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Gonadal steroids regulate aromatase P450 expression in the rat pituitary

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Summary

Rat aromatase immunohistochemical expression is different in male than in female adult rats. In order to analyze if such differences are related to the presence of gonadal steroids, a study was carried out on pituitaries of adult castrated rats and rats castrated and treated with gonadal steroids, using immunohistochemistry, Western blotting and in situ hybridization for rat aromatase P450. Rat aromatase P450 mRNA was detected in the pituitary of male and female rats. Sex-related variations in the mRNA evidence were observed, the mRNA signal was more abundant in males than in females. Moreover, the male pituitaries showed more immunohistochemical positive cells than females and by Western blotting the enzyme was seen to be more abundant in males than in females. With the three methods assayed, ovariectomy elicited a considerable increase in the reaction to aromatase in females. In male rats, castration reduced the number of reactive cells, although the reaction persisted. Treatment with gonadal steroids after castration modified aromatase expression in the sense that in testosterone-treated castrated males the expression of aromatase was increased while in ovariectomised females treated with oestradiol it decreased. Our results demonstrate the synthesis of aromatase in the pituitary gland and its immunohistochemical expression in the gland of adult rats and suggest that the expression of this enzyme is sex-dependent and that it can be modified by castration and gonadal steroid administration. This in turn suggests that aromatase may be involved in the regulation of adenohypophyseal cytology by gonadal steroids.

Key words: Aromatase – Pituitary – Immunohistochemistry – In situ hybridization – Western blotting – Gonadal steroids

Introduction

The presence of aromatase P450, the enzyme responsible for the aromatization of testosterone to oestradiol, has been demonstrated in different regions of the central nervous system, including the hypothalamus (Shinoda et al., 1989, 1990; Balthazart et al., 1990a,b, 1991a,b; Sanghera et al., 1991; Segovia and Guillamón, 1993; Dellovade et al., 1994; Tsuruo et al., 1994; Yamada et al., 1994; Foidart et al., 1994, 1995).

There are discrepancies concerning the presence of aromatase in the pituitary

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(Abdelgadir et al., 1994; Yamada et al., 1994; Roselli et al., 1998). In rats, studies from our laboratory have demonstrated the immunohistochemical expression of aromatase P450 in the anterior pituitary gland and sex-related differences in this expression due to ageing (Carretero et al., 1999a, 2002). Moreover, in teleosts, Gelinas et al. (1998) reported that physiological regulation of aromatase mRNA expression in the natural environment is mediated by aromatization of androgen to estrogen.

Aromatization plays a critical role in the sex-related regions of the central nervous system and could be very important in the andro/estrogenic regulation of the pituitary gland (Boyar et al., 1973; Marynick et al., 1979; Foidart et al., 1994). However, despite the important role played by the pituitary in the gonadal sphere, synthesis of the enzyme in the anterior pituitary and the possibility that the gonadal steroids might regulate the pituitary expression area issues that remain to be elucidated.

The aim of the present work was to confirm by Western blotting, immunohistochemistry and in situ hybridization whether the pituitary of adult rats expresses aromatase P450 and whether castration and gonadal steroids modify its expression.

Materials and Methods

Animals used

Eighty albino Wistar rats, 40 per sex, weighing ~200 grams were used in this study. The animals were chosen randomly and were kept under standard laboratory conditions: 20±2ºC; 55±5% RH; light/dark cycle of 8:00-20:00 h light daily. Free access to food (maintenance diet, Panlab®) and water was provided. Animals were handled according to the guidelines of the European Communities Council Directive (86/609/EEC) and current Spanish legislation for the use and care of laboratory animals (BOE 67/8509-12, 1998). Rats were divided into four experimental groups: Untreated animals: 10 males and 10 females subjected to the above standard conditions and considered as untreated animals; Sham animals, 10 males and 10 females in which castration was surgically simulated under anaesthesia with 2 mg/kg b.w. of Ketolar and which were studied at 30 days after simulation, serving as controls; Castrated animals, 10 males and 10 females surgically castrated under anaesthesia with 2 mg/kg b.w. of Ketolar. These animals were studied at 30 days after castration; Castrated animals treated with gonadal steroids, 10 males and 10 females that were surgically castrated under anaesthesia with 2 mg/kg b.w. of Ketolar and at 15 days post-surgery were treated respectively with 50 µg of testosterone propionate or with 125 µg of oestradiol valerate over 15 days. The substances were administered subcutaneously at 11.00 a.m. and were studied at 30 days after surgery (15 days after steroid administration had started). From each group and sex, 5 animals were used for Western blotting and the other 5 animals for immunohistochemistry and in situ hybridization studies.

Animal sacrifice and sample processing for immunohistochemistry and in situ hybridization

Animals were sacrificed by decapitation after anaesthesia by Florane® inhalation. The hypophyses were carefully dissected out and immediately submerged in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 24 h. Once the hypophyses had been fixed, they were embedded in paraffin after dehydration in ethanol and clearing in xylene to obtain serial coronal section 5 µm thick. The sections were placed on gelatin-chrome-alum-treated slides for in situ hybridization and for immunohistochemical study. Sections for study were taken from all regions of the gland in the rostro-caudal direction.

Immunohistochemistry

The immunohistochemical study was performed using the Strepto-Avidin-Biotin-Peroxidase method. Endogenous peroxidase was blocked by incubation in 0.24% hydrogen peroxide in methanol. Non-specific reactions of swine anti-rabbit IgG were inhibited by incubation with the purified immunoglobulin fraction of non-specific swine serum (Dako®, dilution 1:30). Samples were then incubated with rabbit anti-aromatase serum (Rb-851) diluted 1:500 at 4ºC for 24 h in a humid chamber; purified (98.75%) immunoglobulin fraction of purified swine anti-IgG serum from rabbit serum (Caltag®, diluted 1:350) labelled with biotin at room temperature for 40 min, and soluble strepto-Avidin-Peroxidase complex (Caltag, diluted 1:400) for 40 min at room temperature. The immunohistochemical reaction was visualized

with 0.25% 3-3'-diaminobenzidine in TRIS buffer (0.05M, pH 7.4) with 0.015 ml of freshly prepared 3% H_2O_2.

As a reaction control, the primary serum was replaced by TBS or normal rabbit serum and a preabsorption test was carried out; in all cases the reaction was completely abolished. The specificity of the antibody has been described previously (Carretero et al., 2002). For the preabsorption test, diluted anti-aromatase serum (1:500) was preabsorbed (24h at 4ºC) with the peptide sequence C-EIIFRHIFNTPFLQC (50 µg peptide/ml antibody solution), which had been used to obtain the rabbit polyclonal anti-aromatase serum.

Generation of anti-aromatase antibodies and Western blotting

Rb-871 antibody against aromatase P450 was obtained in a two-week immunization protocol and bleedings were performed in alternative weeks by Sigma GenoSys®. For immunization, the peptide sequence C-EIIFRHIFNTPFLQC corresponding to residues 489-503 of rat cytochrome P450arom of Hickey's isoform (Hickey et al., 1990) was coupled to KLH/MBS carrier protein.

For Western blotting, after sacrifice of the animals, pituitary glands were dissected from adult rats and immediately frozen. Tissues were later disrupted by homogenization in lysis buffer (137 mM NaCl, 10 mM Tris, pH 7.4, 10% glycerol and 1% Triton x100 containing a cocktail of protease inhibitors). Insoluble material was removed from the lysates by centrifugation at 10,000 rpm for 10 min. Protein concentrations were determined using a standard Bradford assay. 50 µg of total protein from each rat sample was separated by 10% SDS-PAGE. Following electrophoresis, proteins were transferred to nitrocellulose and then blocked for 1 hr with 5% non-fat dry milk in PBS. Nitrocellulose membranes were then incubated overnight with either preimmune serum or anti-aromatase antibodies diluted 1:500. Blots were subjected to 3 x 15 min washes with PBS and then incubated for 1 hr with HRP-labelled secondary antibodies (1:10,000 in PBS). Following extensive washing, blots were revealed by ECL (Amersham®). The average exposure time was 2 mm. Densitometric analysis was performed (ScionImage. NIH, USA) to assess relative levels of aromatase expression.

In situ hybridization

This study was performed using a non-isotopic method involving immunocytochemical detection of biotin using the streptavidin-biotin-peroxidase method. To perform in situ hybridization the biotinylated oligonucleotide 5'BIO-gag gat gac gtg att gac ggc tac ccg gtt aaa aag gga act aac atc att ctg aac atc gga,100% specific to rat aromatase P450 according to GenBank data base (accession number: M33986) was used as probe. Slides were prehybridized in Omnibuffer for 30 minutes at 37ºC; hybridization with the biotinylated-probe (100 pg/ml in Omnibuffer) was carried out over night at 37ºC using a Hybaid termocycler. The reaction was stopped by washes in 1xSSC at 54ºC 20 minutes, 1xSSC at room temperature 20 minutes and 20 minutes in PBS (0.01M, pH 7.4, plus 0.8% NaCl). Biotin was detected using monoclonal anti-biotin antibodies (Roche, 1: 250 in TBS: HCl-Trizma, 0.05M, pH 7.4, plus 0.8% NaCl) over night at 4ºC in a humidity chamber, followed by biotinylated goat anti-mouse (Caltag, 1:250 in TBS). The reaction was amplified using the tyramide amplification kit (Dako) according to the manufacturers instructions. The final reaction was developed in brown with 3,3'-diaminobenzidine (0.025M, Sigma®, in 0.05M Tris-HCl buffer, pH 7.4) to which 0.03% H_2O_2 had been added. The slides were counterstained using Mayer's acid haematoxylin. As controls, omission of the probe, pretreatment with Rnase and in situ hybridization with sense probe were performed (Figs. 4g, 4h and 4i, respectively). No reaction was found in any case.

Quantification of aromatase- or mRNA-aromatase-positive cells

The percentage of aromatase-positive cells for immunohistochemistry or in situ hybridization from each animal was quantified following the double-blind procedure. Briefly, four thousand cells (with intact cell and nuclear profiles) were counted from 20 sections separated from one another by at least 50 µm (200 cells/section) chosen randomly from all parts of the gland, then calculating the percentage of reactive cells. These percentages were analyzed statistically and the differences among the means obtained were contrasted by ANOVA, $p<0.05$ in the F-Scheffé test being considered as significant. Results are expressed as arithmetic mean ± standard errors of the mean.

Results

Immunohistochemistry

Untreated animals. The hypophyses of untreated female rats showed very little reaction to aromatase (Fig. 1a), only 0.84±0.22% of the hypophyseal cells being positive (Fig. 2). By contrast, the male rats contained important populations of reactive cells, 34.40±2.93% of hypophyseal cells being aromatase-immunoreactive. Most cells were round or polygonal, some of them with thin and generally short cytoplasmic prolongations. Overall, the reaction was fairly homogeneous and granular, affecting the whole cytoplasm (Fig. 1b) whereas cells with a weaker reaction were infrequent. The percentage of aromatase-positive cells was significantly lower in the females than that found for the males ($p<0.01$).

Sham animals. No significant differences were found in either sex of animal as regards the number and morphological and staining characteristics of aromatase-immunoreactive cells in the hypophyses of these animals. The percentage of aromatase-reactive cells (Fig. 2) was significantly lower ($p<0.01$) in the pituitaries of female rats (0.78±0.31%) than in males (32.87±3.31%).

Castrated animals. Castration elicited an important change in aromatase immunoreactivity in the hypophyses of both sexes. The reactive cells of castrated males were significantly decreased in number; only 17.63±2.84% of them were immunoreactive ($p<0.01$ with respect to intact males). Moreover, cell morphology was altered, the cells becoming smaller and generally polygonal (Fig. 1d). In ovariectomized females, 21.46±2.63% of hypophyseal cells were aromatase-positive ($p<0.01$ with respect to untreated females), a percentage very similar to that observed in the castrated males (there were no significant differences between either sex following castration, Fig. 2). The cells were generally polygonal and their granular reaction was distributed throughout the cytoplasm (Fig. 1c).

Castrated animals treated with gonadal steroids. After treatment with testosterone, the hypophyses of the castrated males showed large amounts of immunoreactive cells (46.52±4.12%, $p<0.01$ with respect to the castrated males, Fig. 2). This percentage was even higher ($p<0.05$) than that observed in the untreated and sham-operated males and the cells were mainly localized in large clusters of reactive cells in the dorsal zones of the gland, close to the pars intermedia (Fig. 1f). Treatment with estradiol inhibited the effect of ovariectomy on the immunohistochemical expression of aromatase in the hypophyses of female rats, only 0.52±0.43% of the cells being reactive ($p<0.01$ with respect to the ovariectomized females, Fig. 2). These cells were weakly reactive (Fig. 1g).

Western blotting

By western blotting, positive blots close to 50kD were found in all animals analyzed (Fig. 3a). However, very important differences in the intensity of the reaction were found depending of the group studied. As can be seen in figure 3b, the blots of untreated males were significantly more reactive than in females (235±8.23 vs 92±3.22, $p<0.01$), lower values were found in the sham animals although without significant differences in comparison with the untreated animals. However the sex-related differences were similar to those described for untreated animals (209±7.32 vs 70±2.45, $p<0.01$). Castration significantly modified the densitometric values, increasing in females (203±7.11, $p<0.01$ with respect to untreated and sham females) and decreasing in males (83±2.91). Very similar values to those found in untreated and sham animals were observed in the castrated animals after steroid replacement (males: 245±8.53 vs females: 65±2.26, $p<0.01$).

In situ hybridization

In all groups studied, the reaction to *in situ* hybridization of rat aromatase mRNA was mainly granular and was located in the cytoplasm of hypopyseal cells (Fig. 4).

Untreated animals. In untreated females scattered cells were observed (Fig. 4a) and the percentage of positive cells was calculated as 18.15±1.41% of the hypophyseal cells (Fig. 5). In untreated male rats, isolated and grouped positive cells were found (Fig. 4b), and these animals showed a significantly higher percentage ($p<0.01$) of positive cells than the females, calculated as 39.02±2.02% of hypophyseal cells (Fig. 5).

Sham animals. The reaction was very similar to that found in untreated animals and no significant differences in the percentages of mRNA-expressing cells were found (females: 17.4±0.61 vs males: 38.6±1.35, $p<0.01$ among both sexes, Fig. 5).

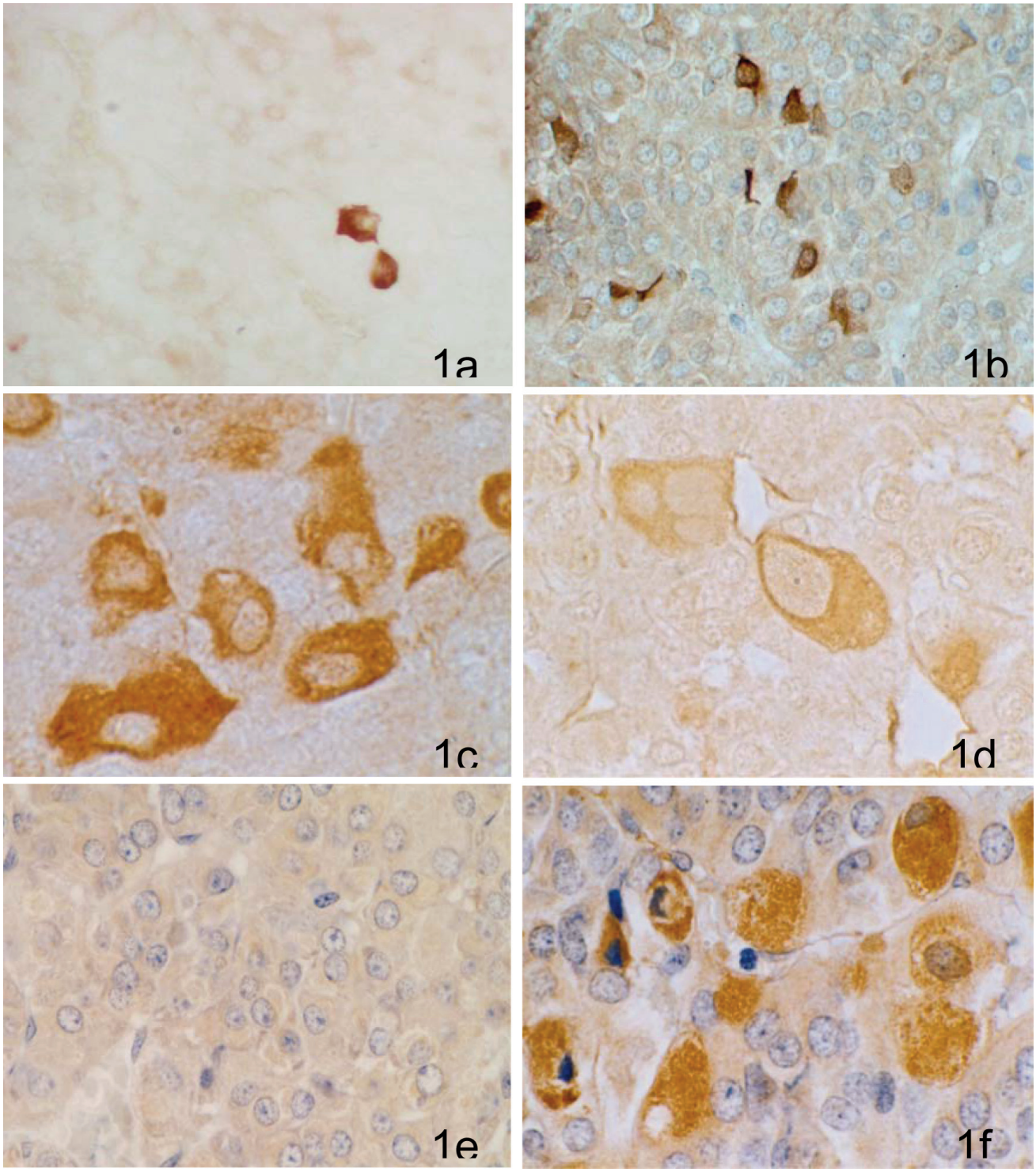

Fig. 1. Morphology and reaction of aromatase immunoreactive cells in the different groups studied. **a:** untreated female, **b:** untreated male, **c:** castrated female, **d:** castrated male, **e:** castrated female treated with estradiol, **f:** castrated male treated with testosterone. (Magnification: a, b: x 250; c, d: x 600; e, f: x 500).

Castrated animals. Ovariectomy induced an important increase in the intensity of the reaction (Fig. 4c) and in the percentage of aromatase mRNA-expressing cells (28.3±4.49, $p<0.01$ with respect to untreated or sham females), while in males (Fig. 4d) castration decreased these percentage (14.6±0.49, $p<0.01$ with respect to untreated or sham males, Fig. 5).

Castrated animals treated with gonadal steroids. Aromatase mRNA-expressing cells appeared similar to those of the untreated and sham animals in the pituitaries of animals castrated and treated with gonadal steroids (Figs. 4e, ef). The percentages of mRNA-expressing-cells were similar to those found in the untreated and sham animals (females: 16.9±0.56, males: 42.56±1.51).

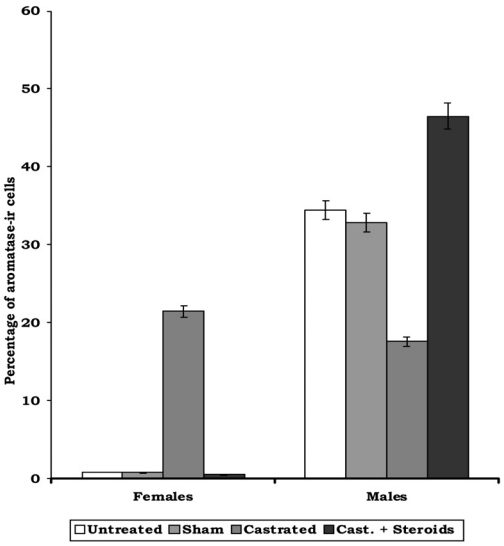

Fig. 2. Plot showing the variations in the percentage of aromatase-immunoreactive cells (aromatase-ir) induced by castration and gonadal steroids.

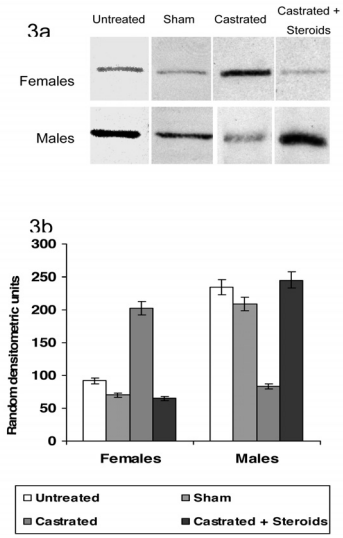

Fig. 3. a: Blots of 52kD obtained by Western blotting in the pituitaries of the different groups analyzed. **b:** Plot showing the differences observed in the intensity of reaction of the blots (random densitometric units) after castration or castration and treatment with gonadal steroids.

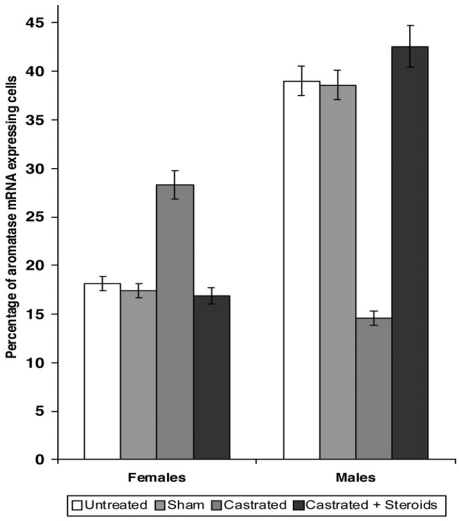

Fig. 5. Plot showing the variations in the percentage of aromatase mRNA-expressing cells after castration or castration and treatment with gonadal steroids.

Discussion

We have previously demonstrated the immunocytochemical expression of aromatase in the rat pituitary gland (Carretero et al., 1999a), variations related to age and sex in this expression (Carretero et al., 1999b, 2003), and the relationship between the aromatase expression and the development of spontaneous prolactinomas in aged rats (Carretero et al., 2002). These studies demonstrated important differences in the immunocytochemical expression of aromatase between male and female adult rats and suggested that gonadal steroids could be involved in the regulation of pituitary aromatase expression.

The present study demonstrates that modifications in the gonadal steroid environment -estradiol or testosterone- as result of castration modify the immunocytochemical expression of the enzyme in the pituitary of adult rats. We also suggest that testosterone would stimulate the expression of the enzyme while estradiol would inhibit it.

Our findings in the pituitary gland are similar to those found upon analyzing the enzymatic activity of hypothalamic aromatase, which is stimulated by testosterone, which also increases the number of hypothalamic cells expressing the enzyme (Steimer and Hutchison, 1981; Roselli et al., 1984, 1987; Hutchison et al., 1991, 1995).

Western blotting revealed that the antibody recognizes a protein of 52kD, which is the molecular weight of the rat aromatase P450, and densitometric analysis of the blots demonstrated that variations of amounts in the enzyme after castration and gonadal steroid replacement follow the same trend as immunocytochemical variations.

To our knowledge this is the first study to demonstrate the presence of aromatase mRNA in the rat pituitary gland using in situ hybridization, in agreement with the results obtained by RT-PCR (Yamada et al., 1994). Moreover, this is the first study to analyze the effects of castration and gonadal steroids on the expression of mRNA aromatase in the anterior pituitary.

Although the percentages of aromatase mRNA-expressing cells were always higher than those obtained by immunocytochemistry, the variations observed after castration or castration and treatment with gonadal steroids were in accordance with those found by immunocytochemistry: Testosterone

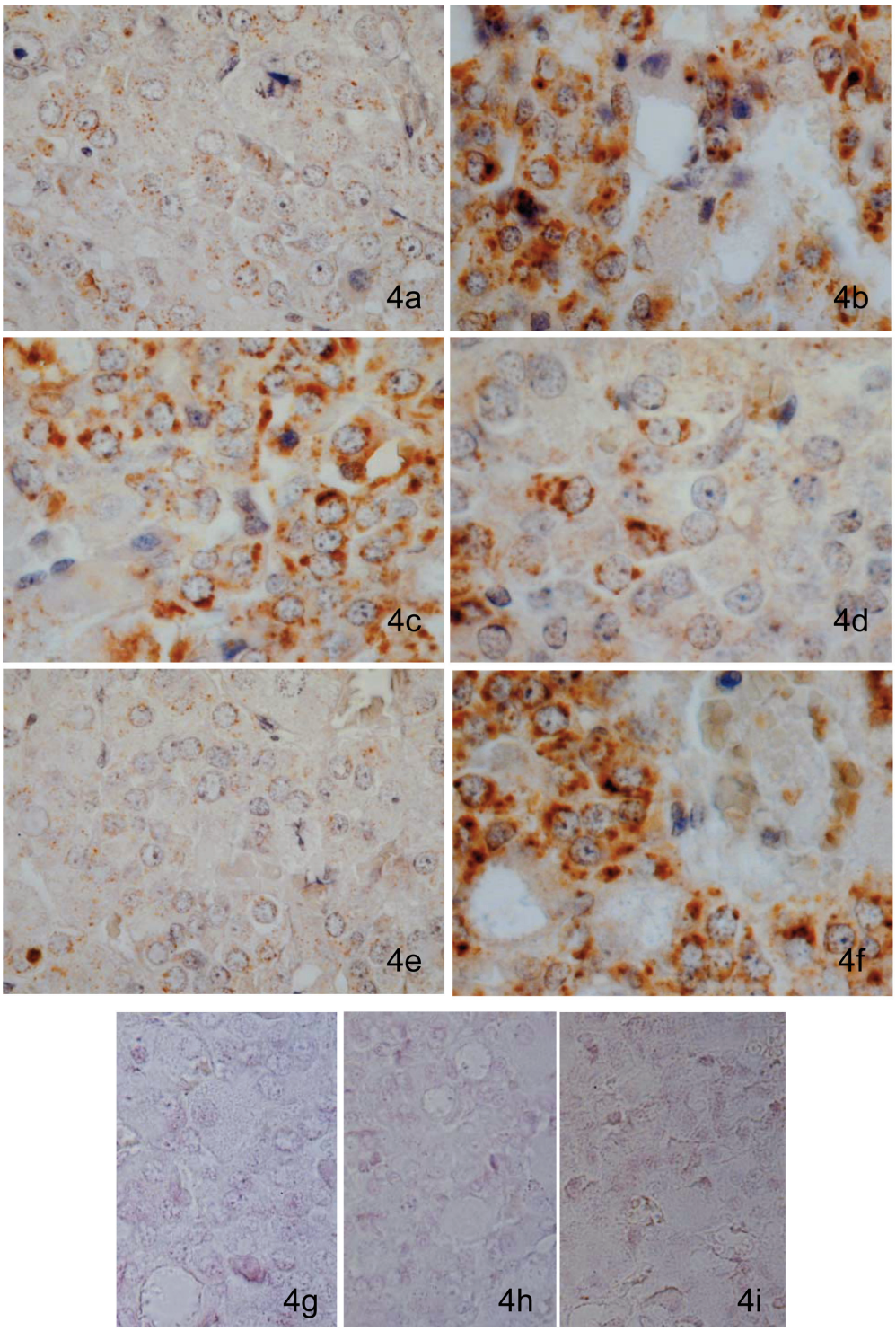

Fig. 4. Morphology and reaction following in situ hybridization for rat aromatase P450 mRNA. **a:** untreated female, **b:** untreated male, **c:** castrated female, **d:** castrated male, **e:** castrated female treated with estradiol, **f:** castrated male treated with testosterone. Controls: **g:** section treated with Rnase prior to developing in situ hybridization, **h:** pituitary in the control following substitution of the probe by hybridization buffer, **i:** section of the pituitary after sense probe in situ hybridization. (Magnification: x 500).

increased the percentage of aromatase mRNA-expressing cells and estradiol decreased it. These results suggest that the effects of gonadal steroids are pre-transcriptional.

The findings of the present study are in accordance with those observed in a previous study (Carretero et al., 1999a), since they demonstrate the presence and synthesis of aromatase in the pituitary of adult rats. In addition, we demonstrate that the presence of this enzyme is sex-dependent, since whereas the pituitaries of the male rats -presumably under the influence of testosterone- had important numbers of aromatase-positive cells, this was not the case in female rats. This suggests stronger enzymatic activity in males than in females, together with a possible sexual dimorphism in hypophyseal aromatase expression.

These differences could be associated with modulation by testosterone, as has been proposed for hypothalamic neurons (Beyer et al., 1994a, 1994b). Moreover, a genetic factor could be involved in aromatase expression because in our study we observed that castration did not lead to the total disappearance of aromatase-immunoreactive cells in the male rat pituitary.

Although a stimulatory effect of oestradiol on brain aromatase activity has been described (Reddy et al., 1973; Schumacher and Balthazar, 1986; Hutchison and Steimer, 1986; Pasmanik et al., 1988; Negri-Cesi et al., 1989), our observations suggest that oestradiol could inhibit the immunohistochemical expression of aromatase in the rat pituitary since the female animals displayed a small amount of aromatase-immunoreactive cells while ovariectomy elicited the onset immunohistochemical expression of the enzyme.

We have previously demonstrated that oestradiol, at doses higher than physiological levels in intact male rats, causes clear effects on hormonal regulation, cell morphology and hypophyseal proliferation (Carretero et al., 1991, 1992, 1995, 1997; Rubio et al., 1992; Sánchez et al., 1993). These effects can now be explained in terms of the presence of aromatase in the male rat pituitary.

In sum, our results demonstrate the presence of the immunohistochemical expression of aromatase in the pituitary of adult male and female rats and suggest that this would be regulated by gonadal steroids.

Acknowledgements

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