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Detection of gonadotropin-releasing hormone receptor in normal human pituitary cells and pituitary adenomas using immunohistochemistry

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Abstract Gonadotropin-releasing hormone (GnRH), which is a well-known regulator of gonadotroph function, has recently been considered to be a paracrine factor involved in the control of somatotroph, lactotroph, and corticotroph cells. GnRH action is initiated by binding to a specific cell surface receptor, the gonadotropin-releasing hormone receptor (GnRHR), which is expressed by follicle-stimulating hormone/luteinizing hormone (FSH/LH) cells. Using in situ hybridization techniques, GnRHR messenger ribonucleic acid (mRNA) has recently been detected in normal human anterior pituitary gland and in various pituitary adenomas, including FSH/LH-cell, growth hormone (GH)-cell, adrenocorticotropic hormone (ACTH)-cell, and null-cell adenomas. However, immunohistochemical studies indicating the specific cell distribution of GnRHR in normal pituitary cells have never been reported. The aim of the present investigation was to evaluate the immunohistochemical expression of GnRHR in different types of normal pituitary cells and related tumors. Using double-label immunohistochemical techniques on formalin-fixed and paraffin-embedded tissues and specific antibodies directed against pituitary hormones and GnRHR, we found GnRHR immunoreactivity not only in FSH/LH cells, but also in GH- and thyroid-stimulating hormone (TSH) cells. GnRHR was detected in FSH/LH-cell, GH-cell, mixed GH- and prolactin (PRL)-cell, and α-subunit (α-SU)/null-cell adenomas. The findings of this study suggest that the interaction between GnRH and GnRHR may play a role in paracrine/autocrine regulation of different types of normal pituitary cells and pituitary adenomas.

Keywords Gonadotropin-releasing hormone receptor · Pituitary gland · Immunohistochemistry · Colocalization

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Introduction

The anterior pituitary gland regulates the functions of several peripheral glands through the secretion of anterior pituitary hormones. It is well known that its secretory activity is controlled by several hypothalamic-releasing hormones (HRHs), each of them modulating the secretion of one adenohypophysial hormone. However, this traditional view of univocal regulation of pituitary hormone secretion by its specific HRH has been revisited in the last 10 years. In fact, recent studies [17, 30, 31] have proven that different functional cell types of the anterior pituitary can respond to a single HRH, and pituitary cells of the same functional type can respond to two or more HRHs. Thyrotropin-releasing hormone (TRH), in addition to regulating thyroid-stimulating hormone (TSH) release, is a potent releaser of prolactin (PRL) [15] and, under some circumstances, of luteinizing hormone (LH) [9] and growth hormone (GH) [12, 32]. Other studies have suggested that gonadotropin-releasing hormone (GnRH), in addition to its actions on gonadotroph functions, might directly interfere with GH secretion [8, 16, 22, 34].

Traditionally, GnRH has been considered to be a hypothalamic hypophysiotropic-releasing hormone that physiologically regulates the secretion of follicle-stimulating hormone (FSH)/LH cell. However, GnRH messenger ribonucleic acid (mRNA) has been identified in normal human adenohypophysial cells, and this finding has suggested that GnRH, produced in the pituitary gland, may be involved in regulation of pituitary activity through a paracrine and/or autocrine mechanism [24, 25, 26]. The activity of GnRH depends on its binding to specific receptors (GnRHRs), which are known to be ex-

pressed by FSH/LH-secreting cells. Although GnRHR mRNA has recently been identified in normal pituitaries using reverse-transcription polymerase chain reaction (RT-PCR) and in situ hybridization (ISH) techniques [24, 26], the distribution of GnRHR in specific cell types of normal adenohypophysis has never been evaluated. Thus, it is unknown whether this receptor is expressed only by FSH/LH cells or also by other functional types of anterior pituitary cells. The hypothesis that cells, other than FSH/LH cells, may express GnRHRs is indirectly suggested by recent findings demonstrating GnRHR mRNA expression in non-gonadotroph pituitary adenomas, including GH-cell, adrenocorticotropic hormone (ACTH)-cell, and null-cell adenomas [26].

Immunohistochemistry, particularly double-label techniques, is useful in demonstrating the presence of two or more antigens within the same cell. The introduction of microwave oven heating in the double-immunostaining technique has eliminated major drawbacks related to antibody cross-reactivity and has provided several advantages over traditional methods [20]. Furthermore, the multiple immunoenzyme-staining technique has been successfully employed in studies demonstrating colocalization of hormones, growth factors, and growth factor receptors in normal endocrine cells and related tumors [18, 19]. This study was designed to evaluate the expression of GnRHRs in different types of normal pituitary cells and in pituitary adenomas using immunohistochemical techniques and to verify whether GnRHRs are expressed in non-gonadotrophic cells.

Materials and methods

We studied five normal pituitary glands obtained at autopsy within 24 h of death from adult subjects without endocrinological abnormalities and 50 well-characterized pituitary adenomas of different types, including 12 FSH/LH-cell, 10 α-SU/null-cell, 10 GH-cell, 3 mixed GH–PRL-cell, 5 PRL-cell, 2 TSH-cell, and 8 ACTH-cell adenomas. Tissue specimens of the pituitary adenomas were obtained during transphenoidal surgery.

Tissues were fixed in buffered formalin (formaldehyde 4% w/v and acetate buffer 0.005 mol/l) and embedded in paraffin. Sections were stained with hematoxylin and eosin, Grimelius' silver impregnation, and alcian blue (1%, pH 2.5)–periodic acid-Schiff (AB-PAS) technique. For immunohistochemical stains, 3-µm-thick sections were mounted on poly-L-lysine slides and then deparaffinized and hydrated through a graded alcohol series, ending with water. Endogenous peroxidase activity was inhibited by treating sections with 3% hydrogen peroxide for 10 min. Primary antibody incubations (Table 1) were done at 4°C for 18–20 h, followed by the avidin-biotin peroxidase complex (ABC) procedure. Sections were then immersed in 0.03% 3,3'-diaminobenzidine tetrahydrochloride and successively counterstained with Harris' hematoxylin. Colocalization studies were performed using double-label immunostains according to Mason [22] and Lan [20].

The anti-GnRHR antibody (clone A9E4), employed in this study, was directed against the 1–29 NH_2 terminal sequence of the extracellular domain of the human gonadotropin-releasing hormone receptor (GnRHR). Sections stained for ACTH were pretreated (10 min) with 0.003% subtilisin (Sigma, P4789; protease type XXVII or Nagarse protease) in 0.05 M tris-buffered saline pH 7.4. Sections stained with anti-FSH antibody were pretreated by immersion into 0.01 M citrate buffer solution, pH 6, and boiled in a microwave oven at 650 W (10 min). GnRHR antigen retrieval was performed using a microwave oven with a pressure cooker. In detail, slides were immersed in a plastic Coplin jar filled with 200 ml citrate buffer (0.01 M), pH 6, and put into a pressure cooker containing 500 ml distilled water. The pressure cooker was placed into the microwave that was set at 800 W for 15 min to achieve boiling, followed by 400 W for another 15 min to maintain boiling. Specificity controls consisted of absorption of each antiserum with 10–20 nM of its homologous antigen per milliliter of diluted antiserum, omission of the first layer, and use of control tissues with or without the pertinent antigen. The specificity of the 1–29 NH_2 terminal region of GnRHR was checked using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI), National Library of Medicine (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast). The BLAST protein database search program indicated that the amino acid sequence 1–29 of human GnRHR does not show any structural homology with other human pituitary hormones and pituitary hormone receptors. On the contrary, a structural homology exists between human GnRHR and GnRHRs of other species, including pig, rat, horse, and sheep.

Results

Normal pituitary

An intense immunoreactivity (IR) for GnRHR was found in numerous glandular cells (about 40%) of the normal anterior pituitary. GnRHR IR was not observed in the neurohypophysial, connective tissue, and endothelial cells. Positive cells showed a diffuse cytoplasmic staining with reinforcement at the membrane level in some cells. Colocalization studies (Fig. 1) demonstrated that GnRHR was present in the cytoplasm of all α-SU-, β-FSH-, β-LH-, and β-TSH-IR cells and of about 70% GH-IR cells. On the contrary, PRL-IR, ACTH-IR, and S100-IR cells did not show IR for GnRHR.

Table 1 Antibodies employed. *ACTH* adrenocorticotropic hormone; *β-FSH* β-follicle-stimulating hormone; *GH* growth hormone; *α-SU* α-subunit; *β-LH* β-luteinizing hormone; *PRL* prolactin; β-TSH β-thyroid-stimulating hormone; *GnRHR* gonadotropin-releasing hormone receptor; *P/M* polyclonal/monoclonal

*See materials and methods for details

Antibodies	P/M(clone)	Tissue treatment*	Dilution	Source
ACTH	P	Subtilisin	Undiluted	Dako, Copenhagen, Denmark
β-FSH	M(C10)	Microwave	1:20	Dako
GH	M(54/92A2)	No	1:100	Biogenex, San Ramon, Calif.
α-SU	M(5E8)	No	1:5000	Prof. Ghielmi, University of Brescia
β-LH	M(3LH5B6YH4)	No	1:100	Biogenex
PRL	M(MIPO203)	No	Undiluted	Biomeda, Foster City, Calif.
β-TSH	M(T-790–02)	No	Undiluted	Biomeda
protein S100	P	Subtilisin	1:500	Dako
GnRHR	M(A9E4)	Pressure cooker	1:20	Novocastra, Newcastle, UK

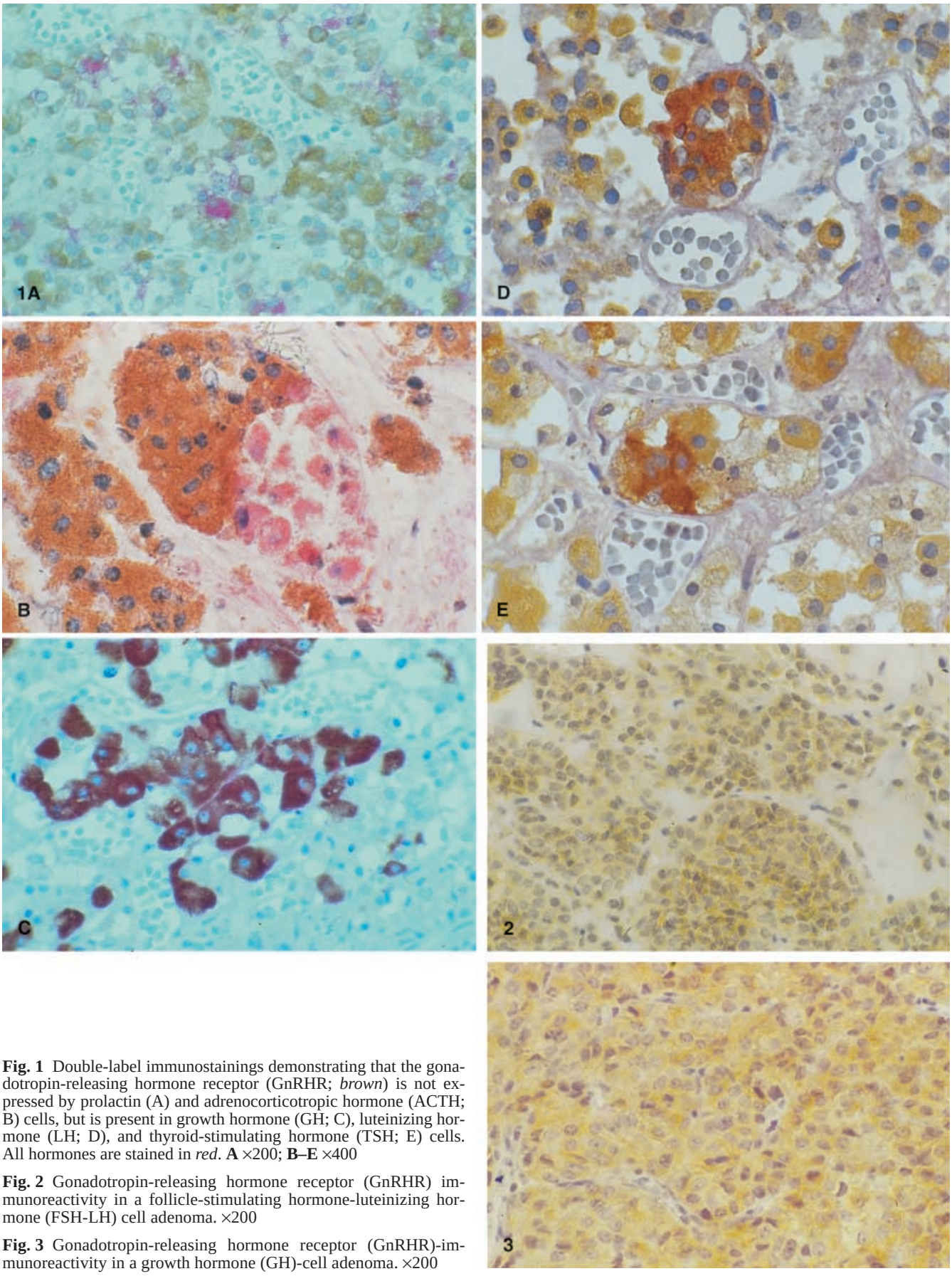

Fig. 1 Double-label immunostainings demonstrating that the gonadotropin-releasing hormone receptor (GnRHR; *brown*) is not expressed by prolactin (A) and adrenocorticotropic hormone (ACTH; B) cells, but is present in growth hormone (GH; C), luteinizing hormone (LH; D), and thyroid-stimulating hormone (TSH; E) cells. All hormones are stained in *red*. **A** ×200; **B–E** ×400

Fig. 2 Gonadotropin-releasing hormone receptor (GnRHR) immunoreactivity in a follicle-stimulating hormone-luteinizing hormone (FSH-LH) cell adenoma. ×200

Fig. 3 Gonadotropin-releasing hormone receptor (GnRHR)-immunoreactivity in a growth hormone (GH)-cell adenoma. ×200

Table 2 Clinico-pathological profile of 50 pituitary adenomas. *GnRHR* gonadotropin-releasing hormone receptor; *n* number of positive tumors; *ACTH* adrenocorticotropic hormone; *FSH* follicle-stimulating hormone; *GH* growth hormone; *α-SU* α-subunit; *LH* luteinizing hormone; *PRL* prolactin; TSH thyroid-stimulating hormone; *MP* mean percentage of positive cells in reactive tumors

Adenomas	Gender Male/female	Age		Specific symptoms	GnRHR	
		Mean	Range		*n* (%)	MP
FSH/LH-cell	6/6	58.4	18–70	0/12	7/12 (58.3)	75.5
α-SU/null-cell	6/4	53.7	16–71	0/10	6/10 (60)	55
GH-cell	2/8	41	26–53	10/10	8/10 (80)	51.2
GH/PRL-cell	2/1	57.3	52–66	3/3	2/3 (66.6)	45
TSH-cell	2/0	40	12–68	0/2	0/2	
ACTH-cell	2/6	45.5	29–58	5/8	0/8	
PRL-cell	0/5	31.2	22–36	5/5	0/5	

Table 3 Gonadotropin-releasing hormone receptor (GnRHR)-protein and GnRHR-messenger ribonucleic acid (mRNA) expression in pituitary adenomas of different series. *IHC* immunohistochemistry; *RT-PCR* reverse transcription polymerase chain reaction; *ISH* in situ hybridization; *FSH/LH* follicle-stimulating hormone/luteinizing hormone; *α-SU* α-subunit; *ACTH* adrenocorticotropic hormone; *PRL* prolactin; *GH* growth hormone; *TSH* thyroid-stimulating hormone

Adenomas	GnRHR protein* IHC	GnRHR mRNA[a] RT-PCR	GnRHR mRNA[b]			GnRHR mRNA[c] RT-PCR
			RT-PCR	ISH	IS-RT-PCR	
FSH/LH-cell	7/12 (58.3%)	8/10 (80%)	5/5 (100%)	3/9 (33.3%)	9/9 (100%)	4/13 (30.7%)
α-SU/null cell	6/10 (60%)		6/6 (100%)	4/12 (33.3%)	10/12 (83.3%)	
ACTH-cell	0/8		1/2 (50%)	1/3 (33.3%)	2/3 (66.6%)	
PRL-cell	0/5		0/2	0/4	0/4	
GH-cell	8/10 (80%)		1/2 (50%)	1/5 (20%)	4/5 (80%)	
GH/PRL-cell	2/3 (66.6%)					
TSH-cell	0/2					

*Present study; [a] Miller et al. 1996 [24]; [b] Sanno et al. 1997 [26]; [c] Alexander et al. 1994 [1]

Pituitary adenomas

The main clinico-pathological features of the 50 pituitary adenomas are reported in Table 2. All tumors were tested with the anti-GnRHR antibody, and an IR was found in the majority of FSH/LH-cell (7 of 12; Fig. 2), α-SU/null-cell (6 of 10), GH-cell (8 of 10; Fig. 3), and mixed GH-PRL-cell (2 of 3) adenomas. Among the GnRHR-positive adenomas, FSH/LH-cell adenomas showed the highest mean percentage of reactive cells. No GnRHR IR was found in TSH-cell, ACTH-cell, and PRL-cell adenomas. The IR for GnRHR did not correlate with gender and age of patients.

Discussion

The immunohistochemical localization of GnRH binding sites in rat pituitary cells with LH-, FSH-, and GH-IR has been reported by Childs et al. [3] and Childs and Unabia [2]. The localization of GnRHR in the human pituitary gland has not yet been investigated extensively. The results of the present study clearly show, for the first time, the presence of immunoreactive GnRHR cells in normal human pituitary glands and in pituitary adenomas.

The IR for GnRHR in normal pituitary cells and for pituitary adenomas was cytoplasmic, with reinforcement at the membrane level in some cells. The membrane localization of GnRHR is in agreement with data reported by Childs et al. [4]. The cytoplasmic GnRHR-IR may represent either neosynthesized or internalized receptors, as suggested previously for other types of receptors, such as somatostatin 2A receptors (sst_{2A}) in specific rat brain neurons with high somatostatin expression [7] and for GH receptors in the human anterior pituitary gland [23]. In this context, it is interesting to recall that GnRHR, after binding with its ligand, is internalized in the cytoplasm [5]. This process has been well demonstrated by Hazum et al. [13, 14], who studied pituitary gonadotrophs and ovarian granulosa cells using immunohistochemical techniques. In addition, Szende et al. [28], using electron microscopic immunohistochemical techniques, have demonstrated that GnRHR was localized in the cytoplasm and nucleus of cells of experimental pancreatic carcinomas of female Syrian Golden hamsters and MXT mouse mammary cancer cells.

In the normal adenohypophysis, GnRHR was immunohistochemically colocalized with α-SU, β-FSH, β-LH, β-TSH, and GH, suggesting that GnRHR is present in gonadotroph, thyrotroph, and somatotroph cells. The finding of GnRHR in gonadotroph cells represents an expected result, since it is well known that GnRH increases the synthesis and release of gonadotropins after binding to specific cell receptors, leading to activation of a specific G protein [6].

The finding of GnRHR expression in GH- and TSH-cells is rather unexpected and more difficult to be ex-

plained in a physiological background. In this context, it is worth noting that Child et al. [4] reported that, in the rat anterior pituitary, about 38% of GH-IR cells bind biotinylated GnRH. This demonstrates that at least a subpopulation of somatotrophs bears GnRH receptors. This subpopulation of rat somatotrophs may represent precursors of some somatogonadotroph cells, and the transition between these cell types, seen from diestrus to proestrus, may be stimulated by activin. Therefore, in rat, the expression of GnRHR by somatotrophs seems to be connected with the transition from somatotrophs to somatogonadotrophs [2]. A similar transition is not reported in human anterior pituitary cells, where normal somatotroph cells are known to co-express GH and the α-SU, but do not co-secrete GH and the β-SU [29]. In human GH cells, GnRHR may be involved in the mechanism of suppression of basal GH release, which may be obtained by administration of GnRH analogues [8, 21, 34].

The possible functional role of GnRHR in thyrotrophs is not clear at the moment, although it has been reported that GnRH is capable of releasing significant amounts of TSH in inhibin-treated ovine pituitary cultures [10]. Our results, demonstrating the lack of GnRHR expression in ACTH cells, are in agreement with the data recently published by Wilson et al. [33], proving that GnRH agonists do not alter the hypothalamic–pituitary–adrenal axis and that, in particular, they do not have a suppressive effect on the release of ACTH by the pituitary gland.

Chronic GnRH-agonist therapy has been found to reduce PRL level in patients with hyperprolactinemia [11]. Therefore, it may be hypothesized that the GnRH suppressive effect on PRL release could be mediated by GnRHRs present in PRL cells. However, our results demonstrate the lack of GnRHR in PRL cells, suggesting that the action of GnRH on PRL cells may be indirect and possibly due to the reduction of estrogen secretion induced by GnRH analogues.

Our results demonstrated that GnRHRs are mostly expressed by somatotroph-, gonadotroph-, and α-SU/null-cell adenomas, while they are not expressed by PRL-cell, TSH-cell, and ACTH-cell adenomas. This distribution of GnRHR well reflects that found in normal pituitary cells, with the exception of thyrotroph adenomas. Our immunohistochemical results concerning the distribution of GnRHR correlate well with the expression of GnRHR mRNA in pituitary adenomas as detected by ISH, RT-PCR, and in situ RT-PCR (IS-RT-PCR), with the exception of ACTH-secreting tumors [1, 24, 26] (Table 3). In fact, the cases of functioning and nonfunctioning corticotroph adenomas that we examined did not show a GnRHR IR. On the contrary, Sanno et al. [26] demonstrated the presence of GnRHR mRNA in a percentage of functioning corticotroph tumors, ranging from 33.3% to 66.6%, depending on the technique employed. The most likely causes of these discrepancies are differences in experimental procedures and in the sensitivity of the methods used. The lack of GnRHR IR in ACTH-cell adenomas may be due to the low sensitivity of immunohistochemical methods or the low or absent expression of receptors despite the expression of GnRHR mRNA. In this context, it is interesting to recall that Sanno et al. [26] detected a lower number of tumor cells with a positive ISH signal for GnRHR in ACTH-cell adenomas than in other types of adenomas.

Our findings, which demonstrate that tumors showing the highest immunohistochemical expression of GnRHR are FSH/LH- and α-SU/null-cell adenomas, are well in agreement with those of Sanno et al. [26] who detected a positive hybridization signal for GnRHR mRNA in the majority of cells of FSH/LH- and null-cell adenomas examined. Interestingly, Miller et al. [24] and Sanno et al. [26], using RT-PCR, ISH, and ISH in combination with RT-PCR, demonstrated that GnRH and GnRHR mRNAs are expressed in various types of human pituitary adenomas, including FSH/LH-cell, GH-cell, ACTH-cell, and null-cell adenomas. The high incidence of GnRH and GnRHR gene expression, found in pituitary adenomas with special reference to gonadotropin-secreting and null-cell adenomas, suggests that locally produced hypothalamic hormones and related receptors may play a role in the regulation of tumor cell growth and function, acting through a paracrine/autocrine mechanism. FSH/LH adenomas showing diffuse IR for GnRHR (7 of 12 of our cases) may correspond to those gonadotroph tumors that are responsive to pulsatile GnRH in vitro and show normal tumor receptor biosynthesis [1]. On the contrary, the FSH/LH adenomas lacking GnRHR IR (5/12 of our cases) may correspond to the subset of gonadotroph tumors with GnRHR biosynthetic defects and GnRH unresponsiveness, described by Alexander and Klibansky [1]. Whether the heterogeneity of the immunohistochemical expression of GnRHR among GH-cell or mixed GH-PRL-cell adenomas has a functional counterpart, it remains to be verified in combined endocrinological and pathological studies.

The lack of GnRHR-IR in our cases of PRL-cell tumors perfectly correlates with results obtained by Sanno et al. [26], who did not find GnRHR mRNA expression in prolactinomas. These results indicate that PRL cells do not bear GnRHRs.

The two TSH-secreting tumors examined in our series were negative for GnRHR, and this result cannot be compared with other cases described in the literature because, as far as we know, investigations on GnRHR or GnRHR mRNA expression in thyrotroph adenomas are not reported. In 1988, Simard et al. [27] demonstrated that GnRH stimulated TSH secretion in cell cultures of a human thyrotroph adenoma, indirectly suggesting that adenomatous TSH cells may bear a GnRHR.

In conclusion, we demonstrated in this study that GnRHRs are widely distributed in normal adenohypophysial cells and are also expressed by several pituitary adenomas. Normal and adenomatous pituitary cells are known to express GnRH. Therefore, it can be concluded that the interaction between GnRH and the GnRHR may play a role in paracrine/autocrine regulation of different types of normal pituitary cells and pituitary adenomas.

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