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Detection of Estrogen Receptor-β Messenger Ribonucleic Acid and ^{125}I-Estrogen Binding Sites in Luteinizing Hormone-Releasing Hormone Neurons of the Rat Brain

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ABSTRACT

Luteinizing hormone-releasing hormone (LHRH) neurons of the forebrain play a pivotal role in the neuroendocrine control of reproduction. Although serum estrogen levels influence many aspects of LHRH neuronal activity in the female, earlier studies were unable to detect estrogen receptors (ERs) within LHRH neurons, thus shaping a consensus view that the effects of estradiol on the LHRH neuronal system are mediated by interneurons and/or the glial matrix. The present studies used dual-label *in situ* hybridization histochemistry (ISHH) and combined LHRH-immunocytochemistry/^{125}I-estrogen binding to readdress the estrogen-receptivity of LHRH neurons in the female rat. In ISHH experiments we found that the majority of LHRH neurons exhibited hybridization signal for the "β" form of ER (ER-β). The degree of colocalization was similar in topographically distinct populations of LHRH neurons and was not significantly altered by estradiol (67.2±1.8 % in ovariectomized and 73.8±4.2 % in ovariectomized and estradiol-treated rats). In contrast, the mRNA encoding the classical ER-α could not be detected within LHRH neurons. In addition, *in vivo* binding studies using ^{125}I-estrogen revealed a subset of LHRH-immunoreactive neurons (8.8%) which accumulated the radioligand, thus providing evidence for the translation of ER protein(s) within these cells. The findings that most LHRH neurons in the female rat express ER-β mRNA and at least some are capable of binding ^{125}I-estrogen challenge the current opinion that estrogen does not exert direct effects upon the LHRH neuronal system.

KEY WORDS

estradiol, estrogen binding, estrogen receptor, gonadotropin-releasing hormone, *in situ* hybridization, luteinizing hormone-releasing hormone

Introduction

Luteinizing hormone-releasing hormone (LHRH) neurons of the forebrain play a pivotal role in the central regulation of female reproduction. Many aspects of LHRH neuronal activity are modulated by circulating estrogens. Estradiol regulates the biosynthesis and secretion of LHRH, and it determines the sexually dimorphic pattern and estrous cycle variations of galanin expression by LHRH neurons (ref. 1 for a review). Previous studies, however, could not detect specific concentration of tritiated estradiol (2), estrogen receptor-α (ER-α) immunoreactivity (3) or ER-β messenger ribonucleic acid (mRNA; 4) in LHRH neurons of the rat. The lack of evidence for ER expression in these cells shaped a consensus view that LHRH neurons are not receptive to estrogen, but rather, the estrogen signal is communicated to them by interneurons and/or the glial matrix. However, somewhat conflicting data suggest that estrogen might have direct actions upon the LHRH neuronal system. For example, estrogen response elements are present within the promoter region of the primate LHRH gene (5) and immortalized LHRH-producing cells take up estradiol (6) and express ER-α mRNA (7). Recent immunocytochemical studies (8) demonstrate ER-α-like immunoreactivity in 17±1% of LHRH neurons of rats. These findings reopened the debate on the presence and putative functions of ERs within LHRH neurons.

In the present studies, we used dual-label *in situ* hybridization histochemistry (ISHH; 9) and combined *in vivo* ^{125}I-estrogen binding/LHRH-immunocytochemistry to revisit the issue of whether the LHRH neuronal system of the rat is directly regulated by estrogen.

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Materials and Methods

All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the University of Massachusetts IAUCC.

Dual-label ISHH studies

Sprague-Dawley rats (n=10; 225-250 g bw) were ovariectomized (OVX), and on postovariectomy day 12 (1000 h) implanted with two subcutaneous Silastic capsules (Dow Corning; l=30 mm, ID=1.58mm, OD=3.18mm) containing either sesame oil (n=5; OVX group) or 17β-estradiol (Sigma; 200μg/ml in sesame oil; n=5; OVX+E_2 group). On day 14 (1000h), all rats were sacrificed and their brains removed and snap-frozen on pulverized dry ice. Then, 12-μm coronal sections through the region containing the organum vasculosum of the lamina terminalis (OVLT) and the medial preoptic area (MPOA) were collected on gelatin-coated slides and processed for dual-label ISHH (9).

The synthesis and application of the digoxigenin-labeled complementary RNA (cRNA) probe to LHRH mRNA, as well as the detection of the non-isotopic ISHH signal with anti-digoxigenin antibodies conjugated to horseradish peroxidase (Boehringer Mannheim; anti-digoxigenin-POD, Fab fragment, 1:200), were described elsewhere (9). For improved detection of LHRH neurons, the peroxidase-catalyzed deposition of biotinylated tyramine (Renaissance kit; NEN), followed by the incubation of sections with the ABC-Elite reagent (Vector) was added to the procedure. Neurons expressing ER-α or ER-β mRNA were targeted with ^{35}S-labeled cRNA probes which were added at 40,000-120,000 cpm/μl/probe concentration to the hybridization buffer (50% formamide, 20% dextran sulfate, 1X Denhardt's solution, 300 mM sodium chloride, 30 mM sodium citrate, 0.5 mg/ml yeast tRNA, 0.5 mg/ml heparin sodium salt, 1 mg/ml sodium pyrophosphate, 80 mg/ml dithiothreitol; pH 7.0). The ER-α-specific cRNA probe was complementary to nucleotides 1224-2090 of the rat ER-α mRNA (10). To detect the expression of ER-β transcripts in LHRH neurons, two strategies were used. In the first set of experiments, dual-label ISHH was performed on proteinase K-pretreated (Sigma; 0.1μg/ml, 15 min) sections using a cRNA probe corresponding to nucleotides 6-1458 of the rat ER-β mRNA (11). A second series of sections was hybridized with a mixture of two shorter ER-β cRNA transcripts targeting nucleotides 52-610 and 1809-2094. The selection of these probe sequences (12) prevented a potential cross-hybridization with the ER-α mRNA. Following the immunocytochemical detection of the digoxigenin-labeled cRNA probe to LHRH mRNA, the slides were dipped in NTB-3 photographic emulsion (Kodak; diluted 1:1 with distilled water), exposed for 6 weeks, then the autoradiograms were developed. Some sections were counterstained with toluidine blue before coverslipping. Effects of E_2 on colocalization were evaluated using Students' t-tests.

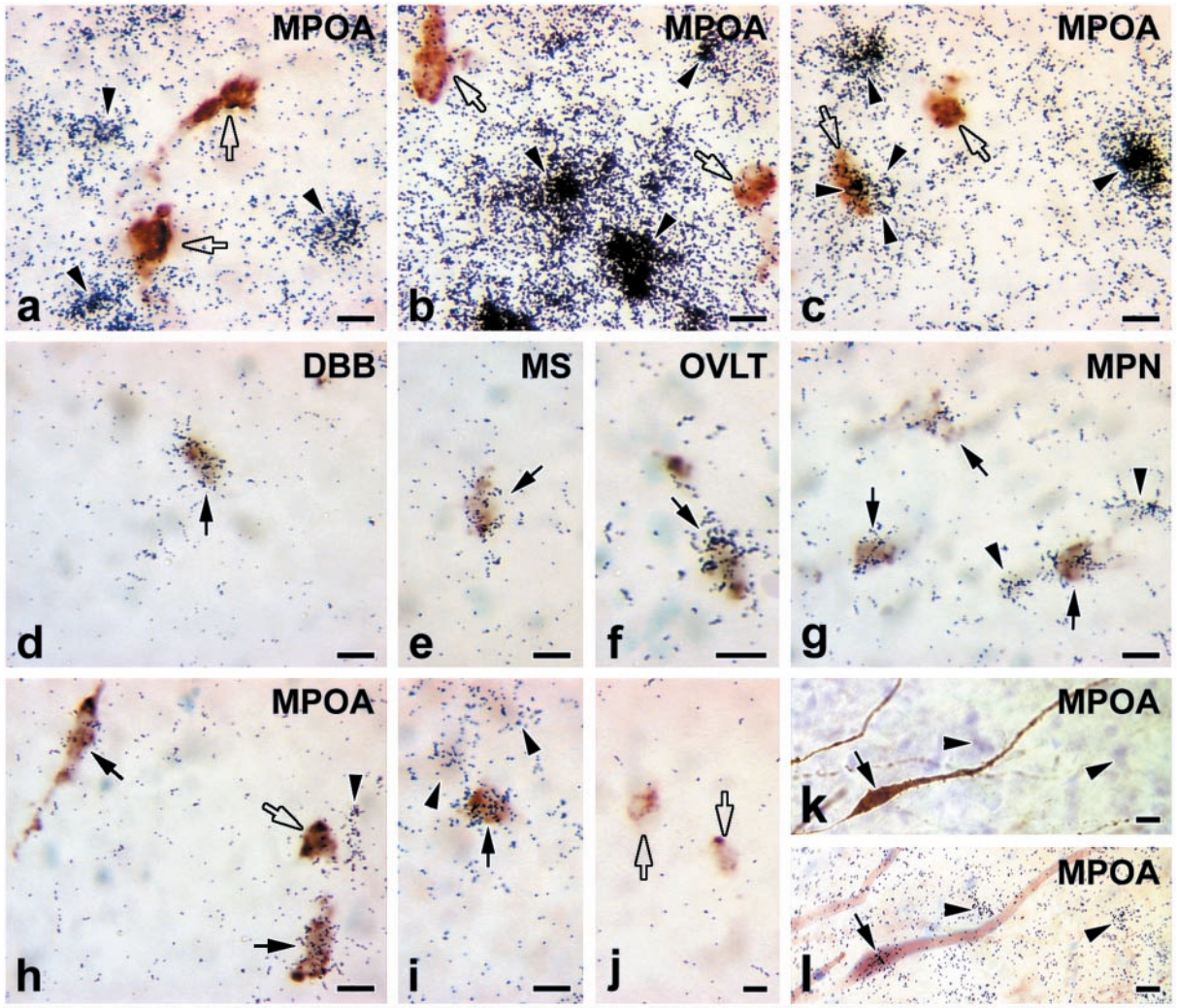
In vivo binding/ ICC studies

On postnatal day 21, female rats (n=7) were ovariectomized. On postovariectomy day 11, five animals were injected subcutaneously with 2 μg/kg bw of 17α-iodovinyl-11β-methoxyestradiol (^{125}I-estrogen, specific activity 2200 Ci/mMol; 13) in 200μl of vehicle (50% DMSO, 50% PBS). Control animals (n=2) were injected with 250 μg/kg bw of 17β-estradiol 1 hour prior to the administration of ^{125}I-estrogen to compete off the radiolabeled compound and verify the specificity of ^{125}I-estrogen uptake in this animal paradigm. Four to six hours after injection of ^{125}I-estrogen, the rats were anesthetized and transcardially perfused with 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde for 20 hours at 4°C. Vibratome sections (35 μm) were collected in ice cold PBS and following pretreatment (13), they were incubated at 4°C with a rabbit polyclonal LHRH antiserum (#H-16) for 18 hours. The detection of LHRH immunoreactivity was completed with the Vector Elite kit. The sections were mounted on gelatin-coated slides, air-dried, then washed (13), dipped in distilled water and allowed to dry at room temperature. Finally, the slides were coated with liquid nuclear emulsion (Kodak; NTB-2, diluted 1:1 with distilled water), air-dried and stored at 4°C in a light-tight desiccator until development (10-20 days).

Results

The results of ISHH studies showed that the mRNA encoding the classical ER-α (Figs a-c) occurred at much higher cellular abundance than ER-β mRNA (Figs d-i) in regions of the preoptic area that contained LHRH neurons. In addition, we found that the autoradiographic signal increased, with slight compromise in background levels, when higher probe concentrations (120,000 cpm/μl, in Figs b and c, vs 40,000 cpm/μl, in Fig a) were used. Most LHRH neurons were entirely devoid of the autoradiographic ISHH signal for ER-α (Figs a-c). In a few instances (4 out of 502 LHRH neurons; <1%) the two signals overlapped partially. Such cases were not interpreted as colocalization (Fig c). In contrast to the lack of evidence for ER-α mRNA in LHRH neurons, a relatively weak autoradiographic signal for ER-β mRNA was frequently detected over the cell bodies of LHRH neurons. The incidence of this colocalization was high in neurons of the diagonal band of Broca (DBB; Fig d), medial septum (MS; Fig e), OVLT (Fig f), median preoptic nucleus (MPN; Fig g) and MPOA (Fig h). We found that 319 of 465 LHRH neurons in OVX, and 539 of 725 neurons in OVX+E_2 rats expressed ER-β mRNA. There was no statistically significant difference between OVX (67.2±1.8%, mean±SEM); and OVX+E_2 (73.8±4.2%) animals in the percentage of colocalization. The finding of ER-β mRNA in LHRH neurons using the 1453-base cRNA probe (Figs d-h) was successfully replicated with the combined application of the two short ER-β probe constructs (Fig i). In further control experiments, the ER-β hybridization signal was absent if the hybridization was performed on sections pretreated with RNAse A (not shown) or if ER-β probes were substituted with ^{35}S-labeled "sense" strand RNA transcripts (Fig j). The absence of grain cluster formation over LHRH profiles using either the antisense probe for ER-α (Figs a-c) or the sense probes for ER-β (Fig j) confirmed our previous observation that DAB does not cause positive chemography on the NTB-3 emulsion (9).

The results of dual *in vivo* binding/ICC experiments showed that a small population of LHRH-immunoreactive neurons (8.8%; 145 out of 1635) in the OVLT/MPOA concentrated the radioactive label in their nuclei (Figs k, l). Only scattered (0-2%) double-labeled cells were observed in the MS. All ^{125}I-estrogen binding to LHRH neurons was eliminated by estradiol pretreatment of the animals, indicating the specificity of radioligand uptake.



Studies of estrogen-receptive luteinizing hormone-releasing hormone (LHRH) neurons using dual-label *in situ* hybridization histochemistry (ISHH; Figs a-j) and combined *in vivo* ^{125}I-estrogen binding/LHRH-immunocytochemistry (Figs k, l). a-c: The autoradiographic ISHH signal for ER-α (clustered silver grains; arrowheads) is heavily expressed in cells of the medial preoptic area (MPOA), but is absent from LHRH neurons (brown histochemical staining; empty arrows). Note the strong ER-α signal and the somewhat compromised autoradiographic background in "**b**" and "**c**", due to the use of higher probe concentrations (120,000 cpm/μl) than in "**a**" (40,000 cpm/μl). The grain cluster in figure "**c**" identified by three arrowheads overlaps only partially with an LHRH profile. **d-h**: Large numbers of LHRH neurons in the diagonal band of Broca (DBB; **d**), medial septum (MS; **e**), organum vasculosum of the lamina terminalis (OVLT; **f**), median preoptic nucleus (MPN; **g**) and MPOA (**h**) express the autoradiographic ISHH signal to ER-β. Dual- (solid arrows) and single-labeled (empty arrow in "**h**") LHRH neurons, in addition to non-LHRH cells expressing ER-β mRNA (arrowheads) are present in ovariectomized (**f**) as well as ovariectomized+estradiol-treated (**d, e, g, h**) animals. The detection of ER-β mRNA was performed with the 1453-base probe. Control studies using the combination of two short ER-β probe constructs in "**i**" confirm the finding of ER-β mRNA expression in LHRH neurons. **j:** Results of dual-label ISHH with the 1453-base sense strand probe for ER-β mRNA show no signal, further confirming the specific binding of the antisense probe and also showing that positive chemographic artifacts do not occur over DAB-stained LHRH neurons (empty arrows). **k, l**: An LHRH-immunoreactive neuron (diaminobenzidine chromogen; brown cytoplasm), photographed at two different focus planes, exhibits *in vivo* accumulation of ^{125}I-estrogen (autoradiographic signal). Note the concentration of silver grains over the cell nucleus of the LHRH neuron (arrows), in addition to the nuclei of non-LHRH cells (arrowheads). Scale bars=10 μm.

Discussion

The results of our dual-label ISHH studies demonstrated that the majority of LHRH neurons in the female rat contain ER-β mRNA. However, a similar methodology failed to detect any expression of ER-α mRNA in these cells. In addition, the observation of *in vivo* ^{125}I-estrogen binding to a subset of LHRH neurons in the OVLT/MPOA provided strong evidence for the translation of functional ER protein(s) at least within some LHRH neurons.

Although much methodological effort, including the application of increased probe concentrations, has been invested successfully to improve the detection of ER mRNAs, our ISHH experiments could not confirm the recent ICC finding of ER-α expression in LHRH neurons of the rat (8). However, we recognize the possibility that very low cellular levels of ER-α mRNA might not be detected in our ISHH studies.

Recently, Laflamme et al. reported the absence of ER-β mRNA in LHRH neurons of the rat (4). In contrast, we detected ER-β mRNA expression in high percentages of LHRH neurons. The different results of our experiments are most likely due to the technical modifications that enhanced the detection sensitivity of the colocalization method. It is noteworthy that we used 4- to 12-fold higher concentration of ER-β hybridization probe and a 4-fold longer autoradiographic exposure than did the cited study.

The *in vivo* uptake of ^{125}I-estrogen indicates the presence of ER protein(s) in a population of LHRH neurons, although LHRH neurons showing accumulation of the radioligand were less numerous then those co-expressing ER-β and LHRH transcripts. This discrepancy might be attributable to differences in sensitivities of the methodologies, together with a functional heterogenity of LHRH neurons in the cellular abundance of translated ER protein(s).

While this manuscript was in preparation, Skynner and co-workers demonstrated ER-α and ER-β mRNA transcripts in individual LHRH neurons of mice using a single-cell multiplex RT-PCR method (14). Because both the RT-PCR and our ISHH approaches are based on the detection of cellular mRNAs, the discrepancies between the results of these two studies are somewhat surprising. The RT-PCR method showed the predominance of ER-α over ER-β mRNA in LHRH neurons in mice, whereas our ISHH experiments could not provide evidence for the presence of ER-α mRNA in rat LHRH neurons. Furthermore, the RT-PCR study detected ER-β expression in a much lower percentage of LHRH neurons in mice (0-19%, the highest in estrous animals), than did our ISHH study in rats (67.2 % in OVX, 73.8 % in OVX+E_2 animals). Finally, the RT-PCR method showed the disappearance of ER-β mRNA from non-LHRH as well as LHRH neurons of the preoptic area in mice at proestrous, when estrogen levels are high. In contrast, we did not observe this dramatic down-regulation of ER-β mRNA by estradiol in the preoptic area; moreover, the incidence of ER-β mRNA expression in LHRH neurons was similar in the presence or absence of E_2. These discrepancies may be attributable to methodological or species differences.

The mechanism whereby estrogen signaling via ER-β within LHRH neurons can modulate reproductive functions will be the subject of further investigation. It is currently impossible to separate direct effects of estrogen from those mediated by interneurons and glial cells. Also, future studies are needed to determine the contributions of the two types of ER to the modulation of LHRH neuronal functions.

In summary, our studies demonstrated the expression of ER-β, but not ER-α, mRNA in large numbers of LHRH neurons and the presence of ^{125}I-estrogen binding sites within a smaller population of these neurons in adult female rats. These data together provide a strong argument against the widely-held view that the LHRH neuronal system is not estrogen-receptive.

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