Journal of Neuroendocrinology, 2004, Vol. 16, 64–71

Gender Differences in the Expression of Galanin and Vasoactive Intestinal Peptide in Oestrogen-Induced Prolactinomas of Fischer 344 Rats

G. G. Piroli,*† L. Pietranera,*† C. A. Grillo*† and A. F. De Nicola*†
*Laboratorio de Bioquímica Neuroendócrina, Instituto de Biología y Medicina Experimental, UBA-CONICET, Buenos Aires, Argentina.
†Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.

Key words: pituitary tumours, diethylstilbestrol, prolactin, lactotroph, testosterone propionate.

Abstract

We have previously described a sexual dimorphism in oestrogen-induced anterior pituitary tumorigenesis in Fischer 344 rats, with female tumours averaging twice the size of those of males. Neonatal androgenization of female Fischer 344 rats with 100 μg of testosterone propionate reverted that effect, causing a 'male-like' phenotype. The peptides galanin and vasoactive intestinal peptide (VIP) are possible mediators of oestrogen effects on the anterior pituitary, including hyperprolactinemia and lactotroph proliferation. To further extend our previous findings, we investigated the expression of galanin and VIP in the anterior pituitary of control and oestrogenized male, female and neonatally androgenized female Fischer 344 rats. At 3 months of age, rats were deprived of their gonads and divided into control and diethylstilbestrol (DES)-treated groups. In the anterior pituitary of control rats, galanin and VIP immunoreactive cells were absent. However, in DES-treated rats, pituitaries from normal ovariectomized females showed higher number of galanin and VIP positive cells than pituitaries from neonatally androgenized ovariectomized females and gonadectomized males. This pattern correlated with changes in anterior pituitary weight and serum prolactin. Our study suggests that sexual differences in oestrogen-induced pituitary tumorigenesis could be due to the differential expression of galanin and VIP. Furthermore, our data support the fact that neonatal exposure to androgens, as in normal males and androgenized females, may condition the response of the pituitary gland to oestrogens in adult life.

Oestrogens increase prolactin synthesis and secretion in the rat (1). In addition, chronic administration of oestrogens induces pituitary tumorigenesis due to lactotroph proliferation, as described by Selye in 1935 (2). Development of these tumours is highly dependent on the rodent strain, with the Fischer 344 (F344) rats being the most susceptible (3). We previously called these tumours DES-T because, in most cases, the synthetic oestrogen diethylstilbestrol (DES) was used as the inducing agent (4). In our experience, F344 rats implanted with a single 20 mg DES pellet usually developed pituitary tumours after 30–45 days, with prolactin titres that may reach a 1000-fold elevation (5). Susceptibility to oestrogen-induced pituitary tumorigenesis is observed both in male and female F344 rats. However, an interesting finding is that a sexual dimorphism in tumour size is present, with female F344 tumours averaging twice the size of males (3). Using neonatally androgenized female rats, we further studied this gender difference, showing that masculinized F344 females develop tumours of the same size of males (6).

In recent years, several peptides have been studied as mediators of oestrogen actions in the anterior pituitary, including pituitary tumorigenesis. One of those peptides is galanin, which is found at low levels in somatotrophs of male and ovariectomized (OVX) rats (7, 8). Oestrogens lead to an increase in galanin expression in the anterior pituitary, both in physiological and pharmacological paradigms, including oestrogen-induced pituitary tumours in F344 rats (7–14). The induction of galanin expression by oestrogens in the pituitary gland is mediated by oestrogen receptors, as shown by *in vitro* experiments in which the oestrogen receptor antagonist tamoxifen was employed (15). Furthermore, oestrogen receptor α but not β appears to be essential for oestrogenic induction of galanin expression in the anterior pituitary, as shown by experiments using mice lacking the α isoform of the oestrogen receptor (16). In rats undergoing oestrogen treatment, galanin colocalizes with prolactin in the same secretory granules (7, 8, 12, 13). Galanin released by some lactotrophs regulates prolactin secretion of the same or

Correspondence to: Dr Gerardo G. Piroli, 1001 True Street Apartment 116, Columbia, SC 29209, USA (e-mail: pirolig@rockefeller.edu).

other lactotrophs in an autocrine/paracrine fashion (17, 18). In addition, other reports also indicate a role of galanin in lactotroph proliferation in oestrogenized rats, linking galanin expression to pituitary tumorigenesis (19, 20).

Another peptide that exerts regulatory actions on prolactin synthesis/secretion is the vasoactive intestinal peptide (VIP). Experiments performed *in vitro* showed that VIP induces prolactin secretion both from incubated hemipituitaries (21) and dispersed pituitary cells (17, 22). Furthermore, antisera against VIP blocks VIP-induced prolactin release (17, 22, 23). The origin of VIP reaching the anterior pituitary *in vivo* was initally assigned to the paraventricular nucleus (24); however, a body of evidence also pointed to a local intrapituitary source of VIP, which could act as a paracrine stimulator of prolactin release. In this sense, VIP was shown to be synthesized in the anterior pituitary and VIP mRNA was also detected in the gland (25). Although the expression of preproVIP mRNA is barely detectable in the pituitary gland of OVX rats, oestrogenization leads to an increase in VIP gene expression and VIP content, which correlates with increments in prolactin secretion (26, 27). Moreover, it has been shown that VIP colocalizes with prolactin in the anterior pituitary of oestrogenized rats (8).

Considering the sexual differences in oestrogen-induced pituitary tumorigenesis and the role of galanin and VIP as mediators of oestrogen actions in the pituitary gland, in the present study, we compared the response of the pituitary gland to oestrogen stimulation in terms of VIP and galanin expression in castrated (GDX) male and OVX female F344 rats, the latter divided into subgroups receiving neonatally vehicle or androgen treatment. Our data suggest that neonatal steroid exposure may be responsible for a differential expression of VIP and galanin which, in turn, could contribute to sexual differences in the growth of the pituitary under oestrogenic stimulus.

Materials and methods

Experimental animals

Animal procedures were in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals (NIH Guide; Instituto de Biología y Medicina Experimental Assurance Certificate No. A5072-01). The protocol employed for neonatal androgenization has been previously described (6). Fischer 344 (F344) rats obtained from the National Atomic Energy Agency of Argentina were housed under controlled temperature (22 ± 1 °C) and a 12 : 12 h light dark cycle (lights on 07.00 h), with *ad libitum* access to food and water. After mating, pregnant rats were housed separately until parturition. Twenty-four hours after birth, a group of female rats received a single subcutaneous injection of 100 µg of testosterone propionate (TP) dissolved in 100 µl of sunflower oil. Other groups of female and male rats received the vehicle only. At 3 months of age, ovaries or testes were removed from all rats and, consequently, the experimental groups were: ovariectomized rats (OVX), OVX rats given TP treatment (OVX + TP) and gonadectomized male rats (GDX). Some of the rats in each group received a single subcutaneous pellet of 20 mg of diethylstilbestrol (DES) to induce pituitary tumours (4–6, 28). These groups were labelled OVX + DES, OVX + TP + DES and GDX + DES, respectively. All rats were used 40 days after the beginning of DES treatment. Rats were decapitated, anterior pituitaries and tumours were removed, weighed and immediately fixed in Bouin's fluid at room temperature for 6 h. Tissues were embedded in paraffin following standard protocols, as already described (6, 28). At the time of killing, trunk blood was collected, allowed to clot and centrifuged to remove serum, which was stored at −20 °C. Prolactin in serum was measured by RIA applying a double antibody technique, with reagents prepared by the NIDDK-NIH (Bethesda, MD, USA) (5, 6, 14, 28). Results were expressed in ng/ml, in terms of the RP3 standard.

Immunocytochemistry for galanin and VIP

Galanin and VIP immunostaining was performed with antibodies from Peninsula Laboratories (San Carlos, CA, USA) (antigalanin, IHC 7141, dilution 1 : 7000, source: rabbit; anti-VIP, IHC 7161, dilution 1 : 500, source: rabbit) following previously published protocols (6, 28). Paraffin sections were cut in a microtome every 4 µm and mounted onto glass slides. Sections were rehydrated, washed in phosphate-buffered saline (PBS) and treated with 0.3% H_2O_2 in methanol for 20 min at room temperature to block endogenous peroxidase. The slides were preincubated in 10% normal goat serum at 37 °C for 10 min, and a solution containing the specific antibodies to galanin or VIP was then added to the sections. After incubating overnight at 4 °C, slides were washed with PBS and incubated at room temperature for 1 h with a biotin-labelled second antibody against rabbit IgG, and then with a preformed ABC complex for 30 min (Vector Laboratories, Burlingame, CA, USA). Subsequently, slides were immersed in a 0.05% 3, 3′-diaminobenzidine (Sigma, St Louis, MO, USA) solution in 0.1 M Tris buffer, pH 7.2 containing 0.01% H_2O_2. After 5 min at room temperature, slides were removed, the reaction was stopped by immersion in PBS, and sections were counterstained with haematoxylin, dehydrated and coverslipped with Permount. Immunoreactive cells were visualized with the aid of an Olympus microscope equipped with a VT-C33ON video camera at a magnification of 400X, and quantified by computerized image analysis (Optimas, Bioscan) as already described (6, 28). Most cells were individually distinguishable due to nuclear haematoxylin counterstaining, and only cells showing a clear nuclear profile were counted. For each rat, approximately 150–200 cells were counted in each section, and at least 10 pituitary sections from different levels of the gland were used for quantification. The total area under study was also measured. Results were pooled for each rat, and the number of galanin or VIP immunoreactive cells was expressed per mm^2 and as a percentage of total cells.

Statistical analysis

Values reported are the mean ± SE of 6–8 rats per group. Results were analysed by one-way analysis of variance, followed by post-hoc comparisons with the Student–Newman–Keuls test, according to the GraphPad Prism V3.0 program (GraphPad Prism, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

We have previously reported the effects of neonatal androgenization on pituitary weight and serum prolactin concentration in rats subjected to chronic oestrogenization (6). As the pituitaries used in the present study were obtained from a new set of rats, we repeated these determinations to be able to correlate any possible change on pituitary weight and serum prolactin with the expression of galanin and VIP in the anterior pituitary of the same rats (Table 1). Confirming our previous report, pituitary weight was low in rats not receiving DES, with no significant differences among the different groups. Chronic DES treatment produced enlargement of the pituitary gland in all the groups; however, the effect was stronger in females than in androgenized females and

TABLE 1. Pituitary Weight and Serum Prolactin in Untreated Rats and Rats Receiving a Diethylstilbestrol Pellet (20 mg) During 40 days.

Experimental group	Pituitary weight (mg)	Serum prolactin (ng/ml)
OVX	10.34 ± 0.94	3.24 ± 0.22
OVX + TP	8.85 ± 0.35	2.68 ± 0.65
GDX	9.25 ± 1.50	2.66 ± 0.67
OVX + DES	83.82 ± 8.04*	1262 ± 97*
OVX + TP + DES	31.32 ± 4.39*†	474 ± 79*†
GDX + DES	34.38 ± 3.88*†	498 ± 61*†

Group labelling: ovariectomized female rats (OVX); neonatally androgenized, ovariectomized females (OVX + TP); and gonadectomized males (GDX); without or with DES pellet implantation (OVX + DES, OVX + TP + DES, GDX + DES, respectively). Results represent the mean ± SE of 6–8 rats per group. *$P < 0.001$ versus corresponding nonestrogenized controls; †$P < 0.001$ versus OVX + DES group.

males (Table 1). Serum prolactin levels were lower than 5 ng/ml in nonestrogenized rats; no differences were observed among these groups. As for pituitary weight, DES treatment induced a huge increment in serum prolactin concentration, which was higher in OVX + DES than in OVX + TP + DES and GDX + DES (Table 1).

Results of galanin immunodetection in the anterior pituitary of rats from the six experimental groups are shown in Figs 1 and 2. In the glands of rats not treated with oestrogens, cells immunoreactive for galanin were almost absent (Fig. 2A). In the oestrogenized rats, galanin expression in the tumours was significantly higher in the OVX + DES group than in OVX + TP + DES and GDX + DES rats, when results were expressed on an area basis (Figs 1A and 2B–D). The difference in galanin expression among the oestrogenized groups was not due to differences in the area of the cells, because they were still present when results were expressed as a percentage of total cells (Fig. 1B).

Our study also included the detection of VIP expression. VIP was not detectable in the glands of nonestrogenized rats (Fig. 4A). When rats were given DES, immunoreactivity for VIP was present in all three groups, though to a lower extent than for galanin (Figs 1 and 3). As for galanin expression, VIP expressing cells were more concentrated in tumours from OVX + DES rats than in those from OVX + TP + DES and GDX + DES rats (Figs 3A and 4B–D). Again, changes were still evident when results were expressed on a percentage basis instead of per area unit, indicating that they were not due to differences in the area of the cells (Fig. 3B).

Remarkably, many cells in the anterior pituitary of oestrogen-deprived rats showed small dark nuclei and small cytoplasm (Fig. 2A and 4A), whereas most of the cells in the tumours from oestrogenized rats presented large pale nuclei and abundant cytoplasm (Figs 2B–D and 4B–D).

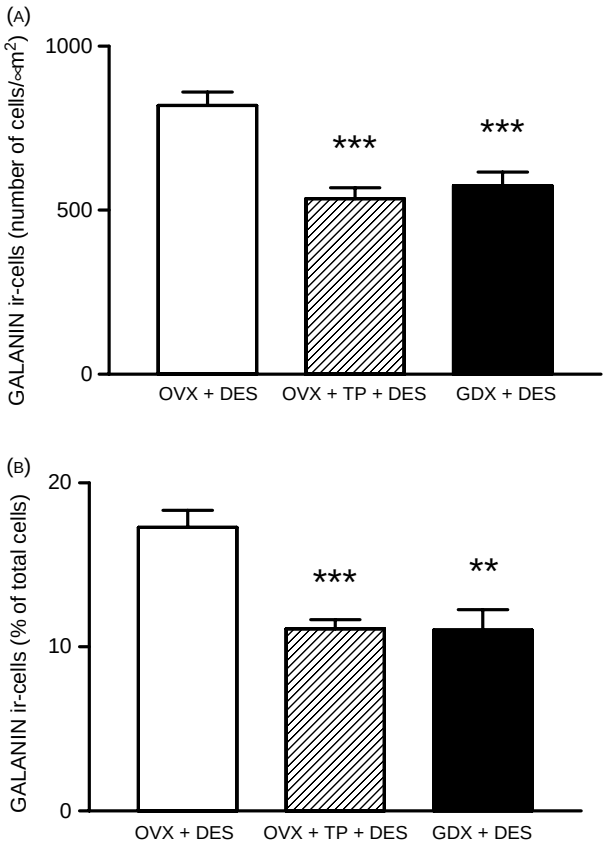

FIG. 1. Number of galanin immunoreactive cells expressed per area unit (A) and as a percentage of total cells (B) in anterior pituitary glands from oestrogenized ovariectomized rats (OVX), OVX rats given testosterone propionate (TP) treatment (OVX + TP) and gonadectomized male rats (GDX). OVX + TP + diethylstilbestrol (DES) and GDX + DES rats showed a lower content of galanin immunoreactive cells compared to OVX + DES rats. $^{**}P < 0.01$; $^{***}P < 0.001$ versus OVX + DES group.

Discussion

Previous reports indicate a sexual dimorphism in oestrogen-induced pituitary tumours in F344 rats, suggesting that the pre-existing hormonal milieu plays an important role in setting the sensitivity to exogenous oestrogen (3). To further explore that hypothesis, we modified the neonatal steroid environment of female rats by early androgenization, a treatment that causes profound changes in the hypothalamic centres that control lactotroph function (29, 30). It has been described that, under basal conditions, neonatally androgenized females show higher levels of serum prolactin (29, 31, 32), and exhibit changes in the arcuate nucleus morphology, which is more similar to that of males than to that of normal females (33). These changes in the arcuate nucleus structure could account for the differences observed in prolactin levels, considering that dopamine, the main prolactin-inhibitory factor, is synthesized in that nucleus (34). However, other factor(s) may be involved, including ovarian steroids, as it has been shown that OVX decreases hyperprolactinemia in neonatally androgenized females, and that oestrogen replacement increases again serum prolactin (29, 30, 32). To avoid possible baseline differences, our rats were deprived of their sexual steroids by OVX or GDX. As a result, serum prolactin was low in all the nonestrogenized groups, without gender differences. However, after chronic oestrogenization, pituitary weight and serum prolactin levels were increased to a lower extent in males and in neonatally androgenized females than in normal female rats, confirming our previous report (6). One possible explanation for this sexual dimorphism is the heterogeneity in the cell population of the pituitary gland observed between sexes. It is known that, after puberty, pituitaries of female rats have a higher percentage of lactotrophs and those of male rats contain a higher percentage of somatotrophs (35). However, if males are neonatally deprived of androgens by castration, the pituitary gland at adulthood resembles more the gland of a female rat, with a higher content of lactotrophs and a lower content of somatotrophs (35). These changes are not reversed by adult treatment with testosterone, suggesting that the ability of the adult anterior pituitary to change its cellular composition in response to gonadal steroids is limited by the neonatal hormonal milieu to which the rat was exposed (35). Consistent with that idea, we found that neonatally androgenized females respond to oestrogens with a male-like phenotype (i.e. smaller tumours and lower serum prolactin levels than normal female rats).

The local expression of galanin and VIP, two possible mediators of oestrogen effects in the anterior pituitary, was then investigated to verify if they were related to the gender differences in pituitary growth and prolactin production.

Galanin has been proposed as the main paracrine/autocrine regulator of prolactin synthesis/secretion. Previous reports

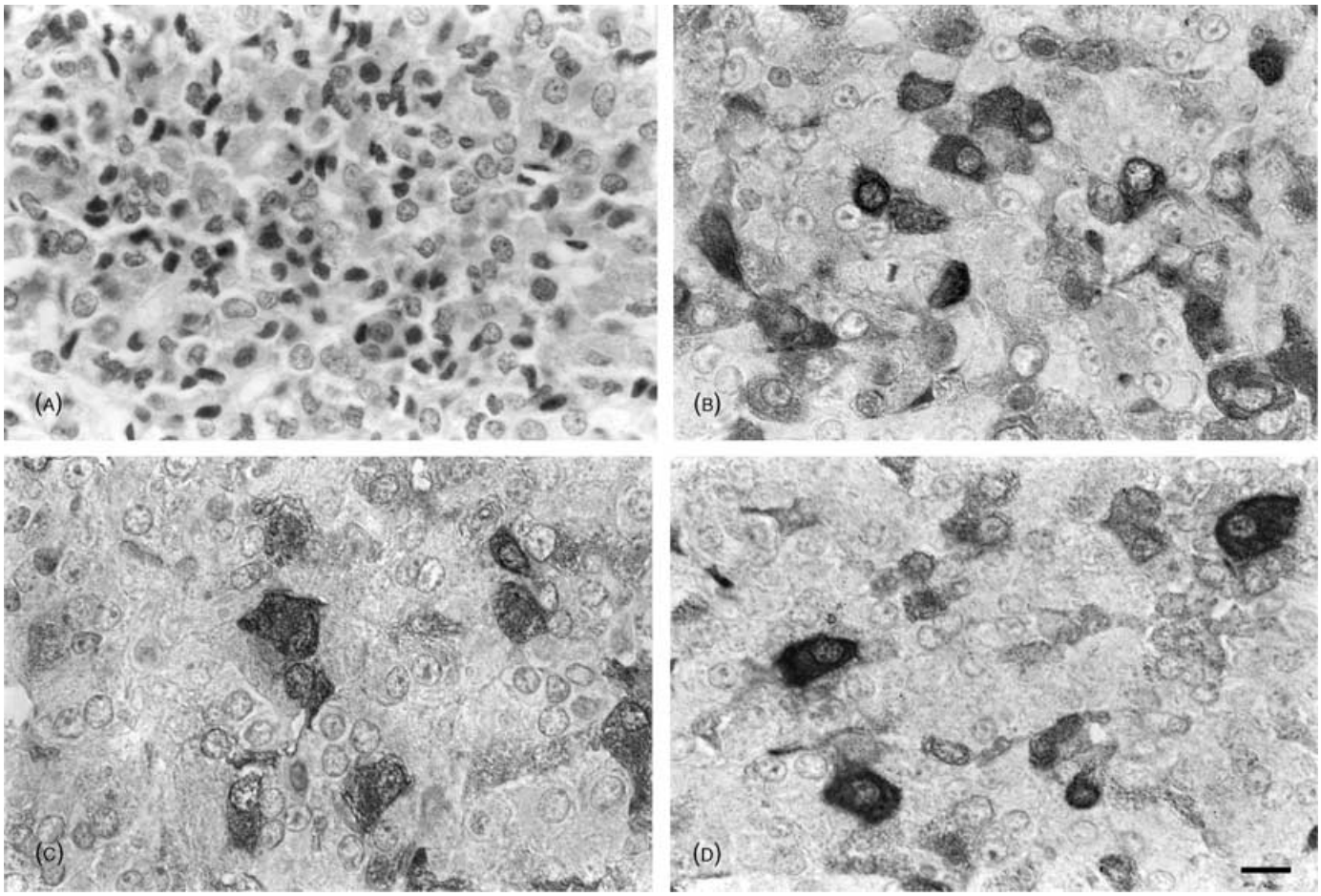

FIG. 2. Representative photomicrographs of galanin immunoreactivity in anterior pituitary glands from (A) ovariectomized rats (OVX), (B) OVX + diethylstilbestrol (DES), (C) OVX + testosterone propionate (TP) + DES and (D) gonadectomized (GDX) + DES rats, respectively. Magnification ×1000. Scale bar = 10 μm.

showed sexual differences in the distribution of galanin immunoreactive cells in the anterior pituitary, with a differential pattern of colocalization of galanin and anterior pituitary hormones. Thus, normal female rats presented galanin positive cells evenly distributed throughout the anterior pituitary; these cells were characterized as lactotrophs (8, 12). After surgical castration or treatment with luteinizing hormone-releasing hormone agonists and anti-oestrogens, a depletion of galanin immunoreactive cells was observed (8, 12). In male rats, galanin immunoreactive cells in the anterior pituitary were less abundant than in normal females; in this case, galanin is present in somatotrophs and thyrotrophs but not in lactotrophs (8, 12). Gonadectomy of male rats also reduced galanin immunoreactivity in the anterior pituitary (12). To our knowledge, no studies on galanin expression in the anterior pituitary of neonatally androgenized females have been carried out. In the present study, we found that cells immunoreactive for galanin were not detectable in the pituitaries from nonestrogenized rats, which is in accordance with the literature (8, 12). It has also been described that oestrogenization leads to increments in galanin expression in the anterior pituitary (7–18, 28). Accordingly, in our present study, galanin expressing cells were clearly visible in pituitary tumours from all three groups of oestrogenized rats. Although we did not perform colocalization studies, previous reports indicated that galanin concentrates with prolactin within the secretory granules of lactotrophs in rats undergoing oestrogen treatment (7, 8, 12, 13, 17, 18). Furthermore, the number of lactotrophs expressing galanin also increased with the oestrogenization (13), although galanin mRNA and peptide are not present in all lactotrophs (7, 13, 17, 18). Taking into account that lactotrophs comprise approximately 80% of the cells in the tumours (6, 28, 36), it is likely that the galanin-expressing cells detected in this study were lactotrophs. We found that the number of cells expressing the peptide was higher in the glands of the OVX + DES rats than in oestrogenized males and neonatally androgenized females. Considering that galanin regulates prolactin synthesis/secretion by acting on the same or on neighbouring lactotrophs (18, 19), it is possible that the higher number of galanin expressing cells in tumours of the OVX + DES group is responsible for the higher levels of serum prolactin observed in these rats. It has also been described that galanin regulates gonadotroph function in normal female rats (37). An interesting observation is that the number of gonadotrophs is lower in the OVX + DES group than in GDX + DES and OVX + TP + DES rats (Piroli *et al.*, unpublished observations), an effect that could also correlate with the increased expression of galanin in OVX + DES rats. On the other hand, we also observed a sexual dimorphism in tumour weight, with OVX + DES rats showing bigger tumours than the GDX + DES and

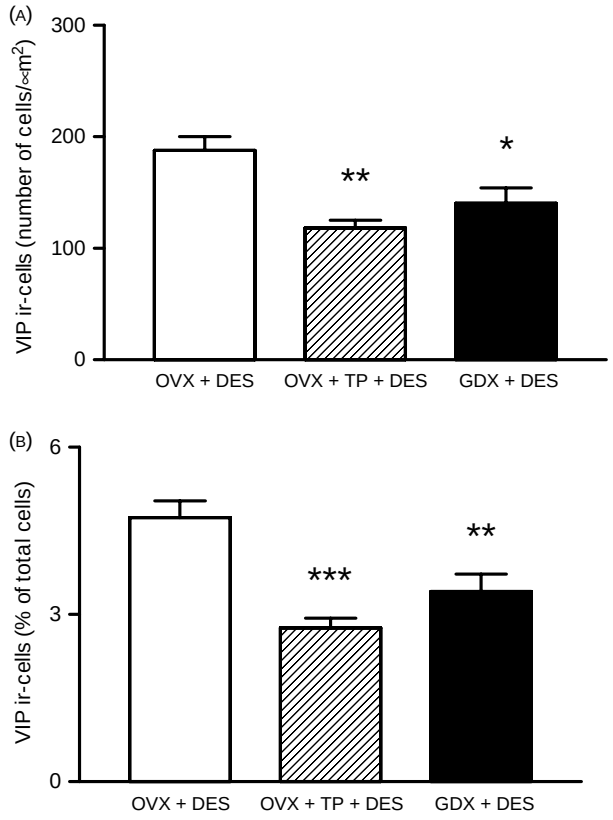

FIG. 3. Number of VIP immunoreactive cells expressed per area unit (A) and as percentage of total cells (B) in anterior pituitary glands from oestrogenized ovariectomized rats (OVX), OVX rats given testosterone propionate (TP) treatment (OVX + TP) and gonadectomized male rats (GDX). OVX + TP + diethylstilbestrol (DES) and GDX + DES rats showed a lower content of VIP immunoreactive cells compared to OVX + DES rats. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus OVX + DES group.

OVX + TP + DES rats. This differential growth of the pituitary gland could also be due to the different expression of galanin. In this context, it has been proposed a role of galanin in lactotroph proliferation *in vitro* (17). More recently, a report in which mice with a loss-of-function mutation of the galanin gene were employed, showed that dispersed pituitary cells from mutant females failed to proliferate under oestrogenic stimulus, while the number of cells from control rats increased by approximately 200% (19). Further evidence in this sense was given by a study in which transgenic mice overexpressing galanin in lactotrophs were developed to enhance the naturally low abundance of galanin in physiological conditions (20). Female transgenic mice exhibited higher cell count and higher weight of the anterior pituitary compared to controls; both effects were suppressed by OVX, showing that galanin-mediated effects were oestrogenic-dependent (20). Additional support was provided by experiments in which treatment with dopaminergic agents or progestins was employed, showing that drugs with an ability to reduce the size of oestrogen-induced pituitary tumours were also able to modulate galanin content and/or release from lactotrophs (28, 38).

As stated above, another peptide that exerts regulatory actions on prolactin synthesis/secretion is VIP. VIP stimulates prolactin secretion both *in vitro* and *in vivo* (17, 21, 22). In addition to hypothalamic sources of VIP that can act on the pituitary gland, local production of VIP in the anterior pituitary was also shown (25). VIP released from lactotrophs exerts an autocrine/paracrine control on prolactin release, and it has been suggested that VIP produced by pituitary cells in culture is the main factor that stimulates prolactin secretion in the absence of hypothalamic influence (39). The exact characterization of VIP-producing cells in the anterior pituitary remains controversial, with most studies supporting a lactotroph localization (8, 26, 40) and others showing presence of VIP in other pituitary cells (41). Our current results show that VIP-immunoreactivity is not detectable in the anterior pituitary gland of gonadectomized rats. Supporting this, previous studies (8, 26) showed that VIP positive cells were almost absent in the anterior pituitary of normal male rats and OVX females, whereas female rats in diestrus showed scarce VIP immunoreactive cells; due to that low count, the characterization of the cellular type was not possible. To our knowledge, no immunocytochemical studies were reported on VIP expression in the anterior pituitary of neonatally androgenized female rats. However, it has been shown that neonatal androgenization with a high dose of 1 mg testosterone per rat increased VIP content in the anterior pituitary gland, whereas at the same dose employed in the present study (100 μg), no changes in VIP content were observed (31). On the other hand, it has been previously shown that long-term oestrogen treatment increased VIP gene expression and VIP content in the anterior pituitary, which correlated with increments in prolactin secretion (27, 41–45). In addition, VIP was shown to colocalize with prolactin in the anterior pituitary of oestrogenized rats (8, 26, 40), and even VIP-galanin colocalization was demonstrated in pituitaries of rats undergoing oestrogen treatment (8). In the current study, we confirmed and extended those observations: VIP immunoreactivity is present in the anterior pituitary of chronically oestrogenized F344 rats, and a sexual dimorphism is present in that VIP immunoreactive cells are more abundant in the glands of OVX rats treated with DES than in those of GDX and OVX + TP rats subjected to oestrogenization. However, sexual dimorphism in VIP expression does not appear to be related to gender differences in anterior pituitary growth caused by oestrogenization. Although it has been suggested that VIP increments could be involved in the proliferating effects of oestradiol on lactotrophs (45), experiments performed *in vitro* showed that 100 nM VIP had no effect on lactotroph proliferation, whereas the same concentration of galanin induced increments of approximately three-fold and two-fold in thymidine incorporation and cell number count, respectively (17). Nonetheless, and as in the case of galanin, the higher expression of VIP could be responsible for the higher serum prolactin levels observed in the OVX + DES group compared to OVX + TP + DES and GDX + DES rats.

As we mentioned previously, DES-T are composed mainly of proliferating lactotrophs, which comprise approximately 80% of total cells (6, 28, 36). In our present study, tumoral cells exhibited large pale nuclei and abundant cytoplasm, in opposition to the small nuclei and cytoplasm present in cells from oestrogen-deprived rats. These features are in complete agreement with previous reports that indicate that, in castrated rats, most of the anterior pituitary cells are small (46–48) with the exception of gonadotrophs (46, 49). However, in chronically oestrogenized rats, the large lactotroph population undergoing hypertrophia has been also extensively described (6, 8, 26, 46, 48, 50, 51). Another important factor is that functionally and structurally different populations of lactotrophs have been described both

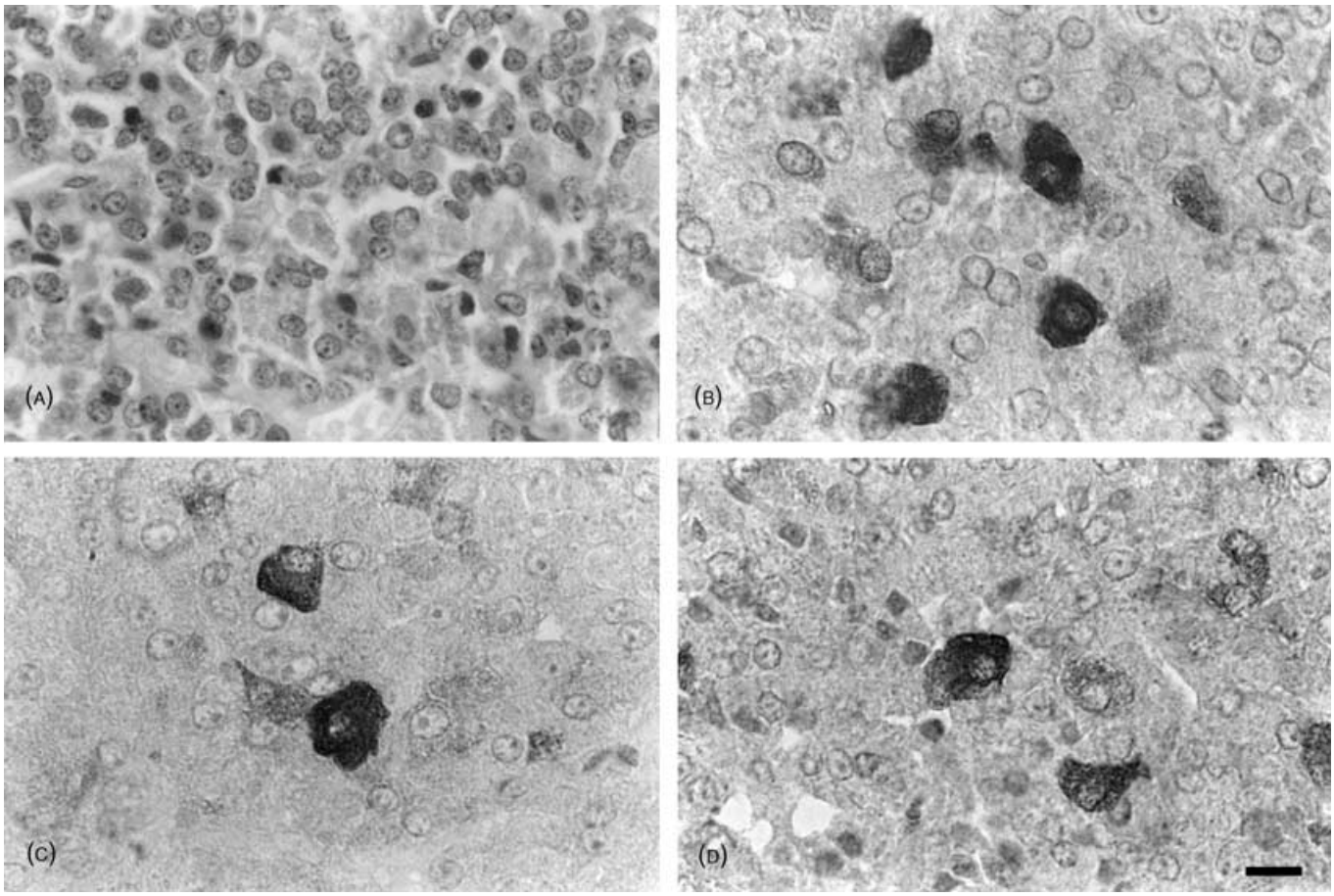

FIG. 4. Representative photomicrographs of VIP immunoreactivity in anterior pituitary glands from (A) ovariectomized rats (OVX), (B) OVX + diethylstilbestrol (DES), (C) OVX + testosterone propionate (TP) + DES and (D) gonadectomized (GDX) + DES rats, respectively. Magnification ×1000. Scale bar = 10 μm.

in normal pituitary glands and in DES-induced pituitary tumours (36, 52–54). In this sense, typical (type I) lactotrophs are characterized by large secretory granules and enhanced biosynthetic and releasing activity, whereas atypical (types II and III) lactotrophs contain smaller granules and lower efficiency for prolactin secretion (52–54). In a recent study, we described the prevalence of typical lactotrophs in tumours from OVX + DES rats, in opposition to tumours from OVX + TP + DES and GDX + DES, in which atypical lactotrophs were also present (6). Accordingly, it could be possible that this different composition accounts for the higher serum prolactin levels observed in OVX + DES rats compared to OVX + TP + DES and GDX + DES rats. Moreover, the possible presence of mammosomatotrophs, a transitional cell that secretes both prolactin and growth hormone (55), is an additional factor to be taken into account. If the population of mammosomatotrophs differs in OVX + DES pituitaries from OVX + TP + DES and GDX + DES glands, it could also explain the differences observed in prolactin secretion. We can speculate that galanin- and VIP-expressing lactotrophs correspond to a certain type of lactotroph and not to all of them, raising the possibility that the differential expression of the peptides described in this study is due to the different population of prolactin-producing cells in tumours from OVX + DES on one side and OVX + TP + DES and GDX + DES on the other side. Further studies will be necessary to ascertain this possibility.

Acknowledgements

This work was supported by grants from Universidad de Buenos Aires (TM13 and JM19), Fundación Alberto Roemmers, CONICET (PIP 4103 and PEI 0005/97) and FONCYT (BID 802 OC AR PICT97 00438). The authors thank Ms Paulina Roig and Analia Lima for their technical assistance, and Dr V. Lux-Lantos for prolactin measurements. The scientific support of Dr Bruce S. McEwen and Dr Lawrence P. Reagan is gratefully acknowledged.

Accepted 13 October 2003

References

1 Lamberts SWJ, MacLeod RM. Regulation of prolactin secretion at the level of the lactotroph. *Physiol Rev* 1990; **70:** 279–318.
2 Selye H, Collip JB, Thomson DL. Effect of estrin on ovaries and adrenals. *Proc Soc Exp Biol Med* 1935; **32:** 1377–1381.
3 Wiklund J, Wertz N, Gorski JA. A comparison of estrogen effects on uterine and pituitary growth and prolactin synthesis in F344 and Holtzman rats. *Endocrinology* 1981; **109:** 1700–1707.
4 Piroli GG, Weisenberg LS, Grillo CA, De Nicola AF. Subcellular distribution of cyclic adenosine 3′,5′-monophosphate-binding protein and estrogen receptors in control pituitaries and estrogen-induced pituitary tumors. *J Natl Cancer Inst* 1990; **82:** 596–601.
5 Piroli GG, Lima AE, Díaz-Torga G, De Nicola AF. Biochemical parameters in the anterior pituitary during the course of tumorigenesis induced by diethylstilbestrol treatment. *J Steroid Biochem Mol Biol* 1994; **51:** 183–189.
6 Piroli GG, Torres A, Pietranera L, Grillo CA, Ferrini MG, Lux-Lantos V, Aoki A, De Nicola AF. Sexual dimorphism in diethylstilbestrol-induced

prolactin pituitary tumors in F344 rats. *Neuroendocrinology* 2000; **72:** 80–90.
7 Hyde JF, Engle MG, Maley BE. Colocalization of galanin and prolactin within secretory granules of anterior pituitary cells in estrogen treated Fischer 344 rats. *Endocrinology* 1991; **129:** 270–276.
8 Steel JH, Gon G, O'Halloran DJ, Jones PM, Yanaihara N, Ishikawa H, Bloom SR, Polak JM. Galanin and vasoactive intestinal polypeptide are colocalised with classical pituitary hormones and show plasticity of expression. *Histochemistry* 1989; **93:** 183–189.
9 Kaplan LM, Gabriel SM, Koenig JI, Sunday ME, Spindel ER, Martin JB, Chin WW. Galanin is an estrogen-inducible, secretory product of the rat anterior pituitary. *Proc Natl Acad Sci USA* 1988; **85:** 7408–7412.
10 Vrontakis ME, Peden LM, Duckworth ML, Friesen HG. Isolation and characterization of a complementary DNA (galanin) clone from estrogen-induced pituitary tumor messenger RNA. *J Biol Chem* 1987; **262:** 16755–16758.
11 Vrontakis ME, Yamamoto T, Schroedter IC, Nagy JI, Friesen HG. Estrogen induction of galanin synthesis in the rat anterior pituitary gland demonstrated by in situ hybridization and immunohistochemistry. *Neurosci Lett* 1989; **100:** 59–64.
12 O'Halloran DJ, Jones PM, Steel JH, Gon G, Giaid A, Gathei MA, Pollack JM, Bloom SR. Effect of endocrine manipulation on anterior pituitary galanin in the rat. *Endocrinology* 1990; **127:** 467–475.
13 Hsu DW, el Azouzi M, Black PM, Chin WW, Hedley Whyte ET, Kaplan LM. Estrogen increases galanin immunoreactivity in hyperplastic prolactin-secreting cells in Fischer 344 rats. *Endocrinology* 1990; **126:** 3159–3167.
14 Piroli GG, Pietranera L, Grillo CA, De Nicola AF. Dehydroepiandrosterone regulation of prolactin gene expression in the anterior pituitary does not depend on galanin induction. *Neuroendocrinol Lett* 2002; **23:** 321–324.
15 Ponchon M, Lause P, Maiter D. In vitro effects of oestradiol on galanin gene expression in rat anterior pituitary cells. *J Neuroendocrinol* 2000; **12:** 559–564.
16 Shen ES, Hardenburg JL, Meade EH, Arey BJ, Merchenthaler I, López FJ. Estradiol induces galanin gene expression in the pituitary of the mouse in an estrogen receptor alpha-dependent manner. *Endocrinology* 1999; **140:** 2628–2631.
17 Wynick D, Hammond PJ, Akinsanya KO, Bloom SR. Galanin regulates basal and oestrogen-stimulated lactotroph function. *Nature* 1993; **364:** 529–532.
18 Cai H, Bowers RC, Moore JP, Hyde JF. Function of galanin in the anterior pituitary of estrogen-treated Fischer 344 rats: autocrine and paracrine regulation of prolactin secretion. *Endocrinology* 1998; **139:** 2452–2458.
19 Wynick D, Small CJ, Bacon A, Holmes FE, Norman M, Ormandy CJ, Kilic E, Kerr NCH, Ghatei M, Talamantes F, Bloom SR, Pachnis V. Galanin regulates prolactin release and lactotroph proliferation. *Proc Natl Acad Sci USA* 1998; **95:** 12671–12676.
20 Cai A, Hayes JD, Patel N, Hyde JF. Targeted overexpression of galanin in lactotrophs of transgenic mice induces hyperprolactinemia and pituitary hyperplasia. *Endocrinology* 1999; **140:** 4955–4964.
21 Enjalbert A, Arancibia S, Ruberg M, Priam M, Bluet-Pajot MT, Rotsztejn WH, Kordon C. Stimulation of *in vitro* prolactin release by vasoactive intestinal peptide. *Neuroendocrinology* 1980; **31:** 200–204.
22 Lam KS, Reichlin S. Pituitary vasoactive intestinal peptide regulates prolactin secretion in the hypothyroid rat. *Neuroendocrinology* 1989; **50:** 524–528.
23 Hagen TC, Arnaout MA, Scherzer WJ, Martinson DR, Garthwaite TL. Antisera to vasoactive intestinal polypeptide inhibit basal prolactin release from dispersed anterior pituitary cells. *Neuroendocrinology* 1986; **43:** 641–645.
24 Mezey E, Kiss JZ. Vasoactive intestinal peptide-containing neurons in the paraventricular nucleus may participate in regulating prolactin secretion. *Proc Natl Acad Sci USA* 1985; **82:** 245–257.
25 Lam KS. Vasoactive intestinal peptide in the hypothalamus and the pituitary. *Neuroendocrinology* 1991; **53** (Suppl. 1): 45–51.
26 Köves K, Gottschall PE, Görks T, Scammell JG, Arimura A. Presence of immunoreactive vasointestinal polypeptide in anterior pituitary of normal male and long term estrogen-treated female rats: a light microscopic immunohistochemical study. *Endocrinology* 1990; **126:** 1756–1763.
27 Lam KS, Srivastava G, Lechan RM, Lee T, Reichlin S. Estrogen regulates the gene expression of vasoactive intestinal peptide in the anterior pituitary. *Neuroendocrinology* 1990; **52:** 417–421.
28 Piroli GG, Cassataro J, Pietranera L, Grillo CA, Ferrini M, Lux-Lantos V, De Nicola AF. Progestin regulation of galanin and prolactin gene expression in oestrogen-induced pituitary tumours. *J Neuroendocrinol* 2001; **13:** 302–309.
29 Vaticon MD, Fernandez Galaz MC, Tejero A, Aguilar E. Alteration of prolactin control in adult rats treated neonatally with sex steroids. *J Endocrinol* 1985; **105:** 429–433.
30 Dyer RG, Mansfield S. Neonatal testosterone treatment potentiates stimulated prolactin secretion in adult oestrogen-primed rats. *J Endocrinol* 1986; **109:** 57–60.
31 Watanobe H, Takebe K. A comparative study of the effects of neonatal androgenization and estrogenization on vasoactive intestinal peptide levels in the anterior pituitary and the hypothalamus of adult female rats. *Neuroendocrinology* 1992; **56:** 653–659.
32 Gala RR. The influence of estrogen administration on plasma prolactin levels in the neonatally androgenized female rat. *Proc Soc Exp Biol Med* 1981; **166:** 216–220.
33 Matsumoto A, Arai Y. Effect of androgen on sexual differentiation of synaptic organization in the hypothalamic arcuate nucleus: an ontogenetic study. *Neuroendocrinology* 1981; **33:** 166–169.
34 Ben-Jonathan N. Dopamine: a prolactin-inhibiting hormone. *Endocr Rev* 1985; **6:** 564–589.
35 Gonzalez Parra S, Argente J, Garcia Segura LM, Chowen J. Cellular composition of the adult rat anterior pituitary is influenced by the neonatal sex steroid environment. *Neuroendocrinology* 1998; **68:** 152–162.
36 Hymer WC, Motter KA. Heterogeneity in mammotrophs prepared from diethylstilbestrol-induced prolactinomas. *Endocrinology* 1988; **122:** 2324–2338.
37 Todd JF, Small CJ, Akinsanya KO, Stanley SA, Smith DM, Bloom SR. Galanin is a paracrine inhibitor of gonadotroph function in the female rat. *Endocrinology* 1998; **139:** 4222–4229.
38 Hyde JF, Keller BK, Howard G. Dopaminergic regulation of galanin gene expression in the rat anterior pituitary gland. *J Neuroendocrinol* 1992; **4:** 449–454.
39 Nagy G, Mulchahey J, Neill JD. Autocrine control of prolactin secretion by vasoactive intestinal peptide. *Endocrinology* 1988; **122:** 364–366.
40 Chew L-J, Seah V, Murphy D, Carter D. Anterior pituitary vasoactive intestinal peptide mRNA is colocalised with prolactin mRNA in hyperoestrogenised rats. *J Mol Endocrinol* 1996; **16:** 211–220.
41 Carrillo AJ, Phelps CJ. Quantification of vasoactive intestinal peptide immunoreactivity in the anterior pituitary glands of intact male and female, ovariectomized, and estradiol benzoate-treated rats. *Endocrinology* 1992; **131:** 964–969.
42 Maletti M, Rostene WH, Carr L, Scherrer H, Rotten D, Kordon C, Rosselin G. Interaction between estradiol and prolactin on vasoactive intestinal peptide concentrations in the hypothalamus and in the anterior pituitary of the female rat. *Neurosci Lett* 1982; **32:** 307–313.
43 Kasper S, Popescu RA, Torsello A, Vrontakis ME, Ikejiani C, Friesen HG. Tissue-specific regulation of vasoactive intestinal peptide messenger ribonucleic acid levels by estrogen in the rat. *Endocrinology* 1992; **130:** 1796–1801.
44 O'Halloran DJ, Jones PM, Gathei MA, Domin J, Bloom SR. The regulation of neuropeptide expression in rat anterior pituitary following chronic manipulation of estrogen status: a comparison between substance P, neuropeptide Y, neurotensin, and vasoactive intestinal peptide. *Endocrinology* 1990; **127:** 1463–1469.
45 Prysor-Jones RA, Silverlight JJ, Kennedy SJ, Jenkins JS. Vasoactive intestinal peptide and the stimulation of lactotroph growth by oestradiol in rats. *J Endocrinol* 1988; **116:** 259–265.
46 Tougard C, Tixier-Vidal A. Lactotropes and gonadotropes. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction*, 2nd edn. New York: Raven Press Ltd, 1994: 1711–1747.
47 Osamura RY, Komatsu N, Izumi S, Yoshimura S, Watanabe K. Ultrastructural localization of prolactin in the rat anterior pitutiary glands by preembedding peroxidase-labeled antibody method: observations in normal, castrated or estrogen-stimulated specimen. *J Histochem Cytochem* 1982; **30:** 919–925.
48 Vrontakis ME, Thliveris JA, Friesen HG. Influence of bromocriptine and oestrogen on prolactin synthesis, secretion and tumour growth in vivo in rats. *J Endocrinol* 1987; **113:** 383–388.
49 Ibrahim SN, Moussa SM, Childs GV. Morphometric studies of rat anterior pituitary cells after gonadectomy: correlation of changes in gonadotropes with the serum levels of gonadotropins. *Endocrinology* 1986; **119:** 629–637.
50 Holtzman S, Stone JP, Shellabarger CJ. Influence of diethylstilbestrol treatment on prolactin cells of female ACI and Sprague-Dawley rats. *Cancer Res* 1979; **39:** 779–784.

51 Phelps C, Hymer WC. Characterization of estrogen-induced adenohypophyseal tumors in the Fischer 344 rat. *Neuroendocrinology* 1983; **37:** 23–31.
52 Takahashi S, Mayatake M. Immuno-electron microscopical study of prolactin cells. *Zool Sci* 1991; **8:** 549–559.
53 De Paul A, Pons P, Aoki A, Torres AI. Heterogeneity of pituitary lactotrophs: immunocytochemical identification of functional subtypes. *Acta Histochem* 1997; **99:** 277–289.
54 Kurosomi K. Ultrastructural modifications in prolactin-producing cells in the adenohypophysis during post-natal development and functional variations. In: Koshino K, ed. *Molecular Biology to Clinical Problems*. Amsterdam: Elsevier, 1988: 289–297.
55 Frawley LS, Boockfor FR. Mammosomatotrophs: Presence and functions in normal and neoplastic pituitary tissue. *Endocr Rev* 1991; **12:** 337–355.