Endocrine, vol. 4, no. 2, 159-163, April 1996 0969-711X/96/4:159-163/$6.25

Gonadotropin-Releasing Hormone Receptor Gene Expression in Rat Anterior Pituitary

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Gonadotropin-releasing hormone (GnRH) effects on the lactotroph function have been widely studied, but they probably result from paracrine interactions. No visual data about GnRH receptor in the pituitary are available. In order to identify the GnRH target cells in the pituitary of adult rats, the cellular distribution of rat GnRH receptor mRNA was investigated by electron microscopy, using *in situ* hybridization on ultrathin pituitary frozen sections. *In situ* hybridization was performed using a digoxigenin-labeled oligonucleotide probe revealed by an indirect immunogold reaction. Gonadotropin-releasing hormone receptor mRNA was found in the cytoplasmic matrix, apposed to the endoplasmic reticulum and the nucleus of the gonadotrophs, which were identified by their ultrastructural characteristics, and by the presence of luteinizing hormone (LH) immunoreactivity. It was also found in the lactotrophs, which were revealed by the immunocytological detection of prolactin. No GnRH receptor mRNA was detected in corticotrophs, somatotrophs, thyrotrophs or hepatocytes. This result, without excluding paracrine effects, clearly showed that in addition to the gonadotrophs, the lactototrophs are likely to be direct target cells for the hypothalamic GnRH.

Key Words: Gonadotropin-releasing hormone receptor; mRNA; rat anterior pituitary; *in situ* hybridization; immunocytology; electron microscopy; paracrine.

Introduction

Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide that acts at the pituitary level to induce the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Clayton, 1989), after binding to its specific receptor (Conn et al., 1986). Moreover, at the pituitary level, GnRH effects on the lactotroph function have also been demonstrated, by stimulating lactotroph differentiation (Begeot et al., 1983; 1984), prolactin (PRL) release (Yen et al., 1980; Casper and Yen, 1981), and PRL gene expression (Vanbael et al., 1994). It has been established that GnRH effects on lactotrophs result from complex paracrine interactions mediated by gonadotroph-stimulated factors (Denef and Andries, 1983; Robberecht et al., 1992). Thus, for example, it has been shown that angiotensin II (A II), which is present in the gonadotrophs (Deschepper et al., 1986), has direct effects on PRL release (Schramme and Denef, 1984; Becuvillalobos et al., 1994; Conn et al., 1986). The recent cloning of the rat pituitary GnRH receptor (Eidne et al., 1992) should make it possible to go further in the determination of GnRH target cells. The aim of the study reported here was to determine the anterior pituitary cell types that express the GnRH-R transcript using ultrastructural *in situ* hybridization (ISH) along with the immunocytological detection of LH and PRL.

Received August 2, 1995; Revised October 19, 1995, December 4, 1995; Accepted December 28, 1995.

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Results

The *in situ* hybridization signal, which indicates the presence of GnRH receptor mRNA (Figs. 1A and B and 2A and B), and the immunocytological signal, which indicates LH- or PRL-like immunoreactivity (Figures 1B and C and 2B and C), were detected with 15-nm and 5-nm gold particles, respectively, which appear in the photographs as regular dark points.

The specificity of the hybridization signal was established as follows:

1. Abolition of the signal when the probe was omitted, or in the presence of an excess of unlabeled GnRH receptor probe at a ratio of 100:1 (Figs. 1C and 2C);
2. Nonmodification of the signal in the presence of an unlabeled heterologous probe for PRL or GH at the same ratio (100:1); and
3. Hybridization of adjacent ultrathin sections with a PRL or GH probe, which produced a signal restricted to the lactotrophs and the somatotrophs, respectively.

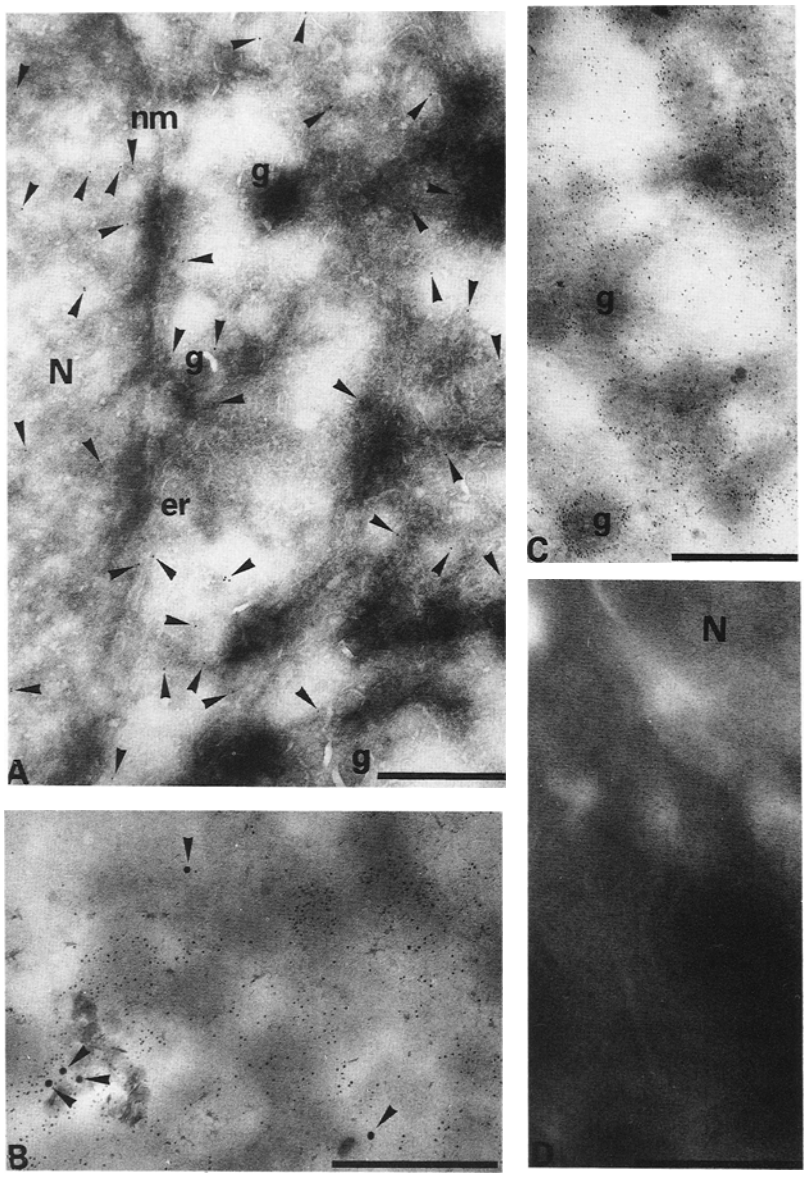

Fig. 1. (A) Localization of GnRH receptor mRNA on ultrathin frozen section of a male rat gonadotroph. The *in situ* hybridization signal indicated by 15-nm gold particles (arrowheads) was localized in the cytoplasmic matrix, and was associated exclusively with the rough endoplasmic reticulum (er) and the nucleus (N). nm: nuclear membrane; g: secretory granule. Original magnification 15,000; Bar: 1 μm. **(B)** Localization of GnRH receptor mRNA (15-nm gold particles) and LH-like immunoreactivity (5-nm gold particles) on ultrathin frozen section of a female rat gonadotroph. The *in situ* hybridization signal (arrowheads) was localized in the cytoplasmic matrix, and the LH-like immunoreactivity was detected as patches in the secretory granules (g). Original magnification 25,000; Bar: 0.5 μm. **(C)** Control of the double-labeling procedure performed on a section of a female rat gonadotroph: *In situ* hybridization reaction was performed with 100-fold excess of unlabeled probe, after immunocytological detection of LH. LH immunoreactivity was detected with 5-nm gold particles present as patches in the secretory granules (g). No 15-nm gold particles used for the *in situ* hybridization revelation of GnRH receptor mRNA were present. Original magnification 25,000; Bar: 0.5 μm. **(D)** Double-labeling procedure performed on a section of a male rat hepatocyte: *In situ* hybridization reaction was performed with 100-fold excess of unlabeled probe, after immunocytological detection of PRL. No 15- or 5-nm gold particles were detected, indicating that liver can be used as a negative control. N: nucleus; m: mitochondria. Original magnification 31,000; Bar: 1 μm.

Moreover a double-labeling procedure performed on ultrathin liver cryosections showed no gold particles; this was used as a negative tissue control (Fig. 1D).

In the anterior pituitary, GnRH receptor mRNA was localized in two cell types: the gonadotrophs, identified by their ultrastructural characteristics (Fig. 1A), and by the presence of LH-like immunoreactivity (Fig. 1B). GnRH receptor mRNA was also detected in the lactotrophs, which were also identified by their ultrastructural characteristics (Fig. 2A) as well as the presence of PRL-like immunoreactivity (Fig. 2B). No gold particle was detected in corticotrophs, somatotrophs, or thyrotrophs.

In the gonadotrophs (Fig. 1A and B) and lactotrophs (Fig. 2A and B), 15-nm gold particles were detected in the

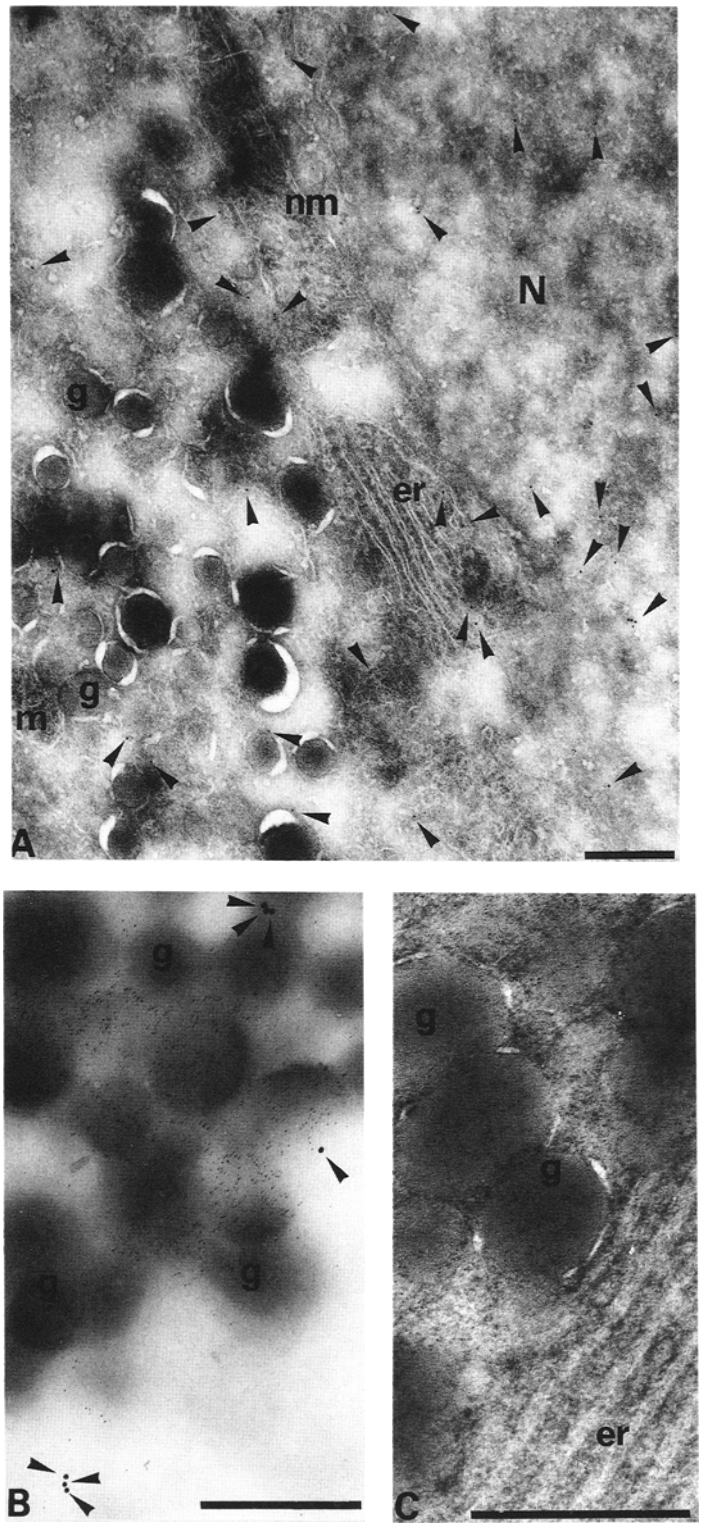

Fig. 2. (A) Localization of GnRH receptor mRNA on ultrathin frozen section of a male rat lactotroph. The *in situ* hybridization signal indicated by 15-nm gold particles (arrowheads) was localized in the cytoplasmic matrix, and was associated with the rough endoplasmic reticulum (er) and the nucleus (N). nm: nuclear membrane; g: secretory granule; m: mitochondrion. Original magnification 15,000; Bar: 1 μm. **(B)** Localization of GnRH receptor mRNA (15-nm gold particles) and PRL-like immunoreactivity (5-nm gold particles) on ultrathin frozen section of a male rat lactotroph. The *in situ* hybridization signal (arrowheads) was localized in the cytoplasmic matrix, and the PRL-like immunoreactivity was detected as patches in the secretory granules (g). Original magnification 25,000; Bar: 1 μm. **(C)** Control of the double-labeling procedure performed on a section of a male rat lactotroph: *In situ* hybridization reaction was performed with 100-fold excess of unlabeled probe, after immunocytological detection of PRL. PRL immunoreactivity was detected with 5-nm gold particles present as patches in the secretory granules (g). No 15-nm gold particles used for the *in situ* hybridization revelation of GnRH receptor mRNA were present, even in the endoplasmic reticulum (er). Original magnification 25,000; Bar: 1 μm.

cytoplasm and the nucleus, indicating the presence of GnRH receptor mRNA. In the cytoplasm, they were localized near the rough endoplasmic reticulum membranes and in the cytoplasmic matrix. In the gonadotrophs (Fig. 1B and C) and lactotrophs (Fig. 1B and C), 5-nm gold particles were identified as patches in the secretory granules, indicating the presence of LH- and PRL-like immunoreactivity.

Discussion

These results, which clearly show the restricted presence of GnRH-R gene expression in gonadotrophs and lactotrophs, were obtained using nonisotopic ISH along with the immunocytological detection of LH or PRL on ultrathin frozen sections. These methods have been shown to be highly sensitive (Hemming et al., 1983; Morel, 1993), and have been used successfully to detect the synthesis of hormone receptor mRNA (Mertani et al., 1994; Morel et al., 1994b). The extreme sensitivity of this technique has been demonstrated by Hemming et al. (1983), who reported that hormone immunoreactivity could be detected on ultrathin frozen sections using specific antibodies diluted 1000 times more than was possible with epoxy resin-embedded sections. In the same way, ISH with nonradioactive probe on ultrathin frozen sections appeared to be the most sensitive of the ISH methods (LeGuellec et al., 1992). In this study, the specificity of the ISH was checked by the highly selective digoxigenin method and that of the immunocytological procedure was also checked. Careful assessment of mRNA distribution showed a preferential localization of mRNA in the cytoplasmic matrix, endoplasmic reticulum and nucleus, and its absence from the mitochondria, Golgi apparatus, and secretory granules. PRL-like immunoreactivity was detected in one particular cell population that had the cytological appearance of lactotrophs and disappeared when the anti-rPRL serum was replaced by a nonimmune serum. Moreover, the visualization of the GnRH-R mRNA signal in the gonadotrophs, which was more intense than the signal obtained from the lactotrophs, and also the absence of a signal in the other pituitary cell populations, as well as in the hepatocytes, provide additional arguments for the validity of this detection method.

This study is the first to visualize GnRH-R mRNA in gonadotrophs, in which effects on gonadotropin secretion are well documented (Clayton, 1989). The detection of GnRH-R mRNA in the lactotrophs is in accordance with previous findings on binding and internalization of GnRH in the lactotrophs using immunocytology (Morel and Dubois, 1982; Morel et al., 1994a), and ultrastructural autoradiography (Morel et al., 1987). The presence of this receptor and its ligand in lactotrophs can be correlated with reported GnRH stimulation of lactotroph differentiation (Begeot et al., 1983, 1984; Heritier and Dubois, 1994), PRL release (Giampietro et al., 1979; Yen et al., 1980; Casper and Yen, 1981), and PRL gene expression (Tilemans et al., 1991). These effects have previously been considered only as resulting from complex paracrine interactions within the pituitary, via GnRH-stimulated factors (Tilemans et al., 1991; Eidne et al., 1992). Such paracrine interactions between lactotrophs (or somatotrophs) and gonadotrophs have been extensively studied by Denef's group (Denef, 1994), but they seems to be highest in cultures of neonatal rat pituitary and in reaggregate pituitary cultures of 2-wk-old rats, compared to pituitary cultures from adult rats (Denef, 1994). Thus, it seems that gonadotrophs play a role during the postnatal development of lactotrophs (or somatotrophs). In conclusion, without ruling out possible paracrine actions of secreted gonadotrophic factors, a direct GnRH effect on lactotroph function can be postulated, since GnRH-R transcript is also present in the lactotrophs.

Materials and Methods

Animals

Normal adult male and female rats (Wistar strain, 250 g) were stunned, and their pituitaries and livers were quickly removed and cut into 1-mm^3 pieces, which were fixed in 4% paraformaldehyde in 0.1*M* phosphate buffer, pH 7.4, for 2 h at 4°C. Ultrathin frozen 80-nm thick sections were obtained as previously described (Morel, 1991).

In Situ *Hybridization*

A 30-mer oligodeoxyribonucleotide probe, complementary to nucleotides 574–603 of the pituitary GnRH-R cDNA sequence (Eidne et al., 1992), was 3'end labeled with digoxigenin-16 dUTP (Boehringer, Mannheim, Germany) as described (Morel, 1993). In the present study, conditions of hybridization and detection were optimal, as previously described (Morel, 1993; Mertani et al., 1994; Morel et al., 1994b). The hybridization buffer contained 30% deionized formamide, 4X standard saline citrate (1X SSC: 0.15*M* NaCl, 0.015*M* sodium citrate, pH 7.0), 1X Denhardt's solution (50X Denhardt: 1% bovine serum albumin, 1% Ficoll 400, 1% polyvinylpyrrolidone), 250 mg/mL yeast tRNA, and 4 pmol of labeled probe/mL of hybridization buffer. After 3 h of incubation of the probe at saturating concentration at 30°C, the grids were washed twice (30 min each) at room temperature in 2X SSC, postfixed in 4% paraformaldehyde for 5 min, and then washed in mono-di-sodium phosphate buffer for 30 min. Hybridized digoxigenin probes were reacted with antidigoxigenin serum (1:40) (Boehringer) conjugated with 15-nm gold particles (BioCell, Cardiff, UK) for 1 h, and washed before processing for electron microscopic observation. ISH controls were performed on sections consecutive to positive controls and included:

1. Omission of the probe;
2. Hybridization with an excess of unlabeled probe in a ratio of 100:1;
3. Hybridization with a 30-mer labeled heterologous probe for GH or PRL, containing the same percentage of G-C bases, and using the same conditions as for the labeled probe; and

4. Hybridization with an excess of heterologous unlabeled probe for GH or PRL, in a ratio of 100:1, using the same buffer conditions as for the experimental probe. Liver sections were also used to determine the specificity of the reactions.

Immunocytology

Antibodies used were rabbit anti-rPRL (1:10 000) (Dako, Trappes, France) and prediluted rabbit anti-hLH, which crossreacted in rat sections (pure) (Dako). Double labeling was performed as previously described (Morel, 1993). Briefly, after the ISH washing steps, the sections were incubated simultaneously with mouse antidigoxigenin (1:100) and rabbit antipituitary hormones in 0.1*M* phosphate buffer containing 0.65*M* NaCl, 0.05% Tween 20, and 1% albumin, pH 7.4, for 30 min. After washing in a phosphate buffer, and then in a buffer containing 0.02*M* Tris, 0.65*M* NaCl, and 0.5% ovalbumin, pH 7.6, the antigen–antibody complexes were revealed by incubation for 30 min with a mixture of goat antimouse IgG (1:40), conjugated to 15-nm gold particles and goat antirabbit IgG (1:40), conjugated to 5-nm gold particles. The grids were finally washed in the same buffer (3 × 10 min), fixed with 2.5% glutaraldehyde, washed with distilled water, and contrasted as above before electron microscopic observation. The specificity of the immunocytological reaction was verified on sections consecutive to positive controls by: (1) omission of the antipituitary hormone sera, and (2) replacement of the antipituitary hormone serum by a nonimmune serum or a negative control reagent (Dako). Liver sections were also used as a negative control.

Observation

The grids were stained with uranyl acetate, embedded in methyl cellulose (Morel, 1991), and examined with a Philips CM 10 electron microscope at 80 kV. In the pituitary cells, whose morphological characteristics after cryoultramicrotomy are similar to those of epoxy resin-embedded tissue (Hemming et al., 1983), the somatotrophs were found to contain secretory granules 350 nm in diameter, and their endoplasmic reticulum consisted of regular and parallel cisternae; the lactotrophs contained secretory granules of irregular shape, measuring 400–700 nm, and their endoplasmic reticulum took the form of regular and parallel cisternae; the gonadotrophs contained secretory granules of 200–300, and occasionally 600–700 nm diameter, and in many cases, their endoplasmic reticulum was vesicular; the thyrotrophs contained dense secretory granules uniformly smaller than 100 nm; the corticotrophs were characterized by their stellate shape and their small (200-nm) secretory granules.

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