

Sex-related effects of hypothyroidism on cellular proliferation in the hypophyseal mammotroph population of the rat

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SUMMARY

Changes in the morphology and number of prolactin-immunoreactive cells have been described in hypothyroidism. However, no analyses of the changes that occur in the proliferation of these cells have been undertaken. In order to determine the proliferation rate of rat hypophyseal prolactin cells in hypothyroidism and whether the observed changes are related to increases in TRH, a double immunohistochemical study for PCNA and prolactin was carried out in thyroidectomised rats and in rats treated with TRH. TRH treatment, but not thyroidectomy, increased the percentage of prolactin-producing cells in both sexes ($p < 0.01$). However, whereas the cellular proliferation of prolactin-producing cells was significantly increased in males ($p < 0.01$) in both experimental designs, in female rats the proliferation of prolactin-producing cells was increased after treatment with TRH, but not in thyroidectomised rats ($p < 0.01$). Our results demonstrate —mainly in male rats— that modifications in the hypophyseal-thyroid axis elicit changes in prolactin-producing cells that are related to the proliferation rate of these cells. These results suggest that sex-related effects of both TRH and thyroid hormones could be involved

in the physiological regulation of the proliferation of pituitary prolactin-producing cells, because the responses to treatment with TRH were different from the changes observed in thyroidectomised rats.

Key words: Mammothroph cells – Cellular proliferation – Hypothyroidism – Methimazole

INTRODUCTION

Hypothyroidism induces an increase in prolactin secretion that is accompanied by a decrease in the hypophyseal content of the hormone (Lam et al., 1989). Morphologically, hypothyroidism elicits an increase in the size of prolactin-immunoreactive cells, increasing the synthesis, and probably the release, of the hormone (Carretero et al., 1992). All this is a consequence of the fact that a deficit of thyroid hormones leads to an increase in the synthesis of TRH and VIP (Segerson et al., 1987, 1989), both of which are well known markers of the synthesis and release of prolactin (Panerai et al., 1977; Schelinger et al., 1978; Pérez-López et al., 1981; Geras and Gershengorn, 1981; Albert and Tashjian, 1984).

In states of hypothyroidism, an increase occurs in the synthesis of TRH (Segerson et al., 1987) and in its release to the portal system (Rondeel et al., 1988); as a result, prolactin and TSH levels increase (Snyder et al., 1973). If this situation is palliated by the administration of thyroid hormones, the levels of both hormones return to normal values (Snyder et al., 1973; Hermite et al., 1974; Yamajo, 1974).

Nevertheless, it remains to be elucidated whether the increase in the activity of prolactin cells in hypothyroidism is exclusively due to the action of TRH or whether the absence of thyroid hormones is also involved.

Although quite a few authors have addressed the secretory response of prolactin in physiological or experimental states that induce such secretion, such as in the case of hypothyroidism, few studies have investigated whether or not the known secretory responses are due (among other causes) to increases in the number of cells able to produce the hormone or whether such possible increases are due to stimulation of the proliferation of that cellular population.

Here we performed a double immunocytochemical labelling for PCNA and prolactin with a view to analysing the effects of hypothyroidism on the proliferation of prolactin cells. In a second phase of the study, aimed at determining whether the effect was due to the increase in TRH brought about by hypothyroidism and not by the deficit in thyroid hormones, we performed the same technique in rats rendered non-hypothyroid by TRH treatment; unlike the hypothyroid animals, these latter showed an increase in thyroid hormone levels in peripheral blood.

MATERIALS AND METHODS

Animals

In this study we used 30 adult albino Sprague-Dawley rats of both sexes. The animals, with an approximate weight of 200 g, were kept under standard stabling conditions ($22 \pm 2^\circ\text{C}$, relative humidity $50 \pm 5\%$, a controlled photoperiod of 14h light/10h darkness), with water and food (Panlab maintenance diet) *ad libitum*. The animals were divided into three groups, each containing 10 animals (5 of each sex). A first control group, considered untreated, contained males and females in the pro-oestrus phase, as deter-

mined by means of vaginal smears. A second group included animals that—under Ketolar anaesthesia—were subjected to bilateral thyroidectomy and the remains of the thyroid gland were blocked by treatment with the antithyroid agent methimazole for 14 days, following the protocol of Stoll (Stoll et al., 1978). The third treatment group received a single dose of 10^{-5} M of TRH suspended in saline intraperitoneally and the animals were sacrificed six hours later. The handling and care of the animals followed European Communities Council Directive (86/609/EEC) and current Spanish legislation regarding the use and treatment of laboratory animals (Official State Bulletin 67/8509-12, 1998).

Animal sacrifice and sample processing

The animals were sacrificed by decapitation following anaesthesia by Forene inhalation. The hypophyses were carefully dissected out and immediately fixed in a solution of 15% saturated picric acid in 4% paraformaldehyde plus 0.1 M phosphate buffer, pH 7.4, for 24 h. Then, they were dehydrated in ethanol, cleared with xylol, and embedded in paraffin in order to obtain serial sections of 5 mm thickness. These were placed on slides treated with gelatin-chrome alum and were then used for the immunohistochemical study.

Immunocytochemistry

To study PCNA-positive cells and to determine the PCNA-Prolactin labelling index, a double-labelling immunohistochemical method for PCNA and prolactin was applied. Endogenous peroxidase was blocked with H_2O_2 in methanol and non-specific reactions of the secondary antibody by incubation in normal goat serum (Dako, diluted 1:30). Sections were incubated overnight at 4°C with mouse anti-PCNA monoclonal antibody (PC10, Dako, diluted 1:2000 in TBS). Biotinylated goat anti-mouse IgG (Dako, diluted 1:100) and Avidin-Biotinylated horseradish peroxidase complex (ABC kit, Dako, diluted 1:100) were successively applied at room temperature for 40 min and 30 min, respectively. The reaction was developed in freshly prepared 3,3'-DAB (0.025% in TRIS buffer containing 0.03% H_2O_2). Following PCNA immunolabelling, the peroxidase-antiperoxidase (PAP) reaction was performed for the detection of prolactin, using as primary serum anti-prolactin rabbit serum at a dilution of 1:800, swine anti-rabbit serum (Dako,

diluted 1:100), and rabbit-PAP complex (Dako, diluted 1:100). Preabsorption tests with prolactin and tests substituting the specific serum by normal rabbit serum abolished the reaction. By ELISA, the specificity of the swine anti-rabbit IgG was lower than 1% for rat and mouse IgG and 100% for rabbit IgG. For the washes and dilutions of the sera, TRIS buffer (0.05 M, pH7.4) containing 0.8% NaCl was used. The reaction was developed in freshly prepared 4-chloro-1-naphthol (1.7×10^{-3} M in 3% absolute ethanol and TRIS-buffer containing 0.3% H_2O_2).

Quantification of prolactin and PCNA-prolactin immunoreactive cells

Using a Nikon Optiphot-2 microscope at a final magnification of x500 and counting 4000 glandular cells, taken from all zones of the gland, from the hypophysis of each animal included in the study, the following labelling indices were determined: 1) cells reactive to PCNA (reactive or not to the hormone), 2) cells reactive to prolactin (reactive or not to PCNA) and 3) cells reactive to both PCNA and prolactin, in all three cases as referred to the number of reactive cells with respect to the total number of cells counted. A calculation was also made of the percentage of cells reactive to both PCNA and prolactin with respect to the total number of cells reactive to the hormone. These indices were obtained for each of the experimental groups.

Statistical analysis

For each parameter evaluated, the values obtained were processed statistically and the differences observed were compared using analysis of variance, accepting p values of <0.05 as significant for the Scheffé F test. The results are expressed as arithmetic means \pm SD.

RESULTS

Hypophyseal cellular proliferation

Untreated animals. Hypophyseal proliferation, determined as the percentage of cells showing PCNA-immunoreactive nuclei, was very low in the untreated animals, and although the values found were slightly higher in males than in females (1.35 ± 0.11 vs. 0.74 ± 0.05) the differences were not statistically significant (Fig. 1).

Effects induced by thyroidectomy. The effects induced by the lack of thyroid hormones after thyroidectomy and treatment with methy-

zole were analysed in both sexes and the results were seen to be completely different (Fig. 1). Thus, whereas in female rats chronic hypothyroidism did not induce variations in the cellular proliferation with respect to the untreated females (1.07 ± 0.08 vs. 0.74 ± 0.05), in the males it led to a significant increase ($p < 0.01$) in comparison with the untreated males (4.69 ± 0.62 vs. 1.35 ± 0.11).

Effects induced by TRH. Unlike the situation in the hypothyroid animals, in both sexes TRH elicited a significant increase in hypophyseal proliferation with respect to the untreated animals (females: 3.11 ± 0.43 vs. 0.74 ± 0.05 , $p < 0.01$; males: 3.47 ± 0.27 vs. 0.74 ± 0.05 , $p < 0.01$). However, in the males, the increase was less marked than that induced after thyroidectomy (3.47 ± 0.27 vs. 4.69 ± 0.62 , $p < 0.05$).

Proliferation in prolactin-producing cells

Untreated animals. The percentage of prolactin-producing cells was very similar in both sexes (Fig. 2) and no statistically significant differences were observed in any case. Thus, in the females $30.22 \pm 0.28\%$ of the glandular cells were prolactin-positive and $30.16 \pm 0.34\%$ of them were so in the males. The situation was similar on considering prolactin cells undergoing proliferation (Fig. 3): $0.48 \pm 0.01\%$ of the hypophyseal cells of the females and $0.56 \pm 0.10\%$ in the males.

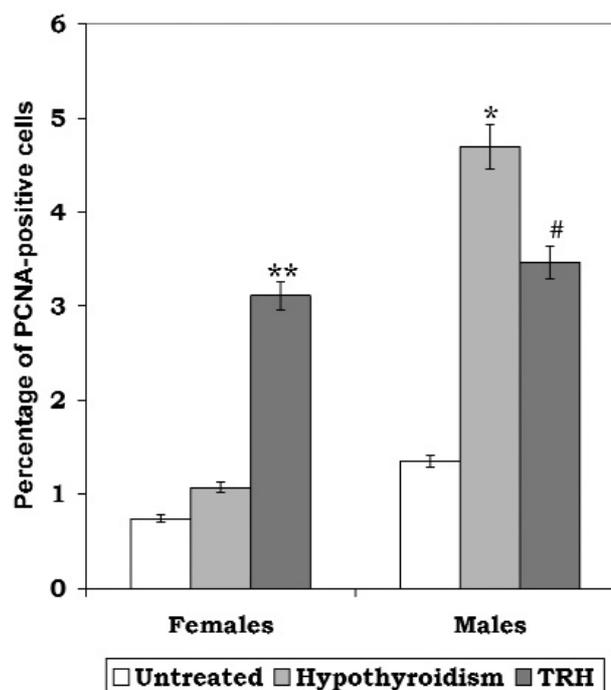


Figure 1. Percentage of cells undergoing proliferation -PCNA-positive- in the hypophysis of the different groups of animals studied (*: $p < 0.01$ with respect to the untreated males; **: $p < 0.01$ with respect to the untreated females; #: $p < 0.01$ with respect to the untreated males and $p < 0.05$ with respect to the hypothyroid males).

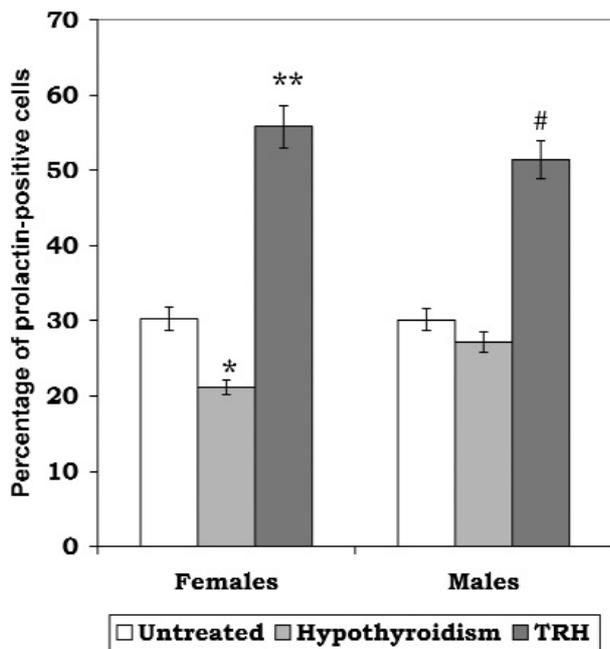


Figure 2. Plot showing percentage of prolactin-positive cells in the hypophysis of the different groups of animals studied (*: $p < 0.05$ with respect to the untreated females; **: $p < 0.01$ with respect to the untreated and hypothyroid females; #: $p < 0.01$ with respect to the untreated and hypothyroid males).

Effects induced by thyroidectomy. In the hypothyroid females, $21.13 \pm 0.13\%$ of the glandular cells of the hypophysis were immunoreactive to the hormone, a significantly lower percentage than that observed in the untreated females (21.13 ± 0.13 vs. 30.22 ± 0.28 , $p < 0.05$). However, no significant differences were observed in cells jointly reactive to prolactin and PCNA (0.48 ± 0.06 vs. 0.44 ± 0.01). In the hypothyroid males, a slight decrease in the percentage of immunoreactive cells was observed with respect to the untreated males, although no significant differences were recorded (27.22 ± 0.91 vs. 30.16 ± 0.34). Nevertheless, regarding the mammoth cells in the cell cycle, in the hypothyroid males the percentage of cells immunoreactive to prolactin and PCNA was greater than that seen in the untreated males (1.66 ± 0.46 vs. 0.56 ± 0.10 , $p < 0.01$). A similar difference was observed with respect to the hypothyroid females (1.66 ± 0.46 vs. 0.48 ± 0.06 , $p < 0.01$).

Effects induced by treatment with TRH. Treatment with TRH induced an increase in the percentage of prolactin-producing cells in both sexes, with respect to untreated rats (females: 55.78 ± 0.48 vs. 30.22 ± 0.28 , $p < 0.01$; males: 51.43 ± 1.20 vs. 30.16 ± 0.34 , $p < 0.01$). These increases were accompanied by

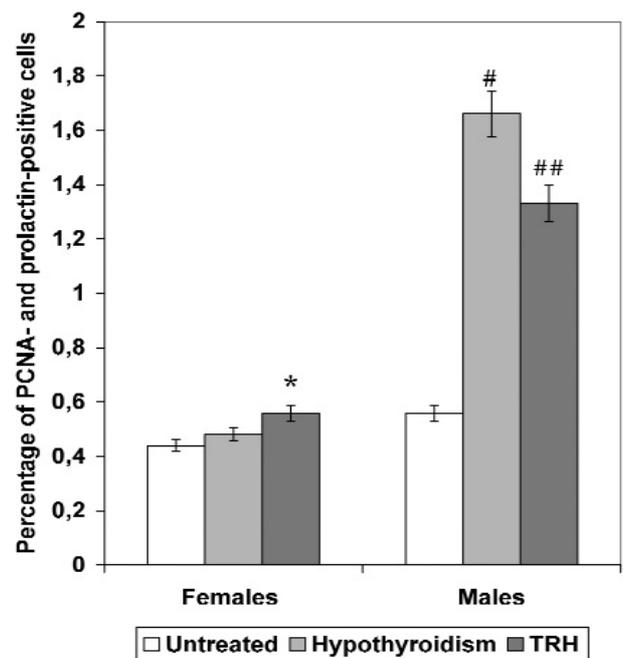


Figure 3. Percentage of prolactin cells in proliferation (prolactin- and PCNA-positive) in the hypophysis of the different groups of animals studied, (*: $p < 0.05$ with respect to the untreated females; #: $p < 0.01$ with respect to the untreated males and the hypothyroid females; ##: $p < 0.01$ with respect to the untreated males and the TRH-treated females).

an increase in the percentage of proliferating prolactin-producing cells. However, this increase was more pronounced in the males ($p < 0.01$ with respect to the untreated males, 1.33 ± 0.21 vs. 0.56 ± 0.10) than in the females ($p < 0.05$, 0.56 ± 0.04 vs. 0.44 ± 0.01) ($p < 0.01$ on comparing the two sexes of animal: 1.33 ± 0.21 vs. 0.56 ± 0.04).

Upon comparing the effects of TRH with those induced by hypothyroidism, it was observed that in both sexes the percentage of cells immunoreactive to prolactin was greater after treatment with TRH ($p < 0.01$, 55.78 ± 0.48 vs. 21.13 ± 0.13 in the females, and $p < 0.01$ in the males, 51.43 ± 1.20 vs. 27.22 ± 0.91). This, however, was not the case upon analysing the percentage of cells jointly immunoreactive to prolactin and PCNA, since a sex-related difference was observed: whereas in the females TRH increased, but not significantly, in comparison with the hypothyroid females (0.56 ± 0.14 vs. 0.48 ± 0.06), in the males the percentage decreased slightly, although not significantly (1.33 ± 0.21 vs. 1.66 ± 0.46).

DISCUSSION

Owing to the high incidence of prolactinomas, the proliferation of prolactin cells has

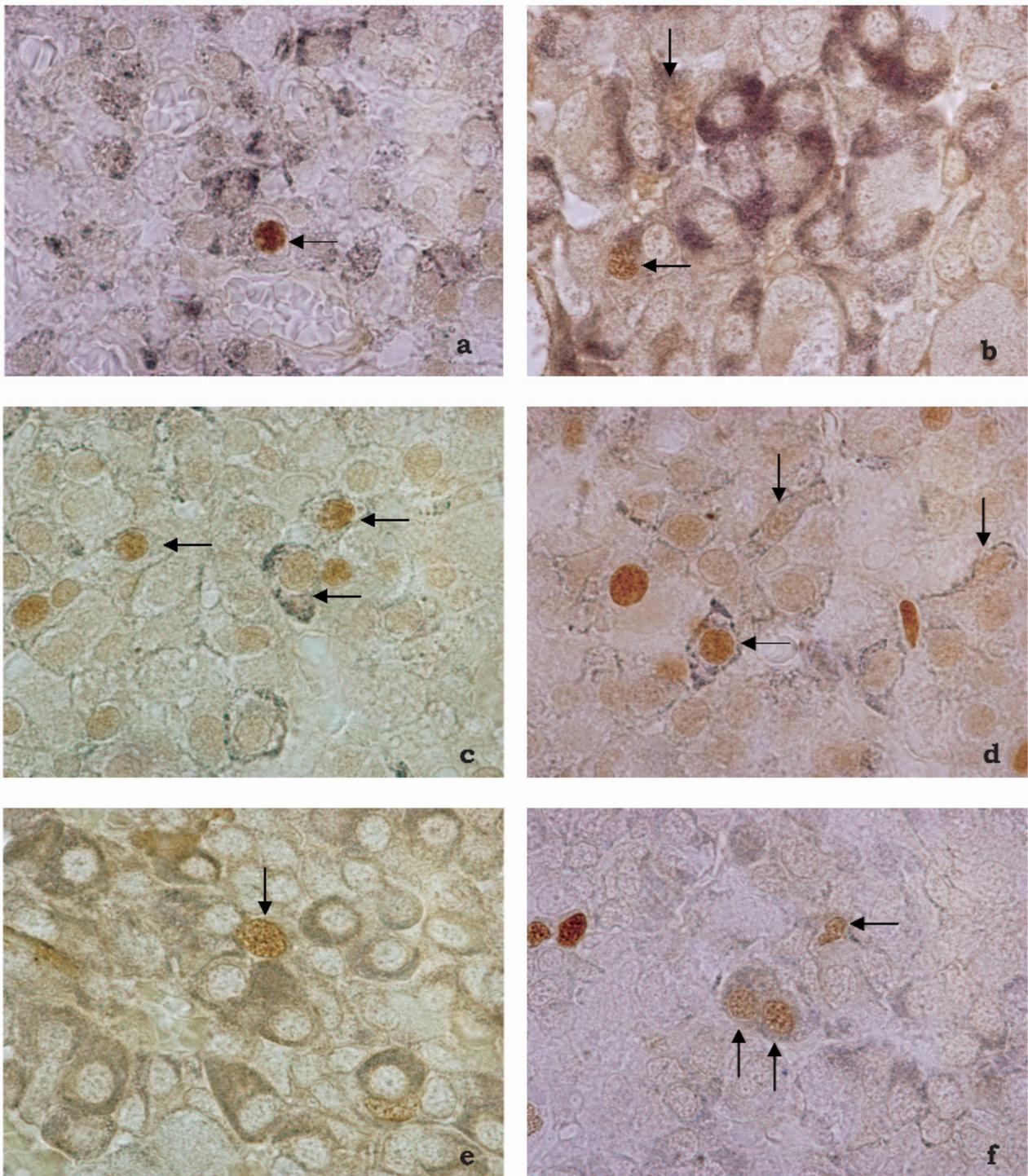


Figure 4. Micrographs showing the typical aspect of the immunocytochemical reactions for prolactin (dark blue) and PCNA (brown) in the untreated rats (a: females, b: males), in the hypothyroid rats (c: females, d: males) and in the TRH-treated rats (e: females, f: males). x 675.

been studied in some depth and most investigations have been carried out addressing treatment with oestradiol, tumour proliferation, *in vitro* proliferation and the use of drugs that are agonists of dopamine (Lloyd et al., 1975; Kalbermann et al., 1979; Burdman et al., 1984; Pérez et al., 1986; Stefanescu et al., 1992; Zhou-Li et al., 1992; Oishi et al., 1993;

Carretero et al., 1995). As far as we are aware, there are no studies that have analysed the proliferation of prolactin-producing cells in situations of hypothyroidism.

Although the absence of experimental work attempting to relate the various states of hypothyroidism with increases in proliferative activity prevents us from contrasting our

results with those of others, in this endocrine state prolactin synthesis may increase up to levels at which, as in other glandular cells, such activity ceases and cellular division shoots up.

It is known that thyroid hormones are necessary for the maintenance of the normal structure and secretory activity of prolactin cells (Meites and Turner, 1947). *In vivo* investigations carried out at our laboratory have shown that in hypothyroidism induced by methimazole the morphology of prolactin cells is modified, with considerable increases in cell and nuclear sizes, a granular immunoreactive deposit, and the appearance of cytoplasmic prolongations, all these being clear signs of hyperactivity (Carretero et al., 1992).

The present findings show a sex-dependent response to thyroidectomy: in females the proliferative activity of prolactin cells was similar in thyroidectomised and in untreated animals; however, in male rats the proliferative activity rose to percentages three-fold higher than those seen in untreated males.

Additionally, TRH is considered to be one of the hypothalamic factors able to stimulate prolactin release (Mueller et al., 1973; Machlin et al., 1974; Drouin et al., 1976; Panerai et al., 1977; Schelinger et al., 1978; Tindal 1978; Van Laudghem and Va de Wiel, 1978; Pérez-López et al., 1981; Gershengorn, 1982, 1985; Batten and Wigham, 1984; Wigham and Batten, 1984; Martin, 1985). Despite this, several authors have questioned its action in male rats following its acute systemic administration (Lu et al., 1972; Stevens and Lawson, 1977; Shin, 1978; Carretero et al., 1992).

Moreover, *in vitro*, TRH increases the release of prolactin. Other findings suggest that TRH would first induce increases in the release and later synthesis of the hormone (Martin and Tashjian, 1977; Tixier-Vidal and Gourdji, 1981; Tougard and Tixier-Vidal, 1988).

The present results, showing a marked increase in the number of prolactin-reactive cells in animals treated with TRH and also in the proliferation of prolactin cells-greater in TRH-treated males than in untreated males or TRH-treated females-, demonstrate that apart from eliciting greater cellular activity, TRH is able to stimulate cell proliferation. However, this proliferative effect is more evident in male than in female rats.

Our results demonstrate that TRH increases the percentage of prolactin-producing cells

in both sexes. However, this increase is induced by cellular proliferation only in male rats.

Moreover, variations in the percentage and proliferation of prolactin-producing cells in thyroidectomized rats are not well correlated with increases of TRH, mainly in female rats, and our results suggest that in these the effects of thyroidectomy would be related to deficits of thyroid hormones.

The fact that we observed an increase in proliferative activity in both thyroidectomised and TRH-treated male animals allows us to conclude not only that the increase in the proliferative activity of prolactin cells in the hypothyroidism of these animals is due to the absence of thyroid hormones but also that the stimulatory effect of TRH plays an important role.

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