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Expression of Functional Estrogen Receptors and Galanin Messenger Ribonucleic Acid in Immortalized Luteinizing Hormone-Releasing Hormone Neurons: Estrogenic Control of Galanin Gene Expression

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ABSTRACT

The activity of estradiol on the LHRH neuronal network is crucial in the regulation of reproduction. *In vivo*, estradiol induces galanin (GAL) gene expression in LHRH neurons and GAL/LHRH colocalization is sexually dimorphic and neonatally determined by steroid exposure. The effects of estradiol on LHRH neurons, however, are considered to be indirect because estrogen receptors (ER) have not been detected in LHRH neurons *in vivo*. Using immortalized mouse LHRH neurons (GT1–7 cells), we demonstrated by RT-PCR and Southern blotting that GT1–7 cells express ER messenger RNA (mRNA). Sequencing of the amplification products indicated that GT1–7 ER is of the α-subtype (ERα). Additionally, estrogen receptors in GT1–7 cells were characterized by competitive radioligand receptor binding and IC_{50} values for 17β-estradiol and ICI-182,780 were found to be 0.24 and 4.1 nM, respectively. The ability of endogenous GT1–7 cell ER to regulate transcription was determined in transient transfection studies using a construct that consisted of a luciferase reporter gene that is driven by tandem estrogen response elements (ERE) and a minimal herpes simplex virus thymidine kinase promoter. 17β-Estradiol was found to enhance luciferase activity by 2.5-fold at physiological concentrations with an ED_{50} value of 47 pM. This induction was completely inhibited by ICI-182,780 which had an IC_{50} value of 4.8 nM. Raloxifene, tamoxifen, 4-hydroxytamoxifen, and droloxifene also fully blocked estrogen-mediated luciferase induction with IC_{50} values of 58.4, 89.2, 33.2, and 49.8 nM, respectively. In addition, GAL mRNA was detected and identified by RT-PCR followed by Southern blotting using a rat GAL complementary DNA (cDNA) probe. The ability of 17β-estradiol to modulate expression of the endogenous GAL gene in immortalized LHRH neurons was also determined. Quantitative RT-PCR demonstrated that physiological concentrations of estrogen increase GAL gene expression by 2-fold with an ED_{50} value of 23 pM. ICI-182,780, raloxifene, and droloxifene completely blocked this induction.

In summary, our data demonstrate the presence of ERα and GAL mRNA in GT1–7 cells. The ER in GT1–7 cells is biologically active because 17β-estradiol enhances both endogenous GAL gene expression and an ERE-driven reporter gene. These results suggest that estrogenic control of GAL gene expression in immortalized LHRH neurons may be transduced by ER. Thus, hypothalamic-derived LHRH neurons appear to have the capacity to be directly regulated by estrogen. (*Endocrinology* **139:** 939–948, 1998)

A VAST literature exists that describes the control of the LHRH neuronal network by estrogens (1). Indeed, estrogen treatment stimulates LHRH neurons as reflected by an induction of c-*fos* expression (2), a hallmark of cellular activity (3). In addition, our group detected a higher incidence of galanin (GAL) gene expression in LHRH neurons of female rats (4, 5), indicating that coexpression of GAL and LHRH represents an estrogen-dependent phenomenon. Further studies have provided evidence to indicate that expression of GAL in LHRH neurons is, indeed, dependent on estradiol exposure (6–9). More importantly, we have demonstrated that colocalization of GAL and LHRH is sexually dimorphic in terms of both GAL expression in LHRH perikarya (10) and copackaging of both peptides within the same secretory vesicles in the median eminence (7). Our studies on GAL expression in LHRH neurons, however, did not determine whether or not estrogens exert these actions by acting directly or indirectly on the LHRH neuron. In fact, studies aimed at evaluating the site of action of steroids on the LHRH neuronal network have been confounded by the intrinsic complexity of current models used to assess the problem. The failure to detect estrogen receptors (ER) in LHRH neurons (11, 12) and the identification of estrogen-concentrating neurons in close proximity to LHRH neurons (13, 14) have provided the grounds to establish the dogma that the actions of steroids on the LHRH neuronal system are mediated through regulatory neuronal systems. These interneurons then act as relay stations to transduce steroid inputs that impinge upon the LHRH neuronal network.

Using targeted tumorigenesis, Mellon *et al*. (15) have established an immortalized LHRH neuronal cell line (GT1 cells), which retains many physiologically relevant features of the LHRH neuron, including regulation of LHRH secretion (16–18). It has been reported that immortalized LHRH neurons (GT1–1 subclone) do indeed express estrogen (19, 20) and androgen receptors (19), suggesting that gonadal steroids could act directly on the LHRH neuronal network.

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These observations challenge the current dogma that estradiol exerts its actions on the LHRH neuronal network via interneurons, and they imply that either the technologies used to detect ER *in vivo* within the LHRH neuronal network may lack sufficient sensitivity, or GT1 cells may not be representative of the LHRH phenotype present *in vivo*.

In our current studies, we have used a radioligand binding assay and RT-PCR to identify ER protein and messenger RNA (mRNA) in GT1–7 cells. The data indicate that [^{125}I]17β-estradiol labels binding sites and that [^{125}I]17β-estradiol is specifically displaced by ICI-182,780 from these sites. GT1–7 cells also express ER mRNA corresponding to the ligand binding domain of the ERα-subtype. In addition, the functional activity of ER in GT1–7 cells was determined by transient transfection studies using a minimal estrogen response element (ERE) to regulate a thymidine kinase promoter (tk)-driven luciferase reporter gene. Induction of luciferase activity was ligand- and ERE-dependent, indicating that the expressed receptor is functionally active to enhance transcription. Furthermore, RT-PCR analysis of GT1–7 cell total RNA demonstrated that immortalized LHRH neurons also express GAL mRNA. Because GAL expression in LHRH neurons is exquisitely regulated by estrogen (21, 22) a quantitative RT-PCR (qRT-PCR) method was developed to evaluate estradiol modulation of GAL gene expression in GT1–7 cells. Our data indicate that not only does estradiol, via interaction with ER, regulate GAL gene expression in immortalized LHRH neurons, but, equally as important, that these cells can be stimulated directly by estrogen. Overall, GT1 cells represent a useful model to evaluate the molecular mechanisms of estradiol-dependent activation of LHRH neurons that may occur during the estrous cycle.

Materials and Methods

Cell culture

GT1–7 cells were kindly provided by Dr. Pamela Mellon (University of CA, San Diego, CA). The cells were cultured under standard conditions as described in Moretto *et al*. (23). Cells were maintained in growth medium that consisted of high-glucose (4.5 g/liter) DMEM (BioWhittaker, Walkersville, MD) supplemented with 5% horse serum (GIBCO-BRL, Grand Island, NY), 5% FCS (Hyclone, Inc., Logan, UT), 2 mM GlutaMax, I (GIBCO-BRL), 100 U penicillin/ml and 100 μg streptomycin/ml (GIBCO-BRL). Cells were grown at 37 C in a humidified incubator in an atmosphere of 5% CO_2:95% air.

ER and GAL mRNA identification in GT1–7 cells

RT-PCR and Southern blotting were used to detect the presence of ER and GAL mRNA in GT1–7 cells. Mouse brain total RNA (Clontech Laboratories, Inc., Palo Alto, CA) was used as a positive control. Total RNA was isolated from GT1–7 cells using TRIzol (GIBCO-BRL) reagent according to the manufacturer's specifications. Mouse brain and GT1–7 total RNA were treated with DNase I (GIBCO-BRL) under the conditions suggested by the manufacturer.

A primer pair that spans two thirds of the mouse ERα ligand binding domain (1079–1635 bp) (24) was used to reverse transcribe and amplify ER mRNA from total GT1–7 RNA by RT-PCR. The sequences of the primers used were 5′ CCAAGCCCTCTTGTGATTAAGCACACTAAG 3′ for the forward primer and 5′ ACACGGTGGATGTGGTCCTTCTCT-TCCAGAG 3′ for the reverse primer. The RT reaction consisted of 0.25 μg mouse brain or GT1–7 cell total RNA, 2 mM $MgCl_2$, 1× PCR buffer (GIBCO-BRL), 1 mM dNTPs, 2 U RNasin (Promega, Madison, WI), 2.5 μM reverse primer, 200 U Superscript II reverse transcriptase (GIBCO-BRL) in a final volume of 20 μl. Reverse transcriptase was omitted in some reactions to serve as negative controls. One drop of mineral oil was overlaid onto each reaction mixture to prevent evaporation. The reaction mixtures were heated in a thermocycler (MiniCycler, MJ Research, Inc., Watertown, MA) at 42 C for 15 min followed by 99 C for 5 min. The reaction mixtures were cooled and maintained at 4 C until the amplification step. Reaction mixtures from the RT step were prepared for DNA amplication by the inclusion of 0.5 μM forward primer, 1.6 mM $MgCl_2$, 1× PCR buffer (GIBCO-BRL), and 5 U *Taq* DNA polymerase (GIBCO-BRL) in a final volume of 100 μl. The reactions were heated at 94 C for 3 min followed by 35 rounds of successive heating steps at 94 C for 30 sec, 62 C for 30 sec, and 72 C for 45 sec. Amplification was followed by a final elongation step at 72 C for 5 min. The amplification products were resolved by electrophoresis in a 1% agarose gel using 0.5× Tris-borate-EDTA buffer.

RT-PCR was also used to determine the presence of GAL mRNA in GT1–7 cell total RNA. DNase treatment, RT, and DNA amplification were performed as described above with the following differences. The sequence for the forward and reverse primers for mGAL were 5′ CCT-GCAAAGGAGAAGAGAGGTTG 3′ and 5′ CAGAGGATTGGCTT-GAGGAGTTG 3′, respectively. For the RT step, the reaction mixtures consisted of 0.25 μg mouse brain total RNA as a positive control and 0.25 or 1 μg GT1–7 total RNA, 2.75 mM $MgCl_2$, and 0.5 μM reverse primer in addition to the components of the DNase I reactions. For the amplification step, the concentrations of the forward primer and $MgCl_2$ were adjusted to 0.1 M and 2.75 mM, respectively.

DNA was transferred from the agarose gels to Hybond-N^+ nylon membrane filters (Amersham, Arlington Heights, IL) by vacuum blotting. The filters were washed in 2× SSC for 2 min and dried under vacuum at 80 C. The filters were pre-wetted in dH_2O and prehybridized for 15 min in 10 ml of Rapid-Hyb (Amersham, Arlington Heights, IL) at 42 C and 65 C for the ERα and GAL blots, respectively. An oligonucleotide was synthesized [1305–1323 bp of the published mouse ERα sequence (24)] to serve as a probe for the 557 bp mouse ER amplification product. The mouse ERα probe was end-labeled using T4 polynucleotide kinase (GIBCO-BRL) and [γ-^{32}P]ATP (3000 Ci/mmol; NEN Life Science Products, Boston, MA) according to standard procedures (25). A cDNA (532 bp) that includes the entire rGAL sequence was labeled with [α-^{32}P]dCTP (3000 Ci/mmol; NEN Life Science Products) by random priming (Random Primers DNA Labeling System; GIBCO-BRL) and used to probe the mGAL amplification product. After addition of the denatured probes, each blot was hybridized for 30 min in 10 ml Rapid-Hyb at the respective prehybridization temperatures. After hybridization, the blot for ER was washed twice in 50 ml 2× SSC with 0.1% SDS for 15 min per wash at room temperature. The mGAL blot was subjected to one 15 min wash in 50 ml 2× SSC with 0.1% SDS at room temperature followed by a more stringent 15 min wash at 65 C in 50 ml 0.5× SSC with 0.1% SDS. After washing, both blots were exposed to a PhosphorImaging screen (Molecular Dynamics, Sunnyvale, CA) and the resulting band intensities visualized using a PhosphorImager and the ImageQuaNT software (Molecular Dynamics).

ER binding analysis

Cells were plated in growth medium at a density of 8×10^6 cells/plate on 150-mm Nunc plates precoated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO) and kept in culture until 90% confluent (approximately 4 days). Forty-eight hours before assay, the growth medium was replaced with phenol red-free, charcoal/dextran-stripped serum medium (low steroid medium) and the cells maintained in culture until harvest.

For the receptor preparation, the medium was removed from the plates and the cells were washed twice with 10 ml D-PBS (without Ca^{++} or Mg^{++}; BioWhittaker). ER binding buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.4, 1 ml per plate) was added to each plate and cells were scraped from the plates. The cell material from each plate was pooled and homogenized (30 sec, setting of 3, PT1200 polytron; Brinkmann Instruments, Westbury, NY). The homogenate was spun at 12,000 × *g* for 10 min. Supernatants were collected, homogenized, and spun at 50,000 × *g* for 1 h. The supernatant fraction was collected and used for receptor binding analysis. Protein concentration was determined on this cytosolic preparation by the Pierce BCA protein assay, using BSA as the standard (26).

Binding reactions were prepared in triplicate in 1.5 ml polypropylene

microcentrifuge tubes. Cytosolic preparation (100 μl) was added to each tube followed by 50 μl ER binding buffer for determining total bound counts, 50 μl 40 μM 17β-estradiol (10 μM final concentration; Sigma Chemical Co., St. Louis, MO) for determining nonspecific binding, or 50 μl competitor. Binding reactions were initiated by the addition of 50 μl 16α-[^{125}I]-iodo-3,17β-estradiol (200 pM final concentration; 2200 Ci/mmol specific activity; NEN Life Science Products) in ER binding buffer for a final reaction volume of 200 μl. Tubes were incubated at room temperature on an orbital shaker for 2 h. Reactions were terminated by adding 200 μl ice-cold ER binding buffer containing 1% (wt/vol) Norit A activated charcoal (Fisher Scientific, Fair Lawn, NJ) and 0.01% (wt/vol) dextran T500 (Pharmacia Biotechnology, Uppsala, Sweden) to trap unbound steroid. Thereafter, tubes were spun for 3 min at 12,000 × *g* at 4 C and radioactivity of a 300 μl aliquot of supernatant was determined in a gamma counter (ICN Micromedic Systems, Huntsville, AL). The amount of specifically bound radioactivity was calculated by subtracting nonspecific counts from total counts after correcting the data for a total sample volume of 400 μl.

Transient transfection and expression of an ER-dependent luciferase reporter gene construct

A plasmid (p294+) containing the luciferase gene (pGL3-Basic, Promega Corporation, Madison, WI) was engineered to express the luciferase gene under the control of the herpes simplex virus tk promoter and two copies of an ERE [the ERE is *underlined* in the following sequence with vector sequence in *lower case letters*: 5′ gctagcGGTCACTGTGACCAAGATCTCGGTCACTGTGACCagatctctcgagCCCCGCCCACTTGTCATTGGCGAATTCGAACACGCAGATGCAGTCGGGGCGGCGCGGTCCGAGGTCCACTTCGCATATTAAGGTGACGCGTGTGGCCTCGAACACCGAGCGaagctt 3′]. Plasmid p290+, which has the same sequence as p294+ with the exception that it does not contain any ERE or intervening sequences [vector sequence is represented by lower case letters 5′ gctagctcgagatctctcgagCCCCGCCCACTTGTCATTGGCGAATTCGAACACGCAGATGCAGTCGGGGCGCGGCGGTCCGAGGTCCACTTCGCATATTAAGGTGACGCGTGTGGCCTCGAACACCGAGCGaagctt 3′], was used as a control to determine any ERE/ER-independent activity.

Forty-eight hours before transfection, cells were plated in poly-L-lysine-coated 24-well culture plates (Nunclon Δ SI, InterMed Nunc, Roskilde, Denmark) at 3 × 10^5 cells/well. On the day following plating, the growth medium was removed by aspiration and the cells were washed twice with 1 ml low steroid medium. One milliliter of low steroid medium was added to each well, and the cells were maintained in a humidified environment at 37 C in the presence of 5% CO_2:95% air for the duration of the studies. After 24 h in low steroid medium, the medium was removed and 1 ml fresh low steroid medium was added to each well. Plasmid DNA was introduced into GT1–7 cells by calcium phosphate-mediated transient transfection that included a glycerol shock (25). Cells were cotransfected with a β-galactosidase reporter gene construct (pSV-β-galactosidase control vector; Promega) to normalize the data for transfection efficiency. Each well of cells received 100 μl of transfection mix that consisted of 1 μg p294+ or p290+ and 0.2 μg pSV-β-galactosidase control vector in 1[times HEPES-buffered saline (HBS; 25 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4, and 6 mM dextrose) containing 125 mM $CaCl_2$. Cells were returned to the tissue culture incubator for 4 h. Thereafter, the culture medium containing the transfection mix was removed and 250 μl of 15% glycerol in 1× HBS was added to each well. After a 90-sec incubation at 37 C, 1 ml of D-PBS (without Ca^{++} or Mg^{++}; GIBCO-BRL) was added to each well and then aspirated. Each monolayer of cells was washed an additional time with 1 ml of D-PBS before 1 ml of fresh low steroid medium was added to each well. Cells were returned to the incubator at 37 C for overnight recovery from the transfection.

The day following the transfection, cells were treated with 17β-estradiol or 0.1% (vol/vol) ethanol in low steroid medium. 17β-Estradiol stock solutions were prepared in absolute ethanol. Each drug solution was serially diluted in ethanol to 1000-fold concentrations of the final doses used in the studies. Treatment was begun by replacing low steroid medium with 1 ml low steroid medium containing ethanol vehicle or steroid at the final designated concentrations for the luciferase studies.

After 20–24 h treatment, each well of cells was washed twice with D-PBS and 100 μl of cell lysis buffer (Promega) was overlaid on the cells according to the manufacturer's recommendations. After a 15-min incubation at room temperature, the cells were scraped off and lysates from each well transferred to individual wells of a 96-well V-bottom tissue culture plate. The cell debris was pelleted by centrifugation and the resulting supernatants were assayed for luciferase (20 μl lysate) and β-galactosidase (5 μl lysate) activities by chemiluminescence using the luciferase (Promega) and the Galacto-Light Plus reporter assay systems (Tropix, Bedford, MA), respectively, following the manufacturers' recommendations.

Measurement of estrogen-induced galanin gene expression in GT1–7 cells

For evaluating the effects of estrogen on GAL gene expression, GT1–7 cells were plated in 6-well plates (1.75 × 10^6 cells/well) in growth medium. On the day following plating, the growth medium was removed, the cells were washed once with low steroid medium, and 3 ml fresh low steroid medium was added to each well. Steroid treatments were prepared as described earlier. Treatment was begun 48 h after plating by replacing low steroid medium with 3 ml low steroid medium containing ethanol vehicle or steroid at the final designated concentrations, and cells were incubated for 48 h. At the end of the treatment period, total RNA was isolated from each well of cells using TRIzol reagent (GIBCO-BRL) according to the manufacturer's recommendations and GAL mRNA concentrations were determined by qRT-PCR as described below.

Galanin mRNA expression analysis: quantitative RT-PCR

Mouse galanin mRNA expression was measured by qRT-PCR using an *in vitro* transcribed mGAL complementary RNA (cRNA) as an amplification standard (GAL sequence: nt 8–40/268–410). The mGAL cDNA used to prepare the standard consisted of an internally truncated form of the full-length mGAL cDNA (deletion of bases 40–268). The mGAL cDNA standard was engineered to conserve the 3′ and 5′ priming sites of the full-length mGAL cDNA while providing a shorter amplification product (bases 8 to 39 + bases 269 to 410) than that of the endogenous mRNA. The mGAL cDNA standard was transcribed *in vitro* using the MEGAscript kit (Ambion, Austin, TX) following the manufacturer's recommendations. After transcription, the truncated mGAL cRNA amplification standard was quantified by UV spectrophotometry and stored as frozen aliquots at −70 C until used. The concentrations of GT1–7 cell total RNA were determined spectrophotometrically according to standard procedures (25). The sequences of the forward and reverse primers used in the RT-PCR reactions are 5′ CAAAGGAGAAGAGAGGTTGGA 3′ and 5′ TTCAAAGCAGAGAACAGAGGA 3′, respectively. These primers yield a 173-bp amplification product from the mGAL amplification standard and a 403-bp product from both mouse brain and GT1–7 cell total RNA.

Two sets of reactions were set up for each sample, one with reverse transcriptase and one without, which served as the negative control. The DNase reactions that would contain reverse transcriptase consisted of 10 μg GT1–7 cell total RNA, 1× DNase I buffer (GIBCO-BRL), 2 U DNase I (GIBCO-BRL), and 40 fg mGAL amplification standard in a final volume of 20 μl. The negative controls that would not contain reverse transcriptase consisted of 2.5 μg GT1–7 cell total RNA, 10 fg mGal amplification standard and 1 U DNase I in 1× DNase buffer in a final volume of 10 μl. [Amounts of sample RNA and cRNA standard added to the reactions were selected based upon preliminary experiments indicating the range of amounts that would produce similar band intensities among the two species. The sample and cRNA standard amounts differ between reactions with and without reverse transcriptase to maintain identical amounts and concentrations in PCR amplification following dilution of reactions containing reverse transcriptase (as described below).] Mouse brain total RNA (500 ng; Clontech, Palo Alto, CA) was substituted for the GT1–7 mRNA as the positive control. The DNase I reactions were incubated at room temperature for 15 min. After the incubation period, 1 and 2 μl of 25 mM EDTA were added to reaction mixtures without and with reverse transcriptase, respectively. All reaction mixtures were incubated at 65 C for 10 min and then held at 4 C in a thermocycler (GeneAmp PCR System 9600, Perkin-Elmer, Norwalk, CT) until used in the RT reactions.

For the RT step, 18 μl of the following reagent mixture were added

to achieve the following concentrations: 0.5× PCR buffer without Mg^{++} (GIBCO-BRL), 3 mM $MgCl_2$, 2.2 mM dNTP mix, 5.6 μM mGAL reverse primer, 5 U/μl Superscript II reverse transcriptase. Each RT-omitted reaction received 9 μl of the reagent mixture described above except with the enzyme replaced with 50% (vol/vol) glycerol to achieve final concentrations identical to those of the RT-containing reactions. All reactions were incubated in a thermocycler (GeneAmp PCR System 9600) at 42 C for 15 min, 99 C for 5 min, and then held at 4 C until used in the PCR reactions.

For the PCR step, RT-containing samples were prepared by using 20 μl (1/2) of each reaction to make five serial 1:1 dilutions resulting in a 1:4 to 1:64 reduction in initial RNA and cRNA amounts, *i.e.* 2.5 μg GT1–7 total RNA and 10 fg mGAL amplification standard in the first tube of the dilution series to 156.3 ng GT1–7 total RNA and 0.625 fg. Reactions without reverse transcriptase were not diluted. Special attention was paid to maintain constant buffer concentrations after the dilutions to the following specification: 0.5× DNase I buffer (GIBCO-BRL), 1.25 mM EDTA, 0.5× PCR buffer (without Mg^{++}, GIBCO-BRL), 3 mM $MgCl_2$, 1 mM dNTP mix, 2.5 μM mGAL reverse primer, 5% (vol/vol) glycerol in a final volume of 20 μl. One PCR Gem50 wax bead (Perkin-Elmer) was added to each reaction tube. All reaction mixtures were heated at 75 C for 5 min and then held at 4 C in a thermocycler. Each tube then received the necessary reagents to achieve the concentrations as indicated in a final volume of 100 μl: 0.1× DNase buffer (GIBCO-BRL), 0.9× PCR buffer without Mg^{++} (GIBCO-BRL), 2 mM $MgCl_2$, 0.5 μM mGAL forward primer, 0.04 μCi/μl [α-^{32}P]dCTP (3000 Ci/mmol; NEN Life Science Products), and 0.025 U/μl *Taq* polymerase (GIBCO-BRL). All tubes were incubated in a thermocycler at 95 C for 3 min. This was followed by 30 cycles of consecutive incubations at 95 C for 20 sec, 56.5 C for 20 sec, and 72 C for 30 sec. After the last cycle, a 5-min elongation step at 72 C was carried out. Under these conditions, target amplification remained in the exponential phase of the amplification [the amount of incorporated nucleotide (cpm) increased with cycle number until cycle number 36. Amount of radioactivity present (cpm; mean ± SEM of three determinations) in the GAL band obtained after PCR cycles number 26, 30, 34, 36, 38, and 40 was as follows: 98,536 ± 2,942; 504,550 ± 2,294; 868,065 ± 25,659; 910,673 ± 33,152; 996,055 ± 56,178 and 916,320 ± 5,697, respectively]. Samples were then held at 4 C until the five sample dilutions were analyzed by PAGE. One-fifth of each PCR reaction was separated on a 1.5 mm-thick 5% polyacrylamide:bis acrylamide gel (19:1, polyacrylamide:bis acrylamide; InstaPAGE-19; Kodak, Rochester, NY) at 40 mA per gel at 4 C. The gels were dried on a gel dryer and exposed to phosphor screens (Molecular Dynamics) for 4 h. Band intensities for GT1–7 mGAL and mGAL amplification standard cDNAs were determined using a PhoshorImager SI and ImageQuaNT software (Molecular Dynamics).

Data analysis and statistics

For the receptor binding characterization, a customized JMP (SAS Institute, Inc., Cary, NC) statistical analysis script was developed (Dr. Krishnendu Ghosh, Biometrics, Wyeth-Ayerst Research) to determine the concentrations at which maximal binding of [^{125}I]-17β-estradiol is reduced 50% (IC_{50}) by the competitor. Square root transformation and Huber weighting were applied to all data before performing nonlinear curve fitting to a four parameter logistic model $y = (A-D)/1+ (x/C)^B) + D$; where y = response, × = nM concentration of drug, A = asymptotic maximum, B = slope factor, C = point of inflection (IC_{50} value), and D = asymptotic minimum). Total counts and nonspecific counts were used in the analysis as zero response dose and maximal response dose, respectively. The affinity constant, K_i, was calculated for each competitor using the Cheng-Prusoff equation (27), $K_i = IC_{50}/(1 +(L/K_d)$ in which L is the concentration of the [^{125}I]17β-estradiol (200 pM) and K_d is the affinity of 17β-estradiol for the receptor (110 pM) (19).

In the transfection experiments, luciferase activity was corrected for transfection efficiency by normalizing to β-galactosidase activity. For the qRT-PCR experiments, the band intensity data for the GT1–7 cell total RNA and the amplification standard were corrected for the difference in cytosine composition between the full length (GT1–7 cell) and truncated (amplification standard) forms of GAL. For each data set from serially diluted samples, two linear regressions (one for GT1–7 cell GAL bands and one for amplification standard bands) were performed on log-transformed data from which the determination coefficient (R^2) and % parallelism between the two generated lines were obtained. The slope for each line was used to calculate a parallelism factor (greater slope/smaller slope × 100). If both lines are perfectly parallel, this factor would be 100%. Further data evaluation was performed only if the parallelism factor was 75% or better. Thereafter, the band intensities for GAL and amplification standards were pooled and the median value calculated. The regression lines were then used to estimate the amount of GT1–7 RNA and the amount of GAL standard that produces the median band intensity. Thus, the amount of GAL mRNA present in the GT1–7 cell samples was defined as being equal to the amount of standard RNA that produced this median band intensity.

For both the transfection and qRT-PCR studies, the mean and SE of the mean were calculated for each group of four determinations. Data were fitted to a four-parameter nonlinear regression model (see above) using the SigmaPlot software package (Jandel Scientific). Statistically significant differences were inferred by ANOVA using JMP statistical software (SAS Institute, Cary, NC). The Dunnett's multiple comparison test was used to identify statistically significant differences *vs.* the control group. A $P < 0.05$ was considered as the minimal criteria to define statistically significant differences.

Results

We were able to detect and identify ERα mRNA in total RNA isolated from GT1–7 cells that were grown under standard culture conditions (23) by RT-PCR in combination with Southern blot hybridization (Fig. 1, *upper panel*). Based on the published mouse ERα sequence (24), the primers that were used produced the expected 557-bp amplification product. The identification of this product as an indicator of the presence of ER mRNA was substantiated by Southern hybridization in which an oligonucleotide that is complementary to region 1305–1323 of the ERα ligand binding domain was used as a probe. The 557-bp amplification product, indicative of mouse ERα mRNA, was detected by Southern hybridization from RT-PCR reactions with mouse brain total RNA or GT1–7 cell total RNA (Fig. 1, *upper panel*, lanes 1 and 3, respectively). No products were detected in reactions in which reverse transcriptase was omitted (Fig. 1, *upper panel*, lanes 2 and 4), demonstrating that the amplification products originated from mRNA and not from any residual sample DNA contamination. Cloning and subsequent sequencing of the 557 bp amplification product indicated perfect homology to the ERα sequence (data not shown).

Competition experiments using radioinert 17β-estradiol and the antiestrogen ICI-182,780 in the presence of [^{125}I]17β-estradiol were performed to characterize the specificity of the estrogen binding site in GT1–7 cells. Both 17β-estradiol and ICI-182,780 displaced [^{125}I]17β-estradiol from binding to the estrogen binding sites resulting in IC_{50} values of 0.24 and 4.1 nM, respectively (Fig. 2). By applying the Cheng-Prusoff equation (27) and using the K_d value for ER obtained with GT1–1 cells and 17β-estradiol (19), the binding constants (K_i values) for 17β-estradiol and ICI-182,780 were estimated to be 0.86 and 1.45 nM, respectively. Slope factors for the competition curves were not statistically different from one, indicating a ligand receptor interaction that could be explained by a one-site binding model.

The detection of the mRNA for the ERα ligand binding domain and estrogen binding sites in LHRH neurons, however, does not affirm the presence of biologically functional ER. Therefore, ER functionality in GT1–7 cells was studied by analyzing its ability to transactivate a heterologous gene. 17β-Estradiol induced luciferase activity in a dose-depen-

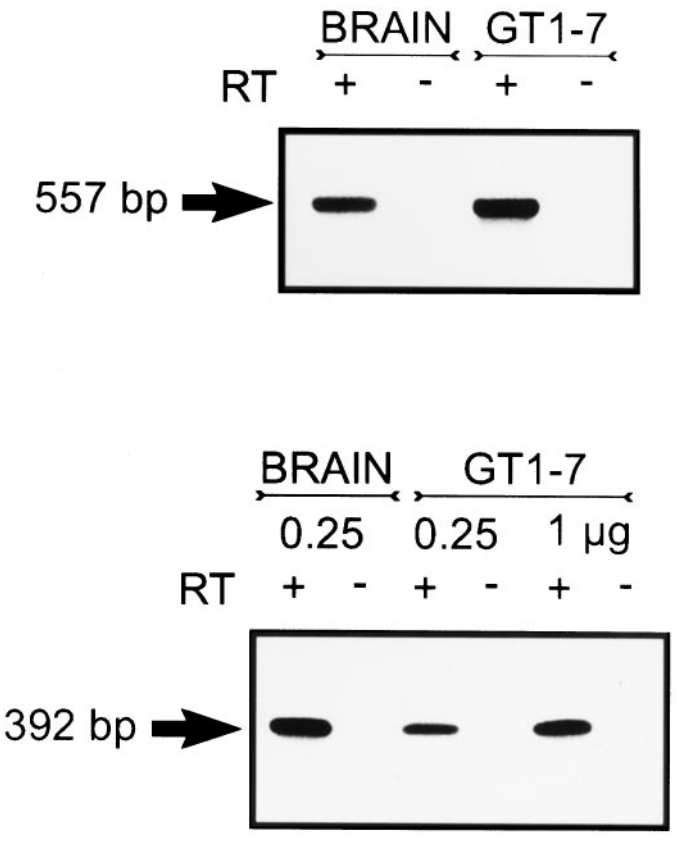

FIG. 1. Immortalized LHRH neurons (GT1–7 cells) produce estrogen receptor subtype-α (ERα) and galanin (GAL) mRNA. Primers specific for mouse ERα or mouse GAL were used to reverse transcribe and amplify by RT-PCR total RNA isolated from mouse brain or GT1–7 cells. Amplification products were resolved by electrophoresis in a 1% agarose gel, transferred to a nylon membrane, and hybridized with an oligonucleotide probe specific for the mouse ERα ligand binding domain or a cDNA probe spanning the entire rGAL sequence (Southern blotting). *Top panel*, RT-PCR followed by Southern blotting for ERα mRNA: Lane 1. RT-PCR of 0.25 μg mouse brain total RNA. Lane 2, RT-PCR of 0.25 μg mouse brain total RNA in the absence of reverse transcriptase. Lane 3, RT-PCR of 0.25 μg GT1–7 cell total RNA. Lane 4, RT-PCR of 0.25 μg GT1–7 cell total RNA in the absence of reverse transcriptase. *Bottom panel*, RT-PCR followed by Southern blotting for GAL mRNA. Lane 1, RT-PCR of 0.25 μg mouse brain total RNA. Lane 2, RT-PCR of 0.25 μg mouse brain total RNA in the absence of reverse transcriptase. Lane 3, RT-PCR of 0.25 μg GT1–7 cell total RNA. Lane 4, RT-PCR of 0.25 μg GT1–7 cell total RNA in the absence of reverse transcriptase. Lane 5, RT-PCR of 1 μg GT1–7 cell total RNA. Lane 6, RT-PCR of 1 μg GT1–7 cell total RNA in the absence of reverse transcriptase.

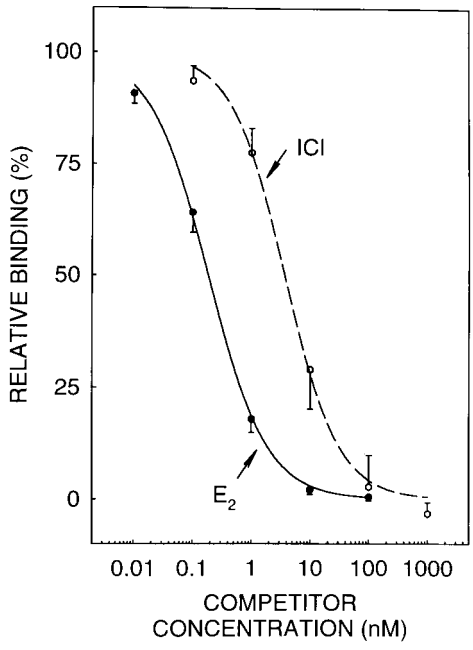

FIG. 2. ER present in GT1–7 cells binds 16α-[^{125}I]-iodo-3,17β-estradiol that is competitively inhibited by 17β-estradiol and ICI-182,780. Binding of 16α-[^{125}I]-iodo-3,17β-estradiol in the presence of 17β-estradiol (*solid circles*) or ICI-182,780 (*open circles*). The data for each competition curve are representative of two independent experiments in which cytosolic fractions prepared from GT1–7 cells were incubated in the presence of 200 pM 16α-[^{125}I]-iodo-3,17β-estradiol and increasing concentrations of competitor. Data were analyzed using a customized JMP software script and are presented as the mean $\pm$ SEM of three determinations for each competitor concentration as described in *Materials and Methods*.

dent manner with a 2- to 3-fold increase over control (Fig. 3, *top panel*). Maximal response was observed at 1–100 nM with an estimated potency (EC_{50}) of 47 pM. 17α-Ethinylestradiol was also effective in inducing luciferase activity with a potency of 17 pM (data not shown). This regulation by estrogens was ERE-dependent because 17β-estradiol did not induce luciferase activity when GT1–7 cells were transfected with the reporter construct lacking the ERE sequence (Table 1). To further substantiate ER involvement in the induction of reporter gene expression, GT1–7 cells were treated with the antiestrogen, ICI-182,780, in the presence of the EC_{80} concentration (0.2 nM) of 17β-estradiol. The 17β-estradiol-mediated luciferase induction could be completely inhibited by ICI-182,780 (Fig. 3, *bottom panel*), with an IC_{50} of 4.8 nM. In contrast, ICI-182,780 treatment alone did not alter luciferase activity when compared with basal levels (Table 1). In addition, other ER blockers such as raloxifene, tamoxifen, 4-hydroxytamoxifen, and droloxifene prevented estradiol-induced luciferase activity with varying potencies (Table 2).

Because GAL gene expression in LHRH neurons is responsive to estrogen exposure and is sexually dimorphic in rats (5), we evaluated the presence of GAL mRNA in immortalized LHRH neurons. The identification of GAL mRNA in GT1–7 cells was accomplished by RT-PCR coupled to Southern blot hybridization (Fig. 1, *lower panel*). The primers used for RT-PCR generated a 392-bp product from both mouse brain total RNA and GT1–7 cell total RNA (Fig. 1, *lower panel*, lanes 1, 3, and 5). No product was detected in reactions in which RT was omitted, which indicates that the original template for the 392-bp product was RNA and not contaminating DNA (Fig. 1, *lower panel*, lanes 2, 4, and 6). The PCR product was identified as a portion of the GAL transcript by Southern blot hybridization using a cDNA probe complementary to rat GAL (rGAL). This rGAL cDNA probe has 95% amino acid sequence homology to the mouse GAL (mGAL) sequence.

To characterize the effects of 17β-estradiol on GAL gene expression in GT1–7 cells, we developed a qRT-PCR method for measuring mGAL mRNA. Quantification by qRT-PCR was deemed necessary because solution hybridization and

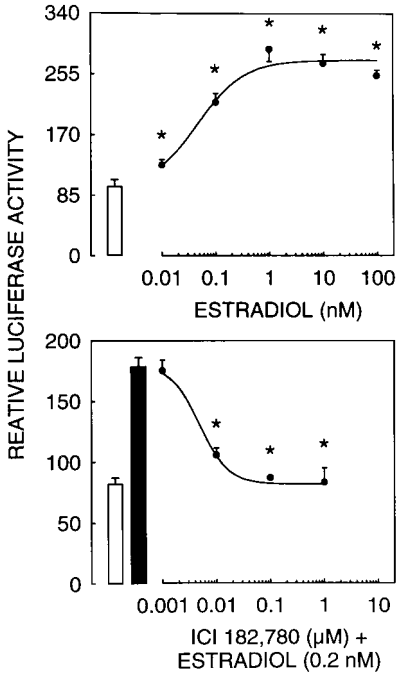

FIG. 3. 17β-Estradiol induces ERE-dependent heterologous gene transcription, which is inhibited by the antiestrogen, ICI-182,780. GT1–7 cells were transiently transfected with an expression vector plasmid containing the luciferase gene driven by an ERE-thymidine kinase promoter. To control for transfection efficiency, all cell samples were cotransfected with a pSVβ-galactosidase control vector. Luciferase and β-galactosidase assays were performed 20–24 h after addition of 17β-estradiol alone (*top panel*) or in combination with ICI-182,780 (*bottom panel*) as described in *Materials and Methods*. Data in each panel are presented as the mean ± SEM of results for quadruplicate transfections from a representative experiment of two separate experiments. *Open bars*, Luciferase activity in the control group. *Filled bar*, Luciferase activity after treatment with 0.2 nM 17β-estradiol in the absence of competitor. *, $P < 0.05$ *vs.* control (ethanol vehicle in *top panel* or 0.2 nM 17β-estradiol treatment in *bottom panel*) by ANOVA followed by the Dunnett's multiple comparison test on log-transformed data.

TABLE 1. 17β-estradiol-induced luciferase activity is ERE-dependent while ICI-182,780 has no effect on luciferase activity or galanin gene expression in immortalized LHRH neurons.

Treatment[a]	Luciferase activity (relative activity)[b,c]		GAL mRNA levels (fg/μg total cellular RNA)[c]
	−ERE	+ERE	
None	101.0 ± 1.5	97.2 ± 10.0	1.52 ±0.07
17β-Estradiol	10.5.0 ± 1.5	252.7 ± 8.4	3.38 ± 0.27
ICI-182,780	97.3 ± 8.6	83.0 ± 11.8	1.44 ± 0.08

[a] Cells were exposed to vehicle [0.1% (vol/vol) ethanol], 17β-estradiol (0.1 μM), or ICI-182,780 (1 μM) in low steroid medium for 20–24 h (luciferase activity) or 48 h (GAL gene expression).

[b] Cell were transiently transfected with a luciferase reported construct that either lacked an ERE (−ERE) or contained two copies of an ERE (+ERE).

[c] At least two independent experiments were done in quadruplicate and data are shown as the mean ± SEM from representative experiments.

Northern blot analyses failed to detect any mGAL mRNA and semiquantitative RT-PCR previously detected 17β-estradiol-induced GAL gene expression in GT1–7 cells with normalization to cyclophilin mRNA levels (22). Mouse GAL mRNA was normalized to total cellular RNA, a convention also used by others (28) because total RNA did not change significantly over the range of 17β-estradiol concentrations used (data not shown). The GAL gene-specific primer set chosen for qRT-PCR amplified a 173-bp product from a truncated cRNA amplification standard and a 403-bp product from GT1–7 cell total RNA (Fig. 4, *upper left panel*). Band intensity for mGAL is increased by 17β-estradiol treatment, which is most evident at the lower sample dilutions, (Fig. 4, *upper right panel*), suggesting that 17β-estradiol increases GAL gene expression in immortalized LHRH neurons. Estrogen-induced GAL gene expression was time dependent. Statistically significant increases in GAL mRNA were detected after as short as 6 h of treatment with 100 nM 17β-estradiol and approached maximal response by 48 h (Table 3). Quantification of band intensities reveal that at physiologically relevant concentrations, 17β-estradiol increased the expression of the GAL gene in a dose-dependent manner (Fig. 4, *middle panel*). Maximal expression was obtained at 0.1 nM with a 2-fold increase over control levels (Fig. 4, *middle panel*) and an EC_{50} value of 23 pM. 17α-Ethinylestradiol also increased GAL gene expression with a potency of 14 pM (data not shown). This induction by estrogens was ER-specific because the antiestrogen ICI-182,780 was completely effective (IC_{50} = 30.2 nM) in blocking the induction of GAL mRNA produced by 1 nM 17β-estradiol (Fig. 4, *lower panel*). ICI-182,780 treatment alone (1 μM) did not alter GAL mRNA concentrations when compared with basal levels (Table 1). Other antiestrogens such as raloxifene and droloxifene also inhibited 17β-estradiol induction of GAL gene expression (Table 2). Thus, it appears that GT1–7 cells produce functionally active ER that may be directly involved in the regulation of GAL gene expression.

TABLE 2. Various antiestrogens block 17β-estradiol-induced luciferase activity and galanin gene expression in immortalized LHRH neurons.

Treatment[a]	IC_{50} values (nM)[b]	
	Luciferase activity	GAL gene expression
Raloxifene	58.4 ± 8.6	39.9 ± 21.0
Tamoxifen	89.2 ± 10.5	N.D.[c]
4-Hydroxytamoxifen	33.2 ± 4.5	N.D.[c]
Droloxifene	49.8 ± 0.1	37.6 ± 26.7

[a] Cells were exposed to 0.2 (luciferase activity) or 1 nM (GAL gene expression) 17β-estradiol in low steroid medium for 20–24 h (luciferase activity) or 48 h (GAL gene expression in the presence of varying concentrations of ER blockers. Control cells represent a series of wells exposed to stripped serum-containing medium and vehicle [0.1 (vol/vol) ethanol] for 48 h.

[b] At least two independent experiments were done in quadruplicate and data are shown as the mean ± SEM from representative experiments.

[c] N.D., Not determined.

Discussion

We have previously demonstrated that GAL is localized in LHRH neurons and that this colocalization is sexually dimorphic and neonatally determined by steroid exposure (10). In this regard, estradiol induces colocalization of GAL in LHRH neurons (4), emphasizing the potential role of estro-

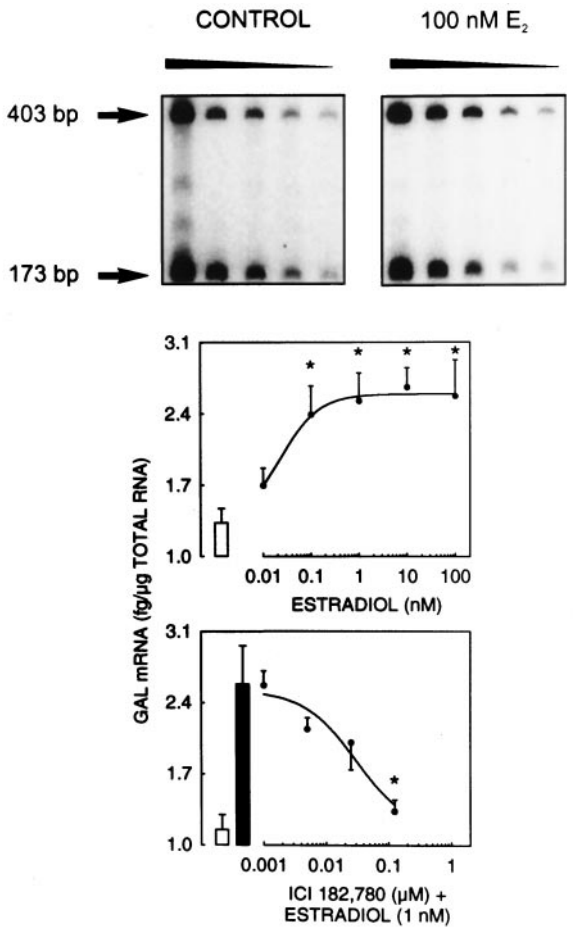

FIG. 4. GAL mRNA in GT1–7 cells is induced by 17β-estradiol, which is competitively inhibited by ICI-182,780. Total RNA was isolated from GT1–7 cells that were treated with 17β-estradiol alone or in conjunction with ICI-182,780. Quantitative RT-PCR was performed on the isolated RNA using specific primers for full-length mouse GAL and an internally truncated amplification standard as described in *Materials and Methods*. All treatments were performed in quadruplicate. Data shown are representative of at least two separate experiments. *Top panel*, Amplification products obtained using total RNA prepared from vehicle-treated (*left panel*) or 17β-estradiol-treated (*right panel*) GT1–7 cells. RT-PCR products were resolved in 5% polyacrylamide:bis acrylamide gels as described in *Materials and Methods*. Band intensities were determined for full-length mGAL (403-bp band) and truncated mGAL amplification standard (173-bp band) by ImageQuaNT analysis. Lanes 1 to 5 represent PCR amplified samples from serially diluted (1:1, vol/vol) reverse transcriptase reactions in increasing dilutions from left to right. *Middle panel*, 17β-estradiol increases GAL mRNA in immortalized LHRH neurons in a dose-dependent manner. Data are presented as the mean ± SEM. *Open bar*, GAL mRNA concentration for vehicle-treated cells (control). *, $P < 0.05$ *vs.* control by ANOVA followed by Dunnett's multiple comparison test on log-transformed data. *Bottom panel*, ICI-182,780 blocks 17β-estradiol-induced GAL mRNA in immortalized LHRH neurons. Data are presented as described for *middle panel*. *Filled bar*, GAL mRNA concentration for 17β-estradiol-treated cells. *, $P < 0.05$ *vs.* 17β-estradiol-treated group by ANOVA followed by Dunnett's multiple comparison test on log-transformed data.

gens in the regulation of GAL gene expression in LHRH neurons. Indeed, GAL expression is sensitive to estrogen in the hypothalamic pituitary axis. The mechanism by which estrogen influences GAL gene expression in LHRH neurons,

TABLE 3. 17β-Estradiol treatment of immortalized LHRH neurons induces galanin gene expression as early as 6 h after steroid exposure

Time after 100 nM 17-β-estradiol (hours)[a]	GAL mRNA levels (fg/μg total cellular RNA)[b]
Control	1.03 ± 0.11
3	1.19 ± 0.09
6	1.53 ± 0.02[c]
12	1.55 ± 0.05[c]
24	1.79 ± 0.13[c]
48	2.19 ± 0.11[c]

[a] Cells were exposed to 100 nM 17β-estradiol for the specified length of time. Control cells represent a series of wells exposed to low steroid medium and vehicle [0.1% (vol/vol) ethanol] for 48 h.

[b] Data represent the mean ± SEM of quadruplicate determinations.

[c] $P < 0.05$ *vs.* control by ANOVA on log-transformed data followed by the Dunnett's test.

however, is poorly understood. Previous efforts by several investigators to demonstrate the presence of ER in LHRH neurons have been hampered by technical limitations and by the paucity and scattered distribution of endogenous LHRH neurons (11, 12). Consequently, the effects of estradiol on the LHRH neuronal network were thought to be indirect. However, the 5′ flanking region of the human LHRH gene was found to confer estrogen responsiveness to a luciferase reporter gene (29). In addition, purified ER from calf uterus was found by DNase footprinting to bind to this region (29). These findings suggest that if LHRH neurons do indeed produce functional ER, the necessary *cis*-acting elements are present in the LHRH gene for ER-dependent regulation. It is extremely difficult from a molecular perspective to delineate the mechanism(s) by which estradiol elicits ER-dependent gene expression *in vivo* in the LHRH neurons. To circumvent these difficulties, we set out to investigate the nature by which estradiol modulates GAL gene expression using immortalized LHRH neurons, *i.e.* GT1–7 cells, and to ascertain any direct involvement of biologically active ER in this event.

By using RT-PCR followed by Southern hybridization we were able to detect and positively identify the presence of ERα mRNA transcripts in GT1–7 cells. The ER present in these immortalized LHRH neurons was determined to be functionally active for both ligand binding and gene transactivation capabilities. In whole cell binding experiments, Poletti *et al*. (19) determined that ERs are present in another subclone of the GT cells (GT1–1). ER in immortalized LHRH neurons are low in abundance (B_{max} value of 6.2 fmol/mg total cell protein) but have a high affinity (K_d value of 0.11 nM) for [^{3}H]estradiol (19). The low binding capacity (B_{max}) of ER in GT1 cells compared with MCF-7 cells (60 fmol/mg cytosolic protein) (30, 31), may help to explain why previous *in vivo* studies failed to detect ER in LHRH neurons (11, 12, 32). Our radioligand binding studies support the findings of Poletti *et al*. (19) by demonstrating a high affinity ER in GT1–7 cells. In addition, we have expanded the characterization of this receptor by demonstrating ligand dependency of the GT1–7 ER through homologous and nonhomologous competition studies using nonradioisotopic 17β-estradiol and ICI-182,780, respectively. Each compound competitively inhibited [^{125}I]17β-estradiol binding with estimated inhibition constants (K_i values) for 17β-estradiol and ICI-182,780 of 0.86

and 1.45 nM, respectively. The corresponding IC_{50} values for 17β-estradiol and ICI-182,780 (0.24 and 4.1 nM, respectively) are within the range of values reported by others [0.05–8.3 and 9.4 nM, respectively (31, 33, 34)]. Because LHRH neurons are not concentrated in distinct nuclei in the hypothalamus and we found that RT-PCR was necessary to detect ERα mRNA, it is possible that the techniques previously employed to identify ER *in vivo* were not sufficiently sensitive to detect this receptor in LHRH neurons. Similar confounding conditions existed in bone biology until ER in estrogen-responsive osteoblast-like cells (200–1600 binding sites per nucleus) were detected by using more sensitive methods than the conventional approaches used in the past (35, 36).

Additional scenarios can be envisioned that reconcile the apparent discrepancies in the detection of ER in hypothalamic LHRH neurons *in vivo* compared with immortalized LHRH neurons. For example, because GT1 cells were isolated from tumors produced through targeted oncogenesis (15), in which the expression of SV40 T-antigen oncogene is directed by the regulatory regions of the LHRH gene, it is possible that GT1 cells represent hypothalamic LHRH neurons during a particular fetal stage when LHRH and ER are coexpressed. These neurons may become less abundant or absent as development progresses and, therefore, the lack of detection of appreciable hypothalamic LHRH neurons that express ER in the adult animal may be an accurate depiction. Alternatively, the number of hypothalamic LHRH neurons that express ER may be low in number throughout development and immortalization of one of these neurons by targeted tumorigenesis was fortuitous. This case, as well, is in concordance with the established *in vivo* findings.

Identification of ERα mRNA and estradiol binding sites does not preclude the existence of ER that are incapable of mediating transcriptional activation. Nonfunctional ERs have been identified that bind steroid but are incapable of nuclear binding and activation of gene transcription (37–39). Therefore, it was of paramount importance to demonstrate that the ERα produced by GT1–7 cells is biologically functional. To this end, we have shown by transient transfection experiments that although endogenous ERα is in low abundance in GT1–7 cells, it can be activated sufficiently by the ligand to drive transcription of the luciferase reporter gene through an ERE-dependent mechanism. Although the construct we used contains a consensus vitellogenin ERE, the fact that receptor activation is mediated by physiological concentrations of estradiol indicates that LHRH neurons may respond to estrogen under *in vivo* conditions by activating or repressing particular genes. The fact that ICI-182,780 and other antiestrogens specifically block this response suggests that this effect is ER dependent. In addition, estrogen treatment of GT1–7 neurons results in elevated androgen receptor binding sites (19), which reinforces the functionality of ER present in immortalized LHRH neurons.

Previous work has demonstrated that GAL expression in LHRH neurons in adult rats is sexually dimorphic with more GAL-containing LHRH neurons present in females than in males (5, 7) as well as higher GAL mRNA content in LHRH neurons in females than males (40). Colocalization of GAL and LHRH is profoundly influenced by estrogen. In rats, ovariectomy reduces the amount of LHRH neurons expressing GAL from 80% in intact female animals to approximately 15% and this reduction is reversed by estrogen treatment (6). Likewise, GAL mRNA concentrations are reduced in LHRH neurons of ovariectomized adult rats, but can be restored to control levels by administration of physiological concentrations of estradiol (8). With the establishment of the presence of ER in both the GT1–7 and GT1–1 cell lines (19), the foundation is partially laid to support the premise that estrogen can directly regulate GAL expression in a subpopulation of LHRH neurons. We found that steroid treatment resulted in elevated GAL gene expression in GT1–7 cells with a detectable increase in GAL mRNA occurring after only 6 h of 17β-estradiol treatment. This rapid response to estrogen treatment suggests that ER-mediated transcriptional activation of the GAL gene may play a role in the increase in GAL mRNA; however, we cannot rule out changes in transcript stability as a possible mechanism for this effect. In addition to 17β-estradiol, several other estrogens, such as estrone, $\Delta^{8,9}$ dehydroestradiol, 17β, $\Delta^{8,9}$ dehydroestradiol, or 17α-ethinylestradiol, increase GAL gene expression (41, 42). In other tissues, estrogen also has been shown to enhance GAL gene expression. For example, GAL produced in the rat anterior pituitary is known to be sensitive to estrogen (43–45). Kaplan and co-workers (43) have demonstrated that pharmacological doses of 17β-estradiol increased pituitary rGAL mRNA in both male and ovariectomized female rats by as much as 4000-fold. Furthermore, the GAL gene was cloned and identified as an estrogen-inducible gene from estrogen-induced pituitary tumor mRNA (44). The mechanism(s) by which estrogen-induced GAL gene transcription occur(s), however, has not been fully elucidated. One can envision that the liganded ERα induces GAL gene activation by directly binding to *cis*-acting enhancer element(s) in the GAL promoter. In this respect, the cow (46), rat (47), and mouse (48) GAL genes have been sequenced recently. Whereas in the rGAL gene promoter region one can identify 3 half-palindromic ERE sites (47), the mGAL and bovine gene promoters appear to contain a single half ERE site (5′ GGTCA 3′) (46, 48). GenBank identifies this half-palindromic ERE site as being located at bases 504–508 (GenBank accession no. L38575) in the mouse sequence. Whether this site is responsible for ER-dependent activation of the GAL gene on its own or in combination with other *trans*-acting entities is currently unknown. In support of a direct ER-DNA interaction resulting in GAL gene activation, Howard *et al*. (49) have reported that, in gel mobility shift assays, one of two ERE consensus regions that they identified in the human GAL gene bound recombinant human ERα as well as nuclear extract protein from either rat anterior pituitary tissue or rat pituitary tumor cells (MtTW). The ability of this ERE consensus region to modulate gene expression was not determined; however, the availability of the regulatory sequences from mouse, rat, and human GAL genes has made it possible to design gel mobility shift assays and heterologous gene transcription activation studies to determine the role of these putative ERE regions in the regulation of GAL gene expression.

The results from our transient transfection studies support a direct role of estrogen in regulating LHRH neuronal gene expression because endogenous ERs present in GT1–7 cells were found to be capable of activating luciferase gene tran-

scription through a conventional ERE-dependent mechanism. Indeed, it has been reported that half-palindromic ERE sites identified in the rat and human GAL gene promoter have ER-dependent transcriptional activation capabilities (50, 51). It is interesting to note that the half-palindrome of the consensus ERE (5′ GGTCA 3′) is present as a functional ERE in the chicken ovalbumin gene promoter (52). This sequence (5′ TGGGTCA 3′), which is present in both the chicken ovalbumin gene (53) and rGAL gene promoters (51), that has been shown to be a binding site for a constitutive protein complex containing c-*fos* and c-*jun* that coactivates gene transcription in the presence of ERα and estrogen (53). Gaub and co-workers found that the ERα-mediated activation of the ovalbumin gene through the *fos/jun* complex does not require the ERα DNA binding domain and, therefore, conclude that direct ERα interaction with genomic elements is not required for this system (53). We can envision several scenarios for ER activation of GAL gene expression. These include a direct activation through receptor-DNA interactions and/or an indirect activation through ER-response modulator(s) complexes that bind to DNA through response modulator(s) contacts. Given these findings, further analyses of the *cis*- and *trans*-acting elements that regulate GAL gene expression are clearly warranted to elucidate the mechanism(s) by which estrogen directly and indirectly regulates gene expression in LHRH neurons and to determine whether or not these effects are tissue specific.

In conclusion, immortalized LHRH neurons possess the characteristics of target cells for estrogen activity. They express ERα mRNA and produce ER that displays specific and saturable binding to 17β-estradiol that is inhibited competitively by the antiestrogen, ICI-182,780. The ER present in GT1–7 was also able to transcriptionally activate a reporter gene through a classical ER-dependent mechanism. In addition, the endogenous GAL gene was found to be induced by 17β-estradiol through an increase in GAL mRNA. Because estrogen was demonstrated to mediate gene transactivation in cultured GT1–7 cells (in the absence of other cell types) and that GT1–7 cells do, indeed, produce functional ER, these findings strongly support the scenario that, in addition to any indirect actions, estrogen may also exert its effects directly on hypothalamic LHRH neurons.

Acknowledgments

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