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Membrane-initiated Estrogen Signaling in Hypothalamic Neurons

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Summary

It is well known that many of the actions of 17 β -estradiol (E2) in the central nervous system are mediated via intracellular receptor/transcription factors that interact with steroid response elements on target genes. However, there is compelling evidence for membrane steroid receptors for estrogen in hypothalamic and other brain neurons. But it is not well understood how estrogen signals via membrane receptors, and how these signals impact not only membrane excitability but also gene transcription in neurons. Indeed, it has been known for sometime that E2 can rapidly alter neuronal activity within seconds, indicating that some cellular effects can occur via membrane delimited events. In addition, E2 can affect second messenger systems including calcium mobilization and a plethora of kinases to alter cell signaling. Therefore, this review will consider our current knowledge of rapid membrane-initiated and intracellular signaling by E2 in the hypothalamus, the nature of receptors involved and how they contribute to homeostatic functions.

Keywords

ER α ; ER β ; GPCR (G protein-coupled receptor); mER (membrane estrogen receptor that is a GPCR)

Electrophysiological studies in the 1960's and 1970's suggested that gonadal steroids could directly modulate the electrical activity of hypothalamic neurons (Barraclough and Cross, 1963, Lincoln, 1967, Lincoln and Cross, 1967, Moss and Law, 1971, Cross and Dyer, 1970, Dyer et al., 1972, Yagi and Sawaki, 1971, Bueno and Pfaff, 1976, Yagi, 1970, Kubo et al., 1975, Yagi, 1973, Whitehead and Ruf, 1974, Dufy et al., 1976). However, it was microelectrophoresis studies that showed that 17 β -estradiol (E2) could rapidly alter neuronal firing activity of hypothalamic neurons within seconds (Kelly et al., 1976, Kelly et al., 1977a, Kelly et al., 1977b, Kelly et al., 1978b, Kelly et al., 1978a). The E2 -induced inhibition of spontaneous activity was stereospecific (17 α -estradiol had no effect) and occurred at low nanomolar concentrations. Extracellular recording of action potentials with application of steroids via microelectrophoresis could not pinpoint the specific target cell of steroid actions to alter electrical activity, but it clearly established that steroids could have rapid actions (within

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seconds) to alter electrophysiological activity similar to classical neurotransmitters (Kelly et al., 1977a). Using intracellular recording techniques, it was found that fast perfusion of estrogen (100 pM) hyperpolarized arcuate neurons within seconds in a hypothalamic slice preparation and that this effect was reversed within minutes of washing out estrogen (Kelly et al., 1980). Similar actions of E2 were also demonstrated in hypothalamic ventromedial nucleus (VMH) and amygdala neurons, even in the presence of protein synthesis inhibitors (Nabekura et al., 1986, Minami et al., 1990). Later, it was found that GnRH neurons, identified *post hoc* using a combination of intracellular dye labeling and immunocytochemical staining, were a target of estrogen actions (Kelly et al., 1984). In these hypothalamic neurons, estrogen rapidly induced hyperpolarization via the opening of K⁺ channels (Kelly et al., 1984).

The rapid electrophysiological effects of E2 suggested that there may be a membrane binding site for E2 and indeed about the same time (Pietras and Szego, 1977, Pietras and Szego, 1979) identified binding sites for E2 on endometrial cells. Several years later relatively high affinity, specific binding of [³H]-17 β -estradiol was demonstrated in synaptosomal membranes prepared from the adult rat brain (Towle and Sze, 1983). These findings were later corroborated using the membrane impermeant 17 β -estradiol-6-[¹²⁵I]-BSA (Zheng and Ramirez, 1997). Furthermore, competition-binding assays of synaptosomal membranes showed that the hypothalamus exhibited a relatively high affinity (3 nM) binding site for E2 and somewhat lower affinity binding sites in the olfactory bulb and cerebellum (Ramirez et al., 1996, Ramirez and Zheng, 1996). The stereospecificity of the binding was demonstrated by displacement of the radiolabeled E2 with cold E2 or E2-BSA, but not by 17 α -estradiol or 17 α -estradiol-BSA even at micromolar concentrations (Ramirez et al., 1996). These biochemical data complemented the electrophysiological findings that gonadal steroid signaling could be initiated at the membrane.

Nuclear-initiated signaling of E2

Estrogen receptors regulate cellular function through at least two signaling pathways previously broadly classified as “genomic” versus “nongenomic” (McEwen and Alves, 1999, Björnström and Sjöberg, 2005). However, recently the FASEB steroid signaling work group suggested that “membrane-initiated steroid signaling” and “nuclear-initiated steroid signaling” are more appropriate terminologies (Hammes and Levin, 2007). The nuclear-effects on a variety of tissues that involves gene stimulation as well as gene repression (Herbison, 1998, Couse and Korach, 1999, Nilsson et al., 2001, Etgen et al., 2001, Stossi et al., 2006, Kininis et al., 2007). In general, this “classical” signaling pathway of estrogen involves steroid-dependent formation of nuclear estrogen receptor homo- or heterodimers and the subsequent binding of this complex with a unique DNA sequence known as an estrogen response element (ERE), in E2-responsive gene promoters (O’Malley and Tsai, 1992, Muramatsu and Inoue, 2000, Gruber et al., 2004). The inactive ER exists in a complex of several proteins that disassociate upon ligand binding, which transforms the receptor to an active state (Couse and Korach, 1999, Gruber et al., 2004). More specifically, recruitment of other nuclear co-activator and co-regulatory proteins and interactions with the transcription machinery results in transactivation of genes that contain EREs (Muramatsu and Inoue, 2000, Gruber et al., 2004).

Several genes in the brain that are clearly estrogen-responsive do not appear to contain ERE sequences (Malyala et al., 2004, Gruber et al., 2004). There is compelling evidence that ER α and ER β can regulate transcription of some of these “estrogen-responsive” genes by interacting with other DNA-bound transcription factors, such as specificity protein-1 (SP-1) and activator protein 1 (AP-1), rather than binding directly to DNA (Paech et al., 1997, Jacobson et al., 2003, Gruber et al., 2004). For example, the ligand-induced responses with ER β , in contrast to ER α , at an AP-1 site illustrates the negative transcriptional regulation by estrogen and strong

positive regulation by antiestrogens like ICI 164,384 (Paech et al., 1997). This provides a mechanism for differential regulation of gene expression by estrogen.

Membrane-initiated signaling of E2

It has been known for a number of years that estrogen has acute, membrane-initiated signaling actions in the brain (for review see (Kelly and Rønnekleiv, 2002, Rønnekleiv and Kelly, 2005, Bryant et al., 2006)). The nature and significance of these actions have been a matter of dispute. However, it is now widely accepted that some of the actions of estrogen are quite rapid and cannot be attributed to the classical nuclear-initiated steroid signaling of ER α or ER β . One view is that both nuclear and plasma membrane-associated ERs might be products of the same genes (Razandi et al., 1999, Boulware et al., 2005, Pedram et al., 2006, Szegő et al., 2006, Dewing et al., 2007). This belief stems primarily from the fact that many of the rapid effects of E2 can be induced by selective ER α or ER β ligands, antagonized by the ER antagonist, ICI 182,780, or are lost in animals bearing mutations in ER α and/or ER β genes (Couse and Korach, 1999, Singer et al., 1999, Dubal et al., 2001, Wade et al., 2001, Abraham et al., 2003, Boulware et al., 2005, Boulware et al., 2007). Another view is that estrogen activates a unique membrane ER (mER) (Gu et al., 1999, Toran-Allerand, 2004, Toran-Allerand, 2005, Qiu et al., 2003, Qiu et al., 2006b).

In hippocampal slices E2 enhances N-methyl-D-aspartate (NMDA)-mediated excitatory postsynaptic potentials (EPSPs) and long-term potentiation (LTP) following Schaffer (collateral) fiber stimulation (Foy et al., 1999). Also, E2 potentiates non-NMDA (kainate)-mediated excitation of hippocampal CA1 pyramidal neurons via activation of a cAMP/PKA pathway (Gu and Moss, 1996, Gu and Moss, 1998). Based on the findings from the hippocampal slice, it was hypothesized that E2 activates a Gs-coupled receptor on the extracellular surface of hippocampal neurons, which operates in concert with an internal action of E2 on cAMP-dependent phosphorylation (Gu and Moss, 1998). Importantly, these rapid actions of E2 on kainate-induced currents in hippocampal neurons are suggesting a novel mechanism (receptor) for the rapid actions of E2 in the hippocampus (Gu et al., 1999). In addition E2 and E2-BSA, when applied acutely to the hippocampus in ovariectomized animals, produce a sustained reduction of the slow I_{AHP} in CA1 pyramidal neurons (Carrer et al., 2003). The slow I_{AHP} is mediated by a Ca²⁺-activated K⁺ conductance. This provides further evidence for the involvement of a membrane ER, although, the mechanism by which E2 regulates Ca²⁺ influx into CA1 neurons is currently unknown. More recent studies in hippocampal CA3-CA1 neuronal cultures have revealed that E2 rapidly stimulates MAPK-dependent cAMP-responsive element binding protein (CREB) phosphorylation. In addition, E2 also decreases L-type calcium channel-mediated CREB phosphorylation, and therefore, has both positive and negative influences on CREB activity (Boulware et al., 2005). Both effects are mimicked by the membrane-impermeable E2-BSA and are inhibited by the antiestrogen ICI 182,780, which collectively suggest that a membrane estrogen receptor is involved with some of the characteristics described by Qiu and coworkers (Qiu et al., 2003). The positive modulation of CREB activity occurs via ER interactions with metabotropic glutamate receptors 1 (mGluR 1) and the negative modulation via mGluR 2/3 (Figure 1A). The type of interaction, however, is currently unknown, but potentially could be similar to that described for mER and 5HT_{2C} receptors which have diverse but convergent signaling pathways in arcuate neurons (Qiu et al., 2007). A similar scenario has been hypothesized for the rapid E2-induced activation of μ -opioid receptors in the medial preoptic area associated with female sex behavior (Dewing et al., 2007). Based on responses to the ER agonists, propylpyrazoletriol (PPT) and diarylpropionitrile (DPN), which are selective for ER α and ER β , respectively, and transfection studies with mutant ER α (Harrington et al., 2003, Lund et al., 2006, Boulware et al., 2007), the membrane-localized receptors in the hippocampus are hypothesized to be ER β and ER β (Boulware et al., 2005, Boulware et al., 2007). However further studies, such as in ER α and/

or ER β knockout mice, are needed to more clearly define these hippocampal (and hypothalamic) ERs (mERs), and whether they are indeed ER α and/or ER β associated with the plasma membrane.

Potential candidates for novel membrane ERs include ER-X and the G-protein-coupled receptors, Gq-mER and GPR30 (Toran-Allerand, 2005, Qiu et al., 2003, Revankar et al., 2005, Qiu et al., 2006b, Filardo and Thomas, 2005, Funakoshi et al., 2006). ER-X is a plasma membrane-associated, putative ER that is enriched in caveolar-like microdomains of postnatal, but not adult, cortical membranes (Toran-Allerand et al., 1999, Toran-Allerand et al., 2002). In organotypic explants of the developing cerebral cortex, E2 induces tyrosine phosphorylation of both ERK1 (extracellular signal-regulated protein kinase 1) and ERK2, an action very similar to a number of growth factors including nerve growth factor (NGF) (Toran-Allerand, 2005). Interestingly, this E2-induced activation is not antagonized by the antiestrogen, ICI 182,780, and occurs in cortical explants from mice lacking ER α . In addition, this putative mER is particularly responsive to 17 α -estradiol as compared to 17 β -estradiol (Singh et al., 1999, Singh et al., 2000, Toran-Allerand et al., 2002). These and other findings have led to the hypothesis that ER-X is a novel ER that is ICI-insensitive (for review see (Toran-Allerand, 2004, Toran-Allerand, 2005)).

An orphan G-protein-coupled receptor, GPR 30, has also received considerable attention because it binds estrogen, albeit in cancer cells, and exhibits binding and signaling characteristics of a membrane ER (Revankar et al., 2005, Thomas et al., 2005). In breast cancer cells that are transfected with GPR30, estrogen activates the mitogen-activated protein kinases (MAPK), ERK1 and ERK2, and these actions are independent of ER α or ER β (Filardo, 2002, Filardo and Thomas, 2005). According to this model (Figure 1B), E2 activates G $\beta\gamma$ -subunits that promote the release and activation of an epidermal growth factor precursor (proHB-EGF). The active HB-EGF binds to the EGF receptor (ErbB) to facilitate receptor dimerization and downstream activation of ERK (Filardo et al., 2000, Filardo et al., 2002, Filardo, 2002). In addition, E2 can also stimulate adenylyl cyclase activity, which leads to PKA-mediated suppression of EGF-induced ERK1/2 activity. Interestingly, the selective estrogen receptor modulator (SERM) tamoxifen and the antiestrogen ICI 182,780 both promote GPR30-dependent transactivation of the EGF receptor and subsequent MAPK activation. Therefore, the pharmacology of GPR30 as defined in cancer cells is different from that of Gq-mER in neurons (Lagrange et al., 1997, Qiu et al., 2003, Qiu et al., 2006b), suggesting that these are different receptors. In fact, the mER-mediated response to estrogen in arcuate neurons is still present in GPR30 KO mice (Qiu et al., 2008). Although GPR30 has been localized in the brain and estrogen binds to this receptor (Funakoshi et al., 2006, Bologna et al., 2006, Brailoiu et al., 2007, Prossnitz et al., 2007), further studies on GPR30 are necessary to delineate its function in the brain.

Substantial evidence has been generated in the support of a G protein-coupled membrane ER. Intracellular and whole cell recording from guinea pig and mouse hypothalamic slices have been used to characterize the mER (Lagrange et al., 1997, Qiu et al., 2003, Qiu et al., 2006b). These studies show that E2 acts stereospecifically with physiologically-relevant concentration dependence ($EC_{50} = 8$ nM) to cause a significant reduction in the potency of μ -opioid and GABA $_B$ agonists to activate an inwardly rectifying K $^+$ conductance (Lagrange et al., 1997, Qiu et al., 2003). Estrogenic modulation of μ -opioid and GABA $_B$ agonists potency is mimicked either by stimulation of adenylyl cyclase with forskolin or by direct PKA activation with Sp-cAMP, in a concentration-dependent manner (Lagrange et al., 1997, Qiu et al., 2003). Furthermore, the selective protein kinase A (PKA) antagonists KT5720 and Rp-cAMP block the effects of E2. More recent data indicate that PKA is downstream in a signaling cascade that is initiated by a G α_q -coupled membrane ER that is linked to activation of phospholipase C-protein kinase C-protein kinase A (Figure 1C)(Qiu et al., 2003, Qiu et al., 2006b). E2, however,

does not compete for the μ -opioid (or GABA_B) receptor or alter the affinity for the receptor. Importantly, the antiestrogens ICI 164,384 or ICI 182,780 block the actions of E2 with subnanomolar affinity that is similar to ICI's affinity (K_i) for ER α (Weatherill et al., 1988, Lagrange et al., 1997). These pharmacological findings clearly define a G-protein-coupled membrane receptor for estrogen.

The mER is not ER α or ER β , since it can be activated with a diphenylacrylamide compound, STX, that does not bind ER α or ER β (Qiu et al., 2003, Qiu et al., 2006b). STX (and E2) selectively target a G α_q -coupled phospholipase C-protein kinase C-protein kinase A pathway in mediating μ -opioid and GABA_B desensitization of hypothalamic neurons in normal animals (Figure 1C). Importantly, STX (and E2) can also activate the Gq signaling pathway in mice lacking both ER α and ER β (Qiu et al., 2006b). As with ER-X, definitive characterization of this Gq-mER awaits cloning of the gene. However, it is evident that estrogen can rapidly alter cell function through ER α , ER β and/or novel ERs.

It has become clear that there are “indirect” genomic actions of estrogen in the brain that do not require nuclear targeting of estrogen receptors and therefore are classified as “membrane-initiated” signaling of estrogen (for review (Kelly and Levin, 2001, Bryant et al., 2006, Hammes and Levin, 2007)). Such signals that are initiated by E2 at the plasma membrane can trigger intracellular signaling events that result in gene transcription. New gene transcription can result from E2 activation of multiple intracellular kinase cascades including MAPK, phosphoinositide 3-kinase (PI3K), cAMP-protein kinase (PKA) and protein kinase C (PKC) pathways (Watters et al., 1997, Bi et al., 2001, Cato et al., 2002, Yang et al., 2003, Deisseroth et al., 2003). These events are often termed rapid signaling cascades because they are initiated at the plasma membrane or in the cytoplasm and occur much faster than ERE-driven events (Bryant et al., 2005, Björnström and Sjöberg, 2005). For example, E2 binding to its known receptors or a novel membrane ER can upregulate cAMP in hypothalamic neurons by increasing adenylyl cyclase activity (Lagrange et al., 1997). Cyclic-AMP activates PKA, which in turn phosphorylates cAMP-responsive element binding protein (CREB) and elicits new gene transcription (Zhou et al., 1996, Gu et al., 1996, Watters and Dorsa, 1998, Abraham et al., 2004). Therefore, genes with CRE binding sites can be activated within a relatively short time course in neurons independent of estrogen receptors interacting with EREs. These actions of estrogen via pCREB result in activation of genes encoding neurotransmitters such as dopamine, enkephalin, dynorphin and neurotensin, which are all critical for hypothalamic function (Gu et al., 1996, Watters and Dorsa, 1998). Moreover, in hypothalamic (mouse) GnRH neurons the rapid phosphorylation of CREB following E2 treatment is observed in ER α but not in ER β knockout mice indicating a role for ER β in the acute activation by pCREB in GnRH neurons (Abraham et al., 2003).

17 β -estradiol, growth factors and reproduction

Systemic administration of E2 in ovariectomized rats activates IGF-I receptors and induces the association between IGF-I receptors and ER α in the hypothalamus (Quesada and Etgen, 2001, Cardona-Gómez et al., 2002, Mendez et al., 2003). Similar to the effects in cortical neurons, there is an interaction (complex formation) between the p85 subunit of PI3K and ER α within 1-3 h, which leads to activation of Akt (Cardona-Gómez et al., 2002, Mendez et al., 2003). Also, the E2-induced activation of IGF-I receptors augments α 1-adrenergic receptor signaling, which is important for reproductive functions (Quesada and Etgen, 2001). On the other hand, blockade of IGF-I receptors during E2 priming prevents E2-induced increases in α 1-adrenergic receptor binding density as well as IGF-I enhancement of noradrenergic receptor signaling (Quesada and Etgen, 2002). Collectively, these findings support functional interactions between E2 and IGF-I. Therefore, these actions of E2 on the IGF-I receptor

signaling pathway may be a key mechanism by which estrogen affects synaptic remodeling and neuronal plasticity during the estrous cycle.

Of particular interest are the findings that intracerebroventricular (i.c.v.) infusion of JB-1, a selective competitive antagonist of IGF-1 autophosphorylation, inhibits the estrogen-induced LH surge and sexual behavior in ovariectomized rats (Quesada and Etgen, 2002). In addition, co-administration (i.c.v.) of blockers of PI3 kinase (wortmannin) and MAPK (PD98059) inhibit the long-term (48 h) effects of E2 to induce the LH surge and facilitate lordosis behavior (Etgen and Acosta-Martinez, 2003). Therefore, facilitation of female sexual behavior by E2 appears to involve activation of both PI3 kinase and MAPK signal transduction pathways. The importance of growth factors for female sexual behavior is further illustrated by observations that epidermal growth factor (EGF) and also IGF-I can, in the absence of estrogen and progesterone (within 1-4 h of i.c.v. administration), induce mating behavior in rats and mice, in part, through an ER α -dependent mechanism (Apostolakis et al., 2000). This relatively rapid, ligand-independent ER action is in striking contrast to the well established finding that estrogen priming over a period of at least 24 h is needed for progesterone induction of female reproductive behavior (Etgen et al., 2001). The cross-talk between estrogen signaling and membrane-initiated growth factor signaling in the hypothalamus is particularly interesting, although it is currently not well understood. The ability of both IGF-I and estradiol to induce female sexual behavior may involve complex interactions between ER α , the IGF-1 receptor and the PI-3 kinase p85 subunit.

17 β -estradiol and GnRH neurosecretion

Despite having been studied extensively for over 25 years, the mechanism(s) by which estrogen regulates gonadotropin releasing hormone (GnRH) neurons is not well understood. It has been obvious for a number of years that GnRH neurons are modulated by estrogen in a complex manner. For example, loss of estrogen by ovariectomy disrupts GnRH regulation of pituitary LH secretion and results in elevated levels of plasma LH. This effect is due to the loss of negative feedback actions of E2. However, both negative and positive (induction of the LH surge) feedback regulation of LH (GnRH) secretion can be restored by replacement with E2. The positive feedback is believed to be by an action of E2 in the anteroventral periventricular (AVPV) nucleus (Herbison, 1998, Han et al., 2005, Smith et al., 2006, Wintermantel et al., 2006). Neurons in the AVPV express kisspeptin, a neuropeptide encoded by the Kiss gene, GABA and opioid peptides, all of which are important for regulation of GnRH neurosecretion (Simerly et al., 1988, Wagner et al., 2001a, Jackson and Kuehl, 2002, DeFazio et al., 2002, Smith et al., 2006, Christian and Moenter, 2007). The AVPV area expresses high levels of ER α and also ER β , and the actions of the gonadal steroids are mediated, in part, via the nuclear-initiated signaling (genomic) mechanism (Shughrue et al., 1997, Wintermantel et al., 2006). However, the AVPV is also sensitive to the rapid actions of gonadal steroids. For example, E2 within 30 min increases the expression of the pCREB in the AVPV (Gu et al., 1996). At the cellular level, individual AVPV neurons including GABA neurons, respond to both β -adrenergic and α -adrenergic input by a reduction in the median afterhyperpolarization current (mI_{AHP}), which increases the action potential firing in these neurons (Wagner et al., 2001b). Moreover, the α_1 -adrenergic, but not β -adrenergic inhibition of the mI_{AHP} is potentiated after acute (15-20 min) exposure to estrogen, which further increases neuronal excitability (Wagner et al., 2001b). The estrogen-induced enhancement of the coupling of the α_1 -adrenergic receptors to their effector calcium-activated K⁺ (SK) channels (underlying the mI_{AHP}) is initiated within 15 min *in vitro* and lasts for at least 24 h following systemic steroid administration, suggesting both rapid and sustained effects (Wagner et al., 2001b). Since SK channels are critical for modulating neuronal firing rate and pattern (Stocker et al., 1999, Sah and Davies, 2000), estrogen-induced modulation of these channels would have significant functional consequences for AVPV neurons and their targets.

Because estrogen receptor α (ER α), the first cloned receptor/transcription factor for E2, has not been localized to native GnRH neurons, the predominant view has been that estrogen affects GnRH neurons through pre-synaptic mechanisms. However, GnRH neurons are rapidly hyperpolarized by estrogen, an effect that inhibits the activity of GnRH neurons in the presence of tetrodotoxin (TTX) which blocks fast Na⁺ channel activity and thus prevents synaptic inputs (Kelly et al., 1984, Condon et al., 1989, Lagrange et al., 1995). These findings, therefore, suggest a direct hyperpolarizing action of estrogen on GnRH neurons via a G $\alpha_{i,o}$ -coupled receptor. Indeed, in GT1-7 cells, an immortalized GnRH neuronal cell line, estrogen inhibits adenylyl cyclase activity (cAMP production) via a pertussis toxin (G $\alpha_{i,o}$ coupling) mechanism (Navarro et al., 2003). The electrophysiological effects are much too rapid to involve transcription through classical ERs, but an estrogen - G $\alpha_{i,o}$ -coupled receptor has not been identified. Interestingly, E2 increases the firing in primate nasal placode GnRH neuronal cultures within 10 min (Abe and Terasawa, 2005). Therefore, E2 may have both direct inhibitory and indirect excitatory effects on GnRH neurons, since the latter experiments were done in the absence of blockade of synaptic input.

An important milestone for understanding estrogen action in GnRH neurons was the discovery of a second ER, ER β , in 1996 and the documentation that this receptor was expressed in GnRH neurons (Kuiper et al., 1996, Hrabovszky et al., 2000, Hrabovszky et al., 2001, Kallo et al., 2001, Herbison and Pape, 2001). The latter findings combined with recent technological advances, such as the development of ER mutants and transgenic animals expressing green fluorescent protein (GFP) in GnRH neurons, have greatly facilitated studies to understand the cellular mechanisms by which GnRH neurons are regulated by estrogen (Spergel et al., 1999, Suter et al., 2000, Kato et al., 2003, Han et al., 2005, Abraham et al., 2003, Smith et al., 2006, Wintermantel et al., 2006, Zhang et al., 2007). A series of recent publications show that in mouse hypothalamic neuronal explants and in primate nasal placode cultures, E2 augments synchronous intracellular Ca²⁺ oscillations in GnRH neurons (Temple et al., 2004, Temple and Wray, 2005, Abe et al., 2007). This relatively rapid effect is observed within 10-30 min of E2 application. The calcium oscillations in the presence of TTX are most likely the result of estrogen action directly on GnRH neurons. Furthermore, E2 conjugated to BSA at the C-17 position (E2-17 BSA) or to a large nondegradable poly(amido)amine-dendrimer macromolecule (Harrington et al., 2006) mimics the effects of E2 on intracellular calcium oscillations (Temple et al., 2004, Temple and Wray, 2005, Abe et al., 2007). However in the mouse explant experiments (Temple et al., 2004, Temple and Wray, 2005), the E2 -induced calcium oscillations are blocked by ICI 182,780 suggesting that an ER is involved. In contrast, E2-induced calcium oscillations in the primate placode cultures are not blocked by ICI 182,780, and the antagonist ICI 182,780 alone has no effect suggesting that an ER may not be involved (Abe et al., 2007). The actions of E2-BSA in mouse GnRH neurons are abrogated by pertussis toxin treatment and not by inhibition of gene transcription, supporting a role for a G protein-coupled membrane receptor (Temple et al., 2004, Temple and Wray, 2005). However, *in vivo* studies have provided evidence for ER β in the E2-induced rapid (15 min) induction of pCREB in mouse GnRH neurons (Abraham et al., 2003). Collectively, these findings indicate that there are multiple acute actions of E2 in GnRH neurons, some of which involve ER β .

Interestingly, mice lacking ER β appear to exhibit normal sexual behavior and reproduce successfully with the exception that their litter size is reduced, presumably due to a decline in ovarian function (Krege et al., 1998, Dupont et al., 2000). In contrast, ER α , which is not expressed in GnRH neurons, is essential for both sexual behavior and fertility (Ogawa et al., 1998, Dupont et al., 2000). Therefore, the functional significance of ER β expression in GnRH neurons remains unclear. In fact, how the rapid actions of the steroids are related to the longer term genomic actions are currently not well understood. For example, recent findings have revealed that GnRH neurons express K-ATP channels and the agonist-induced outward (K-ATP channel) current is increased by approximately two-fold in E2-treated animals (Zhang et

al., 2007). The increased activity, which occurs in the presence of TTX and GABA and glutamate blockade, is not the result of increased expression of the channel based on quantitative real-time PCR results. Therefore, it appears that there is an E2-induced change in signaling molecules (kinases) that impinge on the channel, part of which may be nuclear-initiated steroid signaling and/or acute phosphorylation events. Clearly, further studies are needed to elucidate the regulation of GnRH neurons by E2.

Studies are forthcoming on the cross-talk between rapid membrane-initiated and long-term nuclear-initiated steroid actions (Lagrange et al., 1994, Lagrange et al., 1997, Wagner et al., 2001b, Vasudevan et al., 2001, Kow and Pfaff, 2004, Malyala et al., 2005, Qiu et al., 2006b, Qiu et al., 2006a, Roepke et al., 2007). For example, it has been found that both acute effects of E2 and the transcriptional changes alter excitability of hypothalamic neurons (Kelly et al., 2003, Malyala et al., 2005, Qiu et al., 2006a). In addition, the estrogen-induced membrane actions in the ventromedial nucleus (VMH) of the hypothalamus can potentiate its genomic effects on lordosis behavior (Kow and Pfaff, 2004). Moreover, this membrane effect in the VMH appears to be mediated by signaling pathways involving PKC and PKA (Kow and Pfaff, 2004). These findings are particularly intriguing in view of other findings that estrogen activates a novel membrane ER, other than ER α or ER β that is G α_q -coupled to phospholipase C, PKC δ and PKA to alter cell firing (Figure 1C) (Qiu et al., 2003, Qiu et al., 2006b).

Effects of 17 β -estradiol on VMH and Arcuate Neurons: Role in Regulation of Feeding

In addition to its role in the control of reproduction, estrogen is involved in the regulation of appetite, energy expenditure, body weight, adipose tissue deposition and distribution in females (Milewicz et al., 2000, Geary, 2001, Poehlman, 2002). Ovariectomy induces an increase in food intake and decreases ambulatory and wheel running activities in rodents, all of which are reversed with estrogen replacement (Ahdieh and Wade, 1982, Colvin and Sawyer, 1969, Shimomura et al., 1990, Asarian and Geary, 2002). In fact, hypo-estrogenic states are associated with decreased activity and an increase in body weight in (Czaja and Goy, 1975, Butera and Czaja, 1984, Czaja, 1984, McCaffrey and Czaja, 1989, Jones et al., 2000, Asarian and Geary, 2002, Qiu et al., 2006b, Clegg et al., 2006, Clegg et al., 2007). The anorectic effects of estrogen are thought to be mediated through CNS actions based on the findings that direct injections of E2 into the paraventricular nucleus of the hypothalamus (PVH) or arcuate/ventromedial nucleus are effective to reduce food intake, body weight and increase wheel running activity in females (Colvin and Sawyer, 1969, Ahdieh and Wade, 1982, Butera and Czaja, 1984). It is evident that neurons in these hypothalamic nuclei regulate energy homeostasis and are affected by E2. For example, E2 up-regulates the expression of the peptide β -endorphin in proopiomelanocortin (POMC) neurons in ovariectomized female guinea pigs (Thornton et al., 1994, Bethea et al., 1995). Furthermore, there is a decrease in hypothalamic β -endorphin levels in the hypothalamus of postmenopausal women who do not take hormone replacement that correlates with weight gain (Leal et al., 1998). In contrast, E2 and mER agonist STX reverses the ovariectomy-induced increase in arcuate neuropeptide Y (NPY) mRNA expression in rodents (Shimizu et al., 1996, Roepke et al., unpublished observations). Therefore, it appears that the arcuate nucleus and specifically POMC and NPY neurons are a major target for the anorectic actions of estrogen, which underscores their importance in the control of energy homeostasis. Indeed, POMC neurons are critical for the regulation of feeding and are also involved in the rewarding aspects of food intake (Hayward et al., 2002, Appleyard et al., 2003).

Important inhibitory regulators of POMC neuronal activity are the G-protein activated, inwardly-rectifying potassium (GIRK) channels. Both μ -opioid receptor (e.g., by β -endorphin or by selective μ -opioid agonists) or GABA $_B$ receptor activation of these GIRK channels

directly hyperpolarize and thereby inhibit hypothalamic neurons (Loose et al., 1990, Kelly et al., 1992, Lagrange et al., 1994, Lagrange et al., 1995, Lagrange et al., 1996). Brief (<20 min) application of E2 or BSA-E2 *in vitro* causes a four-fold decrease in the potency of μ -opioid receptor agonists and GABA_B receptor agonists to inhibit POMC neurons (Lagrange et al., 1994, Lagrange et al., 1996, Qiu et al., 2003), indicating that a membrane ER (mER) is involved. As stated previously, this mER is distinct from ER α or ER β (Qiu et al., 2003, Qiu et al., 2006b). Therefore, E2 via a mER-pathway rapidly decreases the potency of μ -opioid and GABA_B ligands at their receptors (desensitization), thus increasing POMC neuronal firing and the release of the POMC products, β -endorphin and melanocyte stimulating hormone (MSH).

Interestingly, inwardly rectifying K⁺ channels are also a point of convergence for the effects of leptin and insulin in POMC neurons (Plum et al., 2006). Both leptin and insulin activate PI3Kinase (via insulin receptor substrate) that leads to the metabolism of phosphatidylinositol (4,5) biphosphate (PIP₂) to phosphatidylinositol (3,4,5)-triphosphate (PIP₃) in POMC neurons (Xu et al., 2005). Activation of GIRK channels requires permissive levels of membrane PIP₂ and increased channel activity results from G $\beta\gamma$ -mediated stabilization of PIP₂-GIRK binding (Huang et al., 1998, Zhang et al., 1999). In fact, the regulation of channel activities by G $\beta\gamma$, PIP₂, or phosphorylation occurs on the time scale of a few seconds which can be resolved by electrophysiological techniques (Suh and Hille, 2002). In recent studies from our lab we have found that PI3K plays a critical role in facilitating the rapid membrane response to estrogen, as elucidated by the use of PI3K inhibitors (Malyala et al., 2008). Indeed, we found that PI3K inhibitors, which block the phosphorylation of PIP₂ and thereby increase the levels of PIP₂ in the membrane, augmented GIRK channel activity and attenuated the effects of E2 on the mER signaling pathway.

As proof of principle of the importance of the membrane-initiated estrogen signaling pathway in the control of energy homeostasis, we used STX to selectively target the mER (i.e., *in vivo* treatment), and found that STX, similar to E2, attenuated the weight gain following ovariectomy (Qiu et al., 2006b). Moreover, the fact that both E2 and STX are fully efficacious in activating this signaling pathway in double-estrogen receptor knock-out mice is further proof for the existence of a novel mER that is involved in critical physiological processes such as the control of energy homeostasis.

Cross talk between membrane actions and genome activation

The gonadal steroid E2 participates in numerous functions including reproduction, feeding, neuroprotection and cognition. It has been known for some time that the main actions of E2 is to regulate gene transcription through binding to and activating nuclear receptors that can stimulate or inhibit gene transcription at specific DNA binding sites. However, recently it has become clear that E2 can exert its action through multiple signaling mechanisms including membrane-initiated, cytoplasmic as well as nuclear-initiated steroid signaling (Figure 1). For example, there is evidence that E2 increases the activity of hypothalamic POMC and dopamine neurons through binding to a membrane-localized receptor distinct from ER α or ER β (Qiu et al., 2003, Qiu et al., 2006b). In addition, E2 conjugated to macromolecules can activate calcium pulses in GnRH neurons by a mechanism that appears to be independent of ER β (Temple and Wray, 2005, Abe et al., 2007). There are many examples in the hypothalamus and other brain regions for an E2-induced upregulation of PKC, PKA, PI3K and MAP kinase activity leading to altered neuronal activity, as well as altered behavioral activities. Although the exact identity of the mER is still not known, we are making substantial progress towards a better understanding of the full complement of E2 actions in the brain. Also, we are beginning to understand the interaction between rapid signaling events and changes in gene expression. However, many challenges lie ahead to fully identify the nature of membrane steroid receptors, functional properties and integration (cross-talk) with nuclear steroid receptors.

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Figure 1. Cellular models of membrane-initiated signaling pathways of E2

(A) E2 interacts with ERα localized at the neuronal (plasma) membrane and activates group 1 and group 2 metabotropic glutamate receptor (mGluR1a, mGluR2/3) signaling. Activation of mGluR1a causes Gαq stimulation of phospholipase C (PLC) which leads to MAPK-induced CREB phosphorylation. Activation of mGluR2/3 causes Gαi/o inhibition of adenylyl cyclase (AC) and PKA which reduces the activity of L-type calcium channels and leads to attenuated CREB phosphorylation.

(B) E2 acts via GPR30 to promote EGF-receptor transactivation. This happens through a Gβγ-subunit pathway that promotes intracellular tyrosine kinase (Src)-mediated metalloproteinase (MMP)-dependent cleavage of proheparin-binding EGF (proHB-EGF) and

release of HB-EGF from the cell surface. EGF redeptor transactivation leads to Ras-dependent ERK1/2 activation and presumably CREB phosphorylation. E2 via GPR30 can also stimulate adenylyl cyclase (AC) activity, which leads to PKA-mediated suppression of EGF-induced ERK1/2 activity.

(C) E2 activates a membrane-associated ER (mER) that is $G\alpha_q$ -coupled to activation of phospholipase C that catalyzes the hydrolysis of membrane-bound phosphatidylinositol 4,5-biphosphate (PIP_2) to inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG). Calcium is released from intracellular stores (endoplasmic reticulum) by IP3, and DAG activates protein kinase C δ (PKC δ). Through phosphorylation, adenylyl cyclase (AC) activity is upregulated by PKC δ . The generation of cAMP activates PKA, which can rapidly uncouple GABA $_B$ and μ -opioid (μ) receptors from their effector system through phosphorylation of a downstream effector molecule (e.g., G protein-coupled, inwardly rectifying K $^+$ (GIRK) channel). The mER-mediated modulation of kinase pathways reduces the capacity of neuromodulators such as GABA and β -endorphin (β -End) to inhibit POMC neuronal excitability. mER-mediated activation of PKA can lead to phosphorylation of cAMP-responsive element binding protein (pCREB), which can then alter gene transcription through its interaction with CREs on genes.