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In vivo studies on cytodifferentiation of pituitary aromatase-immunoreactive cells

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Summary

We have recently described the presence of aromatase P450 in the adult rat hypophysis. This enzyme is responsible for the aromatization of testosterone into estradiol and has been implicated in prenatal cerebral differentiation and evolution. However, the ontogeny of hypophyseal aromatase is completely unknown and no information is available as to whether its appearance is related or not to the differentiation of the classic hypophyseal cell types from Rathke's pouch. Aiming to analyze the ontogeny of hypophyseal aromatase expression and to correlating this with hypophyseal cell differentiation, here we performed an immunocytochemical study of the hypophyses of rat fetuses from day 13.5 to 19 of prenatal development. The immunohistochemical expression of the enzyme was first observed on day E17 and this increased on day 19. The significant increase in cells immunoreactive to the enzyme ($p<0.05$) coincided with an increase in their mitotic index ($p<0.05$) from day 17 to day 19 and with a significant increase ($p<0.05$) in cellular size. Our results demonstrate the prenatal expression of hypophyseal aromatase and suggest a possible relationship between such expression and the differentiation of hormone-producing cells whose secretion is subject to regulation by gonadal steroids.

Key Words: Rat - Pituitary gland - Aromatase - Immunohistochemistry - Ontogeny.

Introduction

Several investigators have focused their attention on clarifying the factors involved in the cytodifferentiation of the pituitary gland. Different in vivo and in vitro studies have afforded a plethora of results, some of them contradictory. Such work has mainly been devoted to elucidating whether the pituitary primordium might have a self-differentiation capacity for cytodifferentiation or whether this depends on the action of hypothalamic factors or peripheral hormones (Nemeskéry et al., 1976; Chatelain et al., 1979; Begeot et al., 1981, 1983; 1984a; Gash et al., 1982; Watanabe, 1982, 1985, 1987; Héritier and Dubois, 1994; Kudo et al., 1994). However, with the exception of several hormones or their subunits (Begeot et al., 1984b; Voss and Rosenfeld, 1992), no intrinsic pituitary factors have yet been addressed.

We have recently described the immunohistochemical expression of aromatase P450 in the rat pituitary and the influence of gender in such expression (Vázquez et al., 1997; Carretero et al., 1998). Although several contradictory findings on the cerebral and hypothalamic ontogenic expression of aromatase have been reported (Tsuruo et al., 1994; Lauber et al., 1997), the ontogenic expression of aromatase in pituitary cells remains to be fully elucidated.

Since of the two main metabolic pathways of androgens —reduction and aromatization— the latter route depends on the presence of the enzyme aromatase P450, and because the presence of aromatase has been shown to be

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important in the sexual differentiation and maturation of the brain (McLusky and Naftolin, 1981; Arnold and Gorski, 1984; Pfaff and Schwart-Giblin, 1988; Sachs and Meisel, 1988), this enzyme could be related to pituitary cytodifferentiation, especially in the case of cells influenced by estrogens and androgens, such as gonadotroph, somatotroph and lactotroph cells.

With a view to determining the cytodifferentiation of pituitary aromatase-immunoreactive cells and the relationship between this and the ontogenesis of cells producing the classic pituitary hormones, we carried out an immunohistochemical study of aromatase expression in pituitary glands from E13.5-E19 rat fetuses.

Materials and Methods

Animals. For this work, 25 Sprague-Dawley rat fetuses were used. The fetuses were divided into 5 groups of five animals each, depending on gestational age: fetuses from 13.5, 14.5, 16.5, 17 and 19 days of gestational age (stages: E-13.5, E-14.5, E-16.5, E-17 and E-19). E-0 was considered the night of copulation. Gestating mothers were kept under standard stabling conditions (temperature 21±2°C; relative humidity of air 20±5%, controlled photoperiod of 14 h light/10 darkness, food and water *ad libitum* with a balanced mouse-rat maintenance diet).

Animal sacrifice and sample processing

Fetuses were obtained by laparotomy under Fluorane® inhalation by the gestating mothers and were sacrificed by decapitation. The heads were carefully removed and immediately submerged in 15% saturated picric acid in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, for 24 h. Once the hypophyses had been fixed, they were embedded in paraffin to obtain serial frontal sections 5µm thick 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 Streptavidin-Biotin-Peroxidase method. Endogenous peroxide 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 fraction of non-specific swine serum (Dako®, dilution 1:30). Samples were then incubated with rabbit anti-aromatase serum diluted 1:800 at 4°C 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; soluble Streptavidin-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 preabsorption test was carried out; in all cases the reaction was completely abolished. The specificity of the antibody has been described previously [Beyer et al., 1994c]. For the preabsorption test, diluted anti-aromatase serum (1:500) was preabsorbed (24h at 4°C) with the synthetic peptide sequence C-EIIFSPRNSDKYLLQQ corresponding to residues 488-502 of mouse cytochrome P450arom -Aromatase- (50 µg peptide/ml antibody solution) which was used to obtain the rabbit polyclonal anti-aromatase serum.

Quantification and morphometry of aromatase-immunoreactive cells

The percentage of cells reactive to aromatase from each animal was quantified by the double blind method. 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) that were chosen randomly from all the parts of the gland, then calculating the percentage of reactive cells. Mitotic metaphasic aromatase-immunoreactive cells were quantified in order to calculate the mitotic index. Using a MIP-2 (IMCO 10) image analyzer, the cellular areas of 100 aromatase-immunoreactive cells from each animal, chosen randomly from all parts of the gland (500 cells per group; E-17 and E-19), were calculated. These percentages were analyzed statistically and the differences among the means obtained were contrasted by ANOVA, $p<0.05$ in the Scheffé-F test being considered significant.

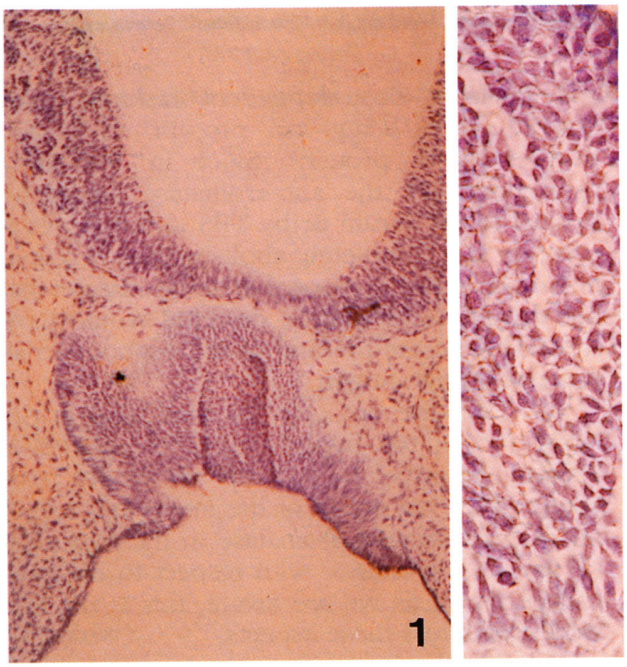
Fig. 1. Panoramic view of Rathke's pouch from an embryo on day E13.5 (x125). Inset: Micrograph showing the absence of immunoreactivity for aromatase in the E13.5 (x500).

Fig. 2. Panoramic view of the pituitary primordium from an embryo on day E14.5 (x125). Inset: Micrograph showing the absence of immunoreactivity for aromatase in the E14.5 (x750).

Fig. 3. Panoramic view of the pituitary gland from an embryo on day E16.5 (x125). Inset: Micrograph showing the absence of immunoreactivity for aromatase in the E16.5 (x750).

Fig. 4. Panoramic view of the pituitary gland of an embryo on day E17 showing cells weakly immunoreactive to aromatase, brown (x250). Inset: Micrograph showing the light immunoreaction to aromatase in these embryos (x 1250).

Figs. 5, 6 and 7. Aromatase-immunoreactive cells (brown) in the pituitary gland of embryos on day E19. Mitotic aromatase-immunoreactive cells (arrows in figures 6 and 7) were evident in the pituitary gland of the embryos on day E19 (x1250).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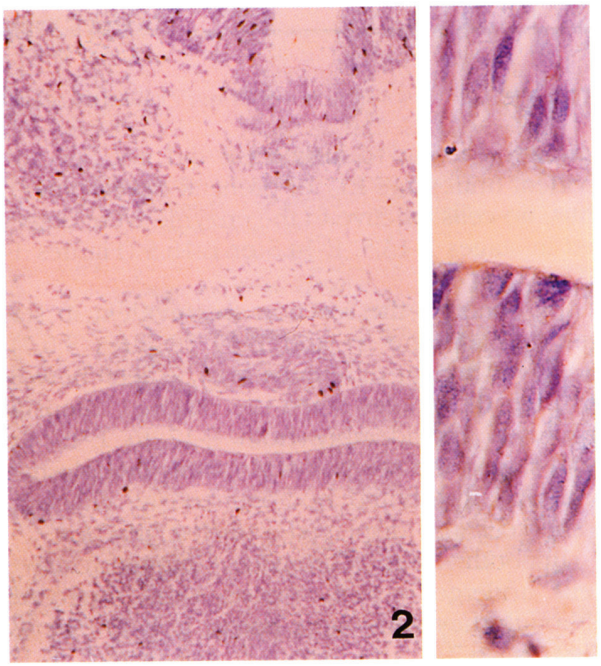
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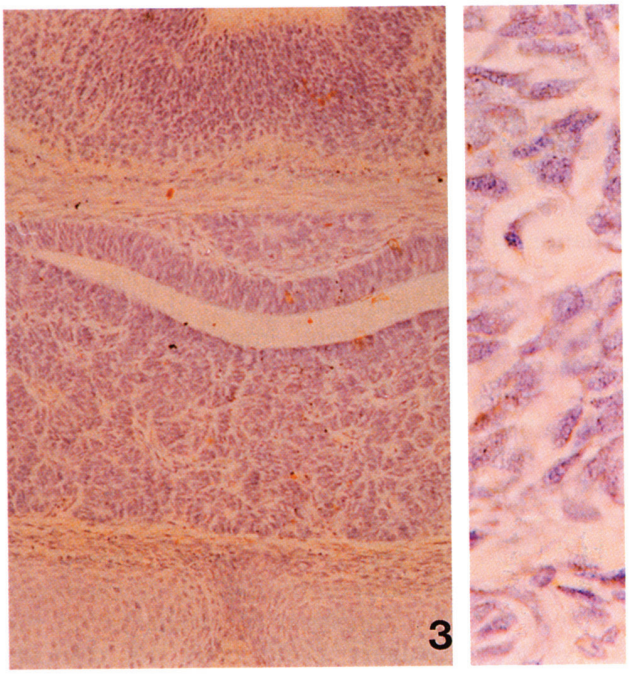
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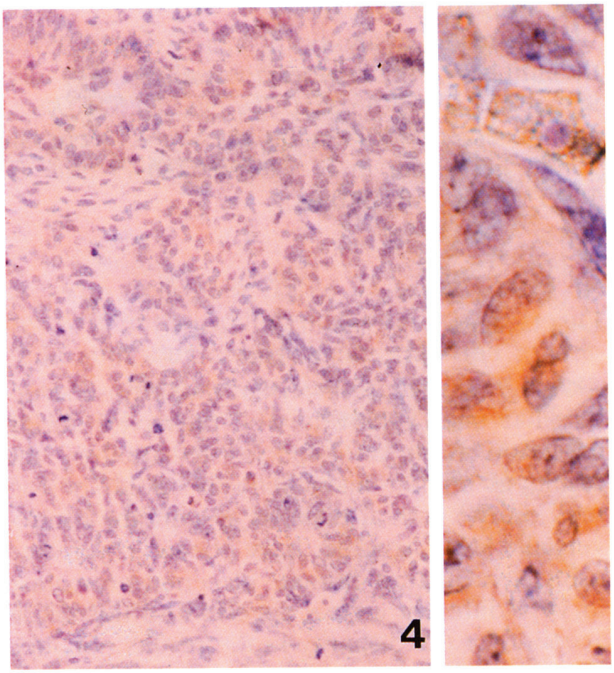
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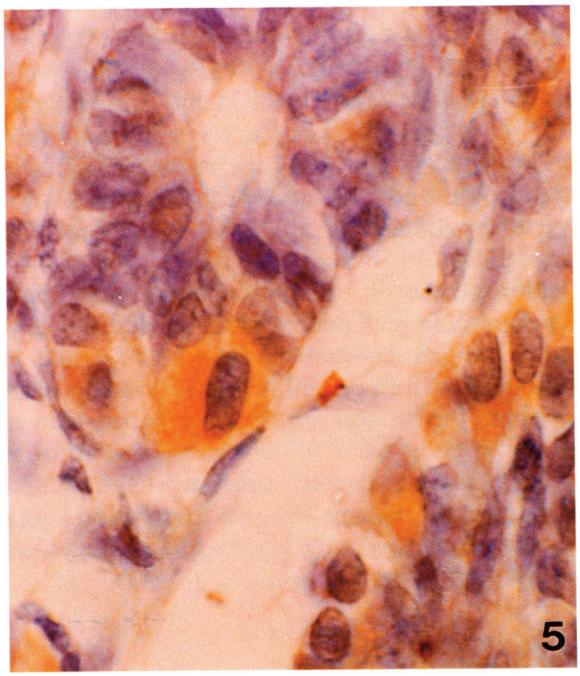
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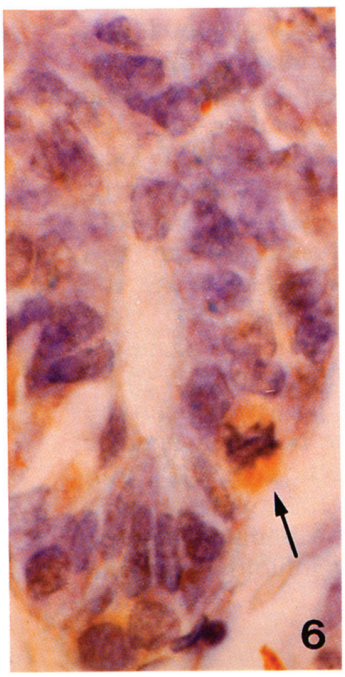
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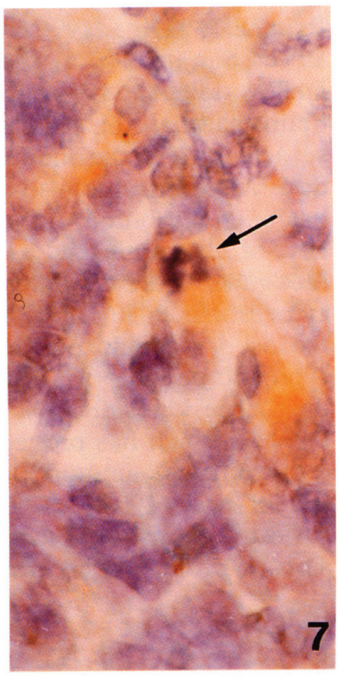
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Results

Immunoreaction to aromatase did not become evident until stage E17 of prenatal development; thus, no cells reactive to the enzyme were detected in stages E-13.5, E-14.5 or E-16.5 (Figures 1-3).

By contrast, the reaction was evident in stages E-17 (Figure 4) and E-19 (Figures 5,6,7). However, a clear difference may be seen between both these ages since the reaction intensity increased considerably from E-17 to E-19. In both stages, the reaction was mainly cytoplasmic and was distributed homogeneously, although some cells also displayed a nuclear reaction.

In both stages, aromatase-immunoreactive cells undergoing cell division were observed, although they were more patent in E-19 (arrow, Figures 6,7), the mitotic index in E-17 (0.19±0.02%) being significantly lower ($p<0.05$) than in E-19 (0.36±0.04%).

As a result, a significant increase ($p<0.05$) in the number of immunoreactive cells in E-19 with respect to E-17 (46.25±0.76% vs. 17.52±0.98%) was observed; additionally, these cells were larger in E-19 than in E-17 (150.67±6.45 μm^2 vs. 109.13±4.28 μm^2, $p<0.05$).

Discussion

Although aromatase is an enzyme whose structure has been relatively well preserved during evolution from one species to another, the results of immunohistochemical studies performed on the central nervous system are controversial. Most studies have been conducted using sera against human placental aromatase in very different animal species and this could account for the disparity in the results obtained [Roselli and Resko, 1984; Harada, 1987; Shinoda et al., 1989a, 1989b, 1989c, 1992a, 1992b; Balthazart et al., 1990a, 1990b, 1990c, 1991a, 1991b; Sanghera et al., 1991; Jakob et al., 1993; Dellovade et al., 1994). In studies carried out in brain material, the results concerning aromatase activity and the immunohistochemical location of the enzyme also conflict. This disparity has been attributed to the possible non-specificity of the antibody used since it can also recognize other similar antigens from the cytochrome P450 family (Shinoda, 1994). Here we used a highly purified antibody against a synthetic peptide of 15 amino acids corresponding to residues 488-502 of mouse cytochrome P450 A, which has proved to be highly specific for aromatase immunodetection in the brain (Beyer et al., 1994a, 1994b, 1994c). Beyer et al. (1994) demonstrated that the immunosuppression of aromatase in ovary inhibits enzymatic activity in a dose-dependent fashion (from 204±26 pmol/h/mg of protein to 21±10 pmol/h/mg of protein). Since in our study replacement of the anti-aromatase serum by normal rabbit serum or by TBS and the preabsorption test completely abolished the reaction, it would appear reasonable to speculate that the immunoreactive cells detected would have resulted from the binding of the antibody to its corresponding antigen.

Although the ontogeny of aromatase in the brain and in the hypothalamus of rodents is known, nevertheless to date no works have analyzed the ontogeny of the immunohistochemical expression of aromatase in the hypophysis and its importance with respect to differentiation. As far as we are aware, this is the first work to address these aspects.

The five classic cells types in the mature anterior pituitary are defined by the trophic factors that they synthesize and secrete. These five types arise in a precise temporal and spatial pattern, but it has not been established what actually triggers the appearance of each cell type from an apparently homogeneous primordium or whether transcription factors or paracrine pituitary substances modulate the cytodifferentiation of these cells types.

The cytogenesis and differentiation of the fetal pituitary gland has been the subject of repeated investigation. Immunohistochemical observations on the developing pituitary gland have been made in rats and a summary of the literature about the differentiation of pituitary cells and the temporal sequence in such differentiation is offered in Table 1. The disparities observed in this table from one author to another could be due to the use of different rat strains. As may be seen from the table, the appearance of the expression of aromatase in the developing pituitary gland does not coincide exactly with any classic pituitary cell type but does coincide with the appearance of the alpha subunit (Stoeckel et al., 1993) and LH- (Watanabe and Daikoku, 1979) and TSH-immunoreactive cells (Begeot et al., 1981; Sétalo and Nakane, 1972, 1976) and a trophic action has been attributed to the glycoprotein hormone a-subunit in the cytodifferentiation and proliferation of prolactin cells (Begeot et al., 1984a; Van Bael and Denef, 1996).

Although the differentiation of hormone-producing pituitary cells takes place in defined portions of the gland —ACTH-, LH- and FSH-cells in the ventral periphery and TSH, GH- and prolactin cells in the central regions- aromatase-immunoreactive cells were widely dispersed throughout the gland and were present in the central and peripheral regions. These observations suggest that aromatase could be expressed in more than one cell type.

Table 1. Embryonic day of appearance of rat pars distalis cell types cited in the literature (*: pars tuberalis).

Cell Type	Embryonic Day	References
α–Subunit	11	Simmons et al. (1990).
	14 *	Stoeckel et al. (1993)*.
	17-18	Stoeckel et al. (1993).
Corticotroph	13.5	Lugo and Pintar (1996).
	15	Watanabe and Daikoku (1979).
	16	Sétálo and Nakane (1972,1976).
	16	Chatelain et al. (1979).
	16	Begeot et al. (1982).
Gonadotroph FSH	18	Tougard et al. (1977).
	19	Watanabe and Daikoku (1979).
	20	Sétálo and Nakane (1976).
LH	16/17	Tougard et al. (1977).
	17	Watanabe and Daikoku (1979).
	18	Sétálo and Nakane (1972).
	18	Begeot et al. (1981,1984a).
Lactotroph	16	Sétálo and Nakane (1972,1976).
	21	Chatelain et al. (1979).
	Undet.	Watanabe and Daikoku (1976,1979).
Somatotroph	18	Watanabe and Daikoku (1979).
	19	Weidner (1972).
	19	Sétálo and Nakane (1972,1976).
	19.5	Chatelain et al. (1979).
Thyrotroph	16.5	Watanabe and Daikoku (1979).
	17	Sétálo and Nakane (1972,1976).
	17	Begeot et al. (1981).

The sequential appearance of the five classic cell types suggests the actions of inducing substances and cell-cell interactions during development. In hypothalamic gender differentiation, aromatase plays an important role (McLusky and Naftolin, 1981; Arnold and Gorski, 1984; Pfaff and Schwart-Gibli, 1988; Sachs and Meisel, 1988) and, possibly, in the pituitary gland aromatase could either be involved in the differentiation of its cell types or could be a final product of differentiation and could perhaps modulate the secretory activity of these cells.

Regarding aromatase, we have found no references in the literature that establish its adenohypophyseal ontogeny. Tsuruo et al. (1994) found the first signs of reaction to aromatase in the hypothalami of rats in the early differentiation stages and established its appearance at day E-13. However, in the study of transient sex differences in aromatase (CYP 19) mRNA expression in the developing rat brain, aromatase mRNA was detected in several preoptic, hypothalamic and limbic regions on gestation days 18 and 20 (Lauber and Lichtensteiger, 1994; Lauber et al., 1997). Our study demonstrates the appearance of the immunohistochemical expression of aromatase in the pituitary gland on gestational day 17.

The date of pituitary aromatase expression is very interesting because it is concomitant or anterior to cytogenesis of the cell types regulated by gonadal steroids —FSH, LH-, GH- and prolactin cells (see Table 1)— and could be involved in their differentiation.

In sum, our results demonstrate the expression of aromatase in the developing pituitary gland and suggest that the enzyme could be involved in the differentiation of pituitary cell types, whose differentiation and secretory activity are modulated by gonadal steroids.

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