Immunocytochemical and morphometric evidence of the existence of different cellular populations reactive to r-GHRH in the rat pituitary gland

J. Carretero [1,2], M. Rubio [1,2], E. Blanco [1,2], J.M. Riesco [1,2], D. Burks [2,3], J.J. Herrero [1], P. Bodego [1], E. Pérez [4] and R. Vázquez [1,2]

1- Department of Human Anatomy and Histology, Faculty of Medicine, University of Salamanca, Spain

2- Laboratory of Neuroendocrinology, Institute of Neurosciences of Castilla y León, University of Salamanca, Spain

3- Joslin Diabetes Center, Harvard University, Mas. USA

4- Department of Surgery, Faculty of Medicine, University of Salamanca, Spain

SUMMARY

In order to characterize the pituitary cells reactive for r-GRF in the adult rat, an immunocytochemicaI and morphometric study was made of the cells immunopositive for r-GHRH and those immunopositive for GH and for GHRH using a double labelling method. The existence of two populations of r-GRF-immunoreactive cells was observed; one of these was only stained with specific serum against r-GRF and the other was immunopositively stained for GH and for r-GHRH. Morphometrically, in both sexes the GH-immunoreactive cells were seen to have a cellular size that was significantly larger ($p<0.01$) than the r-GHRH-immunoreactive cells. The cells that were only stained for r-GHRH were significantly smaller than those immunoreactive for GH and r-GHRH. Our findings suggest the existence of at least two cellular populations in the pituitary gland of the adult rat able to react with anti-r-GHRH serum.

Key Words: Rat – Pituitary gland – r-GRF – Immunocytochemistry – Morphometry

INTRODUCTION

Immunocytochemical and morphometric techniques have proved to be useful tools for determining the intracellular presence of neuropeptides and for evaluating their morphological characteristics and the degree of cellular activity.

The presence of GHRH in the hypophysis has been much debated. Using immunocytochemical procedures, several authors have been unable to demonstrate its existence in the rat (Sawchenko et al., 1985), the monkey (Lechan et al., 1984) and in humans (Pelletier et al., 1986). Other authors, however, have detected its presence in the monkey (Morel et al., 1984), in the pituitaries of transgenic mice (Brar et al., 1989) and in teleosts (Pan et al., 1985). In previous work (Carretero et al., 1991a), in *in vivo* and *in vitro* studies we found GHRH-immunoreactive cells in the hypophysis of the adult rat which increased following intraventricular colchicine treatment.

Despite this, the nature of the GHRH-reactive pituitary cells and their true function remain uncertain. Morel et al. (1984) reported GHRH reactivity only in somatotrophs and suggested that the peptide would participate in the regulation of GH secretion. Pan et al. (1985) described the presence of immunoreactivity in follicle stellate cells and hypothesized a paracrine role in the control of the regulation of prolactin secretion. Brar et al. (1989) detected h-GHRH immunoreactivity in some somatotroph, gonadotroph, thyrotroph and mammotroph cells.

Submitted: November 21, 2001
Accepted: February 26, 2002

Correspondence to:
Prof.Dr. J. Carretero. Laboratorio de Neuroendocrinología, INCYL. Facultad de Medicina, Universidad de Salamanca. Avda. Alfonso X el sabio, s/n. E-37007 Salamanca, Spain.
Fax: 34/923/294559. E-mail: jcar@usal.es

Using double immunocytochemical and morphometrical methods, the aim of the present study was to analyze these cells in the pituitary gland of colchicine-treated rats, comparing the findings from single or double immunostaining methods, and to rule out or confirm the presence of GHRH-immunoreactive cells other than somatotrophs in the adult rat.

Material and Methods

Animals used

Twenty adult Sprague-Dawley rats, 10 per sex, (supplied by Charles-River Inc.) housed under standard conditions (temperature: 20±2ºC, RH: 50±5%, lights on from 8.00 to 20.00h, a balanced diet and water ad libitum) were studied.

Under deep ketamine anaesthesia, the rats were treated with 90 mg of colchicine in the lateral ventricle according to the stereotaxic coordinates of Pellegrino and Cushman (1966). The animals were sacrificed at 24 hours following treatment by decapitation under isoflurane anaesthesia. The pituitary glands were then carefully removed, fixed in 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.4) for 12 hours, and embedded in paraffin to obtain serial frontal sections of 5 mm thickness for immunocytochemical studies.

Immunocytochemistry

The samples were processed with the PAP immunocytochemical method (Sternberger et al., 1970) for r-GHRH or by double staining for both GH and r-GHRH, after inhibition of endogenous peroxidase (Streefkerk, 1972):

Single labelling. This was performed using anti-GH rabbit serum (Dako) at a dilution of 1:1200 or anti-r-GHRH(1-43) rabbit serum at a dilution of 1:1000; swine anti-rabbit serum (Dako, diluted 1:100) and rabbit PAP soluble complex (Dako, diluted 1:100). Saline Tris (0.05M, pH 7.4) with 0.8% NaCl was used for the dilutions and washes. The reaction was visualized by the Graham and Karnovsky procedure (1966).

Double labelling. After performing the first labelling for GH in a similar way to that described previously for single labelling, the immunoglobulins present in the sample were eluted by incubation in glycine-HCl buffer (0.1M, pH 2.2) over 48 h at 4ºC (Vandesande, 1983), thereafter developing the second labelling for r-GHRH(1-43) in a similar way to that described for the single labelling and developing the reaction with benzidine and sodium nitroprussiate in phosphate buffer (0.05M, pH 6.8). Controls to determine the time of immunoglobulin elution were made and estimated to be optimum at 48 hours.

The slices obtained from all portions of the pituitary gland were studied under a Zeiss Axiophot microscope.

The primary anti r-GHRH(1-43) serum was obtained as described previously (Fernández et al., 1989; de los Frailes et al., 1992) and tested for immunocytochemistry (Carretero et al., 1991a). The specificity of the serum was tested against pancreatic polypeptide, insulin, gastrin, glucagon, somatostatin, Arg-Vasopressin, LHRH, cholecystokinin, b-endorphin, secretin and gastrin inhibitory peptide, proving to be highly specific. After substitution or preabsorption tests, the reaction was completely abolished (Figs. 1 and 2). According to radioimmunoassay, the cross-reaction was 100% for r-GHRH (1-43), 0.004% for h-GHRH (1-44) and 0.37% for h-GHRH (1-39). Preabsorption with GH (NIH) (10 nM/100 pL of diluted serum) was unable to abolish the reaction (Fig. 3). By RIA, no binding was found for any of the pituitary hormones.

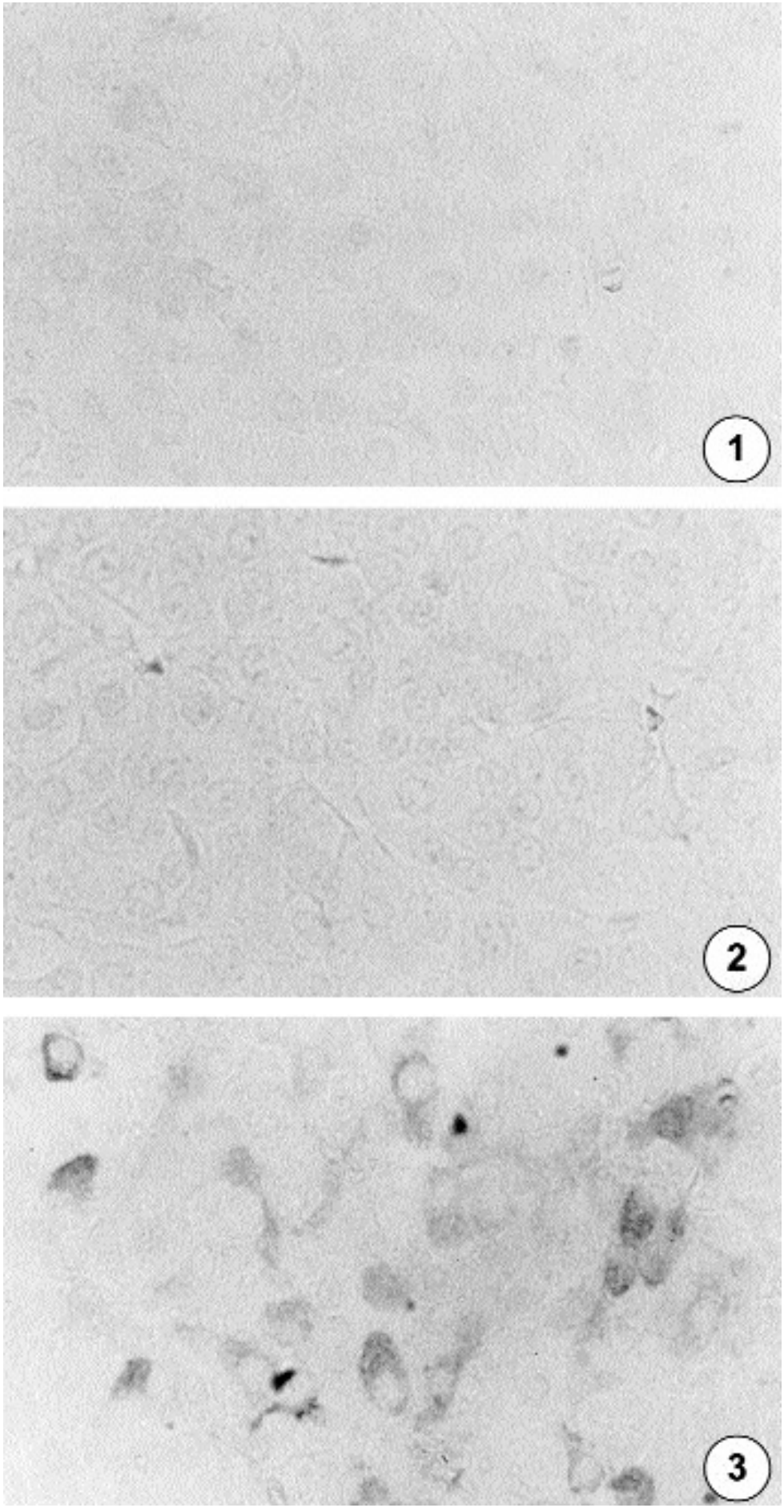
Morphoplanimetry

Using an MIP (IMCO 10) image analyzer connected via an Hitachi video system to a Nikon Optiphot 2 microscope, a total of 1000 reactive cells of each type, per sex, was analysed, calculating in each animal (in the single labelling) the cellular area of 100 GH-reactive cells and of 100 r-GHRH-reactive cells and, in the double labelling (also in each animal), the cellular area of 100 cells reactive to GH and r-GHRH and of 100 cells reactive to only r-GHRH, chosen at random from frontal pituitary sections separated from one another by at least 150 mm from all portions of the gland. The values obtained were analyzed statistically by ANOVA. The differences observed were considered significant for $p<0.05$ (for Scheffé's F-test). The values obtained are shown in Table 1 as arithmetic means (± standard error of the mean).

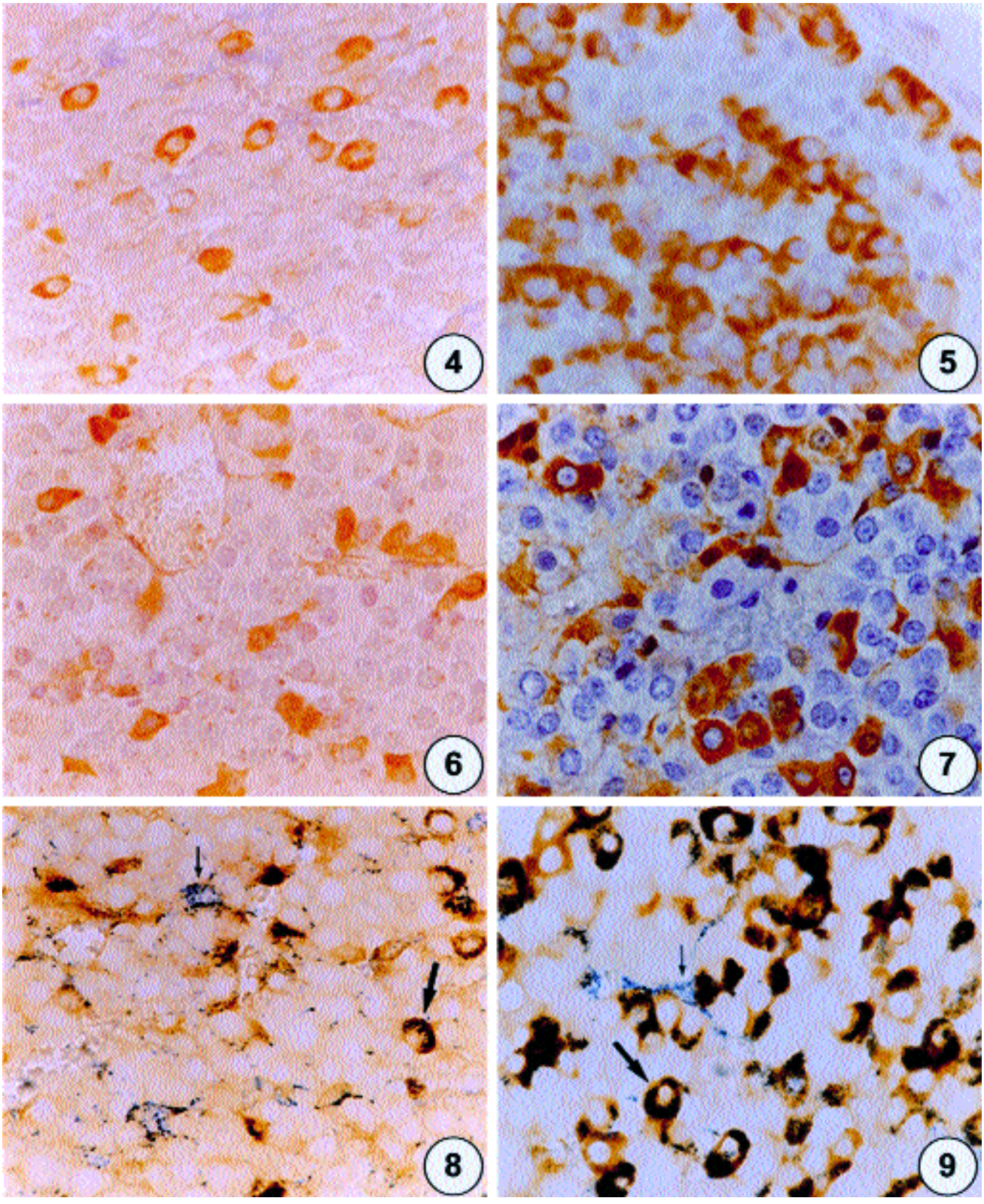
Results

The immunocytochemical reaction for GHRH was abolished following preabsorption with rat-GHRH (Fig. 1) or substitution of anti-GHRH serum by normal rabbit serum (Fig. 2). By contrast, the reaction was conserved after preabsorption with rat GH (Fig. 3).

In the females, the cytoplasm of GH-cells was irregularly immunoreactive (Fig. 4). These cells were either isolated or more frequently formed small clusters, and were apposed to blood vessels. Their morphology varied, with a predominance of oval or polygonal shapes. The morphology of the GH-cells of the males (Fig. 5) was similar to that of the cells of the females except for the cytoplasmic reaction, which appeared with a different pattern in the males from that observed in the females.



Figures 1-3.- Micrographs from the hypophysis of a male rat following preabsorption with rat-GHRH (Fig. **1**), substitution of anti-GHRH serum by normal rabbit serum (Fig. **2**), and following preabsorption with rat GH (Fig. **3**). x 500.



Figures 4-5.- Micrographs from the hypophysis of a female rat (Fig. **4**), showing GH-immunoreactive cells with irregular cytoplasmic staining and from a male rat (Fig. **5**), showing GH-immunoreactive cells stained homogeneously. x 750.

Figures 6-7.- Micrographs from the hypophysis of a female rat (Fig. **6**), showing weakly stained r-GHRH-immunoreactive cells and from a male rat (Fig. **7**), showing strongly stained r-GHRH-immunoreactive cells. x 750.

Figures 8-9.- Micrographs from the hypophysis of a female (Fig. **8**) and a male rat (Fig. **9**) showing, by double labelling, cells immunoreactive to GH and r-GHRH (thick arrows) and cells that were only stained for r-GHRH (thin arrows). x 750.

In both sexes, the rat pituitary gland showed r-GHRH immunoreactive cells distributed throughout the gland and these were preferentially oval or polygonal, although GHRH-reactive cells with cytoplasmic prolongations were also seen (Figs. 6 and 7).

Using the double labelling technique, cells reactive only to GH, both GH and r-GHRH (GH-

GHRH-ir cells) and cells that only reacted to r-GHRH were observed (Figs. 8 and 9). Some of the latter had an irregular morphology and sometimes displayed cytoplasmic prolongations (thin arrows). In other cases, they had an oval or polygonal morphology, and the GH-GHRH-ir cells were generally oval (thick arrows) and resembled the GH-immunoreactive cells observed in the single labelling.

Morphometrically, in the single labelling technique it was seen that in the males the mean size of the r-GHRH-reactive cells was significantly larger than in females ($p<0.01$). The same was the case of the GH-reactive cells. Additionally, the GH-reactive cells were significantly larger ($p<0.01$) than the r-GHRH-reactive cells in both sexes (see Table 1).

In the double staining, in both sexes the morphometry revealed that the sizes of the cells reactive to GH and GHRH were similar to those observed for the GH-reactive cells in the single staining.

The GH and GHRH immunoreactive cells in the double staining were significantly larger ($p<0.01$) than the r-GHRH-reactive cells observed in the single labelling.

By contrast, in the double staining the cells only reactive to r-GHRH were significantly smaller in size ($p<0.01$) than the cells reactive to GH or to r-GHRH, in the single staining, and than the cells reactive jointly to GH and GHRH in the double staining (see Table 1).

Table 1.

Immunopositivity	Females	Males
GH	89.72±3.22	115.28±3.23
GHRH	74.07±1.99*	95.45±3.14**
GH-GHRH	92.86±4.41#	120.98±2.69##
GHRH*	52.93±3.37•	80.88±2.30••

Table1.- Morphometric values (μm^2) of cellular area obtained in the different types of cells and groups of animals studied. GH: Immunoreactive GH-cells in single labelling, GHRH: immunoreactive r-GHRH-cells in single labelling, GH-GHRH: immunoreactive GH- and r-GHRH-cells in double staining, GHRH*: immunoreactive r-GHRH cells in double labelling. *: $p<0.01$ in relation to the GH of female rats. **: $p<0.01$ in relation to GH of male rats. #: $p<0.01$ in relation to r-GHRH (single labelling) of female rats. ##: $p<0.01$ in relation to r-GHRH (single labelling) of male rats. •: $p<0.01$ in relation to other cellular populations from female rats. ••: $p< 0.01$ in relation to other cellular populations from male rats. All cellular types in the males were significantly larger ($p<0.01$) than the corresponding cells in females.

DISCUSSION

GHRH is a hypothalamic neuropeptide that is transported from the nucleus arcuatus (Jacobowitz et al., 1983; Lechan et al., 1984; Bloch et al., 1984; Merchenthaler et al., 1984; Pan et al., 1984; Daikoku et al., 1985; Kita et al., 1985; Pelletier et al., 1986; Ibata et al., 1986; Bruhn et al., 1987; Leidy and Robbins, 1988) to the external layer of the median eminence. From there, it is released into the pituitary portal system (Sawchenko et al., 1985) and reaches the pituitary, where its role is to regulate the synthesis and release of growth hormone (Guillemin et al., 1982; 1984; Rivier et al., 1982; Thorner et al., 1983; Vance et al., 1984; Evans et al., 1985; Sassolas and Guillemin, 1985).

In a previous work, following abolition of the axonal transport of GHRH in the median eminence by intraventricular colchicine administration (Carretero et al., 1991a), it was observed that at 24 hours after treatment r-GHRH immunoreaction was present and was increased in the pituitary gland of adult rats, possibly indicating a local production of GHRH at pituitary level. This was supported by the presence of r-GHRH immunoreactive cells in vitro after seven days of incubation in a medium devoid of GHRH (Carretero et al., 1991a).

The presence of GHRH in the pituitary gland is consistent with the characterization of GHRH from pituitary adenomas (Joubert et al., 1989; Peillon et al., 1989) and the immunocytochemical characterization of GHRH in the pituitary glands of transgenic mice (Brar et al., 1989).

Similarly, the presence of GHRH at pituitary level has been described in other animal species (Morel et al., 1984; Pan et al., 1985; Brar et al., 1989; Peillon et al., 1990). Our findings are also consistent with previous reports of non-hypothalamic pituitary GHRH (Carretero et el., 1991b), as has been described for other neuropeptides such as VIP (Arnaout et al., 1986; Nagy et al., 1988; Hsu et al., 1989; Segerson et al., 1989; Lam et al., 1990; Lam, 1991), LHRH (Li et al., 1984; May et al., 1987), somatostatin (Mesguich et al., 1988; Pagesy et al., 1989; Joubert et al., 1989; Levy and Lightman, 1990) and TRH (Peillon et al., 1989; May et al., 1987; Joubert et al., 1989; Le Dafniet et al., 1990).

In the present study, following the double labelling two types of cells reactive to r-GHRH were observed: cells reactive to GH and GHRH and cells reactive exclusively to GHRH. These populations were confirmed morphometrically on observing that the size of the GHRH cells was different from that of the GH cells in the single staining. Also morphometrically, in the double labelling we confirmed that the sizes of the GH-GHRH-ir cells differed from those of the cells reactive to GHRH alone.

The fact that morphometrically -in the single staining- cells reactive to GHRH appeared that were smaller than those reactive to both GH and GHRH -in the double staining- suggests that the former encompass a pituitary cellular component other than somatotropic cells.

This second component was corroborated on obtaining cells reactive to GHRH but not to GH in the double staining; morphometrically, these were smaller than those reactive to both GH and GHRH.

The morphology of the cells that were only reactive to r-GHRH was different. We observed oval or rounded cells and some cells which had a polygonal soma with cytoplasmic processes. This suggests the possibility of different populations reactive to GHRH, but net to GH, which might play different endocrine roles.

The fact that some authors have been unable to find any reaction for GHRH in the pituitary gland and that there is discordance in the results of different studies addressing the location of GHRH at pituitary level (Morel et al., 1984; Pan et al., 1985; Brar et al., 1989) could derive from the fact that these studies were performed in different animal species (Lechan et al., 1984; Shibasaki et al., 1984; Pelletier et al., 1986), using different immunocytochemical methods (Sawchenko et al., 1985) or different anti-GHRH sera.

The anti-GHRH serum used in our study had been previously tested by RIA for pituitary hormones and GHRH-related neuropeptides and similar controls were made using immunocytochemistry. The findings obtained, together with the abolition of immunocytochemical reaction following GHRH-preabsorption tests, suggest a very high specificity for the serum employed, as has been reported previously (Fernández et al., 1989; Carretero et al., 1991a; de los Frailes et al., 1992).

The morphometric study carried out here points to significant differences in the parameters analyzed, depending on the sex of the animals, which are similar to those described for GH cells (Gross, 1980; Carretero et al., 1990a, 1990b, 1991b) and which, in the present work, appeared in similar proportions among the GH cells and among the r-GHRH-reactive cells.

These differences could be related to the functional role of pituitary GHRH in control of the regulation of GH, which is known to differ according to sex in adult rats (Eden, 1979; Akira et al., 1988; Carretero et al., 1990b).

The fact that a cellular population reactive to r-GHRH but not GH was found could be related to the production of endogenous r-GHRH, which, in a paracrine fashion, might act at pituitary level. Alternatively, it could play a mitogenic or proliferative role, as has been described in pituitary tumours (Billestrup et al., 1986; Peillon et al., 1990).

Our results suggest the existence of different pituitary cellular populations able to express GHRH that can be detected immunocytochemically. One of them is the somatotroph population.

The evidence for the synthesis of r-GHRH in the rat at pituitary level and its functional role in the regulation of the synthesis and release of GH or of other hormones remains to be elucidated and further research should be carried out under different physiological and experimental conditions to clarify these aspects.

ACKNOWLEDGEMENTS

We thank Dr. Sánchez-Franco (Service of Endocrinology I.N.I.C. Carlos III, Madrid, Spain) for the generous gift of r-GHRH antiserum. We thank the FIS (99/1187) and DGICYT (PM88-0159) for financial support.

REFERENCES

AKIRA S, WAKABAYASH T, SUGIHARA H, MINAMI S, TAKAHASHI F and MOYOYAMA A (1988). Effect of testosterone on growth hormone secretion in female rats during a continuous infusion of growth hormone releasing factor. *Neuroendocrinology*, 47: 116-124.

ARNAOUT MA, GARTHWAITE TL, MARTINSON DR and HAGEN T0 (1986). Vasoactive intestinal polypeptide is synthesized in anterior pituitary tissue. *Endocrinology*, 119: 2052-2057.

BILLESTRUP N, SWANSON LW and VALE W (1986). Growth hormone-releasing factor stimulates proliferation of somatotrophs in vitro. *Proc Natl Acad Sci (USA)*, 83: 6854-6857.

BLOCH B, GAILLARD RC, BRAZEAU P, LIN HD and LING N (1984). Topographical and ontogenic study of the neurons producing growth hormone-releasing factor in human hypothalamus. *Reg Pep*, 8: 21-31.

BRAR AK, BRINSTOR RL and FROHMAN LA (1989). Immunohistochemical analysis of human growth hormone-releasing hormone gene expression in transgenic mice. *Endocrinology*, 125: 801-809.

BRUHN TO, ANTHONY EL, WU P and JACKSON IMD (1987). GRF immunoreactive neurons in the paraventricular nucleus of the rat: An immunohistochemical study with monoclonal and polyclonal antibodies. *Brain Res*, 424: 290-298.

CARRETERO J, SÁNCHEZ D, SÁNCHEZ F, MONTERO M, BLANCO E, GONZÁLEZ R and VÁZQUEZ R (1990a). Morphometric study of immunoreactive GH-cells of the adenohypophysis of rats treated with met-enkephalin. *Acta Histochem*, 89: 121-130.

CARRETERO J, SÁNCHEZ F, MONTERO M, BLANCO E, RIESCO JM, CARBAJO E, GONZÁLEZ R and VÁZQUEZ R (1990b). Morphological and functional study of the GH-immunoreactive adenohypophyseal cells in ovariectomized rats. *Histochemical J*, 22: 683-687.

CARRETERO J, SÁNCHEZ F, VÁZQUEZ R, CACICEDO L, SÁNCHEZ-FRANCO F, FERNÁNDEZ G and MONTERO M (1991a). In vivo and in vitro evidence of growth hormone releasing factor-like produced locally in the adenohypophyseal cells of the rat. *Neuropeptides*, 19: 223-229.

CARRETERO J, SÁNCHEZ F, GÓNZALEZ R, BLANCO E, JUANES JA, RIESCO JM and Vázquez R (1991b). Sexually dependent changes in rat somatotropic cells following bilateral adrenalectomy. *Acta Anat*, 142: 19-24.

DAIKOKU S, KAWANO H, NOGUCHI M, TOKUCEN M, CHIHARA K, SAITO H and SHIBASAKI T (1985). Ontogenic appearance of immunoreactive GRF-containing neurons in the rat hypothalamus. *Cell Tissue Res*, 242: 511-518.

DE LOS FRAILES MT, CACICEDO L, FERNÁNDEZ G, TOLÓN RM, LORENZO MJ, AGUADO F and SÁNCHEZ FRANCO F (1992). Role of locally produced growth hormone-releasing fac-

tor in somatostatin regulation by fetal rat brain cells in culture. *Neuroendocrinology*, 55: 221-229.

EDEN S (1979). Age and sex related differences in episodic growth hormone secretion in the rat. *Endocrinology*, 105: 555-560.

EVANS WS, KRIEG RJ, LIMBERG ER, KAISER DL and THORNER MO (1985). Effect of in vivo gonadal environment on in vitro GRF-40 stimulated GH release. *Am J Physiol*, 249: E 276-280.

FERNÁNDEZ G, CACICEDO L, LORENZO MJ, DE LOS FRAILES MT and SÁNCHEZ-FRANCO F (1989). Growth hormone-releasing factor production by fetal rat cerebrocortical and hypothalamic cells in primary culture. *Endocrinology*, 125: 1991-1998.

GRAHAM RC and KARNOVSKY MJ (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. *J Histochem Cytochem*, 14: 291-302.

GROSS DS (1980). Role of somatostatin in the modulation of hypophysial growth hormone production by gonadal steroids. *Am J Anat*, 158: 507-519.

GUILLEMIN R, BRAZEAU P, BOHLEN P, ESCH F, LING N and WEHRENBERG WB (1982). Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. *Science*, 218: 585-587.

GUILLEMIN R, ZEYTIN F, LING N, BÖLEN P, ESCH F, BRAZEAU P, BLOCH B and WEHRENBERG WB (1984). Growth hormone-releasing factor: chemistry and physiology. *Proc Soc Exp Biol Med*, 175: 407-413.

HSU W, RISKIND PN and HEDLEY WHYTE ET (1989). Vasoactive intestinal peptide in the human pituitary gland and adenomas. An immunocytochemicaI study. *Am J Pathol*, 135: 329-338.

IBATA Y, OKAMURA H, MAKINO S, KAWAKAMI F, MORIMOTO N and CHIHARA K (1986). Light and electron microscopic immunocytochemistry of GRF-like immunoreactive neurons and terminals in the rat hypothalamic arquate nucleus and median eminence. *Brain Res*, 370: 136-143.

JACOBOWITZ DM, SCHULTE H, CHROUSOS GP and LORIAUX DL (1983). Localization of GAF-like immunoreactive neurons in the rat brain. *Peptides*, 4: 521-524.

JOUBERT (BRESSION) D, BENLOT O, LAGOGUEY A, GARNIER P, BRANDI AM, GAUTRON JP and PEILLON F (1989). Normal and growth hormone (GH)-secreting adenomatous human pituitaries release somatostatin and GH-releasing hormone. *J Clin Endocrinol Metab*, 68: 572-577.

KITA T, CHIHARA K, ABE H, MINAMITANI N, KAJI H, KODAMA H, KASHIO Y, OKIMURA Y, FUJITA T and LING N (1985). Regional distribution of rat growth hormone releasing factor-like immunoreactivity in rat hypothalamus. *Endocrinology*, 116: 259-262.

LAM KSL (1991). Vasoactive intestinal peptide in the hypothalamus and pituitary. *Neuroendocrinology*, 53 (suppl 1): 45-51.

LAM KSL, SRIVSTAVA G, LECHAN RM, LEE T and REICHLIN S (1990). Estrogen regulates the gene expression of vasoactive intestinal peptide in the anterior pituitary. *Neuroendocrinology*, 52: 417-421.

LE DAFNIET M, LEFEBVRE P, BARRET A, MECHAIN O, FEINSTEIN MC, BRANDI AM and PEILLON F (1990). Normal and adenomatous human pituitaries secrete thyrotropin releasing hormone in vitro: modulation by dopamine, haloperidol and somatostatin. *J Clin Endocrinol Metab*, 71: 480-486.

LECHAN RM, LIN HD, LING N, JACKSON IMD, JACOBSON S and REICHLIN S (1984). Distribution of immunoreactive growth hormone-releasing factor (1-44)-NH in the tuberoinfundibular system of the Rhesus monkey. *Brain Res*, 309: 55-61.

LEIDY JWJR and ROBBINS RJ (1988). Growth hormone-releasing factor immunoreactivity in the hypothalamus and cortex of the rat: in vivo and in vitro studies. *Endocrinology*, 122: 1652-1657.

LEVY A and LIGHTMAN SL (1990). Relationship between somatostatin and growth hormone messenger ribonucleic acid in human pituitary adenomas: an in situ hybridization histochemistry study. *Clin Endocrinol*, 32: 661-668.

LI JY, KNAPP AJ and STERNBERGER LA (1984). Immunocytochemistry of a "private" luteinizing-hormone-releasing hormone system in the pituitary. *Cell Tissue Res*, 253: 263-266.

MAY V, WILBER JF, PRICHARD DC and CHILDS GV (1987). Persistence of immunoreactive TRH and GnRH in long-ten primary anterior pituitary cultures. *Peptides*, 8: 543-558.

MERCHENTHALER T, VAGH S, SCHALLY AV and PETRUSZ P (1984). Immunocytochemical localization of growth hormone-releasing factor in the rat hypothalamus. *Endocrinology*, 114: 1082-1085.

MESGUICH P, BENOIT A, DUBOIS PM and MOREL G (1988). Somatostatin-28- and somatostatin-14-Iike immunoreactivities in the rat pituitary gland. *Cell Tissue Res*, 252: 419-427.

MOREL G, MESGUICH P, DUBOIS MP and DUBOIS PM (1984). Ultrastructural evidence for endogenous growth hormone-releasing factor-like immunoreactivity in the monkey pituitary gland. *Neuroendocrinology*, 38: 123-133.

NAGY G, MULCHAHEY JF and NEILL JD (1988). Autocrine control of prolactin secretion by vasoactive intestinal peptide. *Endocrinology*, 122: 364-366.

PAGESY P, LI Jy, RENTIER-DELRUE F, LE BOUC Y, MARTIAL JA and PEILLON F (1989). Evidence of pre prosomatostatin (pre pro SRIH) messenger ribonucleic acid (mRNA) in human normal and tumoral anterior pituitary gland. *Mol Endocrinol*, 3: 1289-1294.

PAN JX, LECHAN RM, LIN HD, REICHLIN S and JACKSON IMD (1984). Human pancreatic growth hormone-releasing factor (hpGRH) in teleost brain and pituitary: multiple molecular forms and two distinct neuronal systems. In: Abs. 7th International Congress of Endocrinology, pp 1154.

PAN JX, LECHAN M, LIN HD and JACKSON IMD (1985). Immunoreactive neuronal pathways of growth hormone-releasing hormone (GRH) in the brain and pituitary of the teleost *Gadus morhua*. *Cell Tissue Res*, 241: 487-493.

PEILLON F, LE DAFNIET M, GARNIER PH, BRANDI AM, MOYSE E, BIRMAN P, BLUMBERG-TICK J, GROUSELLE D and JOUBERT (BRESSION) D (1989). Receptors and neurohormones studios in human pituitary adenomas. *Hormone Res*, 31: 13-18.

PEILLON F, LE DAFNIET M, PAGESY P, LI JY, BENLOT C, LAGOGUEY A, BRANDI AM and JOUBERT (BRESSION) D (1990). Neuropeptides d'origine antéhypophysaire. *Ann Endocrinol*, 51: 133-136.

PELLETIER G, DESY L, COTE J, LEFEVRE G and VAUDRY H (1986). Light-microscopic immunocytochemicaI localization of growth hormone-releasing factor in the human hypothalamus. *Cell Tissue Res*, 245: 461-463.

RIVIER J, SPIESS J, THORNER M and VALE W (1982). Characterization of a growth hormone-releasing factor from a human pancreatic islet tumor. *Nature*, 300: 276-278.

SASSOLAS G and GUILLEMIN R (1986). Etat actuel de la somatocrinine, ou GRF, facteur hypothalamique de sécrétion de l'hormone de croissance. *Ann Endocrinol*, 47: 332-341.

SAWCHENKO PE, SWANSON LW, RIVIER J and VALE WW (1985). The distribution of growth-hormone-releasing factor (GRF) immunoreactivity in the central nervous system of the rat: An immunohistochemical study using antisera directed against rat hypothalamic GRF. *J Comp Neurol*, 237: 100-115.

SCHULTE SA, SEAL US, PLOTKA ED, VERME LJ, OZOGA JJ and PARSONS JA (1980). Seasonal changes in prolactin and growth hormone cells in the hypophyses of white-tailed deer (*Oddoccoileus virginianus borealis*) studied by

light microscopic immunocytochemistry and radioimmunoassay. *Am J Anat*, 159: 369-377.

SEGERSON TP, LAM KSL, CACICEDO L, MINIMITANI N, FINK S, LECHAN RM and REICHLIN S (1989). Thyroid hormone regulates vasoactive intestinal peptide (VIP) mRNA levels in the rat anterior pituitary gland. *Endocrinology*, 125: 2221-2223.

SHIBASAKI T, KIYOSAWA Y, MASUDA A, NAKAHARA M, IMAKI T, WAKABAYASHI T, DEMURA H, SHIZUME K and LING N (1984). Distribution of growth hormone-releasing hormone-like immunoreactivity in human tissue extracts. *J Clin Endocrinol Metab*, 59: 263-268.

STERNBERGER LA, HARDY PHJR, CUCULIS JJ and MEYER HG (1970). The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase antihorseradish peroxidase) and its use in identification of spirochetes. *J Histochem Cytochem*, 18: 315-333.

STREEFKERK JG (1972). Inhibition of erythrocyte pseudoperoxidase activity by treatment with hydrogen peroxide following methanol. *J Histochem Cytochem*, 20: 829-831.

THORNER MO, RIVIER J, SPIESS J, BORGES JCL, VANCE ML, BLOOM SR, ROGOL AD, CRONIN MJ, KAISER DL, EVANS WS, WEBSTER JD, MACLEOD RM and VALE W (1983). Human pancreatic growth hormone-releasing factor selectively stimulates growth hormone secretion in man. *Lancet*, i: 24-28.

Vance ML, BORGES JL, KAISER DL, EVANS WS, FURLANETTO R, THOMINET JL, FROHMAN L, ROGOL AD, MACLEOD RM, BLOOM S, RIVIER J, VALE W and THORNER MO (1984). Human pancreatic tumor growth hormone-releasing factor: dose-response relationships in normal man. *J Clin Endocrinol Metab*, 58: 838-844.

VANDESANDE F (1983). Immunohistochemical double staining techniques. In: Cuello AC (ed.). *Immunohistochemistry*. IBRO Wiley, Chichester, New York, pp 257-272.