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Diverse Actions of Estradiol on Anorexigenic and Orexigenic Hypothalamic Arcuate Neurons

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Abstract

There is now compelling evidence for membrane-associated estrogen receptors in hypothalamic neurons that are critical for the hypothalamic control of homeostatic functions. It has been known for some time that estradiol (E2) can rapidly alter hypothalamic neuronal activity within seconds, indicating that some cellular effects can occur via membrane initiated events. However, our understanding of how E2 signals via membrane-associated receptors and how these signals impact physiological functions is only just emerging. Thus, E2 can affect second messenger systems including calcium mobilization and a plethora of kinases to alter cell excitability and even gene transcription in hypothalamic neurons. One population of hypothalamic neurons, the anorexigenic proopiomelanocortin (POMC) neurons, has long been considered to be a target of E2's actions based on gene (Pomc) expression studies. However, we now know that E2 can rapidly alter POMC neuronal activity within seconds and activate several intracellular signaling cascades that ultimately affect gene expression, actions which are critical for maintaining sensitivity to insulin in metabolically stressed states. E2 also affects the orexigenic Neuropeptide Y/Agouti-related Peptide (NPY/AgRP) neurons in similarly rapid but antagonistic manner. Therefore, this review will summarize our current state of knowledge of how E2 signals via rapid membrane-initiated and intracellular signaling cascades in POMC and NPY/AgRP neurons to regulate energy homeostasis.

Keywords

β-endorphin; ERα; ERβ; $GABA_B$ receptor; Gαq-mER; GIRK channels; NPY/AgRP neurons; PKA; PKC; POMC neurons

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Estrogen Neurobiology—Classical Signaling

17β-estradiol (E2) modulates hypothalamic neuronal excitability that ultimately regulates reproduction, energy balance, temperature, circadian rhythms, and stress. In addition, E2 is involved in neuronal synaptic plasticity in the hippocampus, striatum and cerebellum (Grove-Strawser et al., 2010; Hedges et al., 2012; Woolley, 2007). E2 signaling in the hypothalamus is the quintessential function that controls reproduction (Kelly and Ronnekleiv, 2008; Kelly and Rønnekleiv, 2015; Kelly et al., 2013; Micevych and Kelly, 2012; Moenter et al., 2003; Sinchak and Wagner, 2012). In females, E2 signaling in the hypothalamus is the basis of positive and negative feedback within the hypothalamic-pituitary-ovarian axis. The endocrine status of gonads is communicated to the brain by circulating E2 that activates hypothalamic circuits that regulate ovulation. E2 both inhibits and stimulates the release of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH), as well as follicle stimulating hormone (FSH) and stimulates sexual behavior. E2 binds to and activates the classical estrogen receptors ERα and ERβ, but also G protein-coupled metabotropic receptors. We now know that many of these actions of E2 are mediated via its presynaptic effects on Kisspeptin (Kiss1) neurons in the anteroventral periventricular/periventricular nuclei (AVPV/PeN) (Clarkson and Herbison, 2009; Smith et al., 2005; Zhang et al., 2015).

Classically, ERs were defined by their ability to bind estrogens and elicit a specific response (Jensen and DeSombre, 1973). They were initially considered cytosolic receptors that upon E2 binding underwent a conformational change and translocation to the nucleus where they interacted with DNA to regulate the expression of targeted genes. Now it is thought that they are found either in the nucleus or associated with the plasma membrane (Levin, 2009). ERα (*ESR1*) and ERβ (*ESR2*) were cloned in the 1980's and 1990's, respectively (Kuiper et al., 1996; Walter et al., 1985). Although they are the product of different genes, ERα and ERβ share a similar modular structure that binds E2 and have significant sequence homology, especially in their DNA and ligand binding domains. Also, ERα and ERβ interact with other transcription factors, such as Fos and Jun, which bind DNA at the activator protein-1 (AP-1) site, to regulate transcription independent of the unique DNA sequences known as estrogen response elements (EREs) (Kushner et al., 2000; Paech et al., 1997).

Early studies utilizing ^{3}H-17β-estradiol identified binding sites in the brain and revealed that estradiol-concentrating neurons were localized in hypothalamic regions including the preoptic (POA), periventricular (PV) and arcuate nuclei (Pfaff and Keiner, 1973; Sar, 1984; Sar and Stumpf, 1975; Tardy and Pasqualini, 1983; Warembourg, 1977). Once ERα and ERβ were cloned, their distribution was thoroughly elucidated using *in situ* hybridization and/or immunocytochemistry (DonCarlos et al., 1991; Gréco et al., 2001; Gundlah et al., 2000; Kruijver et al., 2002, 2003; Laflamme et al., 1998; Osterlund et al., 2000; Sar and Parikh, 1986; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Simerly et al., 1990). ERα is robustly expressed in regions such as the preoptic area (POA), bed nucleus stria terminalis (BNST), amygdala, periventricular nucleus (PeN), ventrolateral part of the ventromedial nucleus of the hypothalamus (VMH) and the arcuate nucleus. ERβ is found in many of the same regions, but is more highly expressed in the BNST, POA, paraventricular nucleus of the hypothalamus (PVH) and supraoptic nuclei (SON), with some notable species

differences (Kruijver et al., 2003; Laflamme et al., 1998; Mitra et al., 2003; Shughrue et al., 1997; Warembourg and Leroy, 2004). ERα and ERβ are also found in other brain regions including the cortex, hippocampus, midbrain, striatum (Merchenthaler et al., 2004; Shughrue et al., 1997) and in dorsal root ganglion neurons (Chaban and Micevych, 2007). Co-localization studies have identified ERα in hypothalamic neurons containing GABA, neurotensin, somatostatin, galanin, dopamine, norepinephrine, neuropeptide Y (NPY), proopiomelanocortin (POMC) and kisspeptin (Flugge et al., 1986; Herbison, 1994; Herbison and Theodosis, 1992; Horvath et al., 1995; Hu et al., 2006; Laflamme et al., 1998; Lehman and Karsch, 1993; Roepke et al., 2007; Skinner and Herbison, 1997). ERβ is expressed in different populations of hypothalamic neurons: GnRH, vasopressin, oxytocin, and nociceptin/orphanin FQ, as well as in midbrain serotonin neurons (Cardona-Gomez et al., 2000; Gundlah et al., 2001; Herbison et al., 2001; Hrabovszky et al., 1998; Hrabovszky et al., 2004; Hrabovszky et al., 2000; Hrabovszky et al., 2001; Isgor et al., 2003; Kallo et al., 2001; Skynner et al., 1999). ERα and ERβ are co-localized in neurons expressing corticotropin releasing hormone and insulin-like growth factor I (IGF-I), as well as in subpopulations of unidentified hypothalamic neurons (Bao et al., 2005; Cardona-Gomez et al., 2000; Gréco et al., 2001; Shughrue et al., 1998).

The nuclear-initiated signaling of estradiol via ERα and ERβ exerts diverse effects in a number of tissues that involve gene stimulation as well as gene repression (Couse and Korach, 1999; Etgen et al., 2001; Herbison, 1998; Kininis et al., 2007; Nilsson et al., 2001; Stossi et al., 2006). In general, the “classical” signaling pathway of E2 involves steroid-dependent formation of nuclear estrogen receptor homo- or heterodimers and the subsequent binding of this complex to an ERE, in E2-responsive gene promoters and enhancers (Gruber et al., 2004; Muramatsu and Inoue, 2000; O’Malley and Tsai, 1992).

However, there are many genes in the brain that are estrogen-responsive that do not appear to contain ERE sequences (Gruber et al., 2004; Malyala et al., 2004). There is compelling evidence that ERα and ERβ 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 (Gruber et al., 2004; Jacobson et al., 2003; Paech et al., 1997). In contrast to ERα, the ligand-induced responses with ERβ at an AP-1 site illustrate the negative transcriptional regulation by estrogens and strong positive regulation by ER antagonists like ICI 164,384 (Paech et al., 1997). In addition, Kiss1 mRNA is differentially regulated by E2 in the AVPV/PeN and arcuate nucleus. Although the positive E2 regulation of Kiss1 mRNA expression in the AVPV is dependent on an ERE-binding site, the down regulation of Kiss1 mRNA in the arcuate nucleus is via an ERE-independent mechanism (Gottsch et al., 2009). Therefore, there are potentially multiple mechanisms for differential regulation of gene expression by E2 via nuclear-initiated signaling.

Another parallel line of research developed in the 1970’s that implicated E2 in rapid, non-genomic actions in numerous neuronal and non-neuronal cells: E2 membrane signaling rapidly increased levels of cAMP in the uterus (Szego and Davis, 1967), altered firing of hypothalamic neurons within seconds (Kelly et al., 1976) and the release of neuropeptides (Sarkar and Fink, 1980). However, the concept of “rapid” non-genomic effects for estrogen

signaling was foreign to neuroendocrinologists. Although E2 elicited effects on hypothalamic and striatal neurons at subnanomolar concentrations, there did not appear to be identifiable steroid receptors associated with the plasma membrane for mediating these rapid actions (Lagrange et al., 1997; Mermelstein et al., 1996). This changed in the 1990's when membrane localization of ERα was documented in pituitary cells and primary cultures of hippocampal CA1 neurons (Clarke et al., 2000; Pappas et al., 1994). Moreover, Razandi *et al.*, (Razandi et al., 1999) discovered that nuclear and membrane receptors were encoded by the same estrogen receptor genes, and ERα and ERβ were shown to complex with G protein signaling cascades. In addition, several groups identified membrane estrogen receptors (mERs) that were not derived from ERα or ERβ transcripts (Qiu et al., 2003; Qiu et al., 2006; Toran-Allerand et al., 2002) including a *bona fide* G protein-coupled receptor, GPR30/GPER1 (Filardo et al., 2000; Revankar et al., 2005). It was evident from the investigation of "non-genomic" signaling that while some of these signaling cascades initiated at the membrane were tied to rapid membrane effects on ion channel activity, others led to the regulation of gene transcription - similar to the membrane-to-nucleus signaling described for many neurotransmitters (Wu et al., 2001). With this caveat in mind, it has been more accurate to differentiate between membrane-initiated signaling and nuclear-initiated signaling when discussing hormone actions in neurons and non-neural cells (Hammes and Levin, 2007). Therefore, this review will focus on the role of mERs in hypothalamic functions with an emphasis on energy homeostasis, keeping in mind that similar membrane-initialed actions of E2 have been documented in other brain structures such as the hippocampus, striatum and cerebellum, CNS structures involved in cognition and motor functions, respectively (Grove-Strawser et al., 2010; Hedges et al., 2012; Woolley, 2007).

Estrogen Neurobiology—Non-classical signaling

Selective membrane binding sites for E2 were first identified on endometrial cells (Pietras and Szego, 1977; Pietras and Szego, 1979), and later studies revealed relatively high affinity, specific binding of [^{3}H]-17β-estradiol to synaptosomal membranes prepared from the adult rat brain (Towle and Sze, 1983). The binding in the central nervous system (CNS) was later corroborated using the membrane impermeant 17β-estradiol-6-[^{125}I]-conjugated to bovine serum albumin (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 and Zheng, 1996; Ramirez et al., 1996). The stereospecificity of the binding was demonstrated by displacement of the radiolabeled E2 with cold E2 or E2-BSA, but not by 17α-estradiol or 17α-estradiol-BSA even at micromolar concentrations (Ramirez et al., 1996).

In parallel electrophysiological studies E2 was shown to have acute, rapid membrane-initiated signaling actions in many CNS structures including the hypothalamus (Kelly et al., 1976; Kelly et al., 1977a, 1978a, b; Kelly et al., 1977b; Kelly and Rønnekleiv, 2002; Kelly et al., 1984; Micevych and Dominguez, 2009; Qiu et al., 2003; Qiu et al., 2006; Ronnekleiv and Kelly, 2005; Smith et al., 2013). Three decades ago the nature and physiological significance of these actions were a matter of debate, but it is now generally accepted that some of the actions of E2 are much too fast to be attributed to the classical nuclear-initiated

steroid signaling of ERα or ERβ. However, ERα and ERβ can associate with signaling complexes in the plasma membrane—*e.g.*, caveolins (Bondar et al., 2009; Boulware et al., 2005; Dewing et al., 2007; Pedram et al., 2006; Razandi et al., 1999; Szegõ et al., 2006). Caveolin-dependent clustering allows ERα to activate an associated metabotropic glutamate receptor (mGluR) (Boulware et al., 2007), altering the phosphorylation of CREB, with protein kinase C acting as an intermediary (Dewing et al., 2008). In addition, mERs can trigger mitogen-activated protein kinase (MAPK) via mGluR1a and phospholipase C (PLC) or inhibit L-type Ca^{2+} channels through mGluR2/3 and decreased cAMP production (Boulware et al., 2005) (see Kelly and Rønnekleiv, 2008 for review). Finally, many of the rapid effects of E2 can be induced by selective ERα or ERβ ligands, antagonized by the ER antagonist ICI 182,780 and abrogated in animals bearing mutations in ERα and/or ERβ genes (Abraham et al., 2003; Boulware et al., 2007; Boulware et al., 2005; Couse and Korach, 1999; Dubal et al., 2001; Singer et al., 1999; Wade et al., 2001).

It is also evident that E2 can activate *bona fide* G protein-coupled receptors (GPCRs), the most notable being GPR30 and a putative Gαq-coupled membrane ER (Gu et al., 1999; Kenealy et al., 2011; Noel et al., 2009; Qiu et al., 2003; Qiu et al., 2006; Toran-Allerand, 2004; Toran-Allerand, 2005). Over the years, evidence has been generated in the support of a novel Gαq-coupled membrane ER (Gαq-mER). Intracellular sharp electrode and whole cell patch recording from guinea pig and mouse hypothalamic slices were used to characterize this Gαq-mER (Lagrange et al., 1997; Qiu et al., 2003; Qiu et al., 2006; Smith et al., 2013). These two independent electrophysiological methods established that E2 acts rapidly and stereospecifically within physiologically-relevant concentrations to significantly reduce the potency of μ-opioid and $GABA_B$ agonists (*i.e.*, heterologous desensitization) to activate G protein-coupled inwardly rectifying K^+ (GIRK) channels (Lagrange et al., 1997; Qiu et al., 2003). Estrogenic desensitization of μ-opioid and $GABA_B$ receptors was mimicked by stimulation of adenylyl cyclase with forskolin or by direct protein kinase A (PKA) activation with the non-hydrolyzable cAMP analog Sp-cAMP, in a concentration-dependent manner (Lagrange et al., 1997; Qiu et al., 2003). Furthermore, the selective PKA antagonists KT5720 and Rp-cAMP blocked the effects of E2. As predicted from the literature on desensitization of GPCRs (Gainetdinov et al., 2004), PKA is downstream in a signaling cascade that is initiated by a Gαq-coupled mER that is linked to activation of phospholipase C (PLC)-protein kinase C (PKC)-protein kinase A (PKA) (Qiu et al., 2003; Qiu et al., 2006). It should be emphasized that E2 does not alter the affinity of the μ-opioid and $GABA_B$ ligands for their respective receptors (Cunningham et al., 1998). In addition, E2 uncouples opioid receptor-like 1 (Mela et al., 2016) and cannabinoid receptor one (Conde et al., 2016) from their respective effector systems in POMC neurons, more specifically A-type K^+ channels and Ca^{2+}-activated K^+ channels. Presynaptic to POMC neurons, E2 rapidly attenuates the ability of cannabinoid signaling to reduce glutamate release (Jeffery et al., 2011; Washburn et al., 2013). The actions of E2 on metabotropic receptors is not restricted to negative modulation as the activity of 5HT2C (Gq-coupled) receptor agonists are augmented by E2 in POMC neurons thereby augmenting the anorexigenic activity of serotonin drugs (Qiu et al., 2007). Therefore, E2 attenuates Gi,o-coupled receptor signaling but augments Gq-coupled receptor signaling in POMC neurons. Furthermore, the ER antagonists ICI 164,384 and ICI 182,780 block the actions of E2 with subnanomolar affinity

(K_i = 0.5 nM), which is similar to K_i for antagonism of ERα (Lagrange et al., 1997; Weatherill et al., 1988). These pharmacological findings clearly argued for a G protein-coupled membrane receptor with high selectivity for E2.

The results from these early physiological and pharmacological experiments led to the design of STX, which is structurally similar to 4-OH tamoxifen, for selectively targeting the Gαq-mER signaling pathway (Qiu et al., 2003). As predicted, STX has greater affinity (~20-fold) for the Gαq-mER than E2, and most importantly, does not bind to ERα or ERβ (Qiu et al., 2006; Tobias et al., 2006). Furthermore, both STX and E2 activate the Gαq signaling pathway in POMC neurons in mice lacking both ERα and ERβ and in GPR30-knockout mice (Qiu et al., 2006; Qiu et al., 2008). More recent studies indicate that STX can rapidly increase neurotransmitter release from POMC neurons onto NPY/AgRP neurons, which is indicative of its presynaptic actions to inhibit the inhibitory $GABA_B$ receptor in POMC nerve terminals (Figure 1) (Stincic et al., 2017). These actions would further enhance the anorexigenic actions of E2 in the POMC-NPY/AgRP circuitry. The ability of STX to robustly mimic the rapid effects of E2 on POMC neuronal activity led to the hypothesis that the putative Gαq-mER has a role in the control of energy homeostasis. Indeed, peripheral administration of STX is found to mimic the effects of E2 in controlling energy homeostasis (Qiu et al., 2006; Roepke et al., 2010; Roepke et al., 2008). Both E2 and STX reduce food intake, body weight gain and abdominal fat accumulation following ovariectomy.

Estradiol Signaling in POMC and NPY/AgRP neurons

Energy Homeostasis

Hypothalamic POMC and neuropeptide Y/agouti-related peptide (NPY/AgRP) neurons reside within the arcuate nucleus and compose a critical circuit for regulating energy homeostasis (Gao and Horvath, 2007). Selective optogenetic stimulation of NPY/AgRP neurons evokes intense feeding (Aponte et al., 2011), and ablation of these neurons in adults causes starvation (Arroyo et al., 2006; Luquet et al., 2005; Wu et al., 2009). Similar genetic approaches have revealed that POMC cells have the exact opposite actions in the control of energy homeostasis (Aponte et al., 2011; Gao et al., 2007; Kavalali et al., 2011; Qiu et al., 2006; Shi et al., 2010; Xu et al., 2011). Moreover, these two populations of neurons regulate feeding through sensing circulating levels of metabolic hormones, thereby altering their firing frequency and the release of peptide and/or amino acid neurotransmitters onto target neurons in the paraventricular nucleus and other hypothalamic nuclei (Gao and Horvath, 2007).

POMC and NPY/AgRP neurons are also sensitive to circulating estrogens. Experiments dating back 30-40 years determined that the anorectic effects of E2 in rodents are mediated through CNS sites of action since direct injections of E2 into the arcuate/ventromedial nucleus were effective to reduce food intake, body weight and increase wheel running activity in females (Ahdieh and Wade, 1982; Butera and Czaja, 1984; Colvin and Sawyer, 1969). Moreover, E2 increases the expression of the peptide β-endorphin in POMC neurons in ovariectomized female guinea pigs (Bethea et al., 1995; Thornton et al., 1994), and in postmortem studies there is a decrease in hypothalamic β-endorphin expression associated with weight gain in postmenopausal women who abstained from hormone replacement

therapy (Leal et al., 1998). More recently it has been shown that E2 signaling via ERα is a critical component in the regulation of energy homeostasis (Geary et al., 2001). In rodents, hypo-estrogenic states are clearly associated with decreased activity and an increase in body weight (Asarian and Geary, 2002; Butera and Czaja, 1984; Clegg et al., 2006; Clegg et al., 2007; Czaja, 1984; Czaja and Goy, 1975; Jones et al., 2000; McCaffrey and Czaja, 1989; Qiu et al., 2006). In humans a loss-of-function mutation in ERα, reported in a case history from one male individual, resulted in a clear metabolic phenotype with expression of type 2 diabetes, hyperinsulinemia and obesity (Smith et al., 1994). However, global reinstatement of an ERα that is lacking the ERE targeting domain is sufficient for "rescuing" the metabolic deficits in mice (Park et al., 2011). These findings suggest an important role for non-ERE mediated E2 signaling, albeit this could be via other transcriptional activity (Hewitt et al., 2014) or membrane-initiated signaling of ERα as in NPY/AgRP neurons (see discussion below). Indeed, a point mutation in ERα (C451A), which precludes palmitoylation and membrane trafficking of ERα globally, creates an obese phenotype with excessive visceral fat deposition in mice fed a normal chow diet (Pedram et al., 2014). Moreover, brain-specific knockout of ERα causes hyperphagia and hypometabolism (Musatov et al., 2007; Xu et al., 2011), and selective knockdown of ERα in POMC neurons recapitulates the hyperphagic phenotype in female mice (Xu et al., 2011). However, one must be cautious in interpreting global and conditional ERα gene deletion experiments since ERα is a transcription factor affecting the expression of hundreds of genes important for cell signaling, and many of these genes are essential for membrane initiated actions of E2 that contribute to POMC excitability and hence control of energy homeostasis (Malyala et al., 2004). With this caveat in mind, it appears that the arcuate nucleus and specifically POMC neurons are major targets for the anorectic actions of estrogens via ERα signaling, which underscores their importance in the control of energy homeostasis. In addition, POMC neurons are also involved in the rewarding aspects of food ingestion (Appleyard et al., 2003; Hayward and Low, 2007; Hayward et al., 2002).

There is also a complementary Gαq-mER-mediated anorexigenic pathway in NPY/AgRP neurons. E2 and the Gαq-mER ligand STX rapidly enhance (sensitize) the $GABA_B$ receptor agonist baclofen activation of GIRK channels, and this effect is blocked by the estrogen receptor antagonist ICI 182,780 (Smith et al., 2013). On the other hand, an ERα-mediated signaling pathway exists that opposes the Gαq-mER signaling cascade: activating ERα with the selective agonist propyl pyrazole triol (PPT) rapidly suppresses $GABA_B$ mediated activation of GIRK channels in NPY/AgRP neurons (Smith et al., 2013). In gonadectomized mice, the "non-selective" ligand E2 can either enhance or suppress $GABA_B$-mediated currents (Smith et al., 2013). However, co-administering phosphatidylinositol 3 Kinase (PI3K) inhibitors, specifically a selective inhibitor of p110β, results in E2 enhancing the $GABA_B$ receptor-mediated response similar to effects of STX. Thus, ERα via PI3K attenuates (desensitizes) the $GABA_B$ receptor–mediated response in NPY/AgRP neurons. Physiologically, the effects of E2 could depend on the relative expression of ERα versus Gαq-mER in these orexigenic cells. NPY/AgRP neurons may serve a metabolic function when Gαq-mER expression predominates, whereas they may serve a reproductive function when more ERα is expressed (Figure 2) (see (Acosta-Martinez et al., 2006) for review).

Systemic treatment with STX, similar to E2, regulates gene transcription in the arcuate nucleus, and many of the genes are involved in the control of neuronal excitability (*e.g.*, the T-type calcium channel transcript Cav3.1) and intracellular signaling cascades in arcuate neurons (Roepke et al., 2008). For example, the PI3K regulatory subunits are regulated by E2 and STX: PI3K p55γ mRNA is increased by E2 treatment (Malyala et al., 2008) and PI3K p85α mRNA is upregulated by STX (Roepke et al., 2008). Therefore, the putative Gαq-mER may also function in the estrogenic control of energy homeostasis through direct excitation of POMC and direct inhibition of NPY/AgRP neurons through a Gαq signaling cascade to alter gene transcription in these anorexigenic and orexigenic neurons, respectively (Figure 2). Indeed, the electrophysiological effects of STX in NPY/AgRP neurons are consistent with the finding that STX down-regulates arcuate NPY mRNA expression in ovariectomized female guinea pigs (Roepke et al., 2008).

Circulating levels of E2 are in the low to high pM range, and these actions of E2 to rapidly alter POMC and NPY/AgRP neuronal activity are in the high picomolar range (K_i of ICI 164,384 = 0.5 nM (Lagrange et al., 1997)). Also, there is evidence that E2 is locally synthesized and released (>1 μg/ml) from the mediobasal hypothalamus of rhesus macaques (Kenealy et al., 2013; Kenealy et al., 2017). Kennealy et al. (Kenealy et al., 2017) suggest that there is an obligatory role for “neuroestradiol” in the estrogen-induced LH surge in the rhesus macaque. Neuroestrogen production has also been measured in the hippocampus, cerebellum and brainstem (see (Terasawa and Kenealy, 2012) for review). Therefore, not only ovarian estrogens but locally produced E2 could activate the Gαq-mER signaling cascade to provide continued excitation of POMC neurons and inhibition of NPY/AgRP neurons. However, food intake is depressed during the preovulatory phase of the menstrual cycle in humans, monkeys and guinea pigs that correlates with peak levels of circulating E2 (see (Dye and Blundell, 1997) for review); and ovariectomy (or menopause) often leads to increased food intake and weight gain, which argues for a substantial role of ovarian E2 feedback in the hypothalamic control of energy homeostasis.

POMC neurons and insulin resistance

The pleiotropic effects of insulin (and leptin) in POMC neurons are vital for both the short term (excitability) and long term (transcriptional) modulation of POMC neuronal activity and the control of food intake and energy homeostasis. POMC and NPY/AgRP neurons are major CNS targets of insulin and leptin actions (Belgardt and Bruning, 2010; Morton et al., 2006; Qiu et al., 2014; Schwartz et al., 2000). Insulin delivered directly into the third ventricle decreases food intake in guinea pigs (Qiu et al., 2014), mice (Benoit et al., 2002; Brown et al., 2006) and rats (Clegg et al., 2011). Insulin depolarizes POMC neurons in both males and females via activation of canonical transient receptor potential (TRPC5) channels, and hyperpolarizes NPY/AgRP neurons via activation of K_{ATP} channels (Qiu et al., 2014), activity that is congruent with the anorexigenic effects of insulin. The increase in POMC cell excitability induced by insulin translates into heightened transcriptional activity—*i.e.*, an increase in Fos expression in the arcuate nucleus and specifically in POMC neurons following *icv* insulin (Qiu et al., 2014).

In POMC neurons the insulin receptor (InsR) couples to PI3K p110β activation (Al-Qassab et al., 2009; Xu et al., 2005), and the InsR-mediated excitation of POMC neurons is abrogated by inhibition of PI3K activity (Al-Qassab et al., 2009; Hill et al., 2008; Qiu et al., 2010; Qiu et al., 2014). Activation of PI3K generates phosphatidylinositol-3,4,5-triphosphate (PIP_3), which stimulates phospholipase C (PLC) and protein kinase B (Akt) (Bae et al., 1998; Falasca et al., 1998; Qiu et al., 2014; Rameh et al., 1998). PLC also hydrolyzes PIP_2, which modulates TRPC5 channel activity (Figure 3) (Qiu et al., 2014; Rodríguez-Menchaca et al., 2012; Zhang et al., 2013). In addition, PI3K increases the insertion of TRPC5 channels into the plasma membrane from intracellular vesicular pools to further boost depolarization and Ca^{2+} entry into POMC neurons (Bezzerides et al., 2004). Collectively, all of these PI3K-mediated effects are involved in the actions of insulin in POMC neurons.

We recently investigated the neuroprotective effects of E2 against the development of central insulin resistance with diet-induced obesity (Qiu et al., 2018). Although obesity produced dramatic alterations in metabolic phenotype in both males and females, E2 was able to protect females from the development of CNS (hypothalamic) insulin resistance. Insulin was fully efficacious to activate TRPC5 channels and depolarize POMC neurons in diet-induce obese (DIO), proestrous and E_2-treated, ovariectomized females but not in ovariectomized female or male DIO mice. Treating ovariectomized females with an estradiol regime that mimics proestrous serum levels of E2 rescued the insulin response in POMC neurons. The neuroprotective effects of E2 were mediated, in part, by upregulation of *Cav3.1* mRNA expression and T-type calcium channel currents and downregulation of *Stim1* (Stromal interaction Molecule-1) mRNA. STIM1 is localized to the endoplasmic membrane and its N-terminal domain contains an EF-hand that protrudes into the lumen of the endoplasmic reticulum to sense changes in endoplasmic reticulum Ca^{2+} concentrations (Salido et al., 2011). Upon depletion of endoplasmic reticulum Ca^{2+}, STIM1 undergoes a conformational change, oligomerizes and then interacts with plasma membrane TRPC channels (Salido et al., 2011; Yuan et al., 2007). Phosphorylation of STIM1 is required for oligomerization, and E2 is known to inhibit the phosphorylation of STIM1 and consequently its interaction with plasma membrane and hence store-operated Ca^{2+} entry (Sheridan et al., 2013). Therefore, in the absence of E2, TRPC channels are more likely to associate with STIM1 and function as store-operated Ca^{2+} channels (Salido et al., 2011; Yuan et al., 2007). Indeed, the insulin-induced TRPC5 current in POMC neurons in ovariectomized females was enhanced in the presence of a store-operated Ca^{2+} channel inhibitor (Qiu et al., 2018), and long-term treatment with E2 or STX down-regulated *Stim1* mRNA expression in the arcuate nucleus of female guinea pigs (Rønnekleiv, *unpublished findings*). In addition, E2 prevented the increase in *Socs3* expression with diet-induced obesity, which is known to inhibit the coupling of the insulin receptor with its downstream signaling cascade. Therefore, E2 protects the coupling of insulin receptors to TRPC5 channels through multiple Gq-mER and ERα signaling mechanisms.

The significance of this cellular protection of insulin signaling in POMC neurons was highlighted by the fact that *icv* insulin was fully efficacious in female, but not male, guinea pigs fed a high-fat diet to reduce food intake and increase energy metabolism (Qiu et al., 2018). Therefore, the insulin receptor signaling cascade in POMC neurons appears to be

augmented by E2 through membrane (Gq-mER)- and nuclear-initiated (ERα)-signaling to help protect females against insulin resistance (Figure 3), and may help explain why premenopausal women are protected against development of insulin resistance in type II diabetes (Janssen et al., 2008; Margolis et al., 2004).

Thermoregulation

Concomitantly with regulation of food intake and energy metabolism, the hypothalamus is critical for the control of thermogenesis (*i.e*, the production of heat energy) and the maintenance of core body temperature (Tc) (Morrison et al., 2014), and POMC neurons are involved in generating heat production by brown adipocytes (Dodd et al., 2015). It is also known that circulating estrogens are critical for the maintenance of Tc (Rance et al., 2013); and approximately eighty percent of perimenopausal/postmenopausal (hypo-estrogenic) women experience hot flashes (Moline et al., 2003), which are characterized by periods of sweating and peripheral vasodilation, often associated with increased environmental temperature (Rapkin, 2007). The majority of hot flashes are preceded by elevation in Tc independent of peripheral vasoconstriction or elevated metabolic rate (Freedman, 1998; Freedman, 2005; Freedman et al., 1995). Therefore, elevated Tc may serve as one trigger of menopausal hot flashes (Freedman and Blacker, 2002; Mittelman-Smith et al., 2012; Rapkin, 2007; Roepke et al., 2010). Recent studies have implicated arcuate Kisspeptin neurons, which co-express express Neurokinin B (NKB) and Dynorphin (*aka*, KNDy neurons) and project to the preoptic temperature sensitive neurons (Mittelman-Smith et al., 2012; Rance et al., 2013). The KNDy neurons also project directly to and activate POMC neurons (Nestor et al., 2016). POMC neurons project directly to the preoptic area, where μ-receptors are highly expressed, and warm-sensitive neurons in this brain area are directly responsive to μ-opioid agonists (Petersen and LaFlamme, 1997; Yakimova et al., 1996; Zhou and Hammer, 1995). Clearly, there is a reduction in the incidence of hot flashes in hypo-estrogenic females with E2 treatment (Brooks et al., 1997; Freedman and Blacker, 2002; Tankersley et al., 1992); and E2 lowers Tc and reduces hot flashes by raising the Tc “sweating threshold” (Brooks et al., 1997; Freedman and Blacker, 2002; Roepke et al., 2010; Tankersley et al., 1992). Furthermore, activation of the medial preoptic GABAergic thermosensitive neurons is responsible for evoking vasomotor responses in rodents (Nakamura and Morrison, 2010), which is thought to correspond to heat dissipation responses (*i.e.*, vasodilatation, sweating) in women experiencing hot flashes. At the cellular level E2 reduces NKB expression in the arcuate Kisspeptin neurons (Rance et al., 2013). Based on the hypothesis that the expression of vasomotor symptoms in menopausal women is due to an elevation in Tc and a reduced thermo-neutral zone in core body temperature (Freedman, 1998; Freedman, 2005; Freedman et al., 1995), we established a guinea pig “hot flash” model and found that both E2 and STX significantly reduce Tc in ovariectomized female guinea pigs compared to animals receiving vehicle injections (Roepke et al., 2010). Similar effects of E2 were reported in rats (Mittelman-Smith et al., 2012), and this was thought to be due to the down-regulation of NKB expression in arcuate Kisspeptin neurons (Ogawa et al., 2003). A complementary increase in POMC neuronal activity (Roepke et al., 2010) would also result in the inhibition of warm sensitive neurons (Petersen and LaFlamme, 1997; Yakimova et al., 1996; Zhou and Hammer, 1995). Therefore, there appears to be a convergence of membrane-initiated

signaling by E2 (excitation of POMC) and E2-driven alterations in gene expression (down-regulation of NKB expression in arcuate kisspeptin neurons) to maintain Tc.

Conclusions

Based on decades of research it is now clear that E2 can signal via metabotropic (G protein-coupled) receptors to alter neuronal excitability and autonomic functions controlled by the hypothalamus. Signals initiated by E2 at the plasma membrane can trigger multiple intracellular signaling cascades (e.g., MAPK, PI3K, and PKC) that result in the phosphorylation of hundreds of proteins that ultimately affect not only cell excitability but also gene transcription. The activation of the Gαq-mER in POMC neurons leads to a rapid increase in excitability and the activation of intracellular signaling cascades that ultimately affects gene transcription. In contrast, engagement of the Gαq-mER in NPY/AgRP neurons generates the opposite effects—*i.e.*, an increase in K^+ channel activation by the $GABA_B$ receptor and downregulation of NPY mRNA expression–which is congruent with the anorexigenic effects of E2. Thus, E2 can act on hypothalamic neurons in a cell-specific manner to generate the appropriate physiological responses by eliciting a combination of rapid changes in membrane excitability accompanied by slower alterations in gene expression. A future challenge will be to identify the putative Gαq-mER, its interaction with other metabotropic receptors and its physiological/behavioral functions not only in the hypothalamus but throughout the CNS.

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Highlights

- E2 rapidly alters POMC neuronal activity that ultimately affects gene expression
- E2 rapidly affects the orexigenic NPY/AgRP in an antagonistic manner
- E2's actions are critical for the control of food intake and energy homeostasis
- E2 maintains the response to insulin in POMC neurons in metabolically stressed states

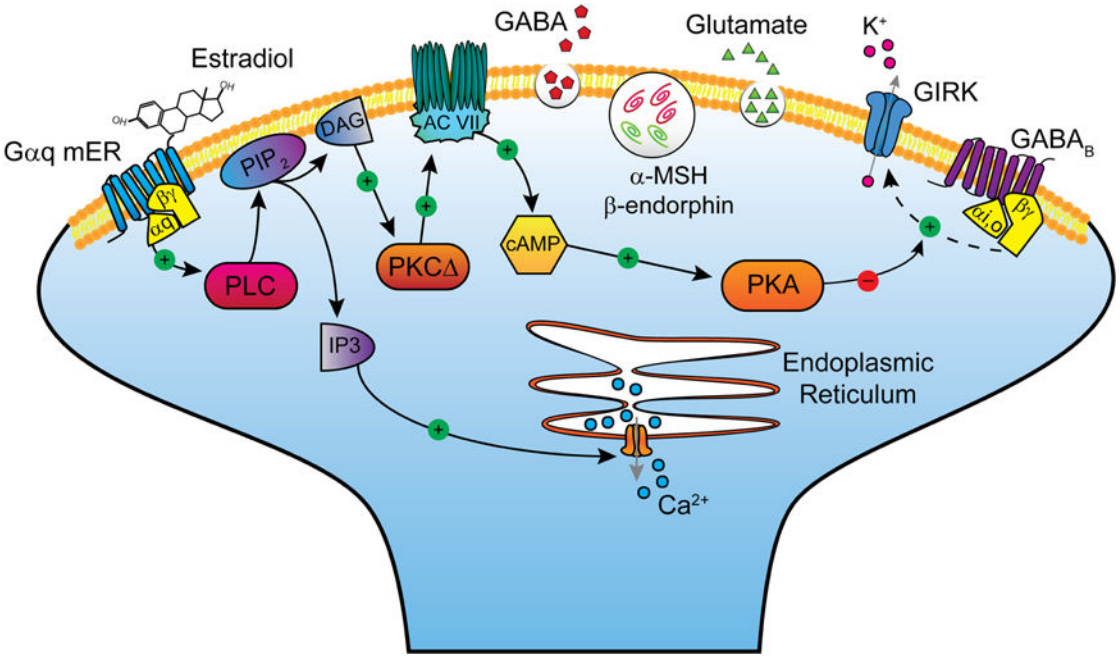

Figure 1. Presynaptic actions of 17β-estradiol (E2) in POMC neurons

Schematic overview of the E2-mediated modulation of Gαi,o-coupled $GABA_B$ receptors via a membrane-associated receptor (mER) in hypothalamic POMC nerve terminals. E2 binds to a mER that is Gαq-coupled to activate phospholipase C and catalyzes the hydrolysis of membrane-bound phosphatidylinositol 4,5-biphosphate (PIP_2) to inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). Calcium is released from intracellular stores (endoplasmic reticulum) by IP_3, and DAG activates protein kinase C Δ (PKCΔ). Through phosphorylation, adenylyl cyclase VII (AC VII) activity is upregulated by PKC. The generation of cAMP activates PKA, which can uncouple (dashed line) $GABA_B$ receptors from their signaling pathway through phosphorylation of a downstream effector molecule (*e.g.*, G protein-coupled, inwardly rectifying K^+, GIRK, channels). Together, elevated intracellular Ca^{2+} and attenuation of $GABA_B$-mediated inhibition will facilitate the release of multiple neurotransmitters—GABA, glutamate, β-endorphin and α-melanocyte stimulating hormone (α-MSH)—from POMC neurons.

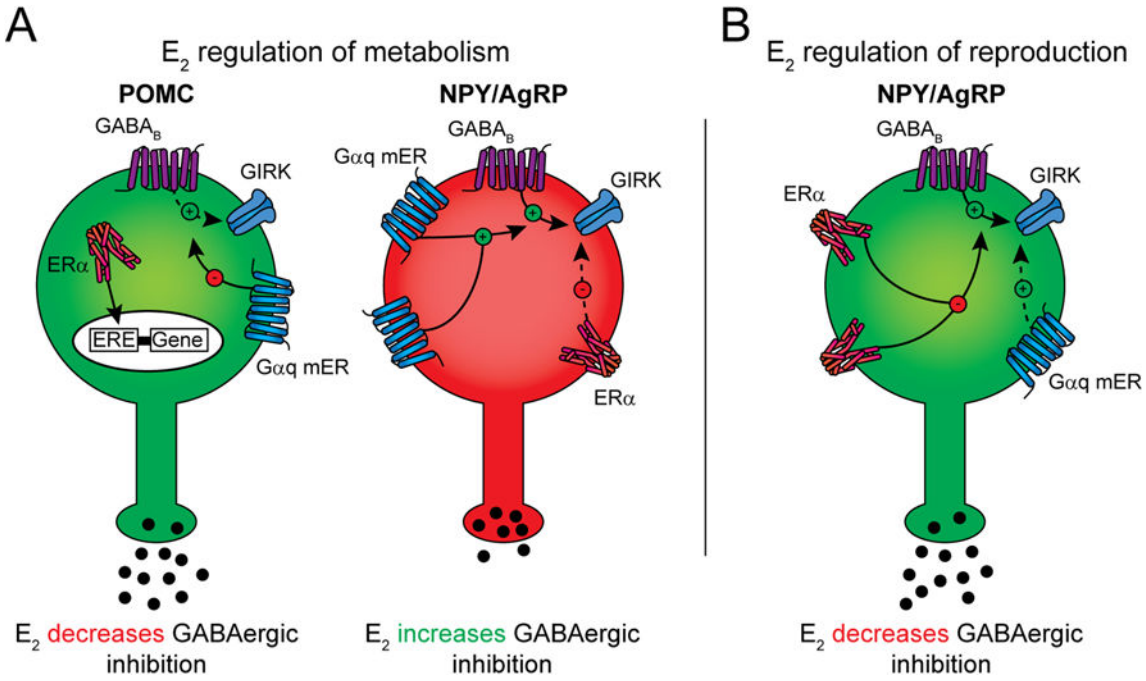

Figure 2. Effects of E2 and STX on hypothalamic POMC and NPY/AgRP neurons

Summary of how E2 excites POMC neurons and inhibits NPYAgRP neurons via a Gαq-mER and ERα mediated signaling pathways to regulate metabolism (A). Activation of the Gαq pathway attenuates the coupling of the $GABA_B$ receptor to GIRK channel activation in POMC neurons, thereby increasing the excitability, but enhances the $GABA_B$ receptor activation of GIRK channels in NPY/AgRP neurons, thus decreasing their excitability. In addition, upon binding E2, ERα inside the cell can activate estrogen response elements (ERE) to initiate changes in gene transcription. However, in NPY/AgRP neurons, ERα is also associated with the membrane, and its activation leads to attenuation of the $GABA_B$ receptor coupling to GIRK channels (B), which is hypothesized to be involved in the control of reproduction. Abbreviations: Gαq-mER, Gαq-coupled membrane estrogen receptor; GIRK, G protein-coupled inwardly rectifying potassium channel; ERα, estrogen receptor α; NPY/AgRP, neuropeptide Y/Agouti-related peptide; POMC, proopiomelanocortin.

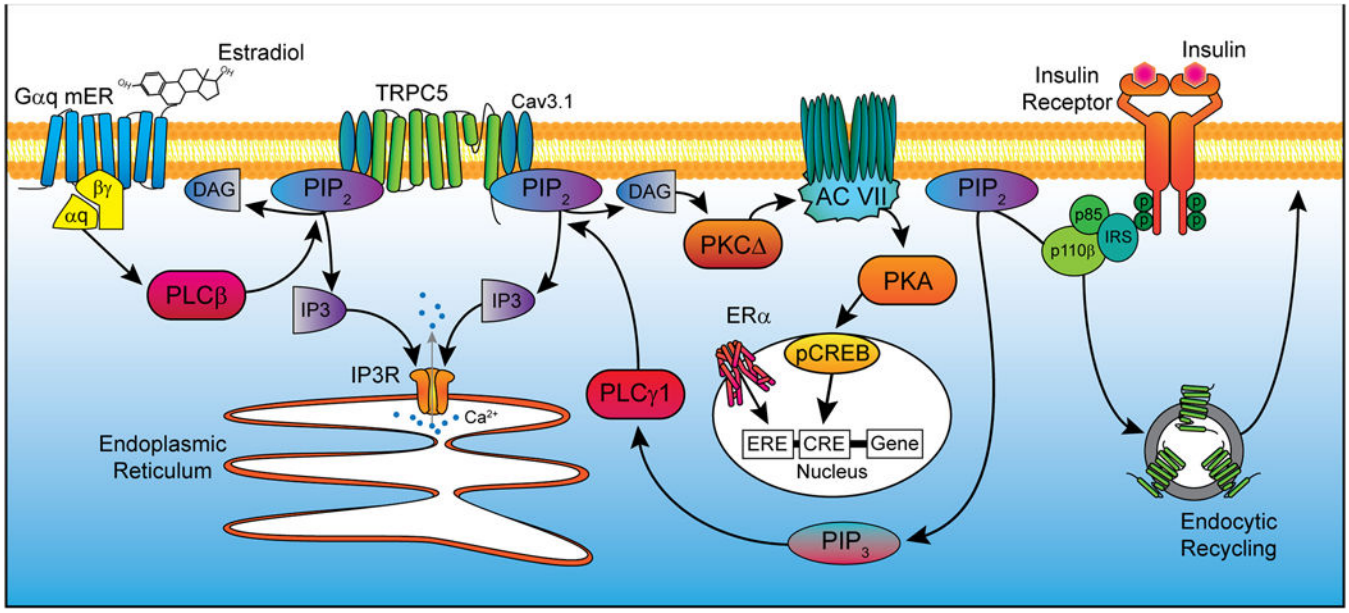

Figure 3. A cellular model of insulin signaling via TRPC5 channel activation in POMC neurons

Activation of a Gq-mER by E2 stimulates phospholipase C β (PLCβ) to catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol and inositol 1,4,5-trisphosphate (IP3). IP3 activates the IP3 receptor, causing release of calcium (Ca^{2+}) from the endoplasmic reticulum. DAG drives protein kinase CΔ to increase the activity of cAMP production by adenylyl cyclase VII which in turn drives protein kinase A activity (PKA). cAMP response element-binding protein (CREB) in the nucleus is phosphorylated, allowing for interactions with certain DNA sequences to alter gene transcription. Alternatively, ERα can dimerize after binding E2 and act on estrogen response elements (ERE) in the nucleus to similarly affect expression of target genes. Insulin signals via insulin receptor substrate-phosphoinositide 3 kinase (IRS-PI3K) to activate TRPC5 channels in POMC neurons, which generates a robust inward cationic current to depolarize POMC neurons and increase their excitability. PI3K (p85/p110) will also accelerate the rapid insertion of TPRC5 channels into the plasma membrane (Bezzerides et al., 2004). E2 facilitates TRPC channel activity through upregulation expression of Cav3.1 (T-type calcium) channels and PLC catabolism of PIP2 to facilitate TRPC5 channel opening via ERα and Gαq-mER, respectively.