

## Postnatal differentiation of the immunohistochemical expression of aromatase P450 in the rat pituitary gland

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**Summary.** At our laboratory, we have recently demonstrated the immunohistochemical expression of aromatase P450 in the pituitary glands of adult rats; this expression was seen to be sex-dependent. In order to determine whether the changes in the expression of the enzyme are related to changes in the gonadal sphere and whether the expression of the enzyme is related to the postnatal differentiation of hypophyseal cytology, in the present work we performed an immunohistochemical study in the rat pituitary gland from birth to old age. The immunohistochemical reaction to aromatase was evident and very generalized at 7 days after birth, with no large differences between the male and female animals. At 14 days the immunohistochemical reaction was decreased in the females, with no changes in the males. At 17 days, aromatase immunoreactivity in the pituitary glands of female rats was very weak whereas the males showed large numbers of reactive cells. These observations were further pronounced at 21 days and 2 months of life. At 24 months, the immunoreactivity found in the pituitary glands of the male rats had almost completely disappeared. Our results show that a postnatal differentiation in the immunohistochemical expression of aromatase occurs; this is tightly linked to sexual activity and is lost in old age. This suggests that hypophyseal aromatase would be related to the mechanisms of action of gonadal steroids on hypophyseal differentiation and secretion.

**Key words:** Rat, Pituitary gland, Postnatal differentiation, Aromatase

### Introduction

The importance of the influence of sex in embryonic development and during the first postnatal stages seems

to be well established and reports have been made of a postnatal sexual differentiation of adenohipophyseal cytology (Childs et al., 1981; Campbell et al., 1987). During the second postnatal week, profound changes occur in the percentages of adenohipophyseal cells that affect gonadotrophs (Siperstein et al., 1954; Denef et al., 1982), and also corticotrophs, somatotrophs and, partially, lactotrophs (Gulyas et al., 1993). Moreover, as from day 15 of life a differentiation between males and females appears in gonadotroph cells (Watanabe, 1986) which is accompanied by an important morphological change (Childs et al., 1981).

Of the two pathways through which androgens are metabolized -reduction and aromatization- the latter depends on the presence of an enzyme -aromatase P450- which belongs to the family of cytochrome P450. At our laboratory we have recently demonstrated the expression of aromatase in the pituitary glands of adult rats; this expression was related to the sex of the animals (Carretero et al., 1997, 1999; Vázquez et al., 1997). However, currently it is not known whether this expression is subject to a postnatal differentiation similar to the rest of the hypophyseal cytology and whether it is related or not to the differentiation of other hypophyseal cells.

Although the pituitary is an essential gland in the regulation of gonadal function and is subject to regulation by gonadal steroids, little effort has been directed to analysing the possible involvement of aromatase in its differentiation, like the situation of the sexual differentiation of hypothalamic nuclei related to physiological functions and the sexual orientation of behaviour (Swaab and Fliers, 1985; LeVay, 1991).

Although the regulatory factors of the gonadal sphere seem to be well established, the finding of aromatase in the pituitary gland suggests that this enzyme would be involved in the action of gonadal steroids on hypophyseal secretion and cytology and also invokes its possible importance in the postnatal sexual differentiation of the pituitary gland. We were thus prompted to conduct a study of the postnatal differentiation of hypophyseal aromatase in the rat with

a view to checking the changes occurring from the first weeks of postnatal life until old age and to correlating these with the results of other authors about the postnatal sexual differentiation of hypophyseal cytology.

## Materials and methods

### Animals

Sixty Sprague-Dawley rats were used. The animals were divided into 6 groups of 10 animals each (5 of each sex) as a function of age: 7, 14, 17, 21 and 60 days and 24 months of age that did not develop spontaneous hypophyseal adenomas. All groups were kept under standard stabling conditions (temperature  $21 \pm 2$  °C, relative humidity  $50 \pm 5\%$ , controlled photoperiod of 14 h light/10h darkness, food and water ad libitum with a balanced rat/mouse maintenance diet (Panlab®). Since in a previous work it was observed that during the oestral cycle of the females the changes in reactivity did not affect the percentage of aromatase-reactive cells and that the aromatase-positive cells could be best seen in the dioestrus phase (Carretero et al., 1999), in this work we studied adult female rats during this latter phase, as determined by vaginal smears.

### Sample collection and processing

Animals were sacrificed between 10.00 and 11.00 h under sodium pentotal anaesthesia. To do so, they were perfused with saline solution (0.08% NaCl in distilled water) and then with 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.4) for 15 min. Following this, the skull was opened and the pituitary gland removed. The organs were postfixed by immersion in the same fixative solution for 24 h at 4 °C and washed in PBS for a further 12 h at 4 °C, dehydrated in ethanol and embedded in paraffin to obtain 5  $\mu\text{m}$  serial sections for immunohistochemical study.

### Immunohistochemistry

To characterize the immunohistochemical expression of aromatase, the PAP method was applied to serial frontal sections separated from one another by 100  $\mu\text{m}$ . After endogenous peroxidase had been blocked by incubation in 0.25% hydrogen peroxide in methanol and the non-specific reactions of the swine anti-rabbit IgG serum had been blocked by incubation with normal swine serum (Dako®, diluted at 1:30), the sections were incubated for 24 h at 4 °C in a humid chamber with anti-aromatase polyclonal antibody (kindly provided by Dr. Hutchison, diluted at 1:500 in TBS) for 30 min at room temperature with swine anti-rabbit IgG (Dako®, diluted at 1:100 in TBS) and for 30 min at room temperature with soluble PAP complex obtained from rabbits (Dako®, diluted at 1:100 in TBS). The reaction was developed with freshly prepared 3-3'-diaminobenzidine (Sigma®, 0.024% in TRIS buffer plus 0.03%  $\text{H}_2\text{O}_2$ ).

TBS (0.05M Trizma base, Sigma®, pH 7.5, with 0.8% NaCl) was used as the washing and dilution buffer. The specificity of the sera employed had been checked in previous works (Beyer et al., 1994; Carretero et al., 1999) and in the present work by substitution of the primary antiserum by assay buffer or normal rabbit or mouse serum; no reactive cells were observed.

Quantification of aromatase-immunoreactive cells. The percentage of aromatase-reactive cells from each animal was quantified by the double-blind method. Briefly, 4000 cells (with intact cell and nuclear membranes) were counted from 20 sections separated from one another by at least 50  $\mu\text{m}$  (200 cells/section) chosen randomly from all the parts of the gland, then the percentage of reactive cells was calculated. These percentages were analyzed statistically and the differences among the means obtained were contrasted by ANOVA,  $p < 0.05$  in the Scheffè test being considered significant.

## Results

Figure 1 summarizes the percentages of immunoreactive cells observed in the different study groups analysed.

The immunohistochemical reaction to aromatase was evident and very generalised at seven days after birth, with no differences between the male and female animals as regards distribution, although in the males the

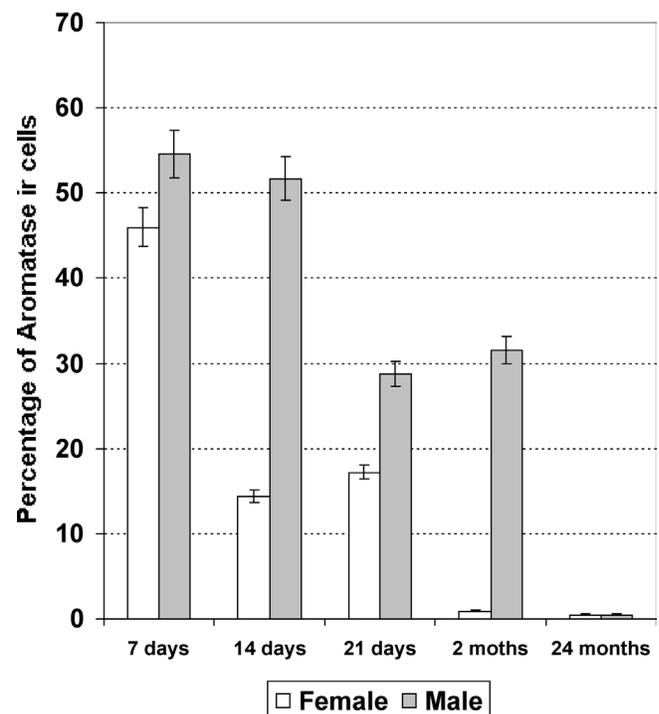


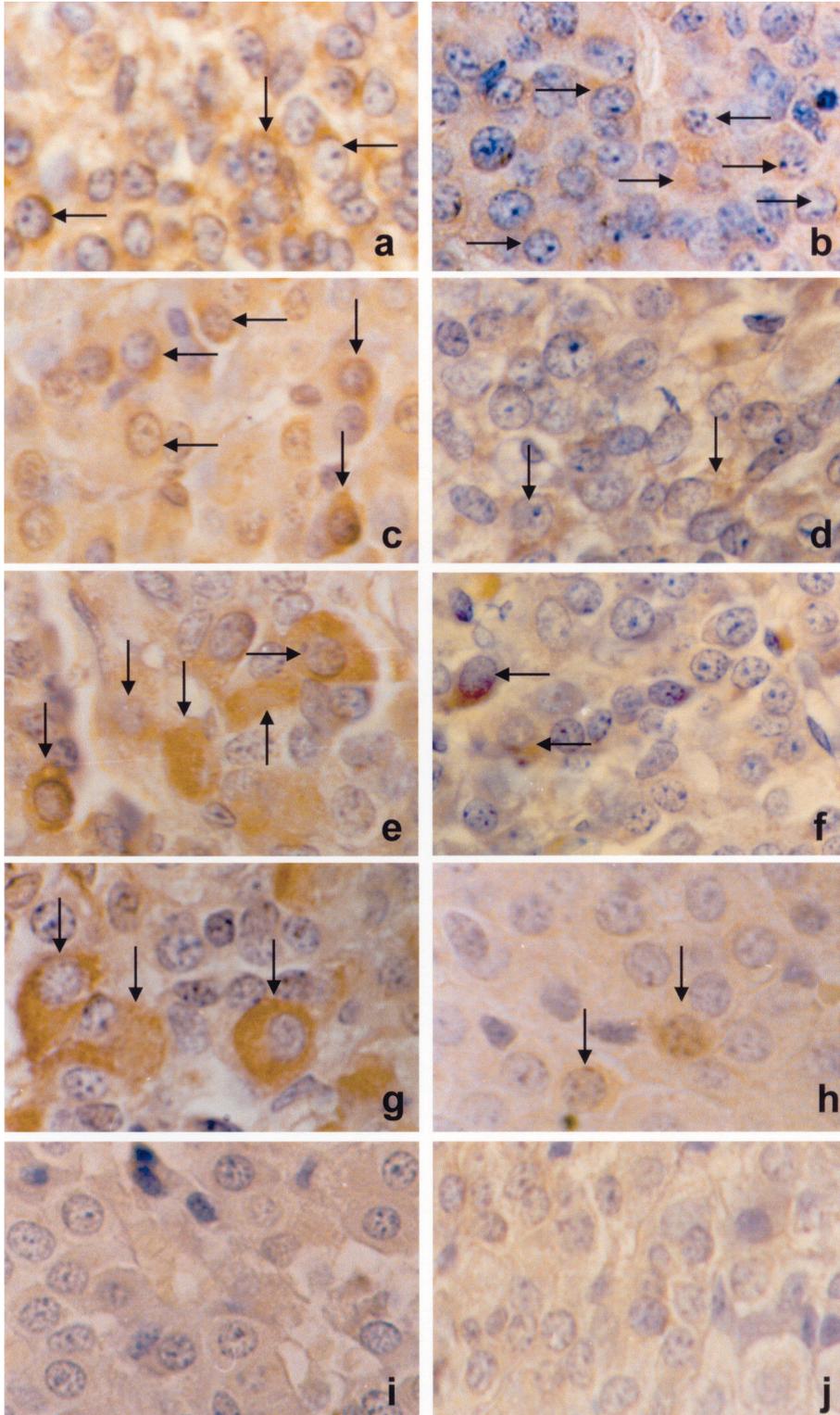
Fig. 1. Plots of percentages of aromatase-immunoreactive cells in the pituitary gland of male and female rats at the different ages studied.

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cells showed a more intense reaction (Fig. 2a, arrows) than in the females (Fig. 2b, arrows).

At 14 and 17 days of age, the most striking

characteristic was a decrease in the aromatase reaction intensity in the hypophyseal cells of the females (Fig. 2d, arrows). In the males, the number of immunoreactive



**Fig. 2.** Micrographs showing aromatase-immunoreactivity (arrows) in the cells of the pituitary gland of female and male rats at the different ages studied. **a.** Male, 7 days. x 600. **b.** Female, 7 days. x 600. **c.** Male, 14 days. x 600. **d.** Female, 14 days. x 600. **e.** Male, 21 days. x 600. **f.** Female, 21 days. x 600. **g.** Male, 2 months. x 600. **h.** Female, 2 months. x 600. **i.** Male, 24 months. x 600. **j.** Female, 24 months. x 600

cells and characteristics of the cytoplasmic reaction were very similar to what was observed at 7 days, the reaction being much more intense than that seen in the females of the same age (Fig. 2c, arrows).

At 21 days of age, the males displayed large numbers of reactive cells, although it was also possible to differentiate some more intensely reactive cells -oval or polygonal- from other less reactive ones, which in general were polygonal (Fig. 2e, arrows). The immunoreactivity of aromatase cells in the pituitary glands of female rats was very weak and almost imperceptible (Fig. 2f, arrows).

In adult rats of 60 days of age, the differences between male and female animals detected at 21 days persisted. The pituitary glands of male rats featured an important population of reactive cells (Fig. 2g, arrows), which were much more frequent in the dorsal region of the gland, although they could be seen throughout it. Most cells were polygonal, some of them with fine, generally short, cytoplasmic prolongations. Adult females displayed very little reaction to aromatase (Fig. 2h, arrows). The scant immunoreactive cells observed were mainly polygonal, with a homogeneous and weakly reactive cytoplasm, and were seen either alone or in loose groups of a few cells.

To analyze the evolution of the expression of aromatase, rats of both sexes of 24 months of age chosen randomly from phenotypically normal animals that did not spontaneously develop pituitary gland adenomas were studied. In both sexes, the pituitary glands of these animals, unlike the situation in males from birth to adulthood, had no aromatase-immunoreactive cells (Fig. 2i,j).

## Discussion

The endocrine function of the mammalian pituitary-gonadal axis begins in utero. This is particularly important for the ontogeny and function of the male reproductive organs. The fetal and postnatal periods of testicular activity have crucial effects on male sexual differentiation (Huhtaniemi, 1995). Postnatally, sex differences in pituitary gonadotroph cells are the most evident and the parallel differentiation during fetal development disappears after birth.

During the second week of postnatal life, a very important increase in the population of gonadotroph cells is observed in both sexes (Denef et al., 1982). However, the increase is more evident in female than in male rats (Siperstein et al., 1954; Denef et al., 1978). These findings are accompanied by differences in LH secretion from the pituitary gland until pubertal phases are reached (Dupon and Schwarz, 1971; Döhler et al., 1977; Watanabe, 1986).

From our observations, an increasing dimorphic expression of aromatase in the pituitary gland that lasts from the second week of life to puberty can be inferred. This begins simultaneously with the sex differentiation of gonadotroph cells and coincides with changes in other

pituitary cells such as somatotrophs or lactotrophs, which are affected morphologically and functionally by gonadal steroids. These considerations suggest that changes in pituitary aromatase levels under hypothalamic and gonadal steroid influx may be related to the start of sex-differentiated pituitary secretory patterns in pubertal and adult rats.

Because the pituitary expression of aromatase after puberty seems to be closely related to the gonadal steroid environment, our findings suggest that such steroids may be involved in the regulation of aromatase expression. Testosterone stimulates aromatase activity in the hypothalamus and increases the numbers of aromatase-immunoreactive neurons (Steimer and Hutchison, 1981; Roselli et al., 1984, 1987; Hutchison et al., 1991a,b, 1995) and the prenatal and postnatal sources of this androgen in the male rat are associated with the neurobehavioural sexual differentiation of the brain but not with the induction of significant alterations in the expression of related behaviours in adulthood (Hermans et al., 1994).

Similar conclusions could be inferred from our results because they demonstrate the expression of the enzyme in the adult pituitary gland with very evident differences between the sexes; aromatase-immunoreactive cells are very numerous in the male pituitary gland but not in that of females.

Although debated, studies carried out in humans have shown that during old age a decrease occurs in serum testosterone levels (Vermeulen et al., 1972; Perke and Doerr, 1973, 1975; Stearns et al., 1974; Rubens et al., 1974; Horton et al., 1975); its binding to transport plasma globulin increases, which means that less testosterone is available (Perke and Doerr, 1973, 1975). Additionally, owing to the peripheral aromatization of the androgen circulating oestradiol levels are increased (Perke and Doerr, 1973, 1975; Horton et al., 1975; Greenblatt et al., 1976; Harman and Tsitouras, 1980).

Since testosterone seems to play an important role in stimulating the immunohistochemical expression of hypophyseal aromatase, whereas estradiol would produce the opposite effect, and since the main defect in old age is located at gonadal and not hypophyseal or hypothalamic level (Vermeulen et al., 1972; Longcope, 1973; Perke and Doerr, 1973, 1975; Rubens et al., 1974; Stearns et al., 1974; Horton et al., 1975; Greenblatt et al., 1976; Kaler and Neaves, 1978; Harman and Tsitouras, 1980; Harman et al., 1982), this would serve as a basis for assuming that the loss of reactivity in aged male rats would be due to a drop in serum testosterone levels and a possible increase in estradiol levels.

Our results reflect a dimorphic sexual differentiation of hypophyseal aromatase expression during the postnatal phase of development that is intimately linked to sexual activity and that is lost in aged rats. This suggests that hypophyseal aromatase may be closely related to the mechanisms of action of gonadal steroids on hypophyseal secretion.

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