Kisspeptin and Gonadotropin-Releasing Hormone Neuronal Excitability: Molecular Mechanisms Driven by 17β-Estradiol

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\textbf{Abstract}
Kisspeptin is a neuropeptide that signals via a Gαq-coupled receptor, GPR54, in gonadotropin-releasing hormone (GnRH) neurons and is essential for pubertal maturation and fertility. Kisspeptin depolarizes and excites GnRH neurons primarily through the activation of canonical transient receptor potential (TRPC) channels and the inhibition of K\textsuperscript{+} channels. The gonadal steroid 17β-estradiol (E\textsubscript{2}) upregulates not only kisspeptin (Kiss1) mRNA but also increases the excitability of the rostral forebrain Kiss1 neurons. In addition, a primary postsynaptic action of E\textsubscript{2} on GnRH neurons is to upregulate the expression of channel transcripts that orchestrate the downstream signaling of kisspeptin in GnRH neurons. These include not only TRPC4 channels but also low-voltage-activated T-type calcium channels and high-voltage-activated L-, N- and R-type calcium channel transcripts. Moreover, E\textsubscript{2} has direct membrane-initiated actions to alter the excitability of GnRH neurons by enhancing ATP-sensitive potassium channel activity, which is critical for maintaining GnRH neurons in a hyperpolarized state for the recruitment of T-type calcium channels that are important for burst firing. Therefore, E\textsubscript{2} modulates the excitability of GnRH neurons as well as of Kiss1 neurons by altering the expression and/or function of ion channels; moreover, kisspeptin provides critical excitatory input to GnRH neurons to facilitate burst firing activity and peptide release.

\textbf{Introduction}
Gonadotropin-releasing hormone (GnRH) neurosecretion and the control of the ovulatory cycle in females is dependent on estrogen feedback, primarily 17β-estradiol (E\textsubscript{2}) secreted from the ovaries which reaches the pituitary and the brain via the circulation. E\textsubscript{2} treatment in ovariectomized females initially inhibits GnRH and luteinizing hormone (LH) secretion by the process known as negative feedback, followed by E\textsubscript{2}-induced super-secretion (positive feedback) in a species-specific manner 12–42 h later [1–3]. With the development of mouse and rat models in which GnRH neurons express enhanced green fluorescent protein, it has been possible to systematically study these neurons in order to evaluate the GnRH neuronal excitability and activity with the ultimate goal of understanding the neuronal activity underlying the different secretory patterns uti-
lized by these cells [4–6]. In addition, kisspeptin neurons, located in the anteroventral and more caudal periventricular preoptic area (AVPV/PeN), express the E_2 receptor-α (ERα), and E_2 stimulates Kiss1 mRNA expression in this brain region [7]. Moreover, kisspeptin is one of the most potent excitatory neurotransmitters of GnRH neurons [8–11].

Based on decades of intracellular sharp electrode and whole-cell recordings in a number of parvocellular hypothalamic neurons, we have modeled currents crucial for rhythm burst firing and have continued to explore the role of these currents in GnRH and, more recently, in Kiss1 neurons [12–14]. These currents include the low-threshold T-type calcium current (I_T), the hyperpolarization-activated, cyclic nucleotide-gated current (I_H), a calcium-dependent, afterhyperpolarization potassium current (I_AHP), as well as a persistent, sodium current (I_NaP), all of which will be further discussed in this review (fig. 1). All of these currents have been studied extensively in thalamic relay neurons, in which the T-current is responsible for low-threshold calcium spikes and the h-current serves as the ‘pacemaker’ to control the rate of rhythmic oscillations in these neurons [15–17]. Recently, several models have been developed that include an ensemble of channels that appear to be critical for burst firing in parvocellular neurosecretory neurons [12, 18–20]. Variations of these models have been described in a number of reviews and primary publications [21–30] and therefore will not be extensively covered in this minireview. Rather, this review will focus on the E_2 modulation of both GnRH neurons and the presynaptic kisspeptin neurons as well as discuss the role of kisspeptin as a unique excitatory neurotransmitter of GnRH neurons.

**E_2 Modulation of GnRH Neuronal Activity through Channel Expression**

As in thalamic neurons, I_T, I_H, I_AHP and I_NaP are important for burst firing in GnRH as well as in AVPV/PeN Kiss1 neurons [14, 15, 17, 22, 25, 31–34]. We and others have continued to explore the modulation of these currents by E_2 in GnRH and Kiss1 neurons in order to elucidate the conductances underlying burst firing activity.

Three subunits of the T-type calcium channel (CaV3.1, 3.2 and 3.3) have been cloned, with the specific gating properties of the channel being dependent on its subunit composition [35, 36]. Thus, the kinetic properties of channels composed of CaV3.1 and CaV3.2 subunits are different from those of CaV3.3 subunits [37]. GnRH neurons express all three of the T-type calcium channel subunits, and the I_T in GnRH neurons shows different types of inactivation kinetics, with the slow CaV3.3 channel being the most prominent of the three [32, 38]. It is of importance that I_T is present in the majority of GnRH neurons, and the membrane potential at which half of the T-type calcium channels are available for activation (i.e., are de-inactivated) is ~80 mV [32]. As found in other neuronal systems, the role of I_T is to initiate transient membrane depolarizations which recruit high-voltage-activated (HVA) calcium channels to increase calcium influx [39, 40]. This is of functional significance since HVA calcium currents are prevalent in GnRH neurons [41], but they can only be activated from a more depolarized state, whereas low-voltage-activated T-type currents are recruited from a more hyperpolarized state (i.e., they provide the signal for setting off burst firing) [32, 38, 41–44]. Importantly, the T-type CaV3.3 channel subunit expression and current density are significantly enhanced with a high LH surge-inducing E_2 treatment, facilitating the excitation of GnRH neurons [32]. Therefore, the E_2-dependent increase in T-type calcium channels may serve to augment the excitability of GnRH neurons in preparation for the GnRH (and LH) surge. In addition, the majority of HVA channel subtypes are expressed in GnRH neurons, and the mRNA levels of the L-type HVA calcium channel CaV1.3, the N-type HVA calcium channel CaV2.2, and the R-type HVA calcium channel CaV2.3 are also increased by LH surge-inducing E_2 treatment [45]. Similarly, L- and N-type currents are increased by E_2 during the afternoon as compared to the morning in an E_2 implant model that induces daily LH surges [41, 46]. Collectively, these findings suggest a prominent role for calcium channels in the E_2-induced excitation of GnRH neurons. GnRH neurons also express a hyperpolarization-activated, cyclic nucleotide-gated cation current (I_H; the pacemaker current), which also contributes to rhythmic firing [18, 33, 47, 48]. Two I_H channel mRNAs, HCNI and HCN2, are highly expressed in mouse GnRH neurons, and the mRNA expression of HCNI is upregulated by E_2 [45]. Although E_2 regulation of the h-current has not been evaluated in female GnRH neurons, I_H is upregulated by E_2 in Kiss1 neurons, an indication that this current is E_2 sensitive [14, 34].

Critical for hyperpolarizing the membrane to de-inactivate and recruit I_T and activate I_H are the inwardly rectifying K^+ (K_{ir}) channels. It is well known that K_{ir} channels are vital for maintaining excitable cells in a hyperpolarized resting state closer to the Nernst equilibrium potential for potassium, but once cells are depolarized,
the channels allow for the quick transition to long depolarizing responses because of their inwardly rectifying properties [49]. With greater membrane hyperpolarization of GnRH neurons, there is an increase in the number of Na⁺ spikes generated during the rebound excitation [Zhang et al., unpubl. obs.]. Indeed, ATP-sensitive potassium (K_{ATP}) and G-protein-coupled inwardly rectifying potassium (GIRK) channels appear to be the critical Kᵢ channels in GnRH neurons for maintaining the membrane in a more negative resting state. Blocking K_{ATP} channels significantly depolarizes GnRH neurons, which is indicative of tonic K_{ATP} channel activity that is significantly augmented with E₂ treatment [48]. Also, GABA, opioids, neuropeptide Y and perhaps melanin-concen-
trating hormone inputs via their respective GABAA, μ-opioid, Y5 and MCH1 receptors activate GIRK channels to hyperpolarize GnRH neurons [47, 50–52]. This combination of hyperpolarizing influences would allow for the recruitment of multiple excitatory conductances that are critical for generating burst firing in central nervous system neurons [15, 18].

Neuronal excitability is also determined by the after-hyperpolarization (AHP) that follows an action potential, and the three kinds of calcium-activated potassium channels that mediate AHPs have been identified in GnRH neurons [25, 53–56]. This includes the fast AHP, which is mediated by the large-conductance Ca2+-activated K+ (BK) channels, the medium AHP (mAHP), which is mediated by small-conductance, calcium-activated K+ (SK) channels, and the slow AHP (sAHP), which is mediated by a yet unidentified potassium channel. The BK channels appear to be involved in the repolarization of action potentials in GnRH neurons [57]. Inhibition of SK channels by apamin directly attenuates the mAHP as well as augments the afterdepolarization in GnRH neurons, and, thus, these channels exert a significant influence on the firing properties of these neurons [58, 59]. Of the SK channel subtypes, only SK3 mRNA exhibits significant expression in GnRH neurons, and, surge-inducing E2 treatments decrease the expression and function of this channel in ovarioctomized females [45, 59]. The channel responsible for the sAHP current has not been identified. However, this current slows or limits the firing frequency (called ‘spike frequency adaptation’) and is a major player controlling neuronal excitability [60]. A reduced AHP current allows a faster trajectory to burst firing of GnRH neurons [25, 56, 59]. Thus, the upregulation of low-voltage-activated (CaV3.3) and HVA calcium (CaV1.3, 2.2, 2.3) and hyperpolarization-activated cation (HCN1) channels and the downregulation of SK3 channels by E2 play key roles in increasing GnRH neuronal excitability.

Rapid E2 Modulation of GnRH Neuronal Activity

The relatively fast (~15 min) inhibition of GnRH and LH secretion by E2 is congruent with its initiation of a membrane signaling cascade. In fact, years ago, it was found that guinea pig GnRH neurons are rapidly hyperpolarized by E2 via activation of an inwardly rectifying GIRK conductance in the presence of tetrodotoxin, which blocks fast Na+ channel activity and isolates GnRH neurons from action potential-driven synaptic inputs [47, 61, 62]. In mice, physiological concentrations (picomolar) of E2 rapidly augment KATP (also of the inwardly rectifying family) channel activity to hyperpolarize GnRH neurons [63]. E2 signals via a protein kinase C (PKC)-protein kinase A (PKA) pathway, and, hence, a selective Gq-membrane-associated estrogen receptor (Gqα-mER) ligand called ‘STX’ are also able to mimic the effects of E2 in GnRH as in other hypothalamic neurons [63–66]. Both the effects of E2 and STX are abrogated by the estrogen receptor antagonist ICI 182,780 with a Ki of 0.5 nM in GnRH neurons [63, 67], which is similar to the Ki for the antagonism of ERα [68]. Interestingly, the membrane-initiated signaling by E2 in GnRH neurons appears to persist given that female mice treated with an E2 implant for 4–7 days similarly exhibit augmentation of the KATP current, which attenuates GnRH neuronal firing [48]. This would imply that membrane-initiated signaling by E2 is involved in negative feedback regulation of GnRH neurons.

In addition, nanomolar concentrations of E2 enhance action potential firing by modulating intrinsic afterhyperpolarizing and afterdepolarizing potentials via a PKA-dependent mechanism involving ERβ [59]. Picomolar concentrations of E2 also inhibit action potential firing via presynaptic ERα-dependent mechanisms [59]. E2 rapidly potentiates HVA Ca2+ currents (L- and R-type Ca2+ channels) via ERβ and a G-protein-coupled receptor of 30-kDa molecular weight (GPR30) suggesting that Ca2+ signaling is also a target for E2 membrane actions in GnRH neurons [41]. However, in slices prepared from GnRH-Pericam transgenic mice, E2 initiates calcium transients within 15 min in a subgroup of GnRH neurons [69]. In this transgenic mouse model, the E2 effects are mediated indirectly via input from GABA neurons that express ERα. The role of ERβ to mediate rapid effects of E2 on GnRH neurons has also been shown following E2 treatment in vivo [70]. E2 rapidly (within 15 min) phosphorylates cAMP response element-binding protein (pCREB) in GnRH neurons, an effect that is lost in ERβ-deleted animals, supporting a direct role for ERβ in mediating rapid E2 signaling in GnRH neurons [70]. However, how pCREB affects gene expression and cell excitability is not known.

In primate and mouse embryonic nasal explants (the source of immature GnRH neurons), E2 modulates Ca2+ oscillations in GnRH neurons [71–74] which synchronize with a periodicity of approximately 60 min in primates, a rhythm that is similar to the pulsatile GnRH release [71, 73–75]. Furthermore, nanomolar concentrations of a membrane-impermeant E2 (E2 dendrimer) and the Gq-mER ligand STX alter the patterns of Ca2+ oscil-
lations in primate GnRH neurons [75, 76]. As found previously with E2, STX also elicits an increase in the frequency and synchronization of Ca2+ oscillations in rhesus macaque GnRH neurons [75, 77]. The STX-induced modulation of Ca2+ oscillations (and GnRH release) from macaque nasal explants is not altered by GPR30 small-interfering RNA transfection, suggesting that GPR30 is not involved. Importantly, the effects of STX are blocked by the ER antagonist ICI 182,780 and by the phospholipase C inhibitor U73122 [75]. This would suggest that STX (E2) is activating Gq-mER (as defined above) in rhesus macaque GnRH neurons, which induces calcium oscillations via the IP3 receptor (fig. 1) and/or a PKC signaling pathway [78]. Therefore, although the specific role of membrane-initiated E2 signaling for GnRH physiology is uncertain, it could potentially play a critical role in sculpting GnRH burst firing activity.

Kisspeptin-GnRH Neuronal Circuitry

Kisspeptin 54 is the endogenous ligand of GPR54 (Kiss1R) [79]. GPR54 is highly expressed in GnRH neurons [45], and mutations in GPR54 cause autosomal recessive idiopathic hypogonadism in humans, and deletion of GPR54 or Kiss1 in mice results in defective sexual development and reproductive failure [80, 81]. The Kiss1 gene encodes a 145-amino-acid protein, which is proteolytically processed to kisspeptin 54 and several other smaller peptide fragments, and centrally administered kisspeptins robustly stimulate GnRH and gonadotropin secretion in both prepubertal and adult animals [82, 83]. Neurons in the AVPV/PeN areas express kisspeptin, GABA and opioid peptides, all of which are important for the regulation of GnRH neurosecretion [84–89]. The AVPV/PeN expresses high levels of ERα and also ERβ, and the actions of the gonadal steroids on kisspeptin neurons are mediated, in part, via nuclear-initiated signaling (genomic) mechanisms [90–92]. Also, Kiss1 mRNA expression is greatly increased in the AVPV/PeN following E2 treatment [7]. These findings, combined with previous observations that lesions or antiestrogen implants of the AVPV/PeN or Kiss1 in rodents abrogate the positive feedback effects of E2 [93–96], have led to the hypothesis that E2 acts on AVPV/PeN Kiss1 neurons to induce positive feedback on GnRH and LH secretion. Notably, an E2 treatment that induces an LH surge increases both the h-current and T-type calcium current in the AVPV/PeN Kiss1 neurons [14, 34].

Kisspeptin excites GnRH neurons primarily through activation of canonical transient receptor potential (TRPC) channels and, to a lesser extent, through inhibition of inwardly rectifying K+ channels (fig. 1) [8–11, 78, 97]. In addition, kisspeptin induces a transient elevation of intracellular calcium in GnRH neurons, which is thought to be due to the release from intracellular calcium stores and has been hypothesized to play an important role in the kisspeptin-mediated depolarization [9]. However, the activation of TRPC channels in GnRH neurons by kisspeptin is not affected by buffering intracellular calcium levels by the calcium chelators EGTA or BAPTA or by depleting intracellular calcium stores [11, 78, 98]. Therefore, the release of calcium from intracellular stores does not appear to play a critical role in the kisspeptin-mediated activation of TRPC channels but may be involved in the Ca2+/calmodulin-dependent inhibition of HVA Ca2+ channels [99]. The kisspeptin-activated TRPC current is attenuated by the general calcium channel blocker Cd2+ and by the low-voltage-activated calcium channel blocker Ni2+ but not by the HVA calcium channel blocker amlodipine [11, 98]. This would indicate that low-voltage-activated (T-type) calcium channels may be involved in facilitating TRPC channel opening. However, reducing extracellular calcium to nominally calcium free has no effect on the kisspeptin-activated TRPC current, an indication that very little calcium is needed to enable the opening of TRPC channels in GnRH neurons. This is consistent with the small, but persistent, T-type calcium channel activity (window current) around ~65 mV in GnRH neurons [32]. Therefore, with a sustained depolarization that exceeds that of classical neurotransmitters (e.g., glutamate), kisspeptin excites GnRH neurons primarily through the opening of a TRPC channel that is independent of intracellular calcium store release but appears to be dependent on transient calcium influx through T-type calcium channels (fig. 1). This ensures a fast and yet sustained depolarization of GnRH neurons. Although GnRH neurons express all of the TRPC channel subunits that are found in the brain (i.e., TRPC1, 3, 4, 5, 6, and 7), quantitative PCR analysis shows that TRPC4 is the main TRPC channel subtype in GnRH neurons and is expressed at levels 4-fold higher than TRPC1 and TRPC5 [11, 45]. In fact, TRPC4 mRNA is increased in high E2-treated mice [45]. Phosphatidylinositol-4,5-bisphosphate (PIP2) is an important regulator of TRPC channels, and depletion of PIP2 is required for kisspeptin-induced TRPC channel activation in GnRH neurons [98]. In addition to PIP2 depletion, kisspeptin activation of TRPC channels is also dependent on the non-receptor tyrosine (cSrc) kinase activation (fig. 1), since both global tyrosine kinase inhibitors such as genistein and the spe-
cSrc kinase inhibitor PP2 attenuate (inhibit) kisspeptin currents in GnRH neurons [98], cSrc kinase directly regulates TRPC4 channel activity through tyrosine phosphorylation, which causes rapid insertion of TRPC4 channels into the plasma membrane [100]. Therefore, cSrc appears to be a key signaling molecule in the kisspeptin-mediated activation of TRPC channels in GnRH neurons.

PKC is an important second messenger stimulated by Gq-coupled receptors. However, PKC appears not to be involved in TRPC channel activation in cell lines expressing cloned TRPC channels [101]. Similarly, in GnRH neurons, PKC activation or inhibition has only a minor or no effect on the kisspeptin-induced inward TRPC current [11, 98].

Since the initial studies showing that kisspeptin has prolonged effects on GnRH neuronal activity [8–11], the question has been why is there very little spike frequency adaptation (slowing of action potential firing due to activation of K+ channels) during kisspeptin-induced sustained firing? Recently, it has been shown that kisspeptin reduces spike frequency adaptation and prolongs firing via the inhibition of a calcium-activated slow AHP [8–11]. Kisspeptin currents in GnRH neurons are activated and neuronal input resistance is high [14, 34]. Thus, I_{NaP} tends to amplify the depolarization induced by T-type calcium channels. Most importantly in terms of providing excitatory drive to GnRH neurons, an E2 treatment that produces an LH surge significantly increases both I_h and I_T in AVPV/PeN Kiss1 neurons by 3.4- and 6-fold, respectively [14, 34]. AVP/PeN Kiss1 neurons rest at a relatively positive resting membrane potential of ~55 mV. Thus, some sort of robust inhibitory synaptic input is necessary for reaching hyperpolarized membrane potentials to recruit a critical mass of CaV3.1 (by removing their inactivation) and HCN1 channels for initiating burst generation [14, 34].

In addition, Kiss1 neurons express a persistent Na+ current (I_{NaP}) that is activated ~10 mV negative to the threshold for the transient sodium current in a membrane potential range in which few voltage-gated channels are activated and neuronal input resistance is high [14]. Thus, I_{NaP} facilitates sustained firing through inhibiting an apamin-insensitive I_{AHP} in GnRH neurons (via PKC). The physiological significance is that although single-action potential-generated calcium influx is sufficient to spark the release of classical neurotransmitters, burst firing or tonic stimulation is required for the release of neuropeptides as first eloquently shown by Wakerly and Lincoln [102] in vivo and then in vitro by others [103–105].

The Role of Pacemaker Currents in Kiss1 Neurons

Kiss1 neurons in the AVPV/PeN appear to be presynaptic pacemaker neurons that drive GnRH neurons. At least 50% of GnRH neurons receive monosynaptic input from the AVPV/PeN neurons, some of which have been identified as Kiss1 neurons [106]. Importantly, high-frequency stimulation of AVPV/PeN neurons elicits a delayed excitatory response in GnRH neurons, which can be blocked by the kisspeptin inhibitory peptide 318 [106]. Recently, the development of Kiss1-CreGFP knock-in mice has allowed the targeting of GFP-expressing Kiss1 neurons for more detailed analysis [107] [for a review, see ref. 30]. Both I_h and I_T are prominently expressed in Kiss1 neurons [14, 34], and the Kiss1 neurons also express the corresponding critical transcripts HCN1 and CaV3.1 [14]. In particular, CaV3.1 calcium channels are highly expressed in AVPV/PeN Kiss1 neurons and are exceptionally sensitive to E2 [14; Zhang et al., in preparation].

The presence of a robust T-type calcium current is essential for the high-frequency rebound bursting that is manifested following a hyperpolarizing stimulus in these neurons [14].

Indeed, GABA via GABAB receptors (Gai/o coupled to GIRQ channels) can provide the hyperpolarizing stimulus for generating burst firing [14]. GABA neurons are abundant in the hypothalamus, and the majority of Kiss1 neurons in the AVPV/PeN express GAD67 (glutamic acid decarboxylase, the GABA synthesizing enzyme) [108]. As such, Kiss1 neurons may themselves be an endogenous source of GABA, whose action could be autosynaptic and perhaps be responsible for hyperpolarizing Kiss1 neurons via GABAB receptors. Although further studies are needed to elucidate all of the physiological mechanisms underlying high-frequency burst firing of AVPV/PeN Kiss1 neurons, it is clear that these neurons express the critical channels and receptors that permit E2-dependent burst firing. Therefore, Kiss1 neurons, similar to thalamocortical neurons, express the critical pacemaker conductances, and, as such, Kiss1 neurons have the capacity to generate bursting activity and excite GnRH neurons, which supports the idea that Kiss1 neurons provide the excitatory drive for GnRH surge secretion.
Conclusion

What is critical for the control of GnRH neuronal excitability and ultimately the control of fertility is the hypothalamic circuitry. This ‘circuitry’ not only includes the synaptic input to kisspeptin and GnRH neurons but also the effects of circulating E$_2$, which conveys vital feedback information about reproductive states, on the excitability of these neurons. All AVPV/PeN kisspeptin neurons express the endogenous burst-generating conductances (I$_h$, I$_T$ and I$_{NaP}$) that allow these vital neurons to generate burst firing and release kisspeptin, which in turn excites GnRH neurons that also express I$_h$, I$_T$ and I$_{NaP}$ [22, 32, 48, 106]. A future challenge is not only to identify all of the key channels and signaling molecules and how they are regulated by E$_2$ but also how these channels fit into the kisspeptin-GnRH neuronal circuitry for the generation of burst firing and peptide release.

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