DR STEPHANIE CONSTANTIN (Orcid ID : 0000-0003-0596-9737)
DR SUZANNE MOENTER (Orcid ID : 0000-0001-9812-0497)
DR RICHARD PIET (Orcid ID : 0000-0001-5160-6027)

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The electrophysiologic properties of gonadotropin-releasing hormone neurons

Stephanie Constantin[1,2*], Suzanne M Moenter[3*♦], Richard Piet[4*♦]

* All authors contributed equally and are listed in alphabetical order

[1] Cellular and Developmental Neurobiology Section, National Institute of Neurological Disorders and Stroke, Bethesda, MD 20892-3703, USA

[2] Section on Cellular Signaling, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, Bethesda, MD 20892, USA

[3] Departments of Molecular & Integrative Physiology, Internal Medicine, Obstetrics & Gynecology, and the Reproductive Sciences Program[4], University of Michigan, Ann Arbor, MI, 48109, USA

[4] Brain Health Research Institute & Department of Biological Sciences, Kent State University, Kent, OH, 44242, USA.

♦ **Corresponding authors**: Richard Piet (rpiet@kent.edu) & Suzanne Moenter (smoenter@umich.edu).

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Abbreviations: ADP, afterdepolarization; AHP, afterhyperpolarization; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AVPV, anteroventral periventricular nucleus; c-Src, protein-tyrosine kinase Src; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CAN, Ca^{2+}-activated mixed cation channel; eCB, endocannabinoid; ERβ, estrogen receptor β; GABA, gamma-aminobutyric acid; GPCR, G protein-coupled receptor; GFP, green fluorescent protein; GnRH, gonadotropin-releasing hormone; *hpg*, hypogonadotropic mutant mouse; I_A, A-type K^+ current; I_h, hyperpolarization-activated current; I_{KCa}, Ca^{2+}-activated K^+ current; $I_{KCa\ slow\ AHP}$, Ca^{2+}-activated K^+ current mediating the slow AHP; I_{Kir}, inward rectifying K^+ current; I_{NaP}, persistent Na^+ current; I_T, transient low-voltage-activated Ca^{2+}; KCC2, K^+ Cl^- cotransporter 2; Kiss1r, kisspeptin receptor; LH, luteinizing hormone; ME, median eminence; NKB, neurokinin B; NKCC1, Na^+ K^+ Cl^- cotransporter 1; NMDA, N-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PGE_2, prostaglandin E_2; PIP2, phosphatidylinositol 4,5-bisphosphate; PI4K, phosphatidylinositol 4-kinase; PLCβ, phospholipase β; PSC, postsynaptic current; RFRP-3, RF amide-related peptide 3; sGC, soluble guanylate cyclase; TRP, transient receptor potential channel

1 **Summary**

2 For about two decades, recordings of identified gonadotropin-releasing hormone (GnRH)
3 neurons have provided a wealth of information on their properties. We describe areas of
4 consensus and debate on the intrinsic electrophysiologic properties of these cells, their
5 response to fast synaptic and neuromodulatory input, Ca^{2+} imaging correlates of action potential
6 firing and signaling pathways regulating these aspects. How steroid feedback and development
7 change these properties, functions of GnRH neuron subcompartments and local networks
8 revealed by chemo- and optogenetic approaches are also considered.

9 **Keywords**

10 Fertility, luteinizing hormone, intrinsic properties, action potential, synaptic transmission,
11 kisspeptin

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Introduction

In the half century since GnRH was sequenced, studies using native GnRH decapeptide and antagonist analogs demonstrated the pattern of GnRH release was vital for normal physiology. The hypogonadal (*hpg*) mouse, a natural GnRH knock-out, established this hormone as the critical final output from the central nervous system to regulate the reproductive system through its effects upon the anterior pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone. A rich history of physiologic studies on the central control of reproduction is reviewed in this special issue. In this chapter, we focus in on the neurobiological mechanisms revealed by studies of identified GnRH neurons in brain slices, nasal explants and *in vivo* to help explain how their intrinsic and synaptic properties change with reproductive state, and how these cells are integrated into steroid-responsive networks.

The before time

For the current and next generations of young scientists, for who fluorescently-identified living neurons are a norm, we briefly visit the time before green fluorescent protein (GFP) and other genetically-encoded marker genes. The first reported recordings of putative GnRH neurons were in 1984 by the laboratory of Martin Kelly[1]. A few statistics from that paper are worth noting. Guinea pig cells were recorded in the arcuate nucleus using electrodes containing procion-yellow, allowing *post hoc* identification of recorded cells and, when combined with immunohistochemistry using an anti-GnRH antibody, identification of the recorded cell's phenotype. Of 102 cells recorded, just four were GnRH-immunopositive. It is important to note that the antibody used in these studies was reported the same year to non-specifically label neurons in the arcuate nucleus in the rat[2]. A later report, using a similar approach with a validated GnRH antibody, revealed that GnRH neurons were sensitive to estradiol and opioid receptor agonists[3]. Later efforts to identify recorded GnRH neurons *post-hoc* employed reverse-transcriptase polymerase chain reaction on the cytosol from single neurons collected after patch-clamp recordings to detect GnRH transcripts[4]. These labor-intensive methods of identification were supplanted by promoter transgenic approaches and have since been abandoned.

Identified GnRH neurons

Near the turn of the century, promoter-driven genetic approaches made it possible to identify specific cell types in mammalian tissue. People studying GnRH neurons were among the first to take advantage of this because of the difficulties of recording from this small, anatomically-

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diffuse population[5]. Early success was likely facilitated by the strength and cell-specificity of the GnRH promoter[6]. GnRH neurons have been identified in living tissue using β-galactosidase substrates[7], Ca^{2+} indicators[8-13] or variations of GFP derived from *Aequorea victoria* in mice[14-16], rats[17], and medaka fish[18]. GFP identification is the method most used and will be the main source of studies covered in this review. Because of space limitations, the reader is referred to the article on non-mammalian systems for discussion of the many substantial contributions to GnRH neuron electrophysiology from work on teleost fish.

Intrinsic properties of GnRH neurons

For explanations of recording approaches, the reader is referred to these reviews[19-21]. Initial studies of GnRH neuron properties suffered from a lack of consistent and rigorous methodology that had been established for patch-clamp investigation; essentially no lab in the field escaped errors ranging from use of inappropriate pipette solutions, to failure to report quality parameters allowing the reader to evaluate data integrity, and to failure to explain how properties (e.g., action potential threshold, resting potential) were defined; these limitations resulted in variable data. We have chosen to focus our review on work done after these initial growing pains.

The majority of GnRH neurons recorded in brain slices exhibit spontaneous action potential firing that is often arranged into short term patterns called bursts, and an array of voltage-gated conductances typical of many neurons, including tetrodotoxin-sensitive Na^{+}[22], multiple K^{+}[10, 23-25], Ca^{2+}[26, 27], hyperpolarization-activated[28], and transient receptor potential (TRP)[29-31]. These conductances enable action potential firing, regulate firing patterns and mediate membrane responses to inputs. From this rather standard list, we highlight four aspects that are important to GnRH neurons: action potential shape, firing patterns, currents regulating action potential initiation, and Cl^{-} homeostasis.

The GnRH neuron action potential: GnRH neurons are often spontaneously active from a basal potential between -75 and -60 mV in brain slices[14, 32]. The GnRH neuron action potential is majestic (Figure 1A). In current-clamp recordings with a pipette solution mimicking intracellular milieu and amplifier settings allowing more precise characterization of these rapid events (e.g., high-frequency (≥10kHz) acquisition and filtering), the amplitude of the first action potential induced by current injection routinely achieves 90 mV from action potential threshold (defined as 1V/s)[23, 33]. Despite this amplitude, the spikes are over quickly, with full width at half maximum averaging under 0.8ms. Spikes are followed by a pronounced afterhyperpolarization potential (AHP) of about 25mV, and show little evidence of frequency adaptation. Finally, a slow (peaking

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76 1-2s post threshold) afterdepolarizing potential (ADP) follows the AHP in many GnRH neurons
77 and can contribute to ongoing firing[34-36]. These characteristics produce a spike profile that
78 distinguishes these cells and is consistent among GnRH neurons (Figure 1). Where these
79 action potentials are initiated is an interesting question. Simultaneous recordings of proximal
80 dendrites and soma indicate that action potentials are initiated in dendrites in some GnRH
81 neurons[37, 38]. Immunoreactivity for ankyrin G, a protein often linked to the site of action potential
82 initiation, revealed that it is typically located within 150 μm from the soma in GnRH neurons[39].
83 Consistent with action potential initiation in the dendrites, ankyrin was located in one of the
84 dendrites of most (75%) GnRH neurons, and in the axon in a small proportion of these cells[39].
85 Future work can investigate if the site of initiation is constant for a particular neuron, or if it is
86 regulated by the type of input being received, and/or can be in other regions such as the
87 terminals.

88 *GnRH neuron firing patterns*: Spontaneous GnRH firing is not dependent upon fast synaptic
89 transmission[40], GnRH itself[41] or kisspeptin[42], a major activator of GnRH neurons. An interesting
90 feature of spontaneous GnRH neuron firing is that it exhibits short-term (burst firing, Figure 1B)
91 and long-term patterning (Figure 1C)[10, 43-46]. GnRH neuron bursts have a longer intraburst
92 interval (~150-400ms) than neurons in the thalamus and cortex[47, 48] or even magnocellular
93 neuroendocrine cells[49], and bursts are short, with typically two to eight spikes per burst[50]. This
94 low spontaneous frequency is curious because GnRH neuron firing can be driven at much
95 higher rates by current injection, suggesting the ionic conductances of these cells are not in and
96 of themselves limiting[23, 36]. As reviewed [20, 21], GnRH neurons recorded in brain slices may be
97 quiescent or spontaneously firing. While most GnRH neurons exhibit irregular bursting, 1-2% of
98 these cells exhibit parabolic bursting riding on marked slow (~0.05Hz) oscillations in membrane
99 potential[51]. Burst firing is linked with peptide release in magnocellular neurons[52] and the higher
100 frequency activity within bursts is likely important for GnRH release[53]. Long-term alterations
101 between peaks and nadirs in firing rate have been observed in both sexes; the frequency of
102 these peaks resembles that of LH release *in vivo* and is likewise modified by gonadal steroid
103 feedback[45, 46]. Short- and long-term patterns may be related. One investigation demonstrated
104 that bursts within a GnRH neuron maintain consistent characteristics between peaks and nadirs,
105 but the interburst interval increases during nadirs[43]. Further studies of this relationship and its
106 modulation, the underlying ionic conductances and excitation-secretion coupling in GnRH
107 neurons are required.

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108 Interestingly, despite demonstration of synaptic connections, bundling and even cytoplasmic
109 bridges among GnRH neurons[54-56], there are not convincing data that GnRH neurons in brain
110 slices are coordinated with one another from dual patch-clamp recordings[51, 57] or calcium
111 imaging[9]. It is important to point out that few attempts have been published despite the
112 fascination of the field with pulsatile release and the presumption that this involves some sort of
113 coordination among these cells. Such studies are technically difficult and the possible caveats of
114 looking for coordination within a reduced slice preparation are many; for example, if connecting
115 fibers are severed during slice preparation, a false negative result will be obtained. This is an
116 important area for future studies, which might take advantage of *in vivo* methods that can
117 simultaneously monitor multiple cells.

118 *Ionic conductances of GnRH neurons*: GnRH neurons express a typical set of voltage-gated ion
119 channels that enables action potential firing. Channels that can be active at subthreshold
120 membrane potentials help sculpt spike initiation. GnRH neurons have a very large fast-transient
121 (A-type) K^+ current that fights depolarization[24, 58], and may contribute to both the narrow width of
122 the spike and the large amplitude AHP. Ca^{2+}-activated K^+ currents in these cells appear to
123 regulate intra and interburst intervals[10] and the amplitude of the ADP[34]. Opposing these outward
124 currents are several smaller magnitude inward currents, specifically hyperpolarization-activated
125 or I_h[24, 28], low-voltage-activated Ca^{2+} or I_T[27], a Ca^{2+}-activated mixed cation or CAN[59], and a
126 tetrodotoxin-sensitive persistent Na^+ current or I_{NaP}[34, 59]. Several of these latter conductances
127 along with Ca^{2+}-activated K^+ currents have been implicated in both burst firing and pacemaker
128 activity in other neurons. Both I_T and I_h contribute to rebound firing after the termination of a
129 hyperpolarizing current injection and CAN and I_{NaP} augment the ADP in these cells.
130 Interestingly, many of these are regulated by estradiol in a manner that would tend to increase
131 GnRH neuron activity during positive feedback, thus may be important in the achievement of
132 that state.

133 *Cl^- homeostasis*: An interesting feature of GnRH neurons that was once controversial is their
134 maintenance of higher intracellular Cl^- levels than is typical of most adult neurons. There is now
135 consensus that the Cl^- reversal potential is depolarized relative to the action potential threshold
136 in these cells[60, 61]. As a result, activation of γ-aminobutyric acid type A ($GABA_A$) receptors, for
137 which Cl^- is the main permeable ion, depolarizes these cells and can trigger action potential
138 firing in preoptic GnRH neurons in rodents and terminal nerve GnRH neurons in teleost fish[60-62].
139 High Cl^- levels are likely attributable to continuing activity of the Cl^--accumulating cation
140 cotransporter NKCC1 into adulthood, in contrast to most neurons in which the Cl^--depleting

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141 cotransporter KCC2 become dominant[63]. At the time of this discovery, GnRH neurons were
142 among a small population of primarily sensory neurons for which excitatory GABA was
143 identified, but this is now recognized in other hypothalamic cells[64, 65] and throughout the adult
144 brain[66].
145
146 The induction of action potentials in response to GABA, widely recognized as the primary mode
147 of fast inhibition in the brain, raises several interesting questions about GnRH neurons. First, is
148 this a phenomenon limited to the perisomatic region where measurements have been made?
149 Differential distribution of Cl^- cotransporters in subcompartments has been observed[67] and may
150 lead to regional functional changes[68]. While expression of NKCC1 is prominent, GnRH neurons
151 do express KCC2, but subcellular localization requires further investigation[61, 69]. Second, is
152 regulating Cl^- homeostasis a possible mechanism for controlling GnRH activity? In this regard,
153 the excitatory response to GABA appears to persist regardless of age, gonadal status, or sex,
154 even in mice in negative energy balance[61, 70]. Reduced excitatory response to GABA was
155 observed in prepubertal mice exposed to androgen *in utero*, a model used to generate a
156 phenotype resembling polycystic ovary syndrome; this blunting was not attributable, however, to
157 a change in the Cl^- reversal potential[71]. In the models studied, regulating intracellular Cl^- thus
158 does not appear to be a major point of physiologic control. Third, is it GABA alone or does the
159 initial depolarization initiated by GABA activate other inward currents that boost the depolarizing
160 response[8, 72]? Fourth, what inhibits GnRH neurons? While no 'fast' synaptic inhibition is known,
161 several neuromodulators, discussed below, may do so.

162 **Fast synaptic transmission to GnRH neurons**

163 In addition to having a non-standard response to activation of the $GABA_A$ receptor, the
164 frequency of spontaneous fast synaptic transmission to GnRH neurons is low compared to, for
165 example, cortex[73], and even other hypothalamic neurons regulating reproduction[74]. Fast
166 synaptic transmission detectable at the cell soma is primarily GABAergic and glutamatergic;
167 blocking α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate
168 (NMDA) and $GABA_A$ receptors eliminates postsynaptic currents (PSCs) and no response is
169 observed to local application of glycine, another Cl^--permeable ionotropic receptor[70].
170 Immunoreactivity for vGLUT2 and vGAT, markers of putative glutamatergic and GABAergic
171 synaptic appositions, respectively, identified the proximal dendrite as the main input target[75]. Of
172 note, knockout of subunits of either ionotropic GABA or glutamate receptors have had little

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173 effect on reproduction[76, 77]. This may indicate fast synaptic transmission is not critically
174 important, or that functional compensatory mechanisms were initiated by the knockout.

175 Curiously, the density of putative glutamatergic synapses detected with immunostaining
176 methods is twice that of putative GABAergic inputs on GnRH neuron somata and proximal
177 dendrites[75] . Functional measures of glutamatergic synaptic transmission directly to GnRH
178 neurons made with patch-clamp, however, indicates it is infrequent. A low percentage of cells
179 responding to glutamate receptor agonists, particularly NMDA receptor agonists, was noted in
180 the first recordings of GFP-identified cells[14] and confirmed in subsequent measurements of
181 glutamatergic transmission[78, 79] and of percent of cells responding to bath application of receptor
182 agonists[8, 80]. The spontaneous AMPA-mediated PSC frequency in mice is typically under 0.1Hz.
183 Suppression by estradiol negative feedback has been reported[78, 79], but should be interpreted
184 with caution as small changes in transmission rate that may not be biologically important can
185 result in large fold changes. Of note, higher rates of AMPA-mediated transmission to GnRH
186 neurons occur in rats, with an additional increase during estradiol positive feedback on
187 proestrus, when Ca^{2+}-permeable AMPA receptors are inserted into the membrane[81]. Positive
188 feedback is also associated with more spines, considered a site for glutamatergic synaptic input,
189 on murine GnRH neurons[82] and with changes in expression of genes encoding glutamate
190 receptor subunits in these cells[83], but it is important to point out that it is not known if these
191 anatomical and molecular changes are associated with functional differences.

192 Despite these latter observations, the low rates of glutamatergic transmission observed may call
193 into question its relevance, but two points are worth making. First, the available data on
194 spontaneous transmission is from somatic recordings. Input occurring at a distance from the
195 soma, such as those impinging onto the very distal processes of GnRH neurons[84], may have
196 either decayed before detection by the somatic recording electrode or been eliminated by brain
197 slice preparation. Second, GnRH neurons may have more glutamatergic input than is
198 appreciated as increasing neurotransmission within a brain slice by blocking K^+ channels
199 markedly increases glutamatergic transmission[79]; this may indicate low initial probability of
200 release at these synapses under most conditions.

201 GABAergic transmission is more prevalent but still rarely exceeds 2Hz, being more typically
202 under 1Hz. Several studies demonstrated that the frequency of GABAergic transmission to
203 these cells correlates with what is expected for GnRH/LH output for that physiologic state.
204 Progesterone negative feedback, estradiol negative feedback and negative energy balance

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205 reduce GnRH/LH pulse frequency and reduce the frequency and sometimes amplitude of
206 GABAergic transmission to GnRH neurons[70, 85-87]. In contrast, states that increase GnRH/LH
207 release, such as mild androgen elevation and estradiol positive feedback typically[85-88], but not
208 always[89], correlate with increased GABAergic transmission and PSC amplitude. Consistent with
209 these correlational studies, electrical stimulation of the anteroventral periventricular nucleus
210 (AVPV) induces GABAergic and glutamatergic evoked PSCs in GnRH neurons[90]; GABA was
211 predominant and required for AVPV stimulation to elicit firing of most GnRH neurons.
212 Optogenetic stimulation specifically of AVPV kisspeptin neurons, which use GABA as a
213 cotransmitter, evokes $GABA_A$ receptor-dependent firing in GnRH neurons[91]. Finally, blocking
214 $GABA_A$ receptors during *in vivo* recordings of GnRH neurons consistently reduced firing[92].
215 These findings consistently point to the action of GABA via the $GABA_A$ receptor as being
216 excitatory to GnRH neurons. Activation of $GABA_B$ receptors, in contrast, inhibits GnRH neuron
217 electrical activity[90, 93, 94], indicating GABA may bidirectionally control GnRH neuron firing
218 depending on the receptor activated.

219 It should be noted that the rates and/or patterns of glutamate and GABA synaptic transmission
220 to GnRH neurons *in vivo* may be substantially different, as brain slice preparation removes
221 many afferent soma. Changes in synaptic transmission detected in brain slices from animals in
222 different physiological or pathological states nevertheless indicate functional synaptic plasticity
223 and are, as such, meaningful. Identification of where fast synaptic inputs originate and the
224 reproductive states and/or phases of a pulse cycle when these inputs are active are interesting
225 future directions.

226 **Recording GnRH neurons *in vivo***

227 The brain slice recordings used in work discussed above and below allow targeting of individual
228 cells and mechanistic studies, but also have limitations. Perhaps most prominent is the reduced
229 nature of these preparations, which maintains only local circuitry, removing distal input soma
230 and even some proximal ones. While this can be mitigated to some extent by using different
231 slice orientations[86, 95], this is still far from the integrative *in vivo* situation in which the regulation
232 of reproduction typically occurs. Recording from GnRH neurons *in vivo* avoids this limitation.
233 This is challenging because the few GnRH neurons are scattered and deep relative to the
234 surface of the brain. One paper has accomplished this by using transpharyngeal approach to
235 expose and record from GnRH neurons under anesthesia[92]. The only area in the mouse brain
236 where GnRH-GFP neurons are superficial enough to be targeted with a recording pipette is

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237 dorsal to the optic chiasm at the ventral-most surface of the anterior hypothalamic area, along
238 the posterior cerebral artery. Due to the nearby beating artery, it was only possible to achieve
239 short duration targeted extracellular recordings. Despite these challenges, this heroic effort
240 nonetheless provided important confirmatory evidence of several observations made in brain
241 slices. Specifically, GnRH neuron firing is variable with spontaneous bursts, and responses to
242 GABA, glutamate and kisspeptin-10 are all excitatory.

243 In sum, there are several aspects of GnRH neuron electrophysiology that are quite consistent
244 among cells. These include basic properties such as a relatively high input resistance
245 (compared to, for example, cortical and hippocampal pyramidal neurons), the observation of
246 spontaneous firing activity, grouping of action potentials into bursts in most cells, action potential
247 properties when recorded with physiologic chloride levels, a predominance of fast GABAergic
248 over fast glutamatergic input in recordings made in the perisomatic region and a
249 depolarizing/excitatory response to GABA. Burst properties can exhibit considerable
250 heterogeneity among cells and the importance of this for neuroendocrine output is something
251 that requires further investigation.

252 **Neuromodulation of GnRH neuron electrical activity**

253 Since the first recordings of GnRH neurons, a large repertoire of neuromodulators altering the
254 electrical activity of GnRH neurons has been compiled. For space reasons, we focused on non-
255 conventional neuromodulators that have emerged as important regulators of GnRH neuron
256 activity, and on RF-amide neuropeptides. For detailed accounts of GnRH neuron regulation by
257 neurotransmitters and neuropeptides, readers are referred to excellent recent reviews[96, 97].

258 ***Non-traditional neuromodulators***

259 Studies of endocannabinoid, nitric oxide and prostaglandin E_2 regulation of GnRH neuron
260 electrical activity are relatively recent. These neuromodulators are not stored in vesicles but
261 rather produced on-demand, acting directly or indirectly on GnRH neurons, or as retrograde
262 transmitters synthesized by GnRH neurons to regulate synaptic transmission.

263 *Endocannabinoids (eCB)*: GnRH neuron membrane potential depolarization or activation of
264 receptors stimulates synthesis and release of 2-arachidonoylglycerol (2-AG), which acts on
265 presynaptic type-1 cannabinoid receptors to decrease GABA release[98-101]. eCB-dependent
266 suppression of GABA release occurs in mice of both sexes and may occur constitutively, at
267 least in brain slices[98, 99, 101]. GnRH neurons also synthesize anandamide, which may signal via

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268 TRP vanilloid channels to suppress constitutive eCB signaling from GnRH neurons[30, 31].
269 Importantly, estradiol via estrogen receptor β (ERβ) rapidly promotes 2-AG synthesis,
270 subsequent suppression of GABA release and decreased GnRH neuron firing in metestrous
271 females[100], suggesting a role of retrograde eCB suppression of GABA release as one
272 mechanism of estradiol negative feedback.

273 *Nitric oxide (NO)*: The gaseous neuromodulator NO may affect GnRH neuron electrical activity
274 through multiple mechanisms with opposing effects. The "NO donor" L-arginine directly
275 suppresses action potential firing in >90% of GnRH neurons from both sexes via neuronal NO
276 synthase (nNOS)-dependent NO synthesis, modulation of soluble guanylyl cyclase (sGC),
277 subsequent suppression of a depolarizing plateau potential and activation of a K^+ current[102]. In
278 addition to these direct inhibitory effects, NO produced in response to activation of specific
279 receptors increases GABA and glutamate release, indirectly increasing GnRH neuron activity[31,
280 103, 104]. Of note estradiol, acting at ERβ, rapidly increases neurotransmitter release and action
281 potential firing in GnRH neurons by mobilizing this pathway in proestrous female mice,
282 suggesting a role in estradiol positive feedback[104]. NO may also directly alter GnRH neuron
283 excitability by accelerating recovery from prior exposure to kisspeptin-10, thereby enabling
284 repeated responses to this neuropeptide (see below)[105] (Figure 2). Whether or not GnRH
285 neurons synthesize NO is controversial. Neither nicotinamide adenine dinucleotide phosphate
286 (NADP)-diaphorase[102] nor nNOS[106] are detectable in GnRH neurons, arguing against synthesis.
287 In contrast, pharmacological experiments and detection of *Nos1* mRNA and nNOS
288 immunoreactivity at the ultrastructural level in GnRH neurons argues for synthesis[31], opening
289 the possibility NO acts as a retrograde or neuromodulatory signal.

290 *Prostaglandin E2 (PGE_2)*: PGE_2 directly depolarizes the membrane potential and increases
291 action potential firing of most GnRH neurons regardless of sex or estrous cycle stage. These
292 effects are mediated via the prostaglandin EP2 receptor, adenylyl cyclase and protein kinase A
293 to open a non-selective cation channel[107]. Ambient PGE_2 may help control GnRH neuron
294 membrane potential and spontaneous firing as blocking its synthesis reduces activity in brain
295 slices[107]. Evidence indicates that astrocytes are a primary source of PGE_2 release[99, 107, 108].

296 These nontraditional neuromodulators directly and indirectly regulate GnRH neurons.
297 Interestingly, there may be crosstalk among these pathways as PGE_2 is required for
298 depolarization-induced, eCB-mediated, suppression of GABA transmission to GnRH neurons[99].

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299 Moreover, additional neuropeptides and hormones may recruit these neuromodulators to
300 produce their effects on GnRH neuron firing, as is the case of eCBs and NO[31, 101, 103].

301 ***RF-amide peptides***

302 Exogenous kisspeptin-10 drives with high potency (low nM EC_{50}) prolonged membrane potential
303 depolarizations, action potential firing and increased intracellular Ca^{2+} ($[Ca^{2+}]_i$) fluctuations in
304 GnRH neurons, as well as GnRH secretion[29, 92, 105, 109-116]. Kisspeptin receptor (Kiss1r)
305 expression in GnRH neurons is both necessary and sufficient for kisspeptin-10 effects on GnRH
306 neuron firing and LH secretion, for puberty and overall fertility[42, 117]. Kisspeptin-10 may also have
307 indirect actions by increasing GABA and glutamate release on GnRH neurons, and by
308 modulating NO-synthesizing neurons in the preoptic area[106, 110]. Kisspeptin-10 actions in GnRH
309 neurons are mediated through inhibition of multiple K^+ currents, including inward-rectifying, A-
310 type and the K^+ current mediating the slow AHP[29, 110, 111, 118-120], and via activation of a non-
311 selective cationic current likely mediated by TRP canonical (TRPC) channels[29, 118]. The role of
312 $[Ca^{2+}]_i$ in GnRH neuron membrane responses to kisspeptin is somewhat unclear. Although
313 kisspeptin-10 increases $[Ca^{2+}]_i$ in GnRH neuron somata, dendrites and terminals[12, 112, 118],
314 kisspeptin-10-induced excitation is resistant to Ca^{2+} buffering[111, 121] and to depletion of Ca^{2+}
315 stores[112, 121] (Figure 2). Interestingly, kisspeptin-10-evoked increases in $[Ca^{2+}]_i$ are required for
316 GnRH secretion at the median eminence (ME)[116, 122]. Activation of phospholipase Cβ (PLCβ)
317 and c-Src tyrosine kinase are necessary for kisspeptin-10 effects[29, 118, 121]. PLCβ-mediated
318 hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), which inhibits TRPC channels in a
319 subset of GnRH neurons, and the ensuing PIP2 depletion may contribute to prolonged
320 activation of these channels[121] (Figure 2).

321 The effects of *exogenous* kisspeptin-10 last many minutes and cannot be repeated at the GnRH
322 neuron soma in brain slice recordings[111, 118]. The prolonged nature of this response is likely the
323 result of PIP2 depletion because inhibition of phosphatidylinositol 4-kinase (PI4K), which
324 synthesizes PIP2, prolongs kisspeptin-10 excitation of GnRH neurons[105, 121]. Of interest in this
325 regard, NO increases PI4K activity[123] and thus PIP2 synthesis and thereby both shortens
326 kisspeptin-10-mediated excitation and enables subsequent kisspeptin-10 stimulations of GnRH
327 neurons[105] (Figure 2). NO-mediated recovery from kisspeptin excitation may provide an "off"
328 signal terminating kisspeptin-10-induced GnRH neuron activity/secretion and might facilitate
329 periodic kisspeptin signaling to promote pulsatile GnRH secretion. Of note, because NO
330 diffusion can potentially affect many GnRH neuronal elements, NO-modulation of kisspeptin

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331 signals might help coordinate GnRH secretion between many terminals[105]. It should be noted
332 that *endogenous* kisspeptin actions may be repeatable as consecutive optogenetic stimulations
333 of kisspeptin neurons at ~10-minute intervals repeatedly evoke action potential firing in GnRH
334 neurons[91] and LH secretion *in vivo*[124], suggesting that "on-off" kisspeptin effects might require
335 sustained activation of the kisspeptin receptor and associated signaling pathways. The impact
336 of NO on kisspeptin signaling has only been assessed so far at the GnRH neuron somata[105] and
337 it remains to be seen if kisspeptin signaling in the ME is similarly regulated by NO. This is
338 particularly important as GnRH neuron subcompartments may operate independently from one
339 another *in vivo*[125] (see below). Lastly, the source of NO remains unknown. Bearing in mind the
340 controversy around nNOS expression discussed above, GnRH neurons remain possible
341 candidates. Alternatively, nNOS-expressing neurons are found in the preoptic area, some of
342 which, interestingly, express Kiss1r, and arcuate nucleus[106].

343 RF amide-related peptide 3 (RFRP-3), the mammalian counterpart to avian gonadotropin-
344 inhibiting hormone[126], inhibits GnRH neuron $[Ca^{2+}]_i$ oscillations in nasal explants and decreases
345 the firing rate of 50-70% of prepubertal and adult male and female GnRH neurons via binding to
346 neuropeptide FF receptor 1 and activation of K^+ currents[127-129]. This inhibition transiently
347 opposes the prolonged actions of kisspeptin-10[128, 129], in keeping with the inhibitory effects of
348 RFRP-3 on GnRH secretion in brain slices[122, 130] and LH secretion *in vivo*[131, 132], making RFRP-3
349 a candidate "off" signal. It should be noted, however, that ≈10% of GnRH neurons increase their
350 firing in response to RFRP-3[127] and that central administration of RFRP-3 may stimulate LH
351 secretion in males[132].

352 **Functional interrogation of native circuits**

353 While the studies reviewed above have been very valuable, at least some of these results
354 should be interpreted with caution. Bath or local agonist and antagonist applications provide
355 important information but have limited interpretative value due to off-target actions at all binding
356 sites present within the preparation. Moreover, many neuromodulators are expressed in several
357 brain areas and these neuronal populations may express multiple cotransmitters and/or be
358 involved in completely different physiological functions. Inferring the functional impact of specific
359 neuromodulatory populations thus requires a combination of agonist-induced changes in
360 electrical activity/$[Ca^{2+}]_i$, neuronal tract-tracing and functional circuit interrogation with opto-
361 and/or chemogenetics.

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362 Applying these approaches to the control of GnRH neurons by kisspeptin revealed that
363 kisspeptin neurons in the preotic area in females potently stimulate GnRH neuron firing and that
364 a subset of preoptic kisspeptin neurons further excite GnRH neurons by co-releasing GABA[91].
365 These types of studies also unveiled an unsuspected indirect mode of communication between
366 arcuate kisspeptin neurons and GnRH neurons, via projections to and stimulation of preoptic
367 kisspeptin neurons; whether this pathway substantially contributes to GnRH and LH secretion *in*
368 *vivo* is not known[133]. It is interesting to note that direct interrogation of the arcuate kisspeptin-to-
369 GnRH pathway in brain slices still eludes researchers, probably because, in rodents, the former
370 neurons project predominantly to the GnRH neuron distal processes near and in the ME[134], a
371 compartment almost inaccessible for conventional electrophysiology. Further studies aimed at
372 determining direct inputs to the GnRH neurons, and their impact on these cells' activity are
373 needed to gain deeper understanding of the regulation of GnRH release patterns.

374 **Function of GnRH neuron compartments**

375 Another area that has progressed over the past 10-15 years has been the functional definition of
376 GnRH neuron compartments. Most patch-clamp recordings have been made of the soma, but
377 dual recordings of soma and proximal processes with dendrite-like morphology revealed that
378 these processes can initiate and actively propagate action potentials in some GnRH neurons[37,
379 38, 135]. In rodents, most GnRH neuron projections to the median eminence exhibit features such
380 as presence of spines, synaptic input, larger diameter and ultrastructural profiles together
381 reminiscent of dendrites[84, 135, 136]. Because of these attributes, which collectively suggest these
382 projections share dendritic and axonal properties, they have been referred to as "dendrons"[135].
383 Near the median eminence (ME), GnRH neuron processes often branch out into multiple axon-
384 like profiles (i.e., smaller diameter) with putative secretory terminals in proximity to blood
385 vessels[84, 135]. Determining if these unusual features of GnRH neuron projections exist beyond
386 mice and rats will be an important future research direction[137].

387 Understanding GnRH neuron subcompartments is particularly relevant because several studies
388 suggest functional differences may exist among GnRH somata and/or proximal processes in the
389 preoptic area and GnRH neuronal elements near the ME. *In vivo* opto- and chemogenetic
390 suppression of GnRH neuron activity in behaving mice revealed that distal GnRH neuron
391 projections may operate independently of activity in their somata and proximal dendrites, and,
392 further, that these latter regions may be sufficient to control pulsatile, but not surge LH
393 secretion[125]. In brain slice studies to look at mechanisms, GnRH release from the perisomatic

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394 and terminal regions is differently regulated[138]. In agreement with this, distal projections near the
395 ME are the overwhelming site of arcuate kisspeptin neuron inputs to GnRH neurons in
396 rodents[134]. This region responds to local kisspeptin-10 application in an action potential-
397 independent manner with increases in $[Ca^{2+}]_i$ and GnRH release[12, 13, 138]. Neurokinin B (NKB),
398 dynorphin A and glutamate, co-expressed in arcuate kisspeptin neurons, do not alter $[Ca^{2+}]_i$
399 dynamics in the distal processes of GnRH neurons, but NKB application to the ME induces
400 GnRH release in a kisspeptin-independent manner[12, 13, 138]. Differences in kisspeptin-10-induced
401 signaling might also exist between GnRH somata and distal processes[12, 122]. These
402 observations are consistent with those made in medial basal hypothalamic preparations from
403 rodent and sheep, which contain arcuate kisspeptin but very few GnRH neuron somata. These
404 preparations release GnRH in response to kisspeptin[114-116] and, in rodents, can release GnRH in
405 a pattern reminiscent of pulsatile LH secretion *in vivo*[139]. This suggests arcuate kisspeptin
406 neurons and their projections to the distal processes of GnRH neurons might be sufficient to
407 support pulsatile secretion. An even more reduced preparation of isolated rat ME exhibited
408 episodic release[140], perhaps indicating only the very distal processes of both GnRH and
409 kisspeptin neurons are needed. Together these observations suggest specialization of different
410 regions of the GnRH neurons; understanding how various neuromodulators influence these
411 functional compartments is a rich area for future studies.

412 **Development of GnRH neuron properties**

413 *Early GnRH secretion*: The ability to release GnRH in a pulsatile manner is a core function of
414 these neurons. GnRH is detectable before these cells migrate from the olfactory placode and
415 neurosecretion appears to arise early in their development[130]. Sexual differentiation of the brain
416 requires a postnatal testosterone surge; this may rely upon GnRH secretion immediately after
417 birth[141], although other work suggests this may be GnRH independent[142]. In mice, connections
418 from the putative pulse generator arcuate kisspeptin neuron and GnRH neurons are established
419 before birth[143, 144]. Consistent with this, fetal MBH isolated from humans[145] and non-human
420 primate[146] release GnRH pulses, as do GnRH neurons derived from primate and rodent
421 olfactory placodes[147-150]. Prenatal preoptic tissue containing GnRH neurons restores LH pulses
422 and/or gonadal function models lacking endogenous GnRH including lesioned adult female
423 monkeys[151] and rats[152] and *hpg* male mice[153, 154], suggesting prenatal tissue either has episodic
424 release as an inherent function or can mature into this role.

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425 Olfactory placode-derived GnRH neurons were an early model used to decipher the
426 mechanisms underlying pulsatility and other aspects of GnRH physiology. An important
427 advantage of this preparation is that the whole GnRH neurons is present, unlike in most brain
428 slice preparations, which cut processes. Ca^{2+} imaging was used to assess both GnRH neuron
429 function and coordination. Bursts of action potentials occur concomitantly with $[Ca^{2+}]_i$ oscillations
430 in placodal GnRH neurons and GnRH neurons in brain slices from adults[10, 12]. Increases in
431 $[Ca^{2+}]_i$ are often equated to action potential firing but, while often related[10, 12], this is not
432 technically accurate. Fluctuations in $[Ca^{2+}]_i$ can reflect any of several phenomena, including
433 changes in action potential-dependent or subthreshold Ca^{2+} entry, receptor-mediated Ca^{2+} influx
434 and/or Ca^{2+} release from internal stores. An important advantage to Ca^{2+} imaging is the ability to
435 monitor simultaneously several cells. In addition, Ca^{2+} imaging makes possible the recording of
436 subcompartments, such as GnRH neuron terminal regions, that are not readily accessible with
437 electrophysiology.

438 Ca^{2+} imaging of primate and mouse GnRH neurons in placode cultures revealed GnRH neurons
439 exhibit $[Ca^{2+}]_i$ oscillations at a higher frequency than is typical for LH release. These high
440 frequency oscillations are typically not coordinated among cells. Intriguingly, however, periodic
441 coordination of these higher frequency oscillations occurred at a frequency similar to LH
442 release; further work showed this coordination is correlated with GnRH pulses[150, 155, 156]. These
443 cultures are devoid of neuronal CNS inputs, suggesting pulsatile GnRH secretion is an
444 endogenous property. Supporting this postulate, the coordination of $[Ca^{2+}]_i$ oscillations in
445 primate placodal cultures is followed by a delay in resumption of $[Ca^{2+}]_i$ oscillations. But there
446 are several things to consider before this postulate is accepted. First, increased frequency of the
447 high-frequency oscillation can be driven by neuromodulators, e.g., GABA[156, 157] and estradiol[158,
448 159]; this increased frequency is associated with increased coordination of oscillations, raising the
449 question of whether this is merely an increase in mathematical probability. Second, non-
450 neuronal cells in placodal cultures are also coordinated with GnRH neuron $[Ca^{2+}]_i$ fluctuations
451 and GnRH release[160]. Third, glial cells in placodal cultures express gap junctions and blocking
452 these reduces coordination and GnRH release[161]. Consistent with this, the putative
453 gliotransmitter ATP induces coordinated elevations in $[Ca^{2+}]_i$ in both GnRH neurons and non-
454 neuronal cells[162]. Fourth, GnRH pulses in placodal cultures develop over time[150], in parallel with
455 the network surrounding GnRH neurons[163]. While these mechanistic questions remain to be
456 resolved, it is clear that GnRH neurons are equipped with the exocytotic machinery for
457 peptidergic secretion and can sustain pulsatile secretion early on.

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458 *Development of GnRH firing and signaling properties*: The literature on the intrinsic properties
459 of GnRH neurons before adulthood is sparse but the firing activity of GnRH neurons before
460 weaning might shape their adult properties[164]. Placodal GnRH neurons display action potentials
461 and are equipped with Na^+, K^+ (delayed rectifier and A-type), and Ca^{2+} (high- and low-voltage
462 activated) conductances[165, 166]. Recordings of GnRH neurons from prepubertal GnRH-GFP mice
463 demonstrate adult-like bursting characteristics develop early and that the mean firing rate is
464 developmentally regulated[44, 50]. Placodal and adult GnRH neurons exhibit other consistent
465 features including excitation by activating $GABA_A$ receptors[156, 167], and less influence of
466 glutamatergic signaling[8, 9]. Early on, GnRH neurons also express G-protein-coupled receptors
467 (GPCRs) and their coupling partners ($G_{i/o}$, $G_{q/11}$, G_s). $G_{i/o}$-coupled receptors provide a robust
468 inhibition, directly via G-protein-gated inwardly rectifying K^+ channels[94, 168, 169]. $G_{q/11}$-coupled
469 receptors provide a robust excitation, via PLC and downstream effectors[29, 112, 118]. In contrast,
470 G_s-coupled receptors provides a mild excitation, via protein kinase A and downstream
471 effectors[11, 120, 170]. Kisspeptin-10 potently elicits GnRH release from placodal cultures[168],
472 neonatal to juvenile[171] and adult preparations[114]. Importantly, the response of GnRH neurons to
473 kisspeptin-10 increases during development[109, 130]. The complexity of GnRH neuron cell
474 signaling described above can be appreciated with the activation of $G_{q/11}$-coupled kisspeptin
475 receptor in both placodal and adult GnRH neurons[29, 110, 112, 118-120]. Many GPCRs identified in
476 adult GnRH neurons[172] have been found in placodal GnRH neurons and linked to a signaling
477 pathway modulating the frequency of GnRH $[Ca^{2+}]_i$ oscillations[162, 168, 173]. The close signaling
478 parallel between placodal and adult GnRH neuron highlights the precocity of GnRH neurons.

479 **Conclusions and perspectives**

480 The above and continued characterization of GnRH neuron intrinsic properties is prerequisite to
481 understanding physiologic and pathophysiologic regulation of their output, for generating
482 computational models and for understanding the entire circuitry underlying GnRH release.
483 There remain several critical and unanswered questions about the electrophysiologic properties
484 of GnRH neurons including how is coordination achieved, how does action potential firing relate
485 to hormone release and is this a point of feedback regulation, does signaling and its functional
486 outcomes differ depending on which domain of the GnRH neuron receives the input, and how
487 do changes in ionic conductances in GnRH neurons contribute to long-term patterns of activity
488 in these cells that correlates with hormone release? Pairing electrophysiologic approaches with
489 opto- and chemogenetic interrogation of brain circuits *in vivo* is a powerful approach to
490 determine the role of specific cell populations in GnRH secretion and will expand our

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491 understanding of the functional GnRH network, generation of the secretory pattern, and the
492 regulation of these elements throughout postnatal development, by physiologic steroid
493 feedback, by internal and external cues and under pathophysiologic conditions.

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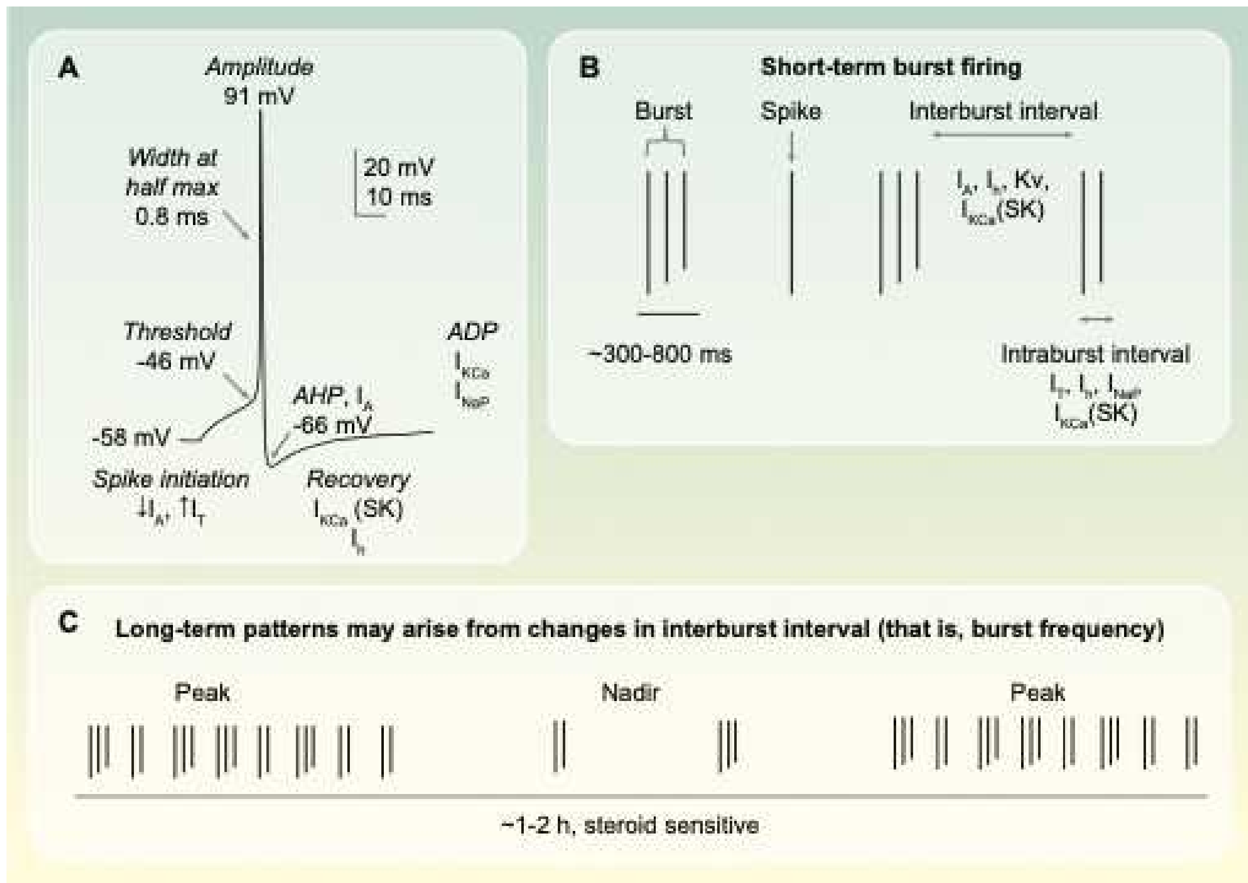

Figure 1: Action potential firing in GnRH neurons. A. GnRH neuron action potential waveform and its regulation by individual conductances. **B**. Short-term burst firing and postulated underlying conductances. C. Postulate for generation of long-term firing patterns from bursts. Current abbreviations: I_A, A-type K^+ current[58]; I_T, T-type Ca current [2+27]; I_{KCa}, Ca^{2+}-activated K^+ current, SK small conductance I_{KCa}[10], I_{NaP}, persistent Na^+ current;I_h, hyperpolarization-activated current [28].

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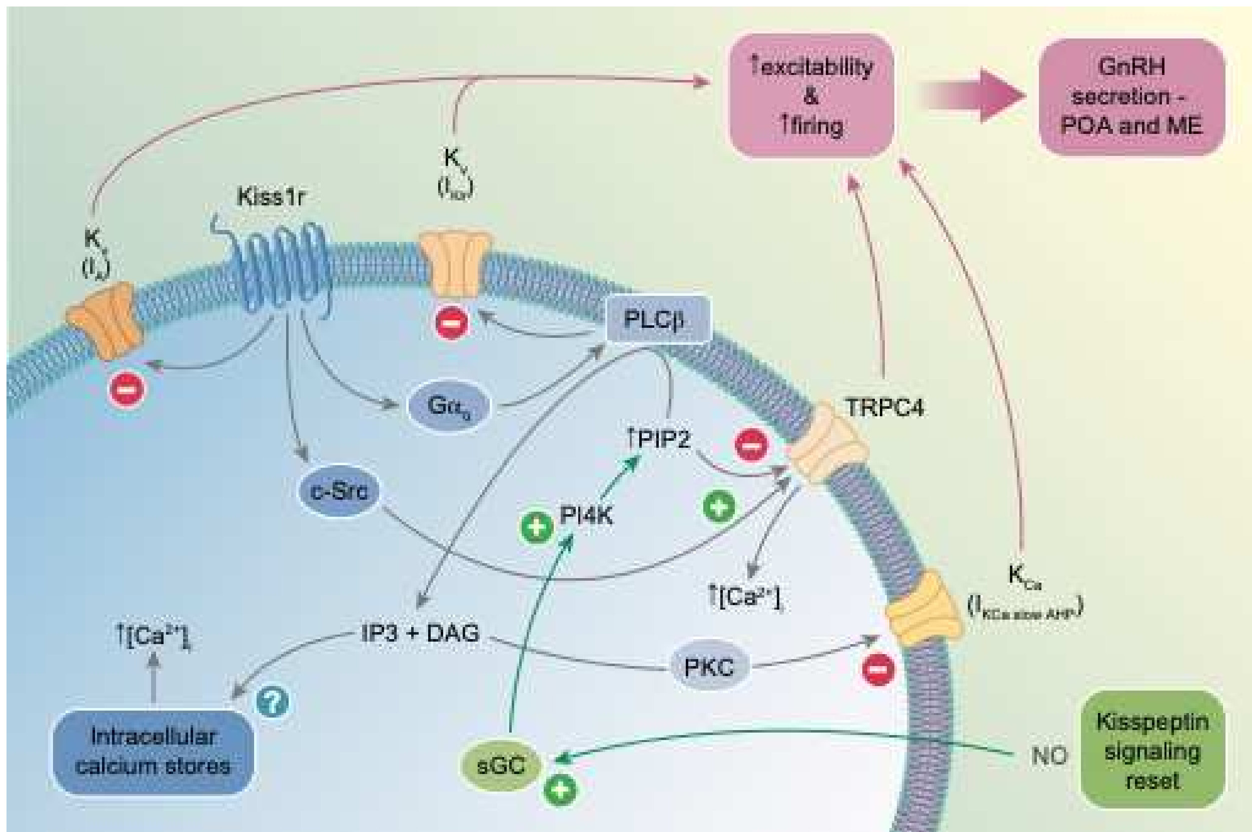

Figure 2: Kisspeptin-Kiss1r signaling in GnRH neurons. Schematic illustration of the main intracellular pathways identified to date that contribute to kisspeptin-10-evoked stimulation of GnRH neurons. The main effects are to inhibit multiple K^+ conductances and to activate non-selective cation channels (TRPC4), resulting in membrane depolarization, further activation of voltage-gated currents and, eventually, action potential firing. See text for details and references. AHP: afterhyperpolarization; c-Src: protein-tyrosine kinase Src; DAG: diacyl glycerol; I_A: A-type K^+ current; $I_{KCa\ slow\ AHP}$: Ca^{2+}-activated K^+ current mediating the slow AHP; I_{Kir}: inward rectifying K^+ current; IP3: inositol triphosphate; K_{Ca}: Ca^{2+}-activated K^+ channel; Kiss1r: kisspeptin receptor; K_v: voltage-gated K^+ channel; ME: median eminence; NO: nitric oxide; PI4K: phosphatidylinositol 4-kinase; PIP2 phosphatidylinositol 4,5-bisphosphate; PKC: protein kinase C; PLCβ, phospholipase β; POA: preoptic area; sGC: soluble guanylate cyclase; TRPC4: transient receptor potential channel 4.

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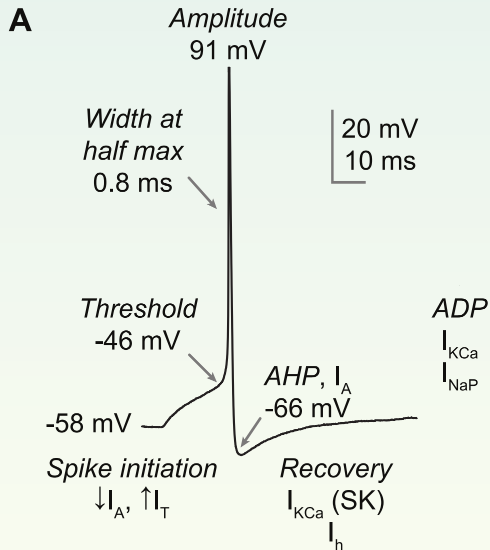

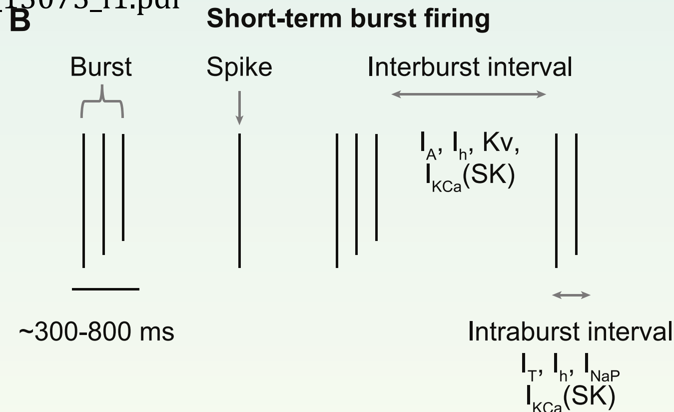

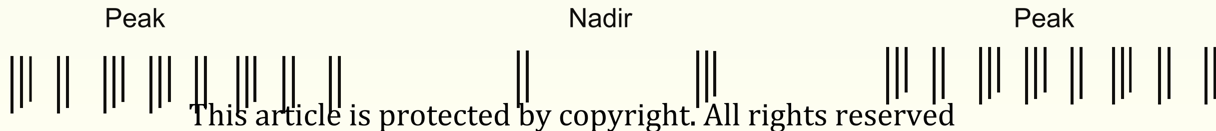

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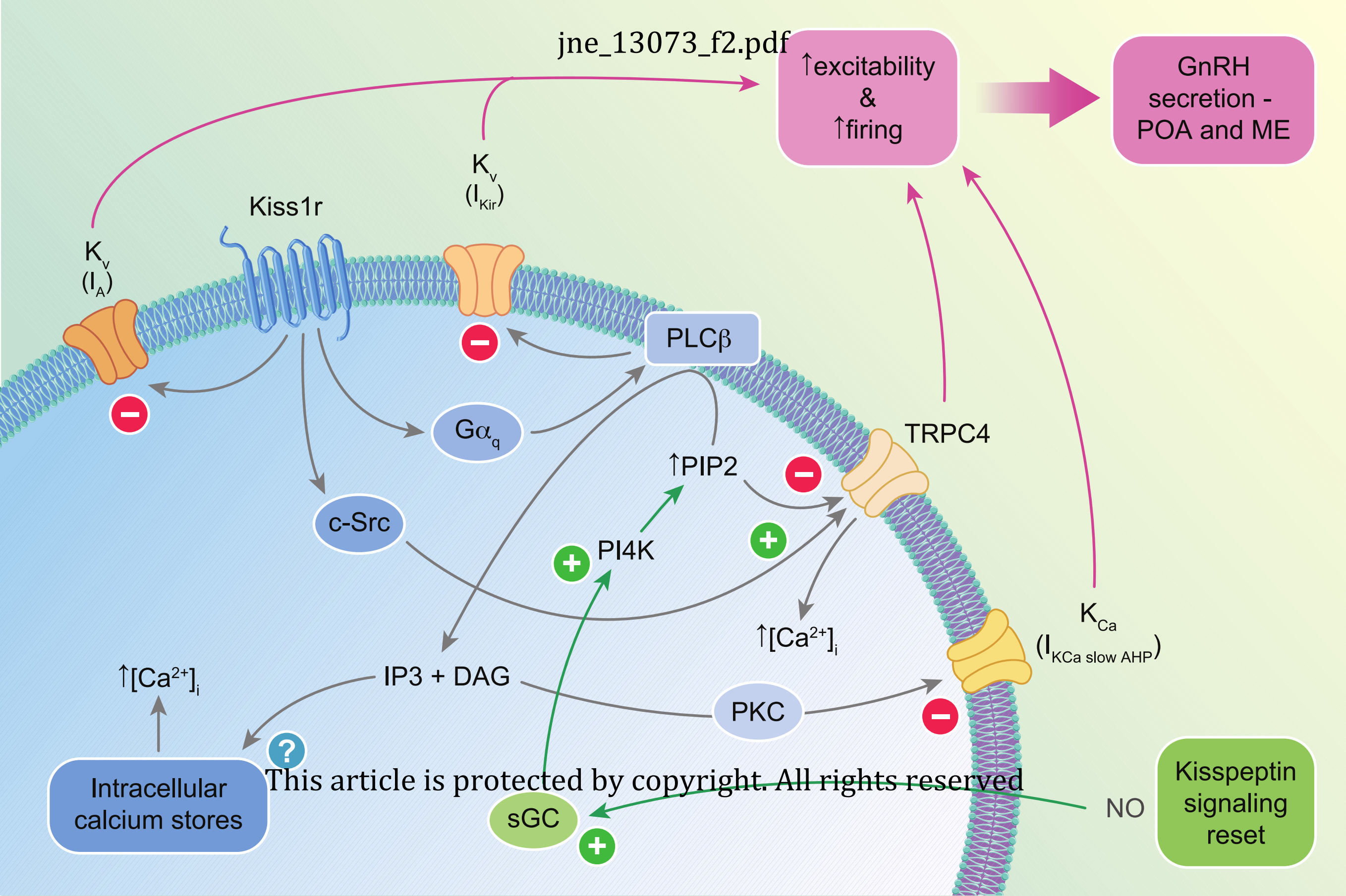

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