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Effects of sex steroids on aromatase mRNA expression in the male and female quail brain

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Abstract

Castrated male quail display intense male-typical copulatory behavior in response to exogenous testosterone but ovariectomized females do not. The behavior of males is largely mediated by the central aromatization of testosterone into estradiol. The lack of behavioral response in females could result from a lower rate of aromatization. This is probably not the case because although the enzymatic sex difference is clearly present in gonadally intact sexually mature birds, it is not reliably found in gonadectomized birds treated with testosterone, in which the behavioral sex difference is always observed. We previously discovered that the higher aromatase activity in sexually mature males as compared to females is not associated with major differences in aromatase mRNA density. A reverse sex difference (females > males) was even detected in the bed nucleus of the stria terminalis. We analyzed here by *in situ* hybridization histochemistry the density of aromatase mRNA in gonadectomized male and female quail that were or were not exposed to a steroid profile typical of their sex. Testosterone and ovarian steroids (presumably estradiol) increased aromatase mRNA concentration in males and females respectively but mRNA density was similar in both sexes. A reverse sex difference in aromatase mRNA density (females > males) was detected in the bed nucleus of subjects exposed to sex steroids. Together these data suggest that although the induction of aromatase activity by testosterone corresponds to an increased transcription of the enzyme, the sex difference in enzymatic activity results largely from post-transcriptional controls that remain to be identified.

Keywords

aromatase; in situ hybridization; Japanese quail; sex differences; preoptic area; aromatase mRNA

1. Introduction

Similar to many other vertebrate species (see [18,24] for reviews), Japanese quail (*Coturnix japonica*) display a pronounced sex difference in their behavioral responsiveness to

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exogenous testosterone. While such a treatment reliably activates the entire sequence of male-typical copulatory behavior in castrated males, the same treatment has no behavioral effect in ovariectomized females [2,14]. It has been established that these behavioral differences in response to testosterone are permanently organized by embryonic sex steroids (for review, see [3,15]). Ovarian estradiol demasculinizes females before embryonic day 12 and makes them unable to display male-typical behaviors in response to testosterone.

In many species, testosterone does not act on neural sites controlling sexual behavior directly as an androgen (see [7] for review). Rather, the conversion of testosterone into 17 β -estradiol, which is catalyzed in the preoptic area of the brain by the enzyme aromatase, is crucial for the activation of male reproductive behavior [7,28]. This conclusion is supported by many studies that have employed a wide range of species including reptiles, birds and mammals. Based on this work it has been demonstrated that: a) behavioral effects of testosterone can be mimicked by estrogens such as estradiol, b) aromatizable androgens such as testosterone or androstenedione fully activate male sexual behavior whereas non-aromatizable androgens such as 5 α -dihydrotestosterone have little or no behavioral effects c) aromatase inhibitors block the behavioral effects of aromatizable androgens and d) similarly injections of anti-estrogens that block the access of estrogens to their specific receptors inhibit testosterone-induced sexual behavior (data reviewed in [5,7,29]).

The distribution of aromatase in the brain has been intensively studied in a range of vertebrate species by quantification of the enzymatic activity in (micro-) dissected brain areas but also by analyzing the neuroanatomical distribution of the enzymatic protein or of the corresponding messenger RNA by immunohistochemistry or *in situ* hybridization respectively. In birds and mammals, aromatase expression can be detected in a variety of hypothalamic and limbic areas including the medial preoptic area, the ventromedial nucleus of the hypothalamus and the amygdala (e.g., [30,34,35,36,39,43,44,45]).

In quail, immunocytochemical and *in situ* hybridization studies have revealed that the preoptic aromatase is specifically expressed in the sexually dimorphic (larger in males than in females) medial preoptic nucleus (POM) [4,12,22,46], a structure where testosterone action is necessary and sufficient for the activation of male sexual behavior [32].

Interestingly, aromatase activity in the hypothalamic-preoptic area of quail is higher in sexually mature, gonadally intact males as compared to sexually mature females [42] and this is the case in rats as well [35]. However, in quail at least, this sex difference in aromatase activity does not appear to be sufficient to explain alone the differential responsiveness to testosterone because this enzymatic difference is not consistently present in males and females treated with the same dose of testosterone [5,13] and, most importantly, because treatment of ovariectomized females with an estrogen, which should bypass the putative enzymatic limiting step related to aromatase, is not sufficient to activate male-typical copulatory behavior while the same treatment is effective in males [40].

After gonadectomy, aromatase activity declines in males and females to baseline levels. While testosterone-treatment fully restores the enzyme activity in males, the same treatment of females was shown to induce a smaller increase in enzymatic activity [42] so that, as on average, aromatase is less active in the brain of ovariectomized testosterone-treated females than in the brain of castrated testosterone-treated males [5]. However, this result is associated with some variability: in some cases the induced enzymatic activity was the same in females as in males [13], in other cases it was significantly lower [5,42]. Given that male-typical copulatory behavior is ALWAYS activated in males but NEVER in females, the differential activation of brain aromatase activity cannot be taken as being solely responsible

for the behavioral sex difference in the ability of testosterone to activate male-typical sexual behavior [15].

We demonstrated recently that the sex difference in aromatase activity which is observed in the preoptic area-hypothalamus of sexually mature gonadally intact males and females is not associated with any major difference in the density of the aromatase mRNA in the corresponding brain areas [46]. A reverse sex difference in mRNA density (females > males) was even detected in the medial part of the bed nucleus of the stria terminalis (BSTM) [46] where males have a higher aromatase activity than females [19]. The sex difference in enzymatic activity thus presumably results from sexually differentiated post-transcriptional events.

In the present study, we investigated by *in situ* hybridization histochemistry methods aromatase transcription as reflected by the density of the corresponding mRNA in gonadectomized male and female quail that were or were not exposed steroids typical of their sex: testosterone in males and ovarian secretions including estradiol in females. The studies described here have three distinct but complementary goals: a) to investigate whether there is a sex difference in the density of aromatase transcripts in gonadectomized subjects that are not exposed to any significant concentrations of sex steroids (difference in basal expression), b) to establish whether the induction of aromatase transcription (presumably causing the increase in mRNA concentration) observed in males that are exposed to testosterone is also taking place (and if so has the same magnitude) in females when exposed to ovarian secretion, and finally c) to determine in subjects exposed to sex steroids whether there is any localized sex difference in aromatase expression that could potentially contribute to explain the dramatically differentiated testosterone effects on the activation of male-typical sexual behaviors.

2. Materials and Methods

2.1. Animals

Male and female Japanese quail (*Coturnix japonica*) were purchased at the age of approximately three weeks from a local breeder in Belgium. Throughout their life in the laboratory, the birds were exposed to a photoperiod simulating stimulatory long days (16 light:8 h dark cycles). Food and water were always available *ad libitum*. Experimental procedures were in agreement with the Belgian laws on "Protection and Welfare of Animals" and on the "Protection of experimental animals" and the International Guiding Principles for Biomedical Research involving Animals published by the Council for International Organizations of Medical Sciences. The protocols were approved by the Ethics Committee for the Use of Animals at the University of Liège.

At the age of approximately four weeks (i.e., just before they reached sexual maturity), 14 males were castrated (CX) and 9 females were ovariectomized (Ovex; see [41] for detail of surgical procedures). Two weeks later, males received two subcutaneous 20 mm-long Silastic™ capsules (Silclear™Tubing, Degania Silicone Ltd, DeganiaBet, 1513, Israel; 1.57 mm i.d.; 2.41 mm o.d.) filled with crystalline testosterone (CX+T; N=7; Sigma, St Louis, MO) or left empty as control (CX; N=7). These testosterone implants restore physiological concentrations typical of sexually mature males and this procedure activates in males the full range of species-typical sexual behaviors [14]. Before implantation, all Silastic™ capsules were incubated overnight in isotonic saline solution to initiate steroid diffusion and avoid an initial surge in steroid release.

Sexual behavior of all males was quantified in the presence of a female during three 5 min tests carried out 2–4 days before sacrifice that took place three weeks after implantation of

the Silastic capsules. In these tests the frequency of neck grabs, mount attempts and cloacal contact movements was recorded (see [26,41]) for procedure and a description of the behaviors that were measured). The area of the cloacal gland, an androgen-sensitive structure [38], was measured in all birds with calipers to the nearest millimeter (area = largest length $\times$ largest width). Their body weight was recorded to the nearest gram. At the time of sacrifice completeness of gonadectomy was checked in all birds. Three females had regrown fully functional ovaries and laid eggs.

2.2. Brain histology

Brains were dissected out of the skull immediately after decapitation, frozen on dry ice and stored at -80°C until used. Frozen brains were cut on a cryostat into 30 μm coronal sections (from the level of the tractus septopallio-mesencephalicus to the third nerve). The plane of the sections was adjusted to match as closely as possible the plane of the quail brain atlas [17]. Sections were mounted onto Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) in five different series, so that one series of slides contained a section every 150 μm . One series of sections was Nissl-stained with thionin blue to provide anatomical landmarks for the interpretation of the *in situ* hybridization signals. *In situ* hybridization for aromatase (ARO) mRNA was carried out on an adjacent series of sections.

2.4. In situ hybridization histochemistry

The expression of ARO in the brain sections was detected with antisense RNA probes labeled with ^{35}S -CTP as described in our previous studies [46,47]. Briefly, cloning of the partial Japanese quail ARO cDNA (GenBank no. AF 533667) was performed in our laboratory and has been described previously [46]. PCR was used to amplify a fragment of the ARO gene from Japanese quail based on sequence information available for quail [6,25]. The synthesis of first-strand cDNA was done with SUPERScript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) and oligo (dT)-primer. The resulting RNA-DNA hybrids were subsequently used in PCR's to generate pieces of the appropriate ARO gene. The forward primer was 5'-GAGATTTCTCTGGATGGGAGT-3' and the reverse primer was 5'-GAGCTTGCCAAGCATCAAAGTA-3'. Amplified fragments were purified, blunt-ended and cloned into the Sma I site of the plasmid vector pGEM7ZF (Promega, Mannheim, Germany). Resultant clones were sequenced to verify the authenticity and fidelity of the amplification. The cloned ARO sequence is 489 bp in length and matches nucleotides 260–748 of the previously cloned ARO sequence of Japanese quail (GenBank no. AF 533667).

Antisense RNA probes were then labeled with ^{35}S -CTP (1250 Ci/mmol; Perkin Elmer, Rodgau, Germany) using the Riboprobe System (Promega). Our *in situ* hybridization procedure followed a previously published protocol [48] with modifications as previously described in detail Gahr and Metzdorf [23]. For signal detection, sections were exposed in x-ray cassettes to autoradiographic film (Kodak Biomax MR, Rochester, NY, USA) for 44 days at room temperature. Sections from different experimental groups were randomly distributed on the different films and cassettes. Films were then developed for 3 min with Kodak D-19 developer (Sigma P-5670) and fixed in Kodak fixer (Sigma P 6557).

2.5. Data analysis

Images from autoradiograms were trans-illuminated with a ChromaPro 45 light source and acquired with a CCD digital camera connected to a Macintosh computer running the image analysis software Image J 1.36b (NIH, USA; see <http://rsb.info.nih.gov/ij/>). Before acquisition the system was calibrated by using a calibrated optical density step tablet (Kodak photographic step tablet no. 3) and a calibration curve was fitted with the Rodbard function of Image J [$y = d + (a - d) / (1 + (x/c)^b)$]. This calibration was applied to all images. Regions of interest in each section (i.e. showing denser signal density than surrounding

areas) were delineated by one of the authors (C.V.) on screen with the computer mouse and their average optical density (OD) and area were calculated by built-in functions of the software. Autoradiograms were analyzed in the order in which sections appeared on each film so that the identity of the bird under study and its experimental group was unknown to the observer.

The volume of brain regions of interest was calculated by summing the area measurements on both sides of the brain and multiplying them by the interval between sections (150 μm). All volumes reported in this paper thus represent the total volume of the structure of interest on both sides of the brain. Background optical density of the film was measured in a rectangular area (2 mm^2) in the same image immediately ventral to the brain section of interest. Final OD measurements were obtained by subtracting the film background OD value from the OD value of the region of interest. Before analysis, sections of all birds were realigned using the commissura anterior (CA) as a landmark. The neuroanatomical nomenclature employed in this paper is based on the quail and chicken brain atlases [17,27] but includes the most recent modifications introduced by the Avian Nomenclature Forum [33].

2.6. Statistical analysis

Statistical analyses were carried out using Graphpad Prism 5 (Graphpad Software, Inc.). Data are presented as means $\pm$ SEM. This experiment was originally planned to compare three experimental groups, CX males, CX+T males and Ovex females. It turned out, however, that some of the Ovex females restored fully functional ovaries (see first section of results). These birds were therefore included in a fourth group of females considered as sham-operated gonadally intact females (Sham-operated group).

Repeated-measures two-way Analyses of Variance (ANOVAs) were first used to analyze the ARO expression in the brain of the four different groups, with the four groups as the independent factor and three brain regions as repeated factor. Subsequent analyses were then performed to identify specific differences related to the sex of the birds or their endocrine condition. They were carried out by additional one-way ANOVAs and corresponding Newman-Keuls *post hoc* tests. More detailed analyses of the ARO mRNA signal in the three regions of interest were also performed along the rostro-caudal axis. These analyses used separate t-tests to compare selected groups at each rostro-caudal level because sample sizes varied at the most rostral and most caudal ends of the nuclei (expression of the mRNA did not extend through the same number of sections in all subjects), which prevented the use of Repeated Measures ANOVA. These tests therefore only have a descriptive value since they are affected by an inflated type I risk associated with multiple testing (see [46] for additional discussion). Behavioral and morphological differences between pairs of groups were analyzed with t-tests. Differences were considered significant for $P < 0.05$.

3. Results

3.1. Morphological measurements

Gonadectomized males and females had similar body weights (CX: 266.3 ± 4.1 g; Ovex: 292.6 ± 16.7 g; $t_{11} = 1.65$, $P = 0.128$) and androgen-dependent cloacal glands (CX: 62.3 ± 5.7 g; Ovex: 57.6 ± 4.3 g; $t_{11} = 0.63$, $P = 0.543$). Further, both groups of males did not differ in body weight (CX+T: 260.4 ± 10.8 g; CX: 266.3 ± 4.1 g; $t_{12} = 0.51$, $P = 0.619$). However, as expected from previous studies [14,40], the cloacal gland was much larger in CX+T males than in CX males (CX+T: 342.7 ± 17.2 mm^2 ; CX: 62.3 ± 5.7 mm^2 ; $t_{12} = 15.5$, $P = 0.0001$). All CX+T males used for the study were sexually active and 6 out of 7 males showed the full sequence of copulatory behavior during the 5-min behavioral tests with a sexually

receptive female. One male showed neck grabs and mount attempts but failed to copulate with the female. This male was discovered to be blind on one side, which could have affected its behavior. None of the CX males showed the complete sequence of male copulatory behavior during the test sessions. Similar to the males, both groups of females did not differ in body weight (Sham-operated: 276.2 ± 4.8 g; Ovex: 292.6 ± 16.7 g; $t_7 = 0.66$, $P = 0.528$) but Sham-operated females had significantly larger cloacal glands than ovariectomized females (Sham-operated: 143.8 ± 10.1 mm²; Ovex: 57.6 ± 4.3 mm²; $t_7 = 9.41$, $P = 0.0001$). Furthermore, cloacal diameter of Sham-operated females was significantly larger than that of ovariectomized females (Sham-operated: 10.4 ± 0.3 mm; Ovex: 6.0 ± 0.4 mm; $t_7 = 7.47$, $P = 0.0001$) indicating that they had been exposed to high concentrations of circulating estrogens [20]. It must also be noted that at autopsy, all Sham-operated females had large numbers of yolky follicles in their ovary with the largest of these follicles nearing ovulation (diameter larger than 10 mm) and these females even laid eggs before the end of the experiment. It can therefore be considered that these subjects were exposed to endocrine condition very similar, if not identical, to those of gonadally intact sexually mature females.

3.2. Distribution of ARO mRNA

The neuroanatomical distribution of ARO mRNA in male and female Japanese quail has been described in detail previously [4,46] and the present results were in full agreement with these previous data. The present analysis focused on the three clusters (POM, BSTM, see Fig. 1 and medio-basal hypothalamus, MBH, see Fig. 2) that show the densest ARO mRNA expression. They also represent the only three brain areas in which dense populations of aromatase-immunoreactive cells are detected by immunohistochemistry in the quail brain ([12,16]

3.3. Quantification of the ARO hybridization signal

Average optical density of the ARO mRNA hybridization signal—The analysis by two-way ANOVA (4 groups as independent factor and 3 brain regions as repeated factor) of the average optical density of the ARO hybridization signal in each nucleus revealed an overall effect of groups ($F_{3,19} = 19.78$, $P = 0.0001$), a significant effect of the brain region ($F_{2,38} = 37.16$, $P = 0.0001$) and a significant interaction between these two factors ($F_{2,38} = 6.76$, $P = 0.0001$; Fig. 3A).

To analyze the origins of the group effect and its interaction with the brain regions, a one-way ANOVA followed by Newman-Keuls *post hoc* tests was carried out for each nucleus separately. Each of these three ANOVAs revealed the presence of significant differences between groups (POM: $F_{3,19} = 19.36$, $P < 0.0001$; BSTM: $F_{3,19} = 16.32$, $P < 0.0001$; MBH: $F_{3,19} = 15.00$, $P < 0.0001$). *Post hoc* comparisons showed, however, that the same differences between groups were not present in the three nuclei. In POM and MBH, ARO expression was denser in CX+T than in CX males and in Sham-operated females than in Ovex females. The signal was similar in CX males and in Ovex females as well as in Sham-operated females and in CX+T males. In contrast, in BSTM, ARO expression was denser in CX+T than in CX males, in Sham-operated than in Ovex females but also in Sham-operated females than in CX+T males. The signal was also similar in CX males and in Ovex females.

Changes in mRNA density along the rostro-caudal axis—In POM, BSTM and MBH, we also analyzed changes in the density of the ARO hybridization signal along the rostro-caudal axis (Fig. 4). Important changes in optical density were observed throughout the extent of this axis in all three nuclei. For comparison of CX+T vs. CX males, analysis by descriptive t-tests (they were not corrected for multiple comparisons and thus have only a descriptive but no predictive value) at specific levels indicated that in POM, CX+T males

had a denser ARO expression in the central and caudal part of the nucleus (CA-2: $t = 3.52$, $df = 12$, $P = 0.004$; CA-1: $t = 10.32$, $df = 12$, $P = 0.0001$; CA: $t = 8.88$, $df = 12$, $P = 0.0001$) but not at the most rostral levels ($P > 0.05$, Fig. 4A). In BSTM, CX+T males had a denser ARO expression throughout most of the nucleus (CA-1: $t = 2.66$, $df = 9$, $P = 0.026$; CA: $t = 5.99$, $df = 12$, $P = 0.0001$; CA+1: $t = 5.60$, $df = 12$, $P = 0.0001$, Fig. 4B). Similarly, throughout most of MBH CX+T males had a denser ARO expression than CX males (CA+7, CA+9 to CA+18, $P < 0.05$, Fig. 4C).

Similar comparisons by descriptive t-tests of Sham-operated vs. Ovex females revealed a denser ARO hybridization signal in Sham-operated females in POM at the central and caudal level (CA-2: $t = 3.48$, $df = 7$, $P = 0.010$; CA-1: $t = 4.54$, $df = 7$, $P = 0.003$; CA: $t = 5.06$, $df = 7$, $P = 0.001$; Fig. 4D) and in BSTM throughout most of the nucleus (CA-1: $t = 4.25$, $df = 6$, $P = 0.005$; CA: $t = 4.51$, $df = 7$, $P = 0.003$; CA+1: $t = 7.88$, $df = 7$, $P = 0.0001$; no difference in CA+2 [$p = 0.057$] but power of the test was very low here due to loss of some sections containing the BSTM, $n = 2$ Sham-operated and $n = 4$ Ovex females, Fig. 4E). Similarly, throughout most of MBH Sham-operated females had a denser ARO expression than Ovex females (CA+10 to CA+15, $P < 0.05$, Fig. 4F).

Analyzing by the same method the potential existence of sex differences in optical density along the rostro-caudal axis revealed that Sham-operated females expressed the ARO mRNA more densely in the central part of BSTM than CX+T males (CA-1: $t = 2.51$, $df = 8$, $P = 0.037$). No such differences were found in POM and MBH. In similar comparisons of CX males vs. Ovex females, a single sex difference was found in MBH with females having a denser ARO expression than males in the central part of the nucleus (CA+15: $t = 3.07$, $df = 9$, $P = 0.013$).

Volumes defined by the dense ARO mRNA signal—We also measured at each level through the 3 different regions the area that expressed ARO more densely than the surrounding tissue and integrated these measures to compute the total volume of the brain regions expressing ARO mRNA. The general two-way ANOVA of these volumes across POM, BSTM and MBH revealed an overall effect of groups ($F_{3,19} = 4.14$, $P = 0.020$), a significant effect of brain regions ($F_{2,38} = 21.24$, $P = 0.0001$) but no significant interaction ($F_{2,38} = 1.17$, $P = 0.343$; Fig. 3B).

The three one-Way ANOVA subsequently performed to analyze this effect of groups, only revealed significant group differences in the POM (POM: $F_{3,19} = 4.97$, $P = 0.010$; BSTM: $F_{3,19} = 3.00$, $P = 0.056$; MBH: *post hoc* comparisons, however, failed to $F_{3,19} = 0.90$, $P = 0.458$). Newman-Keuls identify significant differences between groups in POM although statistical tendencies ($P < 0.10$) were detected in both sexes in the comparisons of gonadectomized birds with birds exposed to steroids).

We finally analyzed along the rostro-caudal axis the change of surface in the three nuclei and detected important variations between the two male groups (Fig. 5).

In CX+T males the surface of POM was significantly larger in the caudal part of the nucleus at the level of the CA compared to CX males (CA: $t = 3.07$, $df = 12$, $P = 0.010$, Fig. 5A). The slightly larger volume of BSTM in CX+T males compared to CX males (not significant in the ANOVA; $P = 0.056$) derived from a surface difference in the central part of the nucleus (CA-1: $t = 2.28$, $df = 9$, $P = 0.049$; CA: $t = 3.09$, $df = 12$, $P = 0.009$, not significant in CA+1 ($P = 0.067$) and CA+2 ($P = 0.09$) due to lower sample size because the nucleus did not display the same rostro-caudal extension in all subjects, Fig. 5B). In MBH, CX males had a larger surface at the caudal level of the nucleus than CX+T males (CA+17: $t = 2.29$, $df = 9$, $P =$

0.048, Fig. 5C). No significant differences were found between the two female groups (data not shown).

Analysis of sex differences along the rostro-caudal axis revealed that CX+T males had a smaller mRNA positive surface than Sham-operated females in the rostral and caudal part of MBH (CA+7: $t=4.30$, $df=8$, $P=0.003$; CA+16: $t=5.35$, $df=6$, $P=0.002$). No differences were found in the other nuclei nor between CX males and Ovex females for all three nuclei considered (data not shown).

Discussion

One fundamental observation of this study is that it confirmed that the anatomical distribution of the aromatase mRNA previously identified by *in situ* hybridization in gonadally intact adult birds [4,46] is also observed in gonadectomized quail and gonadectomized males and females exposed to steroids typical of their sex. There are only three discrete groups of cells that express in a dense manner the aromatase mRNA. They correspond to the medial preoptic nucleus (POM), to the medial part of the bed nucleus of the stria terminalis (BSTM) and to an elongated cell cluster extending through the entire medio-basal hypothalamus (MBH) that does not correspond exactly to any nucleus as defined in Nissl-stained sections but overlaps with the ventro-medial nucleus of the hypothalamus. This distribution additionally matches precisely the distribution of the corresponding protein as identified by immunohistochemistry [12,16,22]. All available information thus converges to indicate that, in the quail brain, aromatase is expressed in a discrete manner in three specific cell groups and accordingly a high aromatase activity is detected by *in vitro* assays in these nuclei dissected by the Palkovits punch technique [31] while little or no enzymatic activity is observed in other parts of the brain [13,43].

Induction of aromatase by sex steroids in males

The present study also confirmed the extensive effects of testosterone on this mRNA expression. In all brain regions investigated (POM, BSTM and MBH), treatment of CX males with testosterone increased the density of ARO mRNA expression and in two of these brain regions (POM and BSTM) the volume covered by a dense ARO mRNA expression was additionally increased. This result was expected based on previous observations indicating that treatment of castrated male quail with testosterone increases aromatase activity [11,13,42] as well as the number of aromatase-immunoreactive cells [16,21]. One previous *in situ* hybridization study had also reported that the ARO mRNA expression is increased by testosterone in the POM and BSTM [4] but this study used a non-radioactive *in situ* hybridization technique (digoxigenine label) and therefore did not permit an accurate quantification of the hybridization signal. In contrast, the present radioactive technique permits an accurate quantification of hybridization signals. In the POM, for example, the nucleus on which the largest number of studies has focused previously [8,32], the ARO mRNA optical density is about 2.5 times higher after testosterone treatment (increase from 0.107 to 0.252). In addition the volume covered by this dense signal is approximately 1.4 times bigger (increase from about 0.5 to 0.7 mm³). Integrating these values would then suggest that the total concentration of ARO mRNA was increased approximately 3.5 times (2.5×1.4) following exposure to testosterone. This induction, as measured by *in situ* hybridization, corresponds very closely to increases in ARO mRNA concentrations that had been previously reported ($\times 3.72$) based on semi-quantitative polymerase chain reaction (PCR) [25]. The PCR studies were performed on blocks of tissue (whole hypothalamus) that incorporated both the POM and BSTM and the similarity of results between the two studies thus suggests that the degree on induction by testosterone is similar in these two nuclei. This is clearly supported by the *in situ* hybridization data presented here. In BSTM, ARO mRNA density increases to 200% of CX values (from 0.071 to 0.142) and volume is about twice

larger (increase from approximately 0.3 to 0.6 mm³) after testosterone. Integrated optical density (See [37,46]) is thus 4 times larger in BSTM after testosterone. This increase in ARO mRNA concentration following testosterone treatment is similar to the increase in aromatase activity [11,42] observed in these conditions but has nevertheless a slightly lower amplitude (see [10] for direct comparisons) suggesting the existence of post-translational controls of aromatase activity by testosterone. We shall come back to this notion later.

... and in females

The group of ovariectomized females in this experiment serendipitously included a few birds in which gonadectomy was incomplete who had thus regrown a fully functional ovary and actually laid eggs. Quite interestingly, ovarian steroids produced by these females also increased aromatase expression, as measured by the density of ARO mRNA. This could be expected based on the finding that aromatase activity is decreased by ovariectomy [42] and that the induction observed after testosterone treatment is largely mediated by estrogens derived from testosterone aromatization [1]. Surprisingly, however, the induction of ARO mRNA by the regenerated ovary displayed almost the same magnitude (with one exception) as the induction by testosterone in CX males. This is unexpected because in sexually mature Sham-operated birds exposed to the endogenous secretions of their gonads aromatase activity is consistently higher in males than in females [11,42]. Three explanations can be offered to explain this lack of difference in ARO mRNA induction by testosterone in CX males and by the regrown ovary in Ovex females. It is first possible that the testosterone treatment did not induce the maximal expression of the ARO mRNA but this appears unlikely since this treatment actually induces a maximal increase in aromatase activity which brings the enzymatic activity to the level observed in gonadally intact sexually mature males [11]. It is useful to note, however, that the POM volume in CX+T birds calculated here was slightly lower than in gonadally intact birds observed in our previous study [46]. Alternatively, one might speculate that the regenerated ovary produced more estrogen than a "normal" ovary and therefore activated aromatase transcription to a larger extent than normally seen in females. This also appears unlikely since in these females the cloacal diameter, an estrogen sensitive index, was smaller than values normally observed in sexually mature females (10.4 mm here compared to 15–17 mm in Delville and Balthazart, [20]). In agreement with this interpretation, however, the ARO mRNA density observed here in Ovex birds that had regrown their ovary was slightly higher than in the intact females studied previously [46] but this small difference does not seem sufficient to explain the lack of sex difference observed here. The final and most likely explanation, therefore, is to postulate once again that the amount of ARO mRNA in a given brain region is not necessarily an accurate representation of the aromatase enzymatic activity that will be displayed by the area (again see final section of this discussion for additional considerations on this discrepancy).

Sex differences

Two sex differences in ARO mRNA expression only were observed in the present study. There was a denser ARO mRNA expression in Sham-operated females than CX+T males in the central part of BSTM in the section just rostral to the anterior commissure (CA-1) and Ovex females had a greater density of ARO mRNA in the central MBH than CX males. Interestingly, our previous *in situ* hybridization study performed on gonadally intact sexually mature males and females had similarly detected very few sex differences in ARO mRNA distribution but a similar difference had been observed in the BSTM with females having a denser expression of ARO mRNA over a broader area than males [46]. This difference in ARO mRNA expression thus seems to be reliable but has surprisingly a direction that is opposite to what would have been expected based on aromatase activity assays. All assays performed previously always pointed, when a sex difference was observed, to a higher enzymatic activity in males than in females. This was the case when

the enzymatic activity was measured in whole preoptic areas or on anterior hypothalamus [42], as well as in punched nuclei that had been dissected by the Palkovits punch technique (Aromatase activity higher in males than in females in POM [13,19] and BSTM [19]. These findings therefore indicate again that there is a discrepancy between local concentration of ARO mRNA and aromatase activity.

One striking feature of these results is that overall there was no major of sex difference in aromatase mRNA density despite the fact that sex differences in aromatase activity have been reported in gonadally intact birds and in some instances in gonadectomized birds treated with exogenous testosterone (see introduction). Immunohistochemical studies quantifying the numbers of aromatase-immunoreactive cells in POM suggested that these differences were quite localized. Foidart and collaborators [21] demonstrated localized differences in the number of aromatase-immunoreactive cells (males>females) in limited sub-regions of the POM ventral to or just rostral to the anterior commissure. These differences, however, disappeared when birds of both sexes were gonadectomized and treated with a same dose of testosterone. Similar analyses were later repeated in much more detail based on the three-dimensional organization of the POM (Foidart et al. [21] only analyzed differences along the rostro-caudal axis) and essentially reached the same conclusion (limited differences in gonadally intact birds, almost no differences in birds gonadectomized and treated or not with testosterone; [16]).

Our detailed analysis of aromatase mRNA expression (density and surface covered by dense signal) along the rostro-caudal axis of the POM should have been sufficient to detect even a localized sex difference if it was present since it is based on the same approach as the immunohistochemical analysis of Foidart *et al.* [21] that was repeated and extended in Balthazart *et al.* [16]. It is therefore very probable that the expression of aromatase mRNA is very similar if not absolutely identical in gonadectomized males and females and that any sex difference in aromatase activity that was previously reported relates to post-transcriptional/post-translational controls of the enzyme or to minor differences in dissections that could have affected one sex more than the other.

Conclusions

In summary, we found that there is essentially no sex difference in the density of aromatase transcripts between gonadectomized subjects that are not exposed to significant concentrations of sex steroids (no difference in basal expression) and, secondly, that the induction of aromatase transcription (presumably causing the increase in mRNA concentration) observed in males exposed to testosterone is also taking place (essentially to the same extent, except in BSTM) in females when exposed to ovarian secretions, most notably 17 β -estradiol. Previous studies in male quail showed that the stimulating effects of testosterone on aromatase activity are mediated by an increase in transcription of the corresponding gene itself largely controlled by the estrogens derived from testosterone aromatization. This was demonstrated at the level of the aromatase mRNA concentration, at the level of the number of aromatase-immunoreactive cells and at the level of the enzyme activity (reviewed in [1]). At the cellular level, estradiol is thus the active metabolite of testosterone for aromatase induction in males. It is likely that the same mechanism is true in females and that the increase in aromatase activity and in the number of aromatase-immunoreactive cells previously observed in ovariectomized females treated with testosterone [16,42]; no study was done on the corresponding mRNA concentrations) is also mediated by estrogenic metabolites of the steroid. This would explain why an active ovary can increase aromatase mRNA concentration in females similar to what a treatment with testosterone does in males.

Multiple discrepancies were however detected between the local amounts of ARO mRNA measured in specific brain regions and the aromatase enzymatic activities that had been previously measured in these areas. Namely, a) the induction of ARO mRNA by testosterone in males had a smaller amplitude than the induction of aromatase activity, b) similar amounts of ARO mRNA were present in CX+T males and in Sham-operated females whereas aromatase activity is usually higher in males than in females, and c) ARO mRNA expression in BSTM is denser in females than in males whereas a sex difference in the opposite direction is usually observed in measures of aromatase activity. All these observations suggest that additional posttranscriptional mechanisms modulate brain aromatase activity in quail without affecting the concentration of its mRNA and probably of the corresponding protein.

We showed during the last few years that in the presence of phosphorylating conditions (presence of ATP, Mg and Ca) aromatase activity is rapidly (within a few min) and reversibly inhibited and that this effect can be blocked by kinase inhibitors [6]. These data indicate that phosphorylation processes are able to transiently modulate aromatase activity without changing the concentration of the enzymatic protein. It is therefore conceivable that all previously published measures of aromatase activity were affected by these processes that could affect enzymatic activity in a more chronic manner than indicated by the currently available studies that focused on the acute changes in activity [9]. These mechanisms could explain the discrepancies between putative measures of aromatase concentration (density of ARO mRNA) and actual values of enzymatic activity. Since many of the observed discrepancies concerned sex differences in ARO mRNA and enzymatic activity, special attention should be paid to the possibility that phosphorylation processes differentially affect the enzyme activity in males and in females.

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Fig.1.

Autoradiograms of coronal sections through the quail brain illustrating the distribution of the ARO mRNA in the preoptic area and adjacent telencephalic regions of castrated testosterone-treated males (A), Sham-operated females (B), castrated males (C) and ovariectomized females (D). Abbreviations: CA, commissura anterior; POM, nucleus preopticus medialis; BSTM, bed nucleus of the stria terminalis, medial part.

Fig. 2.

Autoradiograms of coronal sections through the quail brain illustrating the distribution of the ARO mRNA in the the medio-basal hypothalamus of castrated testosterone-treated males (A), Sham-operated females (B), castrated males (C) and ovariectomized females (D). One dense spot of aromatase mRNA is detected at the level of the ventro-medial nucleus of the hypothalamus (VMN). The third ventricle (VIII) is faintly visible between the left and right VMN.

Fig. 3.

Average optical density of the ARO hybridization signal (A) and total volume covered by the signal (B) at the level of the medial preoptic nucleus (POM), bed nucleus of the stria terminalis medial part (BSTM) and medio-basal hypothalamus (MBH) of male and female quail that had been castrated (CX) or ovariectomized (Ovex) while some of these males had been treated with exogenous testosterone (CX+T males) and Ovex females had regrown a fully functional ovary (Sham). Symbols above the bars indicate the results of the *posthoc* tests comparing within a same sex birds exposed or not to sex steroids (males CX vs. CX+T; females Ovex vs. Sham-operated) or (in BSTM) CX+T males with Sham-operated females. * $P < 0.05$, ***= $P < 0.001$.

Fig. 4. Optical density of the hybridization signal reflecting ARO expression along the rostro-caudal axis in the POM (A, D), BSTM (B, E) and MBH (C,F) in both groups of male (A–C) and female (D–F) quail.

Figure 5. Areas showing dense ARO expression along the rostro-caudal axis of the POM (A), BSTM (B) and MBH (C) in both groups of males.