Detection of Immunoreacting LH-RF Neurons in Rodents*

By

Koji Kami, Tsunehisa Makino, Masaki Shiina and Michitaka Wada

Departments of Anatomy (Prof. T. Mitsui) and Obstetrics and Gynecology (Prof. R. Iizuka) School of Medicine, Keio University Shinanomachi 35, Shinjuku-ku, Tokyo 160, Japan

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Introduction

It is well known that both natural and synthetic luteinizing hormone releasing factor (LH-RF) stimulate the discharge of LH and follicle stimulating hormone (FSH) from the anterior pituitary gland in many mammalian species both in vivo and in vitro. Since the hypothalamic LH-RF was purified from porcine and bovine hypothalamus and it was identified as a decapetide (pyro-Glu-His-Trp-Tyr-Gly-Leu-Arg-Pro-Gly-NH2), this hormone has been widely utilized for many basic animal experiments in neuroendocrinology and has been used a clinical tool in differential diagnosis of pituitary and hypothalamic disorders. Furthermore, sensitive and specific radioimmunoassay for LH-RF have been developed in many laboratories. However, the sites of synthesis and release of this decapetide in the hypothalamus have not been precisely identified, in part because of the lack of direct immunohistochemistry for LH-RF. In addition to LH-RF assays, antiserum raised for LH-RF radioimmunoassay could be utilized to conduct biological studies along these lines. In recent years, the localization of gonadal steroid concentrating neurons in the hypothalamus has been determined by autoradiographic techniques. The availability of antisera to LH-RF for use with indirect immunohistochemical techniques has permitted various attempts to detect the sites of synthesis of this decapetide. However, the localization of the LH-RF immunoreactive neurons is still not known precisely. The present

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paper describes the successful production of a large amount of antiserum against synthetic LH-RF, the measurement of its potency in blocking ovulation, radioimmunoassay of the quantitative distribution of subcellular fractions in the hypothalamus and the localization of immunoreactive LH-RF neurons as determined by means of direct and new immunomorphological techniques.

**Materials and Methods**

Preparation of antigen:

The conjugation of LH-RF with bovine serum albumin (BSA) was carried out as reported previously. Fifty mg of synthetic LH-RF (synthesized by Dr. Baba, Y., Sankyo Pharmaceutical Co. Ltd., Japan) was deamidated without significant peptide bond cleavage by incubation in 0.5 N HCl with continuous stirring for 24 hr at 23°C. After the incubation, the deamidated LH-RF was chromatographed on thin-layer silica gel plates (6060 Silica Gel, Eastman Kodak Co.) using a solvent system of n-butanol : 3%-ammonia solution (100 : 44, v/v). A new ultraviolet absorbing material, presumably the deamidated LH-RF, migrated behind the original synthetic LH-RF. The mixture was neutralized with 0.5N NaOH to pH 7.0-7.3 and lyophilized. The deamidated LH-RF was conjugated with BSA using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC) as a coupling agent. One hundred and seventy mg of BSA (2.5×10⁻⁴ M, “Cohn fraction V”, Sigma Co.) was dissolved in 10 ml of deionized water, then the deamidated and lyophilized LH-RF and 40 mg of EDC (2.5×10⁻⁴ M, Otto Chemical Co.) were added. The pH was adjusted to 6.5-7.0 with 0.5 N NaOH. The reaction mixture was incubated in the dark for 16 hr at room temperature and the conjugate was then dialyzed against phosphate-buffered saline (PBS, 0.01 M, pH 7.2) for 48 hr at 4°C. The dialyzing solutions were changed frequently. The conjugate was diluted in saline to a final volume of 30 ml and stored frozen at -20°C.

Immunization with antigen:

Five- to six-month-old New Zealand white female rabbits (weighing 2.8-3.5 kg) were immunized with an equal volume of emulsified Freund's complete adjuvant (Difco Co.) and the LH-RF-BSA by repeated multiple (10 sites) dorsal intradermal injections in a volume of 1 ml. These injections were carried out at 2-week intervals. Animals were bled from the central ear artery 7 days after the booster. Blood samples were kept at 4°C overnight, then serum was separated by centrifugation and stored at -20°C in 1 ml sealed ampoules.
Detection of immunoreacting LH-RF neurons

Radioiodination of LH-RF:

Synthetic LH-RF was radioiodinated using a modified chloramine T method. Ten μg of synthetic LH-RF and 1 mCi of ¹²⁵I (396 mCi/ml) were mixed in 50 μl of 0.5M tris-acetate buffer (pH 7.3). Twenty-five μl of chloramine T (2.5 mg/ml in 0.2M tris-acetate buffer, pH 7.3) was added and the mixture was agitated. The reaction was stopped exactly 10 seconds later by the addition of 25 μl of sodium metabisulfate (10 mg/ml in tris-acetate buffer). Next, fifty μl of potassium iodine solution (5 mg/ml in tris-acetate buffer) was added, and the mixture was applied to a Sephadex G-10 column (0.9 x 15 cm) using the same buffer as the eluent. Fractions of 0.75 ml were collected in 1.2 x 7.5 cm disposable culture tubes (Corning Co.) containing 25 μl of 0.1% gelatin in the same buffer. Two main radioactive peaks were collected, the first peak corresponding to ¹²⁵I-LH-RF and the second containing free radioiodine. The first peak fractions were used to determine the peptide content in order to calculate the specific activity of labeled product.

Time course study of the incubation for radioimmunoassay:

To determine the optimal incubation time, variously diluted (1 : 100, 1 : 500, 1 : 1000, 1 : 1500, and 1 : 3000) antiserum (200 μl), 100 μl of ¹²⁵I-LH-RF (15,000 cpm/tube, approximately 170 pg), 100 μl of standards diluted from 10 μl to 500 μl with 0.2 M tris-acetate buffer (or unextracted serum samples) and 100 μl of 1% normal rabbit serum (NRS) with 0.05 M EDTA were incubated for 1-6 hr at 4°C. The antigen-antibody complex was precipitated after different periods of the first incubation by adding an excess of sheep or goat anti-rabbit gamma globulin serum (25-50 μl, undiluted) for 16-24 hr using the double antibody technique. After centrifugation at 2000 x g for 20 min at 4°C, the supernatant was decanted off, and the radioactivity of precipitates was counted in a Packard 578 Autogamma Counter.

Specificity of antibody for immunoreactivity:

Thyrotropin-releasing factor (TRF, pyro-Glu-His-Pro-NH₂, Beckman Co.), Substance P (a tri-decapeptide, Beckman Co.), lysine and arginine vasopressin (an octapeptide, Sigma Co.) and oxytocin (an octapeptide, Sigma Co.) were of synthetic origin. Highly purified rat LH (NIAMDD-Rat LH-RP-1), FSH (NIAMDD-Rat FSH-RP-1) and hypothalamic extract (NIAMDD-Rat HE-RP-1) were supplied by the National Institute of Arthritis, Metabolism and Digestive Disease, Rat Hormone Program (prepared by Dr. Parlow, A.F.), U.S.A.. They were tested for cross reactivity to displace ¹²⁵I-LH-RF from the antibody. Precipitin reactions were carried out on Ouchterlony plates using 0.8% Noble agar (Difco Co.).
Biological potency of antiserum:

All animals used in this study were Wistar-Imamichi and Sprague-Dawley male and female rats (weighing 200–230 g) from the Laboratory for Animal Reproduction Co. Ltd., Japan and ICR male and female mice (weighing 18–25 g) from the CLEA JAPAN Co. Ltd., Japan. They were maintained in a room illuminated for 14 hr (05:00–19:00) and allowed water and rat chow ad libitum. Vaginal smears were examined daily. The ability of this antiserum to neutralize endogenous rat LH-RF was determined by i.v. (from femoral vein) or intraperitoneal (i.p.) administration of the antiserum (0.8 ml) to 4-day cycling female S-D rats at 13:00 and 15:00 hr before and during the critical period of LH surge on proestrus that would block ovulation on the next morning. In another group, 0.8 ml of the antiserum was injected into one femoral vein i.v. and, at the same time, 200 ng of LH-RF in 0.2 ml of 0.15 M saline was injected into the contralateral femoral vein. On the morning of estrus, after laparotomy under a light anesthesia, bilateral Fallopian tubes were excised, scrutinized under a stereoscopic microscope (×20) and the ova, if present, were counted. Normal rabbit serum was used in the control group. Blood LH content was measured using the NIAMDD-Rat-LH-assay kit 1–2 hr after each injection to study the effect of antiserum.

Radioimmunoassay in subcellular fractions of rat hypothalamus:

Donor hypothalami of seventeen W-I female rats were hemisected, and weighed immediately after decapitation on the morning of proestrus. Four to five hypothalamic halves were homogenized in a smooth-walled glass or Teflon pestle and mortar (clearance of 0.25 mm) at 10% w/v in 0.32 M sucrose as a suspension medium with a rotational speed (at 14 mm pestle diameter) of 850 rpm with 5 strokes for 1 min at 0°C. The standard fractionation procedures used in our experiments followed those of Whittaker et al. The subcellular fractions were separated by sucrose gradient ultracentrifugation (Hitachi 65-P Automatic Ultracentrifuge) and radioimmunoassayed for LH-RF.

Horseradish peroxidase-labeled antibody preparation:

For the immunohistochemical studies, the antibody to LH-RF was used in W-I rats and ICR mice hypothalami, using the horseradish peroxidase (HRPO) bridge technique, known as the “carbohydrate bridged” enzyme immunohistochemistry method. Ten mg of HRPO (type VI, Sigma Co.) was dissolved in 2 ml of freshly prepared 0.3 M sodium bicarbonate (pH 8.1), 0.2 ml of 1% 2, 4-fluorodinitrobenzene (FD-NB) in ethyl alcohol was added and the mixture was stirred for 1 hr at 22–24°C. In this way, α- and ε-amino groups were completely blocked prior to their reaction with subsequently formed aldehyde groups. The
precipitate formed after 60 min was removed by centrifugation at 15,000 x g for 10 min. To the resulting supernatant, 1 ml of 0.06 M sodium metaperiodate (NaIO₄) in deionized water was added and mixed gently for 30 min at room temperature. The reaction was stopped by the addition of 1 ml of 0.16 M ethylene glycol in deionized water. After one hour the sample was dialyzed against 0.01 M sodium carbonate buffer (pH 9.5) at 4°C. Ten mg of the above HRPO-aldehyde was incubated overnight with 40 mg of anti-LH-RF gamma globulin or goat anti-rabbit gamma globulin at 22-24°C. The conjugate was reacted overnight with sodium borohydride (NaBH₄) at a final concentration of 0.01 M at 4°C. Afterwards, the excess regents were removed on a Sephadex G-25 column preequilibrated with 0.05 M carbonate buffer (pH 9.5). The resulting brownish eluate was absorbed on a DEAE-cellulose column (1.9 x 15 cm) preequilibrated with 0.005 M phosphate buffer (pH 8.0). The column was washed well with 0.005 M phosphate buffer. This eluate contained both non-labeled gamma globulin and native HRPO. Elution was continued with an increasing gradient of phosphate buffer up to 0.05 M (pH 8.0). This eluate contained HRPO-labeled antibody, and the molar ratio of HRPO to gamma globulin was calculated to be about 2 for the 0.05 M buffer eluate.

Immunohistochemical staining for light microscopic observation:

The hypothalami of six male and twelve female W-I rats and five male and seven female ICR mice were rapidly removed after decapitation on the morning of proestrus. They were fixed with Zamboni's fixative for 3-5 hr at 4°C for paraffin embedding, with periodate lysine-paraformaldehyde fixative (PLP) for 3 hr at 4°C for polyethylene glycol embedding and/or frozen with dry ice-acetone for in vacuo freeze-drying (torr gauge: 5 x 10⁻⁴ mmHg for 72 hr at -28 to -35°C; VFD-4 UT unit from Tozai Tsusho Co., Japan) paraffin embedding or cryostat sections. Slide glasses used in this morphological study were coated with 1% aqueous gelatin. After prefixing with 2% paraformaldehyde, freeze-dried paraffin or cryostat sections were postfixed with Zamboni's or PLP solution (these were found to be the most satisfactory). Frontal and sagittal sections of 8-15 μ thickness were washed in PBS for 30 min, and excess PBS around the tissue was wiped off with filter paper. HRPO-labeled or variously diluted anti-LH-RF serum was mounted on the tissues and left for 30-90 min in a humidity chamber at room temperature. The nonlabeled antiserum was used at a dilution of 1: 50-1: 100 with PBS (indirect method). After incubation, in the former case, excess antibody solution was washed off thoroughly with PBS and enzymatic staining for 2-6 min with 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Co.) substrate solution (20 mg DAB in 100 ml of 0.05 M tris-HCl buffer, pH 7.6, and 0.1 ml of 5% H₂O₂ solution)
was carried out. In the latter case, HRPO-labeled goat anti-rabbit gamma globulin was applied in the same manner while processing the tissue for staining (indirect method). After enzymatic stainings, the slides were washed with PBS and counterstained with the mixture solution of toluidine blue and methylene blue (0.15M phosphate buffer, pH 7.4), methyl green, or cresyl violet to identify the nuclear group of neurons, and the tissues were washed, dehydrated, mounted in "Kenvilen" and examined with a light microscope. As a control, NRS, rabbit anti-hen egg white lysozyme serum\textsuperscript{18,19,21} and anti-LH-RF serum absorbed with the LH-RF-BSA were used in place of the specific antibody in both direct and indirect antibody methods.

Results

Biological effect of the deamidated LH-RF:
On thin-layer chromatography of deamidated LH-RF (Rf. 0.04), a new ultraviolet absorbing material migrated behind the synthetic LH-RF (Rf. 0.13). After elution with 0.15M saline, this deamidated LH-RF was shown by radioimmunoassay to release a significant amount of LH from rat pituitary during a 4 hr incubation in vitro\textsuperscript{20}. This release was equal to that of the non-deamidated LH-RF, indicating that the original decapetide form was preserved during deamidation.

Radioiodination of LH-RF and purification of product:
Two main radioactive peaks were observed in Sephadex G-10 fractions of iodination products after treatment with chloramine T. The first peak, eluted with 0.2M tris-acetate buffer, was sharp and contained $^{131}$I-LH-RF. The second peak contained free $^{131}$I. The specific activity of the first peak was 140-160$\mu$Ci/\mu g. This peak was later found to be the most useful for radioimmunoassay.

Binding of LH-RF with antiserum and radioimmunoassay for LH-RF:
Radioiodinized LH-RF and variously diluted antisera were incubated for 1-6 hr at 4°C. After incubation, the antigen-antibody complex was precipitated for 16-24 hr by adding an excess of goat anti-rabbit gamma globulin serum (25-50 \mu l). The optimal time for the first incubation was found to be 3-4 hr. Antiserum diluted to 1:1500 showed a binding ability of 35-40% after 4 hr of first and 16 hr of second incubation. The optimum incubation time for maximum binding was investigated. Radioimmunoassay for LH-RF was performed in a similar manner. A semilogarithmic dose-response curve at 1:1500 antiserum dilution is shown in Fig. 1. A linear response to the addition of cold LH-RF was obtained in the range from 3-2500 pg.
Detection of immunoreacting LH-RF neurons

Specificity for immunoreactivity:
The radioimmunoassay system for LH-RF was tested for cross reaction with other hypothalamic and pituitary hormones. "Relative cross-reactivity" is defined as the molar weight required to replace 30% of $^{125}$I-LH-RF divided by that amount of LH-RF necessary to displace a similar amount (Table 1). No marked displacement of LH-RF was observed with synthetic TRF, Substance P, lysine and arginine.

![Fig. 1 A semilogarithmic dose-response curve for synthetic LH-RF at 1:1500 antiserum dilution. Each performed in duplicate. See details in Materials and Methods.](image)

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Relative cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic LH-RF (Sankyo)</td>
<td>100.0</td>
</tr>
<tr>
<td>Rat Hypothalamic Extract</td>
<td></td>
</tr>
<tr>
<td>(NIAMDD-Rat HE-RP-1)</td>
<td></td>
</tr>
<tr>
<td>Synthetic TRF (Beckman)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Rat LH (NIAMDD-Rat LH-RP-1)</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Rat FSH (NIAMDD-Rat FSH-RP-1)</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Substance P (Beckman)</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td>Lysine-Vasopressin (Sigma)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Arginine-Vasopressin (Sigma)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Oxytocin (Sigma)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* The amount of pure LH-RF is uncertain.
vasopression, oxytocin, rat LH or FSH with this radioimmunoassay system. The amount of pure LH-RF present in "NIAMDD-Rat HE-RP-1" is uncertain. Four μg of "HE-RP-1" was used in this experiment. However, one-tenth of a rat hypothalamus is optimal for assaying LH-RF by radioimmunoassay. A precipitin line was formed in Ouchterlony double diffusion of the antiserum against LH-RF-BSA (Fig. 4-5). This line disappeared after absorption with BSA (10 mg/ml). The antiserum to LH-RF absorbed with BSA, however, showed discernible final reaction products in hypothalamic tissues when used at dilutions ranging from 1:50-1:100 as an indirect immunohistochemistry method.

Prevention of ovulation by antiserum:

The biological potency of the anti-LH-RF serum is shown in Table 2.

Table 2. Effect of administration of anti-LH-RF serum on ovulation of normal cycling female rats. The injections were repeated at 13:00 and 15:00 hr. See details in Materials and Methods.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. rats</th>
<th>Body wt. (g)</th>
<th>No. ova (L)</th>
<th>Uterine wt. (mg)</th>
<th>Ovarian wt. (mg)</th>
<th>Ant. pit. wt. (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7 (7)**</td>
<td>266.3±20.0***</td>
<td>7.3±1.4</td>
<td>6.7±0.9</td>
<td>431.3±13.3</td>
<td>69.2±3.5</td>
</tr>
<tr>
<td>(N.R.S.* i.v.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/S treated</td>
<td>7 (0)</td>
<td>266.7±10.3</td>
<td>0</td>
<td>0</td>
<td>520.6±26.7</td>
<td>59.7±2.7</td>
</tr>
<tr>
<td>(i.v.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/S treated</td>
<td>8 (8)</td>
<td>271.1±14.6</td>
<td>6.8±1.2</td>
<td>7.0±1.3</td>
<td>455.3±30.6</td>
<td>68.8±1.8</td>
</tr>
<tr>
<td>(i.p.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/S+LH-RF</td>
<td>6 (6)</td>
<td>274.3±9.8</td>
<td>8.3±0.9</td>
<td>6.5±1.6</td>
<td>437.6±14.9</td>
<td>63.9±1.7</td>
</tr>
<tr>
<td>(i.v., 200 ng)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* N.R.S. = Normal rabbit serum. **Number of animals which had ovulated in parentheses. *** Organ weights and the number of ova are Mean±S.E.

Table 3. Serum LH levels in proestrus rats treated with anti-LH-RF serum. Rat LH was measured by NIAMDD-Rat-LH-assay kit.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. rats</th>
<th>LH ng/ml Serum 2:00 p.m.</th>
<th>5:00 p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>569.0±204.1</td>
<td>1045.0±218.2</td>
</tr>
<tr>
<td>(N.R.S., i.v.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/S treated</td>
<td>6</td>
<td>443.2±183.2</td>
<td>401.5±170.9</td>
</tr>
<tr>
<td>(0.8 ml×2, i.v.)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The serum was injected i.v. at 13:00 and 15:00 hr on the day of proestrus. Injections of 0.8 ml blocked ovulation completely. In contrast, i.p. injections of the antiserum failed to inhibit ovulation. Nor-
mal ovulation was seen in NRS-treated rats. Simultaneous administration of a large amount of LH-RF (200 ng) reversed the effect of the antiserum. The sera were assayed for rat LH. As indicated in Table 3, LH surge on proestrus was blocked in these antiserum-treated (i.v.) rats, but LH was maintained at basal levels. Control rats injected with NRS showed the normal proestrus peak.

Radioimmunoassay of subcellular fraction for synaptosomes:
The weight of total wet hypothalami of 17 W-I female rats was 1190 mg, whereas the content of whole immunoreactive LH-RF was 47.6 ng. The subcellular fractions for synaptosome separation were radioimmunoassayed for LH-RF, and the percentage contents were as follows: Nuclear Fr. 11%, Mitochondrial Fr. 11%, Synaptosomal Fr. 5%, Myelin Fr. 7%, Microsomal Fr. 1% and Ribosomal (Cytoplasmic) Fr. 65%.

Purification of HRPO-labeled antibody:
The absorbance at 405 nm and 280 nm of each eluate was measured and the ratio of gamma globulin to HRPO (w/w) was calculated as \( \frac{OD_{280\text{nm}} - OD_{200\text{nm}} \times 1.5}{OD_{405\text{nm}} \times 1.5} \), as described by Yamashita et al. The molar ratio of HRPO to gamma globulin was calculated to be about 0.5 for the 0.005M buffer eluate and about 2 for the 0.05M buffer eluate. The 0.05 M buffer eluate contained the specific HRPO-labeled antibody. When the conjugated globulin was purified on Sephadex G-200, it was not easy to separate conjugated globulin from non-labeled globulin, but DEAE-cellulose chromatography gave easy separation in a short time (about 30 min) and it was not necessary to concentrate the eluate before immunohistochemical staining.

Characteristics of immunoreactive neurons:
Immunoreactive hypothalamic LH-RF neurons of male and female rats and mice were localized in tissue sections as brown granular reaction products under normal conditions (without stimulating or inhibiting treatment of the hormonal or reproductive system) by the new and direct immunoperoxidase techniques (Fig. 6-11). These colored neurons were distinct in comparison with the other ones. This deposit as fine grains in perikarya appeared within the cytoplasm filling the cell and beaded axons could be traced as dark-brown strings. The neurons were about 20-25 \( \mu \) in size, mostly pyriform or spindle-shaped, and the apparent volume varied according to the section. These immuno-positive neurons were distinctly smaller than supraoptic (SO) and paraventricular (PV) nuclei classic neurosecretary cells. The nucleus was round with a distinct nucleolus (Fig. 10-11). Specificity controls of the enzyme reaction products of the perikarya and axons were carried out on adjacent serial sections in a corresponding field under conditions that
Figs. 2-3. Cross sections of brain illustrating loci of LH-RF immunoreactive nerve cells. Diamond and club indicate population of larger (20–25μ) and smaller (15–20μ) immunopositive cell bodies, respectively. Also club indicates randomly scattered perikarya. Difference in size (large, middle and small) of symbols is noted in the strength-weakness of immunoreactivity.

Abbreviations: CA, anterior commissura; CO, optic chiasma; FMP, medial forebrain bundle; HI, hippocampus; TO, optic tract; ar, arcuate (AR) nucleus; ha, anterior hypothalamic (AH) nucleus; hpv, paraventricular (PV) nucleus; hvm, ventromedial hypothalamic (VMH) nucleus; pom, medial preoptic (MPO) nucleus; pv, mammilloinfundibular (MI) nucleus or ventrolateral premamillary (PM) nucleus; sc, suprachiasmatic (SC) nucleus; so, supraoptic (SO) nucleus. For other abbreviations, see König and Klippe126.
leave essentially no doubt as to specificity. These control results showed negative or very light staining, confirming the localization of LH-RF neurons and showing that the preparation of this antiserum to LH-RF was satisfactory.

Hypothalamic topography of immunoreactive perikarya and axons:

The rat and mouse frontal and sagittal topography according to the atlases of König et al. and Montemurro et al., respectively,
of such immunopositive cell bodies in the hypothalamus are shown in Fig. 2-3. The perikarya of specifically immunoreactive neurons were observed extensively from the anterior hypothalamus up to the medial and caudal hypothalamus. Most of these cells in the anterior hypothalamus were distributed in the medial preoptic (MPO) and prechiasmatic (PC) nuclei, SO and suprachiasmatic (SC) nuclei. They were also found in the medial area in the anterior hypothalamic (AH), arcuate (AR), ventromedial hypothalamic (VMH) and PV nuclei, and in the caudal area in the mammilloinfundibular or ventrolateral premammillary (MI or PM) nucleus. The perikarya of SO, SC and PV nuclei, however, were randomly scattered. Positive perikarya were never observed in any extrahypothalamic area. The immunopositive perikarya of AR were mostly small (10-15 μ in diameter) and ovoid in shape and generally stained less intensely than those in the others. Occasionally a process containing immunoreactive LH-RF could be traced extending for some short distance, no more than a hundred microns, from the positive perikaryon. Their axons were frequently observed in the lateral portion of the median eminence near the tuberoinfundibular sulci and into the zona palisadica and zona granulosa. Ependymal tanyocytes, on the wall of the third ventricle and with long processes coursing through the AR nucleus into the median eminence, were never found to be immunopositive on absorption of the rabbit anti-LH-RF serum with rat or mouse liver powder prior to immunohistochemical incubation (indirect method). LH-RF was localized in the organum vasculosum of the lamina terminalis (OVLT) (Fig. 9). In that portion of the OVLT cephalic to the third ventricle, the decapeptide was detected in the nervous tissue surrounding the blood vessels. At this time, the other periventricular organs, the subcommissural organ, the subfornical organ and area postrema were not included among the regions studied. No differences were noted in the numbers of positive cells under any of the conditions used. Immunological controls indicated that the reaction product was specific.

Discussion

The synthetic LH-releasing factor, assayed by an in vitro method, released both LH and FSH from rat pituitaries as reported previously by Kerdelhué et al. Since Kerdelhué et al. first reported a radioimmunoassay for LH-RF, successful production of antisera to LH-RF and development of radioimmunoassays for this decapeptide have been reported by many investigators. Though the methods of generating antisera against LH-RF varied, the method of immunization with LH-RF-BSA conjugate, condensation with BSA with carbodiimide reagent by an unspecific mechanism, easily raised antibody to LH-RF.
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with synthetic LH-RF, hypothalamic or pituitary hormones and substances showed no marked displacement ability, indicating that this antiserum appears to possess high specificity for LH-RF, although we have not yet challenged it with many other peptide analogs which have been purified or synthesized. Radioiodination of LH-RF by the modified chloramine T method with reduced time of action did not completely destroy the biological activity of the peptide, which was most suitable for radioimmunoassay. The linear dose-response range against cold LH-RF was extended from 3 pg to 2.5 ng at 1:1500 dilution in comparison with earlier work, though we have not yet attempted enzymatic iodination. The greatest concentration of hypothalamic LH-RF appears to occur on diestrus or proestrus in the rat and mouse. Administration of an antiserum (0.8 ml) raised against synthetic LH-RF to 4-day cycling female rats on the day of proestrus prevented ovulation on the following day. The ovulation-blocking action of the antiserum could be overcome by exogenous LH-RF (200 ng), indicating that this effect of the antiserum was due to the prevention of LH release. A single injection of LH-RF could also induce ovulation in female hamsters treated with antiserum to LH-RF at 12:00 hr on proestrus. Presumably, endogenous LH-RF was neutralized before it reached the anterior pituitary gland. In these rats the LH surge on the evening of proestrus was also blocked, but serum LH was maintained at basal levels. These findings indicate that endogenous LH-RF is necessary for LH-RF surge but the anterior pituitary is capable of secreting basal levels of LH without stimulation by LH-RF. Similar inhibition of ovulation in the rat by passive transfer of antiserum has been reported by some investigators. They also found a decrease of serum FSH as well as serum LH.

Subcellular immunochemical assays of rat hypothalamic tissues were carried out for LH-RF. The synaptosomal pellet was separated by ultracentrifugation and accounted for 5% (2 pg/mg wet tissue). The synthesis, transport and release sites of LH-RF in the hypothalamus, however, have not been precisely determined by these biochemical procedures.

For the immunohistochemical detection of tissue antigen localization, an ideal conjugation reaction would be the labeling of each gamma globulin with one molecule of HRPO without loss of immunologic or enzymatic activities. HRPO was usually coupled to immunoglobulin using bifunctional reagents, such as p, p'-difluoro-m, m-dinitrophenyl sulfone (FNPS), [1-cyclohexyl-3-(2-morpholinoethyl)] carbodiimide metho-p-tolu- enesulfonate, cyanuric chloride, glutaraldehyde, and so on, and usually yielded more globulin polymers than HRPO-immunoglobulin conjugate. Thus the yield of the labeled-antibody indicated very low coupling efficiency. As far as the purification of the labeled antibody is con-
cerned, the conjugate was usually separated from both non-labeled gamma globulin and unreacted HRPO on a Sephadex G-100 or G-200 column, though this technique presents many difficulties\textsuperscript{27}. To facilitate purification, a new method of DEAE-cellulose chromatography was used in this study, as described by Yamashita et al.\textsuperscript{80}. In practice, the 0.05 M phosphate buffer eluate (pH 8.0) was preferred, because this fraction contained only HRPO-labeled gamma globulin and showed high immunoreactivity. We were therefore, able to observe LH-RF neurons and their axons without resorting to stimulation of the reproductive system, and without using inhibitory drugs after stimulating the synthesis of LH-RF. In male and female proestrus rats and mice, we observed extensively reacted materials in the neurons and axons. The topography of neuronal elements in the hypothalamus was similar in rats and mice\textsuperscript{80}. In many species, LH-RF is consistently observed in the external zone of the median eminence. In the rat, Barry and co-workers\textsuperscript{79} demonstrated LH-RF positive cell bodies and their axons after increasing the synthesis of LH-RF by castration or colchicine treatment, principally in the preoptic and septal areas, decreasing in number caudally, particularly beyond the retrochiasmatic area. They postulated a preoptico-infundibular pathway from the preoptic area to the median eminence in the guinea pig\textsuperscript{80}, and concluded that the concentration of LH-RF synthesizing neurons under normal conditions was not sufficient for visualization of the hormone by their immunofluorescent method. Using the same colchicine technique\textsuperscript{50}, Sétálió et al.\textsuperscript{80} failed to find any LH-RF positive perikarya in the rat hypothalamus. Leonardelli et al.\textsuperscript{83} and Baker et al.\textsuperscript{60} were not able to find LH-RF positive cells in intact guinea pigs and rats, respectively. The rat hypothalamus has been observed only in the median eminence and the OVLT; LH-RF was not seen in perikarya with the exception of the report of Naik\textsuperscript{80}. He located positive perikarya in the arcuate nucleus and scattered in more rostral areas. In the mouse, Zimmerman et al.\textsuperscript{63} found LH-RF reactive perikarya only in the arcuate nucleus. Immunoreactive deposits were also seen in the OVLT and tanycytes of the median eminence. They postulated that the hormone in the mouse hypothalamus was mainly concentrated in tanycytes. However, Silverman\textsuperscript{60} and Gross\textsuperscript{10} found that it was absent in nerve cell perikarya, but present in axons widely distributed through the arcuate nucleus. However, the staining of tanycytes appeared to be an artifact by some criteria. First, Gross\textsuperscript{10} reported that the preabsorption of antiserum with mouse liver powder eliminated staining in tanycytes without affecting the reaction in axons in the mouse. Sétálió et al.\textsuperscript{80} were also able to eliminate staining in tanycytes of the rat median eminence by absorption of their anti-LH-RF serum with rat liver powder. The present authors found that the staining of ependymal cells and tanycytes was greatly
Detection of immunoreacting LH-RF neurons

reduced, without altering the immunoreactivity of LH-RF involving neuronal elements, by absorption of the antiserum with rat or mouse liver powder. Similarly, LH-RF was not detected by Baker et al. in the ependyma of three species of animals investigated. It can be concluded that the staining of ependymal cells and tanyocytes was caused by nonspecific binding of rabbit gamma globulin to rat and mouse periventricular glial cells. From our studies it is clear that one can demonstrate the localization of LH-RF in normal rats and mice without stimulating hypothalamic hormone synthesis, provided that a good antibody and a sensitive technique for immunohistochemical localization are used. Most of the immunopositive neurons were observed in the MPO, PC, AR, AH, VMH and MI (or PM) nuclei. Some of the cells were also traced as axons in the median eminence and in the infundibular zone, with delivery to the capillaries of the primary portal vessels. LH-RF was localized around the OVLT of the rat and mouse. It appears, therefore, that there are true species differences in the presence of immunoreactive LH-RF in the hypothalamus or lack of an immunoreactive amino acid residue for conjugation of antigen with carrier protein. The other periventricular organs, the subcommissural organ, the subfornical organ and area postrema were not studied for LH-RF. Our results indicate that the pathway of LH-RF positive neuronal elements coincides completely with the course of the nerve fibers belonging to the tuberoinfundibular tract (hypothalamo-tuberal tract) described by Szentágothai et al. In addition, most of the areas correspond to the hypophysiotropic areas of Halász et al. and to the septoarcuate area of Everett. Their physiological experiments and our present morphological demonstration of LH-RF pathway are similar to those of Leonardelli et al., Barry et al. and Naik. Unfortunately, however, the detection of the LH-RF production site has not yet been achieved by immunohistochemical investigation and will probably require more sensitive methods or better tissue preparation to detect what may be extremely low intracellular concentration. To overcome the effects of species differences, more experimental work is needed to determine the role played by these neurons in the neuroendocrinology and physiology of laboratory animals in reproduction.

Summary

A highly specific antibody to LH-RF was generated in female rabbits by frequent immunization with synthetic LH-RF-BSA conjugate. In vitro incubation gave 35-40% binding of ¹²⁵I-LH-RF and a linear dose-response relationship against cold LH-RF (from 3-2500 pg) using 1:1500 dilutions of the antiserum. This antiserum showed no marked immunocross-reactivity with rat LH and FSH, Substance P, oxytocin,
lysine and arginine vasopressin or synthetic TRF compared with synthetic LH-RF. These substances were less than 0.03% displacement of LH-RF under these conditions. The precipitin line disappeared after absorption by BSA, though the antiserum reacted with the LH-RF-BSA conjugate on the Ouchterlony agar plate test. Intravenous injections of 0.8 ml of this antiserum to 4-day cycling female rats on the day of proestrus blocked ovulation on the following day. In these rats, LH surge on the evening of proestrus was blocked, but LH was maintained at basal levels. Two to five ng of LH-RF was detected in adult mature rat hypothalamus. Subcellular fractions were radioimmunoassayed for LH-RF. The synaptosomal fraction contained 5% LH-RF as compared with whole hypothalamic tissue (47.6 ng in 17 rats). For immunohistochemical demonstrations, the antibody was applied to rats and mice hypothalami by new and direct immunoenzyme techniques. Adult Wistar-Imamichi rats and ICR mice were decapitated and the hypothalami were prefixed with 2% paraformaldehyde and freeze-dried paraffin or cryostat sections were postfixied with Zamboni's or PLP solutions (found to be the most satisfactory). LH-RF was detected in the cytoplasm of some cell bodies close to the ependymal lining in the lower part of the arcuate nucleus. Other specific LH-RF neurons were observed in the medial preoptic, prechiasmatic, anterior hypothalamic, ventromedial hypothalamic and mammilloinfundibular (or ventrolateral premammillary) nuclei. LH-RF was also localized in the organum vasculosum of the lamina terminalis (OVLT). Ependymal tanycytes, however, were never immunoreactive upon absorption of the antiserum with rat or mouse liver powder prior to the immunohistochemical incubation on the rat or mouse brain tissue, respectively, (indirect method). The other periventricular organs were not studied.

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Explanation of figures

Plate I

Fig. 4. The agar gel diffusion test on the Ouchterlony plate. Antiserum (AS) against LH-RF-BSA in the center well is surrounded by six wells filled with the antigen (A: 2mg/ml, double graded dilution are performed from B to F). A precipitin line was found between antiserum and antigen.

Fig. 5. The agar diffusion test (same above). The antiserum (AS) is placed in the center larger well. The surrounding smaller six wells are filled with several materials: wells AG, LH-RF-BSA (the antigen, 2mg/ml); wells LRF, synthetic LH-RF (10mg/ml); well HE, a rat hypothalamic extract in 0.5 ml of 0.2 M acetic acid; well LH, human LH (LER 907, 0.2 mg/ml). A precipitin line (indicated by arrow) was detectable between antiserum and rat hypothalamic extract. No reaction was between antiserum and synthetic LH-RF.

Fig. 6. Frontal section of rat hypothalamus passing through the arcuate nucleus (AR) showing numerous immunopositive neurons. Note that most of the ependyma are LH-RF negative. Cryostat and not counterstained tissue. V3: The 3rd ventricle. (×200)

Fig. 7. Sagittal section of the mouse hypothalamus passing through the anterior hypothalamic nucleus (HA). Many brownish cells are LH-RF positive and are marked by arrows. Cryostat and not counterstained tissue. (×200)

Fig. 8. Frontal section of the rat hypothalamus passing through the paraventricular nucleus (PV), showing the blackened (within osmium tetroxide vapour for 10 minutes) nerve cells are located at subependymal site. Cryostat and not counterstained tissue. (×900)

Fig. 9. Sagittal section of rat hypothalamus passing through the organum vasculosum of the lamina terminalis (OVLT) region. Cryostat and not counterstained tissue.

Fig. 10. Frontal section of the rat hypothalamus through the supraoptic nucleus (SO). Some neurons and their axons show LH-RF positive staining. Note that the blood vessel (indicated by arrow) is with non-specific immunoreaction. Freeze-dried paraffin embedded tissue and counterstained with the mixture of toluidine blue and methylene blue solution for 2 min. (×200)

Fig. 11. Some of the LH-RF positive neurons of mammilloinfundibular nucleus (MI) of rat show highly stained cytoplasmic granules. Only a few axons show LH-RF positive staining and are marked by large arrows. Small arrows indicate the enlarged blood capillaries by some artefacts and show the non-specific staining. Freeze-dried paraffin embedded tissue and counterstained with the mixture of toluidine blue and methylene blue solution for 2 min. (×400)