Molecular mechanisms that drive estradiol-dependent burst firing of Kiss1 neurons in the rostral periventricular preoptic area

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In the RP3V have been reported (9, 11), there have been few studies characterizing the biophysical properties and molecular signature of these cells. A recent report documented the expression of an h-current (Ih) in RP3V neurons (40), but the underlying kinetic properties and channels intrinsic to these unique cells have not been characterized.

Single-action potential-generated calcium influx is sufficient to spark the release of classical neurotransmitters; however, burst firing or tetanic stimulation is required for the release of neuropeptides (1, 32, 46). Burst firing in many CNS neurons is generated primarily by the T-type calcium channel current (IT) (e.g., thalamic relay neurons), and the rhythmicity of the burst firing is dependent on the Ih (30, 53). Ih is mediated by the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel family, which includes channel subtypes 1–4. Ih depolarizes neurons from hyperpolarized states, raising the membrane potential into the range of IT activation (12, 13, 23, 30, 55). IT is mediated by the low-threshold voltage-gated calcium channels Cav3.1–3.3 (37). Upon activation, IT initiates a transient Ca2+-driven depolarization above the threshold of action potential initiation (i.e., a low threshold spike) (27, 49). This depolarization then drives the neuron to fire an ensemble (burst) of Na+-driven action potentials. Since burst firing facilitates neuropeptide release and a “preovulatory” release of Kisspeptin would, in theory, depend on burst firing, we used in vitro recordings and single-cell (sc)RT-PCR of Kiss1 neurons in slice preparation to determine whether Kiss1 neurons in the RP3V exhibit burst firing and display the channels and currents necessary for this special property.

Critical for reaching a hyperpolarized membrane potential for de-inactivating Cav3 channels and activating HCN channels for initiating burst firing are a class of inwardly rectifying K+ channels that are activated by G protein-coupled receptors known as GIRK channels. Opioid receptors are coupled to GIRK channels, and activation of GIRK channels can facilitate rebound burst firing (4, 50). The vast majority of hypothalamic neurons express opioid receptors, and μ- and δ-opioid receptors are coupled to GIRK channels (21, 25, 28, 51, 57). Thus, we argue that rebound burst firing of Kiss1 neurons in the RP3V is facilitated by opioidergic afferents that operate through one or more of the classical opioid receptors expressed by these Kiss1 neurons. We know that the RP3V also expresses a sexually dimorphic, estrogen-sensitive population of endogenous opioid peptides, enkephalin and dynorphin, that activate primarily μ- and δ-opioid, and κ-opioid receptors, respectively (15, 47, 48). Therefore, we also tested the hypothesis that Kiss1...
neurons in the RP3V are the direct targets for regulation by opioidergic afferents. We used whole cell voltage clamp recordings from Kiss1 neurons in slice preparation to assess the effects of opioid agonists on the excitability of Kiss1 neurons in the RP3V. In addition, we used scPCR to verify that the cognate opioid receptor transcripts are expressed in these Kiss1 neurons.

MATERIALS AND METHODS

Animals. All procedures performed with animals were in accord with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by our local committee on animal care and use. Kiss1-Cre GFP (C57BL6/J and S129 background) mice were produced by Dr. Robert Steiner and colleagues at the University of Washington (17), and tyrosine hydroxylase (TH)-EGFP mice (C57BL6/J) were produced by Dr. Kobayashi and colleagues at the Fukushima Medical University in Japan (33, 45). In each case, offspring that carried the transgene were identified by PCR on the genomic DNA extracted from tail biopsies. Transgenic animals were maintained as heterozygous by breeding with WT C57BL6/J mice. Animals were housed under constant temperature and light in a 12:12-h light-dark cycle with lights on at 0600 (zeitgeber time [ZT]0) and lights off at 1800 (ZT12). Food and water was provided ad libitum. Female animals between 6 and 30 wk of age were used for all experiments. Bilateral ovariectomies (OVX) were performed under inhalant isofluorane anesthesia. Carprofen (Rimadyl; Pfizer, NY) was given immediately after surgery at a dose of 4 mg/kg as an analgesic. Following OVX, animals were treated with 17β-estradiol benzoate (E2), demonstrated to induce an LH surge in CB6 (EGFP-GnRH) mice (3). Five days (D5) after OVX, animals were treated with a subcutaneous injection of 0.25 µg E2 in sesame oil at ZT4 (lights on/off at ZT0/ZT12). On the following day (D6), animals were given a subcutaneous injection of 1.5 –2.0 µg of E2 in sesame oil. On the day of the induced surge (D7), E2-treated animals were euthanized at ZT4 (n = 8). E2-treated animals were also euthanized at ZT12 (n = 8). LH levels were 0.35 ± 0.10 ng/ml in the ZT4 E2-treated females (Fig. 1). In the ZT12 E2-treated animals, LH levels were 4.69 ± 1.5 mg/ml (Fig. 1). The low LH levels in the E2-treated animals at ZT4 vs. ZT12 are consistent with the presence of E2-mediated negative and positive feedback.

Animals used for cell harvesting and for the majority of the electrophysiology experiments were treated with the surge-inducing dose of E2 as described above. The E2 injections (high E2) resulted in a uterine weight of 109.7 ± 4.1 mg; n = 28 (the mean uterine weight of proestrus animals in our colony is 135.4 ± 7.2 mg; n = 15). An additional group of animals used for electrophysiology was treated as above but received less E2 as a model for the diestrous stage of the estrus cycle. These animals received injections of 0.08 µg of E2 in sesame oil on D5 and D6 following OVX and were used for experiments on D7. The low-E2 injections (low-E2) resulted in a uterine weight of 56.1 ± 7.4 mg; n = 5 (the mean uterine weight of diestrous animals in our colony is 57.0 ± 2.9 mg; n = 24).

In the Kiss1-CreGFP mice, the fluorescent intensity of the POA neurons was dependent on the circulating E2 levels (as measured by the uterine weights). Therefore, low levels of E2 resulted in only faint Kiss1-GFP neurons. Since we were interested in comparing I1 and Ih under low (diestrus type) and high (proestrus type) E2 states, and Kiss1 and Th mRNAs are coexpressed in RP3V neurons (8), these experiments were done with both Kiss1-CreGFP and TH-EGFP mice. The fluorescent intensity of TH-EGFP neurons is not dependent on circulating levels of E2.

For whole cell recording of I1 and Ih in Kiss1 neurons during low (uterine weight <80 mg) and high (uterine weights >80 mg) E2 states, GFP-expressing cells in the RP3V area were patched, and the electrical activity was recorded for 30–40 min. After whole cell recording, the cell content was harvested for RT-PCR identification of Kiss1 and Th mRNAs (see below).

Radioimmunoassay for LH. Radioimmunoassay (RIA) for mouse LH was performed by the Endocrine Technology and Support Lab at the Oregon National Primate Research Center (Oregon Health and Science University, Beaverton, OR) by using a traditional double-antibody RIA procedure described in Pau et al. (3) and Bosch et al. (36). The detection limit of the assay was 0.2 ng/ml. A mouse serum pool (ET mouse #4) was used in triplicate in each assay as a quality control. The interassay variation (CV) was 14.7% and the intra-assay CV was 3.8%.

Slice preparation. On the day of experimentation, the animal was euthanized by decapitation. Trunk blood was collected, and the brain was removed from the skull. The brain stem was removed, and the resulting block was mounted on a cutting vibratome and submerged in ice-cold oxygenated (95% O2–5% CO2) high-sucrose artificial cerebral spinal fluid (aCSF) (in mM: 208 sucrose, 2 KCl, 1.24 NaH2PO4, 1.25 NaH2PO4, 10 HEPEs, 26 NaHCO3, 10 dextrose, 2 MgSO4, and 1 CaCl2). Two to three 220-µm slices were cut through the RP3V. Slices were transferred to an auxiliary chamber containing oxygenated aCSF at room temperature (in mL: 124 NaCl, 5 KCl, 1.44 NaH2PO4, 5 HEPEs, 10 dextrose, 26 NaHCO3, 2 MgCl2, and 2 CaCl2) and allowed to recover for at least 1 h. The uteri were removed, trimmed of fat, blotted dry, and weighed. Weights were recorded as an indicator of E2 levels.

Cell harvesting and reverse transcription PCR. Slices were individually visualized under a Leica inverted microscope to confirm GFP fluorescence. Two to three slices containing the RP3V were microdissected under a dissecting microscope. The tissue was digested with protease (from Streptomyces griseus, Sigma) at 37°C for 15 min and then washed three times with low-Ca2+ aCSF (1 mM Ca2+) and two times in aCSF. Using flame-polished glass Pasteur pipettes of decreasing sizes, slices were triturated and plated onto a 60-mm cell culture dish with a glass bottom. The cells were allowed to settle for 12 min until being moved to the harvesting microscope. Oxygenated aCSF at room temperature was constantly perfused into the dish at a rate of 2 ml/min. The cells were allowed to rest after perfusion began for at least 15 min. Fully intact, healthy cells that showed uniform fluorescence and were anchored to the glass plate were harvested. Harvesting was done by using a visualized patch-clamping with a standard glass pipette (1.5 mm OD/0.83 mm ID; World Precision Instruments, Sarasota, FL) that had been pulled to a 10-µm tip. Using the XenoWorks microinjector system (Sutter Instruments, Navato, CA), we applied gentle suction to pull the cell off of the plate. Collected cells were ejected from the pipette into a siliconized 0.5-ml tube containing a solution of 1X Invitrogen Superscript III Buffer, 15 U RNAsin (Promega, Madison WI), 10 mM dithiothreitol (DTT), and diethylpyrocarbonate (DEPC)-treated water in a total of 5 µl (single cells) or 8 µl (pools of cells). Cells were harvested individually or as pools of 5
individual cells. Collection tubes were kept in a chilled metal block to avoid freeze/thaw cycling, but each completed pool of cells was frozen as soon as possible on dry ice and stored at −80°C until further processing. Cells were dissociated and harvested as we previously reported (3, 55).

Harvested cells were reverse transcribed according to the manufacturer’s instructions (SuperScript III; Invitrogen, Carlsbad CA) and as described previously (3). The final products were stored at −20°C. Cells and controls underwent reverse transcription within 48 h of harvesting. The positive and negative controls included hypothalamic RNA samples that were subjected to reverse transcription with reverse transcriptase added (+RT) or without reverse transcriptase (−RT), respectively. After conversion to cDNA, individual cells and pools were confirmed to exhibit Kiss1 expression by amplifying Kiss1 mRNA using primers on a 1- to 2-μl template for 35 cycles (Table 1). PCR analysis of individual cells was used to determine the expression of Kiss1 mRNA and Th mRNA in GFP-Kiss1 neurons. PCR analysis of cell pools allowed determining a certain level of Kiss1 expression that was deemed consistent in all harvested pools used for further analysis. Pools that showed a faint or no Kiss1 expression were excluded from analysis. Control samples were run for Kiss1 expression to confirm there had been no contamination during the RT process. Each pool of cells was then evaluated using quantitative real-time PCR (qPCR).

Primer design. PCR primers were designed from the most recent National Center for Biotechnology Information on DNA and mRNA sequences using Clone Manager (SciEd Software, Cary, NC). Primers were designed to produce products between 75 and 200 base pairs (bp) and to be as efficient as possible. To distinguish cDNA from genomic DNA products, all primers crossed an intron/exon boundary. Only primers that demonstrated a single-peak melting curve and had genomic DNA products, all primers crossed an intron/exon boundary. Primer efficiency between 95 and 100% were used (Table 1).

qPCR. Quantitative analysis by PCR was performed using the Power Sybr Green Mastermix method as described previously (55) on an Applied Biosystems 7500 Fast real-time PCR system (Life Technologies, Carlsbad, CA). Primer efficiencies and data were analyzed as described previously (3, 55). All efficiencies are listed in Table 1. cDNA samples from Kiss1 neurons collected from the RP3V were run in duplicates, using 3.5–4 μl of cDNA per duplicate. Melting curves were analyzed for each reaction, and all genes were normalized to β-actin as a reference. One gene from each family (i.e., HCN1, Cav3.1, µ-opioid receptor) was then used as a “calibrator” to compare levels of mRNA among genes. Data were analyzed following the comparative ΔΔCT method described previously (38, 55) and are reported as relative amounts of mRNA expression to the calibrator.

Table 1. Primer sequences for PCR

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<th>Gene</th>
<th>Product Length, bp</th>
<th>Primer Sequence</th>
<th>BP No.</th>
<th>Accession No.</th>
<th>Slope</th>
<th>Efficiency, %</th>
<th>r²</th>
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The sense (forward) primer is listed first, with the antisense (reverse) primer below. HCN, hyperpolarization-activated cyclic nucleotide-gated channel; Cav, voltage-gated calcium channel; OR, opioid receptor; Kiss1, kisspeptin; TH, tyrosine hydroxylase.
examined at resting membrane potentials after a series of hyperpolarization of 1 s duration from −70 mV to −120 mV. The liquid junction potential of −10 mV was corrected for in all analyses. Consecutive current traces were filtered at 2−10 kHz and acquired at a sampling rate of 0.5−5 kHz. Standard whole cell patch clamp recording procedures and pharmacological testing were conducted as described previously (17, 42, 54, 56). Electrophysiological signals were amplified with an Axopatch 200B amplifier and digitized with a Digidata 1440A (Molecular Devices, Foster City, CA) or with an Axopatch 1D amplifier and digitized with a Digidata 1322 (Axon Instruments, Foster City, CA). Data were analyzed with Clampfit software (v. 9.0 or 10.0, Molecular Devices).

After recording, the cell was harvested by applying negative pressure until visualizing the cell content being gently aspirated into the tip of the recording pipette. The cell content was expelled into a 500-µl harvesting tube containing 5 µl of RT solution and stored at −80°C until further processing. All recorded cells that were harvested were reverse transcribed as described above. cDNA was analyzed for Kiss1 and Th mRNAs by using a 4-µl template in a 30-µl PCR reaction as described above. Cells that were negative to both Th and Kiss1 mRNA were further subjected to the detection of β-actin mRNA used as a positive control.

Drugs or chemicals. TTA-P2 (5,3,5-dichloro-N-[2-(2,3-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoroperidin-4-ylmethyl]-benzamide), a gift from Merck) was made up as a 10 mM stock solution in DMSO. ZD-7288 was purchased from Tocris Bioscience (Minneapolis, MN) and was made up as a 10 mM stock solution in DMSO. TTX was a gift from Merck) was made up as a 10 mM stock solution in DMSO. ZD-7288 was purchased from Tocris Bioscience (Minneapolis, MN) and was made up as a 10 mM stock solution in DMSO. TTX was purchased from Alomone Laboratories (Jerusalem, Israel) and was made up as 1 mM in stock in Milli-Q water.

**Data analysis.** The firing pattern of individual neurons was determined with similar criteria as previously reported for RP3V Kiss neurons (11). Silent neurons had low spontaneous spiking activity (mean firing rate <0.5 Hz); tonic neurons fired regularly with a mean firing rate >4 Hz and a coefficient of variation (defined as SD/mean) of the interspike interval (ISI) <0.25. Burst firing was defined as a cluster of spikes occurring with an ISI of ≈250 ms and terminating with an ISI of ≈500 ms. Bursting neurons had more than 50% of their spikes occurring in bursts with intraburst frequency higher than 4 Hz. All other neurons were classified as irregular (CV greater than 0.25). Rebound burst firing was defined as the hyperpolarization-induced high-frequency (>10 Hz) firing with two or more spikes in a cluster. To be consistent, the maximal rebound burst firing frequency was determined from the first two spikes. Data were analyzed with Mini Analysis and Clampfit 9.2 software. Graphs were plotted using GraphPad Prism 4, Sigma Plot 8.0, and Macromedia Freehand 10 software. Comparisons between different treatments were performed using an unpaired Student’s t-test or a one-way or two-way ANOVA with Bonferroni post hoc tests. Differences were considered significant if the probability of error was <5%. All data are presented as means ± SE.

**RESULTS**

**Kiss1-GFP neurons in the RP3V area express Kiss1 mRNA.** Kiss1-CreGFP mice were used to target fluorescent Kiss1 neurons in the RP3V. Kiss1-GFP neurons in the RP3V area were dispersed, harvested individually, and subjected to RT-PCR to confirm Kiss1 mRNA expression. The analysis of 139 cells from five OVX E2-treated (surge model) females revealed that 94% expressed Kiss1 mRNA (Fig. 2A). Kiss1-positive cells were also tested for coexpression with Th mRNA, and 88% expressed Th mRNA in addition to Kiss1 mRNA. Adjacent nonfluorescent cells (n = 10) were negative for Kiss1 or Th mRNA. As reported previously (17), OVX oil-treated control animals had little or no GFP-labeled cells in the RP3V area. Therefore, only E2-treated (low and high dose) animals were used for cell harvesting and electrophysiological recordings.

**Identification of Kiss1 and Kiss1/Th dual phenotype in the slice.** To identify the Kiss1 and Th transcript from recorded Kiss1-GFP and TH-EGFP neurons, cells were harvested after recording for RT-PCR analysis. A total of 63 neurons from 10 Kiss1-GFP mice and 9 TH-EGFP mice were recorded and analyzed. In the high-E2 group (uterine weights >80 mg), we found that 100% (27/27) of fluorescently identified Kiss1-GFP neurons were positive for Kiss1 mRNA. 75% (20/27) of these Kiss1 mRNA-positive neurons (Kiss1-CreGFP mice) coexpressed Th mRNA (Fig. 2B). In addition, 60% (12/20) of the fluorescently identified TH-EGFP neurons were positive for Kiss1 mRNA. There was no difference among Kiss1 neurons that were recorded in the Kiss-GFP mice vs. the TH-EGFP mice in terms of resting membrane potential, cell membrane capacitance, input resistance, spike threshold, and I1 and I8 amplitudes (Table 2). These parameters were also similar in Th-positive and Th-negative Kiss1 neurons. Therefore, the data from the Kiss-CreGFP and TH-EGFP mice were combined for further analysis.

**Spontaneous firing properties of RP3V Kiss1-CreGFP neurons.** In the next series of experiments, we examined the spontaneous firing characteristics of identified RP3V Kiss1 Cre-GFP...
neurons from high-E2-treated animals in coronal slices by using whole cell electrophysiological recordings. We also did cell-attached recording for comparison (17). The expression of GFP was used to identify individual Kiss1 neurons. One to two cells were recorded in each coronal slice. Whole cell current clamp recordings revealed that the mean resting membrane potential of Kiss1 neurons in the RP3V was $-54.7 \pm 0.6$ mV ($n = 29$) from high-E2 animals. The vast majority (78%) of Kiss1 neurons exhibited spontaneous firing at resting membrane potential that could be classified into three types of firing pattern: burst, tonic, and irregular (Fig. 3, A–C). Among the 77 neurons recorded (whole cell) from high-E2-treated animals, ~52% displayed tonic/irregular firing; 26% exhibited burst firing; and 22% were silent during the recording (Fig. 3E). These firing patterns were consistent with those obtained from loose patch recordings, which showed 46% tonic/irregular firing, 29% burst firing, and 25% silent ($n = 63$; Fig. 3E). There was no correlation in firing pattern or frequency between cells recorded in the same slice. For example, we found tonic and irregular firing cells, silent and bursting cells, or tonic and bursting cells in the same slice. Moreover, bath-applied glutamate (100 $\mu$M) or NMDA (40 $\mu$M) depolarized (11.5 $\pm$ 4.3 mV in glutamate, 10.8 $\pm$ 3.2 mV in NMDA, $n = 7$) and increased the firing rate of all RP3V Kiss1 neurons examined (Fig. 3, E and F). Consistent with a previous report (9), the percentage of cells exhibiting spontaneous burst firing was low (26–29%), which indicates that synaptic input and activation of endogenous conductances for burst firing were missing. Indeed, when cells were hyperpolarized to $-100$ mV by constant current injection of $-15$ pA, rebound burst firing was induced (Fig. 3G). This implies that RP3V Kiss1 neurons may express the intrinsic conductances for postinhibitory rebound.

Table 2. Electrotonic properties of RP3V Kiss1+ /TH+ neurons in high-E2-treated TH-EGFP and Kiss-Cre GFP mice

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<th>Mice (cell no)</th>
<th>$C_m$ (pF)</th>
<th>$R_m$ (GΩ)</th>
<th>$I_T$ (pA)</th>
<th>$I_h$ (pA)</th>
<th>AP Threshold (mV)</th>
<th>RMP (mV)</th>
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<td>TH ($n = 12$)</td>
<td>19.1 ± 2.6</td>
<td>2.7 ± 0.5</td>
<td>41.7 ± 10.1</td>
<td>46.0 ± 12.8</td>
<td>$-44.9 \pm 0.7$</td>
<td>$-54.6 \pm 1.1$</td>
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<tr>
<td>Kiss ($n = 14$)</td>
<td>18.5 ± 1.1</td>
<td>2.3 ± 0.6</td>
<td>44.0 ± 4.9</td>
<td>38.4 ± 4.9</td>
<td>$-45.1 \pm 0.9$</td>
<td>$-54.7 \pm 0.6$</td>
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Values are means ± SE. $I_T$, T-type calcium channel current; $I_h$, hyperpolarization-activated h-current.

Fig. 3. Properties of spontaneous firing of Kiss1 neurons in the rostral periventricular area of the 3rd ventricle (RP3V) from E2-treated OVX mice. A–C: representative whole cell, current clamp recordings of spontaneous burst firing (A), irregular firing (B), and tonic firing (C) in RP3V Kiss1 neurons. D: summary of percentage of cells showing different firing pattern in whole cell and loose patch recordings. E and F: representative recordings showing glutamate- or NMDA-induced firing in Kiss1 neurons. G: hyperpolarization-induced spontaneous rebound burst firing recorded from a RP3V Kiss1 neuron. The cell was hyperpolarized by constant current injection of $-15$ pA.
burst firing, and synaptic input may be critical for producing a hyperpolarizing stimulus.

Basic membrane properties and hyperpolarization recruited conductances in RP3V Kiss1 neurons. To examine whether RP3V neurons express intrinsic membrane conductances for burst firing, the basic membrane properties and the conductances recruited through hyperpolarization were examined in voltage clamp recordings from high-E2-treated animals. Since RP3V Kiss1 neurons express K<sub>ATP</sub> channels that will affect the resting membrane potential as the cells are dialyzed with a low (2 mM) ATP internal solution (present findings) (14), the biophysical measurements of the I<sub>h</sub> and I<sub>T</sub> were done under 4 mM ATP (internal) concentrations to minimize the effects of Kir6.2 (K<sub>ATP</sub>) channel activity. In voltage clamp, a series of depolarization and hyperpolarization steps were delivered to examine the input resistance and the hyperpolarization-dependent I<sub>h</sub> and I<sub>T</sub> that contribute to postinhibitory rebound burst in many neurons in the CNS (30, 53). Under voltage clamp conditions, both hyperpolarization-activated I<sub>h</sub> and I<sub>T</sub> were evident in RP3V Kiss1 neurons (Fig. 4A). In the high-E2-treated animal, all fluorescently identified RP3V Kiss1 neurons displayed I<sub>h</sub> greater than 5 pA at −120 mV (39.4 ± 4.9 pA, n = 36; Fig. 4C and Table 3). Eighty-three percent (30/36) of these neurons expressed I<sub>T</sub> with a mean amplitude of 42.9 ± 4.6 pA at −60 mV (Fig. 4C and Table 3); 11% (4/36) of them expressed A-type potassium currents (I<sub>A</sub>) (19.2 ± 4.8 pA, n = 4). Similarly for Kiss1 and TH dual-phenotype (Kiss<sup>+</sup>/TH<sup>+</sup>) neurons, all of them expressed an I<sub>h</sub>, and 84% of them expressed I<sub>T</sub> (Table 3). Moreover, all of the Kiss1 neurons expressed a persistent sodium current (I<sub>NaP</sub>) with a mean amplitude of 35.3 ± 5.2 pA (Fig. 4, B and C). The persistent I<sub>NaP</sub> generated a significant inward current that contributed to the rebound burst firing (see discussion). The slope conductance between −70 and −50 mV was significantly increased from 0.74 ± 0.17 pS to 1.5 ± 0.2 pS by TTX (500 nM), indicative that TTX-sensitive, voltage-gated Na channels are responsible for this robust inward current (43).

RP3V Kiss1/TH neurons express a rapid I<sub>h</sub> that is primarily composed of HCN1. Whole cell voltage clamp recordings revealed that all of the Kiss1 neurons in the RP3V from high-E2-treated animals expressed I<sub>h</sub> (Fig. 5, A–D, and Table 3). I<sub>h</sub> was recognized by its characteristic slowly developing inward current following hyperpolarizing voltage steps with the steady-state current reached early during the more hyperpolarized voltage step (Fig. 5A). Single-cell RT-PCR from recorded cells showed that the majority of these cells expressed HCN1 mRNA and Cav3.1 mRNA (Fig. 5B and see below). I<sub>h</sub> was completely blocked in Kiss1 neurons by the selective blocker ZD-7288 (50 μM; Fig. 5A). I<sub>h</sub> could be evoked in Kiss1 neurons at voltages up to −75 mV (Fig. 5, C and D). qPCR of Kiss1 neurons harvested from the RP3V demonstrated that these neurons express HCN1, −2, and −3 (Fig. 5E); HCN4 was undetectable (data not shown). The expression of HCN2 and HCN3 mRNAs was ~30% of the HCN1 level. The I<sub>h</sub> in Kiss1 neurons displayed fast activation kinetics with a τ = 84 ± 10 ms at −120 mV (Fig. 5A), consistent with the relatively high expression level of HCN1 mRNA (Fig. 5, B and E).

RP3V Kiss1/TH neurons express I<sub>T</sub> that is primarily composed of Cav3.1. Whole cell voltage clamp recordings revealed that 80% of RP3V Kiss1 neurons from the high-E2-treated animals displayed an I<sub>T</sub> larger than 5 pA (fully recovered at −120 mV and activated at −60 mV; Figs. 4 and 6, A and B). All of these neurons also expressed I<sub>h</sub>. The V<sub>T</sub>1/2 for the recovery, as determined by fitting the data to a Boltzmann equation, was −86.4 ± 0.2 mV (Fig. 5E), which was similar to the V<sub>T</sub>1/2 in GnRH neurons (55). Furthermore, the I<sub>T</sub> in these Kiss1 neurons was rapidly inactivated (at −60 mV, τ-fast = 19.4 ± 3.3 ms, τ-slow = 86.3 ± 29.5 ms, n = 8). I<sub>T</sub> in these

Fig. 4. Kiss1 neurons in the RP3V expressed intrinsic conductances for postinhibitory rebound burst firing. A: representative recording showing a series of hyperpolarizing/depolarizing pulses induced current (inset: voltage protocol: holding potential: −60 mV; steps from −45 to −120 mV, duration 500 ms). Single-head arrows indicate where instantaneous and steady-state hyperpolarization-activated h current (I<sub>h</sub>) were measured. Steady-state current (I<sub>NaP</sub>; inset protocol) was taken as an average from the arrow to the end of the pulse. Double-head arrow indicates peak T-type calcium current (I<sub>T</sub>), which was activated when the voltage was stepped back to −60 mV. B: representative recording showing persistent sodium current (I<sub>NaP</sub>) in RP3V Kiss1 neurons. I<sub>NaP</sub> was activated by a slow ramp of voltage (20 mV/s) from −80 to −20 mV (see inset protocol). Measurement of I<sub>NaP</sub> was indicated as the difference between the inward peak at −50 mV and the extrapolated leak current from −80 mV (dashed line). C: scatter plot of amplitude distribution of the I<sub>T</sub>, I<sub>h</sub>, and I<sub>NaP</sub> that were larger than 5 pA in RP3V Kiss1 neurons. (I<sub>T</sub>: 42.9 ± 4.6 pA, n = 30; I<sub>h</sub>: 39.4 ± 4.9 pA, n = 36; I<sub>NaP</sub>: 35.3 ± 5.2 pA, n = 13, respectively). I<sub>h</sub> and I<sub>NaP</sub> were expressed in 100% of Kiss1 neurons; I<sub>T</sub> was expressed in 83% of cells.
Kiss1 neurons were blocked by 50 μM Ni²⁺ (Fig. 6A) and also by the selective T₇ blocker TTA-P2 (10). As shown in Fig. 6B, perfusing TTA-P2 (5 μM) blocked the I₇ within 3 min of bath application by 90.9 ± 5.3% (Fig. 6, C and D). Measurements by qPCR analysis of RP3V Kiss1 neuronal pools revealed that these neurons express primarily Cav3.1 mRNA and to a lesser extent Cav3.2 and Cav3.3 transcripts (Fig. 6F). Cav3.2 and Cav3.3 were significantly less abundant than Cav3.1 transcripts (P < 0.01 and P < 0.05, respectively). These data indicate that Kiss1 neurons in the RP3V preferentially express Cav3.1 channels, which is the T-type Ca²⁺ channel with the fastest kinetics. Despite lower expression, Cav3.2 and Cav3.3 were detectable in the pooled neurons, indicating that they may also contribute to I₇ in Kiss1 neurons in this region.

Hyperpolarization-induced rebound burst firing in RP3V Kiss1 neurons is dependent on expression of I₇. Since T-channels in RP3V Kiss1 neurons were recruited by hyperpolarization, we examined the ability of these neurons to generate rebound burst firing through current injection-induced hyperpolarization. As an example, the Kiss1 neuron in Fig. 7A had a resting membrane potential of −62 mV and fired irregularly at a mean frequency of 0.8 Hz. This cell had a T-current amplitude of 52 pA. The hyperpolarization threshold, defined as the minimum hyperpolarization (or current injection) required to induce rebound burst firing, was −76 mV (or −20 pA), and its corresponding burst firing rate determined by the first interspike interval was 36 Hz. Greater hyperpolarization to −80, −85, and −89 mV or current injection of −30, −40, and −50 pA induced higher firing frequencies of 104, 156, and 172 Hz, respectively (Fig. 7B). The burst firing frequency increased non-linearly, with the largest changes near the hyperpolarization threshold (Fig. 7B). At resting membrane potential, we found that 82% of neurons had a hyperpolarization threshold of −84.7 ± 1.2 mV (n = 18), which, as predicted, is close to the V₁/₂ for de-inactivation

Table 3. Effects of high vs. low dose of E₂ on expressions of I₇ and I₉ in RP3V Kiss1 neurons

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Low E₂</th>
<th>High E₂</th>
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<tr>
<td>Kiss1⁺/TH⁺</td>
<td>Kiss1⁻/TH⁺</td>
<td>Kiss1⁺/TH⁺</td>
</tr>
<tr>
<td>With I₇</td>
<td>3 (33%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>With I₉</td>
<td>7 (78%)</td>
<td>5 (100%)</td>
</tr>
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<td>Cell nos.</td>
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E₂, 17β-estradiol.

Fig. 5. RP3V Kiss1 neurons express ZD-7288-sensitive I₉ and mRNA for HCN channels. A: representative voltage-clamp recording of I₉ with a hyperpolarizing voltage step protocol. Top: characteristic I₉ are visible as slowly activated inward currents at hyperpolarized membrane potentials. Inset: voltage clamp protocol: V₉hold = −60 mV; range from −40 to −120 mV, step size 5 mV, step duration 1 s. Middle: I₉ is blocked by 50 μM ZD-7288, evidenced by lack of slowly developed inward current. Bottom: ZD-7288-sensitive current, which represents the isolated I₉. B: film image of representative Kiss1 neurons that expressed HCN1 and Ca₃.1 transcripts following whole cell recording for 30–40 min. C: I/V relationship of I₉ from A, showing voltage-dependent activation and inward rectification at increasingly hyperpolarized voltages. D: mean I/V relationship of 16 Kiss1 neurons showing instantaneous (filled circles) and steady-state (open circles) whole cell currents. V₉hold = −60 mV. Protocol is indicated in Fig. 4A. The difference between instantaneous and steady-state I/V was due to activation of I₉. E: relative levels of mRNA for HCN1, -2, and -3 in pools of Kiss1 neurons within the RP3V (n = 4–5 animals, 3 pools/animal). Levels of HCN2 and HCN3 mRNA were significantly lower than HCN1 mRNA. Relative expression was calculated by ΔΔCₚ method and normalized to the mean ΔCₚ of HCN1. ***P < 0.001 for HCN2 vs. HCN1 and HCN3 vs. HCN1 (ANOVA).
Fig. 6. RP3V Kiss1 neurons express nickel- and TTA-P2-sensitive \( I_T \) and mRNA for Cav3 channels. A: representative traces showing \( I-V \) relationship of \( I_T \) before (left, control) and after exposure to 50 \( \mu \)M Ni\(^{2+} \) for 10 min (middle, Ni\(^{2+} \)), and the subtracted Ni\(^{2+} \)-sensitive \( I_T \) (right) recorded from a RP3V Kiss1 neuron. Traces were truncated to highlight inward \( I_T \). The insert panel shows the voltage-clamp protocol used to measure \( I_T \). The pulse protocol consisted of 5-mV steps, from −40 to −120 mV, before returning to −60 mV. Pulses were of 1-s duration to ensure complete de-inactivation of the T-channel. B: representative traces showing \( I-V \) relationship of \( I_T \) before (left, control) and after exposure to 5 \( \mu \)M TTA-P2 for 10 min (middle) and the subtracted, TTA-P2-sensitive \( I_T \) (right) recorded from a RP3V Kiss1 neuron. Traces were truncated to highlight the inward \( I_T \) following de-inactivation steps. Bottom: voltage-clamp protocol used to measure \( I_T \) in Kiss1 neurons. C: analysis of time course of TTA-P2 blockade of \( I_T \) in a Kiss1 neuron. D: summary of effects of TTA-P2 on maximum peak amplitude of \( I_T \) at −50 mV. **P < 0.01 vs. control, with Student’s unpaired t-test; \( n = 5 \) cells. Mean inhibition of \( I_T \) by 5 \( \mu \)M TTA-P2 was 90.9 ± 5.5%. E: Boltzmann equation fit of the voltage-dependent de-inactivation of the T-channel. Half-maximum de-inactivation voltage is indicated: \( V_{1/2} = −86.4 ± 0.2 \) mV (\( n = 5, r^2 = 0.999 \)). F: levels of mRNA for Cav3.1, −3.2, and −3.3 derived from qPCR (\( n = 3 \) animals; 3 pools/animal). Relative expression was calculated by \( \Delta \Delta C_T \) method and normalized to mean \( \Delta C_T \) of Cav3.1. *P < 0.05 for Cav3.3 vs. Cav3.1; **P < 0.01, Cav3.2 vs. Cav3.1 (ANOVA).
Fig. 7. Hyperpolarization-induced rebound burst firing in RP3V Kiss1 neurons. A: hyperpolarization-induced rebound burst firing from a Kiss1+/TH+ cell that expressed $I_T$ of 52 pA (activated at −60 mV) and had input resistances of 0.53 and 2.0 GΩ between −80 and −60 mV and between −70 and −50 mV, respectively. C: analysis of the effect of hyperpolarizing steps on maximal burst frequency in 11 cells. Dashed line, mean hyperpolarization threshold for burst firing of 82% of neurons. B: example recording showing that T-channel blocker TTA-P2 (5 μM) blocked the rebound burst firing. This cell had $I_T$ of 67 pA and $I_h$ of 46 pA. Current injections of −30 and −90 pA yielded hyperpolarizations of −89 and −109 mV, respectively. D: summary of effects of TTA-P2 on spike number of rebound burst firing. **P < 0.01, paired Student’s t-test. E: example recording showing that $h$-channel blocker ZD-7288 (50 μM) did not block the rebound burst firing but increased the delay to the first spike. This cell had $I_T$ of 30 pA and $I_h$ of 38 pA. Current injection of −60 pA hyperpolarized the cell membrane to −98 mV.

of the T-channel. Next we examined the contribution of $I_T$ and $I_h$ to the rebound burst firing. Rebound burst firing was clearly blocked by the selective Cav3 channel blocker TTA-P2 (Fig. 7, C and D), whereas rebound burst firing was not blocked by the HCN channel blocker ZD-7288 (Fig. 7E). However, blocking HCN channels did increase the delay for the initiation of first spike (Fig. 7E). Therefore the T-channel and $h$-channel play different roles in the generation of rebound burst firing.

Expressions of $I_T$ and $I_h$ are dependent on circulating levels of E2. Our previous studies showed that the expression of T-type calcium channels and HCN channels in GnRH neurons are regulated by estrogens (2, 3, 55). Therefore, we examined the effects of low-E2 vs. high-E2 treatment on the expression of $I_T$ and $I_h$ in RP3V Kiss1 neurons. To identify the Kiss1 neurons from low-E2-treated OVX females, both TH-EGFP and Kiss-GFP mice were used. For recordings from TH-EGFP mice, cells were targeted by EGFP fluorescence; for recordings from Kiss-GFP mice, cells were blindly targeted. All recorded neurons were harvested for RT-PCR identification of Kiss1 transcripts. A total of 18 neurons were recorded from the low-E2 group, and 10 of them were identified as Kiss1 mRNA positive. We found that, in the low-E2-group, few Kiss1+/TH+ neurons (3 of 10) expressed an $I_T$ (Table 3) with a mean amplitude of 5.8 ± 2.4 pA (n = 10). The majority of Kiss1 neurons in the low-E2 group expressed an $I_h$ (7 of 10) with a mean amplitude of 10.8 ± 2.7 pA (n = 10). In the high-E2 group, the vast majority of Kiss1-positive neurons (83%; total n = 36) expressed an $I_T$ (Table 3) with a mean amplitude of 34.8 ± 4.3 pA (n = 36) and an $I_h$ of mean amplitude of 39.1 ± 4.4 pA (n = 36). We found that there was no difference between the low-E2 Kiss1 neurons and the high-E2 Kiss1 neurons in the resting membrane potential (−54.1 ± 1.5 mV, n = 9 in low-E2 vs. −54.7 ± 0.9 mV, n = 36 in high-E2) or the cell membrane capacitance (18.6 ± 1.5 pF, n = 10 in low-E2 vs. 18.4 ± 1.0 pF, n = 36 in high-E2). The $I_T$ density in the high-E2 group was sixfold higher than in the low-E2 group (1.85 pA ± 0.24 pA/pF vs. 0.31 ± 0.6 pA/pF, P < 0.01), and the $I_h$ density in the high-E2 group was 3.4-fold higher than in the low-E2 group (1.94 pA ± 0.15 pA/pF vs. 0.58 ± 0.13 pA/pF, P < 0.01; Fig. 8). In contrast, only two of nine Kiss1+/TH+ neurons in the high-E2 group expressed an $I_T$ with an overall mean amplitude of 5.8 ± 5.8 pA (n = 9), whereas 89% (n = 8) of them expressed an $I_h$ (Table 3) with mean amplitude of 20.8 ± 6.8 pA. Therefore, the $I_T$ is highly expressed in RP3V Kiss1 neurons and is exquisitely sensitive to E2, whereas the expression of the $I_h$ is less sensitive to E2.

Opioid peptides and GABA$_B$ agonist hyperpolarize RP3V Kiss1 neurons. Clearly, the rebound burst firing is dependent on the hyperpolarization threshold. To search for possible
metabotropic inhibitory inputs onto RP3V Kiss1 neurons, we examined responses of Kiss1 neurons to the \( \mu \)- and \( \kappa \)-opioid receptor agonists by measuring the agonist-activated outward K\(^+\) currents at \(-60\) mV using whole cell voltage clamp recordings (Fig. 9). In Kiss1 neurons in the RP3V (high-E\(_2\) group), the \( \mu \)-opioid receptor agonist DAMGO (1 \( \mu \)M) induced a small outward current (Fig. 9A). The outward current reversed at \( E_K \) and exhibited inward rectification (data not shown). Of the cells tested, 75\% (9 of 12) responded to DAMGO with a mean evoked current of \( 6.7 \pm 0.6 \) pA (Fig. 9D; \( n = 9 \)). On the other hand, the \( \kappa \)-opioid receptor agonist U-69593 (1 \( \mu \)M) induced an outward current of \( 11.8 \pm 2.2 \) pA (Fig. 9, B–D; \( n = 9 \)) that exhibited inward rectification (Fig. 9C), indicative of the opening of GIRK channels. The effects were blocked by the nonselective opioid receptor antagonist naloxone (Fig. 9B). We also examined the \( \kappa \)-opioid receptor agonist U-69593 (1 \( \mu \)M) and GABA\(_B\) receptor agonist baclofen (20 \( \mu \)M)-induced hyperpolarization. As shown in Fig. 9, F–H, U-695993 induced a hyperpolarization of \( 6.8 \pm 1.0 \) mV (\( n = 5 \)), and baclofen induced a similar hyperpolarization (5.8 \( \pm \) 1.4 mV, \( n = 5 \)). Therefore, RP3V Kiss1 neurons, like the vast majority of hypothalamic neurons (21, 23, 29), respond to opioid and GABA\(_B\) agonists. Consistent with these electrophysiological observations, the qPCR analysis from pooled Kiss1 neurons confirmed that expression of the \( \kappa \)-opioid receptor mRNA was significantly greater than either the \( \mu \)- or \( \delta \)-receptor mRNA (Fig. 9E).

**DISCUSSION**

Here, we profile the endogenous electrophysiological properties of Kiss1 neurons in the RP3V of the female mouse. We show that the T-type calcium current is crucial for rebound burst firing and that this phenomenon is estrogen dependent. A significant fraction of these neurons exhibit spontaneous burst firing, and the vast majority of these cells express the pacemaker currents \( I_T \) and \( I_h \). The \( I_h \) displayed rapid kinetics, consistent with the abundant expression of HCN1 channels, which was amplified by E\(_2\). The predominance of the T-type Ca\(^{2+}\) channel Ca\(_{v3.1}\) corroborated the presence of a rapidly inactivating \( I_T \) in these cells, which was increased manyfold by E\(_2\) treatment. The presence of a robust T-current is essential for the high-frequency rebound bursting that is manifested following a hyperpolarizing stimulus. Furthermore, both \( \kappa \)- and \( \mu \)-opioid receptor agonists hyperpolarized Kiss1 neurons, which suggests that opioid synaptic input provides a portion of the stimulus required for reaching the hyperpolarization threshold necessary for recruitment of Ca\(_{v3.1}\) channels. The Kiss1 neurons were also hyperpolarized by the GABA\(_B\) agonist baclofen, which would further contribute to reaching the hyperpolarization threshold. Notably, the majority of the Kiss1 neurons in the RP3V expressed Th mRNA, which is consonant with the observation that these Kiss1 neurons in the RP3V exhibit rebound burst firing reminiscent of dopamine neurons in the arcuate nucleus (29).

Kiss1 neurons in the RP3V are similar to those in the arcuate nucleus (ARC) but differ in important ways. Kiss1 neurons in the ARC (so-called KNDy neurons) coexpress neurokinin B (NKB) and dynorphin (16, 34). In contrast, Kiss1 neurons in the RP3V express neither NKB nor dynorphin but express one or more classical transmitters including dopamine (8, 20, 34; present findings). We have previously shown that Kiss1 neurons in the ARC of mice and guinea pigs are either silent (~50\%) or show tonic/irregular firing (~50\%) (17, 42), and a report in a cross-bred kisspeptin-IRES-Cre \( \times \) ROSA26-CAGS-\( \tau \)GFP mouse described similar findings (9). Although Kiss1 neurons in the ARC and RP3V exhibit ionotropic glutamate-dependent burst firing (17; present findings), one-quarter of the Kiss1 neurons in the RP3V exhibit spontaneous burst firing. This suggests that, although Kiss1 neurons in the ARC and RP3V express a common neuropeptide and share the capacity to exhibit burst firing in response to glutamate, Kiss1 neurons in these two regions exhibit different biophysical properties and/or synaptic inputs.

The “pacemaker” current (\( I_h \)) is required for rhythmic firing, and virtually all Kiss1 neurons in both the ARC and RP3V express this current (present findings; 17, 40, 42). \( I_h \) is a noninactivating, nonselective cation current. This current is activated at hyperpolarized membrane potentials, and it depolarizes the cell into the range required for activation of the low-threshold \( I_T \), which is responsible for generating a burst of action potentials. The activation kinetics of \( I_h \) are determined by the properties of the underlying channels, and the HCN1...
channel exhibits the fastest kinetics (30, 44). The relative abundance of these subunits within a neuronal population can modulate the frequency and pattern of burst firing in these cells. The rapid depolarization mediated by HCN1 channels decreases interburst interval. Therefore, the kinetic properties of HCN1 endow RP3V Kiss1 neurons with the ability to generate rhythmic burst firing. Also contributing to the rhythmicity of burst firing in Kiss1 neurons is the persistent Na\(^+\)/H\(^+\) current, which is active in the range subthreshold to action potential generation (43). A similar persistent Na\(^+\)/H\(^+\) current is recognized to be important for generating a pacemaker potential in GnRH neurons in the telost (35). We found that \(I_{h}\) was regulated by E\(_2\) such that the whole cell current increased 3.4-fold with an E\(_2\) treatment that produced an LH surge in our mice, corroborating a recent report in crossbred kisspeptin-IRES-Cre × ROSA26-CAGS-rGFP mice studied throughout the estrous cycle (40). Most likely, this is attributable to an E\(_2\)-dependent increase in HCN channel expression, as is the case for E\(_2\) regulation of such channels in GnRH neurons (3).

Although \(I_{h}\) is responsible for the rhythmicity of burst firing, \(I_{T}\) is the most critical for generating the depolarizing stimulus (low threshold spike) that supports an ensemble of action potentials (19, 12, 13). \(I_{T}\) is a low-threshold activated Ca\(^{2+}\)/H\(^+\) current, whose primary physiological function is to generate burst firing (5, 37). Since the T-type Ca\(^{2+}\)/H\(^+\) (CaV3) channels are activated by low voltage, they open in a voltage range that induces a strong inward Ca\(^{2+}\)/H\(^+\) current. The primary difference between the various T-type Ca\(^{2+}\)/H\(^+\) channels is their rate of inactivation, with CaV3.1 exhibiting the fastest and CaV3.3 the slowest kinetics (24). In Kiss1 neurons in the RP3V, we found a rapidly inactivating, Ni\(^{2+}\) - and TTA-P2-sensitive T-type current, consistent with the expression of CaV3.1 mRNA (10). The combination of the rapid kinetics of \(I_{h}\) and \(I_{T}\) in Kiss1 neurons in the RP3V would support a higher frequency of burst firing. Previous studies have suggested that Kiss1 neurons in the RP3V must fire at a minimum of 5 Hz (spikes/s) to produce a sustained release of kisspeptin onto GnRH neurons (26), and our data indicate that Kiss1 neurons are capable of firing at much higher frequencies (Fig. 7). Although a rapidly inacti-
vating $I_T$ produced by Cav3.1 channels will produce a shorter duration of Ca$^{2+}$ depolarization, Cav3.1 also de-inactivates more rapidly than other Cav3 channels, which would allow more Cav3.1 channels to be recruited during the hyperpolarization for the next burst. Remarkably, $I_T$ was increased sixfold with an E2 treatment that produced an LH surge in these mice. This would indicate that there is an increase in Cav3 channel density as we have previously demonstrated for the expression of these channels in GnRH neurons (22, 55). This means that, once the cells reach the hyperpolarization threshold for recruiting Cav3 channels, there is greater rebound burst of action potentials. Moreover, we deduce that high-frequency burst firing of Kiss1 neurons in the RP3V has the capacity to drive a sustained output from downstream synaptic targets and may explain the prolonged firing of GnRH neurons associated with the preovulatory surge (6).

Notably, we determined that Kiss1 neurons in the RP3V exhibit a hyperpolarization threshold that is close to the $V_{1/2}$ for de-inactivation of the T-channel, which is the most critical for dictating the rebound burst firing frequency. GIRQ channels are critical for driving the membrane potential into the hyperpolarized state necessary to de-inactivate the Cav3 channels underlying the T-current. The majority of hypothalamic neurons express GABA and opioid receptors, which are coupled to GIRQ channels, and Kiss1 neurons in the RP3V are no exception (present findings; 21, 25, 28, 51, 57). In fact, these cells express $\kappa$-, $\mu$-, and $\delta$-opioid receptor transcripts, and the vast majority of these cells respond to $\kappa$- and $\mu$-agonists. The $\mu$-opioid receptor agonist DAMGO induced an outward current in RP3V Kiss1 neurons, similar to other hypothalamic neurons (present findings; 21, 25, 28, 51). The $\kappa$-opioid receptor agonist U-69593 generated an even greater GIRQ current, which correlated with the relative abundance of the $\kappa$- vs. $\mu$-receptor transcripts in these neurons. GABA and opioid receptors coupled to GIRQ channels in these cells would help provide the hyperpolarization stimulus required to de-inactivate Cav3.1 channels. Also, opioidergic afferents may make direct contact with Kiss1 neurons in the RP3V. Met-enkephalin is coexpressed in 28–38% of Kiss1 neurons in the RP3V area of mice (41). Since met-enkephalin is a potent endogenous agonist for the $\mu$-opioid receptor (52), and Kiss1 neurons respond to the enkephalin analog DAMGO, met-enkephalin may serve an autoregulatory role in hyperpolarizing Kiss1 neurons. Prodynorphin neurons are also abundant in the RP3V and much more prevalent in females than males (47). Moreover, Kiss1 neurons in the RP3V express the $\kappa$-opioid receptor (which mediates the effects of dynorphin), and these cells are robustly hyperpolarized by the $\kappa$-opioid receptor agonist U-69593. Thus, we infer that $\kappa$- and/or $\mu$-receptor-dependent signaling combined with GABAB signaling to Kiss1 neurons in the RP3V provides some of the critical input required for de-inactivating Cav3 channels in Kiss1 neurons. Interestingly, A12 dopamine neurons in the ARC also express $I_h$ and $I_T$. They are hyperpolarized by $\mu$-opioid and GABAB agonists and show similar rebound burst characteristics (29). Since the majority of Kiss1 neurons in the RP3V coexpress $Th$ transcript, their electrophysiological and molecular signatures suggest that these neurons belong to an important class of hypothalamic parvo-cellular “pacemaker” neurons (29, 31).

In summary, we have characterized the endogenous conductances and channels critical for producing burst firing in the Kiss1 neurons in the RP3V. The majority of these neurons colocalized $Th$ mRNA. Kiss1/TH neurons in the RP3V express a rapidly activating $I_h$, mediated primarily through HCN1 channels, and a T-type calcium current, mediated primarily through Cav3.2 channels. We have defined a hyperpolarization threshold that is critical for recruiting Cav3.1 channels for generating a high-frequency rebound burst firing. Importantly, the expression of both $I_T$ and $I_h$ was augmented by an E2 treatment paradigm that generates an LH surge in the ovariectomized female. We have also discovered a robust hyperpolarizing response to GABAB as well as $\kappa$- and $\mu$-opioid receptor agonists, indicative of GABAergic synaptic input and input from opioidergic neurons, most plausibly dynorphin or met-enkephalin. Thus, the biophysical and molecular fingerprints of Kiss1 neurons in the RP3V are consistent with their putative role in generating the sustained, high-frequency burst firing required for driving the preovulatory GnRH surge.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

Burst Firing of Kiss1 Neurons in the RP3V


