

Regulation of Cyclic Adenosine 3',5'-Monophosphate Signaling and Pulsatile Neurosecretion by G_i-coupled Plasma Membrane Estrogen Receptors in Immortalized Gonadotropin-Releasing Hormone Neurons

CARLOS E. NAVARRO, SHEIKH ABDUL SAEED, CYNTHIA MURDOCK, ANTONIO J. MARTINEZ-FUENTES, KRISHAN K. ARORA, LAZAR Z. KRSMANOVIC, AND KEVIN J. CATT

Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-4510

Immortalized GnRH neurons (GT1-7) express receptors for estrogen [estrogen receptor- α and - β (ER α and ER β)] and progesterone (progesterone receptor A) and exhibit positive immunostaining for both intracellular and plasma membrane ERs. Exposure of GT1-7 cells to picomolar estradiol concentrations for 5-60 min caused rapid, sustained, and dose-dependent inhibition of cAMP production. In contrast, treatment with nanomolar estradiol concentrations for 60 min increased cAMP production. The inhibitory and stimulatory actions of estradiol on cAMP formation were abolished by the ER antagonist, ICI 182,780. The estradiol-induced inhibition of cAMP production was prevented by treatment with pertussis toxin, consis-

tent with coupling of the plasma membrane ER to an inhibitory G protein. Coimmunoprecipitation studies demonstrated an estradiol-regulated stimulatory interaction between ER α and G $_{\alpha i3}$ that was prevented by the ER antagonist, ICI 182,780. Exposure of perfused GT1-7 cells and hypothalamic neurons to picomolar estradiol levels increased the GnRH peak interval, shortened peak duration, and increased peak amplitude. These findings indicate that occupancy of the plasma membrane-associated ERs expressed in GT1-7 neurons by physiological estradiol levels causes activation of a G_i protein and modulates cAMP signaling and neuropeptide secretion. (*Molecular Endocrinology* 17: 1792-1804, 2003)

THE EPISODIC SECRETION of pituitary gonadotropin, which is essential for the maintenance of the mammalian reproductive cycle and normal gonadal function, is driven by the periodic release of GnRH from the hypothalamic GnRH neuronal network (1). The principal determinants of GnRH neuronal activity include gonadal steroids, as well as several neuropeptides and neurotransmitters (2, 3). 17 β -Estradiol (E₂) exerts positive and negative feedback control between the ovary and hypothalamic GnRH neurons and has both stimulatory (4, 5) and inhibitory (6) actions on GnRH biosynthesis and secretion. In GnRH neurons, estrogen has been reported to both increase and decrease gene transcription and GnRH mRNA synthesis (7-10). In reproductive tissues, many of the receptor-mediated actions of estrogen are exerted at the nuclear level by transcriptional regulation of specific genes involved in cell metabolism, growth, and differentiation (11).

In addition to its well defined actions in the nucleus, estrogen has rapid regulatory effects on several membrane-associated and intracellular responses that do not depend on changes in gene expression. These include changes in the electrical properties of neurons, as well as alterations in neurotransmitter release (12-14). Furthermore, interactions between estrogen and cAMP can enhance the growth of the mammary gland and breast cancer cells (15), and cAMP can induce estrogen-like uterine growth responses (16). These and other findings have implicated G proteins and second messenger systems in several aspects of estrogen action. Such effects are distinct from the well defined genomic actions of estrogen and are attributable to its interactions with membrane-bound receptors and other cellular components. Such cell-surface receptors for estrogen were first identified by Pietras and Szego (17), and have been recently implicated in variety of rapid signaling responses mediated at the plasma membrane (18). Membrane receptors for estrogen have been observed in the hypothalamus and other brain regions, including the cortex, hippocampus, and brain stem (19).

The extent to which GnRH neurons are directly responsive to steroid hormones has been a controversial

Abbreviations: DPBS, Dulbecco's PBS; E₂, 17 β -estradiol; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; IBMX, 3-isobutyl-1-methylxanthine; P₄, progesterone; PRA, progesterone receptor A; PTX, pertussis toxin; SDS, sodium dodecyl sulfate.

issue in reproductive physiology. However, recent reports have demonstrated that β -isoforms of the estrogen receptor (ER) are present in native GnRH neurons (20). In this study, the expression, binding properties, location, and actions of endogenous ERs were analyzed in immortalized GnRH neurons and cultured rat hypothalamic cells. These cells were found to express plasma membrane as well as nuclear ERs, and to respond to physiological concentrations of E_2 with a G_i -mediated decrease in cAMP production and modulation of the episodic mode of GnRH release.

RESULTS

Expression of ERs and Progesterone Receptors in Cultured Hypothalamic Cells and GT1–7 Neurons

In RT-PCR studies, the expected fragment sizes of 555 bp for ER α and 435 bp for ER β were amplified from RNA isolated from hypothalamic cells, GT1–7 neurons, and pregnant rat uterus. Specific ER α mRNA transcripts were detected in both GT1–7 neurons and hypothalamic cells cultured in the presence (Fig. 1A, lanes b and d) or absence of fetal bovine serum in the culture medium (Fig. 1A, lanes c and e). ER β mRNA products were also expressed in GT1–7 neurons cultured in fetal bovine serum-containing medium (Fig. 1B, lane b) and in GT1–7 neurons and hypothalamic cells cultured in serum-free medium (Fig. 1B, lanes d and e, respectively). Positive ER α mRNA products were detected in rat uterus (Fig. 1A, lane g). There was no amplification of specific products from RNA isolated from the optic nerve (Fig. 1A and 1B, lane f). A specific amplified product of 341 bp for the progesterone A receptor (PRA) was also derived from cultured GT1–7 neurons (Fig. 1C, lane b) cultured with and without fetal bovine serum (lane c) and hypothalamic cells (Fig. 1C, lane d). As expected, positive PRA mRNA products were detected in the rat uterus (Fig. 1C, lane g). There was no amplification of specific products from RNA isolated from the optic nerve (Fig. 1C, lane f). In each case, DNA sequencing of the purified bands confirmed the authenticity of these amplified fragments. The nucleotide sequences matched the published sequences for ER α , ER β , and PRA (data not shown).

Western blot analysis of subcellular fractions of GT1–7 neurons cultured in serum- and phenol red-free medium with specific antibody to ER α (NCL-ER-6F11) revealed expression of ER α in the nuclear fraction (Fig. 1D, lane 1), cytosolic fraction (Fig. 1D, lane 5) and membrane fraction (Fig. 1E, lane 9). During both short-term (5 min) and prolonged (6 h and 24 h) treatment with 1.7 nM E_2 , immunoreactive ER α increased 16% \pm 3.4, 18% \pm 4.1, 22% \pm 5.3 in the nuclear fraction (Fig. 1D, lanes 2, 3, and 4, respectively), decreased 10% \pm 2.4, 14% \pm 1.9, 18% \pm 2.8 in the cytosolic fraction (Fig. 1D lanes 6, 7, and 8, respectively), and remained

unchanged in the membrane fraction (Fig. 1E, lanes 10, 11, and 12, respectively). Molecular markers are labeled as M, and a negative control without ER α is labeled as D.

Saturation binding studies with [3 H] E_2 in immortalized GnRH neurons *in situ* revealed a single high-affinity binding site with dissociation constant (K_d) of 244 \pm 32 pM and B_{max} of 64 \pm 3.5 fmol/mg protein (Fig. 1, F and G). Cultured hypothalamic cells also expressed a single high-affinity binding site with K_d of 146 \pm 39 pM and B_{max} of 1.3 \pm 0.1 fmol/mg protein ($n = 3$, not shown). In addition, a single class of high-affinity E_2 binding sites was detected in membrane fractions of immortalized GnRH neurons using [125 I]-labeled E_2 . Binding parameters were comparable to those of binding to intact cells with K_d of 333 \pm 46 pM and B_{max} of 55 \pm 8.5 pmol/mg protein (Fig. 1, H and I).

Immunocytochemical Characterization of Nuclear and Membrane-Localized ER α and ER β

Characteristic nuclear fluorescence for ER α was present in fixed and permeabilized GT1–7 neurons treated with the H-222 antibody (Fig. 2A). The neuronal processes exhibited granular fluorescence throughout their length and in their terminals (Fig. 2A, arrows). No fluorescence emission was detected when primary antibody (H-222) was pretreated with blocking peptide or substituted with normal rabbit serum (Fig. 2, B and C). Positive ER α immunostaining was also observed in permeabilized GT1–7 neurons with another ER α monoclonal antibody (NCL-ER-6F11) and was distributed in the nucleus, cytosolic compartment, and membrane (Fig. 2D, brown precipitate). Application of goat antimouse IgG (Fab) secondary antibody, which reduces background staining, also revealed positive ER α immunostaining in permeabilized GT1–7 neurons (Fig. 2E). No immunofluorescence was detectable with ER α antibody pretreated with blocking peptide or in the absence of the first antibody (data not shown).

The expression of ER β , as revealed by indirect immunofluorescence, was evident on neuronal bodies as well as the connecting processes (arrow) of permeabilized GT1–7 neurons (Fig. 3A). On confocal microscopy, individual laser sections of the same cell showed widespread distribution of ER β in the cytosol and processes (Fig. 3B, arrows), as well as in the nucleus (Fig. 3C). No staining was observed in the absence of the first antibody and preincubation with blocking peptide (data not shown).

Differentiated GT1–7 neurons cultured on poly-L-lysine-coated coverslips formed interconnected neuronal network that closely resembled the morphology and connections that are characteristic of hypothalamic GnRH neurons in culture. No immunostaining was detectable with preadsorbed ER α antibody and control serum (Fig. 4A). In differentiated nonpermeabilized GT1–7 cells treated with ER α monoclonal antibody (H-222 antibody) showed positive immunostain-

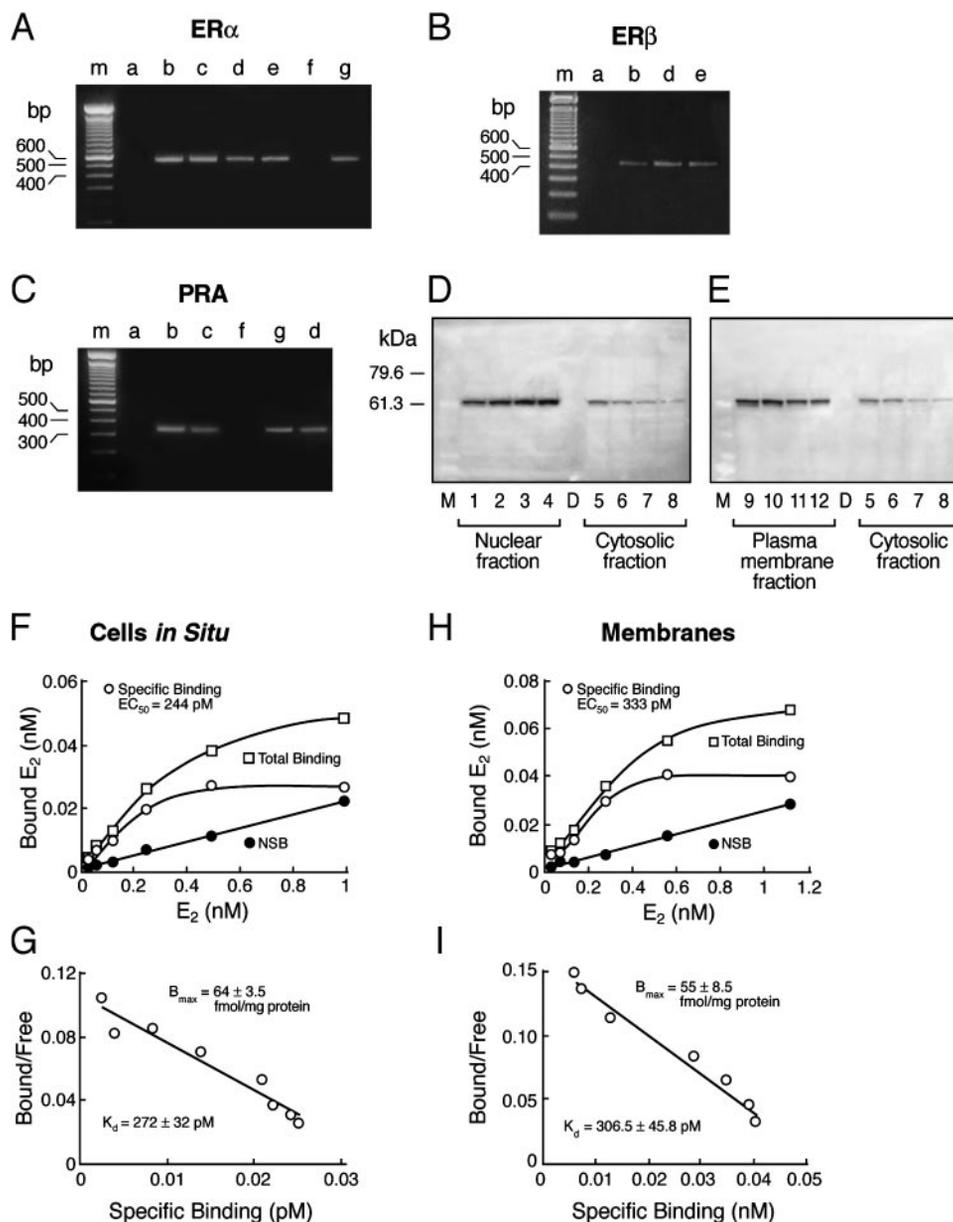


Fig. 1. Expression and Binding Properties of ERs Progesterone Receptors in Cultured Hypothalamic Cells and GT1–7 Neurons

A–C: RT-PCR analysis of ER α (A), ER β (B), and PRA (C) expression. a, Control in the absence of cDNA; b and c, RNA from GT1–7 cells cultured in serum-containing and serum-free medium, respectively; d and e, RNA from hypothalamic cells cultured in serum-containing and serum-free medium; f, Negative control, RNA from optic nerves; g, positive control, rat uterus. D, Western blots of ER α in GT-1 cell nuclei and cytosol (*left*, lanes 1 and 5, and during treatment with 1.7 nM E₂ for 5 min (lanes 2 and 6), 6 h (lanes 3 and 7), and 24 h (lanes 4 and 8). Western blots of ER α plasma membrane and cytosol (lanes 9 and 5) and during treatment with 1.7 nM E₂ for 5 min (lanes 10 and 6), 6 h (lanes 11 and 7), and 24 h (lanes 12 and 8). Lane M is molecular weight marker and lane D is control in absence of ER α protein. F–I, Saturation binding studies and Scatchard analysis of estrogen binding sites in GT1–7 cells (F and G) and membrane fragments thereof (H and I).

ing for ER α on the plasma membrane (Fig. 4B). Positive ER α immunostaining was also observed in nonpermeabilized GT1–7 neurons with another ER α monoclonal antibody (NCL-ER-6F11) and was localized on the plasma membrane (Fig. 4C, *red* precipitate). Membrane-associated ER β staining was also observed in nonpermeabilized GT1–7 neurons analyzed by confocal microscopy (Fig. 4D).

Coupling of ER to the Adenylyl Cyclase Signaling Pathway

Short-term exposure (5 min) of cultured hypothalamic cells and GT1–7 neurons to E₂ concentrations from 2 pM to 100 pM caused a significant decrease in cAMP production, and this was prevented by prior treatment with the competitive ER antagonist, ICI 182,780.

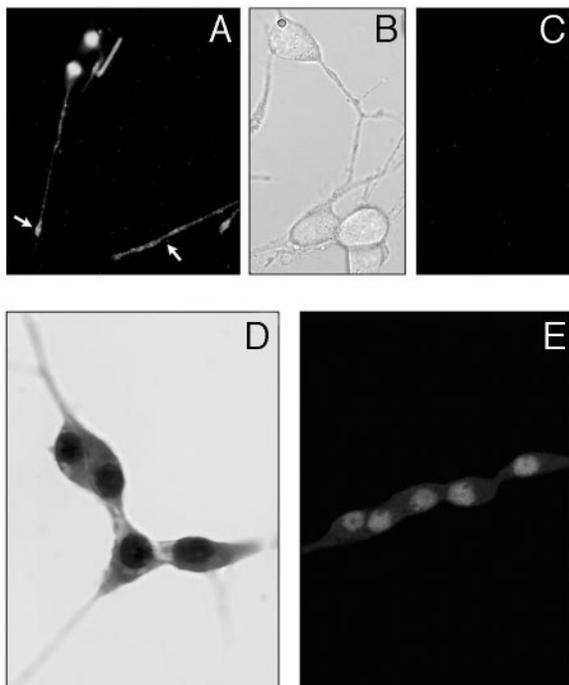


Fig. 2. Immunofluorescence Staining of ER α in GT1–7 Neurons

A, Localization of ER α in the nucleus and neuronal processes (arrows) of a permeabilized GT1–7 neuron. B and C, Lack of fluorescent staining in cells pretreated with blocking peptide or normal rabbit serum. Demonstration of ER α immunostaining (brown immunoprecipitate) with NCL-ER-6F11 monoclonal antibody. C, Blue immunostaining for ER α with a goat antimouse IgG (Fab).

Treatment of cultured hypothalamic cells and GT1–7 neurons with ICI 182,780 alone had no effect on cAMP production (Fig. 5, A and B). A similar rapid (5 min) inhibitory effect on cAMP production was observed when GT1–7 neurons were treated with BSA-conjugated E₂ (Fig. 5C), consistent with membrane-mediated regulation of the adenylate cyclase response. Prolonged treatment (1 h) of both hypothalamic cells and GT1–7 neurons with increasing E₂ concentrations likewise caused inhibition of cAMP production in the picomolar range, with an increase at low nanomolar E₂ concentrations and a decrease to below the control level with high nanomolar E₂ concentrations (Fig. 5, D and E), respectively. Such biphasic dose-dependent cAMP responses to E₂ were also evident after 24 h and were inhibited by pretreatment with the ER antagonist, ICI 182,780 (data not shown). In static cultures of GT1–7 neurons, GnRH secretion paralleled the changes in cAMP production. Treatment with picomolar E₂ concentrations (1 h) inhibited GnRH secretion. However, treatment with nanomolar E₂ concentrations for 1 h stimulated GnRH secretion (Fig. 5F).

Biphasic actions of E₂ on adenylate cyclase activity were also demonstrable in membrane preparations from GT1–7 neurons. Inhibition of cAMP production in the particulate fraction was maximal at 100 pM E₂ (1 h

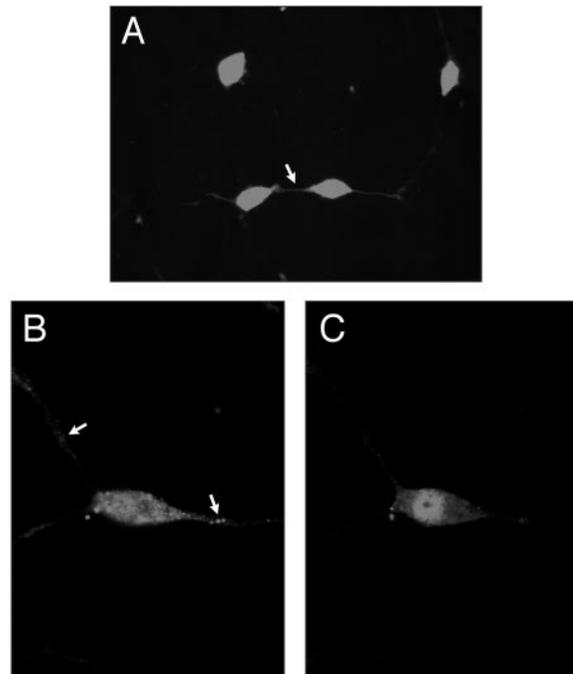


Fig. 3. Immunofluorescence Staining of ER β in GT1–7 Neurons

A, Presence of ER β in the neural bodies and processes (arrow) of GT1–7 neurons. B, Confocal analysis showing granular cytoplasmic localization of ER β in the cytoplasm and neuronal processes of GT1–7 cells. C, Nuclear staining for ER β in another section of the same cell.

treatment), as observed in intact GT1–7 cells, but did not occur at 10 nM E₂ (Fig. 5G). As observed in intact GnRH neurons, pretreatment for 2 h with pertussis toxin (PTX) prevented the E₂-induced fall in cAMP production, again indicating that adenylate cyclase-inhibitory G_{i/o} proteins are involved in the E₂-induced inhibition of cAMP production (Fig. 5, H and I).

Interaction Between ER and Adenylate Cyclase-Inhibitory G Protein

Potential interactions between plasma membrane ER α and G_{ai} proteins were evaluated in coimmunoprecipitation studies using GT1–7 neurons. Immunoprecipitation was performed with ER α antibody on solubilized membrane fractions from control cells and cells treated with 100 pM E₂, or with E₂ in the presence of ICI 182,780. In membrane extracts from untreated cells, G_{ai3} coimmunoprecipitated with the ER α . Interestingly, the amount of G_{ai3} associated with ER α was progressively reduced after 5 min and 30 min exposure to 100 pM E₂ (Fig. 6) and returned toward the control level after 120 min (Fig. 6). Pretreatment of GT1–7 neurons with the ER antagonist ICI 182,780 (30 min, 170 pM) prevented the E₂-induced decrease in membrane-associated G_{ai3} immunoreactivity, indicating that activated ERs are required for the decrease in membrane-associated G_{ai3} immunoreactivity. In fact,

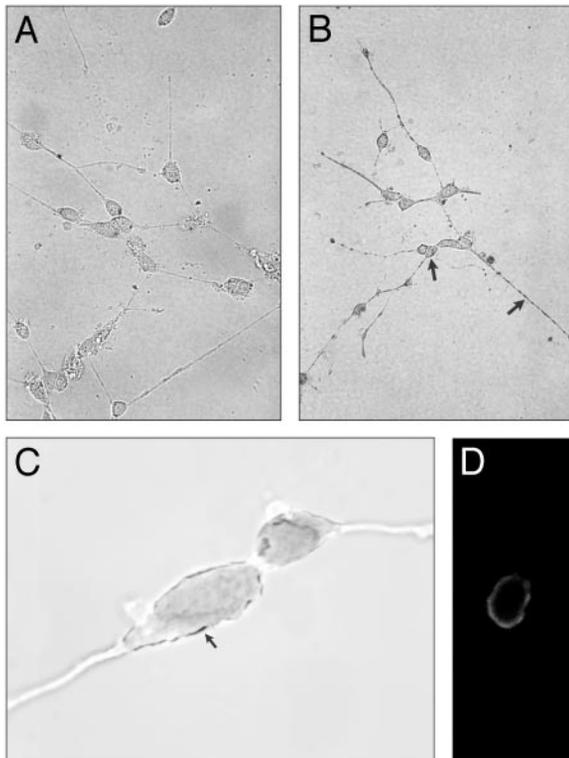


Fig. 4. Membrane Localization of ER α and ER β in GT1-7 Cells

A, Control immunostaining of GT1-7 neurons with antigen preadsorbed H-222 antibody. B, Positive immunostaining (blue) for ER α at the plasma membrane of intact GT1-7 neurons and neuronal processes (arrows). C, Membrane immunostaining of ER α in a nonpermeabilized GT1-7 neuron with specific monoclonal antibody (NCL-ER-6F11). D, Confocal section showing plasma membrane immunostaining for ER β in a nonpermeabilized GT1-7 neuron.

prolonged treatment with the antagonist cause a significant increase in the amount of membrane-associated G α_{i3} .

Regulation of GnRH Secretion by Estrogen and Progesterone in Cultured Hypothalamic Cells and GT1-7 Neurons

Static cultures of GT1-7 neurons and hypothalamic cells were exposed to E $_2$ and progesterone (P $_4$) concentrations similar to those of the rat estrous cycle. Under follicular phase conditions, cells were treated daily for 4 d (duration of rat estrous cycle) with E $_2$ concentrations of 2 μ M and 0.5 nM for serum-free and serum-containing medium, respectively. The samples for GnRH measurement were taken between 1700 and 1800 h on the day corresponding to the *in vitro* proestrous phase, and released GnRH was similar to that of untreated control cells (Fig. 7). Treatment with follicular phase concentrations of progesterone (2 nM in serum-free and 500 nM in serum-containing medium) in the same protocol as for follicular E $_2$ treatment

had no effect on GnRH release, nor did combined treatment with follicular phase concentrations of E $_2$ and P $_4$ (Fig. 7). During cyclic treatment, cells were exposed to follicular phase concentrations of E $_2$ (2 μ M) and P $_4$ (2 nM) for 24 h, then for 24 h with diestrous I concentrations (E $_2$, 6 μ M; and P $_4$, 7 nM), followed by 24 h treatment with diestrous II concentrations (E $_2$, 11 μ M; and P $_4$, 2 nM). Simulation of the preovulatory stage of the estrous cycle, by timed increases in E $_2$ concentrations (17 μ M) and P $_4$ (20 nM), significantly increased GnRH release from both hypothalamic cells and GT1-7 neurons, analogous to the preovulatory surge of GnRH *in vivo* (Fig. 7, panel A, 6.3 \pm 0.3 pg/ml, control vs. 9.5 \pm 0.3, n = 3, P < 0.01 pg/ml, cyclic treatment and panel C, 14.4 \pm 1.2 pg/ml, control vs. 37.2 \pm 2.5 pg/ml cyclic treatment, n = 3, P < 0.01). A doubling of GnRH release was also observed in cultures treated with the higher preovulatory concentrations of E $_2$ and P $_4$ that are required during treatment in serum-containing medium (Fig. 7, panel B, 9.4 \pm 1.47 pg/ml, control vs. 17.8 \pm 0.4, n = 3, P < 0.01 pg/ml, cyclic treatment; and panel D, 17.4 \pm 0.8 pg/ml, control vs. 44.0 \pm 8.1 pg/ml cyclic treatment, n = 3, P < 0.01).

Effects of Estrogen and Progesterone on GnRH Release from Perfused Cells

Dynamic changes in GnRH release during steroid treatment were evaluated in perfused hypothalamic cells and GT1-7 neurons cultured in serum-free medium with added steroids at the concentrations of free estrogen and progesterone found at specific stages of the estrous cycle (Fig. 8). In comparison to control hypothalamic cultures, the cultures treated with an ovulatory level of 17 μ M E $_2$ showed a significant increase in GnRH pulse amplitude and a decrease in pulse frequency. Pulse amplitude rose from 22.2 \pm 1.7 pg/ml in controls to 60.1 \pm 9.1 pg/ml (n = 4; P < 0.01) in 17 μ M E $_2$ -treated cells. The mean peak interval increased from 36.3 \pm 6.4 min to 90 \pm 16.2 min, and pulse duration decreased from 9.4 \pm 1.7 min to 5.6 \pm 1.2 min (n = 4; P < 0.05) (Fig. 8, A and B). Immortalized GnRH-producing neurons also exhibited episodic GnRH release with peak amplitude of 52.3 \pm 1.2 pg/ml, interpeak interval of 46.3 \pm 7.2, and peak duration of 15.4 \pm 3.3 min (Fig. 8C). Treatment of GT1-7 neurons for 24 h with ovulatory E $_2$ levels (17 μ M) increased the GnRH peak interval from 46.3 \pm 7.2 min in control cells to 90.0 \pm 8.16 min (P < 0.01; n = 4). It also shortened peak duration from 15.4 \pm 2.3 min to 5.7 \pm 1.1 and increased peak amplitude from 52.3 \pm 1.2 pg/ml to 118.3 \pm 2.9 pg/ml (P < 0.01; n = 4) (Fig. 8D). An increase in peak amplitude to 79.7 \pm 3.1 pg/ml (P < 0.01 vs. 52.3 \pm 1.2 pg/ml, control) also occurred after treatment of GT1-7 cells with ovulatory phase P $_4$ concentrations (Fig. 8E). Combined treatment of GT1-7 cells with ovulatory phase E $_2$ and P $_4$ concentrations caused a further increase in peak amplitude, from 52.3 \pm 1.2 pg/ml in control cells to 98.3 \pm 3.9

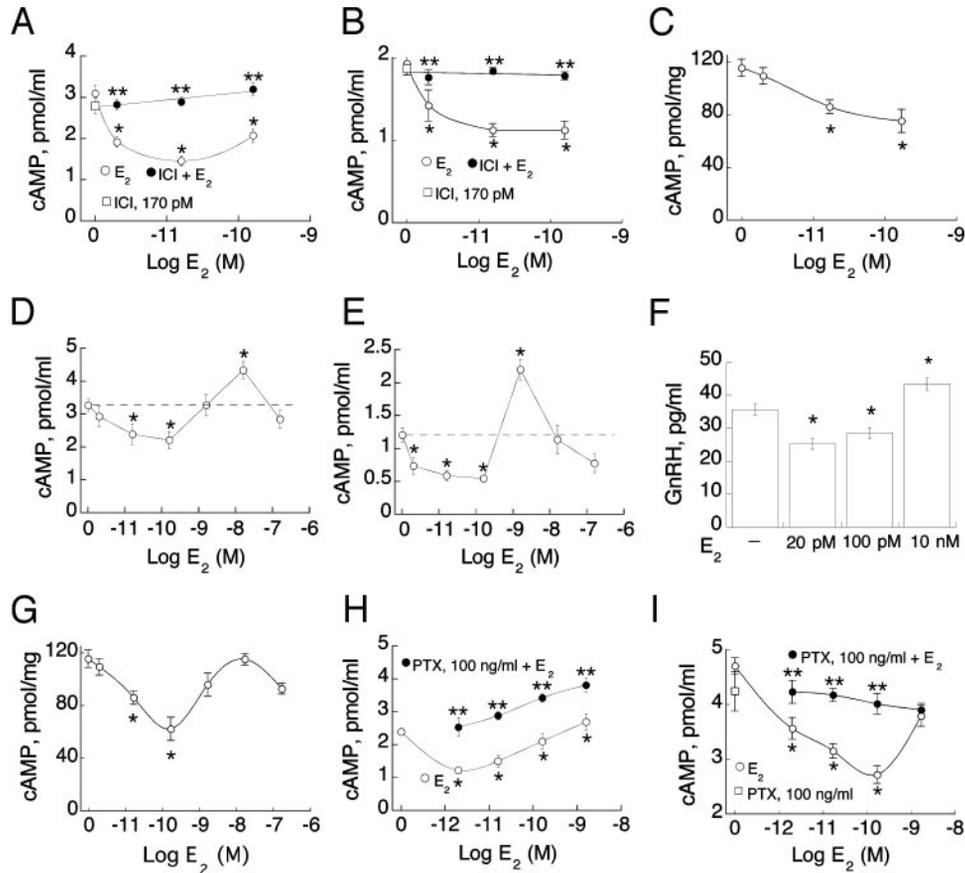


Fig. 5. Time- and Dose-Dependent Effects of E₂ on cAMP Production
 Exposure to E₂ for 5 min consistently reduced cAMP production in both hypothalamic cells (A) and in GT1–7 neurons (B). These inhibitory effects of E₂ were prevented by pretreatment with the specific ER antagonist, ICI 182,780 (solid circles). In GT1–7 cells, inhibition of cAMP production was also evident after 5 min incubation with BSA-conjugated E₂ (C). E₂ treatment of both hypothalamic cells (D) and GT1–7 neurons (E) for 60 min caused inhibition at picomolar levels, with an increase at nanomolar concentrations and a further decrease at higher levels. A correlation between cAMP production and GnRH secretion was observed in GT1–7 neurons treated with E₂ for 6 h in static culture (F). Inhibitory action of E₂ on adenyl cyclase activity in membrane fraction of GT1–7 neurons (G). The inhibitory effect of E₂ on cAMP production was abolished by prior treatment with PTX in both hypothalamic cells (H) and GT1–7 neurons (I). A single asterisk indicates significant difference compared to the control. Double asterisk indicates significant difference between control and treated group.

pg/ml and peak duration from 15.4 ± 2.3 min to 22.5 ± 2.1 min in steroid-treated cells (P < 0.01; n = 4) (Fig. 8F).

DISCUSSION

Pulsatile GnRH release is an intrinsic property of the GnRH neuron and is modulated by neurotransmitters, pituitary hormones, and gonadal steroids. These inputs impinge on the GnRH neuronal network at the hypothalamic level, where E₂ exerts both stimulatory and inhibitory actions on GnRH release. ERs are expressed in several hypothalamic cell types, including galanin-containing, γ-aminobutyric acid, and somatostatin neurons (21, 22). However, despite its prominent role in the regulation of GnRH secretion, the ability of estrogen to exert receptor-mediated actions

directly on the GnRH neuron has only recently been demonstrated.

The inability of earlier studies to detect ERs in hypothalamic GnRH neurons suggested that E₂ exerts indirect actions on the GnRH neuronal network (reviewed in Ref. 20). However, several recent reports have demonstrated that ERs are expressed in both hypothalamic GnRH neurons and their immortalized counterparts, GT1 cells (23–28). Studies using *in situ* hybridization of identified GnRH neurons in the female rat hypothalamus, cultured nasal explants, and immortalized GnRH neurons revealed expression only of ERβ (29, 30). However, we found that cultured rat fetal hypothalamic cells and GT1–7 neurons expressed mRNAs encoding both ERα and ERβ, as well as the PRA and high-affinity estrogen-binding sites.

Immunostaining with the H-222 and NCL-ER-6F11 monoclonal antibodies also revealed ERα expression

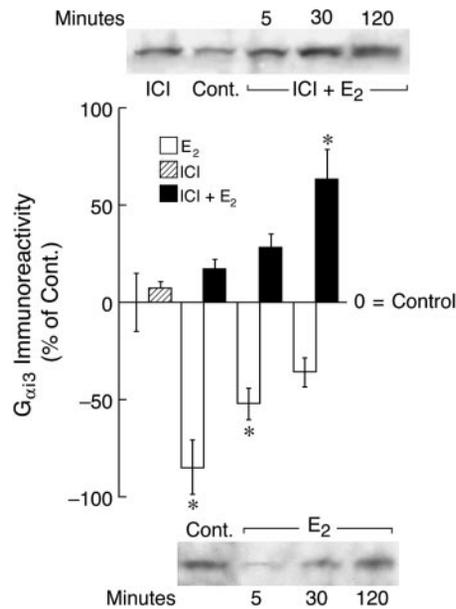


Fig. 6. Estrogen-Induced Regulation of $G_{\alpha i3}$ in GT1-7 Cell Membranes

Cells were treated with 100 pM E_2 for 5 min, 30 min, and 120 min, and then membranes were isolated and solubilized before immunoprecipitation with ER α antibody and Western blot analysis. Treatment with 100 pM E_2 significantly decreased ER α -associated $G_{\alpha i3}$ immunoreactivity (open bars, $P < 0.05$, $n = 4$). Pretreatment of GT1-7 neurons with ICI 182,780 prevented the inhibitory action of E_2 , and during prolonged treatment caused a significant increase in ER α -associated $G_{\alpha i3}$ immunoreactivity (solid bars, $P < 0.05$, $n = 4$). The blots shown are representative of four independent experiments and are quantified below. Asterisks indicate significant differences from the control.

in GT1-7 neurons. In addition to immunostaining in the nucleus, membrane-associated ERs were evident in nonpermeabilized immortalized GnRH neurons. Such receptors have not previously been observed in GnRH neurons, and presumably mediate the rapid nongenomic action of estradiol in these cells. Membrane-localized ER sites were previously found in immortalized pituitary lactotrophs (GH3/B6 cells) (31) and endothelial cells (32). Also, studies in cultured hippocampal neurons (33) have shown ER α immunostaining in close proximity to the plasma membrane, consistent with reports of several rapid estrogen-induced responses in neuronal cells. The plasma membrane ERs identified in the present report include both ER α and ER β isoforms and mediate low-dose inhibitory actions and high-dose stimulatory effects on adenylyl cyclase activity in GnRH neuronal cells. Although the effects of E_2 on GnRH neuronal function are predominantly inhibitory, rapid stimulatory effects of estrogen on neurosecretion have been observed in GnRH neuronal terminals (34, 35).

The ability of E_2 to exert rapid inhibitory actions on hypothalamic neurons has been recognized for more than 25 yr (36). Such responses have been attributed

to hyperpolarization due to the opening of an inwardly rectifying K^+ channel in the plasma membrane of the GnRH neuron, as well as in amygdala and preoptic neurons (37, 38). Also, nanomolar E_2 concentrations were found to activate cAMP signaling in hippocampal neurons (39) and hypothalamic neurons (40).

In our studies, agonist activation of ERs expressed in immortalized GnRH neurons had bidirectional time- and dose-dependent effects on cAMP production. Inhibition of cAMP production by picomolar E_2 concentrations was observed as early as 5 min after receptor activation and persisted for up to 24 h. The ER specificity of this inhibitory action of E_2 was indicated by its prevention by the competitive ER antagonist, ICI 182,780. The ability of E_2 to inhibit cAMP production in the high-speed particulate fraction of GT1-7 neurons indicates that membrane-associated ERs regulate adenylyl cyclase, and that this response does not require the participation of nuclear E_2 receptors. The prevention of such E_2 -induced inhibition of cAMP production by prior treatment with PTX is consistent with its dependence on adenylyl cyclase-inhibitory G protein(s) during agonist activation of the membrane-associated ER. The converse effect, of stimulation of cAMP production by suprananomolar E_2 concentrations, suggests that membrane-associated ER in GnRH neurons can also couple to adenylyl cyclase-stimulatory G_s proteins when activated by high agonist concentrations. These findings indicate that membrane-associated ERs undergo dose-dependent coupling to adenylyl cyclase-inhibitory and -stimulatory G proteins in GnRH neurons. Membrane-localized ER have also been observed in the brain (41) and in ER-transfected COS-7 cells (42). Such receptors have been reported to mediate rapid nongenomic actions of estradiol on both calcium (43) and cAMP signaling pathways (15), and to modulate the conductance of kainate receptors (44, 45).

The observed decreases in ER α -associated $G_{\alpha i3}$ during E_2 treatment, and the increases therein during E_2 antagonist treatment, are consistent with ER-mediated activation and dissociation of inhibitory G_i protein(s) in GT1-7 neurons. These changes are analogous to the prominent decreases in membrane-associated G_{α} subunits that occur during agonist activation of G protein-coupled receptors, including the GnRH receptor (46, 47). Furthermore, the loss of E_2 -induced inhibition of cAMP production during PTX treatment confirms that this effect results from coupling of the activated ER to G_i protein(s). The rapid G_i -mediated action of E_2 on adenylyl cyclase in GT1-7 cells could contribute to its inhibitory effects on GnRH secretion in static and dynamic studies. However, it is likely that G_i -dependent processes other than adenylyl cyclase are involved in regulation of pulsatile GnRH release, as indicated by the studies of Kelly and colleagues (48). Previous studies by Mores *et al.* (49) have demonstrated marked suppression of GnRH secretion during activation of LH receptors in perfused GT1-7 cells. This effect was found to be mediated by a G_i -

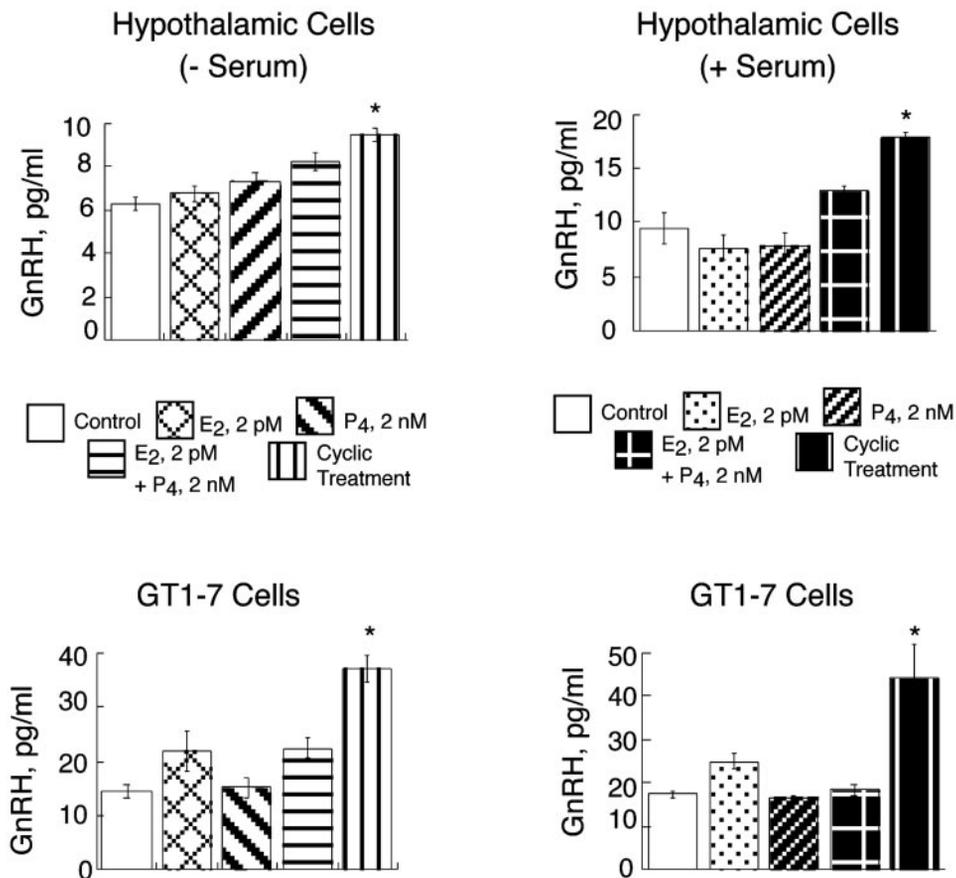


Fig. 7. GnRH Production by Cultured Hypothalamic Cells and Immortalized GnRH Neurons Treated with E₂ and P₄ Concentrations Similar to Those of the Rat Estrous Cycle
 Studies were performed in the absence (A and B) and presence (C and D) of fetal bovine serum in the culture medium. Data are average of three independent experiments of six replicates per treatment. Asterisks indicate significant increases ($P < 0.01$) in GnRH release.

dependent process, possibly inhibition of plasma-membrane ion channel activity, and was not related to changes in cAMP production.

It is of interest that similar decreases in G_{αi3} have been observed in rat pituitary cells and hypothalamic tissue after estrogen administration *in vivo*. In studies by Livingston *et al.* (50), G_{αi3} was substantially reduced in pituitary lactotrobes after 10 d of treatment with E₂, whereas G_{αs} was unchanged. In another report, 2 d of treatment with estradiol benzoate reduced G_{αz}, G_{αi1}, and G_{αi3} levels in the hypothalamus (51). The extent to which such estrogen-induced reductions in G_{αi3} subunit levels *in vivo* are related to altered gene expression has yet to be determined. However, they may also reflect the agonist-induced down-regulation of the G protein α-subunit that we observed during short-term treatment with estradiol *in vitro*.

Observations in several nonneural cell types have identified G_i-mediated actions of E₂ on cytoplasmic Ca²⁺ levels and activation of MAPK (52, 53), as well as transactivation of the epidermal growth factor receptor (54) and activation of endothelial nitric oxide synthase (eNOS) in MCF-7 breast cancer cells (53). Studies on

immortalized ovine endothelial cells also showed coupling of plasma-membrane ER to eNOS through G_i, and an E₂-regulated interaction between ERα and G_{αi} was observed in cells transfected with the two proteins (55). In mouse IC-21 macrophages, a PTX-sensitive E₂-induced rise in Ca²⁺ was mediated by sequesterable receptors that were proposed to be novel G protein-coupled receptor-related ERs (56). In endothelial cells, a subpopulation of ERs appears to be localized to caveolae and to be coupled via G_{αi} to eNOS as a functional signaling module (57).

In the rat, circulating E₂ levels are low during the follicular phase and gradually increase during its progression, whereas progesterone shows a transient increase in the afternoon of diestrus 1. A surge of estrogen precedes the progesterone peak during the ovulatory phase and gradually decreases during the luteal phase. These changes are associated with cyclical variations in GnRH release from the hypothalamus, and of LH and FSH from the pituitary gland, during the estrous cycle (58). We observed that estrogen and progesterone concentrations that mimicked these of the follicular phase had no significant effect

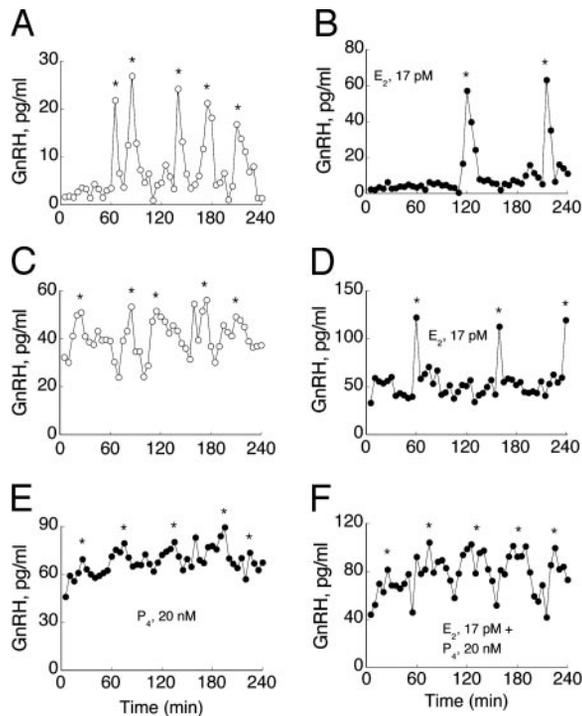


Fig. 8. Pulsatile Release of GnRH from Cultured Hypothalamic Cells (A) and GT1–7 Neurons (C) Perifused with Serum-Free Medium

Treatment with 17 pM E_2 caused a significant reduction in pulse frequency and increased pulse amplitude in both hypothalamic cells (B) and GT1–7 neurons (D). Treatment of GT1–7 neurons with 20 nM P_4 caused an increase in pulse amplitude only (E). Combined treatment with E_2 (17 pM) and P_4 (20 nM) caused less frequent but more prominent episodes of GnRH secretion induced by E_2 alone (F). Representative traces of four independent experiments are shown, and detected GnRH pulses are indicated by an asterisk.

on neuropeptide secretion. However, GnRH release from cultured GnRH neurons was doubled by steroid concentrations that occur during the afternoon of the proestrous phase of the cycle. The modest increase in GnRH release during cyclic steroid treatment could reflect the lack of endogenous afferent inputs on GnRH neurons in cultured hypothalamic cells and GT1–7 cells. These results indicate that cyclic treatment of cultured GnRH neurons with gonadal steroids mimics the *in vivo* pattern of GnRH release and represents a model for studying their direct actions on GnRH release. Our *in vitro* studies are consistent with the existing evidence that E_2 and progesterone act directly at the hypothalamic level to regulate the activity of the neurons that comprise the GnRH pulse generator. They also show that GnRH-producing neurons can directly sense the steroid hormone patterns that occur *in vivo* in generating the pattern of GnRH secretion throughout the estrous cycle.

Pulsatile GnRH secretion is observed in GT1–7 neurons as well as in cultured hypothalamic cells and provides a model for studies on the regulation of GnRH secretion (59–62). Treatment with free plasma

E_2 concentrations typical of the ovulatory phase of the rat estrous cycle increased the GnRH peak interval, shortened peak duration, and increased peak amplitude. Cultured hypothalamic cells showed a more prominent increase in pulse amplitude, with a closely similar secretory profile. In GT1–7 neurons, exposure to ovulatory progesterone concentrations increased both peak amplitude and peak duration. Combined treatment of GT1–7 neurons with ovulatory phase E_2 and P_4 concentrations caused regular and prominent pulses with an increase in peak amplitude and a decrease in pulse frequency. Our analysis of the dynamics of GnRH release *in vitro* demonstrates that ovulatory concentrations of E_2 rapidly increase GnRH pulse amplitude, while progesterone increased both pulse parameters. Furthermore, treatment of GnRH-producing neurons with ovulatory concentrations of E_2 and progesterone elicited the highest rate of GnRH release as manifested by regular and prominent peaks of neuropeptide secretion.

In summary, the ability of cultured hypothalamic cells and immortalized GnRH neurons to respond to physiological E_2 concentrations through activation of a G_i -coupled plasma membrane receptor illustrates an additional and significant aspect of the regulatory action of gonadal steroids on the GnRH pulse generator. Among the many recently recognized examples of rapid steroid-mediated responses that have confirmed the original concept of Pietras and Szego (17), this process is notable for its high sensitivity to circulating E_2 concentrations and its relevance to a fundamental property of the GnRH neuron. In addition to its role in the regulation of GnRH secretion, estrogen-regulated activation of G_i -dependent responses could account for a variety of signaling pathways mediated by G protein α_i - and $\beta\gamma$ -subunits in this and other neuronal systems.

MATERIALS AND METHODS

Tissue and Cell Culture

Hypothalamic tissue removed from fetuses of 17-d pregnant Sprague Dawley rats was subjected to enzymatic dispersion, and the cells were cultured in a 1:1 mixture of DMEM and F12 medium supplemented with 10% heat-inactivated fetal bovine serum. Immortalized GnRH neurons (GT1–7 cells) provided by Dr. R. I. Weiner (University of California at San Francisco) were cultured under the same conditions as primary hypothalamic cells.

Expression of ERs and PRs in Cultured Hypothalamic Cells and GT1–7 Neurons

Total RNA was extracted from fetal hypothalamic and GT1–7 cells, optic nerves, and uteri of 18-d pregnant rats. For ER α , primers were based on the published sequence (63); sense: 5'-GGCCAAGCCCTCTTGTGATTAAG-3' (23 bp); and anti-sense: 5'-CGGTGGATGTGGTCCCTCTCTCCAG-3' (26 bp). For ER β , primers were based on the published sequence (64); sense: 5'-GGATGAGGGGAAGTGCCTAGAA-3' (22 bp);

and antisense: 5'-CCCAGATTGAGGACTTGTAC-3' (21 bp). For PRA, primers were based on the published sequence of the rat PR (65); sense: 5'-AAGGTCGGCGACCAGTCCGG-GACA-3' (24 bp); and antisense: 5'-TTGCTCCAAGGAGAC-CCTAGGAGC-3' (24 bp). The PCR products were amplified for 30 cycles and then analyzed using 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. The identities of the amplified DNA products were confirmed by gel purification (QIAGEN, Chatsworth, CA) and DNA sequencing.

Characterization of Estrogen-Binding Sites in Cultured Hypothalamic Cells and GT1-7 Neurons

Saturation binding studies of [³H]E₂ to hypothalamic cells and GT1-7 cells were performed as previously described (23). Briefly, cells were cultured in 24-well plates at a density of 2 × 10⁶ or 5 × 10⁵ cells per well for primary hypothalamic and GT1-7 cells, respectively. The hypothalamic cells were cultured for 14 d and the GT1-7 neurons were cultured for 2 d in 1:1 DMEM/F12 medium supplemented with heat-inactivated fetal bovine serum. Twenty-four hours before binding assays, the medium was replaced by serum- and phenol red free-medium 1:1 DMEM/F12. Cells were exposed to increasing concentrations of [³H]E₂ for 1 h at 37 C in serum- and phenol red free-medium containing 0.5% BSA and 1 μM triamcinolone acetonide to block progesterin and corticoid receptors. Nonspecific binding was assessed in the presence of 1 μM E₂. After three washes with Dulbecco's PBS (DPBS), cells were collected by trypsinization, and bound radioactivity was determined in a β-spectrometer. ¹²⁵I-labeled E₂ was used for saturation binding studies in the membrane fraction. Membranes were exposed to increasing concentrations of [¹²⁵I]E₂ for 1 h at 37 C in serum- and phenol red-free medium containing 0.5% BSA and 1 μM triamcinolone acetonide to block progesterin and corticoid receptors. Nonspecific binding was assessed in the presence of 1 μM E₂. Bound and free ligand was separated by centrifugation at 4 C for 15 min at 5000 × g. Scatchard analysis was used to characterize the binding properties of ERs in saturation binding studies with radiolabeled E₂.

Subcellular Fractionalization of GT1-7 Cells

GT1-7 cells were plated at a density of 5 × 10⁶ cells in 100-mm dishes and cultured in 1:1 mixture of DMEM/F12 in the presence of heat-inactivated fetal bovine serum. After 2 d, the medium was replaced by serum and phenol red-free medium for a further 24 h before E₂ treatment. Control and E₂-treated cells were washed twice with cold PBS, scraped from the culture dishes in buffer A (50 mM triethanolamine HCl; 25 mM KCl; 5 mM MgCl₂; 0.5 mM dithiothreitol; 10 μM each aprotinin and leupeptin; pH 7.5) in the presence of sterile 0.25 M sucrose, and kept at -70 C. The cells were lysed by nitrogen cavitation at 800 pounds per square inch for 3 min, and the lysate was centrifuged at 500 × g for 5 min at 4 C to remove tissue debris (first pellet). The first supernatant was then centrifuged at 2,000 × g for 15 min at 4 C. The second pellet was saved, and the second supernatant was further centrifuged at 100,000 × g for 1 h at 4 C. The third supernatant was taken as cytosol and the third pellet as the membrane fraction, which was extracted by resuspension in buffer A containing 0.5% (wt/vol) Genapol C-100 and incubating on a rocker at 4 C for 2 h. The second pellet was resuspended in 1 ml of buffer A and mixed with 2 ml of buffer B (buffer A containing 2.3 M sucrose). The mixture was carefully loaded on top of 1 ml of buffer B, and the nuclei were pelleted by centrifugation at 12,400 × g for 1 h at 4 C. The pellet was washed once with buffer A containing 0.25 M sucrose and labeled as nuclear extract (66). The reconstituted fractions were aliquoted and their protein concentra-

tions were determined before Western blot analysis was performed.

Immunoblot Analysis of ERα

Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed on 8–16% acrylamide gels loaded with 30 μg of protein for each subcellular fraction, followed by blotting to polyvinylidene difluoride membranes (0.45 μm pore size) and treatment with a 1:100 dilution of mouse monoclonal antibody to ERα (NCL-ER-6F11, NOVO CASTRA Laboratories). This antibody was raised against a prokaryotic recombinant protein corresponding to the full length of ERα molecule. After exposure to peroxidase-coupled goat-antimouse IgG (H+L) and washing, immunoreactive ER were visualized by chemiluminescence.

Coimmunoprecipitation of ERα and Gαi3

Immortalized GnRH neurons were treated with 100 pM E₂ for 5, 30, and 120 min. Immediately after treatment, cells were rinsed with ice-cold PBS, scraped from the culture dishes with triethanolamine buffer, pH 7.5 (50 mM triethanolamine, 25 mM KCl, 5 mM MgCl₂) with 0.25 M sucrose, 10 μM aprotinin, and leupeptin, and frozen at -70 C. Immunoprecipitation buffer (0.1 mM EDTA in Ca²⁺- and Mg²⁺-free PBS, pH 7.5) was added to 2 mg plasma membrane fractions to a final volume of 500 μl. Membrane fractions were incubated overnight at 4 C with 6 μg of antibody to ER-α (D-12, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Plasma membranes were incubated for an additional 2 h at 4 C with 20 μl protein A agarose beads (Calbiochem, San Diego, CA). Beads were washed twice with 1 ml immunoprecipitation buffer, and immunoprecipitates were eluted by boiling for 5 min in SDS sample buffer. Samples were run on a 12% Tris-glycine SDS-polyacrylamide gel, and proteins were transferred to polyvinylidene difluoride membranes (0.2 μm pore size). Membranes were treated with antibody to G_{αi3} (Calbiochem), and immunoreactivity was visualized by chemiluminescence.

Immunocytochemical Detection of ERα and ERβ in GT1-7 Neurons

Immortalized GnRH neurons were plated on 25-mm glass coverslips coated with 0.01% poly-L-lysine at a density of 5 × 10⁴ cells. After 48 h, the culture medium was replaced with serum- and phenol red-free 1:1 DMEM/F12 for 9 d until cell differentiation was completed. The cells were permeabilized with 0.3% Tween 20 added in blocking buffer for 1 h at room temperature. The H-222 rat monoclonal antibody (1 μg/ml), which recognizes the ligand binding domain of ERα (67), was added for 24 h at 4 C in DPBS containing 1% purified BSA and 0.1% Tween. Rat serum was used in the absence of antibody as a negative control. After washing, nonspecific binding was blocked by incubation with 5% purified BSA. Indirect immunofluorescent analysis was performed by adding fluorescent goat antirat IgG (1:1000) for 2 h at room temperature. The cells were then rinsed and blocked, followed by overnight incubation at 4 C with a polyclonal antibody to GnRH (1:1000). On d 3, after washing and blocking, the cells were treated for 2 h at room temperature with goat antirabbit IgG-biotin conjugate (1:1000) followed by avidin-biotin peroxidase complex (1:350). GnRH staining was visualized with a diaminobenzidine substrate kit for peroxidase (Vector Laboratories, Inc., Burlingame, CA). To detect ERβ, the 1531 mouse monoclonal antibody (1 μg/ml), which reacts with the ligand binding domain of ERβ (provided by Dr. G. L. Greene, the University of Chicago, Chicago, IL), was used in the above protocol. The immunofluorescence of Texas red-conjugated mouse IgG was analyzed by confocal microscopy (Bio-Rad Laboratories, Inc., Hercules, CA).

Membrane-Localized ER α and ER β in GT1–7 Neurons

The presence of membrane-associated ER α in GT1–7 neurons was examined in nonpermeabilized cells treated with either H-222 antibody or rat serum at 4 C for 45 min. After several washes with cold DPBS and fixation with 3.7% formaldehyde, the cultures were washed, blocked, and incubated with goat antirat IgG-biotin conjugate (1:1000, Vector Laboratories, Inc.) and an avidin-biotin alkaline phosphatase complex. ER α antigenic sites were visualized using a Vector Blue Alkaline phosphatase substrate kit. The second staining began with H₂O₂ treatment before washing and incubation with 5% purified BSA in DPBS as a blocking solution, followed by overnight treatment with a rabbit polyclonal GnRH antibody at 4 C. On d 3, goat antirabbit IgG-biotin conjugate was added and GnRH staining was visualized by addition of avidin-biotin peroxidase complex followed by diaminobenzidine substrate. Plasma membrane-localized ER β in GT1–7 neurons were demonstrated by the same strategies using the 1531-mouse monoclonal antibody. The immunofluorescence of Texas red-conjugated mouse IgG was analyzed on a Bio-Rad confocal microscope.

Effects of E₂ and Progesterone on GnRH Release from Cultured Hypothalamic Cells and GT1–7 Neurons

The actions of estrogen and progesterone on GnRH secretion were analyzed in both static and perfusion studies by exposing cells to E₂ and P₄ levels characteristic of the follicular and preovulatory phases of the 4-d rat estrous cycle. In static cultures, follicular phase levels of E₂ (2 pM) and P₄ (2 nM) were maintained by daily treatment with steroids for 4 d. Combined follicular E₂ and P₄ levels were also maintained by daily treatment for the length of one rat estrous cycle. During cyclic treatment, cultured hypothalamic cells and GT1–7 neurons were exposed to steroid hormone levels typical of the estrous phase (E₂, 2 pM; P₄, 2 nM), diestrous I phase (E₂, 6 pM; P₄, 7 nM), and diestrous II phase (E₂, 11 pM; P₄, 2 nM), for 24 h. Morning proestrous phase was mimicked by treatment with E₂ (17 pM) and P₄ (2 nM) for 4 h, followed by 4 h exposure to proestrous afternoon steroid concentrations (E₂, 17 pM; and P₄, 20 nM). Samples for GnRH measurement were taken in the afternoon of the proestrous treatment. For perfusion studies, cells were cultured on cytodex beads in steroid- and phenol red-free medium for 24 h. The cultures were perfused at a flow rate of 10 ml/h at 37 C for at least 1 h to establish a stable baseline before treatment with steroid concentrations typical for a specific phase of rat estrous cycle. The cells were then perfused for 4 h with the corresponding steroid concentrations, and samples for GnRH measurement were collected at 5-min intervals and stored at –20 C before RIA using ¹²⁵I-labeled GnRH, GnRH standards, and primary antibody donated by Dr. V. D. Ramirez (University of Illinois, Urbana, IL). The intra- and interassay coefficients of variation at 50% binding in standard samples (15 pg/ml) were 5% and 7%, respectively. GnRH pulses were identified and their parameters were determined by algorithm cluster analysis. Individual point and SD values were calculated using a power function variance model from the experimental duplicates. A 2 × 2 cluster configuration and a *t* statistic of 2 for the upstroke and downstroke were used to maintain false-positive and false-negative error rates below 10%. The statistical significance of the pulse parameters was tested by one-way ANOVA (68). In static experiments, statistical differences for cAMP production and GnRH release were tested by one-way ANOVA.

Measurement of cAMP Production in Hypothalamic Cells and GT1–7 Neurons

Fetal hypothalamic cells and GT1–7 neurons were grown in 24-well cell culture plates at densities of 2 × 10⁶ cells per

well and 5 × 10⁵ cells per well, respectively, in 1:1 DMEM/F12 with 10% heat-inactivated fetal bovine serum. Forty-eight hours before the experiments were initiated, media were changed to serum- and phenol red-free DMEM/F12 and the cells were treated with steroids, ICI 182,780, or pertussis toxin in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX). The reaction was stopped and intracellular cAMP was extracted by ice-cold ethanol, followed by evaporation and reconstitution of samples in PBS containing 1 mM IBMX. For measurement of cAMP production, cells were washed twice with TE buffer (Tris-HCl 10 mM, EDTA 1 mM, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4), removed from the plates by scraping, and lysed by freeze thawing. After centrifugation at 1,000 × *g* for 5 min at 4 C, the supernatant was recovered and centrifuged at 200,000 × *g* at 2 C for 30 min. The resulting pellets were frozen on dry ice and kept at –70 C. Membrane cAMP production was measured in assay buffer containing 20 mM Tris/HCl, 10 mM MgCl₂, 2 mM CaCl₂, 1 mM IBMX, 20 mM creatine phosphate, 0.1 mM ATP, 10 U/ml creatine phosphate kinase, at 37 C for 5 min. RIA of cAMP was performed as previously described using a specific cAMP antiserum at a final titer of 1:5,000 (69). This assay shows no cross-reaction with cGMP, 2',3'-cAMP, ADP, GDP, CTP, or IBMX.

Materials

[¹²⁵I]GnRH (2200 Ci/mmol), [¹²⁵I]E₂ (2000 Ci/mmol), and [³H]E₂ (70 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL); collagenase (149 U/mg) was from Worthington Biochemical Corp. (Freehold, NJ); Dnase I, trypsin, bacitracin, IBMX, BSA, CTP, GDP, cGMP, 2',3'-cAMP, DMEM/F12 without phenol red, pertussis toxin, E₂, E₂-6(o-carboxy-methyl)oxime-BSA conjugate containing 32 mol of E₂ per mol of BSA, progesterone, triamcinolone acetonide, and forskolin were procured from Sigma Chemical Co. (St. Louis, MO); ICI 182,780 was obtained from Tocris Cookson Inc. (UK); cytodex beads were from Pharmacia Biotech (Piscataway, NJ); DMEM/F12 1:1 powder, eLON-Gase amplification system, SuperScript preamplification system, and Ready-Load 100-bp DNA ladder were from Life Technologies, Inc. (Gaithersburg, MD); QIAEX II gel extraction kit was obtained from QIAGEN (Valencia, CA); the perfusion system was from Acusyst-S Cellex Biosciences (Minneapolis, MN); GnRH was obtained from Peninsula Laboratories, Inc. (Belmont, CA); [¹²⁵I]cAMP was purchased from Covance Laboratories, Inc. (Vienna, VA), and protein assay was from Pierce Chemical Co. Other reagents, if not specified, were obtained from Sigma Chemical Co. A rat monoclonal antibody (H-222) that recognizes the ligand-binding domain of ER α and mouse monoclonal ER β antibody (1531) were generously provided by Dr. G. L. Greene, (The University of Chicago, Chicago, IL). Goat antimouse IgG (Fab) labeled with Cy5 was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Rabbit polyclonal anti-GnRH serum was generously provided by Dr. V. D. Ramirez (University of Illinois, Urbana, IL).

Acknowledgments

Received February 5, 2003. Accepted June 12, 2003.

Address all correspondence and requests for reprints to: Kevin J. Catt, M.D., Ph.D., Endocrinology and Reproduction Research Branch, Building 49, Room 6A-36, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892. E-mail: catt@helix.nih.gov.

REFERENCES

1. Caraty A, Skinner DC 1999 Dynamics of steroid regulation of GnRH secretion during the oestrus cycle of the ewe. *Ann Endocrinol (Paris)* 60:68–78
2. Terasawa E 2001 Luteinizing hormone-releasing hormone (LHRH) neurons: mechanism of pulsatile LHRH release. *Vitam Horm* 63:91–129
3. Karsch FJ, Evans NP 1996 Feedback actions of estradiol on GnRH secretion during the follicular phase of the estrous cycle. *Acta Neurobiol Exp (Warsz)* 56:715–725
4. Sarkar DK, Fink G 1979 Effects of gonadal steroids on output of luteinizing hormone releasing factor into pituitary stalk blood in the female rat. *J Endocrinol* 80:303–313
5. Terasawa E, Rodriguez-Sierra JF, Dierschke DJ, Bridson WE, Goy RW 1980 Positive feedback effect of progesterone on luteinizing hormone (LH) release in cyclic female rhesus monkeys: LH response occurs in two phases. *J Clin Endocrinol Metab* 51:1245–1250
6. Akema T, Tadokoro Y, Kimura F 1984 Regional specificity in the effect of estrogen implantation within the forebrain on the frequency of pulsatile luteinizing hormone secretion in the ovariectomized rat. *Neuroendocrinology* 39:517–523
7. Rothfeld J, Hejtmancik JF, Conn PM, Pfaff DW 1989 *In situ* hybridization for LHRH mRNA following estrogen treatment. *Brain Res Mol Brain Res* 6:121–125
8. Verdi JM, Campagnoni AT 1990 Translational regulation by steroids. Identification of a steroid modulatory element in the 5'-untranslated region of the myelin basic protein messenger RNA. *J Biol Chem* 265:20314–20320
9. Rosie R, Thomson E, Fink G 1990 Oestrogen positive feedback stimulates the synthesis of LHRH mRNA in neurones of the rostral diencephalon of the rat. *J Endocrinol* 124:285–289
10. Hoffman GE, Lee WS, Attardi B, Yann V, Fitzsimmons MD 1990 Luteinizing hormone-releasing hormone neurons express c-fos antigen after steroid activation. *Endocrinology* 126:1736–1741
11. Herbison AE, Dye S 1993 Perinatal and adult factors responsible for the sexually dimorphic calcitonin gene-related peptide-containing cell population in the rat preoptic area. *Neuroscience* 54:991–999
12. Hardy SP, Valverde MA 1994 Novel plasma membrane action of estrogen and antiestrogens revealed by their regulation of a large conductance chloride channel. *FASEB J* 8:760–765
13. Kato A, Hiruma H, Kimura F 1994 Acute estradiol modulation of electrical activity of the LHRH pulse generator in the ovariectomized rat: restoration by naloxone. *Neuroendocrinology* 59:426–431
14. Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR 2000 Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc Natl Acad Sci USA* 97:5930–5935
15. Aronica SM, Kraus WL, Katzenellenbogen BS 1994 Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc Natl Acad Sci USA* 91:8517–8521
16. Yoshioka H, Harada T, Iwabe T, Nagano Y, Taniguchi F, Tanikawa M, Terakawa N 1999 Menstrual cycle-specific inhibition of the proliferation of endometrial stromal cells by interleukin 6 and its soluble receptor. *Am J Obstet Gynecol* 180:1088–1094
17. Pietras RJ, Szego CM 1977 Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature* 265:69–72
18. Kelly MJ, Levin ER 2001 Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 12:152–156
19. Horvat A, Nikezic G, Martinovic JV 1995 Estradiol binding to synaptosomal plasma membranes of rat brain regions. *Experientia* 51:11–15
20. Herbison AE, Pape JR 2001 New evidence for estrogen receptors in gonadotropin-releasing hormone neurons. *Front Neuroendocrinol* 22:292–308
21. Herbison AE 1994 Somatostatin-immunoreactive neurones in the hypothalamic ventromedial nucleus possess oestrogen receptors in the male and female rat. *J Neuroendocrinol* 6:323–328
22. Horvath TL, Leranath C, Kalra SP, Naftolin F 1995 Galanin neurons exhibit estrogen receptor immunoreactivity in the female rat mediobasal hypothalamus. *Brain Res* 675:321–324
23. Poletti A, Melcangi RC, Negri-Cesi P, Maggi R, Martini L 1994 Steroid binding and metabolism in the luteinizing hormone-releasing hormone-producing neuronal cell line GT1-1. *Endocrinology* 135:2623–2628
24. Butler JA, Sjoberg M, Coen CW 1999 Evidence for oestrogen receptor α -immunoreactivity in gonadotrophin-releasing hormone-expressing neurones. *J Neuroendocrinol* 11:331–335
25. Hrabovszky E, Steinhäuser A, Barabas K, Shughrue PJ, Petersen SL, Merchenthaler I, Liposits Z 2001 Estrogen receptor- β immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. *Endocrinology* 142:3261–3264
26. Kallo I, Butler JA, Barkovics-Kallo M, Goubillon ML, Coen CW 2001 Oestrogen receptor β -immunoreactivity in gonadotropin releasing hormone-expressing neurones: regulation by oestrogen. *J Neuroendocrinol* 13:741–748
27. Shen ES, Meade EH, Perez MC, Deecher DC, Negro-Vilar A, Lopez FJ 1998 Expression of functional estrogen receptors and galanin messenger ribonucleic acid in immortalized luteinizing hormone-releasing hormone neurons: estrogenic control of galanin gene expression. *Endocrinology* 139:939–948
28. Roy D, Angelini NL, Belsham DD 1999 Estrogen directly represses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor- α (ER α)- and ER β -expressing GT1-7 GnRH neurons. *Endocrinology* 140:5045–5053
29. Hrabovszky E, Shughrue PJ, Merchenthaler I, Hajszan T, Carpenter CD, Liposits Z, Petersen SL 2000 Detection of estrogen receptor- β messenger ribonucleic acid and 125 I-estrogen binding sites in luteinizing hormone-releasing hormone neurons of the rat brain. *Endocrinology* 141:3506–3509
30. Sharifi N, Reuss AE, Wray S 2002 Prenatal LHRH neurons in nasal explant cultures express estrogen receptor β transcript. *Endocrinology* 143:2503–2507
31. Watson CS, Campbell CH, Gametchu B 1999 Membrane oestrogen receptors on rat pituitary tumour cells: immuno-identification and responses to oestradiol and xenoestrogens. *Exp Physiol* 84:1013–1022
32. Levin ER 2002 Cellular functions of plasma membrane estrogen receptors. *Steroids* 67:471–475
33. Clarke CH, Norfleet AM, Clarke MS, Watson CS, Cunningham KA, Thomas ML 2000 Perimembrane localization of the estrogen receptor α protein in neuronal processes of cultured hippocampal neurons. *Neuroendocrinology* 71:34–42
34. Drouva SV, Laplante E, Gautron JP, Kordon C 1984 Effects of 17 β -estradiol on LH-RH release from rat mediobasal hypothalamic slices. *Neuroendocrinology* 38:152–157
35. Prevot V, Croix D, Rialas CM, Poulain P, Fricchione GL, Stefano GB, Beauvillain JC 1999 Estradiol coupling to endothelial nitric oxide stimulates gonadotropin-releasing hormone release from rat median eminence via a membrane receptor. *Endocrinology* 140:652–659
36. Kelly MJ, Moss RL, Dudley CA 1977 The effects of microelectrophoretically applied estrogen, cortisol and

- acetylcholine on medial preoptic-septal unit activity throughout the estrous cycle of the female rat. *Exp Brain Res* 30:53–64
37. Lagrange AH, Ronnekleiv OK, Kelly MJ 1995 Estradiol-17 β and μ -opioid peptides rapidly hyperpolarize GnRH neurons: a cellular mechanism of negative feedback? *Endocrinology* 136:2341–2344
 38. Nabekura J, Oomura Y, Minami T, Mizuno Y, Fukuda A 1986 Mechanism of the rapid effect of 17 β -estradiol on medial amygdala neurons. *Science* 233:226–228
 39. Gu Q, Moss RL 1996 17 β -Estradiol potentiates kainate-induced currents via activation of the cAMP cascade. *J Neurosci* 16:3620–3629
 40. Kelly MJ, Wagner EJ 1999 Estrogen modulation of G-protein-coupled receptors. *Trends Endocrinol Metab* 10:369–374
 41. Ramirez VD, Zheng J 1996 Membrane sex-steroid receptors in the brain. *Front Neuroendocrinol* 17:402–439
 42. Levin ER 1999 Cellular functions of the plasma membrane estrogen receptor. *Trends Endocrinol Metab* 10:374–377
 43. Benten WP, Lieberherr M, Giese G, Wunderlich F 1998 Estradiol binding to cell surface raises cytosolic free calcium in T cells. *FEBS Lett* 422:349–353
 44. Gu Q, Moss RL 1998 Novel mechanism for non-genomic action of 17 β -oestradiol on kainate-induced currents in isolated rat CA1 hippocampal neurones. *J Physiol (Lond)* 506:745–754
 45. Gu Q, Korach KS, Moss RL 1999 Rapid action of 17 β -estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors. *Endocrinology* 140:660–666
 46. Shah BH, MacEwan DJ, Milligan G 1995 Gonadotrophin-releasing hormone receptor agonist-mediated down-regulation of G α /G11 α (pertussis toxin-insensitive) G proteins in α T3-1 gonadotroph cells reflects increased G protein turnover but not alterations in mRNA levels. *Proc Natl Acad Sci USA* 92:1886–1890
 47. Krsmanovic LZ, Mores N, Navarro CE, Arora KK, Catt KJ 2003 An agonist-induced switch in G protein coupling of the gonadotropin-releasing hormone receptor regulates pulsatile neuropeptide secretion. *Proc Natl Acad Sci USA* 100:2969–2974
 48. Kelly MJ, Ronnekleiv OK, Ibrahim N, Lagrange AH, Wagner, EJ 2002 Estrogen modulation of K(+) channel activity in hypothalamic neurons involved in the control of the reproductive axis. *Steroids* 67:447–456
 49. Mores N, Krsmanovic LZ, Catt KJ 1996 Activation of LH receptors expressed in GnRH neurons stimulates cyclic AMP production and inhibits pulsatile neuropeptide release. *Endocrinology* 137:5731–5734
 50. Livingston JD, Lerant A, Freeman ME 1998 Ovarian steroids modulate responsiveness to dopamine and expression of G-proteins in lactotropes. *Neuroendocrinology* 68:172–179
 51. Rapp DK, DonCarlos L, Garcia F, Muma NA, Wolf WA, Battaglia G, Van de Kar LD 2000 Estrogen desensitizes 5-HT_{1A} receptors and reduces levels of G₂, G₁₁ and G₁₃ proteins in hypothalamus. *Neuropharmacology* 39:1823–1832
 52. Le Mellay V, Lasmoles F, Lieberherr M 1999 G α (q/11) and g β γ proteins and membrane signaling of calcitriol and estradiol. *J Cell Biochem* 75:138–146
 53. Ram PT, Kiefer T, Silverman M, Song Y, Brown GM, Hill SM 1998 Estrogen receptor transactivation in MCF-7 breast cancer cells by melatonin and growth factors. *Mol Cell Endocrinol* 141:53–64
 54. Filardo EJ, Quinn JA, Bland KI, Frackelton Jr AR 2000 Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* 14:1649–1660
 55. Wyckoff MH, Chambliss KL, Mineo C, Yuhanna IS, Mendelsohn ME, Mumby SM, Shaul PW 2001 Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through G α (i). *J Biol Chem* 276:27071–27076
 56. Benten WP, Stephan C, Lieberherr M, Wunderlich F 2001 Estradiol signaling via sequesterable surface receptors. *Endocrinology* 142:1669–1677
 57. Chambliss KL, Shaul PW 2002 Rapid activation of endothelial NO synthase by estrogen: evidence for a steroid receptor fast-action complex (SRFC) in caveolae. *Steroids* 67:413–419
 58. Smith MS, Freeman ME, Neill JD 1975 The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* 96:219–226
 59. Martinez de la Escalera G, Choi AL, Weiner RI 1992 Generation and synchronization of gonadotropin-releasing hormone (GnRH) pulses: intrinsic properties of the GT1-1 GnRH neuronal cell line. *Proc Natl Acad Sci USA* 89:1852–1855
 60. Wetsel WC, Valenca MM, Merchenthaler I, Liposits Z, Lopez FJ, Weiner RI, Mellon PL, Negro-Vilar A 1992 Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. *Proc Natl Acad Sci USA* 89:4149–4153
 61. Krsmanovic LZ, Stojilkovic SS, Merelli F, Dufour SM, Virmani MA, Catt KJ 1992 Calcium signaling and episodic secretion of gonadotropin-releasing hormone in hypothalamic neurons. *Proc Natl Acad Sci USA* 89:8462–8466
 62. Krsmanovic LZ, Martinez-Fuentes AJ, Arora KK, Mores N, Navarro CE, Chen HC, Stojilkovic SS, Catt KJ 1999 Autocrine regulation of gonadotropin-releasing hormone secretion in cultured hypothalamic neurons. *Endocrinology* 140:1423–1431
 63. White R, Lees JA, Needham M, Ham J, Parker M 1987 Structural organization and expression of the mouse estrogen receptor. *Mol Endocrinol* 1:735–744
 64. Pajunen M, Saviranta P, Jauria P, Karp M, Pettersson K, Mantsala P, Lovgren T 1997 Cloning, sequencing, expression and characterization of three anti-estradiol-17 β Fab fragments. *Biochim Biophys Acta* 1351:192–202
 65. Schott DR, Shyamala G, Schneider W, Parry G 1991 Molecular cloning, sequence analyses, and expression of complementary DNA encoding murine progesterone receptor. *Biochemistry* 30:7014–7020
 66. Zhang JH, Barr VA, Mo Y, Rojkova AM, Liu S, Simonds WF 2001 Nuclear localization of G protein β 5 and regulator of G protein signaling 7 in neurons and brain. *J Biol Chem* 276:10284–10289
 67. Greene GL, Sobel NB, King WJ, Jensen EV 1984 Immunohistochemical studies of estrogen receptors. *J Steroid Biochem* 20:51–56
 68. Urban RJ, Johnson ML, Veldhuis JD 1989 *In vivo* biological validation and biophysical modeling of the sensitivity and positive accuracy of endocrine peak detection. I. The LH pulse signal. *Endocrinology* 124:2541–2547
 69. Fujita K, Aguilera G, Catt KJ 1979 The role of cyclic AMP in aldosterone production by isolated zona glomerulosa cells. *J Biol Chem* 254:8567–8574