Published in final edited form as:

Brain Res. 2010 July 23; 1345: 137–145. doi:10.1016/j.brainres.2010.05.031.

Estradiol Attenuates Multiple Tetrodotoxin-Sensitive Sodium Currents in Isolated Gonadotropin-releasing Hormone Neurons

Yong Wang¹, Mona Garro², and M. Cathleen Kuehl-Kovarik²

¹ Department of Biological Engineering, University of Missouri, Dalton Cardiovascular Research Center, 134 Research Park Drive, Columbia, MO 65211

² Department of Biomedical Sciences, University of Missouri, E102 Veterinary Medicine, 1600 E. Rollins, Columbia, MO 65211

Abstract

Secretion from gonadotropin-releasing hormone (GnRH) neurons is necessary for the production of gametes and hormones from the gonads. Subsequently, GnRH release is regulated by steroid feedback. However, the mechanisms by which steroids, specifically estradiol, modulate GnRH secretion are poorly understood. We have previously shown that estradiol administered to the female mouse decreases inward currents in fluorescently-labeled GnRH neurons. The purpose of this study was to examine the contribution of sodium currents in the negative feedback action of estradiol. Electrophysiology was performed on GnRH neurons dissociated from young, middle-aged, or old female mice. All mice were ovariectomized; half were estradiol replaced. The amplitude of the sodium current underlying the action potential was significantly decreased in GnRH neurons from young estradiol-treated animals. In addition, *in vivo* estradiol significantly decreased the transient sodium current amplitude, but prolonged the sodium current inactivation time constant. Estradiol decreased the persistent sodium current amplitude, and induced a significant negative shift in peak current potential. In contrast to results obtained from cells from young reproductive animals, estradiol did not significantly attenuate the sodium current underlying the action potential in cells isolated from middle-aged or old mice. Sodium channels can modulate cell threshold, latency of firing, and action potential characteristics. The reduction of sodium current amplitude by estradiol suggests a negative feedback on GnRH neurons, which could lead to a downregulation of cell excitability and hormone release. The attenuation of estradiol regulation in peripostreproductive and postreproductive animals could lead to dysregulated hormone release with advancing age.

Keywords

GnRH; estradiol; transient sodium current; persistent sodium current; aging

Corresponding author: M. Cathleen Kuehl-Kovarik, Department of Biomedical Sciences, University of Missouri, E102 Veterinary Medicine, 1600 E. Rollins, Columbia, MO 65211, Phone: 573-882-2536, Fax: 573-884-6890, kovaric@mizzou.edu.
Reprint requests should be sent to the corresponding author at the above address

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Reproduction in mammals is critically dependent upon the appropriate neurosecretion of gonadotropin-releasing hormone (GnRH). The pulsatile release of GnRH into the portal bloodstream is necessary for the maintenance of normal reproductive function. In addition, GnRH secretion leads to the release of gonadotropic hormones from the anterior pituitary, which then regulate the production of gametes and hormones from the gonads. Steroid hormones act at the hypothalamus to regulate GnRH release, but the mechanisms are poorly understood (Kelly and Ronnekleiv, 2008). Estradiol typically has a negative effect on GnRH release, but positive feedback occurs to initiate the luteinizing hormone (LH) surge, resulting in ovulation (see (Chappell, 2005) for review).

Multiunit activity, a summation of electrical activity in the hypothalamus, is attenuated following estradiol treatment in the ovariectomized rhesus monkey and rat (Kesner et al., 1987, Kato et al., 1994). Multiunit volleys correlate with pulsatile LH release, suggesting that estradiol inhibits or desynchronizes GnRH neuronal activity during negative feedback. However, the pulsatile release of GnRH appears to persist, possibly even increase, in the presence of increased estradiol during the LH surge (Kesner et al., 1987, Moenter et al., 1990, Clarke, 1993, Evans et al., 1997). Interestingly, recordings from individual GnRH neurons in hypothalamic brain slices from chronically estradiol-treated mice demonstrate an increased duration of quiescent time in the morning compared to recordings from ovariectomized counterparts. Recordings from the same mice demonstrate an increased firing rate and instantaneous firing frequency in the afternoon when compared to ovariectomized animals (Christian et al., 2005). These results suggest that estradiol has varying effects on GnRH neuronal activity that are dependent on the influence of other factors, such as circadian time (Christian et al., 2005, Moenter et al., 2009).

Estradiol may control hormone release through the modulation not only of firing frequency, but also firing pattern in GnRH neurons. The pulsatile secretion of GnRH is thought to depend on repetitive and prolonged bursts of action potentials (Wilson et al., 1984, Hiruma et al., 1994). Thus the modulation of subthreshold currents or ionic currents during the action potential in GnRH neurons could significantly affect hormone release. We have previously shown that GnRH neurons isolated from ovariectomized, estradiol-treated (OVX +E) adult mice have reduced whole-cell inward currents (Wang et al., 2008) when compared to control (OVX) animals. In addition, we have shown that spontaneous firing frequency and spike patterning is significantly reduced in GnRH neurons from aged animals (Wang et al., 2008). Voltage-dependent sodium channels, which contribute significantly to inward cellular currents, play a key role in governing cell excitability (Raman and Bean, 1997, Raman and Bean, 1999, Fry et al., 2007) and repetitive (Kiss, 2008) and spontaneous (Raman and Bean, 1997) firing. The purpose of this study was to examine the contribution of sodium currents to steroid-dependent inhibition in GnRH neurons from young adult and aged animals.

2. Results

Characterization of inward ionic currents underlying the action potential

Most isolated GnRH neurons fire spontaneously, and have a typical burst firing both in whole cell mode and cell attached mode (Kuehl-Kovarik et al., 2002, Kuehl-Kovarik et al., 2005). Burst firing has been shown to contribute to hormone release in magnocellular neurons (Leng et al., 1999) and likely contributes to GnRH release as well. To analyze the ion currents flowing during action potentials, a particularly useful preparation is provided by acutely dissociated cell bodies, in which recorded action potentials are close to true membrane action potentials and where high-resolution voltage-clamp experiments, including action potential clamp experiments, can be performed (see (Bean, 2007) for review). Using

action potential clamp, the contribution of particular currents to the action potential can be isolated with specific blockers (Bean, 2007).

It has previously been demonstrated that whole-cell inward currents in GnRH neurons are significantly reduced when animals are treated with estradiol (Wang et al., 2008). Therefore, action potential clamp was used to characterize the inward currents during action potentials in GnRH-eGFP neurons from OVX and OVX+E mice. Currents were evoked by using a previously recorded spike train (evoked by a current pulse of 200 pA for 1.2 ms) from a GnRH-eGFP neuron as the command voltage waveform (Figure 1A,B). Tetrodotoxin (TTX; 500 nM) was used to identify the inward currents. Tetrodotoxin completely blocked the inward current evoked by the spike train, indicating that the upstroke of the action potential is caused by a TTX-sensitive sodium current without contribution of a voltage-dependent calcium current (Figure 1C; (Raman and Bean, 1999). The amplitude of inward currents was converted to current density (pA/pF), using capacitance to normalize for cell size. Cell capacitance does not vary between OVX and OVX+E animals (young OVX = 10.15 pF, young OVX+E = 9.35 pF, $p = 0.21$, $n = 21/\text{group}$). Sodium current density was significantly attenuated in cells from young animals treated with estradiol compared to cells from OVX animals (Figure 1D,E, $p < 0.002$; $n = 18\text{--}20$ from six animals in each group). There was no significant difference in the width of the inward current at $1/2$ amplitude between cells isolated from the two groups of animals (OVX: 0.44 ± 0.006 ms; OVX+E: 0.48 ± 0.03 ms; $p = 0.25$; $n = 9\text{--}12$). When recordings were divided into AM and PM groups, the time of day that the recording was made had no effect on current amplitude ($p > 0.7$ for both OVX and OVX+E groups). To confirm that the effect of estradiol was specific to GnRH neurons, sodium current density was determined in non-green cells from the same hypothalamic dissociations. Interestingly, inward current density, in response to an action potential stimulus, was consistently smaller in non-green neurons, even though they were undoubtedly a heterogeneous population (Figure 1E; $p < 0.001$; Two-way ANOVA; $F(1,56) = 29.94$; $n = 11\text{--}20$). There was no significant difference in sodium current density between non-GnRH neurons from OVX and OVX+E animals (Figure 1E; $p = 0.36$, $n = 11\text{--}13$), indicating that the effect of estradiol on this sodium current is specific to GnRH neurons.

Estradiol inhibits transient sodium current amplitude while prolonging entry into an inactivated state

The transient sodium current was evoked by 30-ms step depolarizations from -60 mV to $+60$ mV in 10-mV increments. A high TEA extracellular solution and cesium-based intracellular solution were used to block potassium currents. Transient sodium currents started to activate at -40 mV and reached their peak at -10 mV in neurons from both control and estradiol-treated groups (Figure 2A,B). As seen for whole-cell currents (Wang et al., 2008) and sodium currents underlying the action potential, transient sodium current amplitude density was significantly decreased in GnRH neurons from estradiol-treated animals when compared to neurons from OVX animals (Figure 2B; $p < 0.05$, $n = 31\text{--}36$ from 5 animals per group).

Overlying normalized current traces obtained from neurons from OVX and OVX+E animals suggested that the time-course of inactivation was different between the two treatments (Figure 2C). Inactivation was best fit with a single exponential function. The inactivation time constant τ was significantly prolonged at peak currents (Figure 2D; $p < 0.05$, $n = 31\text{--}36$) in neurons from estradiol-treated animals.

Steady-state activation and inactivation were calculated for transient currents. The steady-state activation curve did not vary significantly between OVX and OVX+E animals (Figure 3A). The voltage at half-maximal activation ($V_{1/2}$) was similar (OVX: -23.46 ± 0.58 mV; OVX+E: -21.99 ± 0.66 mV; $p = 0.11$, $n = 31\text{--}36$) and the slope factor (k) did not vary (OVX:

5.26 ± 0.24 mV; OVX+E: 5.68 ± 0.40 mV; $p = 0.20$, $n = 31-36$) between groups. The steady-state inactivation was determined by the protocol demonstrated in Figure 3B. The inactivation curve was not significantly different between cells from the two treatment groups (Figure 3C). Again, $V_{1/2}$ (OVX: -54.25 ± 0.72 mV; OVX+E: -52.41 ± 0.65 mV; $p = 0.06$, $n = 27-29$) and k (OVX: -7.55 ± 0.16 mV; OVX+E: -7.84 ± 0.15 mV; $p = 0.19$, $n = 27-29$) did not change significantly.

Estradiol modulates the persistent sodium current

The persistent sodium current was evoked by depolarizing the membrane from -60 to 0 mV at a velocity of 50 mV/s (Figure 4A). The current in 500 nM TTX was subtracted from the current in control for each cell, to yield a persistent, TTX-sensitive current. Current-voltage curves were constructed for the subtracted currents from control and estradiol groups (Figure 4B). Currents in cells from both OVX and OVX+E groups started to activate at -52 mV, a more negative activation potential than the transient sodium current, which activated around -40 mV (Figure 2B). Persistent currents in neurons from OVX animals reached an average peak at -36.9 ± 0.7 mV. The peak in neurons from OVX+E animals was shifted in a negative direction (-39.4 ± 0.8 mV; $p = 0.03$, $n = 7-9$ from 3 animals per group), resulting in a significantly attenuated current amplitude (OVX: -26.3 ± 2.6 pA; OVX+E: -18.0 ± 1.9 pA; $p = 0.03$; $n = 7-9$).

GnRH neurons do not express a resurgent sodium current

The resurgent sodium current was evoked by a conditioning step (+30 mV) followed by steps to potentials between -60 mV and -20 mV in 10 mV increments, at a holding potential of -60 mV (Grieco et al., 2005). This voltage protocol revealed no resurgent sodium current in either OVX or OVX+E animals (Figure 5, $n = 10$).

The effect of estradiol on sodium currents is attenuated with age

In vivo estradiol significantly reduces peak sodium current density, in response to an action potential stimulus, in cells isolated from young, reproductive animals. To determine if the effect of estradiol is maintained in postreproductive animals, action potential clamp was performed in cells isolated from middle-aged and old, OVX or OVX+E animals. Although the overall effect of estradiol was to attenuate the current response ($p = 0.02$, Two-way ANOVA; $F(1,118) = 5.94$; $n = 15-27$), estradiol did not significantly attenuate sodium current density in cells isolated from 10 month old ($p = 0.60$, $n = 15-17$) or older ($p = 0.27$, $n = 27$) animals (Figure 6). There was a trend towards decreased sodium current density in OVX animals with increasing age. However, it was not significant ($p = 0.65$, $n = 18-27$).

3. Discussion

This is the first time that multiple sodium currents have been documented in adult, native GnRH neurons. A large transient sodium current is observed; the sodium current underlying the action potential is significantly larger in GnRH neurons than in other hypothalamic neurons. In addition, these neurons express a small persistent sodium conductance. *In vivo* estradiol modulates multiple sodium currents in isolated GnRH neurons, but does not affect sodium currents in unidentified, heterogeneous hypothalamic cells. Through the modulation of sodium currents, estradiol could affect cell excitability, firing properties, and vesicle release to facilitate the physiological effects of hormone feedback.

We have previously shown that GnRH neurons isolated from estradiol-treated mice have significantly reduced whole-cell inward currents (Wang et al., 2008). The large, rapidly activating and inactivating transient sodium current contributes significantly to the whole-cell inward current. The present study demonstrates that estradiol attenuates transient

sodium current amplitude at a wide range of voltages. The transient sodium current underlies the rapid regenerative upstroke of the action potential (Fry et al., 2007, Aman et al., 2009). As predicted, the amplitude of the sodium current evoked by an action potential stimulus is also significantly reduced in cells from OVX+E animals. Estradiol also slightly, but significantly, prolongs the fast inactivation time constant (τ) of the transient current. This could prolong the duration of the sodium spike and the duration of the action potential. However, neither the width of the sodium influx nor action potential width (Wang et al., 2008) varies significantly with estradiol treatment. Calcium has also been shown to underlie the generation of the action potential in the GT1 cell line (Van Goor et al., 1999), suggesting that calcium spikes contribute to the inward current underlying the action potential in GnRH neurons. Our data, however, indicate that only TTX-sensitive sodium currents underlie the inward current in adult, native GnRH neurons.

The TTX-sensitive persistent sodium current is a small, inactivating current that has been demonstrated in neurons from a number of brain regions (see (Crill, 1996) for review). The persistent current is typically activated at more negative potentials than the transient current (Crill, 1996, Kiss, 2008), as demonstrated in GnRH neurons. This subthreshold activation may influence firing rates and patterning (Bean, 2007, Kiss, 2008, Aman et al., 2009), and has been shown to play a role in spontaneous activity (Bean, 2007), a hallmark of GnRH neurons (Kuehl-Kovarik et al., 2002). Estradiol does not reduce the persistent current at potentials negative to -40 mV, and thus would not be expected to modulate the effects of subthreshold activation.

Sodium channels consist of a pore-forming α subunit (Na_v 1.1–1.9), sufficient for functional expression, and a β subunit (β 1– β 4) that modifies kinetics and voltage dependence (Catterall et al., 2005). Central neuronal soma primarily express the α subunits Na_v 1.1, 1.3 and 1.6 (Catterall et al., 2005). Both Na_v 1.1 and 1.6 have been shown to contribute to the transient and persistent current in other neuron types (Fry et al., 2007), and we would predict that these subunits contribute to multiple sodium currents in GnRH neurons. The persistent current is likely a result of flow through transient sodium channels at submaximal inactivation, or through channels that fail to inactivate (Aman et al., 2009). Both transient and persistent sodium currents are attenuated by estradiol at voltages positive to -40 mV, suggesting that the expression or function of an α subunit, carrying both currents, is reduced. Estradiol also slows the decay of transient currents, possibly through the modulation of β subunits, which could increase the persistent current. Estradiol decreases the persistent current in GnRH neurons, suggesting that the downregulation of channels predominates.

Chronic estradiol has been shown to evoke daily LH surges in the GnRH-eGFP mouse model, as well as the rat (Legan and Karsch, 1975, Christian et al., 2005). This model is not physiological, as estradiol levels do not change on a circadian basis, and daily LH surges do not represent the normal murine cycle. Nevertheless, chronic estradiol replacement allows us to begin to isolate the effects of estradiol on the GnRH neuronal circuitry. Recordings from GnRH neurons in brain slices from this same OVX+E model demonstrate decreased firing in AM recordings, while firing activity in PM recordings is increased (Christian et al., 2005). Thus, the negative and positive effects of estradiol appear to be dependent upon circadian cues. In the current studies, mice were sacrificed in the AM, a time of decreased activity (Christian et al., 2005). Estradiol decreased sodium current amplitude, suggesting that attenuated sodium currents contribute to steroid-dependent inhibition. Circadian time did not affect sodium currents in the isolated neuronal preparation. Whether this is because estradiol decreases sodium currents irrespective of reproductive status, or because the neurons were removed from exogenous influences for many hours before recordings, is unclear.

An unidentified, TTX-sensitive sodium current appears to underlie the depolarizing afterpotential (DAP) in GnRH neurons (Chu and Moenter, 2006). Interestingly, *in vivo* estradiol *increases* the amplitude of the DAP (Chu and Moenter, 2006) in GnRH neuronal recordings from a slice preparation, although *in vivo* estradiol has no effect on the DAP in isolated GnRH neurons (Wang et al., 2008). This would suggest that the sodium current underlying the DAP is not transient, persistent, or resurgent, as these currents are attenuated by estradiol or are not present in GnRH neurons. Recent data suggest that 10 nM estradiol acutely reduces the afterhyperpolarization potential in GnRH neurons, resulting in the indirect modulation of DAP current amplitude (Chu et al., 2009). Thus, the sodium current underlying the DAP does not appear to be sensitive to modulation by estradiol.

In vivo estradiol has been shown to reduce the potassium conductance I_A , while slowing the decay time constant (DeFazio and Moenter, 2002), and the effect of estradiol on other potassium currents, including the current underlying action potential repolarization, remains to be explored. The physiological balance of multiple currents undoubtedly contributes to the overall control of GnRH neuronal activity and function by estradiol. Interestingly, the ability of estradiol to attenuate the sodium current underlying the action potential in GnRH neurons is lost as animals age. The attenuation of the efficacy of estradiol could result from a number of changes, including loss of estrogen receptors on GnRH neurons or upstream inputs, or loss of regulation at the cellular or network level. The loss of estradiol signaling in peripost- and postreproductive animals could potentially involve multiple ion channels, and would likely result in dysregulated GnRH release. Estrogen negative feedback appears to be impaired in some perimenopausal women (Burger et al., 2007), suggesting that attenuated hormone signaling at the level of the GnRH neuron could contribute to reproductive senescence.

The mechanisms through which estradiol affects GnRH neuronal physiology are not yet clear. There is evidence that estradiol affects GnRH neurons both directly and indirectly (Sun et al., Hu et al., 2008, Chu et al., 2009, Moenter et al., 2009, Noel et al., 2009), that it can decrease or increase firing frequency (Hu et al., 2008, Chu et al., 2009), and that it can act through classical (Sun et al., Chu et al., 2009) and nonclassical (Sun et al., Noel et al., 2009) receptors. A number of channels play a role in the regulation of cell excitability; many are likely modulated by estradiol (Sun et al.). Both the transient and persistent sodium current are attenuated in GnRH neurons by chronic, *in vivo* estradiol treatment. Because these neurons are studied in isolation after an overnight incubation, channels normally expressed in processes may be expressed in the soma. Nevertheless, it is clear that estradiol has a direct, modulatory effect on multiple sodium channels in adult, native GnRH neurons.

4. Experimental procedures

Animals

Adult virgin GnRH-eGFP transgenic mice (Suter et al., 2000) were used for all experiments. Animals were used at 3 months of age (young, reproductive), 10 months of age (middle-aged, peripostreproductive), and 15–18 month of age (old, postreproductive). The reproductive status of the animals at each age has been previously established (Wang et al., 2008). Animals were maintained in a colony at the University of Missouri, under a 12 hour light: dark cycle. Care was taken to minimize pain and discomfort, and The University of Missouri Animal Care and Use Committee approved all procedures. All mice were bilaterally ovariectomized under isoflurane anesthesia. Banamine (0.025 µg/10 g body weight) was administered pre-operatively as an analgesic. At the time of surgery, a silastic capsule was placed subcutaneously. The capsule either contained 0.625 µg β-estradiol (E; OVX+E; Sigma, St. Louis, MO) in 20 µl of tocopherol-stripped corn oil (MP Biomedicals, Solon, OH) or corn oil only (OVX). This concentration of estradiol has previously been

shown to significantly increase serum estradiol concentrations and uterine weights (Wang et al., 2008) although serum estradiol concentrations remain at or below physiological levels (DeFazio and Moenter, 2002, Wang et al., 2008). Mice were sacrificed 5–7 days after steroid replacement to allow sufficient time for the endocrine adjustment to hormone manipulation while avoiding long-term steroid withdrawal (Nunemaker et al., 2002). At least three mice were used for each experimental group.

Neuronal Dissociation and culture

Cells were acutely isolated as described previously (Wang et al., 2008). Briefly, animals were sacrificed and brains were quickly removed and sliced at 400 μm in low-calcium artificial cerebrospinal fluid (ACSF; in mM: 124 NaCl, 3 KCl, 2 MgSO_4 , 1.25 NaH_2PO_4 , 26 NaHCO_3 , 10 dextrose, 0.1 CaCl_2 , pH 7.4; bubbled with 95% O_2 , 5% CO_2). Slicing was always performed at the same time of day (late morning). Isolated regions were enzymatically treated with proteinase K (Sigma; 0.2 mg/ml) followed by trypsin (Sigma Type XI; 1 mg/ml) at 30°C for 22–24 minutes. Slices were triturated with flame-polished Pasteur pipettes and dispersed cells were incubated overnight in Neurobasal A/B-27 (Life Technologies, Inc., Carlsbad, CA) before electrophysiological recordings. The glutamate receptor blockers APV (1 mM) and NBQX (10 μM) were added at the time of dissociation.

Electrophysiology

Electrophysiology was performed 16–26 hours post-dissociation. Fluorescent cells were viewed on a Nikon Diaphot-TMD inverted microscope with a GFP filter. Cells were perfused with normal-calcium ACSF (2.5 mM CaCl_2) at 22°C. Whole-cell recordings were obtained with thin-walled borosilicate glass micropipettes (World Precision Instruments; 1.5–3 M Ω) filled with a potassium gluconate intracellular solution (in mM: 120 potassium gluconate, 1 CaCl_2 , 1 MgCl_2 , 10 HEPES, 1 NaCl, 5 EGTA, 2 ATP, 0.2 GTP, pH 7.2–7.4). All recordings were performed using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), digitized with a Digidata 1322A (Molecular Devices), and stored on a Dell computer (Dell, Round Rock, TX) using pClamp 9.2 software.

Whole-cell configuration was initiated only upon the establishment of a G Ω seal. Only stable recordings with a series resistance of less than 10 M Ω was included. Series resistance was compensated 70–90%. Data was digitized at 20 kHz and low-pass filtered at 4 kHz. To measure the ionic current underlying the action potential, a pre-recorded spike train was used as the voltage command. Tetrodotoxin (TTX; 500 nM; Sigma) was used to isolate the sodium current. Leak current was corrected with P/4 subtraction. All amplitude measurements were taken in reference to the prepulse baseline.

A low-sodium, high tetraethylammonium (TEA) extracellular solution was used to record transient, resurgent and persistent sodium currents (Raman and Bean, 1997, Raman and Bean, 2001), containing (in mM) 50 NaCl, 110 TEA-Cl, 2 BaCl_2 , 0.3 CdCl_2 , and 10 HEPES (pH 7.4 with NaOH). The intracellular solution contained (in mM) 117 CsCl, 9 EGTA, 9 HEPES, 1.8 MgCl_2 , 4 MgATP , 0.2 NaGTP (pH 7.4 with CsOH). The holding potential was –60 mV for all voltage commands. The protocol for recording transient current stepped from –60 to +60 mV in 10-mV increments for 30 ms; the same pulse protocol was used to investigate steady-state activation kinetics. Activation plots were fitted to the Boltzmann equation: $G/G_{\text{max}} = 1 / \{1 + \exp[(V - V_{1/2})/k]\}$, where $G = I / (V - V_k)$, V_k is the reversal potential, G/G_{max} is the normalized conductance, V is the conditioning potential, $V_{1/2}$ is the voltage at half-maximal activation and k is the slope factor. The protocol used to investigate steady-state inactivation kinetics included a prepulse of 200 ms from –120 mV to –10 mV in 10-mV increments, followed by a depolarization to –10 mV for 30 ms. Inactivation plots were fit to the Boltzmann equation: $I/I_{\text{max}} = 1 / \{1 + \exp[(V - V_{1/2})/k]\}$, where I is the current, V is

the conditioning potential, $V_{1/2}$ is the voltage at half-maximal activation and k is the slope factor. The protocol for recording resurgent current (Raman and Bean, 2001, Grieco et al., 2005) included depolarizing steps from -60 mV and repolarizing steps from $+30$ mV to potentials between -60 and -20 mV in 10 -mV increments. The ramp protocol for recording the persistent current depolarized the membrane from -60 to 0 mV at a velocity of 50 mV/s, a slow depolarization to prevent activation of fast or transient sodium channels (Gittis and du Lac, 2008, Liu and Shipley, 2008).

Data Analysis

Data were acquired with pClamp 9.2 software (Molecular Devices) and analyzed with Clampfit, SigmaPlot (Systat Software, Inc, Chicago, IL) and Sigmaplot (Systat Software, Inc) software. Each cell was analyzed as an individual data point. Current density was calculated as current/capacitance (pA/pF). Comparisons between hormone treatments were made with unpaired t-tests for normally distributed data, and Mann-Whitney rank sum tests for nonparametric data. One- and two-way ANOVAs were used to analyze interactions of age and hormone treatments, and green vs. non-green neurons. All values are expressed as mean $\pm$ SEM.

Acknowledgments

These studies were supported by NIH AG023139 to M.C.K.-K.

Abbreviations

ACSF	artificial cerebrospinal fluid
E	estradiol
eGFP	enhanced green fluorescent protein
GnRH	gonadotropin-releasing hormone
LH	luteinizing hormone
OVX	ovariectomized
OVX+E	ovariectomized plus estradiol replaced
TEA	tetraethylammonium chloride (potassium channel antagonist)
TTX	tetrodotoxin (sodium channel antagonist)

References

- Aman TK, Grieco-Calub TM, Chen C, Rusconi R, Slat EA, Isom LL, Raman IM. Regulation of persistent Na current by interactions between beta subunits of voltage-gated Na channels. *J Neurosci.* 2009; 29:2027–2042. [PubMed: 19228957]
- Bean BP. The action potential in mammalian central neurons. *Nat Rev Neurosci.* 2007; 8:451–465. [PubMed: 17514198]
- Burger HG, Hale GE, Robertson DM, Dennerstein L. A review of hormonal changes during the menopausal transition: focus on findings from the Melbourne Women's Midlife Health Project. *Hum Reprod Update.* 2007; 13:559–565. [PubMed: 17630397]
- Catterall WA, Goldin AL, Waxman SG. International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol Rev.* 2005; 57:397–409. [PubMed: 16382098]
- Chappell PE. Clocks and the black box: circadian influences on gonadotropin-releasing hormone secretion. *J Neuroendocrinol.* 2005; 17:119–130. [PubMed: 15796763]

- Christian CA, Mobley JL, Moenter SM. Diurnal and estradiol-dependent changes in gonadotropin-releasing hormone neuron firing activity. *Proc Natl Acad Sci U S A*. 2005; 102:15682–15687. [PubMed: 16230634]
- Chu Z, Andrade J, Shupnik MA, Moenter SM. Differential regulation of gonadotropin-releasing hormone neuron activity and membrane properties by acutely applied estradiol: dependence on dose and estrogen receptor subtype. *J Neurosci*. 2009; 29:5616–5627. [PubMed: 19403828]
- Chu Z, Moenter SM. Physiologic regulation of a tetrodotoxin-sensitive sodium influx that mediates a slow afterdepolarization potential in gonadotropin-releasing hormone neurons: possible implications for the central regulation of fertility. *J Neurosci*. 2006; 26:11961–11973. [PubMed: 17108170]
- Clarke IJ. Variable patterns of gonadotropin-releasing hormone secretion during the estrogen-induced luteinizing hormone surge in ovariectomized ewes. *Endocrinology*. 1993; 133:1624–1632. [PubMed: 8404603]
- Crill WE. Persistent sodium current in mammalian central neurons. *Annu Rev Physiol*. 1996; 58:349–362. [PubMed: 8815799]
- DeFazio RA, Moenter SM. Estradiol feedback alters potassium currents and firing properties of gonadotropin-releasing hormone neurons. *Mol Endocrinol*. 2002; 16:2255–2265. [PubMed: 12351691]
- Evans NP, Dahl GE, Padmanabhan V, Thrun LA, Karsch FJ. Estradiol requirements for induction and maintenance of the gonadotropin-releasing hormone surge: implications for neuroendocrine processing of the estradiol signal. *Endocrinology*. 1997; 138:5408–5414. [PubMed: 9389526]
- Fry M, Boegle AK, Maue RA. Differentiated pattern of sodium channel expression in dissociated Purkinje neurons maintained in long-term culture. *J Neurochem*. 2007; 101:737–748. [PubMed: 17448145]
- Gittis AH, du Lac S. Similar properties of transient, persistent, and resurgent Na currents in GABAergic and non-GABAergic vestibular nucleus neurons. *J Neurophysiol*. 2008; 99:2060–2065. [PubMed: 18287543]
- Grieco TM, Malhotra JD, Chen C, Isom LL, Raman IM. Open-channel block by the cytoplasmic tail of sodium channel beta4 as a mechanism for resurgent sodium current. *Neuron*. 2005; 45:233–244. [PubMed: 15664175]
- Hiruma H, Sano A, Kimura F. Injection of bicuculline elicits firing of luteinizing hormone releasing hormone pulse generator in muscimol-treated ovariectomized rats. *Brain Res*. 1994; 641:191–197. [PubMed: 8012820]
- Hu L, Gustafson RL, Feng H, Leung PK, Mores N, Krsmanovic LZ, Catt KJ. Converse regulatory functions of estrogen receptor-alpha and -beta subtypes expressed in hypothalamic gonadotropin-releasing hormone neurons. *Mol Endocrinol*. 2008; 22:2250–2259. [PubMed: 18701637]
- Kato A, Hiruma H, Kimura F. Acute estradiol modulation of electrical activity of the LHRH pulse generator in the ovariectomized rat: restoration by naloxone. *Neuroendocrinology*. 1994; 59:426–431. [PubMed: 8022518]
- Kelly MJ, Ronnekleiv OK. Membrane-initiated estrogen signaling in hypothalamic neurons. *Mol Cell Endocrinol*. 2008; 290:14–23. [PubMed: 18538919]
- Kesner JS, Wilson RC, Kaufman JM, Hotchkiss J, Chen Y, Yamamoto H, Pardo RR, Knobil E. Unexpected responses of the hypothalamic gonadotropin-releasing hormone “pulse generator” to physiological estradiol inputs in the absence of the ovary. *Proc Natl Acad Sci U S A*. 1987; 84:8745–8749. [PubMed: 3317420]
- Kiss T. Persistent Na-channels: origin and function. A review. *Acta Biol Hung*. 2008; 59(Suppl):1–12. [PubMed: 18652365]
- Kuehl-Kovarik MC, Partin KM, Handa RJ, Dudek FE. Spike-dependent depolarizing afterpotentials contribute to endogenous bursting in gonadotropin releasing hormone neurons. *Neuroscience*. 2005; 134:295–300. [PubMed: 15961246]
- Kuehl-Kovarik MC, Pouliot WA, Halterman GL, Handa RJ, Dudek FE, Partin KM. Episodic bursting activity and response to excitatory amino acids in acutely dissociated gonadotropin-releasing hormone neurons genetically targeted with green fluorescent protein. *J Neurosci*. 2002; 22:2313–2322. [PubMed: 11896170]

- Legan SJ, Karsch FJ. A daily signal for the LH surge in the rat. *Endocrinology*. 1975; 96:57–62. [PubMed: 1167356]
- Leng G, Brown CH, Russell JA. Physiological pathways regulating the activity of magnocellular neurosecretory cells. *Prog Neurobiol*. 1999; 57:625–655. [PubMed: 10221785]
- Liu S, Shipley MT. Multiple conductances cooperatively regulate spontaneous bursting in mouse olfactory bulb external tufted cells. *J Neurosci*. 2008; 28:1625–1639. [PubMed: 18272683]
- Moenter SM, Caraty A, Karsch FJ. The estradiol-induced surge of gonadotropin-releasing hormone in the ewe. *Endocrinology*. 1990; 127:1375–1384. [PubMed: 2201536]
- Moenter SM, Chu Z, Christian CA. Neurobiological mechanisms underlying oestradiol negative and positive feedback regulation of gonadotrophin-releasing hormone neurones. *J Neuroendocrinol*. 2009; 21:327–333. [PubMed: 19207821]
- Noel SD, Keen KL, Baumann DI, Filardo EJ, Terasawa E. Involvement of G protein-coupled receptor 30 (GPR30) in rapid action of estrogen in primate LHRH neurons. *Mol Endocrinol*. 2009; 23:349–359. [PubMed: 19131510]
- Nunemaker CS, DeFazio RA, Moenter SM. Estradiol-sensitive afferents modulate long-term episodic firing patterns of GnRH neurons. *Endocrinology*. 2002; 143:2284–2292. [PubMed: 12021193]
- Raman IM, Bean BP. Resurgent sodium current and action potential formation in dissociated cerebellar Purkinje neurons. *J Neurosci*. 1997; 17:4517–4526. [PubMed: 9169512]
- Raman IM, Bean BP. Ionic currents underlying spontaneous action potentials in isolated cerebellar Purkinje neurons. *J Neurosci*. 1999; 19:1663–1674. [PubMed: 10024353]
- Raman IM, Bean BP. Inactivation and recovery of sodium currents in cerebellar Purkinje neurons: evidence for two mechanisms. *Biophys J*. 2001; 80:729–737. [PubMed: 11159440]
- Sun J, Chu Z, Moenter SM. Diurnal in vivo and rapid in vitro effects of estradiol on voltage-gated calcium channels in gonadotropin-releasing hormone neurons. *J Neurosci*. 30:3912–3923. [PubMed: 20237262]
- Suter KJ, Song WJ, Sampson TL, Wuarin JP, Saunders JT, Dudek FE, Moenter SM. Genetic targeting of green fluorescent protein to gonadotropin-releasing hormone neurons: characterization of whole-cell electrophysiological properties and morphology. *Endocrinology*. 2000; 141:412–419. [PubMed: 10614664]
- Van Goor F, Krsmanovic LZ, Catt KJ, Stojilkovic SS. Control of action potential-driven calcium influx in GT1 neurons by the activation status of sodium and calcium channels. *Mol Endocrinol*. 1999; 13:587–603. [PubMed: 10194765]
- Wang Y, Garro M, Dantzler HA, Taylor JA, Kline DD, Kuehl-Kovarik MC. Age affects spontaneous activity and depolarizing afterpotentials in isolated gonadotropin-releasing hormone neurons. *Endocrinology*. 2008; 149:4938–4947. [PubMed: 18583421]
- Wilson RC, Kesner JS, Kaufman JM, Uemura T, Akema T, Knobil E. Central electrophysiologic correlates of pulsatile luteinizing hormone secretion in the rhesus monkey. *Neuroendocrinology*. 1984; 39:256–260. [PubMed: 6504270]

Figure 1. Estradiol reduces the inward ionic current underlying the action potential

(A) Action potential command waveform, consisting of multiple previously recorded action potentials in GnRH neurons. The first spike is evoked, the following spikes are spontaneous. (B) Representative current response to action potential voltage command. The current demarked by the rectangle was used for all analyses. (C) Representative current traces after TTX application (upper panel) and subtracted TTX-sensitive sodium currents (lower panel). (D) Representative traces (average of 10 records) from cells isolated from an ovariectomized (OVX) or estradiol-replaced (OVX+E) animal (D1); Cells were of similar capacitance (9–10 pF). The traces are overlapped in D2; note the attenuated current amplitude in the OVX+E animal. Traces are normalized in D3 to demonstrate similar kinetics. The scale bar in D2 applies to all traces. (E) The sodium current underlying the action potential is significantly reduced in GnRH neurons from estradiol-treated animals (OVX+E; *, $p < 0.002$, $n = 18$ –20). *In vivo* estradiol does not significantly decrease sodium current density in non-green (non-GnRH) neurons isolated from the same dissociation ($p = 0.36$, $n = 11$ –13). Sodium currents in non-green neurons are significantly smaller than currents in GnRH neurons (a, $p < 0.001$).

Figure 2. Estradiol decreases transient sodium current amplitude, but prolongs the inactivation time constant

(A) Representative current traces from cells isolated from an ovariectomized (OVX) or estradiol replaced (OVX+E) animal at step voltages from -60 mV to 60 mV for 30 ms. Cells had similar capacitances (~9 pF). (B) Current-voltage curves demonstrate that the transient sodium current amplitude is attenuated at all voltages in neurons from OVX+E animals (*, $p < 0.05$; **, $p < 0.005$; $n = 31-36$). (C) Representative normalized traces recorded at 0 mV suggest that sodium current inactivation is prolonged in neurons from estradiol-treated animals when compared to neurons from OVX animals. (D) The inactivation time constant Tau is significantly prolonged in neurons from estradiol-treated animals (*, $p < 0.05$; **, $p < 0.001$; $n = 31-36$).

Figure 3. Estradiol does not affect steady-state activation or inactivation

(A) Steady-state activation curves do not vary significantly between GnRH neurons from control (OVX) and estradiol-treated (OVX+E) animals ($p > 0.05$, $n = 31-36$). (B) Representative current traces, evoked by the inactivation protocol, recorded from a cell isolated from an OVX animal. The 230-ms traces have been shortened (hash lines) to better demonstrate the current responses. The box illustrates the tail current from which inactivation data is calculated. The inactivation protocol is inset; every other step is included in both the protocol and the responses for clarity. (C) Steady-state inactivation curves do not vary significantly between GnRH neurons from control (OVX) and estradiol-treated (OVX +E) animals ($p > 0.05$, $n = 27-29$).

Figure 4. Estradiol modulates the persistent sodium current

(A) Ramp protocol and corresponding average current traces in GnRH neurons from control (OVX) and estradiol-treated (OVX+E) animals ($n = 10-16$). Traces have been normalized.

(B) Current voltage relationship of the TTX-subtracted persistent current in neurons from control and estradiol-treated animals. The current in OVX+E animals peaks at a significantly more negative voltage ($p = 0.03$), resulting in an attenuated peak current ($p = 0.03$; $n=7-9$).

Figure 5. GnRH neurons do not express a resurgent sodium current

Representative current responses (**B**) to a protocol (**A**) designed to evoke a resurgent sodium current in a GnRH neuron isolated from a young control (OVX) mouse. Currents were elicited by a 2-ms conditioning step to +30 mV, followed by step repolarizations (**A**). Under this protocol, a resurgent sodium current would be expected to peak approximately 5 ms following the conditioning step (arrow), and last tens of milliseconds (Raman and Bean, 2001). No resurgent current was evoked in GnRH neurons. The current response to the conditioning step (3.5 nA) is truncated.

Figure 6. The effect of estradiol on sodium currents is attenuated with age

(A) Representative traces (average of 10 records) from cells isolated from a 15–18 month old ovariectomized (OVX) or estradiol-replaced (OVX+E) animal. The two traces are overlapped to demonstrate the similar sodium current amplitude. The capacitance of both cells was similar (~11 pF). (B) *In vivo* estradiol significantly decreases sodium current density only in neurons isolated from young animals. *: $p = 0.002$ compared to young OVX, $n = 18–20$. Differences in sodium current amplitude between GnRH neurons isolated from OVX and OVX+E animals were not significant in middle-aged (MA; $p = 0.60$, $n = 15–17$) or old ($p = 0.27$, $n = 27$) animals.