17 β -Estradiol Increases Arcuate KNDy Neuronal Sensitivity to Ghrelin Inhibition of the M-Current in Female Mice

Kristie Conde^a Troy A. Roepke^{a, b}

^aGraduate Program in Neuroscience, Rutgers University Robert Wood Johnson Medical School, The State University of New Jersey, New Brunswick, NJ, USA; ^bDepartment of Animal Sciences, School of Environmental and Biological Sciences, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA

Keywords

Estrogen receptor α · Arcuate nucleus · M-current · Electrophysiology · Ghrelin · Growth hormone secretagogue receptor

Abstract

Obesity and anorexia result in dysregulation of the hypothalamic–pituitary–gonadal axis, negatively impacting reproduction. Ghrelin, secreted from the stomach, potentially mediates negative energy states and neuroendocrine control of reproduction by acting through the growth hormone secretagogue receptor (GHSR). GHSR is expressed in hypothalamic arcuate (ARC) Kisspeptin/Neurokinin B (*Tac2*)/Dynorphin (KNDy) neurons. Ghrelin is known to inhibit the M-current produced by KCNQ channels in other ARC neurons. In addition, we have shown 17 β -estradiol (E2) increases *Ghsr* expression in KNDy neurons 6-fold and increases the M-current in NPY neurons. We hypothesize that E2 increases GHSR expression in KNDy neurons to increase ghrelin sensitivity during negative energy states. Furthermore, we suspect ghrelin targets the M-current in KNDy neurons to control reproduction and energy homeostasis. We utilized ovariectomized *Tac2*-EGFP adult female mice, pretreated with estradiol ben-

zoate (EB) or oil vehicle and performed whole-cell-patch-clamp recordings to elicit the M-current in KNDy neurons using standard activation protocols in voltage-clamp. Using the selective KCNQ channel blocker XE-991 (40 μ M) to target the M-current, oil- and EB-treated mice showed a decrease in the maximum peak current by 75.7 ± 13.8 pA ($n = 10$) and 68.0 ± 14.7 pA ($n = 11$), respectively. To determine the actions of ghrelin on the M-current, ghrelin was perfused (100 nM) in oil- and EB-treated mice resulting in the suppression of the maximum peak current by 58.5 ± 15.8 pA ($n = 9$) and 59.2 ± 11.9 pA ($n = 9$), respectively. KNDy neurons appeared more sensitive to ghrelin when pretreated with EB, revealing that ARC KNDy neurons are more sensitive to ghrelin during states of high E2.

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Introduction

Periods of undernutrition caused by food scarcity as well as overnutrition caused by an abundance of food are both associated with an imbalance in energy status that leads to a suppressed hypothalamic–pituitary–gonadal (HPG) axis, resulting in cessation of reproduction. While

obesity has been linked to coronary heart disease and type 2 diabetes mellitus [1], it also negatively impacts reproduction. In women, obesity leads to irregular menses, infertility, and miscarriages, among other complications [2, 3]. These problems in reproductive health extend to individuals suffering from undernutrition. Amenorrhea is prominent in athletes and anorexia nervosa patients, largely due to their decreased body mass index [4]. These perturbations in energy balance can lead to problems in the HPG axis, though the precise mechanisms remain unclear.

Energy balance and reproduction are centrally regulated processes that are controlled, in part, by neurons in the arcuate nucleus of the hypothalamus (ARC). Regulation of reproduction is controlled by negative and positive feedback of 17 β -estradiol (E2) on the HPG axis mediated, to some extent though not entirely, by neurons expressing kisspeptin. There are 2 main groups of kisspeptin neurons located in the rodent brain [5]. Kisspeptin is expressed in neurons in the anteroventral periventricular nucleus (AVPV) and ARC. ARC kisspeptin neurons co-express neurokinin B (*Tac2*) and dynorphin (*Pdyn*) and are therefore termed KNDy neurons. KNDy neurons mediate the negative feedback of E2 and progesterone on the HPG axis in females [6, 7]. Neurokinin B and dynorphin in KNDy neurons act as positive and negative autoregulators of KNDy neuronal excitability, respectively. Their combined actions produce the pulse generator that controls gonadotropin-releasing hormone (GnRH) release into the median eminence and subsequent release of luteinizing hormone (LH) and follicle-stimulating hormone from the anterior pituitary [8–12]. Both AVPV and ARC kisspeptin regulate the HPG axis by binding to its receptor *Kiss1r* expressed on the soma and axons of GnRH neurons [7–9, 13]. KNDy neurons also contribute to the control of energy homeostasis, especially in females. Ablation of KNDy neurons abrogates the post-ovariectomy weight gain associated with E2 in rats, suggesting that KNDy neurons mediate, in part, the anorectic effects of E2 [14]. One pathway for KNDy neurons to control energy balance is by directly depolarizing POMC neurons via kisspeptin and/or glutamate [15, 16].

Although the neuroendocrine mechanisms that link reproduction and energy balance are not well understood, it is known that ghrelin suppresses the frequency of pulsatile LH release in male and female rats as well as humans, in part, due to activation of β -endorphin (POMC) signaling [17–19]. Ghrelin is a brain-gut peptide hormone secreted from the stomach to stimulate food intake by acting on its receptor, growth hormone secreta-

gogue receptor (GHSR) expressed in the hypothalamus [20, 21]. GHSR is expressed throughout the brain particularly in NPY/AgRP neurons in the ARC, while only 10% of POMC neurons express GHSR [22, 23]. Furthermore, ghrelin activation of GHSR excites NPY neurons and controls calcium homeostasis [23, 24] by activating calcium channels [25, 26] and inhibiting the M-current, a hyperpolarizing, inwardly rectifying potassium channel current [26, 27]. Recently, we reported that E2 increases *Ghsr* expression in the ARC, but not in NPY neurons [26, 28]. While ghrelin actions on NPY neurons are well characterized, only a few studies have examined the actions of ghrelin on KNDy neurons. In one study, central ghrelin administration had no effect on ARC *Kiss1* expression in a fed or fasted state [29] and, in second study, ghrelin depolarized more KNDy neurons in E2-treated ovariectomized (OVX) females than in oil-treated females [29, 30]. These data suggest that direct effects of ghrelin on KNDy neuronal excitability, and not kisspeptin transcription, mediate the suppression of LH pulse frequency. Furthermore, we recently demonstrated that E2 increases *Ghsr* expression by 6-fold in KNDy (*Tac2*-GFP) neurons [30], which can potentially increase KNDy neuronal sensitivity to ghrelin.

The M-current is a subthreshold voltage-gated potassium current generated by KCNQ channels, which modulate firing frequency in neurons. KCNQ channels are expressed in numerous brain regions including the hippocampus, the cortex, and the hypothalamus [27]. Additionally, KCNQ channels are negatively modulated by Gq-coupled G-protein coupled receptors (GPCR), including GHSR. The binding of ghrelin to GHSR activates the alpha subunit, which activates the phospholipase C (PLC) signaling cascade. Once PLC becomes activated, it hydrolyzes the membrane phospholipid, phosphatidylinositol 4, 5-bisphosphate (PIP₂) for inositol triphosphate (IP₃), and diacylglycerol (DAG). IP₃ is released into the cytosol that goes on to increase intracellular calcium. Additionally, PLC activation of the membrane-associated DAG can activate protein kinase C (PKC), which then decreases KCNQ channel (or M-current) conductance [27, 31]. Our previous work has shown that the M-current can be modulated by fasting and 17 β -estradiol in NPY neurons of the hypothalamus [32]. In addition, activation of the 5-HT_{2c} receptors inhibits the M-current in POMC neurons of the hypothalamus [33]. Because KNDy neurons can directly depolarize POMC neurons via kisspeptin and glutamate [15, 16] and because fasting (increasing ghrelin) suppresses the M-current in NPY neurons [32], we speculate that KNDy neuronal ghrelin sen-

sitivity and the M-current play a significant role in the control of energy balance and reproduction.

Because E2, ghrelin, and KNDy neurons all control reproduction and energy homeostasis [14, 34–36], we hypothesize one potential mechanism could be that steroids (E2) increase ghrelin sensitivity in KNDy neurons by augmenting KCNQ channel conductance and *Ghsr* expression to modulate the control of reproduction and other hypothalamic pathways. During states of elevated E2 (proestrus or ovariectomy with E2 replacement), GHSR expression is increased in KNDy neurons, augmenting KNDy sensitivity to ghrelin. These transcriptional effects produce KNDy neurons more sensitive to states of elevated ghrelin (fasting, caloric restriction), increasing KNDy neuronal output, kisspeptin or glutamate release onto ARC POMC and NPY neurons [37]. POMC activation would increase the release of β -endorphin on GnRH neurons to suppress LH pulsatility [23]. To begin to elucidate this circuit and the interactions of E2 and ghrelin in KNDy neurons, we used whole-cell patch-clamp electrophysiology in a *Tac2*-GFP mouse model and determined the impact of E2 and ghrelin on M-current activity and excitatory postsynaptic currents.

Materials and Methods

Animals

All animal procedures were completed in compliance with institutional guidelines based on National Institutes of Health standards and were performed with Institutional Animal Care and Use Committee approval at Rutgers University. Adult mice (6–12 weeks of age) were housed under constant photoperiod conditions (12/12 h light/dark cycle), with lights on/off at 7.00 and 19.00 h and maintained at a controlled temperature (25°C). Animals were given food (LabDiet 5V75) and water ad libitum. Animals were weaned at postnatal day 21 (PD21). Sexually mature *Tac2*-EGFP females were used for all electrophysiology experiments. Genotype was determined by using PCR products of extracted DNA from ear clippings. *Tac2*-EGFP mice were generated by mating either positive EGFP males to positive EGFP females or positive EGFP males to WT EGFP females.

Surgical Procedure

To elucidate the interactions of E2 and the M-current, adult females were bilaterally OVX under isoflurane anesthesia using sterile no-touch technique according to the NIH Guidelines for Survival Rodent Surgery. Animals were given a dose of analgesic (4 mg/kg carprofen [Rimadyl®]) 1 day following surgery for pain management. Animals typically lost 1–2 g of weight 1 day after surgery. Following OVX, females were separated into 2 treatment groups – oil and estradiol benzoate (EB; $n = 8$ –10 cells, 7–8 mice per group). An EB injection protocol was used that has previously been shown to alter gene expression in the hypothalamus and mimic a proestrus state [13, 38]. Animals were injected subcutane-

ously at 10.00 h on post-OVX day 5 with either 0.25 μ g of EB or oil-vehicle. On post-OVX day 6, a 1.5 μ g dose of EB or oil was injected at 10.00 h. On post-OVX day 7, mice were rapidly decapitated, and the brain was prepared for electrophysiology.

Drugs

All drugs were purchased from Tocris unless otherwise specified. Tetrodotoxin (TTX, 1 mM stock), Ghrelin, a GHSR agonist, (100 mM stock), and the GHSR antagonist (D-Lys3)-GHRP-6 (100 mM stock) were all dissolved in ultrapure H₂O. The PKA inhibitor H-89 dihydrochloride (10 mM stock), the PKC inhibitor rottlerin (10 mM stock), the PLC inhibitor U73122 (20 mM stock), and 10, 10-bis (4-pyridinylmethyl)-9(10 h)-anthracenone dihydrochloride (XE-991), a KCNQ channel blocker (40 mM stock) were all dissolved in DMSO. Aliquots of the stock solutions were stored at –20°C until needed. EB was purchased from Steraloids (Newport, RI, USA) and dissolved in ethanol (1 mg/mL) prior to mixing in sesame oil (Sigma-Aldrich). Ethanol was allowed to evaporate off for 24 h prior to storage. EB was stored at 4°C until needed.

Tissue Preparation

Animals were rapidly decapitated on day 7 following OVX. The brain was quickly removed from the skull, and a block containing the hypothalamus was immediately dissected and submerged in cold (4°C), oxygenated (95% O₂, 5% CO₂), high-sucrose artificial cerebrospinal fluid (aCSF) consisting of 208 mM sucrose, 2 mM KCl, 26 mM NaHCO₃, 10 mM glucose, 1.25 mM NaH₂PO₄, 2 mM MgSO₄, 1 mM MgCl₂, 2 mM CaCl₂, and 1 mM HEPES (pH 7.3; 300 mOsm). Coronal slices (250 μ m) were cut on a vibratome at 4°C. The slices were transferred to an auxiliary chamber where they were kept at room temperature (25°C) in aCSF consisting of 124 mM NaCl, 5 mM KCl, 2.6 mM NaH₂PO₄, 2 mM MgCl₂, 2 mM CaCl₂, 26 mM NaHCO₃, and 10 mM glucose (pH 7.3; 310 mOsm) until recording (recovery for 1–2 h). A single slice was transferred to the recording chamber mounted on an Olympus BX51W1 upright microscope. The slice was continually perfused with warm (35°C), oxygenated aCSF at 1.5 mL/min and allowed to acclimate for 15 min prior to patching. Targeted neurons were viewed with an Olympus $\times 40$ water-immersion lens. Uteri were removed, and wet weight was recorded to confirm the effect of oil or EB treatment. Mean uterine weights from oil-treated females were 0.041 ± 0.0013 g, while EB-treated females were 0.15 ± 0.0043 g ($p < 0.0001$).

Electrophysiology Recordings

Electrophysiology was performed as described previously [32, 33, 39]. In hypothalamic slices, standard whole-cell patch-clamp recording procedures and pharmacological testing were used. Whole-cell patch-clamp recordings were performed using pipettes made of borosilicate glass and pulled using a vertical PC-10 Narishige Glass Micropipette Puller (Narishige Group, Japan). An Axopatch 700B amplifier, Digidata 1322A Data Acquisition System, and pCLAMP software (version 10.2; Molecular Devices) were used for data acquisition and analysis.

Current–voltage (I–V) plots were constructed by voltage steps from –50 to –140 mV at 10-mV increments applied at 1-s intervals from a holding potential of –60 mV. The input resistance (R_{in}) was determined from the slope of each I–V plot in the range between –60 and –80 mV. Input R_{in} , series R_{in} , and membrane capacitance were monitored throughout the experiments. Only cells with stable series R_{in} (<30 M Ω ; $<20\%$ change over the course of the re-

Fig. 1. **a** Representative current-clamp trace of the modest depolarization caused by 40 μM XE-991 in an oil-treated OVX female. **b** Representative trace of the depolarization caused by 100 nM Ghrelin in an intact, diestrus female. **c** M-current deactivation protocol. **d** I-V plot from -75 to -30 mV after 1 μM TTX for 0, 10, 20, and 30 min. After a time period of 20–30 min, the M-current ran down by $\sim 50\%$. All subsequent data were collected in under 20 min. Data were analyzed by 2-way ANOVA and post hoc Bonferroni's multiple comparison tests. (b = $p < 0.01$; c = $p < 0.001$; d = $p < 0.0001$). Dark blue letters indicate comparison between 10 and 30 min, light blue letters indicate a comparison between 10 and 20 min. $N = 7$ cells.

cording) and input R_{in} (>500 M Ω) were used for analysis. Low-pass filtering of the currents was conducted at a frequency of 2 kHz. The liquid junction potential was calculated to be -10 mV and corrected for during data analysis using pClamp software. All voltage-clamp recordings were performed from a holding potential of -60 mV.

To record M-currents, pipettes (3- to 5 M Ω R_{in}) were filled with an internal solution containing 10 mM NaCl, 128 mM K-glucuronate, 1 mM MgCl₂, 10 mM HEPES, 1 mM ATP, 1.1 mM EGTA,

and 0.25 mM GTP (pH 7.3; 300 mOsm). In voltage-clamp, a standard deactivation protocol [32, 33] was used to measure potassium currents elicited during 500-ms voltage steps from -30 to -75 mV in 5-mV increments after a 300-millisecond prepulse to -20 mV. The amplitude of the M-current relaxation or deactivation was measured as the difference between initial (<10 ms) and sustained current (>475 ms) of the current trace in the control conditions (1 μM TTX; 5 min). After baseline recording (~ 5 min), XE-991 (40 μM with 1 μM TTX) or ghrelin (100 nM with 1 μM TTX) was perfused for 10 min, and the protocol was repeated. Deactivation protocol was repeated twice for each perfusion condition and averaged for analysis.

The ghrelin dose response was recorded in current- and voltage-clamp, where continuous recording was monitored ~ 2 min before perfusion to establish a baseline. Ghrelin was perfused at a range of concentrations (0.1–1,000 nM; 3 concentrations per neuron for 5 min each in the presence of 1 μM TTX) to determine if there is a change in sensitivity to ghrelin (cells depolarize at lower concentrations of ghrelin). Miniature excitatory post-synaptic current (mEPSC) frequency and amplitude were analyzed from voltage-clamp recordings using pCLAMP software (version 10.2; Molecular Devices). Briefly, using event detection, for each voltage-clamp trace, a mEPSC template was chosen. Once a template was chosen, event detection was utilized to select mEPSCs, and each individual mEPSC was manually accepted or rejected. At the end of the trace, the total number of mEPSCs was used to determine frequency. To determine amplitude, mEPSCs were averaged, and the resulting trace was measured for amplitude. This process was repeated for each concentration of ghrelin.

To determine the second messenger pathway, the same M-current protocol was utilized where control conditions were 1 μM TTX + inhibitor for 5 min followed by 1 μM TTX + inhibitor + 100 nM ghrelin for 10 min. For this experiment, all Tac2-EGFP females were gonadally intact and in diestrus, determined by vaginal cytology the morning of the experiment. All uteri were dissected and weighed for confirmation.

Statistical Analysis

Comparisons of the I-V plots between control and subsequent drug conditions were performed at each voltage (-30 to -75 mV) using a repeated-measures 2-way ANOVA with post hoc Bonferroni's multiple comparison test. Maximum current at -35 mV was analyzed with paired Student t test. All statistical analyses were conducted using GraphPad Prism (GraphPad). Data were considered significant when $p < 0.05$. All data values were presented as mean $\pm$ SEM. Each n represents the number of cells examined.

Results

Rundown of the M-current

Because the KCNQ channel current (M-current) in KNDy neurons have not been previously studied, we initially established M-current activity using the selective KCNQ channel blocker XE-991, 40 μM a dose based on previous investigation [32, 39]. To establish efficacy, we performed current-clamp recordings from Tac2-EGFP

Table 1. RMP and input Rin from Figures 2 and 3 in both control (before) and after either XE-991 (40 μ M) or Ghrelin (100 nM) perfusion conditions

	Figure 2: control	XE-991 (40 μ M)	Figure 3: control	Ghrelin (100 nM)
Oil				
RMP (mV)	-56.4 ± 6.2	-40.2 ± 7.41	-58.22 ± 4.7	-47.67 ± 5.69
Rin (G Ω)	-0.76 ± 0.16	-0.46 ± 0.08	-1.42 ± 0.39	-1.70 ± 0.73
E2				
RMP (mV)	-54.82 ± 5.04	-46.82 ± 5.39	-52 ± 4.11	-46.33 ± 4.72
Rin (G Ω)	-0.93 ± 0.25	-0.37 ± 0.31	-1.57 ± 0.67	-0.82 ± 0.15
RMP, resting membrane potentials; Rin, resistance.				

neurons and applied XE-991 to the bath solution. This resulted in an average depolarization of 3.5 ± 0.86 mV ($n = 4$ cells), confirming that XE-991 was able to block KCNQ channels in these neurons (Fig. 1a), the average resting membrane potential (RMP) was -66.75 ± 1.31 mV. To explore whether ghrelin would do the same, we repeated the current-clamp recordings and applied ghrelin (100 nM) to the bath solution. This resulted in an average depolarization of 10.33 ± 4.63 mV ($n = 3$ cells), confirming that ghrelin does depolarize these neurons potentially through blocking KCNQ channels (Fig. 1b), the average RMP was -72.33 ± 2.03 mV. To evaluate the M-current in voltage-clamp, we measured the deactivation or relaxation of the whole-cell K^+ currents elicited by an established protocol [40, 41]. M-current was calculated by subtracting the current relaxation (the difference between the instantaneous and steady state; arrows; Fig. 1c) during continuous extracellular perfusion of TTX (1 μ M). Because the M-current has been previously reported to show a decrease or “rundown” after cell dialysis in whole-cell recordings in some neuronal cell types, but not all [32, 42], the whole-cell K^+ currents evoked by the deactivation protocol were monitored over a period of 30 min to determine the change in the relaxation currents over time. In Tac2 neurons from oil-treated, OVX females, the outward K^+ currents evoked decreased by approximately 50% over a period of 20 min ($p < 0.01$; Fig. 1d). Therefore, all subsequent recordings were restricted <20 min to limit the rundown and ensure that any changes observed were due to drug application [32].

Regulation of M-Current Activity by E2

To determine if estrogen replacement alters M-current activity in KNDy neurons, we examined the effects of XE-991 in Tac2 neurons from oil- and EB-treated OVX Tac2-GFP females. In the presence of TTX (1 μ M) to

block Na^+ -spike-dependent synaptic input, XE-991 (40 μ M) suppressed evoked currents in both oil- and EB-treated OVX females (Fig. 2a, b). XE-991 application resulted in an inhibition of the M-current in the range of -60 to -30 mV in both oil- and EB-treated females ($p < 0.05$; Fig. 2c, d). However, there was no difference between steroid conditions in the amount of XE-991-sensitive current (Fig. 2e). The change in maximum peak current at -35 mV after XE-991 perfusion was 75.7 ± 13.8 pA ($p < 0.001$, $n = 10$ cells, Fig. 2f) in oil-treated females and 68.0 ± 14.7 pA ($p < 0.001$, $n = 11$ cells, Fig. 2f) in EB-treated females. In addition, steroid condition did not alter the mean or change in RMP or Rin due to XE-991 perfusion (Fig. 2g, h). The RMP mean for oil-treated females before XE-991 was -56.4 ± 6.2 mV, after XE-991 was -40.2 ± 7.41 mV. The average change in RMP after XE-991 was 16.2 ± 5.9 mV. The RMP mean for EB-treated females before XE-991 was -54.82 ± 5.04 mV, after XE-991 was -46.82 ± 5.39 mV. The average change in RMP after XE-991 was 8.0 ± 3.43 mV. The Rin mean for oil-treated females before XE-991 was 0.76 ± 0.16 G Ω , after XE-991 was 0.46 ± 0.08 G Ω . The average change in Rin after XE-991 was a decrease of 0.31 ± 0.13 G Ω . The Rin mean for EB-treated females before XE-991 was 0.93 ± 0.25 G Ω , after XE-991 was 0.62 ± 0.16 G Ω . The average change in Rin after XE-991 was a decrease of 0.37 ± 0.31 G Ω . RMP and Rin values are summarized in Table 1.

Suppression of the M-Current by Ghrelin

Our previous data in arcuate NPY neurons indicate that the M-current is a target for the peptide hormone ghrelin. To ascertain if ghrelin also suppresses the M-current in Tac2 (KNDy) neurons, ghrelin (100 nM) was perfused for 10 min after an initial (control) deactivation protocol. Ghrelin inhibited the M-current in EB-treated, OVX females with a more robust effect compared to oil-

Fig. 2. Representative current traces of the M-current inhibition caused by 40 μ M XE-991 in (a) oil- and (b) EB-treated OVX female mice. I-V plot from -75 to -30 mV after XE-991 perfusion in (c) oil- and (d) EB-treated OVX female mice. e I-V plot from -75 to -30 mV of the XE-991-sensitive M-current in oil- and EB-treated OVX female mice. f XE-991 reduced the maximum peak current

in oil- and EB-treated mice. g, h The mean RMP and Input Rin. c-f Data were analyzed by 2-way ANOVA with Bonferroni's multiple comparison tests. g, h Data were analyzed by unpaired *t* test (a = *p* < 0.05; b = *p* < 0.01; c = *p* < 0.001; d = *p* < 0.0001). Sample sizes for c-h were oil *n* = 10 cells and EB *n* = 11 cells. EB, estradiol benzoate; RMP, resting membrane potential; Rin, resistance.

treated, OVX females (Fig. 3a-d, EB: [*F*(1, 16) = 9.690, *p* < 0.0067]), although steroid condition did not change the magnitude of M-current inhibition by ghrelin (Fig. 3e). The change in maximum peak current at -35 mV after ghrelin perfusion was 58.5 ± 15.8 pA (*p* < 0.05, *n* = 9 cells, Fig. 3f) in oil-treated females and 59.2 ± 11.9 pA (*p* < 0.01, *n* = 9 cells, Fig. 3f). In addition, steroid con-

dition did not alter the ghrelin-induced change in RMP or Rin (Fig. 3g, h). The RMP mean for oil-treated females before ghrelin was -58.22 ± 4.7 mV, after ghrelin was -47.67 ± 5.69 mV. The average change in RMP after ghrelin was 10.56 ± 3.17 mV. The RMP mean for EB-treated females before ghrelin was -52 ± 4.11 mV, after ghrelin was -46.33 ± 4.72 mV. The average change in RMP after

Fig. 3. Representative current traces of the M-current inhibition caused by 100 nM ghrelin in (a) oil- and (b) EB-treated OVX female mice. I-V plot from -75 to -30 mV after ghrelin perfusion in (c) oil- and (d) EB-treated OVX female mice. e I-V plot from -75 to -30 mV of the ghrelin-sensitive M-current in oil- and EB-treated OVX female mice. f Ghrelin reduced the maximum peak cur-

rent in oil- and EB-treated mice. g, h The mean RMP and Input Rin. c-f Data were analyzed by 2-way ANOVA with Bonferroni's multiple comparison tests. g, h Data were analyzed by unpaired *t* test (a = *p* < 0.05; b = *p* < 0.01; c = *p* < 0.001; d = *p* < 0.0001). Sample sizes for (c-h) were oil *n* = 9 cells and EB *n* = 9 cells. EB, estradiol benzoate; RMP, resting membrane potential; Rin, resistance.

ghrelin was 5.67 ± 1.82 mV. The Rin mean for oil-treated females before ghrelin was 1.42 ± 0.39 G Ω , after ghrelin was 1.70 ± 0.73 G Ω . The average change in Rin after ghrelin was an increase of 0.25 ± 0.77 G Ω . The Rin mean for EB-treated females before ghrelin was 1.57 ± 0.67 G Ω , after ghrelin was 0.82 ± 0.15 G Ω . The average change in Rin after ghrelin was a decrease of 0.75 ± 0.7 G Ω . RMP and Rin values are summarized in Table 1.

Ghrelin Dose Response and Tac2 Neuronal Activity

Because of our previous findings demonstrating that E2 increases GHSR expression in KNDy neurons [30], we hypothesize that E2 increases KNDy neuronal sensitivity to ghrelin. As we have established that ghrelin can inhibit the M-current more robustly with EB-treatment, we perfused ghrelin at increasing doses (0.01–1,000 nM) and monitored cell excitability and depolarization in current-

Fig. 4. Representative current-clamp traces of an estrogen-dependent increase in neuronal sensitivity to increasing doses of ghrelin (0.01–100 nM) in (a) oil- and (b) EB-treated OVX female mice. Average RMP for all cells before ghrelin application was -65 ± 6.2 mV (c) Table indicating the number of cells from voltage-clamp experiments that responded with either an inward current, outward current or no response. d Ghrelin dose response curve. Representative voltage-clamp traces of an estrogen-dependent de-

crease in mEPSC frequency in (e) oil- and (f) EB-treated OVX female mice. mEPSC frequency (g) and amplitude (h) before (baseline) and after 100 nM ghrelin. Data were analyzed by 2-way ANOVA with Holm-Sidak's multiple comparison tests. g, h were analyzed by unpaired *t* test ($a = p < 0.05$). Sample sizes are indicated in table (c). EB, estradiol benzoate; TTX, tetrodotoxin; mEPSC, miniature excitatory post-synaptic current.

and voltage-clamp, respectively. During current-clamp recordings, Tac2 neurons from EB-treated, OVX females responded to ghrelin at much lower concentrations eliciting action potential (AP) firing at lower doses compared to Tac2 neurons from oil-treated females (Fig. 4a, b). The average RMP for all cells before drug application was -65 ± 6.2 mV. However, not all neurons responded to ghrelin in the same way. In voltage-clamp, cells exhibited an in-

ward current, an outward current, or did not respond at all (Fig. 4c). Cells were considered responsive if they showed more than an 8% change in current from the maximum current observed. On average the inward currents for 100 nM ghrelin, the only dose where a difference was observed, in oil-treated females was -4.5 ± 0.88 pA, while EB-treated females were -11.43 ± 2.8 pA ($p = 0.0503$). Because there was not an outward current for

Fig. 5. I–V plots from -75 to -30 mV before (black) and after (green) ghrelin (100 nM) perfusion in diestrus *Tac2* female mice with GHSR antagonist, (D-Lys³)-GHRP-6 (50 μ M) $n = 5$ cells (a), PLC inhibitor, U73122 (10 μ M) $n = 6$ cells (b), PKC inhibitor, Rottlerin (5 μ M) $n = 6$ cells (c), and PKA inhibitor H89 (10 μ M) $n = 7$ cells (d). Data were analyzed by 2-way ANOVA with Bonferroni's multiple comparison tests. TTX, tetrodotoxin; GHSR, growth hormone secretagogue receptor.

every dose of ghrelin, the average outward currents across all ghrelin doses from oil-treated females were 4.2 ± 1.62 pA, while EB-treated females were 7.86 ± 2.19 pA. In oil-treated females, 54 cells responded to ghrelin with an inward current, 5 responded with an outward current, and 14 had no response to ghrelin. In EB-treated females, 66 cells responded to ghrelin with an inward current, 7 responded with an outward current, and 10 had no response to ghrelin. Among the cells that responded with an inward current, ghrelin perfusion (100 nM) elicited a more robust current in EB-treated females compared to oil-treated females ($p < 0.05$; Fig. 4d). Interestingly, perfusion of $1,000$ nM ghrelin in EB-treated females did not elicit a stronger current, potentially due to desensitization of GHSR [43]. During our voltage-clamp recordings (Fig. 4e, f), we observed a potential effect of steroid on mEPSC. Analysis of these traces revealed a reduction of mEPSC frequency, but not amplitude, in EB-treated females compared to oil-treated ($p < 0.05$; Fig. 4g, h), indicating an interaction of E2 on presynaptic glutamate release.

Ghrelin Signals through a PLC-Mediated Pathway

Previous publications have established KCNQ channels are negatively modulated by Gq-coupled GPCR, including GHSR [27]. The binding of ghrelin to GHSR activates a PLC signaling cascade that hydrolyzes PIP₂ for IP₃ and DAG. IP₃ is released into the cytosol and increases release of intracellular calcium from internal stores. DAG activates PKC that then either directly decreases KCNQ channel (M-current) conductance [27, 31] or activates PKA [39]. To confirm the role of this pathway (PLC-PKC) in attenuating the M-current in *Tac2* neurons, we applied a series of specific pharmacological interventions. First, to confirm the role of GHSR, we co-perfused a specific GHSR antagonist (D-Lys³)-GHRP-6 (50 μ M) with ghrelin (100 nM) and TTX (1 μ M; Fig. 5a). The GHSR antagonist eliminated the effect of ghrelin on the M-current, suggesting the M-current inhibition by ghrelin is indeed mediated by GHSR. Next, we inhibited PLC with U73122 (10 μ M) and the PKC inhibitor, rottlerin (5 μ M) in separate experiments (Fig. 5b, c). Both U73122 and rottlerin blocked the actions of ghrelin, confirming previous reports. GHSR signaling also may acti-

vate PKA as a second messenger [25], which blocks Gq-GPCR inhibition of the M-current in hypothalamic neurons [39]. Using H89, the selective PKA inhibitor (10 μ M) observed a reduction of the ghrelin-induced suppression of the M-current (Fig. 5d), indicating that PKA may play a role in Tac2 neurons.

Discussion

The results of the present study confirm previous findings that 17 β -estradiol upregulates *Ghsr* expression [30], increases KNDy neuronal sensitivity to ghrelin, and subsequently enhances the ghrelin-induced inhibition of the M-current via a PLC-PKA pathway in ARC KNDy neurons. These conclusions are based on the following observations: (1) 100 nM Ghrelin was effective in inhibiting the M-current similar to XE-991, the selective KCNQ channel inhibitor; (2) ghrelin was able to elicit APs at a lower dose in EB-treated females; (3) there was a more robust inward current in response to ghrelin in EB-treated females compared to oil-treated controls; and (4) blocking the PLC-PKA pathway blocked the M-current inhibition by ghrelin.

The 17 β -estradiol-induced increase in KNDy sensitivity to ghrelin is consistent with previous findings [30] and confirmed with our electrophysiological assessment. Additionally, we have confirmed and corroborated that ghrelin binds to GHSR, a Gq-coupled GPCR, activating PLC to hydrolyze PIP₂ for IP₃ and DAG. DAG in turn activates PKC that decreases KCNQ channel conductance (M-current inhibition) and this results in a more depolarized and excitable cell [27, 31]. Ghrelin inhibition of KCNQ channels in KNDy neurons is similar to our previous research on the M-current in other ARC neurons [32, 33, 44]. This is further supported by the identification of the second messenger pathway (PLC-PKC) in which ghrelin has previously been described to inhibit the M-current [27].

The current study is also an initial characterization of the M-current in KNDy neurons as XE-991 substantially blocked the current and depolarized all KNDy neurons examined. Interestingly, we found that the M-current in ARC KNDy neurons rundown in <20 min, which has not been observed in other ARC neurons, like NPY or POMC [32]. This could be due to cell dialysis during whole-cell patch clamp recordings over time [42] or differences in intracellular signaling that impinges on KCNQ activity [42, 45, 46]. Because the M-current is a common target for Gq-coupled GPCR such as 5HT_{2c} serotonergic and

M_{1/5} muscarinic receptors in many types of hypothalamic neurons [33], robust M-current activity in KNDy neurons may be a key, although not the only mechanism for modulation of KNDy activity.

We have also observed that with EB treatment, lower doses of ghrelin-induced APs and at higher doses of ghrelin exhibited reduced APs, possibly due to overexcitation or internalization of the receptor, a common mechanism for Gq-coupled GPCRs [43]. The EB-induced effects on ghrelin sensitivity are clearly indicated at the 100 nM dose of ghrelin where an increase in the percent maximum inward current is augmented in EB-treated females. Novaira et al. [47] found that not all *Tac2* neurons are active and some are quiescent. Furthermore, not all *Tac2* neurons express detectable levels of GHSR [30]. Thus, a significant subpopulation of KNDy neurons responds to ghrelin and in sufficient numbers to elicit known physiological control of the HPG axis [36, 48].

Last, we observed that EB treatment reduces mEPSC frequency, but not the amplitude, indicating that there is a decrease in the probability of release from presynaptic neurons. This could be due to a reduction in glutamate signaling from neurons that project to KNDy neurons, including other KNDy neurons. Previous studies have demonstrated that ARC KNDy neurons exhibited increased glutamatergic transmission when ER α was knocked down in these neurons [49], which support our study with proestrous levels of E2. Furthermore, it is critical to consider that high E2 inhibits neuropeptide expression in these neurons [30], and EB treatment can increase negative feedback via dynorphin in these neurons, ultimately reducing excitability. It is important to note that we only saw a reduction in mEPSC with the 100 nM dose. As mEPSC amplitude did not change, we expect that there is no change in the expression of NMDA or AMPA receptors in KNDy neurons due to E2 [50].

While the effects of E2 and ghrelin in ARC KNDy neurons have become more defined, it is critical to keep in mind that KNDy neurons are one node of a large circuit within the ARC. KNDy neurons not only project locally to POMC and NPY neurons but also to preoptic GnRH and kisspeptin neurons of the AVPV, playing an important role in puberty and ovarian functions [37, 47]. KNDy neurons activate POMC neurons via kisspeptin and glutamate release and inhibit NPY neurons directly via glutamate and indirectly through kisspeptin [16, 37]. As all 3 neurons modulate the excitability of the other 2 neuronal populations and respond differently to peripheral peptide hormones, any modulation of one neuronal population by a hormone will have disparate effects on the other 2 popu-

lations. Therefore, we hypothesize that ghrelin's excitation of KNDy neurons and its potentiation by E2 would reduce ghrelin-induced food intake via NPY neurons in female mice due to the excitation of POMC and the inhibition of NPY. Our hypothesis is supported by previous data demonstrating that E2 suppresses ghrelin-induced food intake in female rats [51]. This pathway may be sensitive to energy states, both positive (obesity) and negative (fasting/caloric restriction), as it is known that obesity can cause ghrelin Rin in NPY/AgRP neurons [52] and a high fat diet can reduce the amount of plasma ghrelin in females [26]. We are currently exploring the effects of diet-induced obesity on ghrelin's actions in KNDy neurons.

Furthermore, as ghrelin administration suppresses LH pulsatility through β -endorphin produced by POMC neurons in fed female rats [18, 29], ghrelin activation of KNDy neurons may be involved, as GHSR is not highly expressed in POMC neurons [23]. Indeed, KNDy neurons can excite POMC neurons via glutamate during states of elevated E2 [37], which may lead to greater β -endorphin release onto GnRH terminals, inhibiting LH pulsatility. Hence, during states of elevated ghrelin (fasting, food scarcity, etc.), the HPG axis would be suppressed diverting motivation toward food intake and away from reproduction. Because of this, the role of ghrelin signaling in KNDy neurons may prove to be relevant in the control of reproduction, energy homeostasis, and other physiological processes controlled by KNDy neurons. Future experiments will explore if androgens or E2 in males also regulate *Ghsr* expression in KNDy neurons and if ghrelin activation of KNDy neurons alters the downstream modulation of POMC or NPY neurons utilizing additional transgenic mouse models.

In conclusion, during the normal reproductive cycle, E2 fluctuates to control the growth of ovarian follicles via negative feedback regulated by ARC KNDy neurons. We have shown that when E2 is high (proestrus or OVX + E2), *Ghsr* expression is increased in KNDy neurons [30]

to augment their sensitivity to states of elevated ghrelin (starvation/caloric restriction) to suppress the HPG axis. Ghrelin, in turn, suppressed the M-current with greater potency leading to depolarization and neurotransmitter (glutamate) release to potentially suppress the HPG axis during periods of food scarcity in females.

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Statement of Ethics

The research presented in the manuscript was ethically conducted in accordance with institutional guidelines based on National Institutes of Health standards, and all animal experiments were performed with Institutional Animal Care and Use Committee approval at Rutgers University.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

K.C. and T.A.R. conceived and designed the project. K.C. performed the experiments. K.C. and T.A.R. analyzed and interpreted data and drafted the manuscript.

References

- 1 Allison DB, Wang C, Redden DT, Westfall AO, Fontaine KR. Obesity and Years of Life Lost. *JAMA*. 2003;289(14):1777.
- 2 Moran LJ, Dodd J, Nisenblatt V, Norman RJ. Obesity and reproductive dysfunction in women. *Endocrinol Metab Clin North Am*. 2011 Dec;40(4):895–906.
- 3 Clark AM, Thornley B, Tomlinson L, Galletley C, Norman RJ. Weight loss in obese infertile women results in improvement in reproductive outcome for all forms of fertility treatment. *Hum Reprod*. 1998 Jun;13(6):1502–5.
- 4 Sundgot-Borgen J. Risk and trigger factors for the development of eating disorders in female elite athletes. *Med Sci Sports Exerc*. 1994 Apr; 26(4):414–9.
- 5 Brock O, Bakker J. The two kisspeptin neuronal populations are differentially organized and activated by estradiol in mice. *Endocrinology*. 2013 Aug;154(8):2739–49.
- 6 Navarro VM, Castellano JM, McConkey SM, Pineda R, Ruiz-Pino F, Pinilla L, et al. Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. *Am J Physiol Endocrinol Metab*. 2011 Jan; 300(1):E202–10.

- 7 Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. *Endocr Rev*. 2009 Oct;30(6):713–43.
- 8 de Croft S, Boehm U, Herbison AE. Neurokinin B activates arcuate kisspeptin neurons through multiple tachykinin receptors in the male mouse. *Endocrinology*. 2013 Aug;154(8):2750–60.
- 9 Ruka KA, Burger LL, Moenter SM. Regulation of arcuate neurons coexpressing kisspeptin, neurokinin B, and dynorphin by modulators of neurokinin 3 and κ -opioid receptors in adult male mice. *Endocrinology*. 2013 Aug;154(8):2761–71.
- 10 Uenoyama Y, Tsukamura H, Maeda K. KNDy neuron as a gatekeeper of puberty onset. *J Obstet Gynaecol Res*. 2014 Jun;40(6):1518–26.
- 11 Weems PW, Witty CF, Amstalden M, Coolen LM, Goodman RL, Lehman MN. κ -opioid receptor is colocalized in GnRH and KNDy cells in the female ovine and rat brain. *Endocrinology*. 2016 Jun;157(6):2367–79.
- 12 Goodman RL, Hileman SM, Nestor CC, Porter KL, Connors JM, Hardy SL, et al. Kisspeptin, neurokinin B, and dynorphin act in the arcuate nucleus to control activity of the GnRH pulse generator in ewes. *Endocrinology*. 2013 Nov;154(11):4259–69.
- 13 Bosch MA, Tonsfeldt KJ, Rønnekleiv OK. mRNA expression of ion channels in GnRH neurons: subtype-specific regulation by 17β -estradiol. *Mol Cell Endocrinol*. 2013 Mar;367(1-2):85–97.
- 14 Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, Lai J, Ciofi P, McMullen NT, et al. Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. *Endocrinology*. 2012 Jun;153(6):2800–12.
- 15 Nestor CC, Qiu J, Padilla SL, Zhang C, Bosch MA, Fan W, et al. Optogenetic stimulation of arcuate nucleus kiss1 neurons reveals a steroid-dependent glutamatergic input to POMC and AgRP neurons in male mice. *Mol Endocrinol*. 2016 Jun;30(6):630–44.
- 16 Fu LY, van den Pol AN. Kisspeptin directly excites anorexigenic proopiomelanocortin neurons but inhibits orexigenic neuropeptide Y cells by an indirect synaptic mechanism. *J Neurosci*. 2010 Jul;30(30):10205–19.
- 17 Fernández-Fernández R, Tena-Sempere M, Navarro VM, Barreiro ML, Castellano JM, Aguilar E, et al. Effects of ghrelin upon gonadotropin-releasing hormone and gonadotropin secretion in adult female rats: in vivo and in vitro studies. *Neuroendocrinology*. 2005;82(5-6):245–55.
- 18 Fernández-Fernández R, Tena-Sempere M, Aguilar E, Pinilla L. Ghrelin effects on gonadotropin secretion in male and female rats. *Neurosci Lett*. 2004 May;362(2):103–7.
- 19 Messini CI, Dafopoulos K, Malandri M, Georgoulas P, Anifandis G, Messinis IE. Inhibitory effect of submaximal doses of ghrelin on gonadotropin secretion in women. *Horm Metab Res*. 2014 Jan;46(1):36–40.
- 20 Ma X, Lin L, Qin G, Lu X, Fiorotto M, Dixit VD, et al. Ablations of ghrelin and ghrelin receptor exhibit differential metabolic phenotypes and thermogenic capacity during aging. *PLoS One*. 2011 Jan;6(1):e16391.
- 21 Sun Y, Butte NF, Garcia JM, Smith RG. Characterization of adult ghrelin and ghrelin receptor knockout mice under positive and negative energy balance. *Endocrinology*. 2008 Feb;149(2):843–50.
- 22 Willesen MG, Kristensen P, Rømer J. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology*. 1999 Nov;70(5):306–16.
- 23 Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL, et al. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron*. 2003 Feb;37(4):649–61.
- 24 Andrews ZB, Liu ZW, Wallingford N, Erion DM, Borok E, Friedman JM, et al. UCP2 mediates ghrelin's action on NPY/AgRP neurons by lowering free radicals. *Nature*. 2008 Aug;454(7206):846–51.
- 25 Kohno D, Gao HZ, Muroya S, Kikuyama S, Yada T. Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca^{2+} signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes*. 2003 Apr;52(4):948–56.
- 26 Yasrebi A, Hsieh A, Mamounis KJ, Krumm EA, Yang JA, Magby J, et al. Differential gene regulation of GHSR signaling pathway in the arcuate nucleus and NPY neurons by fasting, diet-induced obesity, and 17β -estradiol. *Mol Cell Endocrinol*. 2016 Feb;422:42–56.
- 27 Shi L, Bian X, Qu Z, Ma Z, Zhou Y, Wang K, et al. Peptide hormone ghrelin enhances neuronal excitability by inhibition of Kv7/KCNQ channels. *Nat Commun*. 2013;4(2):1435.
- 28 Frazao R, Dungan Lemko HM, da Silva RP, Ratra DV, Lee CE, Williams KW, et al. Estradiol modulates Kiss1 neuronal response to ghrelin. *Am J Physiol Endocrinol Metab*. 2014 Mar;306(6):E606–14.
- 29 Forbes S, Li XF, Kinsey-Jones J, O'Byrne K. Effects of ghrelin on Kisspeptin mRNA expression in the hypothalamic medial preoptic area and pulsatile luteinizing hormone secretion in the female rat. *Neurosci Lett*. 2009 Aug;460(2):143–7.
- 30 Yang JA, Yasrebi A, Snyder M, Roepke TA. The interaction of fasting, caloric restriction, and diet-induced obesity with 17β -estradiol on the expression of KNDy neuropeptides and their receptors in the female mouse. *Mol Cell Endocrinol*. 2016 Dec;437:35–50.
- 31 Zhang H, Craciun LC, Mirshahi T, Rohács T, Lopes CM, Jin T, et al. PIP(2) activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron*. 2003 Mar;37(6):963–75.
- 32 Roepke TA, Qiu J, Smith AW, Rønnekleiv OK, Kelly MJ. Fasting and 17β -estradiol differentially modulate the M-current in neuropeptide Y neurons. *J Neurosci*. 2011 Aug;31(33):11825–35.
- 33 Roepke TA, Smith AW, Rønnekleiv OK, Kelly MJ. Serotonin 5-HT_{2C} receptor-mediated inhibition of the M-current in hypothalamic POMC neurons. *Am J Physiol Endocrinol Metab*. 2012 Jun;302(11):E1399–406.
- 34 Qiu J, Nestor CC, Zhang C, Padilla SL, Palmiter RD, Kelly MJ, et al. High-frequency stimulation-induced peptide release synchronizes arcuate kisspeptin neurons and excites GnRH neurons. *eLife*. 2016 Aug;5:e16246.
- 35 De Bond JP, Tolson KP, Nasamran C, Kauffman AS, Smith JT. Unaltered Hypothalamic Metabolic Gene Expression in Kiss1r Knock-out Mice Despite Obesity and Reduced Energy Expenditure. *J Neuroendocrinol*. 2016 Oct;28(10).
- 36 Andrews ZB. Central mechanisms involved in the orexigenic actions of ghrelin. *Peptides*. 2011 Nov;32(11):2248–55.
- 37 Qiu J, Rivera HM, Bosch MA, Padilla SL, Stincic TL, Palmiter RD, et al. Estrogenic-dependent glutamatergic neurotransmission from kisspeptin neurons governs feeding circuits in females. *eLife*. 2018 Aug;7:e35656.
- 38 Yang JA, Mamounis KJ, Yasrebi A, Roepke TA. Regulation of gene expression by 17β -estradiol in the arcuate nucleus of the mouse through ERE-dependent and ERE-independent mechanisms. *Steroids*. 2016 Mar;107:128–38.
- 39 Hu P, Liu J, Yasrebi A, Gotthardt JD, Bello NT, Pang ZP, et al. Gq Protein-Coupled Membrane-Initiated Estrogen Signaling Rapidly Excites Corticotropin-Releasing Hormone Neurons in the Hypothalamic Paraventricular Nucleus in Female Mice. *Endocrinology*. 2016 Sep;157(9):3604–20.
- 40 Schweitzer P. Cannabinoids decrease the K(+) M-current in hippocampal CA1 neurons. *J Neurosci*. 2000 Jan;20(1):51–8.
- 41 Xu C, Roepke TA, Zhang C, Rønnekleiv OK, Kelly MJ. Gonadotropin-Releasing Hormone (GnRH) Activates the M-Current in GnRH Neurons: An Autoregulatory Negative Feedback Mechanism? *Endocrinology*. 2008 May;149(5):2459–66.
- 42 Shen W, Hamilton SE, Nathanson NM, Surmeier DJ. Cholinergic suppression of KCNQ channel currents enhances excitability of striatal medium spiny neurons. *J Neurosci*. 2005 Aug;25(32):7449–58.
- 43 Yin Y, Li Y, Zhang W. The growth hormone secretagogue receptor: its intracellular signaling and regulation. *Int J Mol Sci*. 2014 Mar;15(3):4837–55.
- 44 Xu C, Roepke TA, Zhang C, Rønnekleiv OK, Kelly MJ. Gonadotropin-releasing hormone (GnRH) activates the m-current in GnRH neurons: an autoregulatory negative feedback mechanism? *Endocrinology*. 2008 May;149(5):2459–66.

- 45 Delmas P, Brown DA. Pathways modulating neural KCNQ/M (Kv7) potassium channels. [Nat Rev Neurosci](#). 2005 Nov;6(11):850–62.
- 46 Robbins J. KCNQ potassium channels: physiology, pathophysiology, and pharmacology. [Pharmacol Ther](#). 2001 Apr;90(1):1–19.
- 47 Novaira HJ, Sonko ML, Hoffman G, Koo Y, Ko C, Wolfe A, et al. Disrupted kisspeptin signaling in GnRH neurons leads to hypogonadotropic hypogonadism. [Mol Endocrinol](#). 2014 Feb;28(2):225–38.
- 48 Ogata R, Matsuzaki T, Iwasa T, Kiyokawa M, Tanaka N, Kuwahara A, et al. Hypothalamic Ghrelin suppresses pulsatile secretion of luteinizing hormone via β -endorphin in ovariectomized rats. [Neuroendocrinology](#). 2009; 90(4):364–70.
- 49 Wang L, Vanacker C, Burger LL, Barnes T, Shah YM, Myers MG, et al. Genetic dissection of the different roles of hypothalamic kisspeptin neurons in regulating female reproduction. [eLife](#). 2019 Apr;8:e43999.
- 50 Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB. Activity-dependent scaling of quantal amplitude in neocortical neurons. [Nature](#). 1998 Feb;391(6670):892–6.
- 51 Clegg DJ, Brown LM, Zigman JM, Kemp CJ, Strader AD, Benoit SC, et al. Estradiol-dependent decrease in the orexigenic potency of ghrelin in female rats. [Diabetes](#). 2007 Apr; 56(4):1051–8.
- 52 Briggs DI, Enriori PJ, Lemus MB, Cowley MA, Andrews ZB. Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. [Endocrinology](#). 2010 Oct; 151(10):4745–55.