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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.
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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).

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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