Growth-Associated Protein-43 Messenger Ribonucleic Acid Expression in Gonadotropin-Releasing Hormone Neurons during the Rat Estrous Cycle

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ABSTRACT

We have shown previously at the ultrastructural level that morphological changes occur in the external zone of the median eminence allowing certain GnRH nerve terminals to contact the pericapillary space on the day of proestrus. The present study was designed to determine whether the intrinsic determinant of neuronal outgrowth, growth-associated protein-43 (GAP-43), was expressed in GnRH neurons of adult female rats, and whether its expression varied throughout the estrous cycle. To accomplish this, we perfusion-fixed groups of adult female rats at 0800 and 1600 h on diestrous day 2 (diestrous II), at 0800 h and 1600 h on proestrus, and at 0800 and 1600 h on estrus (n = 4 rats/group) and used double labeling in situ hybridization and quantification to compare the levels of GAP-43 messenger RNA (mRNA) in cells coexpressing GnRH mRNA. GnRH mRNA was detected with an antisense complementary RNA (cRNA) probe labeled with the hapten digoxigenin, whereas the GAP-43 cRNA probe was labeled with 35S and detected by autoradiography. In addition, GAP-43 protein was identified with immunohistochemistry in the median eminence. The results show that many GnRH neurons expressed GAP-43 mRNA and that GAP-43 protein was present in many GnRH axon terminals in the outer layer of the median eminence. The number of GnRH neurons expressing GAP-43 mRNA was significantly higher on proestrus (64 ± 5%) than on diestrous II (40 ± 2%; P < 0.001) or on estrus (45 ± 8%; P < 0.05), and the GAP-43 mRNA levels in GnRH neurons also varied as a function of time of death during the estrous cycle. The GAP-43 mRNA levels in GnRH neurons were higher on proestrus and estrus than on diestrous II (P < 0.05). These data show that 1) GAP-43 is expressed in adult GnRH neurons; 2) GAP-43 mRNA expression in GnRH neurons fluctuates during the estrous cycle; and 3) GAP-43 mRNA content in GnRH neurons is highest on the day of proestrus, before and during the onset of the LH surge. These observations suggest that the increased GAP-43 mRNA expression in GnRH neurons on the day of proestrus could promote the outgrowth of GnRH axon terminals to establish direct neurovascular contacts in the external zone of the median eminence and thus facilitate GnRH release into the pituitary portal blood. (Endocrinology 141: 1648–1657, 2000)

THE PREOVULATORY surge of LH is initiated by an abrupt increase in the release of GnRH (1, 2). This event is linked to an activation of the GnRH perikarya in the preoptic area (3, 4) and to morphological changes at the site of GnRH release, i.e. the median eminence, allowing GnRH axon terminals to contact the perivascular space directly (5, 6). Actual physical contacts between GnRH nerve endings and the parenchymatous basal lamina surrounding the perivascular space can be seen only during proestrus, whereas glial elements are interposed during the other stages of the cycle. These observations suggest that GnRH neurosecretory endings could be able to sprout spontaneously and thus reach the basal lamina to facilitate the secretion of the neurohormone into the portal blood during the preovulatory surge.

As the regulation of neurite outgrowth and structural plasticity involves the expression of growth-associated factors, the present study was designed to determine whether growth associated protein-43 (GAP-43) was expressed in adult GnRH neurons and whether GAP-43 protein was present in GnRH axon terminals in the median eminence. GAP-43 stimulates neurons to sprout new terminals even in the adult and can therefore be considered an intrinsic determinant of the neuron’s growth state (7, 8). The present study examines the periovulatory patterns of GAP-43 gene expression in GnRH neurons to determine the temporal relationships with the GnRH/LH surge. Our results show that adult GnRH neurons express GAP-43 messenger RNA (mRNA), that GAP-43 mRNA expression in GnRH neurons varies during the rat estrous cycle, and that GAP-43 protein is transported to the GnRH axon terminals.

Materials and Methods

Animals

Twenty-four adult female Wistar rats (235–250 g; CERJ, Saint Berthevius, France) were maintained on a 14-h light, 10-h dark cycle (lights on at 0500 h), with food and water available ad libitum. The estrous cycle was monitored by daily inspection of vaginal cytology. After at least 2 complete 4-day cycles, the 24 animals were divided into 6 groups,
representing different phases of the periovulatory period: diestrous day 2 (diestrous II) 0800 h (n = 4), diestrous II 1600 h (n = 4), proestrus 0800 h (n = 4), proestrus 1600 h (n = 4), estrus 0800 h (n = 4), and estrus 1600 h (n = 4). Groups were chosen to coincide with times when plastic changes in the GnRH nerve terminal level in the median eminence have been described (proestrus 0800 h and proestrus 1600 h) (5), and when the GnRH release in the portal blood reaches its peak (proestrus 1600 h) (9) as well as for comparison with other stages of the estrous cycle (diestrous 0800 and 1600 h; estrus 0800 and 1600 h) when GnRH release is maintained at a basal rate (9) and when no morphological change occurs at the GnRH nerve endings (5). The time points were also chosen to represent cycle stages in which the steroid environment is different (5, 10): before preovulatory estrogen secretion (diestrus 0800 and 1600 h), during the preovulatory estrogen surge (proestrus 0800 and 1600 h), during the preovulatory estrogen secretion (estrus 0800 and 1600 h), and after preovulatory estrogen and progesterone secretion (estrus 0800 and 1600 h).

**Tissue preparation**

Animals were anesthetized with ketamine (20 mg/kg) and xylazine (0.2 ml/kg). Blood was collected, and plasma was stored at −20 C for the estradiol, progesterone, and LH RIAs. Animals were perfused transcardially with 5–10 ml saline followed by 500 ml 4% paraformaldehyde in 0.1 m phosphate buffer. The brains were removed and immersed in the same fixative for 2 h. They were then washed overnight in 0.05 m Coon’s veronal buffer (pH 7.4) containing 20% sucrose, embedded in Tissue-Tek (Miles Laboratories, Naperville, CA), and frozen in liquid nitrogen. Frozen 14-μm coronal sections were collected from the level of −0.45 to −0.46 mm relative to the bregma, according to the atlas of Swanson (11). The sections were mounted onto gelatin-coated slides and stored at −80 C until used for *in situ* hybridization. All experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), regarding mammalian research.

**35S-Labeled GAP-43 cRNA probes**

The plasmid vector PGEM3Z containing a PstI/EcoRI fragment of 743 bp corresponding to bases 552-1295 of the rat GAP-43 complementary DNA (cDNA) (12) was used. PstI and T7 RNA polymerase were used to synthesize the antisense probe, and EcoRI with T3 RNA polymerase were used to synthesize the sense probe. The radioactively labeled probes were generated in the presence of 200 μCl [35S]TP (Amersham Pharmacia Biotech, Les Ulis, France) using 1 μl of the appropriate RNA polymerase in a 40-μl transcription reaction volume containing 8 μl 5 × transcription buffer, 2 μl 0.1 m dithiothreitol (DTT), 1 μg linearized plasmid (50 ng/μl), 1 μl Escherichia coli transfer RNA (5 mg/ml), 20 U RNasin, and 1 μl of a 10 mm stock solution of ATP, GTP, and UTP. The transcription reagents were incubated for 4 h at 39 C. Labeled probes were extracted with phenol-chloroform and purified on a Sephadex G-50 column. The 35S-labeled riboprobes were diluted with hybridization buffer to a final concentration of 30,000 dpm/μl.

**Digoxigenin-labeled cRNA GnRH probe**

The plasmid vector GST7 containing the 330-bp BamHI/HindIII insert of GnRH cDNA was linearized with HindIII for antisense and with BamHI for sense probes. The riboprobes were synthesized *in vitro* with 1 μg linearized GnRH cDNA, 1 × digoxigenin RNA labeling mixture (Roche, Meylan, France), RNA polymerase (T7 for antisense and SP6 for sense), and 1 × transcription buffer. This mixture was incubated at 37 C (T7) or at 39 C (SP6) for 2 h. Residual DNA was digested with deoxyribonuclease. The probes were diluted 1:1000 with hybridization buffer.

**Dual labeling in situ hybridization**

Sections were removed from storage at −80 C, thawed, and placed into 0.1 ml glycine-0.2 m Tris-HCl (pH 7.4) for 10 min before treatment with proteinase K (1 mg/ml in 100 mM Tris, pH 8.0, and 50 mM EDTA) for 15 min at 37 C. Slides were then immersed in 4% paraformaldehyde/0.1 m phosphate buffer for 15 min, and treated with 0.1 m triethanolamine (pH 8.0) for 10 min, followed by 0.25% acetic anhy-
at 37°C and rinsed in 2× SSC for 15 min at 60°C and 0.1× SSC for 15 min at 60°C. The sections were then washed in buffer 1 (100 mM Tris-HCl and 150 mM NaCl, pH 7.4) and incubated for 30 min in blocking buffer (1% Roche blocking agent in buffer 1). Sections were incubated for 4 h in buffer 1 containing antidigoxigenin Fab conjugated to alkaline phosphatase (Roche) which was diluted 1:250 with buffer 1 containing 1% normal sheep serum and 2.4 mg levamisole/10 ml. After rinsing for 10 min in buffer 1 and for 10 min in buffer 2 (100 mM Tris-HCl, 50 mM MgCl₂, and 100 mM NaCl, pH 9.5), sections were incubated in chromogen solution (buffer 2 containing tetrazolium

FIG. 2. Darkfield microphotographs (a, c, and e) and line drawings (b, d, and f) of the distribution of GAP-43 mRNA-expressing cells in the rostral (a and b) and caudal (c and d) preoptic regions and in the supraoptic nucleus (SO; e and f). GAP-43 mRNA is highly expressed in the median preoptic nucleus (MEPO; a–d) and in the medial preoptic nucleus (MPN; c and d). Note that labeling is absent in the SO (e and f). aco, Anterior commissure; AVP, anteroventral preoptic nucleus; AVPV, anteroventral periventricular nucleus; MPO, medial preoptic area; och, optic chiasma; opt, optic tract; PVpo, preoptic periventricular nucleus; sch, suprachiasmatic nucleus. Magnification, ×100.
chloride and 5-bromo-4-chloro-3-indolyl phosphate). The reaction was stopped after 3 h by rinsing twice for 15 min each time in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The slides were dehydrated in 70% ethanol containing ammonium acetate (300 mM) and 100% ethanol, and dipped in Ilford K5 emulsion (Saint-Priest, France). All sections were developed after a 20-day exposure.

**Controls**

Specificity controls included incubation of the sections with 35S- and digoxigenin-labeled sense probe (Fig. 1a), pretreatment with ribonuclease, and coincubation with a 100-fold excess of unlabeled antisense probe. No labeling was observed on the control sections.

**Quantitative analysis**

Sixteen sections of the diagonal band of Broca (DBB)/rostral preoptic area/medial preoptic area were analyzed per animal. During the first analysis, the grain density corresponding to GAP-43 mRNA levels in non-GnRH cells was quantified by using the DensiRag computerized program of Biocom (Les Ulis, France). The boundaries of the hypothalamic nuclei studied were determined from observation of the corresponding azure blue-stained sections. Sections from DBB, anteroventral periventricular nucleus (AVPV), median preoptic nucleus (MEPO) and medial preoptic nucleus (MPN) were viewed under a 360 epiillumination darkfield objective. Video images were obtained with a camera attached to the microscope. The OD over the third ventricle adjacent to the preoptic area and over the supraoptic nucleus that did not contain hybridization signals was measured, averaged, and used to calculate the mean background density, which was subtracted from the OD measurement of signals over the GAP-43 cells. Cells were identified as labeled with GAP-43 probe if the OD over the perikaryon was at least 5 times higher than the background. About 30 cells per each hypothalamic nuclei and per section were analyzed.

During the second analysis, quantification of GAP-43 mRNA in GnRH neurons was studied. Firstly, GnRH mRNA-expressing cells were observed under a brightfield illumination, and GAP-43 mRNA-expressing...
between the groups were regarded as significant when percentage of GnRH neurons expressing GAP-43 mRNA. Differences among estrous cycle stages were assessed with one-way ANOVA followed by Bonferroni’s post-hoc test to compare the intra- and interassay variances were 6% and 8.5%, respectively.

Plasma estradiol was measured using a RIA kit optimized for the direct quantitative determination of very low concentrations of 17β-estradiol in human serum plasma (e.g. in children), purchased from SORIN Biomedica (Antony, France). Assay sensitivity was 0.2 pg/tube, and intra- and interassay variances were 5.6% and 7.3%, respectively.

Progesterone levels were measured in plasma samples without extraction, using a RIA kit purchased from SORIN Biomedica. Assay sensitivity was 5 pg/tube, and intra- and interassay variances were 5.5% and 8.1%, respectively.

Results

Distribution of GAP-43 mRNA-expressing cells

High levels of GAP-43 mRNA hybridization signal were observed in certain cells of the DBB (Fig. 1, b and c) and in several nuclei of the rostral and medial preoptic regions, e.g. the AVPV, MEPO, and MPN (Fig. 2, a and c). GAP-43 mRNA levels were very low in the supraoptic nucleus (Fig. 2e).

Cellular labeling intensities for GAP-43 mRNA during the estrous cycle

The average GAP-43 labeling density was calculated over cells in the DBB, AVPV, MEPO, and MPN at each of the six time points. Figure 3 depicts average brightness data from the different nuclei. Statistical comparisons revealed that the GAP-43 mRNA levels were higher in the DBB on the day of proestrus than on diestrous II (P < 0.05; Fig. 3A), whereas GAP-43 mRNA levels did not vary throughout the estrous cycle in the AVPV (Fig. 3B). In the MEPO, GAP-43 mRNA levels were slightly higher in diestrus and proestrus than in estrus (Fig. 3C), whereas in the MPN, GAP-43 mRNA levels...
were elevated only at 1600 h on proestrus compared with those at 1600 h on estrus ($P < 0.05$; Fig. 3D).

**Distribution of GAP-43 mRNA-expressing GnRH neurons**

The distribution of neurons expressing GnRH mRNA was similar to the description of previous *in situ* hybridization studies (16–19). The greatest numbers of labeled GnRH neurons were seen in the rostral preoptic area, followed in decreasing order by the medial preoptic area and the DBB.

GAP-43 mRNA was expressed in numerous GnRH neurons (Fig. 4) without preferential distribution according to the different areas.

**Changes in the number of GnRH cells expressing GAP-43 mRNA during the estrous cycle**

GAP-43 mRNA was expressed in many GnRH neurons throughout the estrous cycle; however, the percentage of dual labeled neurons changed significantly depending upon

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**Fig. 5.** Percentage of GnRH neurons expressing GAP-43 mRNA (A) and relative amount of GAP-43 mRNA in GnRH neurons (B) throughout the rat estrous cycle. The values are the mean ± SEM. The number of GnRH neurons that express GAP-43 mRNA is highest during proestrus, as is the relative amount of GAP-43 cRNA hybridization signal per GnRH neuron. Significant differences ($P < 0.05$) among the average values for the different time points are indicated as a vs. b and c vs. d.
the stage of the cycle (Fig. 5A). The percentage of GnRH neurons that expressed GAP-43 mRNA was lowest during diestrous II (40 ± 2%; P < 0.001); it peaked during proestrus (64 ± 5%) and returned to basal on estrus (45 ± 8%; P < 0.05).

Changes in the GAP-43 mRNA labeling intensities in the GnRH neurons during the estrous cycle

The average GAP-43 labeling density per GnRH cell was 29 ± 1 when all animal groups were averaged. Dual labeled GnRH neurons generally expressed lower GAP-43 mRNA than single labeled GAP-43 cells. Figure 5B lists the average density of grains per GnRH neuron during different stages of the estrous cycle. Statistical comparisons revealed differences between several time points. GAP-43 mRNA hybridization signal in GnRH cells was low at 1600 h on diestrous II; it was increased significantly by 0800 h on proestrus and was maintained at high levels through 1600 h on estrus (Fig. 6). When the morning and afternoon data were grouped, GAP-43 mRNA levels were elevated on proestrus (density of grains per cell, 29 ± 1) and on estrus (density of grains per cell, 32 ± 2) compared with those on diestrous II (density of grains per cell, 25 ± 1; P < 0.01; Fig. 5B).

GAP-43 protein is expressed in GnRH nerve terminals in the median eminence of adult animals

In the median eminence, GAP-43 labeling was observed in the same regions where most GnRH axons and terminals were located. Dual immunohistochemistry for GnRH and GAP-43 showed that numerous GnRH-positive axons and nerve endings were also GAP-43 positive (Fig. 7). In addition, many GAP-43-positive elements did not contain GnRH (Fig. 7).

Periovulatory hormone secretions

Average plasma LH, estrogen, and progesterone levels were typical for the different time points of the estrous cycle (Table 1). Thus, LH levels were high only during the preovulatory surge at 1600 h on proestrus, and estrogen showed the expected high values during proestrus and had returned to baseline values by 0800–1600 h on estrus. Average progesterone levels reached two peaks during the estrous cycle. The first peak was observed at 0800 h on diestrous II, and the second peak began during the afternoon of proestrus. These results are in accordance with our previous results (5) and those of Butcher et al. (10).

Discussion

The results of the present study show that GAP-43 mRNA and protein are expressed in adult GnRH neurons and that the percentage of GnRH neurons expressing GAP-43 changes...
GAP-43 mRNA EXPRESSION IN GnRH NEURONS DURING RAT ESTROUS CYCLE

The percentage is the highest during the day of the preovulatory LH surge. Similarly, GAP-43 mRNA content in individual GnRH neurons varies during estrous cycle. Thus, GAP-43 mRNA levels in GnRH neurons are the lowest during diestrous II, and they increase by 50% during proestrus. These high levels of GAP-43 mRNA are maintained for at least 36 h (until 1600 h of estrus). Together, these results suggest that GnRH neurons show an increase in GAP-43 mRNA levels just before the preovulatory gonadotropin surge, i.e., in proestrus.

It is well established that the proestrous surge of GnRH (1, 2) provides the neural trigger for the release of the preovulatory gonadotropin surge that, in turn, induces ovulation (20). Although it is known that GnRH neurons are acutely activated under the preovulatory hormonal conditions, i.e. by high estradiol levels during the morning and afternoon of proestrus (5, 10) and by rising progesterone levels during the afternoon of proestrus (21, 22), the mechanisms by which gonadal and circadian (23) cues are conveyed to the GnRH neurons remain unclear. It appears that both the GnRH perikaryon as well as the axon terminals undergo specific changes in response to elevated estradiol levels. Thus, we recently reported that physical contacts between GnRH nerve endings and the perivascular space in the external zone of the median eminence were observed only on the day of proestrus, whereas during the other stages of the cycle, glial elements prevented direct access of GnRH terminals to the capillaries (6). This neuro-glio-endothelial plasticity allows direct access of the GnRH nerve terminals to the fenestrated capillaries only during the time of high secretory activity during which the GnRH nerve terminals project filipodia-like extensions toward the basal lamina (5, 6). The results of the present study suggest that the outgrowth of GnRH nerve terminals on the day of proestrus is stimulated by GAP-43, which is an important regulator of neurite outgrowth in many neuronal systems. Indeed, both the percentage of GnRH cells expressing GAP-43 mRNA and the GAP-43 mRNA levels in GnRH cells are highest at this estrous cycle stage. As GAP-43 protein levels appear to be proportional to the mRNA levels (12, 24 – 29), it is suggested that increased expression of the GAP-43 mRNA in GnRH neurons leads to a rapid accumulation of GAP-43 protein in the GnRH axon terminals in the external zone of the median eminence (30 – 32). The presence of GAP-43 protein in this brain region has been reported (13), and the results of the present study show that this protein is colocalized with many GnRH nerve fibers and terminals. GAP-43 could cause an enhanced signal for growth (7, 8), such that GnRH neuroendocrine axons sprout toward the basal lamina, which would facilitate GnRH release into the portal blood. This scenario is

### TABLE 1

<table>
<thead>
<tr>
<th>Stage</th>
<th>LH (ng/ml)</th>
<th>Estradiol (pg/ml)</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diestrous II, 0800 h</td>
<td>0.59 ± 0.17</td>
<td>11.28 ± 2.38</td>
<td>12.53 ± 1.81</td>
</tr>
<tr>
<td>Diestrous II, 1600 h</td>
<td>0.49 ± 0.1</td>
<td>8.36 ± 2.84</td>
<td>12.88 ± 1.05</td>
</tr>
<tr>
<td>Proestrus, 0800 h</td>
<td>1.26 ± 0.31</td>
<td>31.95 ± 6.20</td>
<td>11.08 ± 1.33</td>
</tr>
<tr>
<td>Proestrus, 1600 h</td>
<td>4.24 ± 1.57</td>
<td>62.48 ± 9.51</td>
<td>23.24 ± 5.31</td>
</tr>
<tr>
<td>Estrus, 0800 h</td>
<td>0.49 ± 0.12</td>
<td>3.15 ± 0.25</td>
<td>15.60 ± 1.72</td>
</tr>
<tr>
<td>Estrus, 1600 h</td>
<td>0.38 ± 0.08</td>
<td>4.98 ± 2.61</td>
<td>15.45 ± 1.84</td>
</tr>
</tbody>
</table>

* Significant difference from the other estrous stages (P < 0.05), except from c.
* Significant difference from a and the other estrous cycle stages (P < 0.05).

![Fig. 7](https://academic.oup.com/endo/article-abstract/141/5/1648/2988134)

**Fig. 7.** Confocal images of 40-μm thick frontal section through the median eminence. Double immunostaining for GAP-43 (left panel) and GnRH (right panel) shows that the two immunostainings are colocalized within certain axons and nerve terminals (arrows) located in the external layer of the median eminence. Magnification, ×400.
similar to that for neurons of transgenic mice, in which an enhanced expression of GAP-43 induces and potentiates nerve sprouting in the adult animal by promoting growth cone guidance and the formation and growth of sprouts (7). It is likely that circulating gonadal steroids provide important endocrine cues for the regulation of GAP-43 gene expression in GnRH neurons and also in many surrounding cells. Thus, estrogen exerts dramatic effects on the growth and phenotype of basal forebrain neurons, particularly in the preoptic area and hypothalamus (33) where estrogen receptor-containing cells are abundant (34). The trophic actions of estrogen include enhancement of neurite outgrowth (35), dendritic spine formation (36), and synaptic density (37). Previous in situ hybridization studies have shown that treatment of ovariectomized adult rats with estrogen elevates GAP-43 mRNA in the medial preoptic area and hypothalamus (38–40). Shughrue and Dorsa (38) showed that GAP-43 mRNA levels in the medial preoptic area were elevated in estrogen-treated ovariectomized animals compared with those in ovariectomized animals. Our high resolution autoradiograms indicate that under physiological conditions, such as during the estrous cycle, the GAP-43 mRNA levels in non-GnRH cells of the preoptic area do not vary as markedly, whereas greater fluctuations in GAP-43 mRNA levels were observed in the DdB. In most nuclei analyzed in this study, lower cellular levels of GAP-43 mRNA were observed during the stages of the estrous cycle that are characterized by low plasma estrogen levels. However, no variation of GAP-43 gene expression was observed in the AVPV, which is a critical brain region where estrogen is thought to act as an inducer of the GnRH/LH surge (34). With respect to the GnRH neurons themselves, although an action of progesterone cannot be excluded, it is suggested that the dramatic increase in plasma estrogen levels between diestrus and proestrus is responsible for the elevation of GAP-43 mRNA levels and the percentage of GnRH neurons expressing GAP-43 mRNA. As GnRH neurons do not express estrogen receptors (41), estrogen could regulate GAP-43 gene expression in these neurons indirectly through stimulation of estradiol target neurons, which, in turn, convey the steroid signal to the GnRH neurons (34).

In conclusion, the results of this study indicate that GAP-43 is expressed in adult GnRH neurons and that the GAP-43 mRNA expression rate varies during the rat estrous cycle. Elevated levels of GAP-43 mRNA in GnRH neurons on the day of proestrus may lead to an accumulation of GAP-43 protein in GnRH nerve terminals at the median eminence. This phenomenon could contribute to an enhanced responsiveness of GnRH nerve endings to growth factors produced locally in the median eminence and promote sprouting of GnRH nerve terminals toward the pericapillary space. This would permit neurovascular contacts and thus facilitate GnRH release into the pituitary portal blood.

Acknowledgments

The authors thank Mrs. G. Mortreux for her excellent technical assistance with RIA realization, and the NIDDK for the rat LH RIA material. We also thank Mrs. R. Bogaert and Mrs. Christine Declerck (Leica Corp. Microsystem SA, Rueil-Malmaison, France) for assistance with making the figures.

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