel, the differential distribution of cells is represented by a three-dimensional graph similar to those presented above, but in which the vertical coordinate now represents the mean difference in the number of ARO-ir cells between males and females at each specific location. Positive values (above the zero horizontal plane) indicate a higher density of cells in the male group, negative values indicate that more cells were present at the specific location considered in the female group (redrawn from data in [66]).

not been suspected on the basis of the more simple analysis of the distribution in the rostro-caudal axis. In gonadally intact subjects as well as in birds treated with T, a unimodal distribution of cells in space was always observed, with the highest frequency of immunoreactive cells being present in the dorso-lateral part of the POM (see Fig. 6). This distribution was markedly affected by castration, which drastically decreased the number of ARO-ir cells in the lateral POM, but had little or no effect in its medial part (or even increased their number in the rostral POM, see below). As a result of these anatomically specific changes, the ARO-ir cell distribution was bimodal in castrated birds, with two frequency peaks of approximately equal magnitude located, one in the periventricular position and the other in the most lateral part of the nucleus.

Interestingly, the effects of endocrine manipulations varied markedly as a function of the position in the rostro-caudal axis. In the caudal part of the POM, intact subjects displayed the unimodal distribution of cells (peak of cell frequency in the dorso-lateral POM) and castrates the bimodal distribution described above, with a second peak of cell frequency clearly visible in the periventricular position. In the more rostral parts of the nucleus, the magnitude of this medial peak markedly increased whereas the dorso-lateral peak progressively diminished to disappear finally at the rostral tip of the nucleus. This marked rostro-caudal difference is illustrated in Fig. 7 by two matrices of data, representing the difference between numbers of cells in intact and castrated males at the level of the anterior commissure and four sections more rostral (level CA-4, i.e. approximately 360 μm more rostral).

This presentation clearly demonstrates that, at the level of the anterior commissure, intact males had more ARO-ir cells than castrated males in most parts of the POM. Differences in the opposite direction were, however, present along the third ventricle (medial part of the POM) in the more rostral sections (illustrated here by level CA-4). Treatment of castrated males with T restored a distribution of ARO-ir cells that was very similar to the distribution present in intact males. The differential analysis of the distributions in the MCX and MCX + T groups therefore gave a mirror image of the results presented above.

It is obvious that despite the overall decrease in cell numbers in castrates (as a result of a widely spread change in the dorsal and lateral parts of the nucleus),

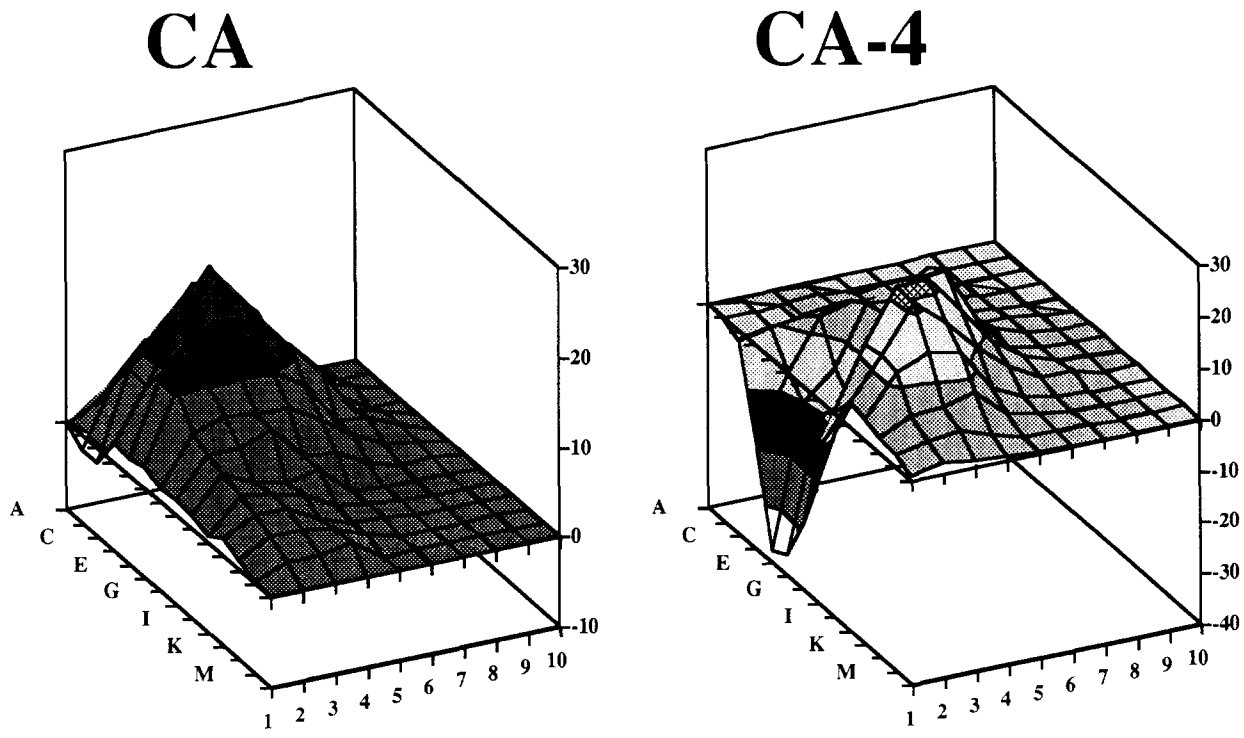

Fig. 7. Effect of castration on the distribution in space of ARO-ir cells in the POM of male quail as observed at the level of the anterior commissure (CA) and in the fourth section rostral to this level (CA-4; see text) that was analysed. In each panel, the differential distribution of cells (MI–MCX) is indicated by a tridimensional graph, in which the two horizontal coordinates (X and Y), respectively, represent the medio-lateral and the dorso-ventral axis of the POM, whereas the vertical coordinate represents the mean difference in number of ARO-ir cells present at each specific location. Positive values (above the zero horizontal plane) indicate a higher density of cells in the MI group, negative values indicate that more cells were present at the specific location considered in the MCX than in the MI group (redrawn from data in [66]).

a localized increase in cell numbers is present in the periventricular position at the rostral end of the POM (level CA-4 essentially). Two independent mechanisms could potentially explain this castration-induced increase in cell density in the medial part of the POM. On the one hand it is known that the entire volume of the POM decreases in castrated birds [58, 59]. The exact mechanism underlying these changes has not been identified. The well-documented decrease in neuronal size observed in castrated males [59, 71] obviously should contribute to these modifications, but a general displacement of cells towards the walls of the third ventricle also presumably takes place. This does not imply that ARO-ir cells specifically migrate towards the ventricle while other non-immunoreactive cells would stay at the same position, but simply that there may be a general shrinkage of this area towards the ventricle. This cell movement may concern an important fraction of the aromatase cells, and it could be responsible for the local increase of the density of ARO-ir neurons in the medial part of the nucleus.

On the other hand, there is indirect experimental evidence suggesting that steroids may have opposite effects on ARO-ir cells in the medial and lateral parts of the POM. In this hypothesis, castration would increase the density of ARO-ir cells at the edges of the third ventricle but decrease it in the lateral part of the POM. As summarized above, treatment of castrated quail with T increases the aromatase mRNA concentration, the number of ARO-ir cells and the aromatase activity in the quail POA [15, 17, 23, 25, 36]. These observations indicate that the steroid enhances the synthesis of new enzyme molecules. Similar effects can be observed after a treatment with estrogens or blocked by the concomitant administration of an aromatase inhibitor, which indicates that T acts through its estrogenic metabolites at the cellular level [24–26, 28, 35].

Several studies show, however, that in quail and in rodents, ARO-ir cells of the POA are devoid of estrogen receptors [44, 45, 72, 73]. This suggests that estrogens may not act as transcription factors to promote aromatase synthesis directly in the aromatase-containing cells of the POM. These data previously led us to postulate that aromatase synthesis and activity are controlled trans-synaptically in the quail POM, and that the catecholamines norepinephrine or dopamine may be important effectors of this control [5, 7, 46]. This interpretation is supported by a number of anatomical, biochemical and pharmacological experiments.

Anatomical relationships have been demonstrated between ARO-ir cells and catecholamine-synthesizing elements, and in particular it has been shown that aromatase is colocalized with tyrosine hydroxylase (TH) in the ventro-medial part of the POA [74] that presumably corresponds to the antero-ventral periventricular nucleus (AVPv) of the rat brain [75, 76], and that ARO-ir cells of the POM receive an apparent innervation by TH and dopamine β-hydroxylase (DBH) fibres [7, 74]. Recent *in vitro* biochemical work demonstrates major interactions between aromatase and dopaminergic compounds that could potentially play an important role in the control of aromatase activity in physiological conditions (Baillien and Balthazart, unpublished data).

Preliminary *in vivo* pharmacological experiments also support the notion that catecholamines may control aromatase. They suggest that norepinephrine may inhibit aromatase activity (and presumably synthesis [7, 77]) whereas dopamine would rather be stimulatory [5, 7, 46]. It is interesting to note, in this context, that the medial part of the POM (where castration increases the number of ARO-ir cells) receives a relatively dense noradrenergic innervation (identified by the presence of DBH fibres), whereas the dopaminergic innervation of this nucleus (identified by TH fibres or by a direct dopamine visualization) is more widespread and certainly covers its lateral parts [7, 78] (Balthazart and Absil, unpublished data; also see [79] for similar results in chicken). On the basis of this anatomical and pharmacological evidence, it is therefore conceivable that estrogens modulate aromatase synthesis and activity through their control of noradrenergic and dopaminergic systems, a proposition which is also indirectly supported by the fact that steroids modulate the noradrenergic and dopaminergic activity in a variety of biological systems [80–82]. The opposite effects of castration on the ARO-ir cells in the medial and lateral POM would then reflect differences in the neurochemical mechanisms that control this enzyme at different locations (noradrenergic inhibition vs dopaminergic stimulation). This hypothesis should be investigated in future experiments. It is also surprising that the periventricular accumulation of ARO-ir cells preferentially takes place at a specific level in the rostro-caudal axis (level CA-4). Whether this corresponds to a particularly dense noradrenergic innervation (second interpretation) or to a convergence point for the migrating neurons (first interpretation) should be experimentally analysed.

CONCLUSION: DIRECT AND INDIRECT CONTROLS OF AROMATASE

In conclusion, the studies summarized in this paper demonstrate that different aromatase inhibitors can have divergent effects on brain aromatase, despite the fact that they all inhibit to the same extent aromatase activity and male copulatory behaviour. These results suggest the presence of additional control mechanisms, which in addition to estrogens, modulate the synthesis and activity of the enzyme and could be

specifically affected by the non-steroidal or by the steroidal aromatase inhibitors. Additional studies should therefore be carried out to investigate these unidentified controls. The analysis by immunocytochemical methods of the spatial distribution of ARO-ir cells and of their sexual dimorphism and control by steroids also suggests anatomically specialized mechanisms of control that may implicate a transynaptic regulation by the catecholaminergic neurotransmitters. Future studies should be devoted to the elucidation of the physiological significance of these anatomically specialized ARO-ir cell populations by a combination of anatomical, neurochemical, pharmacological and biochemical techniques.

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