Lack of sex differences in gonadotropin-releasing hormone (GnRH) neuron potassium currents and excitability

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Abbreviated title: Excitability and K^+ currents in GnRH neurons

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Number of figures: 5
Number of tables: 6
Number of words: abstract 248, introduction 627, discussion 1685.

Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; 4AP, 4-aminopyridine; OVX, ovariectomized; ORC, orchidectomized; GDX, gonadectomized; AHP, afterhyperpolarization, FWHM, full width at half maximum

Acknowledgements: We thank Elizabeth Wagenmaker and Laura Burger for expert technical assistance and Joseph Starrett, Elizabeth Wagenmaker and Jennifer Jaime Alvarez for editorial comments. We thank James L. Kenyon, University of Nevada, Reno, for the Excel spreadsheet used to calculate junction potentials.

Conflict of interest: The authors declare no competing financial interests.

Grant support: Supported by National Institute of Health/Eunice Kennedy Shriver National Institute of Child Health and Human Development R37HD34860 to SMM

Abstract

Gonadotropin-releasing hormone (GnRH) drives pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone, which in turn regulate gonadal functions including steroidogenesis. The pattern of GnRH release and thus fertility depend on gonadal steroid feedback. Under homeostatic (negative) feedback conditions, removal of the gonads from either females or males increases the amplitude and frequency of GnRH release and alters the long-term firing pattern of these neurons in brain slices. The neurobiological mechanisms intrinsic to GnRH neurons that are altered by homeostatic feedback are not well studied and have not been compared between sexes. During estradiol positive feedback, which is unique to females, there are correlated changes in voltage-gated potassium currents and neuronal excitability. We thus hypothesized these same mechanisms would be engaged in homeostatic negative feedback. Voltage-gated potassium channels play a direct role in setting excitability and action potential properties. Whole-cell patch-clamp recordings of GFP-identified GnRH neurons in brain slices from sham-operated and castrated adult female and male mice were made to assess fast (I_A) and slow (I_K) inactivating potassium currents as well as action potential properties. Surprisingly, no changes were observed in most potassium current properties, input resistance or capacitance and this was reflected in a lack of differences in excitability and specific action potential properties. These results support the concept that, in contrast to positive feedback, steroid negative feedback regulation of GnRH neurons in both sexes is likely conveyed to GnRH neurons via mechanisms that do not induce major changes in the biophysical properties of these cells.

Significance Statement

The pattern of activity of gonadotropin-releasing hormone (GnRH) neurons is crucial to reproductive success in both males and females. Direct comparison of GnRH neurons from mice of both sexes during negative feedback and after gonadectomy revealed few differences in potassium currents, excitability, and action potential properties. These results support the hypothesis that neurons presynaptic to GnRH neurons communicate negative feedback to these cells in a manner that does not alter their intrinsic biophysical properties.

Introduction

The episodic release of GnRH from the brain is key to successful reproduction in both sexes. GnRH regulates pituitary release of luteinizing hormone (LH) and follicle-stimulating hormone, which activate gonadal functions including production of sex steroids. These steroids feed back to regulate the pattern of GnRH release and pituitary response to GnRH. Aspects of this feedback system are well established as sexually differentiated, for example estradiol positive feedback is exclusive to females under normal physiologic conditions and induces the preovulatory surge of GnRH (Docke and Dorner, 1965; Sarkar et al., 1976; Moenter et al., 1991). Other aspects appear similar between the sexes, including homeostatic negative feedback that regulates episodic GnRH release and is critical for maintaining most reproductive processes in both males and females. Gonadectomy increases GnRH and LH release (Leipheimer et al., 1985; Levine et al., 1985; Karsch et al., 1987; Caraty and Locatelli, 1988; Condon et al., 1988; Jackson and Kuehl, 2000; Czieselsky et al., 2016). The main circulating sex steroids providing homeostatic feedback are sexually differentiated, specifically estradiol and progesterone in females, depending on stage of the reproductive cycle, and testosterone in males. The differences in circulating steroids are reduced in part in the brain by conversion of testosterone to estradiol (Fisher et al., 1998; Sharma et al., 1999), and the efficacy of androgens and estrogens in eliciting negative feedback in males varies with species (Plant, 1982; Levine and Duffy, 1988; Tilbrook et al., 1999).

Regulation of episodic GnRH release is a key potential intervention point for the very different goals of reversibly inhibiting reproduction for contraception and ameliorating central infertility. Despite a similar hormonal response to the removal of peripheral sex steroid feedback by gonadectomy, whether or not the underlying intrinsic changes in GnRH neurons that lead to these increases are sexually differentiated is unknown. Studies in females have focused on the mechanisms underlying estradiol positive feedback that is critical for inducing ovulation; many of these have compared gonadectomized animals with open feedback loops to those with specific steroid replacement (Wagner et al., 2001; Christian and Moenter, 2010; Dror et al., 2013; Liu et al., 2017; Adams et al., 2018b; Adams et al., 2019; Wang et al., 2019). These studies have revealed changes in both intrinsic GnRH neuron properties and fast synaptic input to these cells. Studies of homeostatic negative feedback suggest that the firing pattern of GnRH neurons varies during the reproductive cycle in females and when steroid feedback is disrupted by

gonadectomy in both sexes (Pielecka and Moenter, 2006; Pielecka et al., 2006; Silveira et al., 2017). There are no direct comparisons, however, of GnRH neuron intrinsic properties between intact males and females or how these are affected by gonadectomy.

A basic measure of the intrinsic biophysical properties of neurons is their excitability, defined as the number of action potentials generated in response to varying current injections. In females, this varies with cycle stage and with induction of daily LH surges by estradiol in ovariectomized mice (Adams et al., 2018b; Adams et al., 2019) but this parameter has not been characterized in males. Voltage-gated potassium channels are widely recognized as regulators of excitability (Johnston et al., 2010) and have been targeted clinically. For example, blocking a fraction of potassium channels with 4-aminopyridine (4AP) increases the excitability of motor neurons in amyotropic lateral sclerosis and multiple sclerosis and provides relief from the symptoms of these diseases (Bakirtzis et al., 2018; Peikert et al., 2019). In many neurons, 4AP increases input resistance and diminishes the ability of voltage-gated potassium channels to blunt action potential firing and/or the membrane potential changes in response to synaptic inputs (Hoffman, 2013; DeFazio et al., 2019). Here we tested the hypotheses that voltage-gated potassium currents are reduced and excitability of GnRH neurons increased by gonadectomy in both sexes.

Materials and Methods

All chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA) unless noted.

Animals. Mice expressing GFP under control of the GnRH promoter (Tg(GnRH1-EGFP)51Sumo MGI:6158457, GnRH-GFP mice, JAX 033639) were propagated in our colony. All mice had *ad libitum* access to Harlan 2916 chow and water and were held at 21-23 °C on a 14L:10D light cycle with lights on at 0300 eastern standard time. Adult mice 82-146 days old were ovariectomized (OVX, females), orchidectomized (ORX, males) or sham operated under isoflurane anesthesia with bupivacaine as a local analgesic. Studies were performed 5-7 days after surgery. In sham-operated females, estrous cycles were monitored by vaginal cytology for at least 10 days before experiments and diestrous mice were selected. Body, uterine and seminal vesicle mass were recorded at the time of brain slice preparation to verify steroid status. The Institutional Animal Care and Use Committee of the University of Michigan approved all procedures.

90 *Brain slice preparation.* All extracellular solutions were bubbled with 95% O_2/5% CO_2 throughout
91 the experiments and for at least 30 min before exposure to tissue. Coronal brain slices (300 μm)
92 containing the preoptic area (POA) were prepared with a Leica VT1200S (Leica Biosystems)
93 using modifications of previously described methods (DeFazio and Moenter, 2002). Brain slices
94 were obtained between 0730-0830 EST; recordings obtained between 0830-1430 EST. The
95 brain was rapidly removed and placed in ice-cold sucrose saline solution containing (in mM):
96 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$
97 (350 mOsm). Slices were incubated for 30 min at room temperature (~21–23°C) in 50% sucrose
98 saline and 50% artificial cerebrospinal fluid (ACSF, containing (in mM): 135 NaCl, 3.5 KCl, 26
99 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 315 mOsm, pH 7.4). Slices were
100 then transferred to 100% ACSF solution at room temperature for 0.5–5h before recording. One
101 to three recordings were obtained per mouse with a minimum of 5 mice studied per group;
102 variation within a mouse was not less than that within a group.

103 *Recording solutions and data acquisition.* The pipette solution consisted of (in mM): 125 K
104 gluconate, 20 KCl, 10 HEPES, 5 EGTA, 0.1 $CaCl_2$, 4 MgATP and 0.4 NaGTP, 305 mOsm, pH
105 7.2 with NaOH; this solution is based on the native intracellular chloride concentration in GnRH
106 neurons determined using gramicidin perforated-patch recordings (DeFazio et al., 2002). A 14.5
107 mV liquid junction potential was negated before each recording (Barry, 1994). During all
108 recordings, slices were continuously superfused at 2 ml/min with carboxygenated ACSF kept at
109 30-31°C with an inline-heating unit (Warner Instruments). GFP-positive cells were visualized
110 with a combination of infrared differential interference contrast and fluorescence microscopy on
111 an Olympus BX50WI microscope. Recordings were made with an EPC-10 patch clamp amplifier
112 and a computer running PATCHMASTER software (HEKA Elektronik). For current-clamp
113 experiments, membrane voltage was acquired at 20 kHz and filtered at 10 kHz; for voltage-
114 clamp experiments, current was acquired at 10 kHz and filtered at 5 kHz. Input resistance,
115 series resistance, baseline current, and capacitance were monitored throughout experiments
116 from the membrane current response to a 20 ms, 5 mV hyperpolarizing voltage step from -65
117 mV to monitor recording quality. All recordings with input resistances (R_{in}) <0.5 GΩ, series
118 resistances (R_s) >20 MΩ, or unstable membrane capacitance (C_m) were rejected. Results did
119 not depend on anatomical location of GnRH neurons within the POA.

120 *Experimental Design.* Potassium currents, action potential properties and passive properties
121 were characterized in GFP-identified GnRH neurons in the preoptic area of brain slices
122 prepared from adult gonad intact and castrated mice of both sexes. Castrated mice were
123 studied 5-7 days post surgery; intact females were in diestrus.

124 *Voltage-gated potassium current characterization.* Potassium currents were isolated
125 pharmacologically during whole-cell voltage-clamp recordings by blocking fast sodium and
126 calcium channels as well as ionotropic receptors for GABA and glutamate (2 μM tetrodotoxin
127 (TTX, Tocris), 300 μM $NiCl_2$, 20 μM D-APV (Tocris), 10 μM CNQX, and 100 μM picrotoxin).
128 Membrane potential was held at -65 mV between voltage-clamp protocols. Series resistance
129 was monitored before compensation using the current response to a 5 mV hyperpolarizing step.
130 Cells with R_s>20 MΩ without compensation were discarded. Two potassium currents, I_A and I_K,
131 were distinguished based on voltage dependence and time course. I_A is a typical, rapidly-
132 inactivating potassium current that can be activated at membrane potentials that are
133 hyperpolarized to the threshold for action potential firing. I_K also displays voltage-dependent
134 inactivation but this is restricted to more depolarized potentials and has a slower time course of
135 inactivation.

136 *Activation and inactivation of I_A*. Preliminary studies on I_A showed that a 500 ms prepulse at -40
137 mV induced full inactivation, and a prepulse at -100 mV for 500 ms completely removed
138 inactivation. These two prepulses were combined with a series of voltage steps to isolate and
139 characterize I_A. To measure total potassium current, a 500 ms prepulse at -100 mV was first
140 applied to remove the inactivation of the fast transient component, followed by a family of
141 voltage steps (500 ms, 10 mV intervals) from -100 mV to +50 mV, and then a final step to -10
142 mV for 100 ms. To inactivate I_A, the same step protocol was applied but with the 500 ms
143 prepulse set at -40 mV to inactivate the fast component while leaving I_K mostly unchanged
144 attributable to its very slow inactivation (see below). The fast I_A component was then isolated by
145 subtracting the family of currents obtained with the -40 mV prepulse from that obtained with the
146 -100 mV prepulse. Activation of I_A was quantified by measuring the peak current reached during
147 each voltage step in the family (see Analysis section below). Inactivation of I_A was estimated
148 from the peak current during the final step to -10 mV that followed the family of voltage steps. All
149 protocols for I_A analysis were leak subtracted using the online -P/8 (average of 8 sweeps, 1/8

150 the size of the voltage step in the opposite direction, from a baseline potential of -65 mV)
151 (Bezanilla and Armstrong, 1977).

152 Time course of inactivation of I_A was characterized by stepping the membrane potential to -100
153 mV for 500 ms to remove inactivation, then stepping to the inactivation potential (-40 mV) for 0,
154 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 ms, followed by a test pulse at -10 mV to assess
155 the peak current. The non-inactivating component during the test pulse after 1024 ms
156 inactivation was subtracted from each of the other traces to isolate the transient current.
157 Similarly, the time course of recovery from inactivation of I_A was characterized by stepping the
158 membrane potential to -40 mV for 500 ms to fully inactivate I_A, and then stepping to -100 mV for
159 the durations above, followed by a test pulse at -10 mV to assess the peak current. The non-
160 inactivating component at 0 ms recovery was subtracted from each of the other traces to isolate
161 the transient current.

162 To study I_K, a separate set of recordings was required because of the slow inactivation of this
163 component. Indeed full inactivation and recovery required >10 s at +50 mV and -100 mV,
164 respectively. Cells did not remain stable upon repeated exposure to these potentials, thus more
165 moderate potentials were used to permit estimation of these properties within the command
166 potentials and durations the cells tolerated.

167 *Activation and inactivation of I_K.* To quantify activation, a double prepulse protocol was used: an
168 initial prepulse to -75 mV for 10 s was used to remove inactivation from I_K, then a second
169 prepulse to -50 mV for 1 s was used to inactivate I_A. After these two prepulses, test pulses of 10
170 s from -50 to +50 mV in 10 mV increments were used to measure the peak I_K current. A final
171 step to +50 mV for 100 ms was used following each test pulse to measure inactivation of I_K. The
172 long duration voltage steps required to characterize this current makes traditional online leak
173 subtraction unrealistic. For the activation of I_K, leak currents were subtracted offline using the
174 approach of (Kimm and Bean, 2014). The shape of the leak response was acquired from the
175 average of 16 steps of -5 mV for 50 ms from the holding potential of -65 mV. Offline, the
176 average was scaled by the voltage difference and subtracted from the portion of the raw current
177 sweep containing the activation test pulses. The leak steps were run immediately before each
178 voltage-clamp protocol. Prior to each leak acquisition, passive properties were recorded and
179 both slow capacitance and series resistance compensation (50-70%) adjusted.

180 Time course of inactivation of I_K was characterized by first removing inactivation by holding at -
181 75 mV for 10s, then I_A was inactivated by stepping to -50mV for 1 s. This was followed by a
182 variable duration inactivation pulse (0.1, 0.21, 0.42, 0.83, 1.64, 3.25, 6.46, 12.87, 25.68, 51.29 s)
183 at -30 mV, followed by a test pulse at +50 mV. Because inactivation was incomplete even after
184 51.29 s at -30 mV, this remaining current was not subtracted and the inactivation graphs level
185 off at ~30%. Recovery from inactivation for I_K was studied using an inactivating prepulse of +50
186 mV for 10 s, followed by a variable duration recovery pulse at -80 mV (0.01, 0.03, 0.06, 0.11,
187 0.2, 0.37, 0.7, 1.35, 2.64, 5.21, 10.34, 20.59 s), a brief pulse to inactivate I_A (-50 mV for 1 s),
188 and a test pulse at +50 mV for 100 ms. The peak current was plotted as a function of the
189 recovery prepulse duration. Since inactivation was incomplete, the non-inactivated current was
190 not subtracted (recovery graphs start at ~30%).

191 *Analysis* Current density was calculated by dividing current by capacitance for each cell. To
192 assess the voltage-dependence of activation, both I_A and I_K were divided by the driving force
193 derived from the Goldman-Hodgkin-Katz (GHK) current equation (Clay, 2000, 2009). To
194 estimate $V_{0.5activation}$, I_A and I_K were divided by the GHK driving force, normalized to the maximum
195 value, plotted as a function of step potential and fit with the Boltzmann equation:

196 $$I(V) = 1 / 1 + e^{-kF/RT(V-V_{0.5})}$$

197 V is the command potential of the step, $V_{0.5}$ is the potential at half maximum, and k is the "slope
198 factor" (k has no units attributable to the F/RT factor). Voltage dependence of inactivation was
199 characterized using the same equation to fit the normalized current measured during the
200 inactivation test pulse.

201 *Action potential properties.* To characterize action potential properties, current-clamp recordings
202 were obtained in the presence of antagonists of receptors for fast synaptic transmission (20 μM
203 D-APV, 10 μM CNQX, 100 μM picrotoxin). Cells were maintained at -65 mV by current injection
204 (<50 pA) in current-clamp after bridge compensation of series resistance by at least 95%.
205 Current steps (5 pA increments, 500 ms, -10 pA to +50 pA, 2.5 s interval near -65 mV between
206 sweeps) were delivered to test the membrane potential response. The first current step to
207 display an action potential was defined as the rheobase and the first spike analyzed in detail.
208 Action potential threshold was defined as the potential at which the membrane potential slope

209 exceeded 1 V/s. Action potential latency was defined as the time from start of the current
210 injection to threshold. Rate of rise was defined as the maximum of the voltage trace derivative
211 from threshold to peak. Full width of the action potential at half maximum (FWHM), and
212 afterhyperpolarization (AHP) time and amplitude were measured relative to threshold.

213 *Statistics.* Data are reported as mean ± SEM, with individual values shown where practical.
214 Statistical comparisons were made using Prism 9 (GraphPad Software). Data were tested for
215 normal distribution with Shapiro-Wilk. Tests were chosen appropriate for the experimental
216 design and data distribution as specified in the results. For two-way ANOVA, the Bonferroni *post*
217 *hoc* is considered sufficiently robust to use with non-normally distributed data (Underwood,
218 1996). Significance was set at $p<0.05$.

219 **Results**

220 *Verification of gonadectomy* Short-term gonadectomy had no effect on body mass within either
221 sex (females n=12 sham diestrus 21.4±0.6 g, n=14 OVX 21.8±0.3 g; males n=13 sham 26.1±0.7
222 g, n=13 ORX 25.4±0.6 g, two-way ANOVA gonadal status: $F(1, 48) = 0.07108$; $P=0.7909$). As
223 expected, males were heavier than females (two-way ANOVA sex: $F(1, 48) = 53.80$; $P<0.0001$).
224 In females, OVX reduced uterine mass (sham diestrus 63.4±5.0 mg, OVX 29.6±1.3 mg,
225 unpaired two-tailed Student's t test: $t=7.001$, $df=24$, $P<0.0001$). In males, ORX reduced seminal
226 vesicle mass (sham 217.2±11.6 mg, ORX 56.6±4.4 mg, unpaired two-tailed Student's t test:
227 $t=12.97$, $df=24$, $P<0.0001$).

228 *Passive properties of GnRH neurons do not vary with sex or gonadal status under conditions*
229 *used to characterize potassium currents* Critical to evaluating of current properties among
230 treatments is comparison of similar quality recordings. No difference was observed among
231 groups for uncompensated series resistance (Figure 1A). Nor were any differences in the
232 passive cellular properties of input resistance (Figure 1B), capacitance (Figure 1C) or holding
233 current (Figure 1D) attributable to either sex or gonadal status. These latter measures offer a
234 combined view of recording quality as well as insight into potential biological changes, such as
235 cell size or membrane conductance (two-way ANOVA, Table 1).

236 *Neither sex nor gonadal status affect voltage-gated potassium currents of GnRH neurons* The
237 output of GnRH neurons in terms of spontaneous firing pattern and hormone release is
238 increased by removal of homeostatic gonadal steroid feedback accomplished via gonadectomy.
239 We hypothesized that removing gonadal steroid feedback would reduce voltage-gated
240 potassium currents. Two components of this current were examined, a fast I_A-like current and a
241 slowly-inactivating I_K-like current. No measured property of either current differed with sex or
242 gonadal status (Figures 2, 3; Tables 3, 4). Only the parameters calculated from the Boltzmann
243 fit, specifically the inactivation slope factor for I_A and both inactivation and activation slope
244 factors for I_K exhibited weakly significant P values; shifts of this magnitude are unlikely to have a
245 substantial biological effect.

246 *Passive properties of GnRH neurons do not vary with sex or gonadal status under conditions*
247 *used to characterize action potentials* To record action potential properties, the blockers of
248 voltage-gated calcium and sodium channels needed to isolate potassium currents were omitted;
249 this may alter the passive properties of the cells, thus these parameters are reported separately
250 for the two types of recordings. There was a weak interaction between sex and gonadal status
251 for uncompensated series resistance (Figure 4A, P=0.0436). This difference is unlikely to
252 account for a difference in measured values as bridge balance was in effect to compensate
253 series resistance by >95% during action potential recordings. As was observed for recordings of
254 potassium currents, there were no differences in the passive cellular properties of input
255 resistance (Figure 4B), capacitance (Figure 4C) or holding current (Figure 4D) attributable to
256 either sex or gonadal status.

257 *Neither excitability nor first action potential properties are altered by sex or gonadal status.*
258 Current-clamp recordings of action potential properties were used to assess possible general
259 differences in the intrinsic properties of GnRH neurons in the experimental groups (Figure 5).
260 No differences were observed in the firing response to current injection attributable to sex or
261 gonadal status (three-way repeated-measures ANOVA, Table 5). Post-analysis consolidation of
262 these data to examine effects of either sex or gonadal status by two-way repeated measures
263 ANOVA revealed a mild interaction between sex and current injection, but no post hoc
264 significance (Bonferroni); there was no effect of gonadectomy. There were also no differences
265 observed in any action potential property examined (two-way ANOVA, Table 6). Together these

266 observations suggest that homeostatic feedback does not induce major changes in action
267 potentials of GnRH neurons.

268 **Discussion**

269 A century ago, early studies of pituitary ablation and replacement revealed a link between the
270 emerging science of neuroendocrinology and reproductive processes (Evans HM, 1921; Smith,
271 1927). Around the same time, the reverse link was being made by observing the effects of
272 orchidectomy upon the pituitary, and amelioration of these effects by replacement with fat-
273 soluble extracts of the testes (McCullagh, 1932). It is now well established that reproduction
274 revolves around the stimulatory and feedback interactions of the hypothalamo-pituitary-gonadal
275 axis, with GnRH neurons serving as the final common pathway for central signaling to the
276 pituitary. These interactions are homeostatic in males and homeostatic throughout most of the
277 reproductive cycle in females; removal of the gonads opens these homeostatic feedback loops
278 and results in increased GnRH neuron activity, GnRH release and LH release in both sexes
279 (Leipheimer et al., 1985; Levine et al., 1985; Karsch et al., 1987; Caraty and Locatelli, 1988;
280 Condon et al., 1988; Jackson and Kuehl, 2000; Han et al., 2020). Despite the similarity in
281 steady-state endocrine response, it is possible that latent sex differences in underlying
282 neurobiological mechanisms exist (Jain et al., 2019). We tested the hypotheses that removal of
283 negative homeostatic feedback would reduce potassium currents and increase the excitability of
284 GnRH neurons. Based on the parameters we quantified, no substantial differences were
285 observed that could be attributed to sex or feedback condition. We thus reject these hypotheses.

286 The present data showing no changes in the intrinsic properties of GnRH neurons in females
287 between diestrous and OVX mice support prior work assessing excitability of GnRH neurons in
288 OVX vs OVX+E mice exhibiting diurnal changes between negative and positive feedback
289 (Christian et al., 2005). In this model, GnRH neuron activity and LH release are suppressed in
290 the morning (AM) in OVX+E mice relative to OVX mice, demonstrating homeostatic negative
291 feedback, but increased in the afternoon (PM) in cells from OVX+E mice, demonstrating
292 estradiol positive feedback. Interestingly there were no differences in GnRH neuron excitability
293 between OVX mice at either time of day and OVX+E mice in the AM (negative feedback),
294 whereas GnRH neurons from OVX+E mice recorded in the PM during positive feedback were
295 more excitable (Adams et al., 2018b). The present work extends these data to include both a

296 comparison between the sexes and a comparison using the open loop OVX/ORX condition vs
297 sham-operated controls to ameliorate caveats associated with steroid hormone replacement.
298 The lack of effect of removing homeostatic feedback suggests a majority of the changes that
299 lead to increased GnRH output in gonadectomized animals are processed presynaptic to these
300 cells. In and of itself, this concept is not new as GnRH neurons do not appear to express
301 detectable levels of steroid hormone receptors other than the beta isoform of the estradiol
302 receptor (Herbison et al., 1996; Hrabovszky et al., 2000; Hrabovszky et al., 2007). There has
303 thus long been a relative consensus in the field that steroid feedback is integrated via upstream,
304 steroid-responsive cells (Wintermantel et al., 2006). The lack of observed changes in GnRH
305 neuron properties examined suggests homeostatic negative feedback signals are conveyed
306 from these afferents in a manner that alters the output of GnRH neurons without substantial
307 biophysical changes at the level of the cell soma. Of note, there is increased frequency of
308 $GABA_A$-receptor-mediated postsynaptic currents, which can excite GnRH neurons (DeFazio et
309 al., 2002; Herbison and Moenter, 2011), observed in GnRH neurons from ORX vs intact males
310 (Chen and Moenter, 2009), and in cells from OVX vs OVX+E females during negative feedback
311 (Christian and Moenter, 2007). Postsynaptic currents have relatively short-lived effects on
312 membrane potential that may not lead to changes in overall excitability. Interestingly, changing
313 homeostatic negative feedback does engage mechanisms affecting excitability in arcuate
314 kisspeptin neurons, also known as KNDY neurons for their coexpression of three neuropeptides:
315 kisspeptin, neurokinin B and dynorphin (Oakley et al., 2009; Moore et al., 2018). KNDy neurons
316 have been postulated to be key afferent inputs to GnRH neurons to drive episodic release from
317 these cells (Qiu et al., 2016; Clarkson et al., 2017; Vanacker et al., 2017). In KNDy neurons
318 from OVX+E vs OVX females examined during negative feedback in the morning, estradiol
319 reduced fast transient I_A (DeFazio et al., 2019), and I_A modifies action potential patterns in these
320 cells (Mendonca et al., 2018).

321 The lack of changes in the homeostatic vs open loop models studied in the present work stand
322 in marked contrast to changes that occur in GnRH neurons from females during estradiol
323 positive feedback (Adams et al., 2018a; Adams et al., 2018b; Adams et al., 2019). In addition to
324 the increase in GnRH neuron excitability observed during positive feedback mentioned above,
325 GnRH neurons exhibit reduced transient potassium currents and increased high-voltage-
326 activated calcium conductances in OVX+E mice during positive feedback (DeFazio and Moenter,
327 2002; Sun et al., 2010). While both the removal of negative feedback by gonadectomy and
328 induction of positive feedback both increase GnRH release, the nature of this increase is quite

329 different. Removal of negative feedback maintains the episodic GnRH/LH release pattern that is
330 characteristic in males and most of the reproductive cycle in females (Leipheimer et al., 1985;
331 Levine et al., 1985; Karsch et al., 1987; Caraty and Locatelli, 1988; Condon et al., 1988;
332 Jackson and Kuehl, 2000; Han et al., 2020). Pulse frequency and amplitude are typically
333 increased. Even in long-term castrated rams, when the pulsatile nature of LH release is no
334 longer evident, high frequency GnRH pulses are clearly observed (Caraty and Locatelli, 1988).
335 Induction of positive feedback, however, shifts the pattern from episodic to a continual elevation
336 above baseline that lasts for several hours (Sarkar et al., 1976; Levine and Ramirez, 1982;
337 Clarke et al., 1987; Moenter et al., 1990; Moenter et al., 1991; Moenter et al., 1992a; Moenter et
338 al., 1992b; Xia et al., 1992). Given the fundamental differences in how the output of GnRH
339 neurons is altered in these two circumstances, it is reasonable to postulate that more extensive
340 changes are required for successful positive feedback, and that this is in part accomplished by
341 extending the mechanisms engaged to the alteration of GnRH neuron intrinsic properties to
342 allow continuous secretion to be maintained. Conceptually, pulse frequency can be rapidly
343 modulated by homeostatic perturbances that influence reproduction. For example, inflammatory
344 stress (Battaglia et al., 1998), hypoglycemia (Chen et al., 1996), and naloxone antagonism of
345 opiates (Caraty et al., 1987) all rapidly induce changes in the pulse pattern of GnRH or multiunit
346 activity associated with LH in the hypothalamus. In contrast, estradiol induction of positive
347 feedback is a process with a substantial (typically >12h) delay to the increased release and this
348 increase appears to be all or none as it is not dependent upon continued presence of estradiol
349 (Evans et al., 1997).

350 The apparently different postsynaptic effects of removing negative feedback and inducing
351 positive feedback raise some interesting questions for future contemplation. This is particularly
352 true given that the prevailing view in the field is that the neuromodulator kisspeptin plays an
353 important role in activating both episodic and surge modes of GnRH release (Porteous and
354 Herbison, 2019; Wang et al., 2019). Kisspeptin application in brain slices reduces I_A in a manner
355 similar to induction of estradiol positive feedback (Pielecka-Fortuna et al., 2011), and also
356 increases excitability of GnRH neurons (Adams et al., 2018b), indicating it can alter the intrinsic
357 properties of these cells. The two hypothalamic populations of kisspeptin neurons, the
358 aforementioned KNDy neurons in the arcuate and those in the anteroventral periventricular area
359 (AVPV) are thought to mediate pulsatile and surge modes of GnRH release, respectively. These
360 populations possess different cotransmitters and mediators (Cravo et al., 2011; Skrapits et al.,
361 2015); if co-released with kisspeptin these could produce counteracting effects in the

362 postsynaptic GnRH neuron. Alternatively other afferent populations may have critical roles. In
363 this regard, the increase in GnRH neuron firing induced by kisspeptin application is longer in
364 duration than the typical pulse (Moenter et al., 1992b; Evans et al., 1996; Han et al., 2005;
365 Pielecka-Fortuna et al., 2008). The neuromodulator known as gonadotropin-inhibitory hormone
366 in birds and RFRP3 in mammals can counteract the activating effects of applied kisspeptin on
367 GnRH neuron firing rate in brain slices by activating a potassium current (Wu et al., 2009).
368 While speculative, an interplay of kisspeptin and RFRP3 may have opposing effects on intrinsic
369 GnRH properties.

370 While we have confidence in the lack of effect of sex or removing homeostatic feedback on the
371 properties examined in the present work, it is important to acknowledge alternative possibilities
372 and mechanisms. It is possible that removing negative feedback induces equal and opposite
373 changes in intrinsic properties resulting in masking of these changes in the current-clamp
374 recordings. This seems unlikely given the different kinetic properties of the voltage-gated
375 channels likely to mediate changes in action potential firing and properties. It is also possible
376 that changes are induced in the biophysical properties of GnRH neurons that are distal to the
377 soma and not possible to monitor in our brain slice preparation. For example, arcuate kisspeptin
378 neurons can interact with GnRH neurons via kisspeptin and neurokinin B at the level of the
379 terminals in the median eminence (Gaskins et al., 2013; Glanowska and Moenter, 2015; Yip et
380 al., 2015). Examining these properties at a different point post gonadectomy may have revealed
381 sex differences. While both males and females increase GnRH and LH release following
382 gonadectomy, the increase is somewhat delayed in females. At the time point we investigated of
383 5-7 days post gonadectomy, clear increases in LH have occurred in both sexes (Yamamoto et
384 al., 1970). It is also possible that removing homeostatic feedback alters other aspects of GnRH
385 neurons to increase GnRH output, such as increasing GnRH mRNA (Finn et al., 1998; Gore,
386 1998), altering ionotropic receptors that produce brief changes in membrane potential, or
387 changing excitation secretion coupling to make it more effective. Any of these alternative
388 mechanisms could be sexually differentiated.

389 In sum, the present work rejected the hypotheses that removing homeostatic gonadal feedback
390 induces changes in potassium currents and excitability of GnRH neurons. In so doing, we
391 provide evidence for different mechanistic strategies to regulate the output of GnRH neurons
392 during homeostatic vs positive feedback.

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Tables

Table 1 Statistical parameters from two-way ANOVA for passive properties from potassium current recordings (Figure 1)

Property	*Sex*	*Gonadal Status*	*Interaction*
Series resistance	F(1, 56) = 1.512; P=0.2240	F(1, 56) = 3.308; P=0.0743	F(1, 56) = 0.1171; P=0.7335
Input resistance	F(1, 56) = 0.03673; P=0.8487	F(1, 56) = 0.3186; P=0.5747	F(1, 56) = 0.4542; P=0.5031
Capacitance	F(1, 56) = 0.1963; P=0.6594	F(1, 56) = 2.540; P=0.1166	F(1, 56) = 0.08991; P=0.7654
Holding current	F(1, 56) = 2.592; P=0.1130	F(1, 56) = 0.6304; P=0.4306	F(1, 56) = 1.496; P=0.2263

Table 2. Two-way ANOVA statistical parameters for I_A. Bold indicates P<0.05.

Property	*Sex*	*Gonadal Status*	*Interaction*
V0.5 activation	F(1, 31) = 2.921; P=0.0974	F(1, 31) = 2.361; P=0.1345	F(1, 31) = 0.1956; P=0.6614
V0.5 inactivation	**F(1, 31) = 4.473; P=0.0426**	F(1, 31) = 0.4313; P=0.5162	F(1, 31) = 0.008208; P=0.9284
activation slope factor	F(1, 31) = 0.004146; P=0.9491	F(1, 31) = 0.01815; P=0.8937	F(1, 31) = 0.02354; P=0.8791
inactivation slope factor	F(1, 31) = 0.02076; P=0.8864	F(1, 31) = 0.07103; P=0.7916	**F(1, 31) = 6.872; P=0.0134**
Imax	F(1, 31) = 0.5863; P=0.4496	F(1, 31) = 0.9055; P=0.3487	F(1, 31) = 0.02845; P=0.8671
Imax density	F(1, 31) = 1.576; P=0.2188	F(1, 31) = 0.1613; P=0.6907	F(1, 31) = 0.006205; P=0.9377

Table 3. Two-way ANOVA statistical parameters for I_K. Bold indicates P<0.05.

Property	*Sex*	*Gonadal Status*	*Interaction*
V0.5 activation	F(1, 30) = 0.09013; P=0.7661	F(1, 30) = 0.0003841; P=0.9845	F(1, 30) = 0.006304; P=0.9372
V0.5 inactivation	F(1, 30) = 0.006266; P=0.9374	F(1, 30) = 1.247; P=0.2731	F(1, 30) = 0.1239; P=0.7273
activation slope factor	**F(1, 30) = 4.294; P=0.0469**	F(1, 30) = 1.170; P=0.2880	F(1, 30) = 2.197; P=0.1487
inactivation slope factor	F(1, 30) = 3.971; P=0.0554	**F(1, 30) = 7.378; P=0.0109**	F(1, 30) = 3.864; P=0.0587
Imax	F(1, 30) = 0.2979; P=0.5892	F(1, 30) = 1.019; P=0.3208	F(1, 30) = 2.259; P=0.1433
Imax density	F(1, 30) = 0.4582; P=0.5036	F(1, 30) = 1.725; P=0.1990	F(1, 30) = 2.988; P=0.0942

Table 4 Two-way ANOVA statistical parameters for passive properties from action potential recordings (Figure 5). Bold indicates P<0.05.

Property	*Sex*	*Gonadal status*	*Interaction*
Series resistance	F(1, 37) = 0.004601; P=0.9463	F(1, 37) = 0.3899; P=0.5362	**F(1, 37) = 4.366; P=0.0436**
Input resistance	F(1, 37) = 0.03866; P=0.8452	F(1, 37) = 0.1832; P=0.6712	F(1, 37) = 0.4448; P=0.5089
Capacitance	F(1, 37) = 0.1299; P=0.7205	F(1, 37) = 1.521; P=0.2253	F(1, 37) = 1.343; P=0.2539
Holding current	F(1, 37) = 1.555; P=0.2202	F(1, 37) = 0.5610; P=0.4586	F(1, 37) = 0.2843; P=0.5971

Table 5. Three-way repeated measures parameters for action potential firing as a function of current injection. Bold indicates P<0.05.

	3-way	2-way (sex consolidated)	2-way (gonadectomized (GDX) consolidated)
current step (pA)	**F(2.554, 91.96) = 641.8; P<0.0001**	**F(10, 380) = 653; P<0.0001**	**F(10, 380) = 636; P<0.0001**
sex	F(1, 36) = 2.447; P=0.1265	F(1, 38) = 2.6; P=0.1119	
GDX	F(1, 36) = 0.4993; P=0.4844		F(1, 38) = 0.64; P=0.4271
pA x sex	F(10, 360) = 1.758; P=0.0668	**F(10, 380) = 1.9; P=0.0477**	
pA x GDX	F(10, 360) = 0.7544; P=0.6728		F(10, 380) = 0.88; P=0.5513
sex x GDX	F(1, 36) = 0.2066; P=0.6522		
pA x sex x GDX	F(10, 360) = 0.5547; P=0.8503		

Table 6. Two-way ANOVA parameters for action potential properties

Property	*Sex*	*Gonadal Status*	*Interaction*
max frequency	F(1, 37) = 1.463; P=0.2341	F(1, 37) = 0.7278; P=0.3991	F(1, 37) = 0.6354; P=0.4305
baseline	F(1, 37) = 0.6163; P=0.4374	F(1, 37) = 0.1470; P=0.7036	F(1, 37) = 0.2182; P=0.6431
threshold	F(1, 37) = 0.7224; P=0.4008	F(1, 37) = 0.001493; P=0.9694	F(1, 37) = 0.002371; P=0.9614
rheobase	F(1, 37) = 2.778; P=0.1040	F(1, 37) = 7.015e-005; P=0.9934	F(1, 37) = 0.8335; P=0.3672
AP latency	F(1, 37) = 0.1120; P=0.7398	F(1, 37) = 0.1392; P=0.7112	F(1, 37) = 0.1060; P=0.7466
AP amplitude	F(1, 37) = 1.173; P=0.2858	F(1, 37) = 0.9688; P=0.3314	F(1, 37) = 0.4683; P=0.4980
AP FWHM	F(1, 37) = 2.817; P=0.1017	F(1, 37) = 3.069; P=0.0881	F(1, 37) = 2.858; P=0.0993
AP rate of rise	F(1, 37) = 0.07317; P=0.7883	F(1, 37) = 0.2856; P=0.5962	F(1, 37) = 0.2910; P=0.5928
AHP amp	F(1, 37) = 3.374; P=0.0743	F(1, 37) = 0.3165; P=0.5771	F(1, 37) = 0.1254; P=0.7253

Figures and Legends

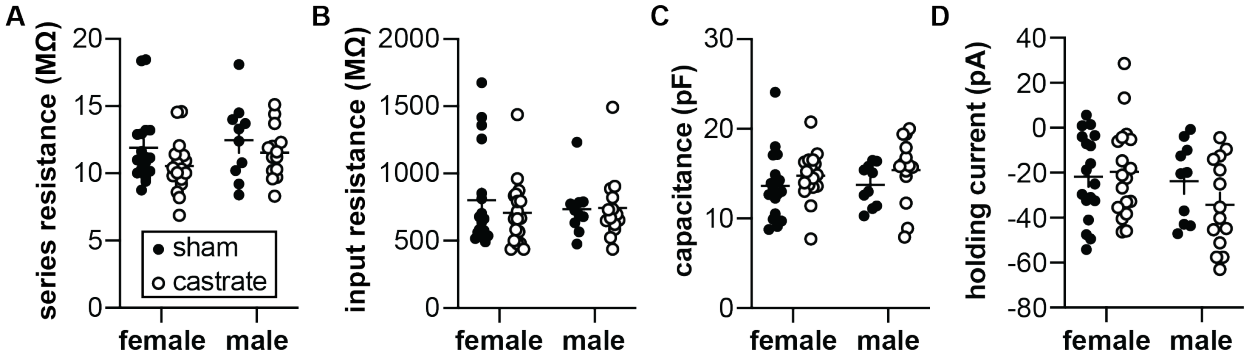

Figure 1. Passive properties of GnRH neurons in the potassium current experiments. No differences attributable to sex or gonadal status were detected in (A) series resistance, (B) input resistance, (C) capacitance or (D) holding current using two-way ANOVA (Table 1).

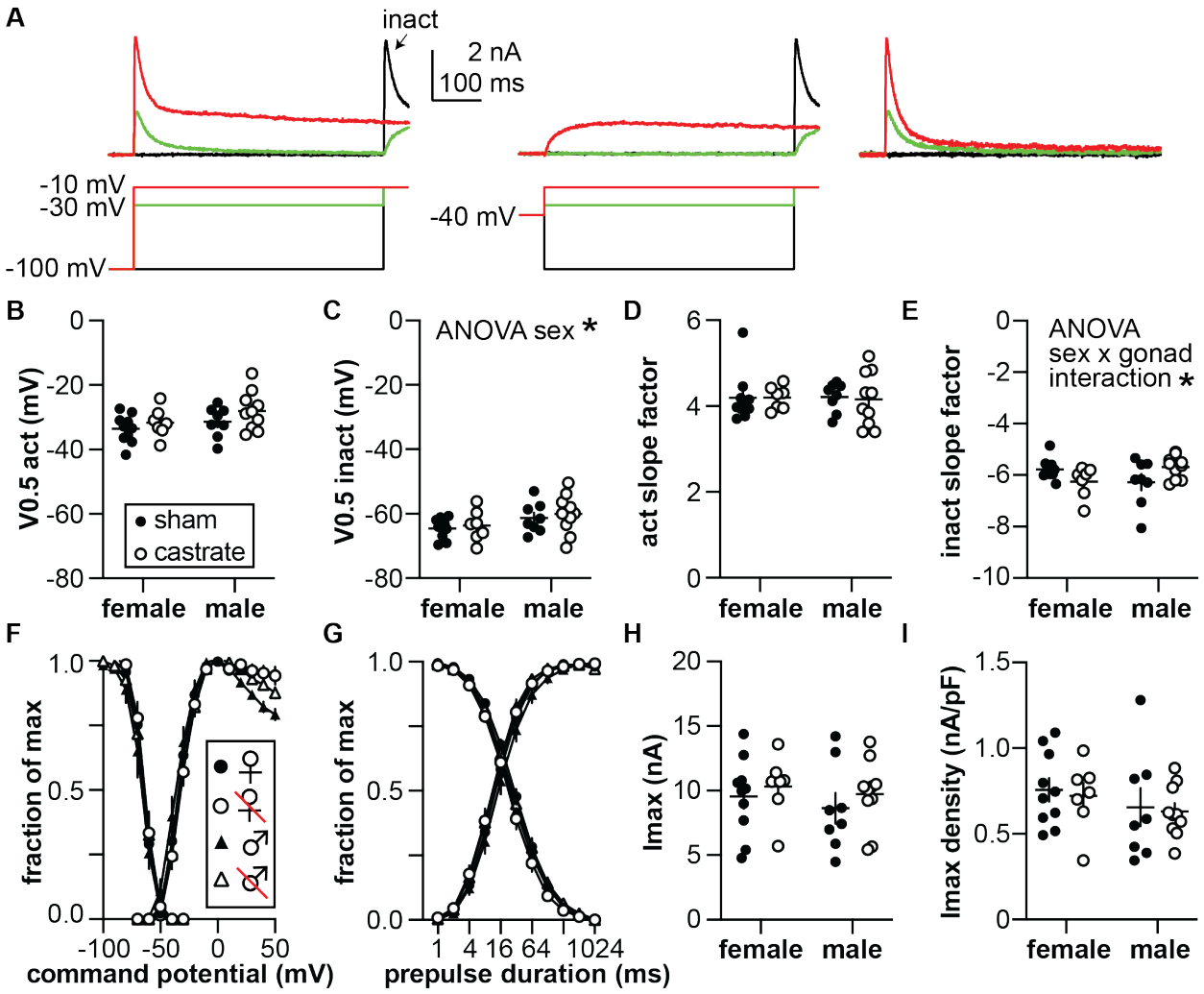

Figure 2. Characterization of the I_A potassium current. (A) Representative traces illustrating the isolation of the rapidly inactivating I_A (top) and the voltage protocols used (bottom). The 500 ms 100 mV prepulse was truncated for illustration. The arrow labeled "inact" indicates the -10 mV test pulse at the end of the activation voltage family used to calculate the voltage dependence of inactivation. Only three steps from the voltage family from -100 to +50 mV are shown for clarity. The right panel shows I_A isolated by subtracting the -40 mV prepulse traces in the middle panel from the -100 mV prepulse traces in the left panel. (B, C) membrane potential at which half of the current is activated (V0.5act) (B) or inactivated (V0.5inact) (C). (D, E) activation (act) (D) and inactivation (inact) (E) slope factors. (F) Voltage dependence of activation and inactivation, normalized by maximum conductance and maximum current respectively. (G) Time course of recovery and inactivation, normalized by maximum current. (H, I) Maximum current (Imax) (H) and current density (I). Statistical parameters are in Table 2.

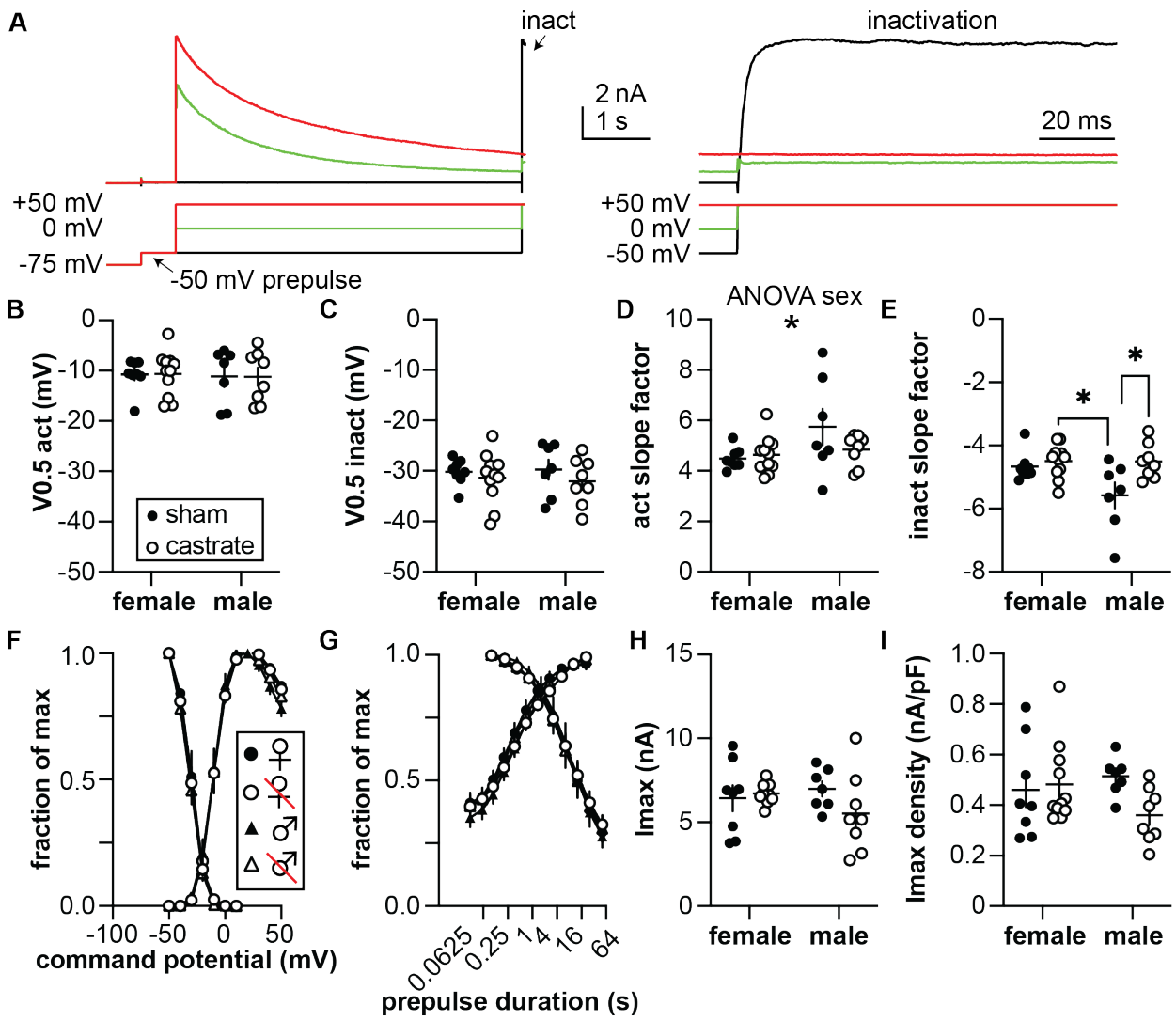

Figure 3. Characterization of the I_k potassium current. (A) Representative traces illustrating the activation and inactivation of I_K, (top) and the voltage protocols used (bottom). The 10 s -75 mV prepulse was truncated in the left panel for illustration. The arrow labeled "inact" on the left panel indicates the region expanded on the right. Only three steps of the voltage family from -100 to +50 mV are shown for clarity. (B, C) membrane potential at which half of the current is activated (V0.5act) (B) or inactivated (V0.5inact) (C). (D, E) activation (act) (D) and inactivation (inact) (E) slope factors. (F) Voltage dependence of activation and inactivation, normalized by maximum conductance and maximum current respectively. (G) Time course of recovery and inactivation, normalized by maximum current. (H, I) Maximum current (Imax) (H) and current density (I). Statistical parameters are in Table 3.

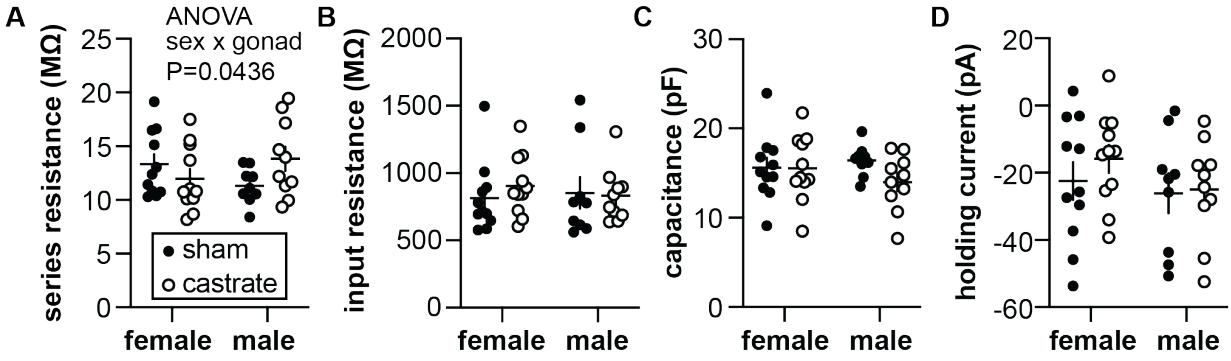

Figure 4. Passive properties of recordings for the action potential study. (A) series resistance (no post hoc comparisons were significant); (B) input resistance; (C) capacitance; (D) holding current at -65 mV.

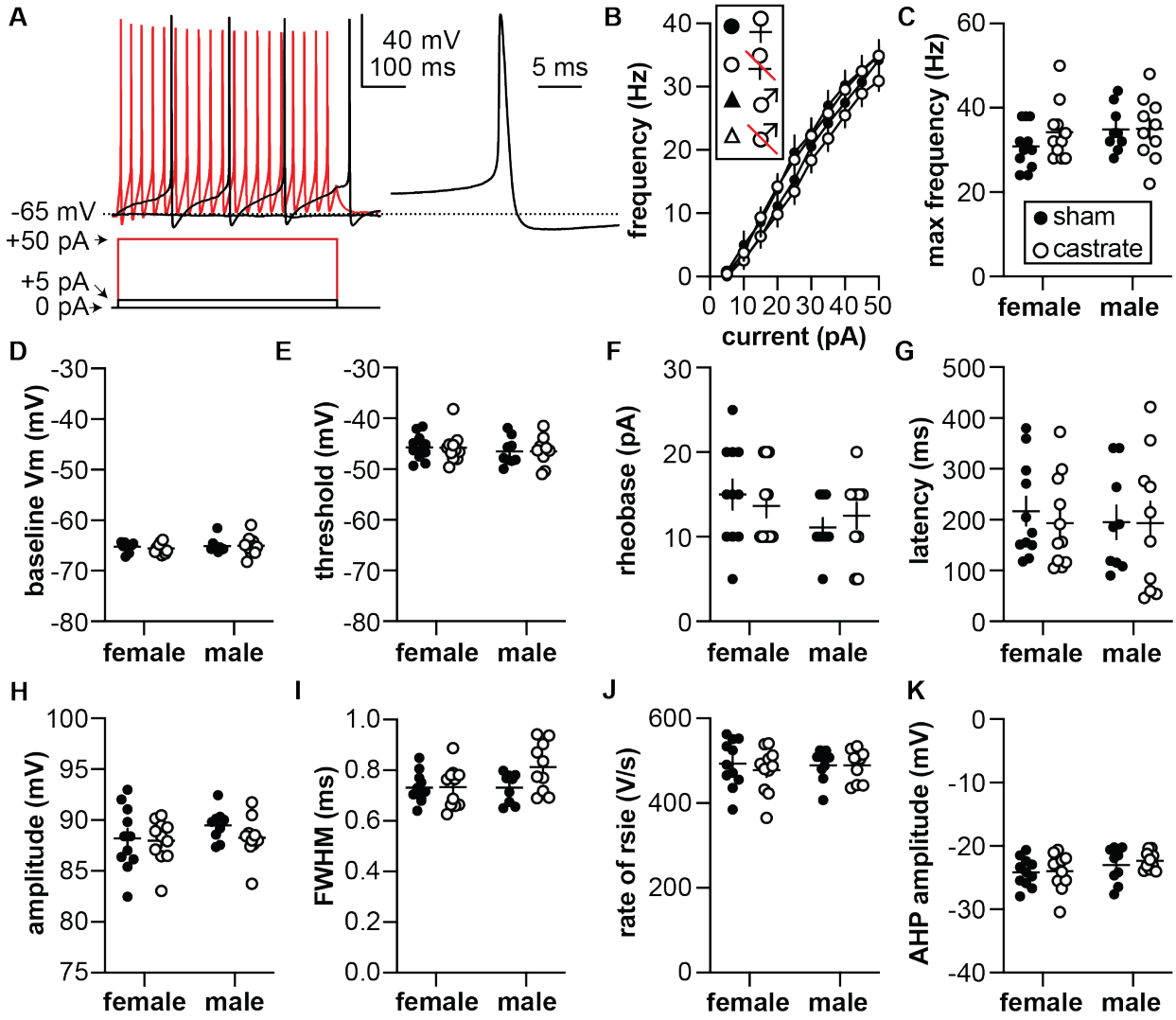

Figure 5. Action potential properties. (A) (left) Representative membrane voltage (top) responses to current injections (bottom); only three steps are shown for clarity. (Right) Expanded first action potential waveform. (B) Frequency of action potentials as a function of current injection. (C) Maximum action potential frequency. (D) Baseline membrane potential before rheobase current injection. (E) Action potential threshold. (F) Rheobase (minimum current to produce an action potential). (G) Action potential latency. (H) Action potential amplitude. (I) Action potential full width at half maximum (FWHM). (J) Maximum rate of rise of the action potential. (K) Amplitude of the afterhyperpolarization. Note y-axes of D, E, and H do not start or end at zero. Statistical parameters are in Tables 5 and 6.