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GnRH agonist reduces estrogen receptor dimerization in GT1-7 cells: Evidence for cross-talk between membrane-initiated estrogen and GnRH signaling

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Abstract

17β-estradiol (E_2), a key participant on the initiation of the LH surge, exerts both positive and negative feedback on GnRH neurons. We sought to investigate potential interactions between estrogen receptors alpha (ERα) and beta (ERβ) and gonadotropin releasing hormone receptor (GnRH-R) in GT1-7 cells. Radioligand binding studies demonstrated a significant decrease in saturation E_2 binding in cells treated with GnRH agonist. Conversely, there was a significant reduction in GnRH binding in GT1-7 cells treated with E_2. In BRET[1] experiments, ERα–ERα dimerization was suppressed in GT1-7 cells treated with GnRH agonist ($p < 0.05$). There was no evidence of direct interaction between ERs and GnRH-R. This study provides the first evidence of reduced ERα homodimerization by GnRH agonist. Collectively, these findings demonstrate significant cross-talk between membrane-initiated GnRH and E_2 signaling in GT1-7 cells.

Keywords

GT1-7 cells; Estradiol; Non-classical estrogen signaling; Estrogen receptor alpha; Estrogen receptor beta; GnRH receptor

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1. Introduction

Gonadotropin-releasing hormone (GnRH) neurons episodically release gonadotropin-releasing hormone, which drives the pulsatile secretion of pituitary gonadotropins and controls the normal reproductive cycle. Gonadal steroids are principal mediators of GnRH pulsatility and, ultimately, of an appropriately timed LH surge to induce successful ovulation. In particular, 17β-estradiol (E_2) is a key determinant and exerts both negative and positive feedback on GnRH neurons. Not only does E_2 signal to GnRH neurons indirectly through various neural afferent networks and neuromodulators (Christian and Moenter, 2010; Garcia-Galiano et al., 2012), but E_2 also exerts feedback directly on GnRH neurons through both classic nuclear and rapid membrane-associated responses that involve G proteins (Hardy and Valverde, 1994; Hoffman et al., 1990; Kato et al., 1994; Navarro et al., 2003; Rosie et al., 1990; Rothfeld et al., 1989; Russell et al., 2000). However, the mechanism(s) of membrane-initiated E_2 signaling in GnRH neurons have not yet been fully elucidated.

Studies of rapid estrogen signaling support the presence of a membrane-associated estrogen receptor, but it remains uncertain whether E_2 exerts its rapid action at the plasma membrane through binding with the classical estrogen receptors (ERα and ERβ) or with a distinct membrane receptor. Both ERα and ERβ are expressed in the murine hypothalamus, GnRH neurons, and immortalized GnRH neurons (GT1-7 cells) (Couse et al., 1997; Hu et al., 2008; Navarro et al., 2003). Prior studies in GT1-7 cells and cultured rat hypothalamic GnRH neurons have also demonstrated the presence of ERα and ERβ at the plasma membrane where E_2 binding altered pulsatile GnRH secretion via G protein signaling pathways (Hu et al., 2008; Navarro et al., 2003). ERα and ERβ contain a highly conserved motif within the ligand binding domain that facilitates association with caveolin proteins via palmitoylation and allows for translocation to the plasma membrane (Pedram et al., 2007). ERα and ERβ have been shown to translocate to the neuronal membrane where E_2 binding induces rapid signaling via interactions between these ERs and metabotropic glutamate receptors (mGluR), which are G protein coupled receptors (GPCR) (Boulware et al., 2005).

The membrane-initiated actions of E_2 in GnRH neurons have rapid regulatory effects including changes in electrical properties (Kato et al., 1994), dose-dependent changes in cAMP production, changes in GnRH release, activation of G_i proteins (Navarro et al., 2003), and phosphorylation of cAMP response element binding protein (Kato et al., 1994; Kwakowsky et al., 2014; Navarro et al., 2003). However, while it is clear that E_2 exerts both positive and negative feedback on GnRH release, the underpinnings of membrane-initiated estrogen signaling in GnRH neurons remain unclear. Furthermore, a body of literature describes the existence of G-protein coupled receptor (GPCR) heterodimers and oligomers; these associations provide additional mechanisms for the regulation of downstream signaling (Gurevich and Gurevich, 2008; Kaczor and Selent, 2011; Mercier et al., 2002; Wilson et al., 2013). Thus, evidence suggests that E_2 could initiate rapid actions on GnRH pulsatility through membrane-initiated binding with ERα and/or ERβ and subsequent direct association of the activated ER with internal residues on the GnRH-R to affect GnRH release, which could further modulate estrogen feedback to GnRH neurons.

Bioluminescence resonance energy transfer (BRET[1]) is a technique that enables the investigation of protein–protein interactions in live cells and has been used for the study of GPCRs (Ayoub and Pfleger, 2010; Pfleger and Eidne, 2003; Wu and Brand, 1994). BRET[1] utilizes the transfer of excited energy from the natural bioluminescence produced during oxidation of the substrate coelenterazine by *Renilla* luciferase (Rluc), the donor molecule, to enhanced yellow fluorescent protein (YFP), the acceptor molecule (Hart et al., 1978; Pfleger and Eidne, 2003; Xu et al., 1999). The energy transfer can only occur at distances less than 100 Å (10 nm) with an efficiency that is inversely proportional to the distance, thus it is useful for studying molecular interactions *in vivo* (Gurevich and Gurevich, 2008; Wu and Brand, 1994). Specifically, while ERα and ERβ translocate to the neuronal cell membrane and have been shown to interact with a GPCR to exert some of their rapid signal transduction, no studies have examined their potential association with the GnRH-R. Therefore, we hypothesized that the estrogen receptors, ERα and ERβ, might directly interact with GnRH receptors in GT1-7 cells. To test this hypothesis, we used ER and GnRH-R fusion constructs with BRET[1].

2. Materials and methods

2.1. Cell culture

Immortalized GnRH neurons (GT1-7 cells) were provided by Dr. Richard Weiner (University of California at San Francisco). Cells were cultured in Dulbecco's modified Eagles' medium with F12 medium (DMEM/F12) supplemented with 10% heat-inactivated fetal bovine serum. Twenty-four hours before assays, media was replaced by serum- and phenol red-free 1:1 DMEM/F12. HEK293 cells were cultured in a 1:1 mixture of DMEM/F12 containing 10% heat-inactivated fetal bovine serum. All cells were cultured at 37 °C in a humidified incubator containing 5% CO_2 and were cultured for at least 7 days prior to use in experiments.

2.2. Plasmid construction

BRET[1] fusion constructs were designed as shown. ERα (NM_007956) and ERβ (NM_207707) were extracted from ORFEXPRESS Gateway PLUS Shuttle Clones (GeneCopoeia) by transformation into GCI-5α *E. coli* cells followed by amplification by PCR, restriction enzyme digestion, and DNA purification using QIAquick Purification Kit. Human ERα-YFP and ERα-BRET constructs contained a linker between the two fusion proteins of 10 amino acids (GGGGSGGGGS). Linker-Rluc and Linker-YFP genes were PCR-amplified using designed primers and cloned into pcDNA3.1-V5-HIS (Invitrogen) at Bam H1 and Age 1 sites for ERα constructs and at Not I and Age I sites for ERβ constructs. ERα-Rluc and ERα-YFP BRET[1] constructs were created by ligating the coding sequence of ERα into the Kpn 1 and BamH1 sites of both pcDNA-Rluc and pcDNA-YFP (Kang et al., 2011). ERβ-Rluc, ERβ-YFP, and YFP-ERβ were generated by ligating the coding sequence of ERβ into the Eco R1 and Not 1 sites of both pcDNA-Rluc and pcDNA-YFP. The YFP moiety was attached to both the C and N terminus of ERβ to provide an alternate acceptor configuration, as this can impact BRET[1] signal strength. A single nucleotide was then added to the linker of the ERα constructs to maintain amino acid frame using the QuikChange II Site-Directed Mutagenesis Kit. The GnRH-R-Rluc construct had been previously prepared

and was used to generate GnRH-R-YFP constructs in a similar fashion as described above. All fusion constructs were verified by direct DNA sequencing, Western Blot analysis, and BRET^1 functionality testing (Kang et al., 2011; Neithardt et al., 2006).

2.3. Analysis of construct functionality in HEK293 cells

HEK293 cells were seeded at a density of 3.2×10^5 cells on 12-well tissue culture plates. After 16–18 hours in culture at 60–80% confluence, cells were transfected with 0, 0.2, or 0.8 μg of BRET^1 construct and empty pcDNA3.1 to maintain total DNA of 1.0 μg using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, cells were detached with phosphate-buffered saline (PBS) with 0.05% trypsin, washed twice with PBS, suspended in culture media and seeded in 96-well plates at a density of 50,000 cells per well. Twenty-four hours later, cells were washed with PBS, incubated with 5 μM coelenterazine in PBS, and assessed for light emitted between 400 and 600 nm using a Mithras LB940 (Berthold Technologies). Specifically, the total fluorescence and luminescence were evaluated to confirm protein expression and BRET^1 functionality of each fusion construct.

2.4. BRET^1 analysis in GT1-7 cells

GT1-7 cells were seeded at a density of 8×10^5 cells on 6-well tissue culture plates. After 24 hours in culture, the cells were transfected with various combinations of BRET^1 constructs (ERα-YFP, ERα-Rluc, ERβ-YFP, YFP-ERβ, ERβ-Rluc, GnRH-R-Rluc, GnRH-R-YFP), as described above. After 48 hours of transfection, the cells were detached with Versene, suspended in BRET buffer (PBS + 0.1% glucose), and distributed on transparent 96-well plates at a density of 50,000 cells. To assess for interaction between donor and acceptor molecules, cells were incubated with a final concentration of 5 μM coelenterazine in PBS and BRET^1 readings were taken immediately.

2.5. Radioligand binding and displacement studies

Saturation binding studies of E_2 in GT1-7 cells were performed as previously described (Navarro et al., 2003; Poletti et al., 1994). GT1-7 cells were cultured for 24 hours in 24-well plates at a density of 5×10^5 cells per well. Cells were washed once then treated with serial dilutions of [^{3}H]E_2 (70 Ci/mmol, Amersham Pharmacia Biotech, Arlington Heights, IL) for 1 hour at room temperature in serum- and phenol red-free medium containing 0.5% BSA and 1 μM triamcinolone acetonide (Navarro et al., 2003). Cells were then transferred to ice and washed with ice-cold PBS three times, solubilized, and radioligand binding was measured using a scintillation counter. Membrane fractions were treated with serial dilutions of [^{125}I]E_2 (2000 Ci/mmol, Amersham Pharmacia Biotech) in a similar fashion and radioligand binding was measured by γ-spectrometry. Cells were either treated with the GnRH antagonist [D-pGlu^1, D-Phe^2, D-Trp^{3,6}]GnRH (D-pGlu), or GnRH agonist des-Gly^{10}-[D-Ala^6]GnRH B-ethylamide (D-Ala^6), at 100 nM and compared with untreated cells with saturation binding curves.

GnRH displacement assays were performed as previously described (Nett et al., 1981). Control GT1-7 cells were washed once then treated with [^{125}I]D-Ala^6 (2200 Ci/mmol, Amersham Pharmacia Biotech) and non-radioactive D-Ala^6 in 100 μl aliquots then incubated for 1 hour at room temperature. Treated cells were washed then treated first with

17 pM of E_2 followed by radioligand and non-radioactive agonist. Cells were then rapidly washed with ice-cold PBS three times, solubilized and then analyzed for total radioactive binding by γ-spectrometry.

2.6. Statistical analysis

The total fluorescence and luminescence were used as relative measures of the expression level of the acceptor (YFP) and donor (Rluc) proteins, respectively. For comparison of total fluorescence and luminescence, one-way ANOVA and the linear trend test was used to assess for significant difference in expression level between different construct concentrations. The BRET ratio was calculated using the following formula: (emission at 535 nm) – (emission at 485 nm) × C*f*/(emission at 485 nm), where C*f* corresponds to (emission at 535 nm)/(emission at 485 nm) for the receptor-Rluc construct expressed alone. To evaluate the specificity of the receptor interactions, saturation assays were performed in which cells were co-transfected with a stable amount of Rluc construct and increasing amounts of YFP construct. Saturation curves were plotted with GraphPad Prism4 software using a nonlinear repression curve assuming one-site binding. Statistical analysis of differences between curves was assessed using a comparison of fits with set alpha of 0.05 and the null hypothesis that one curve was adequate for all data sets and the alternative hypothesis that different curves were best suited for each data set.

3. Results

3.1. Radioligand binding and displacement assays

Radioligand binding and displacement assays were performed to evaluate for changes in E_2 or GnRH binding properties in GT1-7 cells treated with labeled GnRH agonist or E_2, respectively. Membrane fractions revealed an attenuation in E_2 binding sites when cells were co-treated with D-Ala^6 (GnRH agonist), consistent with the conclusion that ligand-induced activation of the GnRH-R reduced E_2 binding (Fig. 1A). Saturation binding studies in GT1-7 cells demonstrated a single high-affinity estrogen binding site with EC_{50} of 201 pM, similar to previous findings (Navarro et al., 2003), that was unchanged by treatment with GnRH antagonist (Fig. 1B). Additionally, displacement-binding studies exhibited specific, high-affinity binding of [^{125}I]-D-Ala^6 that was inhibited by GnRH in a dose-, time-, and temperature-dependent manner. When treated concomitantly with the GnRH agonist and E_2, the number of GnRH-binding sites available at saturation was significantly decreased from 467 ± 21.2 fmol/mg protein to 262.7 ± 42.7 fmol/mg protein with unchanged EC_{50} (Fig. 1C). These experiments indicate reciprocal modulation of the binding properties of E_2 and GnRH; e.g., each agonist led to a reduction in the binding sites available at saturation. We interpret the findings to suggest possible cross-talk between the ER and GnRH-R signaling pathways, which might be important for an appropriately timed LH surge.

3.2. Constitutive and ligand-induced interactions between estrogen receptors

To evaluate this possibility, we used BRET^1 fusion constructs for ERα, ERβ, and GnRH-R. As a control, we tested for the expected dimerization interaction between ERs in order to confirm BRET^1 construct functionality. BRET^1 fusion construct expression and

functionality (Fig. 2A) was assessed by BRET^1 analysis of total luminescence and fluorescence in HEK293 cells transiently transfected with the BRET^1 ERα-Rluc fusion construct (Supplementary Fig. S1A–B). A constitutive ERα BRET^1 signal was observed in GT1-7 cells after transient transfection with ERα-Rluc and ERα-YFP (Fig. 2B). BRET^1 measurements were taken in triplicate and mean total luminescence was significantly increased based on amount of construct added. YFP constructs were similarly tested and mean net YFP fluorescence was also confirmed to be dose-dependent (Kang et al., 2011). Other constructs demonstrated similar expression and dose-dependent functionality (data not shown).

In saturation assay studies, transient transfection of constant amounts of ERα-Rluc with increasing concentrations of ERα-YFP (Fig. 2C) or ERα-Rluc with YFP-ERβ (Fig. 2D) showed a specific BRET^1 signal, demonstrating homodimer and heterodimer formation, respectively. Addition of 100 nM E_2 for 30 minutes resulted in a significant increase in the $BRET_{max}$ for both homodimer (0.725 ± 0.013 to 0.114 ± 0.006, $p < 0.01$) and heterodimer (0.228 ± 0.037 to 0.335 ± 0.021, $p < 0.05$) formation, suggesting an increase in dimerization (Fig. 2C and D).

3.3. Evidence of cross-talk between GnRH and ER signaling

Based on findings that GnRH agonist suppressed E_2 saturation binding in GT1-7 cells (Fig. 1A), BRET^1 studies were performed to evaluate for an effect of GnRH upon ER dimerization. Saturation assay studies resulted in a specific BRET^1 signal for all samples, again demonstrating ERα homodimerization (Fig. 3). Notably, cotreatment with GnRH agonist resulted in suppression of the E_2-induced increase in signal (dotted line) as denoted by the change in $BRET_{max}$ (0.311 ± 0.046 vs. 0.507 ± 0.045 vs. 0.336 ± 0.025, $p = 0.018$). These findings suggest a rapid mechanism of cross-talk between the GnRH and E_2 signaling pathways such that GnRH agonist treatment resulted in a decrease in ligand-induced ERα–ERα interaction. We interpret the results to indicate that GnRH treatment decreased estrogen-dependent ERα homodimerization.

3.4. Constitutive and ligand-induced interactions between GnRH receptors

Next, BRET^1 experiments were performed to test for interaction between GnRH-R fusion constructs in GT1-7 cells based on previous observations of GnRH-R microaggregation in other cell types (Cornea and Conn, 2002; Cornea et al., 2001). As expected due to the inherent ability of GT1-7 cells to secrete GnRH, a constitutive GnRH-R BRET signal was observed in GT1-7 cells after transient transfection with GnRH-R-Rluc and GnRH-R-YFP. In saturation assay studies, transient transfection with GnRH-R-Rluc and increasing concentrations of GnRH-R-YFP resulted in a specific BRET^1 signal. Treatment with 100 nM GnRH for 30 minutes resulted in a non-significant decrease in the maximum BRET ratio (0.060 ± 0.013 vs. 0.048 ± 0.011, $p = 0.129$) (Fig. 4).

3.5. Analysis for direct interaction between GnRH-Rs and ERs

Given the effect of the GnRH ligand on ER dimerization, experiments were next performed to evaluate for direct interaction between GnRH-R and ERα or ERβ. An extremely small BRET^1 signal was observed in GT1-7 cells after transient transfection with GnRH-R-Rluc

and ERα-YFP. The signal was unchanged by treatment with E_2 100 nM, (Fig. 5A). Similarly small BRET1 signals were observed at baseline in saturation assays performed on cells transfected with GnRH-R and ERβ-YFP or YFP-ERβ, detecting no evidence of a specific constitutive interaction (Fig. 5B–C). In studies evaluating for protein–protein interactions between GnRH-R-Rluc and ERβ-YFP, treatment with either E_2 or GnRH resulted in no change in the $BRET_{max}$ (0.009 ± 0.002 vs. 0.038 ± 0.067 vs. 0.023 ± 0.012, p = 0.614) demonstrating no ligand-induced interaction between these two receptors (Fig. 5B). Therefore, these experiments did not provide evidence for a direct interaction between either ERα or ERβ with GnRH-R in transiently transfected GT1-7 cells either in the presence or absence of E_2 or GnRH agonist.

4. Discussion

Considerable evidence indicates that E_2 affects GnRH release both by positive and negative feedback upon GnRH-secreting neurons, leading to the mid-cycle LH surge. The mechanism(s) by which E_2 exerts direct feedback on GnRH neurons are not completely understood. Here, we tested the mechanism of membrane-initiated E_2 signaling and sought to determine whether ERα or ERβ might interact directly with the GnRH-R in GT1-7 cells. With radioligand studies, we found that available E_2 binding sites were reduced in the presence of GnRH. In addition, GnRH binding saturation kinetics were suppressed by E_2 in GT1-7 cells. Furthermore, BRET1 experiments demonstrated a reduction of ERα–ERα dimerization in GT1-7 cells treated with GnRH agonist. However, we did not detect direct interaction between either of the ERs and the GnRH-R. We interpret these findings to indicate that there is significant cross-talk between membrane-initiated GnRH and E_2 signaling, but the mechanism does not appear to occur via a direct interaction between these two receptor types. Rather, downstream signaling events initiated by GnRH binding appear to reduce ER dimerization.

Currently, there are only three known sequenced estrogen receptors – ERα, ERβ, and G protein-coupled estrogen receptor (GPER1 – formerly known as GPR30) (Barton, 2012; Vrtačnik et al., 2014). ERα is known to have at least three and ERβ at least five different isoforms (Irsik et al., 2013; Vrtačnik et al., 2014) encoded by alternatively spliced transcripts of the *ESR1* and *ESR2* genes such as ESR36 (Wang et al., 2005). Furthermore, membrane subpopulations of ERα and ERβ (*m*ERα, *m*ERβ) have been proposed (Barton, 2012). Each ER isoform has different ligand binding, dimerization, or transcriptional abilities, leading to various E2 mediated effects. GPER1, specifically, is located on the cell surface (Kenealy and Terasawa, 2012) and studies using GPER1 agonist G1 (Bologa et al., 2006), antagonist G15 (Dennis et al., 2009), and siRNA knockdown of GPER1 reveal that GPER1 is at least partly responsible for rapid excitatory E2 action (Kenealy and Terasawa, 2012). Estrogen receptor X (ER-X), and novel membrane ER STX (STX-R) sensitive to the diphenylacrylamide compound (Chakraborty and Roy, 2013) have also been found to mediate rapid excitatory actions of estradiol and directly modify GnRH neuronal activity in primates independently of ERα or ERβ (Cheong et al., 2014; Terasawa and Kenealy, 2012; Terasawa et al., 2009). In our study, classic ERα or ERβ and associated constructs were transfected exclusively (without intentional isoforms) in the GT1-7 cells. However our results reflect rapid-action of E2 signaling on GnRH in line with the finding that classical

ERs may also mediate rapid signals induced by E2 (Ding et al., 2014; Pedram et al., 2006; Prossnitz and Barton, 2011).

The majority of studies on E_2 and GnRH regulation describe effects by inhibition or stimulation on the other receptor via both signaling pathways and G-protein-coupled receptors (Barton, 2012; Hu et al., 2008; Kelly and Levin, 2001; Kenealy et al., 2011; Leclercq et al., 2006; Navarro et al., 2003; Perrett and McArdle, 2013; Terasawa and Kenealy, 2012) with outcome measures including changes in subsequent GnRH secretion, pulsatility/frequency, or ER signaling (Boulware et al., 2005; Chu et al., 2009; Hu et al., 2008; Kenealy and Terasawa, 2012; Kwakowsky et al., 2014). Other studies report effects of reciprocal rate of expression of receptor mRNA when treated with either E_2 or GnRH (Ng et al., 2009; Otani et al., 2009). These outcome measures have important implications in the scheme of the E_2 and GnRH regulatory system; however, our results also highlight the possibility that an additional component of the feedback between E_2 and GnRH could be by rapid alteration of receptor number and/or ligand or protein induced changes in ER ligand binding capability. While estrogen receptors are subjected to conformational changes depending on the nature of the molecules with which they transiently interact, some degree of difference in available binding sites is expected at all times (Leclercq et al., 2006). However, our findings imply a rapid GnRH-induced change in ER availability beyond what would be expected by these natural and transient changes in structure.

The mechanism responsible for these findings is unclear, but may be due to consequent changes in estrogen receptor degradation and/or post-transcriptional modifications induced by GnRH via second messengers with consequent conformational changes. Studies in ER degradation reveal biphasic kinetics, first a slow, then more rapid decline – indicating a large population of stable receptors at baseline that are progressively converted into a more labile form subject to degradation (Leclercq et al., 2006). Studies with estrogen agonists and antagonists suggest that this conversion from stable to labile is regulated via cross-talk with other signal transduction pathways (Leclercq et al., 2006). Furthermore, studies in breast cancer cell lines (MCF-7 breast cancer cells) have shown that ligands can induce ER degradation without direct ligand binding (Borras et al., 1996; Leclercq et al., 2006). The mechanism of ligand/second messenger-induced ER turnover is a possible regulatory mechanism that is worthy of more exploration in regard to estrogen and GnRH feedback.

Given this possible mode of interaction and our dimerization findings, we tested the mechanism of membrane-initiated E_2 signaling and sought to determine whether ERα or ERβ directly interact with the GnRH-R in GT1-7 cells. We did this by measuring $BRET^1$ signals in GT1-7 cells transfected with GnRH-R-Rluc and ERα-YFP and GnRH-R-Rluc with ERβ-YFP or YFP-ERβ. $BRET^1$ signaling is non-discriminatory of receptor location: cell membrane, nuclear, or other compartments. ERs are highly mobile proteins continuously shuttling between cellular compartments (Leclercq et al., 2007; Levin, 2002). In addition, estrogen receptor dimerization is increased by estradiol, but also by post-translational modification, as has been shown by phosphorylation induced by signal transduction other than the cognate hormone (Kavarthapu et al., 2014). Post-translational estrogen receptor modification could be inhibitory by activation of phosphatases, or via

other changes. In efforts to minimize the error in our study we created two ERβ constructs with YFP at the N and C terminuses, but did not detect any difference in BRET[1] signals.

Our findings of decreased ERα–ERα dimerization in GT1-7 cells treated with GnRH agonist has not been reported and may comprise one of the negative or inhibitory feedback mechanisms of GnRH on the estradiol-related cellular pathways. The fact that the most stable dimer configuration (ERα homodimer) is inhibited by addition of GnRH agonist implies that a powerful and key step in the dimerization process is at least partially inhibited or suppressed by presumably increased degradation or conformational changes. It is also uncertain as to whether our findings reflect de-novo dimerization rate of strictly the population of unliganded ERs or if stability was altered in existing dimers.

Overall, estrogen and GnRH signaling in GnRH neurons is complex and involves dose, time, and receptor dependent changes in signaling to alter GnRH secretion patterns and receptor availability. Our data indicate that there is significant cross-talk between membrane-initiated GnRH and E_2 signaling, but it does not appear to occur via a direct interaction between these two receptors. Rather, downstream signaling events initiated by GnRH agonist influence ER dimerization and/or availability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.01.023.

Highlights

- GnRH binding saturation kinetics were suppressed by E_2 in GT1-7 cell extracts.
- E_2 binding sites and ERα homodimerization decreased following GnRH agonist treatment.
- Fusion constructs and $BRET_1$ suggested no direct interaction between ERs and GnRH-R.

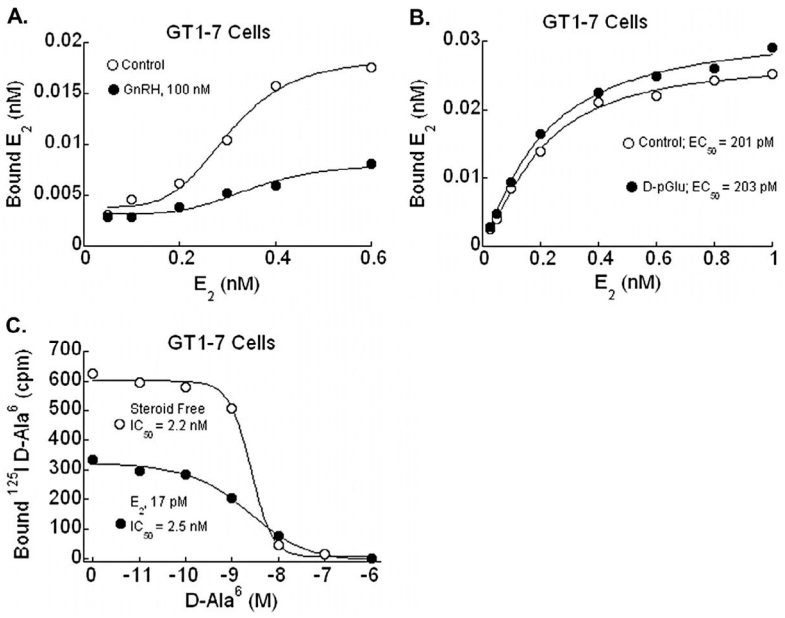

Fig. 1.

Radioligand saturation binding curves in cell or membrane lysates from GT1-7 cells. (A) Saturation binding studies of E_2 in control and GnRH agonist-treated GT1-7 cell membrane fractions. Concomitant treatment of GT1-7 cells with GnRH agonist des-Gly^{10}-[D-Ala^6]GnRH B-ethylamide (D-Ala^6), at 100 nM results in a significant blunting of number of E_2 binding sites. (B) Saturation binding studies of E_2 in control and GnRH-antagonist [D-pGlu^1, D-Phe^2, D-Trp^{3,6}]GnRH (D-pGlu) treated GT1-7 cell lysates at 100 nM. E_2 binding affinity in GT1-7 cells was unchanged in the presence of D-pGlu, a GnRH-antagonist (EC_{50} 201 vs 203 pM). (**C**) Displacement binding studies of GnRH agonist analog in control and estrogen treated GT1-7 cells. In samples treated with E_2 at 17 pM in addition to the unlabeled GnRH agonist, the number of GnRH binding sites was suppressed (467 ± 21.2 fmol/mg protein vs 262.7 ± 42.7 fmol/mg protein).

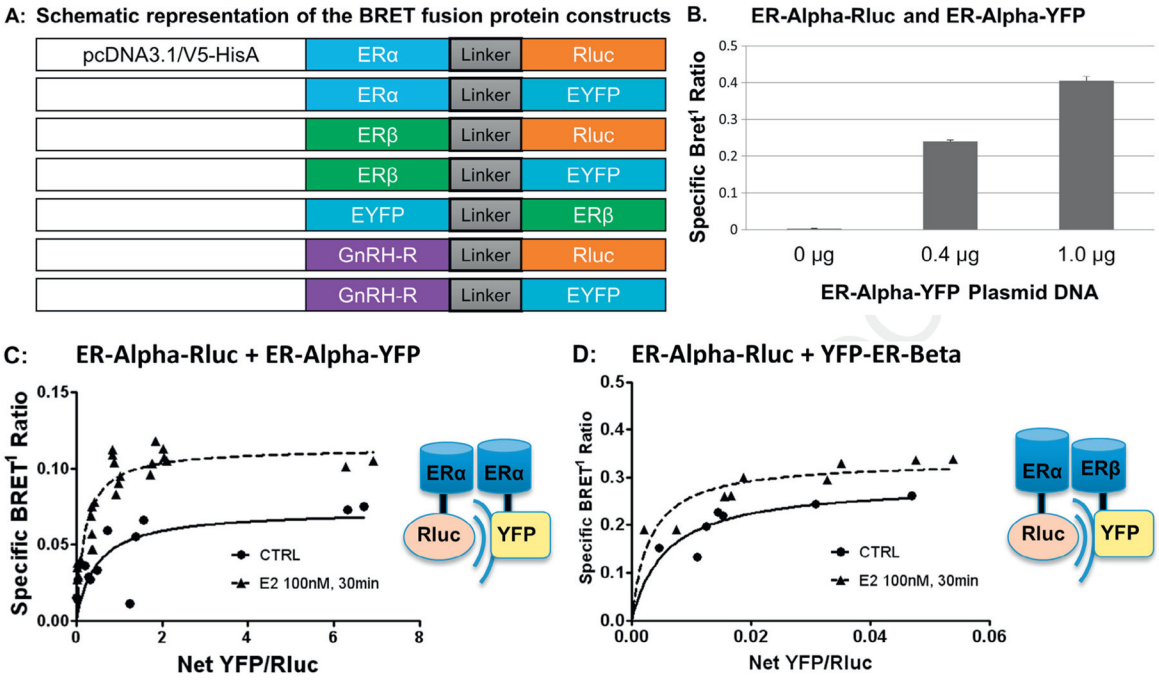

Fig. 2.

Design of BRET^1 fusion constructs for ER and GnRH-R and saturation binding studies. (A) Schematic representation of the BRET fusion protein constructs. Acceptor and donor molecules, *Renilla* luciferase (Rluc) and enhanced yellow fluorescent protein (YFP) were used with linker of 10 amino acids (GGGGSGGGGS) between the two fusion proteins. Linker-Rluc and Linker-YFP genes were PCR-amplified using designed primers and cloned into pcDNA3.1-V5-HIS (Invitrogen) at Bam H1 and Age 1 sites for ERα constructs and at Not I and Age I sites for ERβ constructs. ERα-Rluc and ERα-YFP BRET^1 constructs were created by ligating the coding sequence of ERα into the Kpn 1 and BamH1 sites of both pcDNA-Rluc and pcDNA-YFP. ERβ-Rluc, ERβ-YFP and YFP-ERβ were generated by ligating the coding sequence of ERβ into the Eco R1 and Not 1 sites of both pcDNA-Rluc and pcDNA-YFP. The YFP moiety was attached to both the C and N terminus of ERβ to provide an alternate acceptor configuration, as this can impact BRET^1 signal strength. (B) ERα-Rluc + ERα-YFP: Specific BRET^1 ratio between ERα-Rluc donor and ERα-YFP acceptor in GT1-7 cells transiently transfected with constant amount of ERα-Rluc and increasing amounts ERα-YFP plasmid DNA. The specific BRET^1 ratio increased with increasing amounts of plasmid DNA. (C) ERα-Rluc + ERα-YFP: BRET^1 saturation binding assay performed in GT1-7 cells transiently transfected with 0.2 µg ERα-Rluc and 0–1.0 µg of ERα-YFP. BRET^1 measurements were then obtained in triplicate in both untreated and E_2 treated cells (100 nM for 30 minutes) and saturation curves were plotted, demonstrating a hyperbolic curve that is characteristic of a specific protein–protein interaction. E_2 treatment of 100 nM for 30 minutes resulted in a significant increase in the maximum BRET^1 signal (0.725 ± 0.013 to 0.114 ± 0.006, $p < 0.01$) with unchanged $BRET_{50}$. (D) ERα-Rluc + YFP-ERβ: BRET^1 saturation binding assay performed in GT1-7 cells transiently transfected with ERα-Rluc and YFP-ERβ. Saturation curves also demonstrate a specific BRET^1 signal that increased with E_2 treatment ($BRET_{max}$ 0.228 ± 0.037 to 0.335 ± 0.021, $p = 0.0048$).

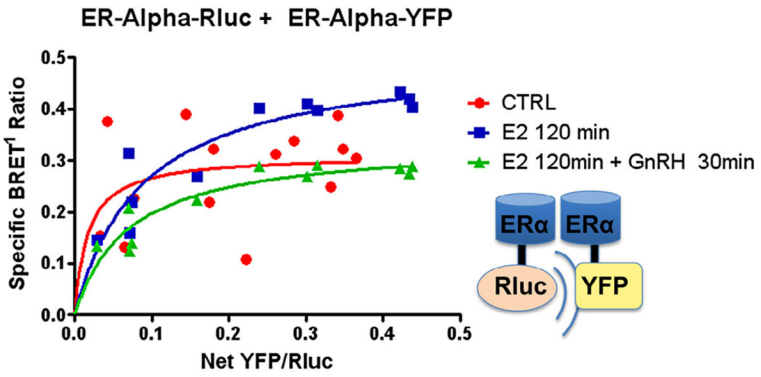

Fig. 3.

Effect of GnRH on ERα dimerization. ERα-Rluc + ERα-YFP: GT1-7 cells in serum-and phenol red-free 1:1 DMEM/F12 medium were transiently transfected with 0.2 μg of ERα-Rluc and increasing amounts from 0 to 1.0 μg of ERα-YFP (*y*-axis). $BRET^1$ measurements were then taken in triplicate of untreated cells (*x*-axis), cells treated with E_2 100 nM for 2 hours or cells treated with E_2 100 nM for a total of 120 min, with GnRH agonist 100 nM added for the last 30 min of incubation. Saturation curves were then plotted as shown, demonstrating a decrease in ligand-induced ERα–ERα dimerization (dotted line, square boxes) when cells were also treated with GnRH ($BRET_{max}$ 0.311 ± 0.046 vs. 0.507 ± 0.045 vs 0.336 ± 0.025, p = 0.018).

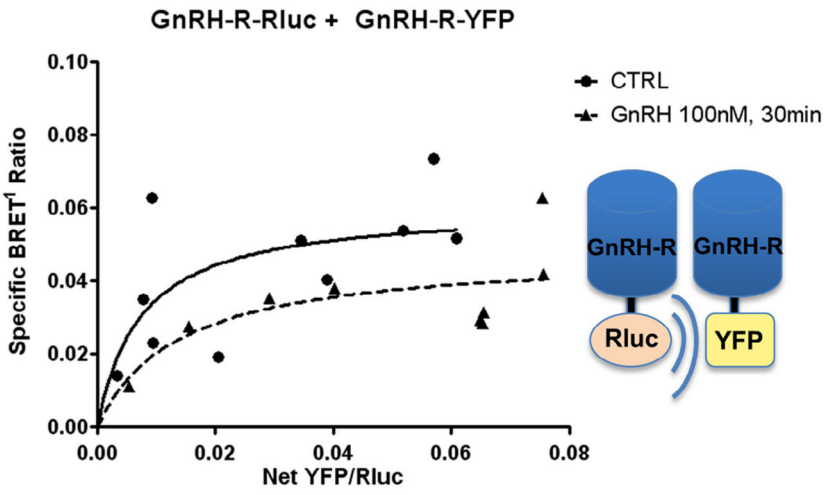

Fig. 4.

GnRH-R fusion construct expression and functionality. GnRH-R-Rluc + GnRH-R-YFP: Saturation binding assays were performed with GnRH-R-Rluc and GnRH-R-YFP. The hyperbolic curves demonstrate specific protein–protein interaction between BRET constructs, confirming construct functionality and GnRH-R homodimerization in GT1-7 cells. We did not detect a significant change with GnRH agonist treatment at 100 nM for 30 minutes ($BRET_{max}$ 0.060 ± 0.013 vs 0.048 ± 0.011, p = 0.129).

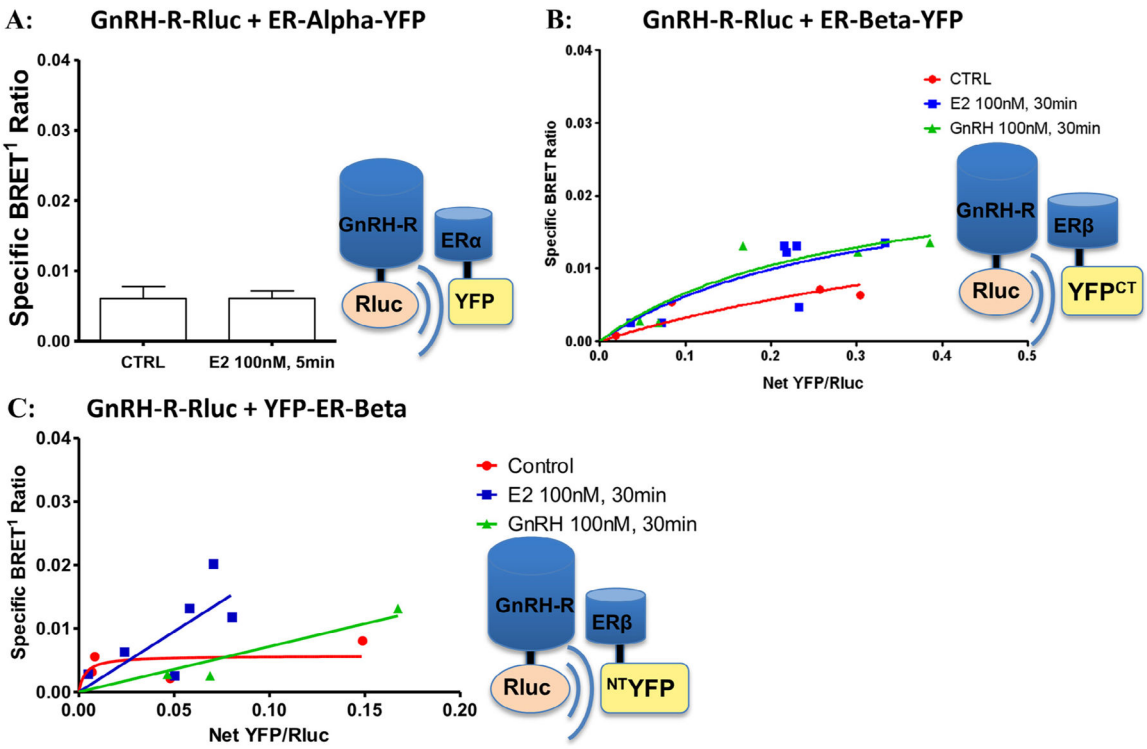

Fig. 5.

No association between ERs and GnRH-R by BRET^1. (A) GnRH-R-Rluc + ERα-YFP: BRET^1 measurements in cells transiently transfected with GnRH-R-Rluc and ERα-YFP demonstrated only a very small constitutive signal that was unchanged by treatment with E_2 at 100 nM. (B–C) GnRH-R-Rluc + ERβ-YFP (ERβ CT-YFP) and GnRH-R-Rluc + YFP-ERβ (ERβ NT-YFP): Saturation binding assays performed in GT1-7 cells transiently transfected with GnRH-R-Rluc and increasing amounts (0–1.0 μg) of either ERβ-YFP (CT-YFP) or YFP-ERβ (NT-FFP) demonstrate almost undetectable BRET^1 signals and absence of a hyperbolic curve. Treatment with either E_2 or GnRH agonist (100 nM for 30 minutes) demonstrated similarly low signals ($BRET_{max}$ 0.009 ± 0.002 vs 0.038 ± 0.067 vs 0.023 ± 0.012, p = 0.614). Thus, studies did not suggest a constitutive or ligand-induced specific interaction between GnRH-R and ERβ. Findings were similar in cells transfected with GnRH-R and ERα (not shown).