Behavioral/Systems/Cognitive

Hyperpolarization-Activated Currents in Gonadotropin-Releasing Hormone (GnRH) Neurons Contribute to Intrinsic Excitability and Are Regulated by Gonadal Steroid Feedback

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Pulsatile release of gonadotropin-releasing hormone (GnRH) is required for fertility and is regulated by steroid feedback. Hyperpolarization-activated currents (I_h) play a critical role in many rhythmic neurons. We examined the contribution of I_h to the membrane and firing properties of GnRH neurons and the modulation of this current by steroid milieu. Whole-cell voltage- and current-clamp recordings were made of GFP-identified GnRH neurons in brain slices from male mice that were gonad-intact, castrated, or castrated and treated with estradiol implants. APV, CNQX, and bicuculline were included to block fast synaptic transmission. GnRH neurons (47%) expressed a hyperpolarization-activated current with pharmacological and biophysical characteristics of I_h. The I_h-specific blocker ZD7288 reduced hyperpolarization-induced sag and rebound potential, decreased GnRH neuron excitability and action potential firing, and hyperpolarized membrane potential in some cells. ZD7288 also altered the pattern of burst firing and reduced the slope of recovery from the after-hyperpolarization potential. Activation of I_h by hyperpolarization increased spike frequency, whereas inactivation of I_h by depolarization reduced spike frequency. Castration increased I_h compared with that in gonad-intact males. This effect was reversed by *in vivo* estradiol replacement. Together, these data indicate I_h provides an excitatory drive in GnRH neurons that contributes to action potential burst firing and that estradiol regulates I_h in these cells. As estradiol is the primary central negative feedback hormone on GnRH neuron firing in males, this provides insight into the mechanisms by which steroid hormones potentially alter the intrinsic properties of GnRH neurons to change their activity.

Introduction

Gonadotropin-releasing hormone (GnRH) neurons form the final common pathway determining fertility in all vertebrate species. These neurons generate pulsatile hormone release that is frequency modulated by gonadal steroid feedback (Leipheimer et al., 1984; Levine et al., 1985a; Clarke et al., 1987; Levine and Duffy, 1988; Barrell et al., 1992). Changes in GnRH pulse frequency differentially regulate the synthesis and secretion of pituitary hormones in the target cells (Wildt et al., 1981; Shupnik, 1990). In brain slices and in culture, GnRH neurons are spontaneously active in an episodic manner (Terasawa, 1998; Kuehl-Kovarik et al., 2002; Moenter et al., 2003), but the mechanisms underlying intrinsic spike generation and episodic patterning are not well understood. One possibility is that this episodic release arises from an intrinsic pacemaker mechanism. GnRH neurons express a variety of intrinsic conductances that shape their firing patterns (Bosma, 1993; Kusano et al., 1995; DeFazio and Moenter, 2002; Kelly et al., 2002; Moenter et al., 2003; Chu and Moenter, 2006), some of which are known to be regulated by gonadal hormone feedback (DeFazio and Moenter, 2002; Chu and Moenter, 2006; Zhang et al., 2009).

The hyperpolarization-activated current (I_h) is an inward current activated by hyperpolarization from typical neuronal resting membrane potentials (Crepel and Penit-Soria, 1986; McCormick and Pape, 1990; Erickson et al., 1993; Clarençon et al., 1996). I_h is crucial for regulating general membrane phenomena, including oscillatory activity and generating phasic burst firing in, for example, thalamic neurons (McCormick and Pape, 1990; Pape, 1996; Lüthi et al., 1998), hippocampal CA1 interneurons (Maccaferri and McBain, 1996), pyramidal neurons (Gasparini and DiFrancesco, 1997), and entorhinal cortical neurons (Nolan et al., 2007). Hyperpolarization-activated nonselective cation (HCN) channels conduct I_h (Robinson and Siegelbaum, 2003). *In situ* hybridization studies demonstrated that all four HCN channel subunits are expressed in the adult mouse and rat hypothalamus (Biel et al., 1999) as well as in immortalized GT1–7 GnRH neurons (Arroyo et al., 2006). A hyperpolarization-activated current was reported in GnRH neurons in brain slices (Zhang et al., 2007), but the pharmacology, contributions of this current to the firing properties of GnRH neurons, and the regulation of this current by steroid-hormone feedback have not been previously investigated. In the current study, we examined the function of I_h in changing intrinsic properties and firing pattern of GnRH neurons, and the regulation of these cells by gonadal hormone feedback.

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Materials and Methods

Animals. Adult (2–3 months of age) male mice expressing enhanced GFP (Clontech) under the control of the GnRH promoter were used to facilitate identification of GnRH neurons (Suter et al., 2000). A total 407 GFP-positive cells from 210 mice were used in these studies. Mice were maintained under a 14 h light:10 h dark photoperiod with Harlan 2916 chow (Harlan) and water available *ad libitum*. To study the influence of gonadal hormones, 184 mice were castrated (CAS) under isoflurane anesthesia (Burns Veterinary Supply) 5–9 d before experimentation; time after gonadectomy within this range did not affect results. The long-acting local anesthetic bupivacaine (0.25%; Abbott Labs) was applied locally to surgical sites to minimize postoperative pain and distress. The steroid hormone estradiol, which appears to be the main testosterone metabolite providing negative feedback on GnRH neuron activity in male mice (Pielecka and Moenter, 2006), was replaced in 29 of the 145 mice via SILASTIC (Dow Corning) implants containing 0.625 μg of estradiol dissolved in sesame oil. These implants were placed subcutaneously in the scapular region at the time of castration, eliminating the need for a second anesthesia. They produce low physiological levels of estradiol (DeFazio and Moenter, 2002; Christian et al., 2005). In addition, 36 gonadal intact mice were studied. The Animal Care and Use Committee of the University of Virginia approved all procedures.

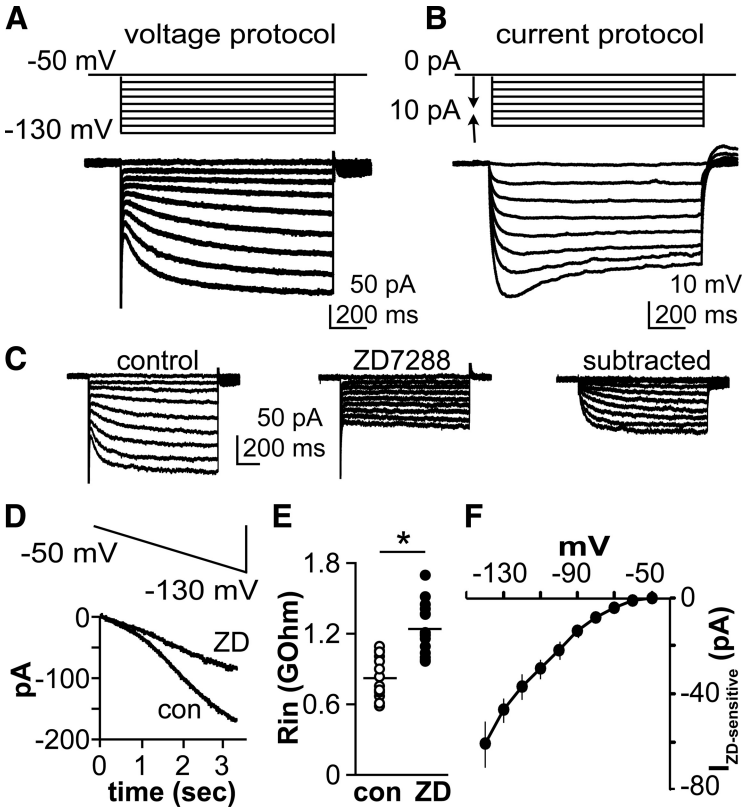

Figure 1. A subpopulation of GnRH neurons exhibit a hyperpolarization-activated current. ***A***, Top, voltage protocol; bottom, representative voltage-clamp recording showing current response with slowly developing inward current. ***B***, Top, current injection protocol; bottom, representative current-clamp recording showing membrane response including voltage-dependent sag. ***C***, Effect of ZD7288 on inward current generated by hyperpolarizing membrane potential steps. ***D***, A ZD-sensitive current is also revealed by a slow hyperpolarizing voltage ramp. ***E***, Effect of ZD7288 on input resistance ($n = 12$, $p < 0.01$). ***F***, I–V relationship of ZD-sensitive current ($n = 16$, $^*p < 0.01$). con, Control.

Brain-slice preparation. Brain slices were prepared as previously described (Nunemaker et al., 2003a; Chu and Moenter, 2005). All solutions were bubbled with 95% O_2 and 5% CO_2 to maintain pH and oxygenation for at least 15 min before use and throughout experiments. In brief, brains were quickly removed and immersed immediately for 30–60 s in ice-cold sucrose buffer containing the following (in mM): 250 sucrose, 26 $NaHCO_3$, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, 10 glucose, 3.5 KCl, and 2.5 $MgCl_2$. Sagittal brain slices (300 μm) through the preoptic area and hypothalamus were cut using either a Vibratome 1000 or 3000 (Technical Products International). Slices were immediately transferred into a holding chamber and incubated at 31–33°C for a 30 min recovery period in a mixture of 50% sucrose saline and 50% artificial CSF (ACSF) containing the following (in mM): 135 NaCl, 26 $NaHCO_3$, 1,25 Na_2HPO_4, 1.2 $MgSO_4$, 10 D-glucose, 3.5 KCl, 2.5 $CaCl_2$, pH 7.4. Slices were then transferred to 100% ACSF and maintained at room temperature (~21–23°C) until study (30 min to 8 h).

Data acquisition. Slices were transferred to a recording chamber mounted on the stage of an upright microscope (BX50WI, Olympus) and stabilized in the chamber at least 5 min before recording. The chamber was continuously perfused with ACSF at a rate of 4–5 ml/min at 31–32°C. Pipettes (3–4 MΩ) were pulled from borosilicate glass capillaries (outer diameter, 1.65 mm; inner diameter,1.12 mm; World Precision Instruments) using a Flaming/Brown P-97 (Sutter Instruments). GnRH-GFP neurons from the preoptic area and ventral hypothalamus were identified by brief illumination at 470 nm. Data were acquired using an EPC-8 (HEKA Electronics) with an ITC-18 interface (Instrutech) controlled by the PulseControl XOP (Instrutech) running in IgorPro (Watermetrics) or using one headstage of an EPC-10 dual amplifier controlled by PatchMaster (both from HEKA Electronics). There were no differences attributable to the acquisition system. Signals were low-pass filtered at 10 kHz. During whole-cell recordings, input resistance (Rin), series resistance (Rs), and membrane capacitance (Cm) were continually measured from averaged membrane response to 5 mV hyperpolarizing voltage steps. Only recordings with stable Rin >500 MΩ and Rs <20 MΩ and stable Cm were used for analysis. Fast transients recorded after formation of the gigaohm seal in the cell-attached configuration were subtracted from the membrane response in the whole-cell configuration to correct for incomplete compensation of electrode capacitance. Data were further examined to make sure changes in Rin or Rs within acceptable limits did not influence results. Calculated liquid junction potential error, estimated to be −13 mV, was not corrected (Barry, 1994).

In all experiments, fast synaptic transmission to GnRH neurons was blocked by antagonists to ionotropic transmitter receptors ($GABA_A$, 20 μM bicuculline methobromide; AMPA, 20 μM CNQX; NMDA, 20 μM APV). To characterize biophysical properties of I_h, tetrodotoxin (TTX) and 4-aminopyridine (4-AP) were used to block Na^+ current and A-type current (I_A), respectively, in voltage-clamp experiments. Nickel (100 μM) was used to block low-voltage-activated (LVA) calcium channels.

Voltage-clamp recordings. Whole-cell voltage-clamp was used to study current activated by membrane hyperpolarization. Patch electrodes for voltage-clamp contained the following (in mM): 125 K gluconate, 20 KCl, 10 HEPES, 5 EGTA, 4.0 MgATP, 0.4 NaGTP, 0.1 $CaCl_2$, pH 7.3, 290 mOsm. Membrane potential was held at −50 mV. Current response to hyperpolarizing voltage steps (1–1.2 s duration, 10 mV interval) was evaluated. The protocol was repeated three times and traces were averaged for analysis. Additionally, a slow (1 mV/50 ms) ramp protocol from −50 to −130 mV was used to characterize I_h. Reversal potential of I_h was

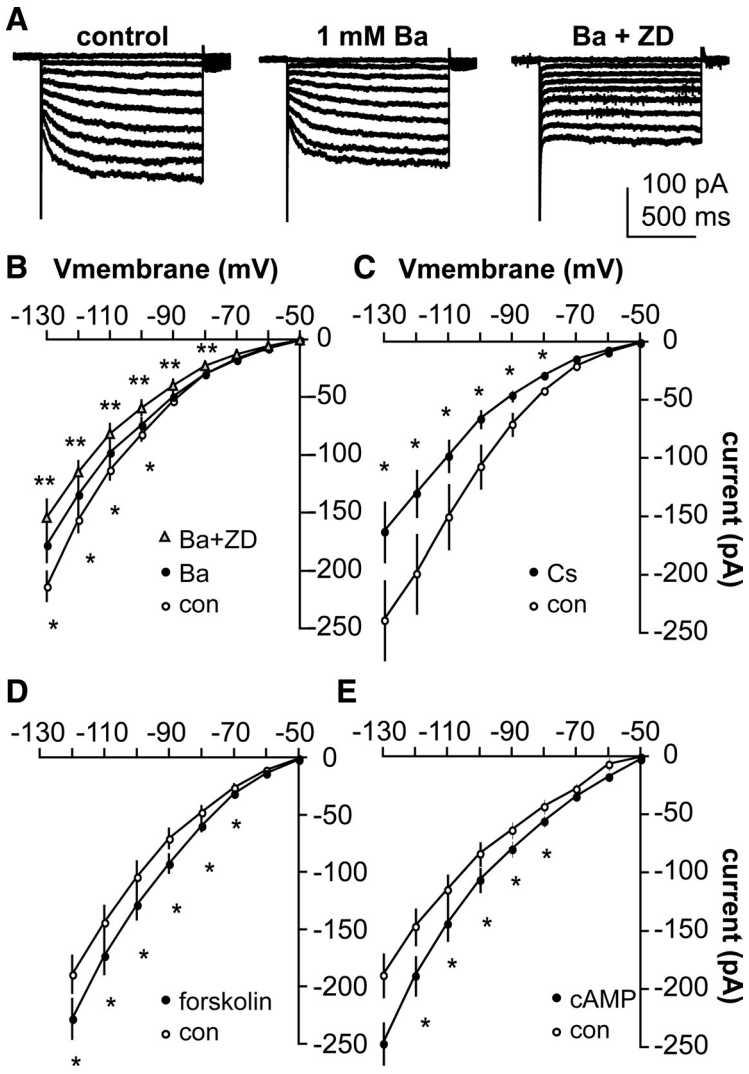

Figure 2. Pharmacological profile of the hyperpolarization-activated current in GnRH neurons indicates it is I_h. ***A***, Representative voltage-clamp recordings showing control (left), treatment with 1 mM Ba^{2+} (center), and Ba^{2+} plus 50 μM ZD7288 (right). ***B–E***, Changes in I–V relationship for 1 mM Ba^{2+} plus 50 μM ZD7288 (***B***, $n = 9$), 3 mM Cs^+ (***C***, $n = 6$), 10 μM forskolin (***D***, $n = 9$), and 100 μM cAMP (***E***, $n = 7$). *$p < 0.05$ versus control (con), **$p < 0.05$ versus Ba.

estimated using a protocol in which membrane potential was held at −50 mV, then stepped to −120 mV for 1 s, following which tail currents were measured at −70 mV to −120 mV (−10 mV interval, 1 s duration). The amplitude of the evoked current was measured from instantaneous and steady-state voltage response to prestep baseline level. Subtraction of traces before and after treatment with ZD7288 (ZD) was used to quantify ZD7288-sensitive current and thus identify I_h. Ion substitution was made as follows: to increase extracellular K^+, KCl was substituted for NaCl in the ACSF; to reduce extracellular Na^+, tetraethylammonium chloride was substituted for NaCl.

Current-clamp recordings. Whole-cell current clamp was used to study membrane potential changes using the same pipette solution as in voltage-clamp studies. Current-clamp recordings were made using bridge balance and capacitance compensation, using the same pipette solution as above. All cells had an initial membrane potential negative to −55 mV without current injection and action potential amplitude of >90 mV. Direct current injection (<±10 pA) was used to normalize membrane potential to facilitate comparison during firing frequency studies. To study the voltage change induced by activating I_h, current injections (1–1.2 s, 0−−80 pA, −10 pA intervals) were given. In some cells, action potential firing rate was monitored; changes in firing rate of at least 20% were used to classify cells as responding to treatment.

To examine the effects of the I_h blocker ZD7288 on GnRH neuronal excitability, positive current injections (600 ms, 0–50 pA) were applied, and the number of spikes before and after treatment quantified. Sag and rebound potential amplitudes were measured from the precurrent injection membrane potential. Latency was the time from end of the current injection to the peak of the rebound depolarization potential. Depolarizing and hyperpolarizing current pulses (1–2 s) were used for activating and deactivating I_h; their effects on subsequent spontaneous action potential firing were determined by comparing spike frequency before current injection to that during the 3 s following termination of the current injection. The input resistance of GnRH neurons was determined from the steady-state voltage response to a hyperpolarizing pulse (10–15 pA producing ~10 mV change in membrane potential). Series of action potentials (2–6 spikes) were considered to be components of a single burst when there was a steady depolarization from the peak of the after-hyperpolarizing potential to the initiation of the subsequent spike, and when they were separated by no more than 250 ms. The latter criterion was based on the duration of the slow after-depolarizing potential in GnRH neurons, which peaks ~200 ms after spike initiation (Chu and Moenter, 2006). Single spikes and spikes per burst before and after ZD7288 were manually determined. To determine whether I_h participates in subthreshold depolarization of GnRH neurons, the slope from the peak of the after-hyperpolarizing potential of single spikes or the first spike of a two-spike burst to 200 ms thereafter was determined before and during treatment with ZD7288. To determine whether I_h alters slow after-depolarizing potential (sADP) following evoked action potentials, brief higher amplitude current injections were delivered (200–300 pA, 3 ms).

Drug treatments. All chemicals were purchased from Sigma unless otherwise noted. All treatments were bath-applied. The specific antagonist 4-(*N*-ethyl-*N*-phenylamino)-1,2-dimethyl-6-(methylamino) pyrimidinium chloride (ZD7288, 50 μM; Tocris Bioscience) was used to identify I_h in all experiments. To characterize biophysical properties of I_h, sodium channels were blocked with TTX (0.5 μM, Calbiochem) and dominant I_A was blocked with 4-AP (3–5 mM; Tocris Bioscience). Response of hyperpolarization-activated currents to cAMP (100 μM), an important signal carrier for biological response of neurons to activation of some metabotropic receptors, and forskolin (10 nM), a compound to increase the intracellular cAMP concentrations, was also characterized, as was inhibition by 1 mM Ba^{2+} (a nonspecific blocker of inward rectifier potassium channels, $BaCl_2$) and 3 mM extracellular Cs^+ (which blocks I_h among other conductances and transporters, CsCl; Fluka). To distinguish between I_h and LVA calcium currents, Ni^{2+} was applied first to block LVA followed by subsequent treatment with Ni^{2+} and ZD7288 to block LVA and I_h.

Statistical analyses. Statistical analyses were performed using Instat or Prism (Graphpad Software). Data values are expressed as mean ± SEM. Each cell served as its own control except in comparisons among animal models. Statistical comparisons were made using paired two-tailed Student's *t* test for comparisons within cells, or ANOVA followed by Bonferroni's multiple-comparison test. Significance was set at $p < 0.05$; all nonsignificant *p* values were >0.1, unless otherwise specified.

Results

GnRH neurons exhibit hyperpolarization-activated currents (I_h)

To examine hyperpolarization-activated currents in GnRH neurons, whole-cell recordings were made from GFP-positive neurons in brain slices from castrated male mice. Cells were isolated from the influence of fast synaptic transmission by blocking ionotropic GABA and glutamate receptors and fast sodium and A-type potassium conductances. As the membrane was stepped from a holding potential of −50 mV to more hyperpolarized potentials in 10 mV increments, an inward current was activated beginning between −60 and −70 mV (Fig. 1*A*). The current activated and inactivated slowly and was observed in 47% of recorded cells (193 of 407). There was no difference in initial resting potential between cells exhibiting I_h and those not exhibiting I_h (61.4 ± 1.1 mV with I_h, $n = 16$; 62.2 ± 1.1 mV without I_h, $n = 18$; $p > 0.05$). In current clamp, injection of hyperpolarizing current produced an initial membrane hyperpolarization followed by a return toward resting potential, or sag, with larger current injections (Fig. 1*B*). To test whether this hyperpolarization-activated current was conducted through HCN channels, the specific blocker ZD7288 (50 μM) was applied in voltage-clamp experiments. ZD7288 reduced the inward current within 5–8 min (Fig. 1*C*,*F*). When membrane potential was slowly (1 mV/50 ms) ramped from −50 to −130 mV, an inward current also developed over time and was reduced by ZD7288 (Fig. 1*D*). ZD7288 also increased input resistance of cells at membrane potentials near −80 mV (850.6 ± 44.9 MΩ in control vs 1248.0 ± 52.8 MΩ in ZD7288, $n = 16$, $p < 0.01$), indicating a reduction in membrane conductance (Fig. 1*E*) and reduced slope conductance from 1.19 ± 0.12 to 0.50 ± 0.03 nS ($p < 0.05$). Input resistance decreased with membrane hyperpolarization from −70 to −90 mV (949 ± 38 MΩ vs 700 ± 27 MΩ, $n = 12$, $p < 0.01$), suggesting opening of voltage-gated channels.

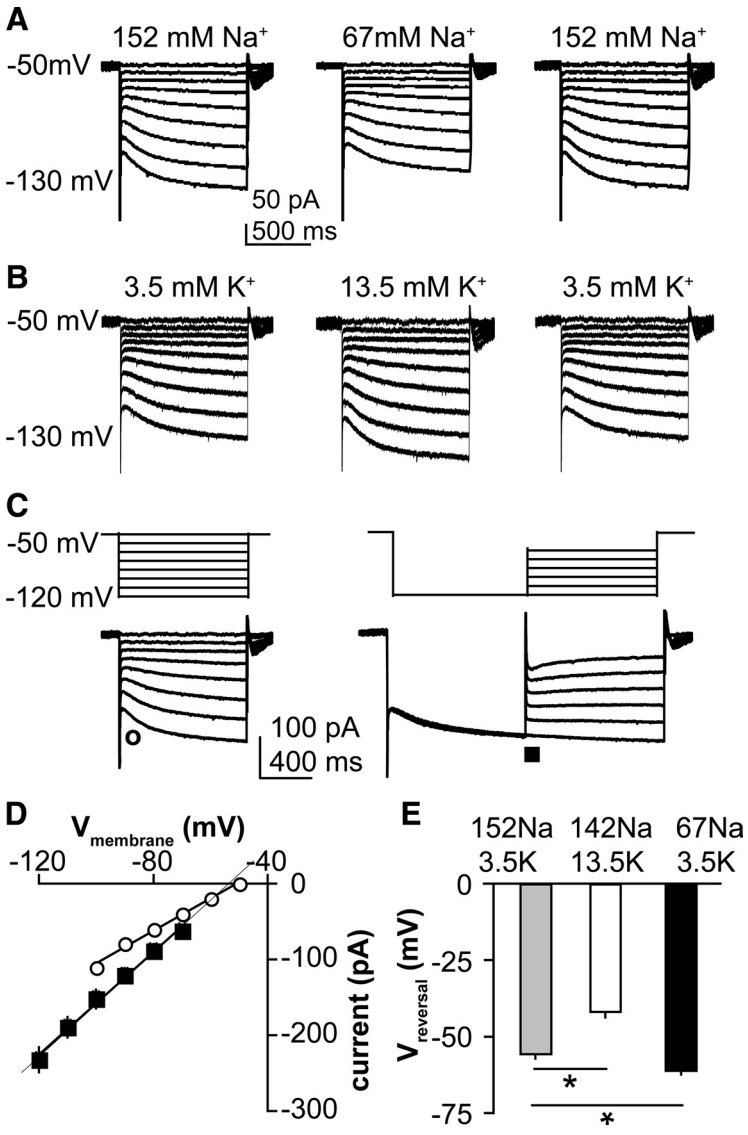

Figure 3. The hyperpolarization-activated current in GnRH neurons is carried by both Na^+ and K^+ ions. ***A***, ***B***, Representative voltage-clamp recordings showing current changes in response to reduction of extracellular Na^+ (***A***, $n = 6$) or increase in extracellular K^+ (***B***, $n = 5$). ***C***, Top, Protocols used to establish reversal potential; bottom, representative voltage-clamp recording showing time of measurement of instantaneous current (○, left) and tail current (■, right). ***D***, Calculation of reversal potential by linear fit of average voltages from ***C*** ($n = 11$). ***E***, Effect of ionic manipulations on reversal potential ($V_{reversal}$). *$p < 0.05$.

Pharmacological properties of hyperpolarization-activated currents in GnRH neurons

In addition to HCN channels, inwardly rectifying potassium channels (I_{Kir}) can be activated by hyperpolarization. The potassium channel blocker Ba^{2+} (1 mM) was able to block a component of hyperpolarization-activated current ($n = 9$, $p < 0.05$ vs control) (Fig. 2*A*,*B*). However, in most of these cells, ZD7288 was able to further reduce hyperpolarization-activated current ($p < 0.05$ vs Ba^{2+} alone) (Fig. 2*A*,*B*). Extracellular Cs^+ (3 mM CsCl) also reduced the hyperpolarization-activated current (Fig. 2*C*) ($n = 6$, $p < 0.01$); although Cs^+ can also block potassium channels, it has been reported as a blocker of HCN-mediated currents (Halliwell and Adams, 1982; Crepel and Penit-Soria, 1986; Spain et al., 1987; McCormick and Pape, 1990; Bayliss et al., 1994; Pape, 1996). The voltage dependence of HCN channel gating is strongly modified by hormones, neurotransmitters, and second messengers, making these channels exquisitely sensitive to changes in cellular environment (Pape and McCormick, 1989; Ludwig et al., 1998; Biel et al., 1999). The adenylyl cyclase activator forskolin (Fig. 2*D*) (10 μM, $n = 9$, $p < 0.01$) and the second messenger cAMP (100 μM) increased the hyperpolarization-activated inward current in GnRH neurons (Fig. 2*E*) ($n = 7$, $p < 0.01$). Together, these pharmacological and biophysical properties suggest the hyperpolarization-activated current in GnRH neurons is mediated by HCN channels and is I_h.

Ion exchange and reversal potential estimation

HCN channels pass both Na^+ and K^+ ions (Mayer and Westbrook, 1983; Crepel and Penit-Soria, 1986; Spain et al., 1987; McCormick and Pape, 1990). In ion exchange experiments, membrane current was reversibly reduced by a reduction in extracellular Na^+

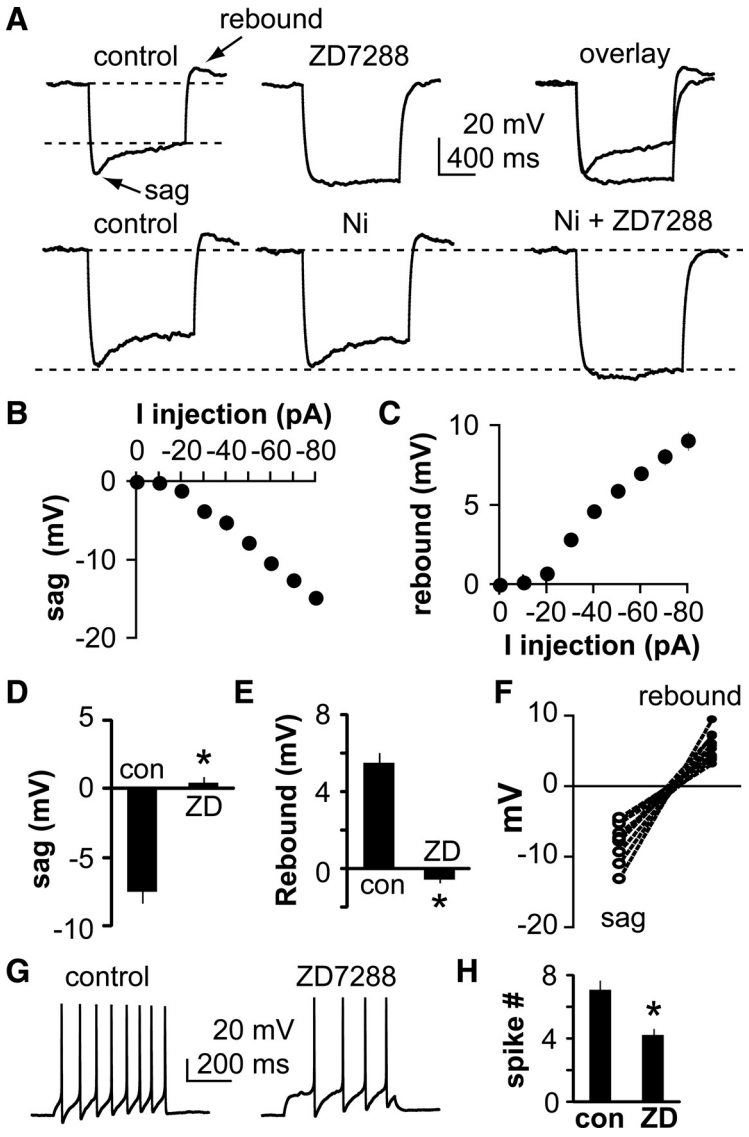

Figure 4. Blocking I_h alters membrane properties of GnRH neurons. ***A***, Top, Representative current-clamp recordings showing the effect of a −50 pA current injection on GnRH neuron membrane potential. A characteristic sag develops during the injection and there is a rebound in membrane potential after termination of the current injection. ZD7288 blocks both the sag and rebound potentials. Bottom, Nickel fails to block the rebound, which is subsequently blocked by ZD7288. ***B***, ***C***, Voltage dependence of sag (***B***) and rebound (***C***) potentials in GnRH neurons ($n = 11$). ***D***, ***E***, Quantification of sag (***D***) and rebound (***E***) potentials before and during treatment with ZD7288 ($n = 11$). ***F***, Relationship between sag and rebound potential in all cells studied. ***G***, Response of GnRH neuron to 20 pA current injection under control (left) and ZD7288 (right) conditions showing that blocking I_h reduces excitability. ***H***, Mean ± SEM spikes induced by a 40 pA current injection ($n = 14$, $^*p < 0.01$). con, Control.

concentration (from 152 to 67 mM; 238.6 ± 22.9 pA to189.0 ± 19.3 pA, $n = 6$, $p < 0.01$) (Fig. 3*A*) and was increased by an increase in extracellular K^+ concentration (from 3.5 mM to 13.5 mM; 226.3 ± 17.3 pA to 274.4 ± 13.1 pA, $n = 5$, $p < 0.01$) (Fig. 3*B*) when measured at a membrane potential of −120 mV. The reversal potential was determined using a protocol described by Bayliss (Bayliss et al., 1994; Funahashi et al., 2003). Instantaneous currents were measured immediately following the capacitive transient (Fig. 3*C*, open circle) after stepping from a potential at which I_h is not activated (−50 mV). In the same cell, I_h was activated by a 1 s prepulse at −120 mV, then the tail current was measured at the onset of test steps to potentials from −120 mV to −70 mV (10 mV increments) (Fig. 3*C*, right). Both resulting I–V plots were fit with linear regression and the reversal potential calculated from the point of crossover ($n = 11$) (Fig. 3*D*). The calculated reversal potential was similar to previously reported values in neurons (Spain et al., 1987; Schlichter et al., 1991), and was sensitive to changes in concentration of either Na^+ or K^+ ions in the extracellular solution (both manipulations, $p < 0.05$) (Fig. 3*E*).

Blocking I_h decreases GnRH neuronal excitability and action potential firing

To examine the action of I_h in GnRH neuronal function, whole-cell current-clamp recordings were made. Injection of hyperpolarizing current pulses (10–80 pA, 600 ms) elicited a hyperpolarization during which a depolarizing sag developed over 800 ms, a time course consistent with the slow kinetics of I_h, allowing this depolarization to be distinguished from that which might be caused by a T-type calcium current (Sun et al., 2010). After termination of a hyperpolarizing current injection (−50 pA), a prominent rebound depolarization potential was observed (Fig. 4*A*, top). Nickel (100 μM) did not block the rebound (control, 4.8 ± 0.5 mV; Ni^{2+}, 4.3 ± 0.4 mV; $n = 8$) (Fig. 4, bottom). Both sag and rebound potentials increased with increasing hyperpolarizing current injection (Fig. 4*B*,*C*), and were reduced by ZD7288 ($n = 11$, $p < 0.01$) (Fig. 4*A*,*D*,*E*). Further, there was a direct association between amplitude of the sag and that of the rebound potential, suggesting a common mechanism (Fig. 4*F*). Finally, blocking I_h with ZD7288 decreased GnRH neuron excitability upon injection of a 20 pA, 600 ms depolarizing current pulse response to depolarizing current injection ($p < 0.01$, $n = 14$) (Fig. 4*G*,*H*).

In recordings of spontaneous action potential firing in the presence of APV, CNQX, and picrotoxin to block ionotropic receptors for GABA and glutamate, blocking I_h decreased action potential frequency in ~50% of cells, which is similar to the percentage exhibiting I_h in voltage-clamp studies ($n = 9$, $p < 0.01$) (Fig. 5). Visible membrane hyperpolarization was observed in 24% of cells (4 of 17 cells, −6.1 ± 0.5 mV). In the presence of TTX to block action potential firing, frank hyperpolarization was observed in 5 of 16 cells (4.8 ± 0.5 mV) with the remaining 11 being within 1 mV of the initial membrane potential (data not shown). ZD7288 reduced the overall action potential firing of GnRH neurons (11.5 ± 1.3 spikes/min in control vs 7.5 ± 1.2 spikes/min in ZD, $n = 17$, $p < 0.01$). For those cells that responded to ZD7288 with a reduction in overall firing rate, further analysis of single spikes and bursts was conducted (Fig. 5*D*,*E*). Blocking I_h altered the pattern of burst firing, reducing both single spikes and the number of spikes per burst ($p < 0.01$ for single spikes, two and three spike bursts, $p < 0.05$ for four spike bursts; statistics not performed on five, six, or seven spike bursts as these were not observed after ZD7288 treatment).

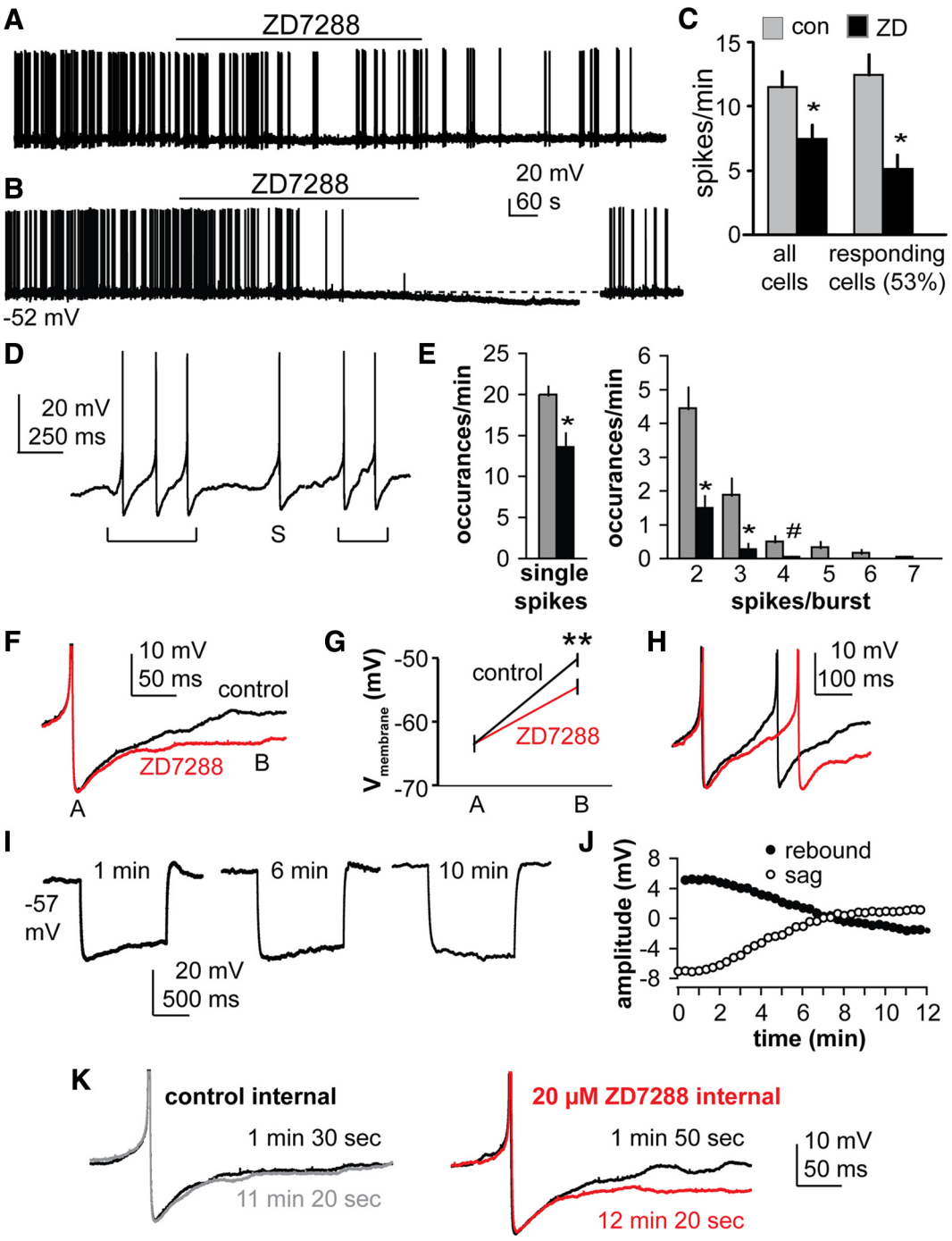

Figure 5. Blocking I_h reduces the spontaneous firing rate of GnRH neurons. ***A***, ***B***, Effect of ZD7288 on firing activity of GnRH neurons. Membrane potential under the trace indicates potential at onset of trace. ***B***, Some cells exhibited frank hyperpolarization in addition to a reduction in firing activity. A 10 pA current injection shown at the end of the trace in ***B*** illustrates the cell is still capable of generating action potentials. ***C***, Firing rate before (gray bars) and during (black bars) ZD7288 treatment in all GnRH neurons (left, $n = 17$) and in only responding GnRH neurons (right, $n = 9$). ***D***, Examples of single spike and burst firing; brackets below trace indicate bursts. S, Single spike. ***E***, Blocking I_h reduces both single spikes and the number of spikes/burst. $^*p < 0.01$, $^{\#}p < 0.05$. ***F***, Representative example of the effect of ZD7288 (red) on the slope of the recovery from the AHP following single spikes ($n = 16$). $^{**}p < 0.001$. ***G***, Mean ± SEM membrane potential at points A (AHP amplitude) and B (200 ms post AHP peak) in ***F*** ($n = 10$). ***H***, Example of interspike membrane potential dynamics in a two-spike burst; red trace is after ZD7288. ***I***, Representative current-clamp recordings with ZD7288 included in the pipette solution showing response to a 50 pA hyperpolarizing current injection; rebound and sag disappear over time. ***J***, Mean ± SEM amplitude of sag and rebound over time with ZD7288 in the pipette. ***K***, Representative recordings illustrating action potential waveform at times indicated with control internal (left) and ZD7288 internal (right).

Blocking I_h also reduced the slope of the recovery from the after-hyperpolarization potential (AHP). For single spikes, there was no difference in the absolute peak value of the AHP (control, −63.5 ± 1.3 mV; ZD, −63.4 ± 1.4 mV, $n = 10$), but 200 ms after the peak of the AHP, control neurons had depolarized more (control, −50.2 ± 1.0 mV; ZD, −54.5 ± 1.1 mV, $p < 0.001$) (Fig. 5*F*,*G*). Within two spike bursts, a similar phenomenon was observed; ZD7288 delayed initiation of the second spike (Fig. 5*H*). Previous work indicated sADPs following evoked action potentials were not different in the presence or absence of ZD7288 in GnRH neurons from female mice (Chu and Moenter, 2006). To determine whether this is a

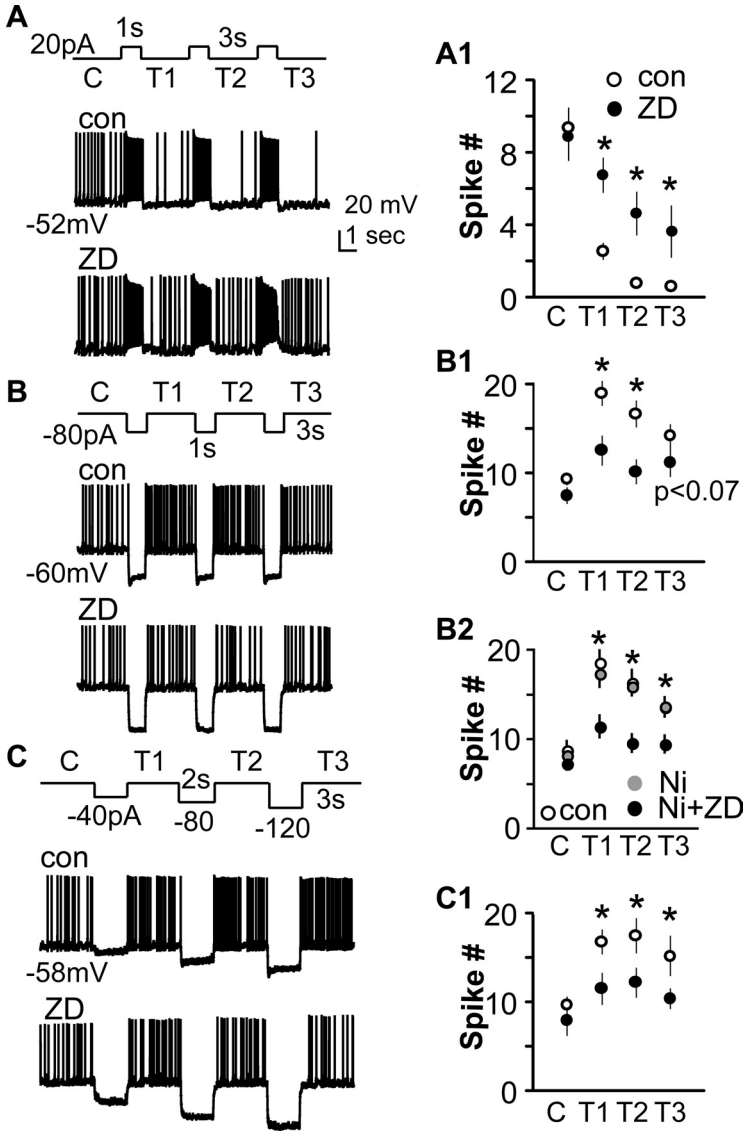

Figure 6. Manipulation of I_h by changes in membrane potential alters firing properties of GnRH neurons. ***A–C***, Membrane potential under the trace indicates potential at onset of trace. ***A***, Current injection protocol. ***B***, Membrane response under control (con) conditions. ***C***, Membrane response during ZD7288 treatment. ***A1–C1***, Quantification. ***A***, Deactivating I_h by membrane depolarization reduces spontaneous firing in GnRH neurons in a ZD-dependent manner ($n = 16$). ***B***, ***C***, Activating I_h by membrane hyperpolarization increases spike frequency ($n = 20$ and 17, respectively). ***A1–C1***, Mean ± SEM spike number for ***A–C***. ***B2***, $n = 9$ control and Ni; $n = 7$ Ni plus ZD. *$p < 0.05$. C, Control; con, control; T, trial (1, 2, or 3).

sex difference or a methodological difference, we examined sADPs following evoked action potentials (200–300 pA, 3 ms) in male mice. Similar to findings in females, the sADP following evoked action potentials (two spikes, 50 ms interval) was not affected by blockade of I_h with ZD7288 (control, 3.8 ± 0.1 mV; ZD7288, 3.7 ± 0.2 mV; $n = 14$; $p < 0.05$). This indicates the spontaneous action potential/AHP waveform is more effective at activating I_h than the evoked waveform. Together, these data suggest I_h plays a role in the pattern of GnRH neuron firing as well as the subthreshold depolarization of these neurons to action potential threshold.

Although fast synaptic transmission via ionotropic GABA and glutamate receptors are blocked in these studies, we cannot rule out the role of neuromodulation in the effects of I_h on action potential waveform. To limit the range of the blocker to the GnRH neuron itself, we made recordings with ZD7288 in the pipette solution, as this blocker has an intracellular site of action (Shin et al., 2001). A 50 pA hyperpolarizing current pulse was given every 20 s and membrane response monitored over time. Intracellular ZD7288 (20 μM) was able to block sag and rebound beginning ~2 min after initiation of the whole-cell configuration, reaching a plateau at ~10 min ($n = 7$ cells selected for exhibiting evidence of sag in initial recordings) (Fig. 5*I*,*J*); recordings without ZD7288 within the pipette were stable over this time (recordings with Ni^{2+} at 8–10 min after achieving whole-cell configuration) (Fig. 4*A*, bottom). Examination of action potential waveform within the first 2 min of recordings of spontaneous firing with ZD7288 within the pipette before the drug became effective compared with after blockade of I_h revealed no change in waveform over time with control internal solution, but a similar suppression of rate of repolarization after the AHP when ZD7288 was in the internal solution (Fig. 5*K*). These data suggest that I_h directly within GnRH neurons contributes to altering the membrane properties.

Activation of I_h by hyperpolarization increases GnRH firing

To further demonstrate the functional properties of I_h in GnRH neurons, whole-cell current-clamp recordings were made. Depolarizing current (20 pA, 1 s) was injected to deactivate I_h by membrane depolarization. These current injections increased firing rate in these GnRH neurons for the duration of injection, with little evidence of spike frequency adaptation (Fig. 6*A*,*A1*). Upon termination of the current injection, firing rate fell below preinjection values ($n = 16$, $p < 0.01$). This suppression was attenuated by ZD7288 ($p < 0.01$). Hyperpolarizing current injections were given to activate I_h. Depolarizing sag during current injection ranged from −7.8 to −15.3 mV. Upon termination of these injections, spike frequency was increased (Fig. 6*B*,*B1*) ($n = 20$, $p < 0.05$) in a ZD-sensitive manner. Treatment with Ni^{2+} to block T-channel-mediated rebound firing had no effect on rebound spike frequency, in contrast to the effect of the I_h blocker ZD7288 ($n = 9$ for control, Ni^{2+}; $n = 7$ for Ni^{2+} plus ZD) (Fig. 6*B2*). Increasing the magnitude of hyperpolarization within the physiological range also produced a voltage-dependent increase in both depolarizing sag and rebound spike frequency (Fig. 6*C*) ($n = 17$, $p < 0.05$). These data suggest I_h within GnRH neurons contributes directly to altering membrane properties.

Gonadal steroid modulation of I_h currents in GnRH neurons

Gonadal steroids provide critical homeostatic feedback to regulate GnRH neurons that involve both changes in synaptic transmission to GnRH neurons and altered intrinsic excitability

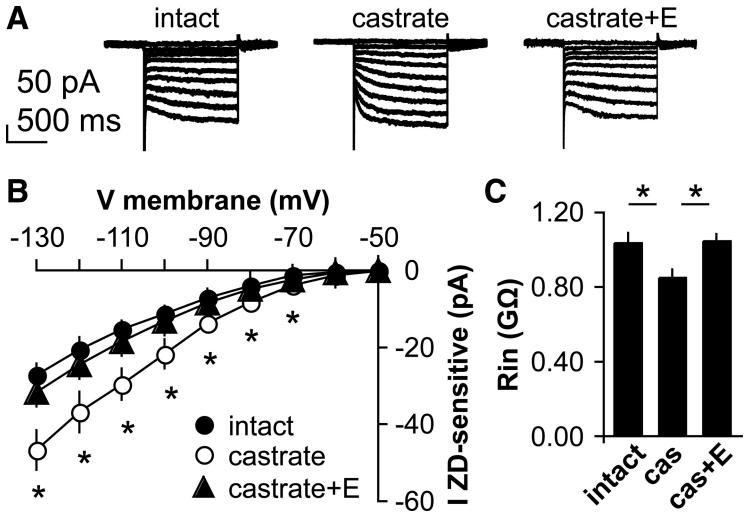

Figure 7. I_h is regulated by steroid milieu. ***A***, Representative voltage-clamp recordings showing hyperpolarization-activated current in GnRH neurons from gonad-intact (left), castrate (middle), and castrate plus estradiol (right) groups. ***B***, I–V curve of ZD7288-sensitive current in these three animal models. ***C***, Input resistance of GnRH neurons in male mice is altered by steroid milieu. *$p < 0.001$ versus intact ($n = 11$ and 16, respectively); $p < 0.05$ versus castrate plus estradiol ($n = 12$).

(DeFazio and Moenter, 2002; Nunemaker et al., 2002; Abe and Terasawa, 2005; Chu and Moenter, 2006; Wintermantel et al., 2006; Christian and Moenter, 2007; Romanò et al., 2008; Chen and Moenter, 2009; Chu et al., 2009). The involvement of I_h in increasing firing activity and excitability of GnRH neurons led us to hypothesize that this current is regulated by steroid feedback. To test this, voltage-clamp experiments were used to characterize the ZD7288-sensitive current in GnRH neurons from mice in different steroid feedback conditions. There was no difference in initial resting potential among cells from the different groups [intact, -61.4 ± 1.1 mV, $n = 11$; castrated, -59.8 ± 0.8 mV, $n = 16$; castrated plus estradiol (E), -60.3 ± 1.0 mV $n = 12$; $p > 0.05$]. Castration, which increases GnRH neuron firing activity (Pielecka and Moenter, 2006), increased the amplitude of I_h in GnRH neurons compared with that observed in gonad-intact males ($n = 11$ and 16, respectively, $p < 0.001$) (Fig. 7*A*,*B*). Estradiol, a metabolite of testosterone, is the main hormone providing negative feedback upon GnRH neuron firing rate in male mice (Pielecka and Moenter, 2006). In castrate mice treated with estradiol (CAS+E), I_h is restored to intact values ($n = 12$, $p < 0.05$ vs castrate). Further, castration and steroid replacement alter input resistance of GnRH neurons in a manner consistent with increased conductance in castrated animals relative to the other two conditions (Fig. 7*C*). Likewise, the slope conductance of I_h in cells from castrated mice was increased ($p < 0.001$) compared with that from both intact and CAS+E mice (intact, -0.34 ± 0.03; CAS, $-.60 \pm 0.05$; CAS+E, -0.40 ± 0.04 pA/mV). Together, these data suggest that steroid-sensitive changes in I_h may contribute to changes in firing of GnRH neurons in different feedback states.

Discussion

The episodic release of GnRH is critical to fertility, as is the modulation of release frequency by gonadal steroid feedback (Knobil, 1972; Karsch, 1987). I_h is associated with rhythmic activity in pacemaker cells (Ludwig et al., 1998; Biel et al., 1999). By providing a persistent inward current at membrane potentials that are hyperpolarized relative to action potential threshold, I_h depolarizes the cell's membrane potential, allowing activation of other channels that generate additional inward current until action potential threshold is reached (Lupica et al., 2001; Robinson and Siegelbaum, 2003; Bean, 2007). Here, we demonstrate that in GnRH neurons I_h plays roles in setting excitability, spontaneous action potential firing, and, potentially, steroid feedback.

Similar to females (Zhang et al., 2007), ~50% of GnRH neurons from male mice exhibited a hyperpolarization-activated current with characteristics indicating it is conducted via HCN channels. The current had a rapidly activating component, but was slow to activate completely and to deactivate, and did not inactivate. It was increased by cAMP, an allosteric activator of I_h (Bobker and Williams, 1989; DiFrancesco and Tortora, 1991; Banks et al., 1993; Erickson et al., 1993; Chen et al., 2001), was blocked by ZD7288 and Cs^+, and was permeable to Na^+ and K^+ ions. Ba^{2+} blocked a component of the hyperpolarization-activated current, indicating GnRH neurons from male mice, like female mice, also exhibit I_{Kir} (Constanti and Galvan, 1983; Zhang et al., 2007; Zhang et al., 2008). However, a ZD7288-sensitive current persisted after Ba^{2+} blockade. The pharmacological properties of the current strongly indicate it is I_h.

Activation and deactivation of I_h altered the functional properties of GnRH neurons. Rebound depolarization potentials are critical to controlling the spike patterns of several types of neurons exhibiting rhythmic activity (Bal et al., 1995). Activation of I_h during membrane hyperpolarization helps bring the cell back toward the threshold for action potential initiation. Likewise, deactivation of I_h during depolarization stabilizes membrane potential. Manipulations of membrane potential that would activate and deactivate I_h lead to increased and decreased action potential firing in GnRH neurons, respectively. Further, I_h contributes to the spontaneous activity of GnRH neurons; blocking I_h with ZD7288 reduced or eliminated action potential firing in GnRH neurons and caused frank hyperpolarization in some cells. This effect appears to be at least in part directly on GnRH neurons, as hyperpolarization persisted in some cells after blockade of both fast synaptic transmission and action potential initiation, and effects on GnRH neuron physiology were observed when the I_h blocker ZD7288 was applied intracellularly via the patch pipette, thus markedly limiting its interaction with upstream neurons. Blocking I_h also slowed the rate of recovery from the AHP, as has been observed in thalamic and hippocampal neurons (McCormick and Pape, 1990; Maccaferri et al., 1993; Nolan et al., 2007). This suggests I_h provides a subthreshold inward current in GnRH neurons that can contribute to action potential firing in these cells. Of note, these responses were observed in cells maintained within ~10 mV hyperpolarized relative to the measured reversal potential for I_h, which would provide minimal activation for this channel and little driving force for current flow through activated channels. The marked effects of such a small current are likely attributable to the high input resistance of GnRH neurons (Sim et al., 2001; DeFazio and Moenter, 2002; Kuehl-Kovarik et al., 2002); this allows even small currents to impact substantially upon the firing properties of these cells (Chu and Moenter, 2006).

Generation of both bursts and single spikes appear to be fundamental characteristics of GnRH neurons. Blocking I_h reduced the ability of GnRH neurons to generate single spikes and decreased the number of spikes per burst. This is attributable, in part, to a reduction in the rate of recovery from the AHP; although I_h is slow to activate completely, a substantial part of this current activates on a time course enabling it to contribute to sculpting membrane potential between spikes in a burst. I_h may also interact with the current underlying after-depolarizing potentials in GnRH neurons, which is carried by TTX-sensitive sodium channels (Chu and Moenter, 2006). The persistence of this latter current after blockade of I_h, as well as contributions by other channels activated at membrane potentials more hyperpolarized than threshold (Zhang et al., 2009), likely accounts for the continued, albeit reduced, firing rate of many GnRH neurons after blockade of I_h. Burst firing in neuroendocrine systems increases hormone release (Dutton and Dyball, 1979), suggesting the changes in firing produced by I_h in GnRH neurons can impact upon functional hormone release from these cells. Because of its slow activation and deactivation properties, I_h is often considered to stabilize membrane potential. The present observations that blocking I_h in GnRH neurons leads to reduced activity suggests that the activity-generating aspects of this current may be predominant in these cells at least near the interspike membrane potential.

Gonadal steroids provide critical homeostatic feedback to regulate of GnRH neurons. This regulation likely involves both changes in synaptic transmission to GnRH neurons and altered intrinsic excitability (DeFazio et al., 2002; Nunemaker et al., 2002; Abe and Terasawa, 2005; Chu and Moenter, 2006; Wintermantel et al., 2006; Christian and Moenter, 2007; Dungan et al., 2007; Romanò et al., 2008; Chen and Moenter, 2009). In the present study, castration, which increases GnRH release, markedly increased I_h in GnRH neurons. In males, there is substantial conversion within the brain of circulating testosterone from the gonads to estradiol (Woolley, 2007), which provides the primary negative feedback signal to reduce the GnRH-dependent secretion of gonadotropins from the pituitary, as well as the activity of GnRH neurons (Roselli and Resko, 1990; Scott et al., 1997; Fisher et al., 1998; Pielecka and Moenter, 2006). Treatment of castrated male mice with estradiol restored I_h to levels observed in gonadal-intact mice, suggesting that estradiol-induced changes in I_h are a component of this negative feedback mechanism. Whether this is a direct or indirect action on GnRH neurons remains to be determined.

The present data suggest I_h plays important functional roles in GnRH neurons. A broader question is how to integrate these observations related to high-frequency activity in single cells with the overall pattern of hormone release from the GnRH neuronal network. This question awaits more data for a full answer, but some speculation is possible based on the present findings. GnRH neurons exhibit repeating bouts of firing activity that have been classified by fast Fourier transform into three rough period time domains: bursts (repeating with a period $<$100 s), clusters (100–1000 s), and episodes ($>$1000 s) (Nunemaker et al., 2003a, 2003b; Abe and Terasawa, 2005). The present study suggests that I_h contributes to the highest frequency of these domains—that of burst firing. Empirical evidence for how bursts are organized into longer period patterns is limited, thus any role of I_h in these processes is speculative; recent data suggest a molecularly uncharacterized calcium-activated potassium current may contribute to this organization (Lee et al., 2010). Both the number of spikes per burst and the interval between bursts are contributors to overall firing rate and these were altered when I_h was blocked. The long-term pattern of GnRH release (on the order of every 30 min in rodents) indicates alterations in network activity. An intriguing possibility is that intrinsic properties of individual GnRH neurons switch between relatively excitable and relatively quiescent states. It is possible that secondary to changes in neuromodulation, channel trafficking, or posttranslational modification, I_h is detectable only during the relatively excitable state. This might account for it being observed in only half of the neurons. Alternatively, HCN subunits may only be expressed in a subpopulation of GnRH neurons and thus render them spontaneously active driver cells for the network.

The overall pattern of GnRH release is likely sculpted by both intrinsic and synaptic mechanisms, but the emerging consensus is that episodic activity of GnRH neurons arises either at individual GnRH neurons or within networks of these cells (Moenter et al., 2003). Supporting observations include spontaneous firing in physically isolated GnRH neurons (Kuehl-Kovarik et al., 2002), pulsatile GnRH release from pure cultures of immortalized GnRH neurons (Catt et al., 1985; Martínez de la Escalera et al., 1992; Pitts et al., 2001), and spontaneous changes in the firing pattern of GnRH neurons in adult brain slices that is reflective of the pattern of GnRH and/or downstream pituitary hormone release from similarly treated animal (Levine et al., 1985b; Karsch, 1987; Moenter et al., 1992, 2003; Christian et al., 2005; Pielecka and Moenter, 2006; Pielecka et al., 2006). Episodic activity does not appear to involve macromolecular synthesis (Pitts et al., 2001) and appears to coordinate within networks of these cells at intervals that are similar to the occurrence of physiologically relevant hormone release in this system (Terasawa et al., 1999; Nunemaker et al., 2001). Together, the present data point to an important functional role for I_h in generating spontaneous activity in GnRH neurons, extending burst duration, enhancing recovery from the AHP, and being one intrinsic property potentially modulated by steroids to provide homeostatic feedback on GnRH neuronal activity.

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