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Rapid Action of Estrogen in LHRH neurons: The Role of GPR30

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Abstract

Previously, we have shown that 17 β -estradiol (E₂) induces an increase in firing activity and modifies the pattern of intracellular calcium ([Ca²⁺]_i) oscillations with a latency less than 1 min in primate luteinizing hormone releasing hormone (LHRH) neurons. A recent study also indicates that E₂, the nuclear membrane impermeable estrogen, estrogen- dendrimer conjugate (EDC), and the plasma membrane impermeable estrogen, E₂-BSA conjugate, all similarly stimulated LHRH release within 10 min of exposure in primate LHRH neurons, indicating that the rapid action of E₂ is caused by membrane signaling. The results from a series of studies further suggest that the rapid E₂ action in primate LHRH neurons appears to be mediated by GPR30. Whereas the estrogen receptor antagonist, ICI 182,780, neither blocked the E₂-induced LHRH release nor the E₂-induced changes in [Ca²⁺]_i oscillations, E₂ application to cells treated with pertussis toxin (PTX) failed to result in these changes in primate LHRH neurons. Moreover, knockdown of GPR30 in primate LHRH neurons by transfection with human siRNA for GPR30 completely abrogated the E₂-induced changes in [Ca²⁺]_i oscillations, whereas transfection with control siRNA did not. Finally, the GPR30 agonist, G1, resulted in changes in [Ca²⁺]_i oscillations similar to those observed with E₂. In this review the authors discuss the possible role of G-protein coupled receptors in the rapid action of estrogen in neuronal cells.

Keywords

GnRH neurons; rapid action of estrogen; GPR30; GPCR; primates

Introduction

In mammalian females proper interplay between LHRH neurons and gonadal steroid hormones maintains normal reproductive function. However, the mechanism of gonadal steroid hormone action, especially estrogen action, in LHRH neurons remains elusive. During the 1980s – 1990s, a large number of studies describing estrogen action in LHRH neurons through interneurons, such as GABA, glutamate, alpha-adrenergic, dopaminergic, opiate, and neuropeptide Y neurons, were reported (see 1,2). In fact, earlier studies with in situ hybridization, immunocytochemistry, and electron microscopy showed that LHRH neurons do not express estrogen receptors (3), whereas those interneurons do (4, 5, see also 6). Entering into the 21st century, direct action of estrogens in LHRH neuronal activities has been suggested with the aid of new cellular and molecular approaches (7–9). In support of direct estrogen action in LHRH neurons, colocalization of ER β in humans, sheep, and mice (10–13) or both ER α and ER β in rats (14,15) has been reported. The absence of ER α in rat LHRH neurons has

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also been reported (16,17). In addition, LHRH neuronal activity appears to be influenced by gonadal steroid hormones through glia (18).

Recent studies by several research groups suggest that in neurons estrogen causes a novel rapid action through the plasma membrane, rather than interacting with the nuclear receptors ER α and ER β . Although the concept of rapid action of estrogens is not particularly new, the question of which receptors mediate their action remains controversial. For example, in LHRH neurons, a series of recent studies from our laboratory indicate that G-protein coupled receptor 30 (GPR30) appears to be involved in estrogen action in primate LHRH neurons (19), whereas other groups showed that ER β mediates rapid E₂ action in LHRH neurons (7,8). Therefore, the main purpose of this article is to review the role of membrane receptors mediating rapid E₂ actions in LHRH neurons with emphasis on the role of GPR30. In addition, we discuss rapid E₂ action through other G-protein coupled receptors in the brain, as there are common features among G-protein coupled receptors involved in E₂ action.

1. 17 β -estradiol (E₂) induces firing activity of primate LHRH neurons

In the first study we investigated whether E₂ modifies single unit activity of LHRH neurons. Patch clamp electrodes were placed on cultured LHRH neurons derived from the primate olfactory placode (20) and single unit electrical activity was recorded. E₂ infusion rapidly (within a minute) increased the firing frequency, spikes per burst, and burst duration of primate LHRH neurons (9). Similar to E₂, the membrane impermeable analog of E₂, E₂-BSA (E₂ 17-hemisuccinate-BSA), also induced rapid action (9). These observations suggest that E₂ causes a rapid stimulatory action in primate LHRH neurons and this rapid effect of E₂ on firing activity does not require E₂ entry into the cell, but perhaps occurs at the cell membrane level.

2. Estradiol increases intracellular calcium, [Ca²⁺]_i, oscillations in primate LHRH neurons

Previous studies from our laboratory have shown that cultured primate LHRH neurons exhibit [Ca²⁺]_i oscillations at an interval of 5–8 min and [Ca²⁺]_i oscillations synchronize periodically at an interval of ~60 min (21). Interestingly, the synchronization interval of [Ca²⁺]_i oscillations was very similar to that of pulsatile LHRH release *in vitro* (22) as well as *in vivo* (23). Using the same setup described previously (21), in the second study we investigated whether E₂ modifies the pattern of [Ca²⁺]_i oscillations in LHRH neurons. Infusion of E₂ (1 nM) for 10 min increased the frequency of [Ca²⁺]_i oscillations within a few minutes. E₂ also increased the frequency of [Ca²⁺]_i synchronization among LHRH neurons occurring at an interval of ~30 min. Similar E₂ effects on the frequency of [Ca²⁺]_i oscillations were observed under the presence of TTX, indicating that estrogen causes direct action in LHRH neurons (19). Moreover, application of a nuclear membrane impermeable estrogen dendrimer conjugate (EDC), described by Harrington et al. (24), resulted in a robust increase in the frequencies of [Ca²⁺]_i oscillations and synchronizations, indicating that effects estrogens on [Ca²⁺]_i oscillations and their synchronizations do not require their entry into the cell nucleus. Exposure of cells to E₂ in the presence of the estrogen receptor (ER) antagonist, ICI 182,780 at 0.1 μ M, did not change the E₂-induced increase in the frequency of [Ca²⁺]_i oscillations, nor the E₂-induced increase in the synchronization frequency (19).

3. Estradiol stimulates LHRH release in primate LHRH neurons

To determine the physiological consequence of E₂ stimulation on firing activity and [Ca²⁺]_i oscillations and their synchronizations, we next examined the effects of E₂ on LHRH release in primate LHRH neurons *in vitro* (25). The results indicate that infusion of E₂ (1 nM) for 20 min stimulated LHRH release: The E₂-induced LHRH increase started to occur within 10 min and lasted an additional 20 min after the termination of E₂ infusion. Moreover, EDC and the plasma membrane impermeable estrogen, E₂-BSA conjugate, both stimulated LHRH release within 10 min of exposure. Interestingly, the E₂-induced increase in LHRH release was not

blocked by the estrogen receptor antagonist, ICI 182,780 at 0.1 μM (25). These results indicate that E_2 causes its action at the membrane level and that $\text{ER}\alpha$ and $\text{ER}\beta$ are not involved in rapid E_2 action in primate LHRH neurons.

4. A possible role of GPR30 in E_2 action

As stated above, the classical estrogen receptors, $\text{ER}\alpha$ and $\text{ER}\beta$, are not likely involved in the rapid action of E_2 in primate LHRH neurons. To investigate the mechanism of E_2 action in primate LHRH neurons, we examined whether E_2 -induced $[\text{Ca}^{2+}]_i$ oscillations and LHRH release can be seen in LHRH neurons treated with pertussis toxin (PTX). PTX is a broad spectral G-protein coupled receptor blocker (26). Results suggest that E_2 application to cells treated with PTX failed to induce both $[\text{Ca}^{2+}]_i$ oscillations and LHRH release (25), indicating that E_2 action in primate LHRH neurons is mediated by a G-protein coupled receptor. Thus, the search for a potential mediator of the rapid action of estrogen through a G-protein coupled receptor began.

Because it has been suggested that estrogen action in cancer cells is mediated by GPR30 (27–29), we decided to examine the role of GPR30 in estrogen action in LHRH neurons. First, we needed to find whether GPR30 is present in primate LHRH neurons. With RT-PCR we found that GPR30 mRNA was expressed in the olfactory placode, from which LHRH neurons originate. Subsequent cloning and amplification of the amplicon from the gel suggested that the deduced amino acid sequence from cDNA of the monkey olfactory placode was identical to the GPR30 described in the human genome (GenBank accession numbers AF027956). In addition, GPR30 protein was expressed in a subset of LHRH neurons in adult monkey hypothalamus (25).

Subsequently, we examined whether E_2 stimulates primate LHRH neurons in which GPR30 was knocked down by transfection with human small interference (si)RNA for GPR30. The results indicate that transfection of LHRH neurons with siRNA for GPR30 completely abrogated the E_2 -induced changes in $[\text{Ca}^{2+}]_i$ oscillations, whereas transfection with control siRNA did not. Similarly, the EDC-induced increase in $[\text{Ca}^{2+}]_i$ oscillations also failed to occur in LHRH neurons transfected with GPR30 siRNA. Finally, we examined whether G1, a GPR30 agonist (30), can induce an effect similar to E_2 . Results suggest that G1 at 10 nM stimulated the frequency of $[\text{Ca}^{2+}]_i$ oscillations, as were observed with E_2 , although it did not cause an increase in synchronizability of $[\text{Ca}^{2+}]_i$ oscillations (25). Preliminary data in our lab further indicated that G1 at 100 nM also stimulated LHRH release (Shiel and Terasawa, unpublished observation). These results suggest that GPR30, at least in part, mediates the rapid action of E_2 in primate LHRH neurons.

5. $\text{ER}\beta$ mediating the rapid action of E_2 in mouse LHRH neurons

Similar to our studies in non-human primates, E_2 or E_2 -BSA at 100 nM exposed to cultured LHRH neurons derived from the mouse olfactory placode induced an increase in the frequency of $[\text{Ca}^{2+}]_i$ oscillations and their synchronization with a latency of ~28 min in the presence of TTX (8). However, unlike primate LHRH neurons, the E_2 -induced stimulation of $[\text{Ca}^{2+}]_i$ oscillations and their synchronization in mouse LHRH was blocked by ICI 182,780 (8). Since mouse LHRH neurons contain $\text{ER}\beta$, not $\text{ER}\alpha$, (10,11), these authors conclude that E_2 action in mouse LHRH neurons is mediated by $\text{ER}\beta$.

Another group also found that *in vivo* exposure to E_2 (1–10 μg) for 15 min in mice resulted in an increase in phosphorylation of CREB and this response was mediated by $\text{ER}\beta$, not $\text{ER}\alpha$ (7). These authors, however, did not examine whether similar effects can be induced by an E_2 exposure of less than 15 min. Recently, the same group reported that stimulatory action of E_2 (100 nM) on $[\text{Ca}^{2+}]_i$ transients in LHRH-Pericam mice occurred with a latency of ~15 min

in a subset of LHRH neurons and that this E₂ action was mediated by presynaptic GABA neurons through ER α , not ER β (31). The difference in mouse studies and non-human primates could be due to species difference or the E₂ doses (there is a 100 fold difference in the dose), because in primate LHRH neurons E₂ at 1 nM readily induces stimulatory action within a couple of minutes. Comparisons between laboratories need to be made with caution, however, as methodologies used in these studies are not the same: Susan Wray's lab and this lab used cultured LHRH neurons of embryonic origin, whereas Allan Herbison's lab used LHRH neurons in sliced preoptic area from adult transgenic mice. Nonetheless, the mechanism of E₂ action in primate LHRH neurons appears to differ from mice LHRH neurons, as the E₂-induced frequency and synchronization of [Ca²⁺]_i oscillations are not blocked by ICI 182,780 in our study.

6. E₂ action through other G-protein coupled receptors

Besides a possible role of membrane associated ER β and/or ER α , several candidates for membrane associated receptors mediating estrogen action have been reported. Reviewing the literature, common features are revealed. As summarized in Table 1, all membrane bound estrogen receptors are seven transmembrane (7TM) G-protein coupled receptors (GPCR). First, Toran-Allerand and colleagues have shown that E₂ elicits rapid and sustained phosphorylation and activation of the mitogen-activated protein kinase (MAPK) isoforms, the extracellular signal-related kinases (ERK1 and ERK2) in neocortical neurons obtained from neonatal mice (32). Because this E₂ action is not blocked by ICI 182,780, yet similar E₂ action can be induced in cortical neurons from ER α knockout mice, these authors designated it as ER-X. Apparently, ER-X is present in association with caveolar-like microdomains (CLMs) of neuronal plasma membranes (32).

Second, Boulware et al. (33) reported that E₂ binding to ER α and ER β modified signaling of metabolic glutamate receptors (mGluRs), a family of GPCRs in hippocampal pyramidal neurons of female rats. In a series of studies, the authors reported that a 5 min exposure of hippocampal pyramidal neurons to E₂ induced MAPK-dependent phosphorylation of cAMP response element-binding protein (CREB) through ER α or ER β and that ER α interacted with mGluR1 coupled to G α_q , whereas ER β interacted with mGluR2/3 coupled to G α_i/o (33). Moreover, E₂-induced MAPK-dependent CREB phosphorylation was activated by membrane estrogen receptor stimulation of mGluR1, whereas L-type calcium channel-dependent CREB phosphorylation was attenuated by estrogen receptor stimulation of mGluR2/3 (33). Finally, these authors found that estrogen receptor interactions with mGluRs were dependent upon caveolin proteins (34): ER α activation of mGluR1 is dependent on caveolin-1 (CAV1), whereas caveolin-3 (CAV3) is necessary for ER α and ER β activation of mGluR2/3. Caveolin proteins are significantly important for trafficking and clustering of signaling molecules (35). A similar mechanism of E₂ signaling through ER α in association with mGluR1a in arcuate nucleus neurons, which regulates lordosis behavior in female rats, is also reported (36) and it is assumed that CAV1 is also involved in this mechanism.

Third, for more than a decade Martin Kelly's group has been proposing the presence of membrane estrogen receptors in hypothalamic neurons containing preproimelanocortin (POMC) or dopamine (see 37). E₂ can rapidly reduce the potency of the GABA_B receptor-coupled inwardly rectifying K⁺ channels, thereby increasing the excitability of POMC and dopamine neurons (38). This E₂ effect is a membrane-mediated action, as the membrane impermeable E₂-BSA causes similar effects. The E₂ effect was blocked by ICI 182,780, but the receptor mediating this E₂ effect appears to be different from ER α and ER β , as a non-steroidal ligand synthesized by the Kelly' group, named STX, causes a similar effect as E₂, not only in wild type mouse, but also in ER α and ER β knockout mice (39). A recent study by the same authors further suggests that STX-sensitive estrogen receptors are associated with

Gαq resulting in phospholipase C activation and subsequent up-regulation of PKC and PKA activity (40).

Fourth, the results from our studies indicate that GPR30 appears to be involved in a rapid E₂ action. There is consistent skepticism on the role of GPR30 in mediating E₂ action because of controversy regarding the subcellular localization of GPR30. Filardo and collaborators (28) have shown the presence of GPR30 in the plasma membrane, whereas Prossnitz and collaborators have shown the presence of GPR30 in the endoplasmic reticulum, Golgi apparatus, and nuclear membrane (41). More recently Filardo and colleagues (42) have suggested that GPR30 is located at the cell surface and upon E₂ stimulation GPR30 is sequestered from the cell surface resulting in codistribution with clathrin-coated vesicles, which makes it difficult to demonstrate the presence of GPR30 in the plasma membrane. Internalization of 7TM GPCRs commonly occurs as a consequence of receptor endocytosis following ligand binding (43). Subcellular localization of GPR30 in primate LHRH neurons is unknown at this time.

We believe that E₂ in primate LHRH neurons appears to signal from the plasma membrane based on the following reasons: EDC caused stimulatory action in LHRH neurons within 10 min and plasma membrane impermeable E₂-BSA also stimulated LHRH release, similar to EDC. It is possible that subcellular localization of GPR30 may be dependent on the cell type, such that GPR30 is in the membrane of hippocampal neurons, whereas in cancer cells GPR30 was present in the endoplasmic reticulum, as suggested by Funakoshi et al. (44). However, at this point controversy on the subcellular location of GPR30 appears unsettled, as newer studies (45,46) report that GPR30 is found in the endoplasmic reticulum and Golgi apparatus in hippocampal neurons.

7. Physiological significance of rapid estrogen action in LHRH neurons

It is a well established concept that LHRH neurons *in vivo* are controlled by the negative and positive feedback actions of E₂. E₂ suppresses release of LHRH (and LH) *in vivo* with a latency of 1–2 h (47,48), whereas E₂ stimulates release of LHRH (and LH) *in vivo* with a latency of anywhere from 12 to 48 h (depending on species) after its administration (see 49,50). As discussed above, the results of our *in vitro* studies indicate that E₂ stimulates LHRH neuronal activity within a min, leading to LHRH neurosecretion. Is this *in vitro* finding not parallel with the well-established *in vivo* concept? There are several differences in the observations from *in vitro* and *in vivo* studies. First, the effect of E₂ reported in this study is direct action to LHRH neurons, not mediated by other interneurons. Previously we reported that our cultures contain no glia and almost no other neurons (20,51). Moreover, the results that excitatory effects of E₂ on LHRH neurons can be induced in the presence of TTX (19), exclude any possible involvement of non-LHRH neurons in culture. In contrast, in *in vivo* studies E₂ action is likely mediated by interneurons or glia, and not direct to LHRH neurons. Wintermantel et al. (52) have shown that the presence of ERα in the brain is sufficient for the positive feedback effects of E₂ and mouse LHRH neurons contain only ERβ, not ERα (10), whereas interneurons, such as kisspeptin, neuropeptide-Y, opioid, glutamate, and GABA neurons, as well as glia contain ERα (50). Two recent studies in transgenic mice further suggest that the positive feedback effect of E₂ requires ERα, which interacts with ERE, whereas the negative feedback action of E₂ is in part mediated by an ERE-independent ERα signaling mechanism (52,53). Presently, whether primate LHRH neurons express ERα is unknown, although the presence of ERβ in primate LHRH neurons has been reported (12). Nonetheless, in primates as well, interneurons and glia may play a significant role in E₂ feedback mechanisms *in vivo*.

Second, it is possible that the E₂-induced excitatory effect in primate LHRH neurons is a completely different phenomenon from the one described as the positive feedback effect of E₂ *in vivo*. In fact, exposure of cultured primate LHRH neurons to E₂ elicits an excitatory effect

with a significantly shorter time domain (within minutes) compare to *in vivo* (more than 12 hours) and the excitatory E₂ action in cultured primate LHRH neurons is, at least in part, mediated through GPR30, an extranuclear ER, rather than nuclear ER. Although it is unknown whether the ultra-short excitatory E₂ effect occurs *in vivo*, a series of studies in our laboratory illustrate a novel mechanism of E₂ action in the brain.

Conclusion

Using cultured LHRH neurons derived from embryonic olfactory placode, we found that E₂ induced a rapid action in primate LHRH neurons and that this rapid action of E₂ was, at least in part, mediated by GPR30. Rapid action of E₂ in neuronal cells in all four cases reviewed above appears to have an involvement of 7TM GPCRs (e.g., ER-X, mGluRs, STX sensitive R, and GPR30). Structures of ER-X and STX-sensitive R are yet to be determined. Are these different forms of membrane estrogen receptors cell type specific, or brain region specific, or species specific? Is there a common binding site (or pocket) for E₂ among these 7TM GPCRs? Many questions remain to be answered. Nonetheless, when we compare the E₂ action through the nuclear receptor ER α and ER β , E₂ action through various GPCRs in neuronal cells would provide a rapid signal transduction mechanism necessary to neuronal function. Moreover, it appears that different types of GPCRs for E₂ action among different neuronal cell types are advantageous for a variety of neuronal functions, because they can make available flexible responses.

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Table 1
Oestrodial sensitive seven transmembrane G-protein coupled receptors (7TM GPCRs)

Name of Membrane Receptors	Cells/Tissue	Antagonized by ICI182,780	G-protein coupled	Association with membrane proteins	Signal transduction	References
ER-X	Neocortex in neonatal mice (culture)	No (ERαKO mice have this receptor)	Yes (Gαq)	Caveolar-like microdomains	MAPK (ERK1/2)	Toran-Allerand et al. (32)
mGluR1	Hippocampal pyramidal neurons in neonatal rats	Yes (Interact with ERα)	Yes (Gαq)	Calveolin 1	PLC → IP ₃ and PKC → MAPK (ERK)	Boulware et al. (33,34)
mGluR2/3	Hippocampal pyramidal neurons in neonatal rats	Yes (Interact with ERβ)	Yes (Gαi/o)	Calveolin 3	cAMP → PKA → L-type Ca ²⁺ channel	Boulware et al. (33,34)
mGluR1a	Arcuate nucleus in adult rats	Yes (Interact with ERα)	Yes	Likely Calveolin 1	MAPK (ERK)	Dewing et al. (36)
STX-sensitive Receptor	POMC and dopamine neurons in adult guinea pig and mouse hypothalamus	Yes (Independent of ERα and ERβ, as ERαKO, ERβKO, ERαβKO mice have this receptor)	Yes (Gαq/Gβγ)	Unknown	PLC → IP ₃ , PKC, and PKA → GIRK channel and GABA _B	Qiu et al. (39) Roepke et al. (40)
GPR30	Cancer cells	No (ICI182,780 is an agonist)	Yes (Gαs and Gβγ)	clathrin	adenylyl cyclase → cAMP → PKA Src → Ras → MAPK (ERK1/2)	Filardo et al. (26,42) Filardo et al. (26,42)
GPR30	LHRH neurons obtained from monkey fetuses (culture)	No (ICI182,780 is an agonist)	Yes	Unknown	Not examined yet	Abe et al. (19) Noel et al. (25)

ERK, extracellular signal-regulated kinase; GIRK, G-protein-gated inwardly-rectifying K⁺; IP₃, inositol trisphosphate; LHRH, luteinizing hormone-releasing hormone; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; POMC, preopiomelanocortin; Ras is a small GTPase; Src is a tyrosine kinase.