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Cross-talk between reproduction and energy homeostasis: central impact of estrogens, leptin and kisspeptin signaling

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Abstract

The central nervous system receives hormonal cues (e.g., estrogens and leptin, among others) that influence reproduction and energy homeostasis. 17β-estradiol (E_2) is known to regulate gonadotropin-releasing hormone (GnRH) secretion via classical steroid signaling and rapid non-classical membrane-initiated signaling. Because GnRH neurons are void of leptin receptors, the actions of leptin on these neurons must be indirect. Although it is clear that the arcuate nucleus of the hypothalamus is the primary site of overlap between these two systems, it is still unclear which neural network(s) participate in the cross-talk of E_2 and leptin, two hormones essential for reproductive function and metabolism. Herein we review the progress made in understanding the interactions between reproduction and energy homeostasis by focusing on the advances made to understand the cellular signaling of E_2 and leptin on three neural networks: kisspeptin, pro-opiomelanocortin (POMC) and neuropeptide Y (NPY). Although critical in mediating the actions of E_2 and leptin, considerable work still remains to uncover how these neural networks interact in vivo.

Keywords

17β-estradiol; kisspeptin; leptin; neuropeptide Y; pro-opiomelanocortin

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Introduction

The central nervous system (CNS) regulates several physiological processes critical for continuation of the species (e.g., reproduction) and survival of an individual (e.g., food intake). In reproduction, the classical role of gonadal steroids in the mammalian CNS is the negative and positive feedback actions on the hypothalamic pituitary axis. In all mammalian species, disruption of the feedback loop by ovariectomy results in rising levels of luteinizing hormone (LH) and follicle stimulating hormone within 1 or 2 days. Restoring the feedback loop with doses of exogenous 17β-estradiol (E_2), results in a rapid (<20–30 min) decline in plasma gonadotropin levels. Following this initial inhibition, high levels of E_2 induces an LH surge in the ovariectomized female, the specific nature of which varies across species [1–5]. The effects of E_2 on the hypothalamus and anterior pituitary act in concert with its effects on other tissues (ovary, uterus, etc.) to ensure a single ovulatory event that is precisely timed.

In addition, it has been known for a number of years that E_2 has acute, membrane-initiated signaling actions in the brain [6–8]. A decade ago the nature and physiological significance of these actions were a matter of debate, but it is now widely accepted that some of the actions of E_2 are quite rapid and cannot be attributed to the classical nuclear-initiated steroid signaling of estrogen receptor α (ERα) or ERβ. One explanation for the rapid steroid actions of E_2 is that ERα and ERβ can associate with signaling complexes in the plasma membrane [9–14]. Many of the rapid effects of E_2 can be induced by selective ERα or ERβ ligands, antagonized by the ER antagonist ICI 182,780 and are absent in animals bearing mutations in ERα and/or ERβ genes [10, 15–21]. As a second means of signaling it is also evident that E_2 can activate *bona fide* G-protein-coupled receptors (GPCRs) such as GPR30 and a putative Gαq-coupled membrane ER (Gαq-mER) [21–29]. A substantial amount of evidence has been generated in the support of a novel Gαq-mER using intracellular sharp electrode and whole-cell patch recording from guinea pig and mouse hypothalamic slices [25, 26, 30]. Therefore, at least two forms of E_2 signaling are known to exist: nuclear-initiated (classical) signaling and membrane-initiated (non-classical) signaling.

In addition to its role in the control of reproduction, E_2 is involved in the regulation of appetite, energy expenditure, body weight, adipose tissue deposition and distribution in females [26, 31–34]. Elimination of E_2 by removal of the ovaries induces an increase in food intake and decreases ambulatory and wheel running activities in rodents, which is reversed with estrogen replacement [26, 35–39]. In fact, hypo-estrogenic states are associated with decreased activity and an increase in body weight in females [26, 34, 38, 40–43]. The anorexigenic actions of E_2 are critical throughout the lifespan of women, but are particularly important at the time of menopause when women often develop central adiposity, insulin resistance and cardiovascular disease [44]. Although E_2 replacement can help reverse these effects, E_2 also increases the risk for cancer and stroke [45, 46]. Interestingly, selective activation of a Gαq-mER elicits robust anorexigenic effects without the systemic risks associated with activating the transcription factors ERα and ERβ [25, 26], opening the door for the development of potential new therapeutics. The anorexigenic effects of estrogens are thought to be mediated through CNS actions, based on findings that injections of E_2 into the third ventricle or directly into the paraventricular nucleus of the hypothalamus (PVH) or the arcuate/ventromedial nuclei are effective in reducing food intake, body weight, and

increasing wheel running activity in females [35, 36, 41, 47]. Furthermore, it is evident that neurons within the hypothalamus regulate energy homeostasis and are affected by E_2. For example, estrogens up-regulate the expression of β-endorphin protein in pro-opiomelanocortin (POMC) neurons in ovariectomized female guinea pigs [48, 49]. In contrast, E_2 reverses the ovariectomy-induced increase in neuropeptide Y (NPY) mRNA expression in the rat [50]. Therefore, it appears that neurons in the arcuate nucleus, more specifically POMC and NPY neurons, are major targets for the anorexigenic actions of estrogens, which emphasize their importance in energy homeostasis. The role of POMC and NPY neurons will be further expanded on below.

Since the middle of the last century, it has been known that a mutation in a single gene can lead to obesity and infertility in mice [51]. In 1994, the gene that encoded for this factor was cloned [52] and shortly thereafter named ‘ leptin’ [53]. This 167 amino acid protein is primarily expressed in white adipose tissue and circulates in its biologically active free form but also bound to leptin-binding proteins [54–56]. Leptin plays a key role in energy homeostasis and reproduction, in particular, this hormone has an important role in the neuroendocrine adaptation to starvation [57]. Studies reveal that low leptin concentrations are important for signaling energy deficits to the hypothalamic-gonadal axis, whereas high leptin concentrations in obesity are often associated with leptin resistance [57]. Furthermore, arcuate nucleus lesions result in an obese phenotype [58, 59], while chemical lesions of the arcuate nucleus that do not impinge on the ventromedial nucleus (VMH) result in the inability of leptin to reduce body weight in leptin-deficient ob/ob mice [60]. In 1995 using RT-PCR, Tartaglia and colleagues identified and cloned the leptin receptor [61]. Although there are several isoforms of the leptin receptor, leptin signaling occurs via the long isoform, from here on referred to as LRb [62]. LRb is expressed abundantly in the hypothalamic arcuate, ventromedial and dorsomedial nuclei [62–69]. Neuron-specific deletion of LRb leads to obesity in mice [70, 71], while neuron-specific replacement of LRb in mice globally lacking LRb can dramatically prevent this obese phenotype [71, 72]. LRb is a member of the class I cytokine receptor family and signals through activation of Janus 2 tyrosine kinase [61, 73]. Leptin binding to its receptor activates (phosphorylates) Jak2 tyrosine kinase, which mediates leptin signaling via several pathways, of which Tyr1138 phosphorylation of LRb and subsequent activation of signal transducer and activator of transcription 3 (STAT3) is particularly important for gene activation [73–78].

17β-estradiol and leptin regulation of GnRH neurons

17β-estradiol

Despite having been studied extensively for many years, the mechanisms by which estrogens regulate GnRH neurons are not well understood. It has been obvious for a number of years that GnRH neurons are modulated by E_2 in a complex manner. For example, loss of gonadal steroids by ovariectomy disrupts GnRH secretion and GnRH regulation of pituitary LH secretion and results in elevated pulses of plasma LH that are synchronized by pulses of GnRH in hypophyseal portal blood [3]. This effect is caused in part by the loss of negative feedback actions of E_2. However, both negative and positive feedback regulation of GnRH and LH secretion can be restored by replacement with E_2 [2, 3, 79–81]. Genetic models have

been generated from ERα knockout (KO) animals that possess an ER-knock-in mutation, which allows in vivo distinction between estrogen response element (ERE)-dependent and ERE-independent mechanisms of E_2 action [82, 83]. Given that GnRH neurons are void of ERα and the role of ERβ in GnRH neurons is uncertain [84–86], the ERE-dependent mechanisms of action presumably occur via afferent neurons. Based on studies using loose cell attached recordings to address the ERE-independent mechanism in GnRH neurons, it was concluded that an E_2-induced decrease in GnRH neuronal firing during the morning (negative feedback model) as well as increased neuronal firing during the afternoon (positive feedback model) are both dependent on ERα binding to ERE [87]. In contrast, based on measurements of E_2-induced inhibition of plasma LH in ovariectomized wildtype, ERαKO and ER-knock-in mutant animals, negative feedback regulation of LH (and presumably GnRH) is at least in part dependent on a mechanism other than ER binding to ERE [82].

In rodents, steroid positive feedback is believed to be by an action of E_2 in the anteroventral periventricular (AVPV) nucleus [4, 88, 89]. AVPV neurons express abundant levels of transcription factors ERα and ERβ and the actions of E_2 are mediated, in part, via nuclear-initiated signaling mechanism [89–91]. Knockout of ERα, but not ERβ, receptor in forebrain neurons including neurons in the AVPV region abrogates the positive feedback effects of E_2 on GnRH neurons [84]. While E_2 actions in the AVPV are in part caused by nuclear-initiated signaling mechanisms, these neurons also appear to be sensitive to rapid actions of E_2 as seen with increased expression of pCREB, a neural activation marker, within as little as 30 min of E_2 administration [92]. In terms of negative feedback by E_2, a rapid inhibition of GnRH and LH secretion (~15 min) [93, 94] is congruent with a membrane-initiated signaling of E_2. In fact, years ago it was found that guinea pig GnRH neurons are rapidly hyperpolarized by E_2 via activation of an G protein-coupled inwardly rectifying potassium (GIRK) channel conductance in the presence of tetrodotoxin, which blocks fast Na^+ channel activity and essentially ‘electrically isolates’ GnRH neurons from synaptic inputs [95–97]. In mice, physiological concentrations (picomolar) of E_2 rapidly augments K_{ATP} channel (also of the inwardly rectifying family) activity to hyperpolarize GnRH neurons (Figure 1) [28, 98]. E_2 activates a protein kinase C (PKC)-protein kinase A signaling pathway and hence the selective Gαq-mER ligand STX (see below) is also able to mimic the actions of E_2 [28]. Both the effects of E_2 and STX are abrogated by ICI 182,780 with a Ki of 0.5 nM [30] (Zhang et al., unpublished observations). These data would indicate that feedback regulation of E_2 to inhibit or excite GnRH neurons is quite complex involving multiple receptors and both pre- and post-synaptic actions of E_2.

Leptin

Serum leptin concentrations have been shown to fluctuate during the menstrual cycle in women, with the highest concentration of leptin coinciding with the time of ovulation [99]. In primates an intravenous administration results in an increase in serum LH concentrations [100]. Furthermore, in vitro analysis of both hypothalamic explants as well as dispersed hypothalamic neurons reveals a stimulatory role of leptin on GnRH secretion [101]. Although this led to the initial hypothesis that leptin could directly regulate GnRH secretion, for several reasons the action of leptin is thought to be indirect via afferent neurons that synapse onto GnRH neurons. Firstly, it has been shown in mice that GnRH neurons are void

of LRb [100, 102]. Secondly, using the premise that expression of LRb might be too low to detect using in situ hybridization, mice genetically engineered to contain a fluorescent tag in the presence of LRb displayed GnRH neurons without LRb fluorescence, indicating a lack of LRb in GnRH cells [103]. Lastly, electrophysiological recording from the hypothalamic slice preparation reveals no direct action of leptin on GnRH neurons [104]. Therefore, the general consensus is that central actions of leptin on the hypothalamic pituitary gonadal axis are upstream of GnRH neurons. Therefore, there are three potential neural networks (kisspeptin, POMC and NPY) that mediate the actions of leptin and E_2 (see below).

Kisspeptin neurons: Regulation of GnRH neurons

Kisspeptin, also termed metastin, was discovered in 1996 [105] and became intimately associated with reproduction in 2003 when it was reported that mutations in the kisspeptin receptor, GPR54 (also known as Kiss1R), cause autosomal recessive idiopathic hypogonadism in humans, and deletion of GPR54 in mice results in defective sexual development and reproductive failure [106, 107]. The *Kiss-1* gene encodes a 145 amino acid protein, which is proteolytically processed to Kisspeptin-54 and several other smaller peptide fragments collectively referred to as 'kisspeptins' [108]. Kisspeptin-54 is the endogenous ligand of GPR54, a receptor that is highly expressed in GnRH neurons [88, 108–110] and when administered centrally, kisspeptin robustly stimulates GnRH and gonadotropin secretion in both prepubertal and adult animals [111–115]. All kisspeptins bind with low nanomolar affinities to rat and human GPR54 expressed in Chinese hamster ovary cells and stimulate PIP2 hydrolysis, Ca^{2+} mobilization, arachidonic acid release, and increased phosphorylation of extracellular signal-regulated protein kinase (ERK) 1, ERK2 and p38 MAP kinase [108, 116]. In native GnRH neurons, kisspeptin causes excitation primarily through activation of transient receptor potential canonical (TRPC) channels and to a lesser extent inhibition of inwardly rectifying K^+ channels [117–121]. In addition, kisspeptin induces a transient elevation of intracellular calcium in GnRH neurons, which is thought to be caused by intracellular calcium store release and has been hypothesized to play an important role in the kisspeptin-mediated depolarization [119]. However, the activation of TRPC channels by kisspeptin in GnRH neurons is not affected by buffering intracellular calcium levels by EGTA or BAPTA or by calcium store depletion [122]. Therefore, a rise in intracellular calcium does not appear to play a critical role in the kisspeptin-mediated activation of TRPC channels, but may be involved in Ca^{2+}/calmodulin-dependent inhibition of high voltage-gated Ca^{2+} channels [123]. Conversely, the kisspeptin-activated TRPC current is attenuated by the calcium channel blockers Cd^{2+} and Ni^{2+}, but not by the high voltage-activated calcium channel blocker amlodipine [117, 122]. This would indicate that T-type calcium channels may be involved. However, reducing extracellular calcium to nominally calcium free has no effect on the kisspeptin-activated TRPC current [117, 122], which is an indication that very little calcium is needed to spark the opening of TRPC channels in GnRH neurons. Therefore, with a sustained depolarization which exceeds that of classical neurotransmitters (e.g., glutamate), kisspeptin excites GnRH neurons primarily through the opening of a cation selective (TRPC) channel that is independent of intracellular calcium store release.

Phosphatidylinositol 4,5-bisphosphate (PIP2) is an important regulator of TRPC channels [124–127]. Heteromeric channels expressing TRPC1 are activated by PIP2 [124, 125]. However, homomeric TRPC4 channels, composed of the full-length TRPC4α, but not the truncated TRPC4β splice variant, are inhibited by PIP2 in HEK cells and vascular smooth muscle cells [128]. Quanitative PCR analysis shows that TRPC4 is the main transcript in GnRH neurons, which is 4-fold higher than TRPC1 and TRPC5 (Figure 2) [110]. Although TRPC4 α is expressed in a subpopulation of GnRH neurons, intracellular dialysis with DiC8-PIP2 (synthesized short chain PIP2) robustly inhibits the kisspeptin-activated TRPC current in essentially all GnRH neurons. Therefore, one would deduce that the full-length isoform TRPC4α is responsible for kisspeptin activation of the TRPC current in the majority of GnRH neurons. In the presence of micromolar concentrations of wortmannin, which inhibits the regeneration of PIP2 via antagonizing PI4K [129], the recovery of TRPC channels following kisspeptin activation is significantly prolonged, which would indicate that the depletion of PIP2 is required for kisspeptin-induced TRPC channel activation in GnRH neurons. Therefore, PIP2 may be a critical point of physiological regulation of TRPC channels in GnRH neurons [130, 131]. In addition to PIP2 depletion, kisspeptin activation of TRPC channels is also dependent on cSrc kinase activation, as both global tyrosine kinase inhibitors such as genistein [132] and the specific cSrc kinase inhibitor PP2 [133, 134] attenuate kisspeptin currents in GnRH neurons [122]. Because cSrc kinase directly regulates TPRC4 channel activity through tyrosine phosphorylation, which also causes rapid insertion of TRPC4 channels into the plasma membrane [135], cSrc appears to be a key signaling molecule in the kisspeptin-mediated activation of TRPC channels in GnRH neurons (Figure 3).

It has been shown numerous times that kisspeptin has prolonged excitatory effects on GnRH neuronal activity [136]. The question has been why is there very little spike frequency adaptation during kisspeptin-induced sustained firing? Recently it was illustrated that kisspeptin reduces spike frequency adaptation and prolongs firing in GnRH neurons via the inhibition of a calcium-activated slow after hyperpolarization current (IsAHP). GnRH neurons express two distinct IsAHP, a kisspeptin-sensitive and an apamin-sensitive IsAHP [137–139]. The kisspeptin-mediated inhibition of IsAHP is abrogated by the PKC inhibitor calphostin C, and the PKC activator phorbol 12,13-dibutyrate mimics and occludes any further effects of kisspeptin on IsAHP [139]. Therefore, in addition to increasing the firing rate through an overt depolarization, kisspeptin facilitates sustained firing through inhibiting an apamin-insensitive IsAHP in GnRH neurons via a PKC-dependent mechanism.

Kisspeptin neurons: reproduction and energy homeostasis

17β-estradiol regulation

Neurons expressing kisspeptin predominantly exist in two distinct areas of the forebrain: the AVPV and adjacent periventricular areas and the arcuate nucleus of the hypothalamus [81, 91, 108, 140–146]. As mentioned before, neurons in the AVPV region including the more caudal periventricular preoptic area expresses high levels of ERα, and the actions of the gonadal steroids are mediated, in part, via the nuclear-initiated signaling (genomic) mechanism [84, 90, 147]. ERα colocalizes with the majority of both AVPV and arcuate

kisspeptin neurons [89, 148]. Also, kisspeptin mRNA expression is greatly increased in the periventricular preoptic area following E_2 treatment [81, 91]. These findings combined with previous observations that the AVPV area is necessary for E_2 positive feedback [84] has led to the conclusion that in rodents E_2 acts on kisspeptin neurons in the AVPV to induce positive feedback. Conversely, kisspeptin expression in the arcuate nucleus is negatively regulated by estradiol. This is evident in ovariectomized adult animals with an increase in arcuate kisspeptin mRNA expression, which can readily be reduced by E_2 replacement [81, 91, 143, 144]. Therefore, kisspeptin neurons in the arcuate nucleus are strongly inhibited by E_2. While this inhibition by E_2 may utilize, at least in part, a non-ERE signaling pathway [91, 149, 150], it is now generally believed that the inhibition of arcuate kisspeptin mRNA expression by E_2 may represent an important contribution to negative feedback, although the exact mechanisms remain to be elucidated.

Leptin regulation

In addition to the E_2 regulation, kisspeptin neurons are also controlled by leptin and might play a pivotal role in integrating energy homeostasis with reproduction. Evidence for this can be seen following food restriction, which results in a reduction of kisspeptin mRNA expression [151–156] as well as reduced GPR54 mRNA expression [154]. Also, an intracerebroventricular administration of leptin in food restricted sheep increases kisspeptin mRNA expression [156]. Furthermore, a global deletion of either leptin or LRb causes reduction in kisspeptin mRNA expression [157, 158]. Additionally, kisspeptin neuron-specific deletion of LRb results in mice that fail to go through puberty and experience increased weight gain [159]. Unlike GnRH neurons, the action of leptin on kisspeptin neurons appears to be direct given that 36% of kisspeptin cells in female guinea pigs [104] and 40% in female mice [157] express LRb. Although one report in male mice indicates that activated p-STAT3 immunoreactivity, a common marker for direct leptin effects, occurs in only 6% of arcuate kisspeptin cells [103], this might represent a difference in leptin signaling between males and females. As final confirmation of direct leptin action on kisspeptin cells, with the use of electrophysiological recording from the hypothalamic slice, leptin was shown to depolarize 82% of recorded kisspeptin neurons, which occurs via activation of TRPC channels [104]. These findings support the hypothesis that kisspeptin neurons are a direct target in mediating leptin action on the hypothalamic pituitary gonadal axis (Figure 4).

POMC neurons: energy homeostasis and reproduction

POMC neurons are also a prime candidate for integration of energy homeostasis and reproduction. POMC is a prohormone located in two areas of the brain, the arcuate nucleus and the nucleus tractus solitarius [160]. While fasting decreases POMC gene expression [161–164], mice with a congenital deletion of POMC results in significant weight gain and elevated plasma leptin concentrations [165]. Furthermore, ablation of POMC neurons in the adult mouse causes significant weight gain [166], which strengthens the argument for a dominant role for the POMC network in controlling energy homeostasis. As POMC is cleaved into multiple bioactive compounds, the one that appears to be important in energy homeostasis is α-melanocyte stimulating hormone (α-MSH). An intraperitoneal injection of

α-MSH can cause weight loss in mice [165]. Given that the anorexigenic actions of an MSH-like agonist, MTII, failed in mice lacking the melanocortin-4 receptor, but not in mice lacking the melanocortin-3 receptor, the effect of α-MSH most likely occurs via the melanocortin-4 receptor [167–170]. As for a role for POMC in reproduction, another bioactive compound produced from cleavage of POMC is an endogenous opioid, β-endorphin, and it is also thought to be involved given that naltrexone, an opioid receptor antagonist, has been used to restore normal menstrual cyclicity in women with hypothalamic amenorrhea [171]. More specifically, in rats it has been shown that β-endorphin can both inhibit tonic LH secretion [172] and can block the LH surge [173]. In addition, cocaine- and amphetamine-regulated transcript, also expressed in POMC neurons, may be involved to communicate energy status to GnRH neurons [174]. Given the influence of energy homeostasis on POMC and the POMC influence on reproduction, this network is most likely a common site where leptin and estradiol overlap.

The neural networks that mediate the action of leptin have been the topic of intense debate for several years and POMC cells have been at the forefront of this research for several reasons. Leptin administration, either given by route of an intraperitoneal [163, 164, 175] or an intracerebroventricular injection [163] in mice lacking leptin (ob/ob mice), results in an increase in POMC mRNA expression. When given intrahypothalamically leptin has been shown to stimulate the release of α MSH in push-pull purfusate solutions of rats [176]. As mentioned before, the arcuate nucleus has a concentrated expression of LRb, and approximately 50% of POMC cells express LRb [177, 178]. Neuron-specific replacement of the LRb in mice lacking all forms of the leptin receptor, stimulates an increase in POMC mRNA expression [71]. Furthermore, LRb KO in POMC cells results in an increase in body weight compared to controls [179]. Electrophysiolical recordings demonstrate that leptin depolarizes POMC cells [180] and this effect of leptin on POMC can be abolished by inhibition of PI3K [181]. Subsequently it was demonstrated that leptin activation of POMC cells is via a Janus 2 tyrosine kinase-dependent pathway, which activates PI3 kinase and PLCγ to augment TRPC channel activity (Figure 5) [104, 182]. Altogether, leptin acts directly on POMC cells to regulate energy homeostasis.

Like leptin, E_2 is also thought to act directly on POMC neurons based on several observations. First, E_2 stimulates POMC mRNA expression in the arcuate [183] and can increase the number of POMC cells (as measured using β-endorphin immunocytochemistry) in the guinea pig arcuate nucleus [48]. Furthermore, with the use of a common marker for neuronal activation, c-fos, it has been shown that E_2 increases POMC activity [184]. Therefore, E_2 not only increases the number of POMC cells, but also enhances their activity. Evidence for direct action is seen in studies reporting that 20%–74% of POMC neurons express ERα [185, 186]. Although, POMC specific LRb KO results in increased body weight, but normal fertility [179], loss of ERα in POMC neurons causes increased weight gain in females as well as abnormal estrous cycles indicative of altered negative feedback regulation [187]. Electrophysiological studies using the hypothalamic slice have established that E_2 acts rapidly and stereospecifically within physiologically-relevant concentrations (EC_{50}=7.5 nM) to significantly reduce the potency of μ-opioid and $GABA_B$ agonists to activate an inwardly rectifying K^+ conductance and thereby increase the activity of hypothalamic neurons including POMC neurons [25, 30]. Importantly, the ER antagonists

ICI 164,384 and ICI 182,780 block the actions of E_2 with subnanomolar affinity (K_i=0.5 nM) that is similar to K_i for antagonism of ERα [30, 188]. These pharmacological findings clearly argue for a novel G-protein-coupled membrane receptor with high selectivity for E_2. About a decade ago a diphenylacrylamide compound, STX, that does not bind ERα or ERβ [25, 26] was developed to selectively target the Gαq-mER and its downstream signaling cascade – phospholipase Cβ-protein kinase Cδ-protein kinase A pathway – that mediates μ-opioid and $GABA_B$ desensitization in hypothalamic neurons. The design arose out of studies in which E_2 was shown to stereospecifically (17α-estradiol is not active) activate the Gαq-mER signaling pathway, and these actions were blocked by the ER antagonist ICI 182,780 [25, 26, 30]. Of high significance is that both STX and E_2 activate this Gαq signaling pathway in mice lacking both ERα and ERβ and in GPR30-knockout mice [26, 189]. Definitive characterization (i.e., cloning) of this novel Gαq-mER is currently a work in progress. The importance of this membrane-initiated signaling is that estrogens and STX can rapidly alter the activity of neurons. including arcuate POMC neurons to quickly influence behaviors such as feeding and reproduction (Figure 6).

NPY neurons: energy homeostasis and reproduction

NPY is a 36 amino acid peptide that was first identified from the porcine brain [190] and has been shown to significantly influence energy homeostasis. Some of the first reports of NPY action on energy homeostasis were in rats where an intracerebroventricle administration of this peptide resulted in increased food consumption [191, 192]. Since then, studies have shown compelling evidence for NPY action on energy homeostasis through two G-protein-coupled receptor subtypes, Y1 and Y5. It has been shown through the central administration that selective agonists for these receptor subtypes cause an increase in food intake [193, 194], while receptor antagonists have the opposite effect resulting in reduced food intake [195–197]. Interestingly, arcuate NPY neurons also contain another neuropeptide involved in energy homeostasis, agouti-related peptide (AgRP) [198] that acts as an inverse agonist to melanocortin-3 and -4 receptors [199, 200]. Despite this evidence, congenital deletion of NPY has no phenotypical effect on body weight, which is presumably due to compensatory mechanisms. Support of a critical role of these NPY/AgRP neurons comes from reports using a genetic model that inserts the diphtheria toxin receptor into NPY/AgRP neurons where injection of diphtheria toxin causes neuron-specific ablation in the adult mouse and results in rapid starvation [166, 201–203]. This phenotypic effect appears to be the result of GABA release from the NPY/AgRP neurons in the brainstem, given that an infusion of a $GABA_A$ receptor partial agonist, bretazenil, into the parabrachial nucleus prevents the starvation induced response caused by NPY/AgRP ablation [203]. In most of these studies, reproductive viability was not assessed, but one can envision that with a 20% reduction in body weight occurring within as little as 6 days, the reproductive function will undoubtedly be compromised. Furthermore, the importance of NPY/AgRP neurons to stimulate feeding is further supported by recent studies using optogenetics and selective photostimulation of NPY/AgRP neurons in mice to evoke voracious feeding within minutes [204]. Collectively, these results clearly define NPY/AgRP neurons as orexigenic. Although the role for NPY on food intake is concise, the role for NPY in reproduction involves both negative and positive feedback. NPY (and AgRP) mRNA expression decrease with food intake across the estrous

cycle with the lowest level during proestrus/estrus [205]. In agreement with this, are reports in female rats [206, 207] and ewes [208, 209] where intracerebroventricular administration of NPY inhibits LH secretion. However, it is of interest to note that NPY may be partially responsible for the LH surge given that mice lacking NPY have an LH surge with dramatically reduced amplitude [210].

As would be expected for a role in energy homeostasis, leptin has profound effects on hypothalamic NPY neurons. First, fasting causes a reduction in circulating concentrations of leptin and an increase in NPY mRNA expression [161, 164]. Secondly, subcutaneous [211] and intraperitoneal [164, 212] administration of leptin decreases NPY mRNA expression. This effect is thought to be directly on NPY neurons given they express LRb [63, 178]. At the cellular level, leptin has been shown to hyperpolarize NPY cells in rats [213] and guinea pigs [104], most likely due to activation of $K\text{-}_{ATP}$ channels [214, 215] and inhibition of calcium currents [213]. This supports the idea that leptin' s action is exerted directly on NPY cells.

As for an effect of estradiol on the NPY network, it has been shown that E_2 suppresses NPY mRNA in the arcuate nucleus [183, 216], which can occur directly on these neurons as up to 20% of NPY neurons in the arcuate nucleus express ERα (Figure 7) [217–219]. Electrophysiological recordings reveal that estradiol hyperpolarizes NPY neurons through activation of the M-current and upregulation of KCNQ potassium channel expression [21]. This effect can be overridden by overnight fasting (caloric restriction), which supports the idea that NPY is a central regulator in energy homeostasis [219]. In addition, E_2 both enhances and attenuates the $GABA_B$ receptor-GIRK channel coupling in NPY neurons, while the selective Gαq-mER ligand STX always enhances the coupling in an ICI 182,780-sensitive manner [21]. Moreover, E_2 and the selective ERα agonist PPT attenuates $GABA_B$ receptor-GIRK channel coupling (Figure 8). These data collectively suggest that E_2 suppresses or augments $GABA_B$-mediated currents in these orexigenic neurons through binding ERα or a putative Gq-mER, respectively. The pathway by which E_2-ERα suppresses $GABA_B$ signaling in NPY neurons appears to require PI3K, specifically the catalytic p110β subunit [21]. Because the selective ERα agonist PPT mimics the inhibitory effects of E_2 on the coupling, presumably increasing membrane excitability, the PI3K signaling pathway may underlie the stimulatory effects of NPY on GnRH and LH secretion in females [220, 221]. Indeed, NPY mRNA expression increases in the arcuate nucleus at the time of the preovulatory surge in female rats [222]. The Gαq-mER signaling pathway in NPY neurons may be specific for the control of energy homeostasis, whereas the ERα-PI3K pathway in NPY neurons may be exclusive for the reproductive pathway. These data suggests that E_2 and STX via a putative Gαq-mER rapidly enhances the coupling of $GABA_B$ receptors to GIRK channels in NPY neurons, thereby increasing the inhibitory tone of these orexigenic cells. Previous work has shown that E_2 and STX exerts the exact opposite effect on POMC neurons [25, 26], which serve an opposing role in the control of energy homeostasis.

Expert opinion

POMC and NPY networks form the bedrock of energy homeostasis, whereas kisspeptin and GnRH neurons are the centerpiece in reproduction. Importantly, kisspeptin is the most potent and efficacious neuropeptide/neurotransmitter to excite GnRH neurons.

E_2 and leptin (and insulin) exert potent effects on kisspeptin, POMC and NPY neurons within the arcuate nucleus. The development of *Kiss1-CreGFP* knock-in mice, as well as mice expressing GFP-tagged GnRH, NPY and POMC neurons, have allowed the direct targeting of these hypothalamic neurons for electrophysiological and molecular biological studies.

These studies have allowed us to clearly define single cell signaling characteristics, such as receptor and ion channel expression and responses to various stimuli. In addition, gene knockout and knock-in mice models have been used to evaluate the significance of discrete hypothalamic neuronal populations and signaling molecules for a number of functions including energy homeostasis and positive and negative feedback regulation of GnRH neurons. Therefore, although our understanding of the convergence of E_2 and leptin on hypothalamic functions continues to progress, many questions still remain.

Outlook (next 5–10 years)

Within the next decade, considerable progress should be made to understand how these three neural networks interact and where the confluence of E_2 and leptin (and insulin) actions is within the CNS to control energy homeostasis and reproduction. The continued use of genetically engineered mice combined with truly innovative techniques such as neural-specific targeting with chemical ablation and optogenetics will aide in defining how these neural networks interact with one another in vivo. An even greater challenge will be to understand these neural networks and the confluence of E_2 and leptin actions on energy homeostasis and reproduction in animal species such as non-human primates, which are more closely related to human. For example, is kisspeptin downstream of NPY and POMC neurons with regard to reproduction, and are NPY and POMC downstream of kisspeptin with regard to control of energy homeostasis in all species? Clearly, obesity with leptin and insulin resistance is rampant worldwide and rationale treatment strategies are needed.

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Highlights

- Feedback of E_2 to inhibit or excite GnRH neurons involves multiple receptors and both pre- and post-synaptic actions.
- Central actions of leptin on the hypothalamic pituitary gonadal axis are upstream of GnRH neurons, e.g., kisspeptin neurons.
- Leptin depolarizes kisspeptin neurons via activation of TRPC channels.
- E_2 both positively and negatively regulates AVPV and arcuate kisspeptin neurons, through altering the expression of critical ion channels.
- Kisspeptin potently excites GnRH neurons through activating TRPC channels and inhibiting potassium channels.
- Leptin depolarizes POMC neurons via activation of TRPC channels.
- E_2 and the selective Gαq-mER ligand STX desensitize μ-opioid and $GABA_B$ receptors in POMC neurons to increase their excitability.
- Leptin hyperpolarizes NPY cells via activation of K_{ATP} channels.
- STX enhances the coupling of $GABA_B$ receptors to GIRK channels in NPY neurons, thereby increasing the inhibitory tone of these orexigenic cells.

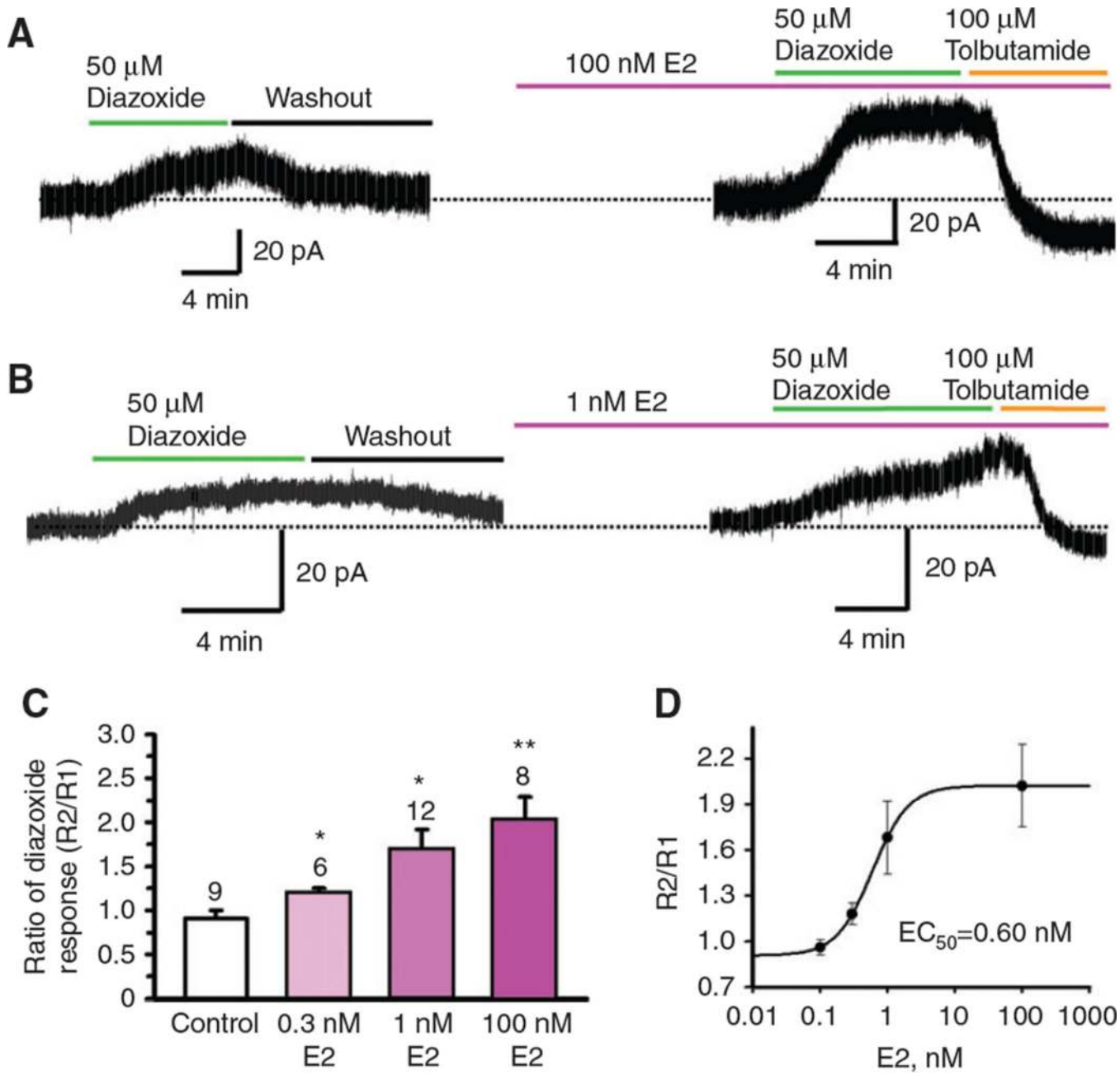

Figure 1.

E2 increases the whole-cell K_{ATP} channel current in GnRH neurons. (A) A representative recording showing that 100 nM E2 acutely enhanced the diazoxide-induced K_{ATP} channel current by 2.7-fold. The 50 µm diazoxide-induced currents were measured at a holding potential of −60 mV. After the first applications of diazoxide for 8 min, cells were washed with artificial cerebrospinal fluid for 15 min and then treated with E2 for 15 min before the second application of diazoxide. Tolbutamide was applied at the end to verify that the current was from the opening of K_{ATP} (Kir6.2/SUR1) channels. (B) A representative recording showing that 1 nM E2 acutely enhanced the diazoxide-induced K_{ATP} channel current by 1.5-fold. (C) Summary of the acute effects of E2 on the diazoxide-activated K_{ATP}

channel currents. The potentiating effects of E2 on the KATP currents were expressed as the ratio of the second diazoxide application-induced current to the first one. *p<0.05, **p<0.01, compared with control. Cell numbers are indicated above the bars. (D) The concentration-response relationship from C was fitted with logistic equation (r^2=0.999), which yielded an EC50 of 0.60 nM. From Zhang et al., 2010 [28]; reprinted with permission from the Endocrine Society.

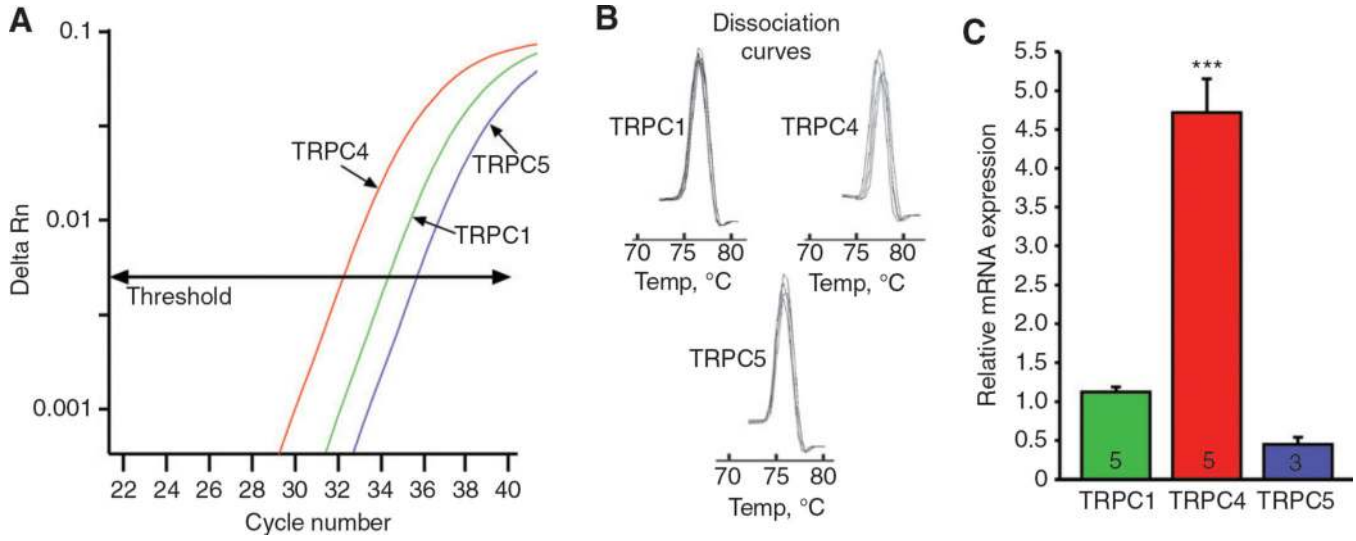

Figure 2.

TRPC channel subtype distribution by real-time PCR. (A) qPCR assay with amplification curves for TRPC1, TRPC4 and TRPC5 subunits. (TRPC1 and TRPC4 were analyzed in five-cell pools and TRPC5 in ten-cell pools). Cycle number was plotted against the normalized fluorescence intensity (delta Rn) to visualize the PCR amplification. The cycle threshold (CT, arrow) is the point in the amplification at which the sample values were calculated. (B) Melting curves depict single-product melting at 77, 78, and 81°C for TRPC1, TRPC4 and TRPC5, respectively, illustrating that only one product was formed for each transcript in GnRH neuronal pools. (C) Bar graphs illustrating the relative mRNA expression of TRPC1, TRPC4 and TRPC5 (***$p<0.001$, TRPC4 compared to TRPC1 and TRPC5). The number of animals is indicated. From Bosch et al., 2013 [110]; reprinted with permission from Elsevier.

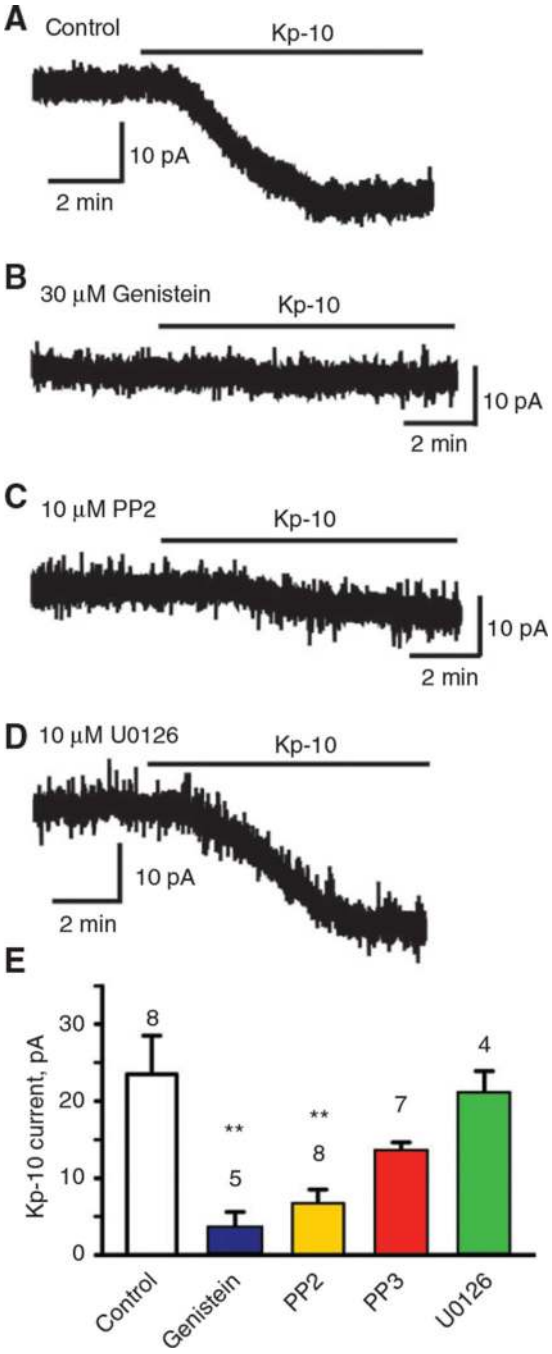

Figure 3.

Src kinase inhibitors abrogate the kisspeptin activation of TRPC channels in GnRH neurons. (A–D) Representative recordings showing that the kisspeptin (Kp-10)-activated inward currents were inhibited by tyrosine kinase inhibitor genistein (30 μM) and the cSrc kinase inhibitor PP2 (10 μM) but not by MAPK inhibitor U0126. Vhold =−65 mV. E, Summary of the effects of genistein, PP2, PP3, and the MAPK inhibitor U0126 on the kisspeptin-induced currents. The control represents kisspeptin in the presence of vehicle. **p<0.01, genistein

vs. control and PP2 vs. control (one-way ANOVA). From Zhang et al., 2013 [122]; reprinted with permission from the Endocrine Society.

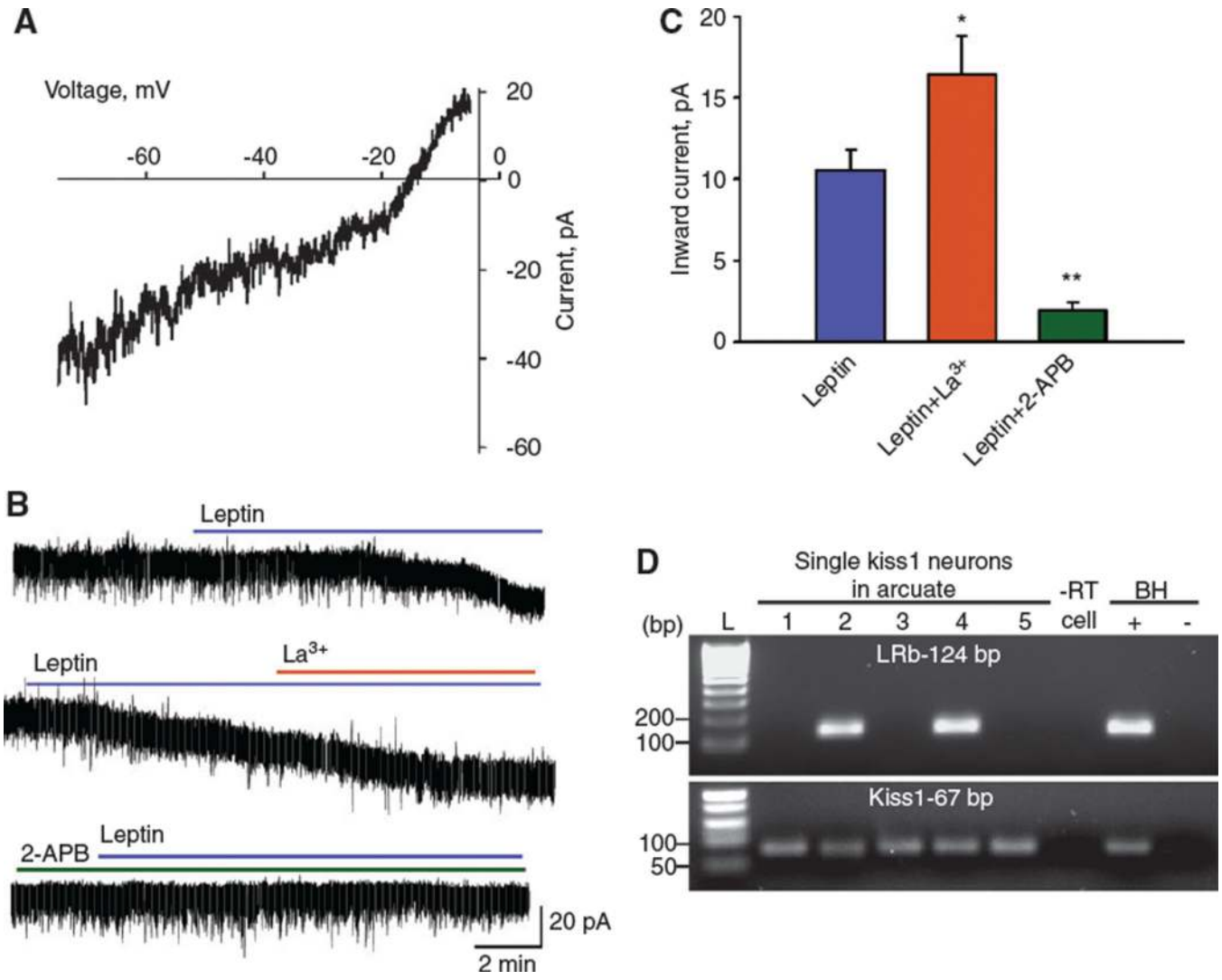

Figure 4.

Leptin activation of nonselective cation current. (A) The I–V relationship for the leptin-induced current was obtained by digital subtraction of the control I–V from the I–V in the presence of leptin (100 nM) using a Cs^+-based internal solution and K^+ channel blockers in the extracellular cerebrospinal fluid. The reversal potential of the nonselective cation current was −15 mV. B, Representative traces of the leptin-induced currents in the presence or absence of the TRPC4,5 activator La^{3+} (100 μM) or the relatively selective TRPC channel blocker 2-APB (100 μM). In voltage clamp, leptin induced an inward current in kisspeptin neurons (upper trace, 10.4 ±1.3 pA), which was potentiated by La^{3+} (middle trace, 16.4±2.4 pA). In another kisspeptin neuron, leptin induced an inward current that was abrogated by 2-APB (lower trace, 1.9±0.5 pA), applied 15 min before the application of leptin (100 nM). (C) Summary of the effects of 2-APB and La^{3+} on the leptin-induced inward currents in guinea pig arcuate (including kisspeptin) neurons. *p<0.05, **p<0.01, significantly different from the maximum current induced by leptin alone. Cell numbers are indicated. (D) Representative gel illustrating LRb mRNA expression in kisspeptin neurons. –RT cell and

BH+, BH− represent controls processed with (+) without (−) RT. From Qiu et al., 2011 [104]; reprinted with permission from the Endocrine Society.

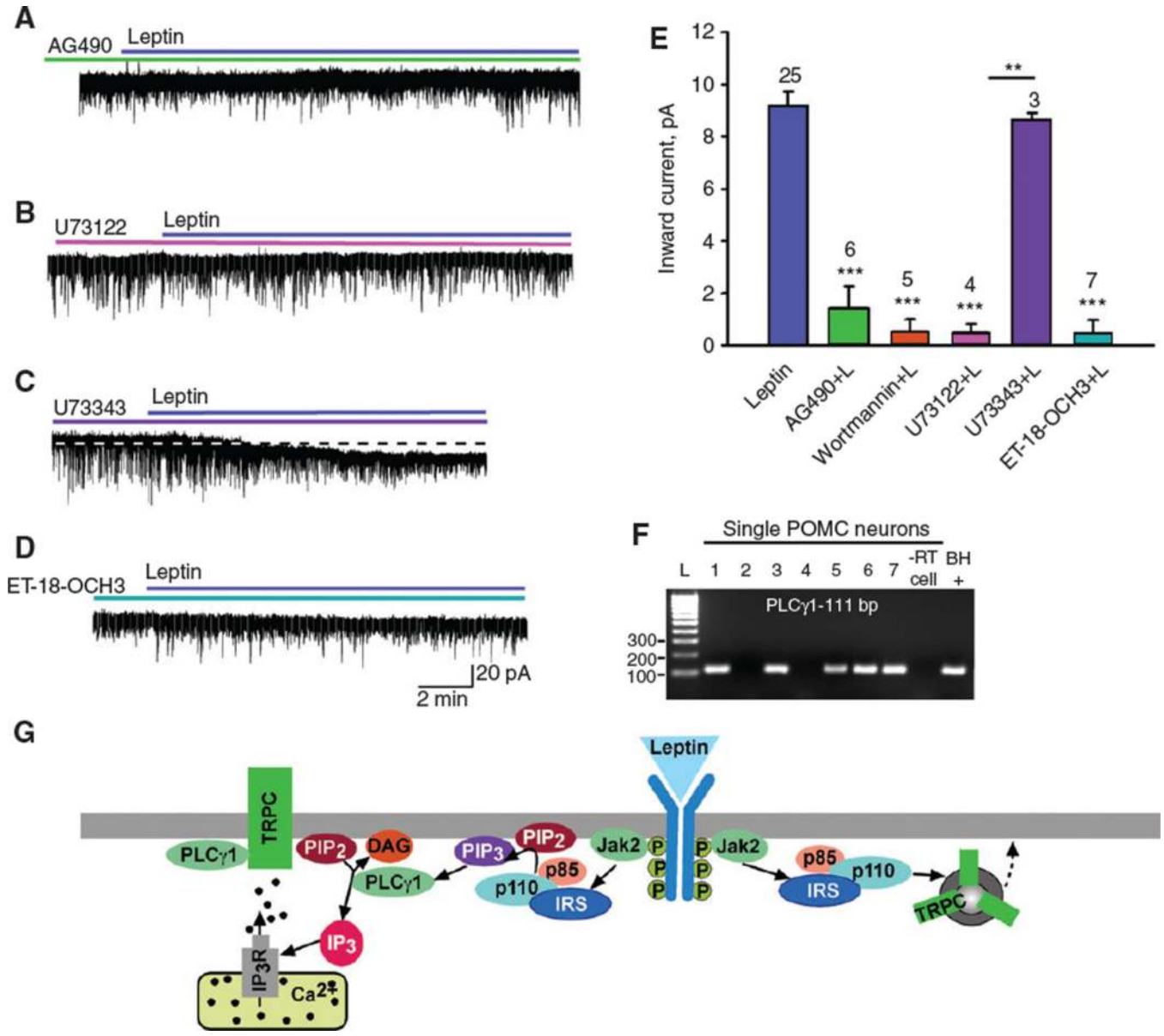

Figure 5.

The leptin response requires Jak2, PI3 Kinase and PLCγ activation. (A–D) Representative traces of the leptin-induced currents in the presence or absence of kinase inhibitors. (E) Summary of the effects of the Jak2 inhibitor AG490 (10 μM), PI3 kinase inhibitor wortmannin (100 nM), PLC inhibitor U73,122 (20 μM) and its inactive analog U73,343 (20 μM), and the PLCγ inhibitor ET-18-OCH3 (15 μM) on the leptin-induced inward current. Blockers were applied for 15 min before the application of leptin (100 nM). Vhold =−60 mV. **p<0.01, U73122 vs. U73343 group; ***p<0.001, significantly different from the leptin control group. Cell numbers tested are indicated. (F) Representative gel illustrating PLCγ1 mRNA expression in POMC neurons.−RT cell and BH+, processed without and with RT. (G) A cellular model of leptin ′s signaling and TRPC channel activation in the POMC neurons. Based on our findings and other published data, we propose that leptin binds to its

receptor (LRb) to activate Jak2, which phosphorylates IRS proteins and in turn activates PI3 kinase. PI3 kinase subsequently activates PLC γ 1 to augment TRPC channel activity. PI3 kinase also stimulates rapid incorporation of functional TRPC channels into the plasma membrane. All of these signaling events enhance POMC neuronal excitability. From Qiu et al., 2010 [182]; reprinted with permission from the Society for Neuroscience.

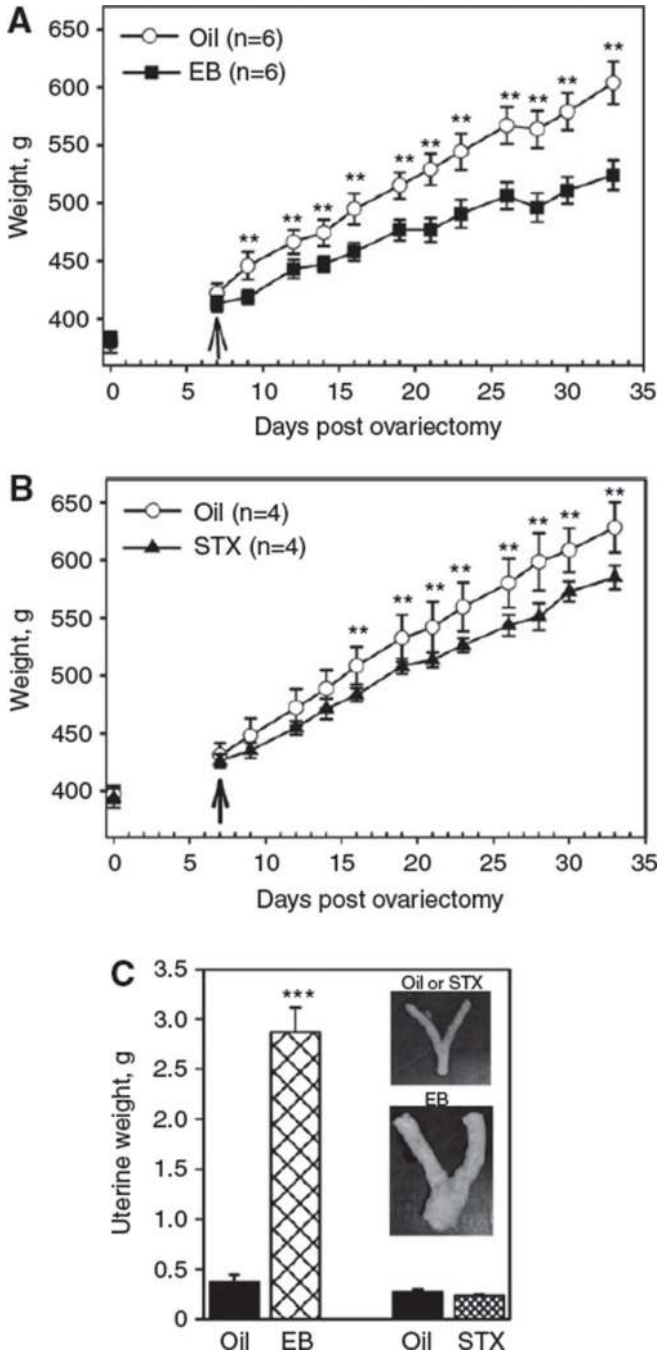

Figure 6.

(A) Estrogen and (B) STX significantly attenuate the body weight gain in female guinea pigs after ovariectomy. The female guinea pigs were ovariectomized (on day 0) and allowed to recover for 1 week before being given bi-daily subcutaneous injections of oil (OIL), estradiol benzoate (EB), or STX (see Materials and methods). A two-way ANOVA (repeated measures) revealed an overall significant effect of both estrogen and STX ($p < 0.001$), and post-hoc Newman-Keuls analysis revealed daily significant differences between estrogen and oil-treated, and STX and oil-treated groups (**$p<0.01$). Bars represent the mean±SEM

of six and four animals per group for estrogen and STX treatment, respectively. (C) Uteri are enlarged after estradiol, but not after STX or oil-vehicle treatment (inset). After the treatment period, the uteri of the guinea pigs were harvested and examined. There was a significant increase in uterine size after EB, compared with oil-vehicle or STX treatment. Bar graphs represent mean uterine weights. ***$p<0.001$, EB vs. oil-treated females; n=6 guinea pigs/group. From Qiu et al., 2006 [26]; reprinted with permission from the Society for Neuroscience.

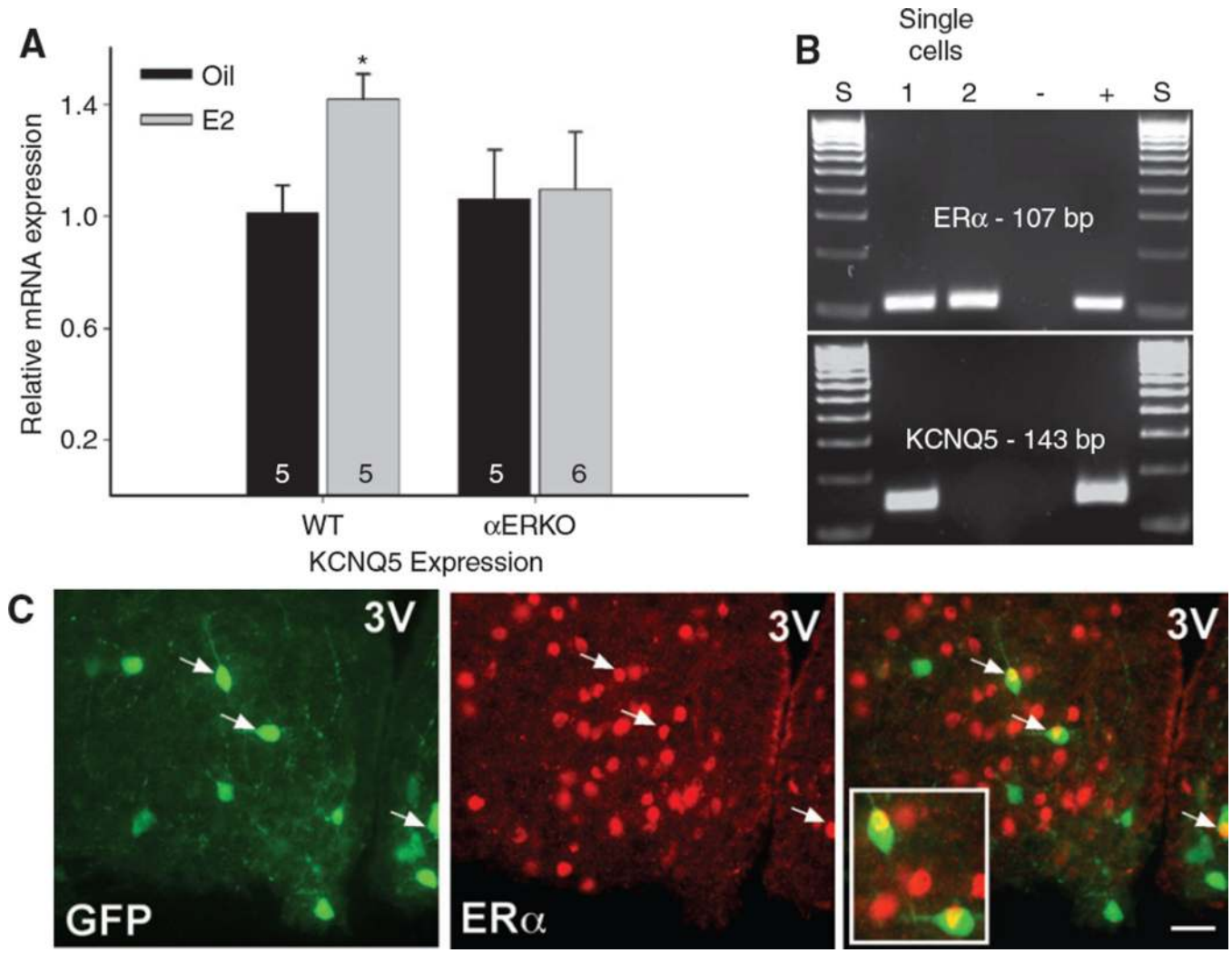

Figure 7.

17β-estradiol regulation of KCNQ5 expression is ERα-dependent and a small population of NPY neurons express ERα. (A) E2 treatment in ovariectomized, wild-type females increased the mRNA expression of KCNQ5 in the arcuate nucleus but failed to regulate KCNQ5 expression in ERα knockout mice. *p<0.05. The number in the column equals the number of animals per treatment. (B) A representative gel illustrating the expression of ERα in NPY neurons harvested from ovariectomized females and the co-expression of KCNQ5. (C) Immunocytochemistry showed a small population of GFP-NPY neurons co-localizing ERα: NPY-GFP neurons to the left and ERα-immunoreactive neurons in the middle with an overlay illustrating co-localization (indicated by arrows) to the right. 99% of GFP-labeled neurons express NPY mRNA. 3V demarks the third ventricle. White bar in the overlay represents 25 μm. From Roepke et al., 2011 [219]; reprinted with permission from the Society for Neuroscience.

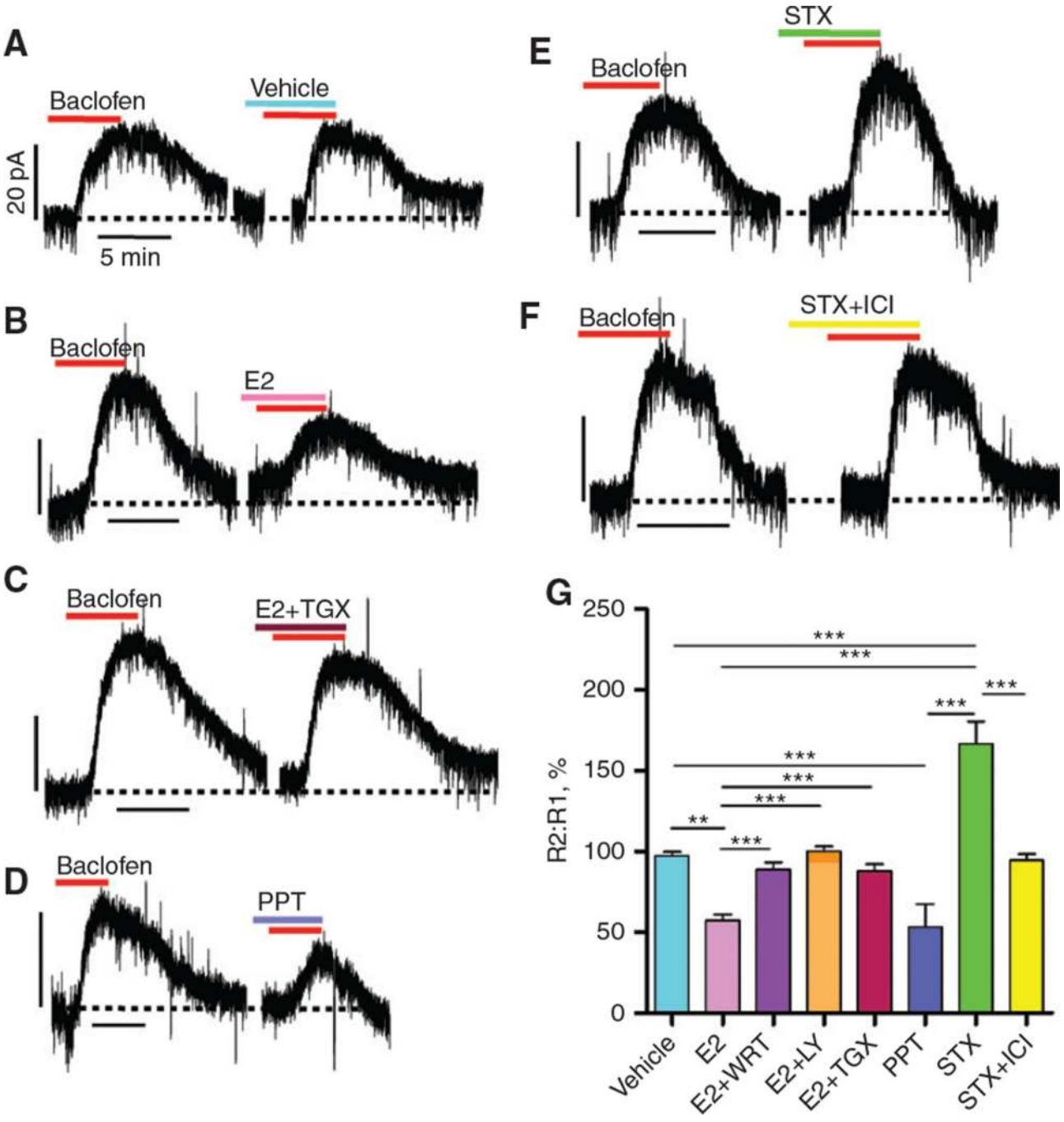

Figure 8.

The ERα ligand PPT and the Gq-mER ligand, STX, differentially modulate the GABAB response in NPY/AgRP cells from intact male mice. (A–F) Representative traces of GABAB responses before and after application of E2, PPT or STX, with or without additional pharmacological manipulations (see below). The dotted line represents the baseline current. Vhold =– 50 mV. All vertical scale bars represent 20 pA, and all horizontal bars represent 5 min. For illustrative purposes, most of the 15-min vehicle or treatment period between GABAB responses (R1 and R2) is removed. Other small breaks in the recording signify

removal of slightly prolonged return to baseline current following baclofen application. (G) Bar graphs summarizing the effects of E2, STX or PPT (all 100 nM) on the GABAB response (baclofen, 10 μM) in NPY/AgRP neurons from intact males. Baclofen elicited two equal-amplitude responses during perfusion of vehicle (n=7), but E2 suppressed the response (n=8). Coperfusing general PI3K inhibitors (WRT =wortmannin, 100 nM, n=5; LY=LY294002, 10 μM, n=4) or the p110β inhibitor TGX-221 (TGX, 11 nM, n=6) with E2 reversed this effect. PPT mimicked the effects of E2 (n=4). STX augmented the response (n=5), but was rendered ineffective by co-perfusing an estrogen receptor antagonist (ICI=ICI 182, 780, 1 μM, n=4). **p<0.01; ***p<0.001, vs. vehicle control group. Smith et al., 2013 [21]; reprinted with permission from the American Physiological Society.