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Cross-talk between membrane-initiated and nuclear-initiated oestrogen signaling in the hypothalamus

Troy A. Roepke[1], **Jian Qiu**[1], **Martha A. Bosch**[1], **Oline K. Rønnekleiv**[1,2,3], and **Martin J. Kelly**[1]

[1]Department of Physiology and Pharmacology, Oregon Health & Science University, Portland, OR 97239

[2]Anesthesiology and Perioperative Medicine, Oregon Health & Science University, Portland, OR 97239

[3]Division of Neuroscience, Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR 97006

Abstract

It is increasingly evident that 17β-oestradiol (E2) via a distinct membrane oestrogen receptor (Gq-mER) can rapidly activate kinase pathways to have multiple downstream actions in CNS neurons. We have found that E2 can rapidly reduce the potency of the $GABA_B$ receptor agonist baclofen and mu-opioid receptor agonist DAMGO to activate G protein-coupled, inwardly rectifying K^+ (GIRK) channels in hypothalamic neurons, thereby increasing the excitability (firing activity) of POMC and dopamine neurons. These effects are mimicked by the membrane impermeant E2-BSA and a new ligand (STX) that is selective for the Gq-mER that does not bind to ERα or ERβ. Both E2 and STX are fully efficacious in attenuating the $GABA_B$ response in ERα, ERβ and GPR 30 knockout mice in an ICI 182,780 reversible manner. These findings are further proof that E2 signals through a unique plasma membrane ER. We have characterised the coupling of this Gq-mER to a Gq-mediated activation of phospholipase C leading to the up-regulation of protein kinase Cδ and protein kinase A activity in these neurons, which ultimately alters gene transcription. Finally as proof of principle, we have found that STX, like E2, reduces food intake and body weight gain in ovariectomised females. STX, presumably via the Gq-mER, also regulates gene expression of a number of relevant targets including cation channels and signalling molecules that are critical for regulating (as a prime example) POMC neuronal excitability. Therefore, E2 can activate multiple receptor-mediated pathways to modulate excitability and gene transcription in CNS neurons that are critical for controlling homeostasis and motivated behaviors.

Keywords

hypothalamus; ER; Gq-mER; signaling pathways; gene expression

Introduction

Oestrogen receptors (ER) regulate cellular function through at least two signalling pathways previously broadly classified as “genomic” versus “nongenomic” (1,[2]). Recently, a more appropriate terminology was suggested. Hammes and Levin suggested “nuclear-initiated

Corresponding Author: Martin J. Kelly, Department of Physiology and Pharmacology, L334, Oregon Health & Science University, 3181 S.W. Sam Jackson Park Road, Portland, OR97239-3098, USA. kellym@ohsu.edu; fax 503-494-4352, phone 503-494-5840.

steroid signalling" for genomic signalling pathways, and "membrane-initiated steroid signalling" for rapid, nongenomic pathways (3). Under nuclear-initiated signalling, oestrogen (E2) via ERα and ERβ exerts diverse effects on a variety of tissues that involves gene stimulation as well as gene repression (4–[9]). In general, this "classical" signalling pathway of E2 involves steroid-dependent formation of nuclear oestrogen receptor (ER) homo- or heterodimers and the subsequent binding of this complex with a unique DNA sequence known as an oestrogen response element (ERE), in E2-responsive gene promoters (10–[12]). There is also compelling evidence that ERα and ERβ can regulate transcription of some of these "oestrogen-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 (12–[14]).

However, over thirty years ago rapid electrophysiological effects of E2 were documented (15) and at the same time E2 membrane binding sites on endometrial cells were identified (16,[17]). Subsequently, high affinity binding of [3H]-17β-oestradiol was demonstrated in synaptosomal membranes prepared from the adult rat brain (18). It is now generally accepted that membrane-initiated signalling of E2 in the brain does not require nuclear targeting of ERs (for review (3,[19],[20])). This type of E2 signalling occurs at the plasma membrane to trigger intracellular signalling events that result in gene transcription and alterations in neuronal activity. New gene transcription results from E2 activation of multiple intracellular kinase cascades including mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase (PI3K), cAMP-protein kinase (PKA) and protein kinase C (PKC) pathways (21–[25]). For example, E2 rapidly up-regulates cAMP in hypothalamic neurons by increasing adenylyl cyclase activity (26), which in turn activates PKA. PKA phosphorylates cAMP-responsive element binding protein (CREB) and elicits new gene transcription (27–[30]). Genes with CRE binding sites are activated rapidly in neurones independent of ER interacting with EREs including genes encoding neurotransmitters such as dopamine, enkephalin, dynorphin and neurotensin (28,[29]). In this review, we describe a novel, putative membrane ER that is a GPCR functionally characterised in arcuate POMC neurones and how the classical ER and this novel Gq-mER interact through nuclear-initiated and membrane-initiated signalling pathways. Recent data suggests that oestrogenic gene regulation and channel modulation is a multiple-receptor mediated mechanism, which synergistically controls hypothalamic functions during the ovulatory cycle.

Membrane-initiating signalling of E2 on hypothalamic arcuate neurones

Previous reviews have described the acute, membrane-initiated signalling actions of E2 in the brain through multiple signalling pathways (20,[31],[32]) including G-protein-activated pathways (33). At least some of these rapid actions of E2 cannot be attributed to classical nuclear-initiated steroid signalling of ERα or ERβ. There are several potential candidates for novel membrane ERs including ER-X and two G-protein-coupled receptors, GPR30 and Gq-mER (34–[39]); therefore, it is evident that E2 can rapidly alter cell function through ERα, ERβ and/or novel ERs.

Compelling evidence has been generated in the support of a Gq-linked membrane ER (Gq-mER). Intracellular and whole cell recording from guinea pig and mouse hypothalamic slices have been used to characterise the Gq-mER (26,[35],[37]). In these studies, E2 acts stereospecifically to significantly attenuate the potency of μ-opioid and $GABA_B$ agonists in activating an inwardly rectifying K^+ conductance within physiologically-relevant concentrations (EC_{50} = 8 nM) in arcuate POMC neurones (26,[35]). Oestrogenic 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 (26,[35]). Furthermore, selective antagonists of PKA block the effects of E2. The activation of PKA is downstream in a signalling

cascade that is initiated by the Gq-mER that is linked to activation of phospholipase C (PLC) and protein kinase C (PKC) (35,[37]). Importantly, the anti-oestrogens ICI 164,384 and ICI 182,780 block the actions of E2 with sub-nanomolar affinity that is similar to ICI's affinity (Ki) for ERα (26,[40]). The SERMs, 4-OH tamoxifen and raloxifene all behave like E2 in mediating this response.

We have formulated the signal transduction pathway for the rapid response to E2 in arcuate neurones. The sequence of events in this model are as follows (See Figure 1): (1) E2 binds to a novel transmembrane oestrogen receptor and activates Gαq; (2) activated Gαq in turn activates PLC which hydrolyzes PIP_2 and liberates DAG; (3) free DAG stimulates PKCδ, and PKCδ activates adenylyl cyclase (VII); (4) cAMP levels are elevated and stimulates PKA, which through phosphorylation uncouples the inhibitory $GABA_B$ and μ-opioid receptors from activation of GIRK channels (35). Furthermore, activated PKA will phosphorylate CREB (cAMP response element-binding protein) to initiated gene expression via cAMP response element (CRE) (5) and IP_3 may release calcium through the IP_3 receptor on the endoplasmic reticulum (6). Oestradiol will also bind to nuclear receptors and activate ERE-dependent transcription (7). Other transcriptional pathways that the Gq-mER may activate include the MAPK and calcium-sensitive pathways through PKC and IP_3. Recently, we have characterised the signalling of a non-steroidal compound, STX, that specifically targets the G-protein-coupled signalling pathway in both male and female arcuate neurones. In fact, STX has a greater affinity (~20-fold) for the Gq-mER than E2 (37).

Because many of the rapid effects of E2 can be induced by selective ERα or ERβ ligands, antagonised by the ER antagonist, ICI 182,780, or are lost in animals bearing mutations in ERα and/or ERβ genes (5,[41]–[45]), it has been suggested that the membrane-associated ERs might be derived from the same genes as ERα and ERβ (45–[48]). However, the Gq-mER is not ERα or ERβ, since it is activated by STX, which does not bind to ERα or ERβ (35,[37]) and has no proliferative effects on reproductive organs (37). STX (and E2) also activates the Gαq signalling pathway in mice lacking either ERα, ERβ or both of these nuclear receptors (37). Our findings indicate that the Gq-mER in hypothalamic arcuate (POMC) neurones is distinct from ERα or ERβ. Definitive characterisation of this Gq-mER awaits cloning of the gene.

Toran-Allerand et al. identified a high-affinity, saturable oestrogen receptor, ER-X, that is associated with caveolar-like microdomains in developing neocortical neurones (49). 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) (34). Interestingly, ER-X also has a distinct pharmacology in that 17α-oestradiol is equipotent as E2 in activating the MAPK/ERK pathway (43,[49]). However, 17α-oestradiol has no effect on the $GABA_B$ response (35), nor on the μ-opioid response, which are both coupled to the same family of GIRK channels in hypothalamic neurones (50). Similarly, Gu and Moss (51) found that 17α-oestradiol did not mimic the actions of E2 in the hippocampus to potentiate the glutamate (kainate)-mediated currents in CA1 pyramidal neurones. Therefore, it appears that the Gq-mER that modulates channel activity in neurones via the PKC-PKA pathway is pharmacologically distinct from the receptor (ER-X) that is coupled to activation of ERK1 and ERK2 to promote growth and survival.

The orphan GPCR, GPR30, is a new oestrogen receptor involved in the rapid actions elicited by E2 in peripheral reproductive tissue (36,[52]). In transfected breast cancer cells, E2 activates ERK1 and ERK2 independently of ERα or ERβ (38,[53]). In these cells, E2 activates Gβγ-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 dimerisation and downstream activation of ERK (53–[55]). GPR30 is localised to endoplasmic

reticulum and binds E2 with nanomolar affinity (36). Previously, the expression of GPR30 in the brain including the hypothalamus has been reported (39,[56],[56]–[59]). In recent studies, GPR30 mRNA was detected in the paraventricular nucleus and supraoptic nucleus (59) and in magnocellular optic tract neurones (60). However, the pharmacology of GPR30 in these neurones has not been characterised. Previously, we have established that E2 is fully efficacious in attenuating the $GABA_B$ response in arcuate (POMC) neurones from GPR30 knock-out mice suggesting that GPR30 is not the Gq-mER signalling in hypothalamic POMC neurones (61).

Activation of the Gq-mER in the arcuate nucleus affects energy homeostasis and gene expression

Oestradiol is involved in the regulation of appetite, energy expenditure, body weight, adipose tissue deposition and distribution in females (62–[64]). Ovariectomy induces an increase in food intake which is reversed with E2 replacement (65–[68]). In fact, hypo-oestrogenic states are associated with an increase in body weight in many rodent models (37,[68]–[75]). The anorectic effects of E2 are partially mediated through actions in the hypothalamus because direct injections of E2 into the paraventricular nucleus of the hypothalamus (PVN) or arcuate/ventromedial nucleus are effective in reducing food intake and body weight (65,[66],[70]). Arcuate POMC neurones through direct synaptic contacts modulate the excitability of hypothalamic neurones that regulate energy homeostasis and are also affected by E2 (76). E2 up-regulates the expression of the peptide β-endorphin in proopiomelanocortin (POMC) neurones in ovariectomised female guinea pigs (77,[78]) and increases the expression of this gene in the arcuate nucleus after chronic treatment (79). Because the Gq-mER is found in the anorectic POMC neurones, we have hypothesised that activation of this receptor would have a physiological effect on energy homeostasis. Indeed, the Gq-mER agonist STX attenuates the post-ovariectomy body weight gain in female guinea pigs. At doses similar to other SERMs, STX (2 or 6 mg/kg) lowers the body weight gain after ovariectomy similar to systemic treatment with oestradiol benzoate in a dose-dependent manner (37,[79]). Therefore, there are at least two receptors involved in the control of energy homeostasis, ERα and the Gq-mER (80), although we cannot rule out that some of the effects of the Gq-mER on energy homeostasis involve synergistic actions with ERα.

Oestradiol may also control energy homeostasis by regulating gene expression of neuropeptides, cation channels and/or channel modulators (signalling molecules) via both nuclear-initiated (ERα/β) and membrane-initiated signalling (Gq-mER). The potential cross-talk between rapid, membrane-initiated effects of E2 on signalling pathways (and channels) and oestrogenic gene regulation of neuropeptides, channels and signalling mechanisms ultimately determines neuronal function and activity. One probable mechanism for the control of gene expression by STX through the Gq-mER is the activation of CREB by PKA (Figure 1). Genes containing a CREB response element in their promoter regions are potential targets for STX-induced gene expression. However, recent data also suggests that cAMP can activate ERα and initiate ERE-mediated transcription via ERα associations with CREB and CREB binding protein (81,[82]). Other potential transcriptional mechanisms include MAPK activation via PKC, CaM-CaMKII activation via IP_3-induced calcium release (Figure 1) and PI3K-Akt activation (83). To measure gene regulation via the Gq-mER using STX, we employed custom gene microarray analysis of arcuate tissue from STX-treated female guinea pigs coupled to quantitative real-time PCR. The analysis of gene expression with STX was used to evaluate the membrane-initiated versus nuclear-initiated signalling of E2. Because the Gq-mER activates signalling molecules that may impinge on channel function (PLC, PI3K, PKC and PKA) (35,[83]), this study was focused on the gene regulation of cation channels, signaling molecules and associated genes. As predicted, several relevant genes in the arcuate nucleus were regulated by long-term STX treatment.

Of particular interest in the context of energy homeostasis is the regulation of NPY from these orexigenic neurones. We have recently demonstrated that STX significantly reduces NPY mRNA expression in the arcuate nucleus (79) suggesting that STX exerts some of its anorectic effects by suppressing NPY. While the cellular mechanism is currently unknown, the anorectic effects of STX may be mediated, in part, by channel modulation in POMC neurones and control of gene expression in NPY neurones. However, in immortalised NPY neurones, E2 can suppress NPY gene expression via membrane-mediated PI3K-Akt signalling and via CREB-interactions with ERα at the CRE promoter half-site (84). STX is potentially activating this CREB-CRE mediated suppression of NPY gene expression through the activation of PKA. Another possible indirect effect on NPY activity and gene expression is the increase in $GABA_A$ receptor function, which is inhibitory. GABA neurotransmission relies on appropriate clustering of $GABA_A$ receptors to the post-synaptic neuronal membrane. In particular, $GABA_A$ receptor targeting to the membrane is facilitated by the GEC-1 protein, which connects the receptors to the cytoskeleton and facilitates translocation to the membrane (85). Indeed, long-term E2 and STX treatment both increased the expression of *gec*1, in the arcuate nucleus (79), confirming previous findings that E2 increases *gec*1 mRNA expression (86). An increase in *gec*1 gene expression could potentiate the $GABA_A$-mediated activity in NPY neurones. The indirect modulation of GABA neurotransmission by E2 (and STX) may be an inhibitory oestrogenic mechanism in hypothalamic neurones.

The second indirect mechanism to control neuronal function is the E2-induced gene regulation of cation channels that control neuronal excitability and neurotransmitter release. We have previously shown that E2 induces gene expression of a Ca^{2+} channel ($Ca_v3.1$) subunit in the arcuate nucleus after 24 hr treatment (87) and after long-term E2 treatment (30 day) (79). The E2-induced increase in $Ca_v3.1$ subunit expression has previously been demonstrated to increase the peak T-type Ca^{2+} current by two-fold in arcuate neurones including POMC neurones (87) and increased the magnitude of voltage-dependent calcium currents in unidentified neurones in the ventromedial hypothalamus (88). Furthermore, the increase in $Ca_v3.1$ expression in the arcuate nucleus from E2 treatment does not occur in αERKO mice (89) indicating that the expression is under the control of the nuclear receptor. However, STX also significantly up-regulates the $Ca_v3.1$ subunit in the arcuate nucleus after long-term treatment (79) suggesting that these two receptor-mediated transcriptional effects converge at the $Ca_v3.1$ gene. Since the Gq-mER signalling pathway is found in POMC neurones, one can postulate that the up-regulation after STX-treatment is occurring in these neurones.

Furthermore, the increase in the T-type Ca^{2+} current facilitates the burst firing of neurones and causes an increase in neurotransmitter and secretory protein (α-melanocyte-stimulating hormone) release. (90). However, in order to deinactivate the T-type current, the neurones must be hyperpolarised below their resting membrane potential. An obvious mechanism to generate a hyperpolarised state is to up-regulate the expression of inhibitory ionotropic receptors and/or K^+ channels. One such ionotropic receptor that STX up-regulates is the glycine receptor (91). STX increased the expression of the glycine receptor β subunit, which is the structural subunit for this ligand-gated Cl^- channel. Glycine is a major inhibitory neurotransmitter and initiates a post-synaptic increase in chloride conductance through this receptor (92). K^+ channels also function to hyperpolarise neurones. We have previously reported that E2 treatment will increase the gene expression of K^+ channel subunits that control neuronal excitability in the guinea pig arcuate nucleus (91). E2 treatment increases the mRNA expression of the KCNQ5 channel subunit that underlies the M-current and the inwardly-rectifying K^+ (Kir) channel Kir2.4 subunit. The M-current controls neuronal excitability by constitutively hyperpolarising the cell membrane (93) while the Kir2 family is the prime determinant of the resting membrane potential (94,[95]). Both of these currents are modulated by GPCR that activate the PIP_2-PLC-PKC and PKA signalling pathways (93–[98]). The modulation of K^+ channels by PKA is controlled by docking with the scaffold protein, A-kinase anchoring proteins (AKAP)

(50). In the arcuate nucleus, E2 down-regulated AKAP11 after 24 hr and long-term treatment (79,[91]); however, long-term STX treatment increases the expression of the AKAP11 (79). The regulation of this gene by E2 or STX may affect PKA regulation of numerous K^+ channels including those discussed in this review.

As stated, STX via the Gq-mER potentially activates a host of signalling pathways. Many of these signalling proteins, which are involved in the modulation of channel activity and gene expression, are also transcriptional targets for both the nuclear ER and Gq-mER receptors. For example, genes involved in calcium signalling pathways are regulated by E2 and STX treatment. Oestradiol treatment, both 24 hr and long-term, increases the expression of calmodulin-1 in the arcuate nucleus (79,[91]). STX does not regulate calmodulin but does regulate the calmodulin-dependent kinase, CaM kinase II. CaMK II is a modulator of ion channels (Ca^{2+}, K^+, Na^+) (99) and is required for the Ca^{2+}-sensitive production of long-term potentiation (LTP) in neurones from the hypothalamus (100). The regulation of these calcium signalling molecules may have multiple effects on gene expression, neuronal excitability and synaptic neurotransmitter release.

The PI3K pathway and its associated proteins are also a target for gene regulation by both E2 and STX. Peripheral signals such as leptin and insulin activate phoshatidylinositide 3-kinase (PI3K) via insulin receptor substrate in POMC neurones (101). Furthermore, the rapid attenuation of GIRK channel activity in POMC neurones by E2 involves the activation of PI3K (83). Oestradiol has recently been shown to up-regulate PI3K p85α in the dorsomedial portion of the ventromedial hypothalamic nucleus (adjacent to the arcuate nucleus) after 24 hr treatment but not in the arcuate nucleus. However, E2 up-regulated p55γ expression in the arcuate nucleus (83). Conversely, STX up-regulates the p85α subunit of PI3K and phosphatidylinositol transfer protein β (PITPβ) in the arcuate nucleus. PITPβ transports lipids (phosphatidylinositols) from their site of synthesis (endoplasmic reticulum) to the cellular membrane where they are the preferred substrates for the lipid kinases (PI3K, PI4K, etc.,) (102). Not only is PITPβ activity required for PI3K, this protein is also necessary for PLC-mediated signalling (103). Since PI3K- and PLC-mediated signalling are implicated in the membrane-mediated effects of E2 and other factors that control energy homeostasis in POMC neurones (leptin, insulin, etc.,), any changes in activity or expression of the transfer proteins may be another indirect mechanism for E2 to potentiate the effects of these peripheral signals on energy homeostasis.

Conclusions and Significance

The gonadal steroid E2 participates in numerous functions including reproduction, feeding, neuroprotection and cognition. The classical actions of E2 are to regulate gene transcription through binding to and activating nuclear receptors that stimulate or inhibit gene transcription at specific DNA binding sites. However, recently it has become clear that E2 can exert its action through multiple signalling mechanisms including membrane-initiated steroid signalling. In this review, we have summarised the evidence for E2 activating a novel hypothalamic signal transduction pathway for the control of neuronal excitability and gene transcription that is initiated by a Gq-mER. This novel pathway is in addition to the classical ERE-mediated and non-ERE-mediated transcriptional pathways that have been extensively investigated in central and peripheral tissues. The activation of the Gq-mER provides an additional mechanism for E2 to control gene expression not involving the classical ERs. Activations of the putative hypothalamic Gq-mER via STX is associated not only with new gene transcription but with attenuation in post-ovariectomy body weight gain in a dose-dependent manner. STX is currently being examined in other homeostatic functions and may be an excellent tool to delineate the effects of the Gq-mER on these hypothalamic functions. The Gq-mER clearly is a coupling mechanism between the control of gene transcription and rapid signalling events that together will ultimately determine the effects of E2 on hypothalamic functions. Also, we

are beginning to understand the cross-talk between rapid signalling events and changes in gene expression. However, many challenges lie ahead to fully identify the nature of membrane steroid receptors, functional properties and integration with nuclear steroid receptors.

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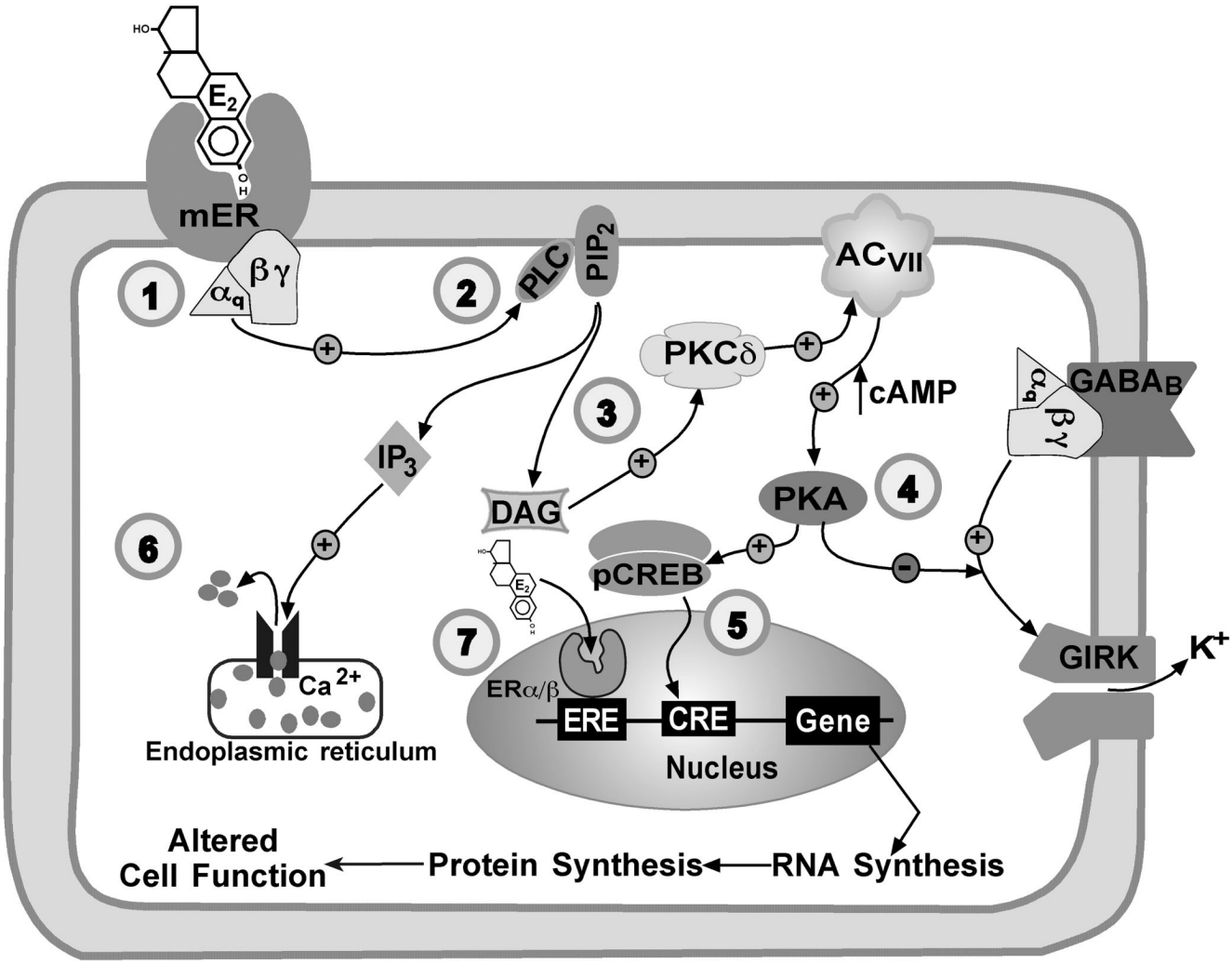

Figure 1. Activation of an oestrogen-responsive G-protein-coupled receptor (GPCR) and the associated signalling pathways in arcuate neurons

(1) The putative Gq-coupled membrane oestrogen receptor (mER) is a G-protein coupled receptor linked to $G\alpha_{q/11}$ protein. Oestrogen binding activates the $G\alpha_{q/11}$ **(2)** which in turn activates (+) phospholipase C (PLC) and initiates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). PLC hydrolyzes PIP_2 into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). **(3)** DAG activates protein kinase Cδ (PKCδ) which activates adenylate cyclase VII (AC VII). **(4)** AC VII increases cAMP production subsequently stimulating protein kinase A (PKA), which through phosphorylation uncouples the inhibitory $GABA_B$ and μ-opioid receptors from activation of G-protein coupled inwardly rectifying K^+ channels (GIRK) channels. **(5)** Activation of PKA will also phosphorylate cAMP-response element binding protein (pCREB) and control gene expression through the cAMP response element (CRE). **(6)** IP_3 produced from the hydrolysis of PIP_2 activates Ca^{2+} release from the endoplasmic reticulum that can activate calcium-dependent signalling. **(7)** Oestradiol will also bind to nuclear receptors and activate oestrogen response element (ERE)-dependent transcription. E_2, 17β-oestradiol.