Original Article

MOLECULAR METABOLISM

17β-estradiol promotes acute refeeding in hungry mice via membrane-initiated ERα signaling

Kaifan Yu [1,2,6], Yanlin He [1,5,6], Ilirjana Hyseni [1], Zhou Pei [1], Yongjie Yang [1], Pingwen Xu [1], Xing Cai [1], Hesong Liu [1], Na Qu [1], Hailan Liu [1], Yang He [1], Meng Yu [1], Chen Liang [1], Tingting Yang [1], Julia Wang [1], Pierre Gourdy [3], Jean-Francois Arnal [3], Francoise Lenfant [3], Yong Xu [1,4,], Chunmei Wang [1,*]**

ABSTRACT

Objective: Estrogen protects animals from obesity through estrogen receptor α (ERα), partially by inhibiting overeating in animals fed ad libitum. However, the effects of estrogen on feeding behavior in hungry animals remain unclear. In this study, we examined the roles of 17β-estradiol (E2) and ERα in the regulation of feeding in hungry female animals and explored the underlying mechanisms.

Methods: Wild-type female mice with surgical depletion of endogenous estrogens were used to examine the effects of E2 supplementation on acute refeeding behavior after starvation. ERα-C451A mutant mice deficient in membrane-bound ERα activity and ERα-AF2^0 mutant mice lacking ERα transcriptional activity were used to further examine mechanisms underlying acute feeding triggered by either fasting or central glucopenia (induced by intracerebroventricular injections of 2-deoxy-D-glucose). We also used electrophysiology to explore the impact of these ERα mutations on the neural activities of ERα neurons in the hypothalamus.

Results: In the wild-type female mice, ovariectomy reduced fasting-induced refeeding, which was restored by E2 supplementation. The ERα-C451A mutation, but not the ERα-AF2^0 mutation, attenuated acute feeding induced by either fasting or central glucopenia. The ERα-C451A mutation consistently impaired the neural responses of hypothalamic ERα neurons to hypoglycemia.

Conclusion: In addition to previous evidence that estrogen reduces deviations in energy balance by inhibiting eating at a satiated state, our findings demonstrate the unexpected role of E2 that promotes eating in hungry mice, also contributing to the stability of energy homeostasis. This latter effect specifically requires membrane-bound ERα activity.

Keywords E2; Feeding; ERα; Hypothalamus; Glucose-sensing

1. INTRODUCTION

17β-estradiol (E2) plays an essential role in the regulation of energy homeostasis in females. Depletion of endogenous estrogen by ovariectomy (OVX) in female animals leads to increased food intake and body weight gain, which can be prevented by E2 treatment [1–6]. The anti-obesity effect of E2 is largely mediated by ERα [7–9], as mice with whole-body ERα knockout develop obesity [3,10–15]. We and others demonstrated that ERα expressed by hypothalamic neurons, including in the arcuate nucleus (ARH) and ventrolateral subdivision of the ventromedial hypothalamic nucleus (vlVMH), is essential to mediate estrogenic actions to prevent body weight gain in females [16–19]. Notably, the ARH and vlVMH are also enriched with glucose-sensing neurons [20–25], which play important roles in energy homeostasis [20,21,25–28]. In particular, we recently found that ERα neurons in the vlVMH (ERα^{vlVMH} neurons) have strong glucose-sensing capability and can maintain normal glucose balance in female mice [29].

As a classic nuclear receptor, ERα can function in the nucleus to regulate gene transcription. ERα regulates gene transcription through two activation functions (ERα-AF1 and ERα-AF2). AF-1 is located in the N-terminal and AF-2 in the C-terminal portion of ERα. Both ERα-AF1^0 mutant mice [30,31] and ERα-AF2^0 mutant mice [32–34] were generated to ablate the transcriptional activity of ERα, but only the ERα-AF2^0 mutant mice recaptured the obese phenotype of whole-body

[1]USDA/ARS Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, One Baylor Plaza, Houston, TX, 77030, USA [2]College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu, 210095, China [3]I2MC, Inserm U1048, CHU de Toulouse and Université de Toulouse III, Toulouse, France [4]Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX, 77030, USA [5]Pennington Biomedical Research Center, Brain Glycemic and Metabolism Control Department, Louisiana State University, Baton Rouge, LA, 70808, USA

[6] Kaifan Yu and Yanlin He contributed equally.

*Corresponding authors. E-mail: chunmeiw@bcm.edu (C. Wang).

**Corresponding author. USDA/ARS Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, One Baylor Plaza, 1100 Bates Street #8066, Houston, TX 77030, USA. Tel.: +(713) 798 719. Fax: +(713) 798 7187. E-mail: yongx@bcm.edu (Y. Xu).

Received May 9, 2020 • Revision received July 17, 2020 • Accepted July 19, 2020 • Available online 23 July 2020

https://doi.org/10.1016/j.molmet.2020.101053

ERα knockout mice [35]. In addition to the transcriptional activity, a sub-population of ERα molecules was reported to be extra-nuclear, essentially bound to the cytomembrane in several types of cultured cells, and is able to initiate rapid actions within seconds or minutes [36]. More recently, we and others [37,38] reported that the point mutation at the palmitoylation site of ERα (C451A-ERα) in mice specifically abolished membrane-bound ERα activity, while the transcriptional activity of ERα was preserved.

Most previous studies focused on the role of E2 in regulating feeding behavior in animals under ad libitum feeding conditions. However, the role of E2 in hungry animals is not well understood. In this study, we first used OVX female mice with or without E2 supplement to examine the effects of endogenous E2 on feeding behavior in hungry female mice. Furthermore, mice carrying ERα-C451A or ERα-AF2^0 mutations were used to examine the roles of membrane-bound ERα vs the transcriptional activity of ERα in feeding control in hungry mice. We also explored the impact of these mutations on neural activity of ERα neurons in the ARH and vlVMH.

2. METHODS

2.1. Mice

ERα-C451A or ERα-AF2^0 heterozygous mice [38] were crossed with ERα-C451A or ERα-AF2^0 heterozygous mice, respectively, to generate ERα-C451A homozygous or ERα-AF2^0 homozygous mice as mutant groups. Mice heterozygous for ERα-C451A or ERα-AF2^0 showed comparable phenotypes as their respective wild-type littermates and therefore were pooled as control groups. The mouse ERα-ZsGreen transgene [39] was introduced to these crosses to label ERα neurons with ZsGreen. C57BL/6J female mice were purchased from the local mouse facility at Baylor College of Medicine. Female mice were used throughout this study.

Care of all of the animals and the procedures were approved by Baylor College of Medicine's Institutional Animal Care and Use Committees. The mice were housed in a temperature-controlled environment in groups of two to four at 22–24 °C using a 12-h light and 12-h dark cycle. The mice were fed a standard chow diet (Cat# 2920, Harlan Teklad) and water was provided ad libitum.

2.2. OVX surgery

C57BL/6J female mice (16 weeks of age) were anesthetized with inhaled isoflurane. As previously described, bilateral OVX or sham surgeries were performed [17,40,41]. One group of these OVX mice received subcutaneous (s.c.) injections of E2 (E8875, Sigma; 2 μg in 50 μL of sesame oil, OVX-E) every 4 days; the other group of OVX and sham mice received vehicle (50 μL of sesame oil, OVX-V, or sham) every 4 days for 80 days.

2.3. Ad libitum feeding and fasting-induced refeeding

The sham, OVX-V, and OVX-E mice were fed ad libitum for 10 weeks after surgery. After a scheduled s. c. injection of vehicle or E2, the body weight and food intake were measured daily for continuous 3 days. The mice were then fasted from the night before their next scheduled s. c. injection. The next morning, the mice received s. c. injection of sesame oil (sham and OVX-V) or E2 (OVX-E) at 9:30 am. Food was provided at 11:30 am and food intake was measured after 2 h and again at 24 h time points. Similarly, ERα-C451A mutant mice, ERα-AF2^0 mutant mice, and their respective littermate controls (16 weeks of age) were fasted overnight. Food was provided at 11:30 am the next morning, and food intake was measured for 2 h.

2.4. Intracerebroventricular injections

Female ERα-C451A mutant mice, ERα-AF2^0 mutant mice, and their respective littermate controls (16 weeks of age) were anesthetized with inhaled isoflurane, and stainless steel cannulas (Plastics One) were inserted into the lateral ventricles (0.34 mm caudal and 1 mm lateral from the bregma; 2.3 mm depth) to establish intracerebroventricular (ICV) cannulation [42,43]. One week after surgery, ICV cannulation was confirmed by demonstration of increased drinking and grooming behavior within 5 min after administration of 10 ng angiotensin II (A9525, Sigma). Four weeks after the surgeries, the mice were briefly fasted for 2 h from 9:30 am in the morning to empty their stomach. At 11:30 am, the mice received ICV injection of saline or 2-DG (1 mg in 2 μL of saline), and food was provided. Food intake was measured at 15, 30, 60, and 120 min after the injections.

2.5. Electrophysiological recordings

Female ERα-ZsGreen, ERα-ZsGreen/ERα-C451A mutant, and ERα-ZsGreen/ERα-AF2^0 mutant mice (12 weeks of age) were used for electrophysiological recordings. The mice were deeply anesthetized with isoflurane and transcardially perfused with a modified ice-cold sucrose-based cutting solution (pH 7.3) containing 10 mM NaCl, 25 mM $NaHCO_3$, 195 mM sucrose, 5 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM Na-pyruvate, 0.5 mM $CaCl_2$, and 7 mM $MgCl_2$ bubbled continuously with 95% O_2 and 5% CO_2 [29]. The mice were then decapitated, and the entire brain was removed and immediately submerged in cutting solution. Slices (250 μm) were cut with a Microm HM 650 V vibratome (Thermo Fisher Scientific). Three brain slices containing the ARH and vlVMH were obtained from each animal (bregma −2.06 mm to −1.46 mm; interaural 1.74 mm–2.34 mm). The slices were recovered for 1 h at 34 °C and then maintained at room temperature in artificial cerebrospinal fluid (aCSF, pH 7.3) containing 126 mM NaCl, 2.5 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 5.0 mM glucose, and 21.4 mM $NaHCO_3$ saturated with 95% O_2 and 5% CO_2 before recording.

The slices were transferred to a recording chamber and allowed to equilibrate for at least 10 min before recording. The slices were superfused at 34 °C in oxygenated aCSF at a flow rate of 1.8–2 mL/min. ZsGreen-labeled neurons in the ARH and vlVMH were visualized using epifluorescence and IR-DIC imaging on an upright microscope (Eclipse FN-1, Nikon) equipped with a movable stage (MP-285, Sutter Instruments). Patch pipettes with resistances of 3–5 MΩ were filled with intracellular solution (pH 7.3) containing 128 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 0.1 mM EGTA, 2 mM $MgCl_2$, 0.05 mM Na-GTP, and 0.05 mM Mg-ATP. Recordings were produced using a MultiClamp 700B amplifier (Axon Instruments), sampled using Digidata 1440A, and analyzed offline with pClamp 10.3 software (Axon Instruments). Series resistance was monitored during the recording, and the values were generally $<$10 MΩ and were not compensated. Data were excluded if the series resistance increased dramatically during the experiment or without overshoot for action potential. Currents were amplified, filtered at 1 kHz, and digitized at 20 kHz. To examine the acute neural responses to propyl pyrazole triol (PPT), a selective ERα agonist [44], the current clamp was engaged to measure the neural firing rate before and after a 500 ms puff of 100 nM PPT.

To examine the glucose-sensing functions, the neurons were recorded under the current clamp mode in response to a 5 → 1 → 5 mM extracellular glucose fluctuation protocol [45]. The firing rate values were averaged within a 2-min bin in the 5 mM glucose or 1 mM glucose aCSF condition. Neurons that were inhibited ($>$10% decrease of firing rate) in response to hypoglycemia (5 → 1 mM glucose) were identified as glucose-excited (GE) neurons. Neurons that were

activated (>10% increase in the firing rate) in response to hypoglycemia were identified as glucose-inhibited (GI) neurons. Neurons that showed responses at a less than 10% change in the firing rate were identified as non-glucose-sensing neurons.

2.6. Statistical analyses

The data are presented as mean ± SEM (standard error of the mean). Statistical analyses were conducted using GraphPad Prism 7.0 to evaluate the normal distribution and variations within and among groups. The methods of statistical analyses were chosen based on the design of each experiment and are indicated in the figure legends or main text. $P < 0.05$ was considered statistically significant.

2.7. Study approval

Care of all of the animals and the procedures were approved by the Baylor College of Medicine's Institutional Animal Care and Use Committee.

3. RESULTS

3.1. E2 promotes acute adaptive refeeding after starvation

Consistent with previous reports [3,5,6,41], we demonstrated that the OVX-V mice, when fed ad libitum, displayed higher body weight and daily food intake compared to the sham mice, and these increases were rescued by the E2 supplement in the OVX-E mice (Figure 1A–B). However, after overnight fasting, the OVX-V mice showed significantly lower 2-hour food intake than the sham mice, while the OVX-E mice demonstrated a 2-hour refeeding response comparable to the sham mice (Figure 1C). Notably, there was no significant difference in 24-hour food intake after fasting among the three groups (Supplemental Figure 1). The heavier OVX-V mice may have lost relatively less energy storage after the same overnight fasting, which may have contributed to the reduced 2-hour refeeding response. Thus, we analyzed the correlation between the relative body weight loss and the 2-hour refeeding response in all 3 groups of mice. We found no significant correlation (Figure 1D; $p = 0.9120$ and $r^2 = 0.00098$), suggesting that altered 2-hour refeeding behavior was not likely due to different weight loss. Thus, these results indicated that endogenous E2 is required to maintain acute fasting-induced refeeding, an important adaptive behavior in response to the shortage of energy storage.

3.2. Membrane-bound ERα activity is required for acute adaptive refeeding after starvation

To determine if the effect of E2 on acute refeeding was mediated by membrane-bound ERα functions or transcriptional activity of ERα, we used ERα-C451A mutant mice that lacked membrane-bound ERα activity and ERα-AF2^0 mutant mice that were deficient in ERα transcriptional activity. The ERα-C451A mutant mice showed similar body weight compared to the control mice (Figure 2A). However, after overnight fasting, the ERα-C451A mutant mice showed decreased food intake during the acute 2-hour refeeding period, recapitulating the phenotype of the OVX-V mice (Figure 2B). Importantly, there was no significant correlation between the relative body weight loss and 2-hour refeeding response in these 2 groups of mice (Figure 2C; $p = 0.0773$ and $r^2 = 0.3392$). These results indicated that membrane-bound ERα activity was required to maintain acute adaptive refeeding after starvation in the female mice.

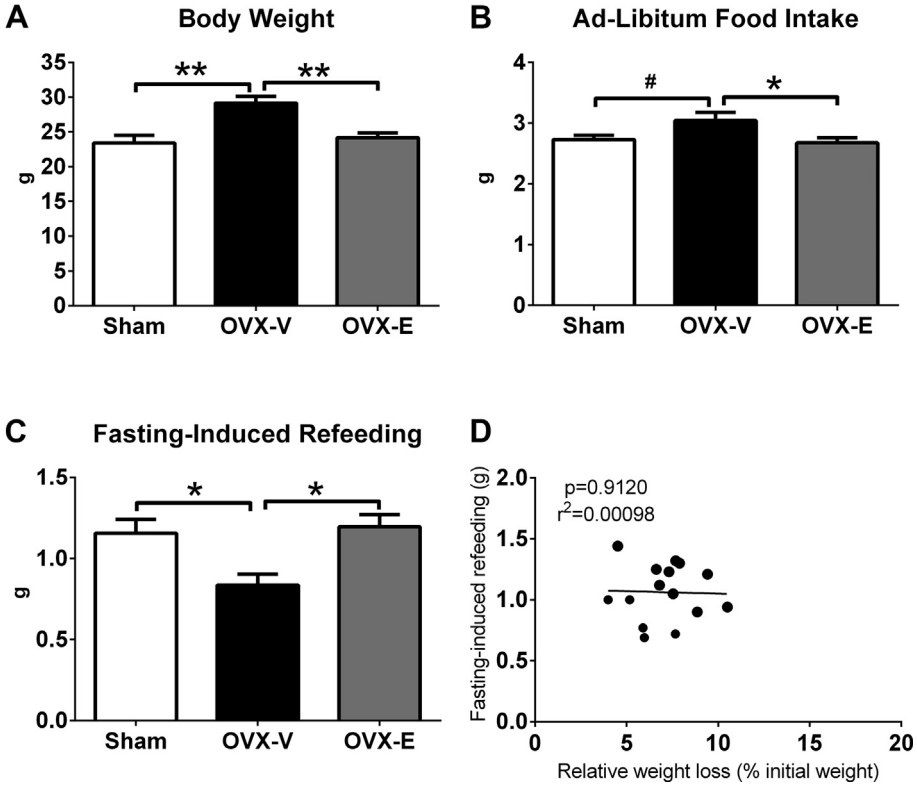

Figure 1: E2 promotes acute adaptive refeeding after starvation. Body weight (A), daily ad libitum food intake (B), and 2-hour fasting-induced refeeding (C) in the sham, OVX-V, and OVX-E mice measured 10 weeks after surgery. Data are presented as mean ± SEM. N = 5 mice per group. *$p < 0.05$ and **$p < 0.01$ in one-way ANOVA followed by post hoc Tukey's multiple comparisons; #$p < 0.05$ in the two-sided t-test. (D) Correlation of the relative body weight loss and 2-hour fasting-induced refeeding in the sham, OVX-V, and OVX-E mice.

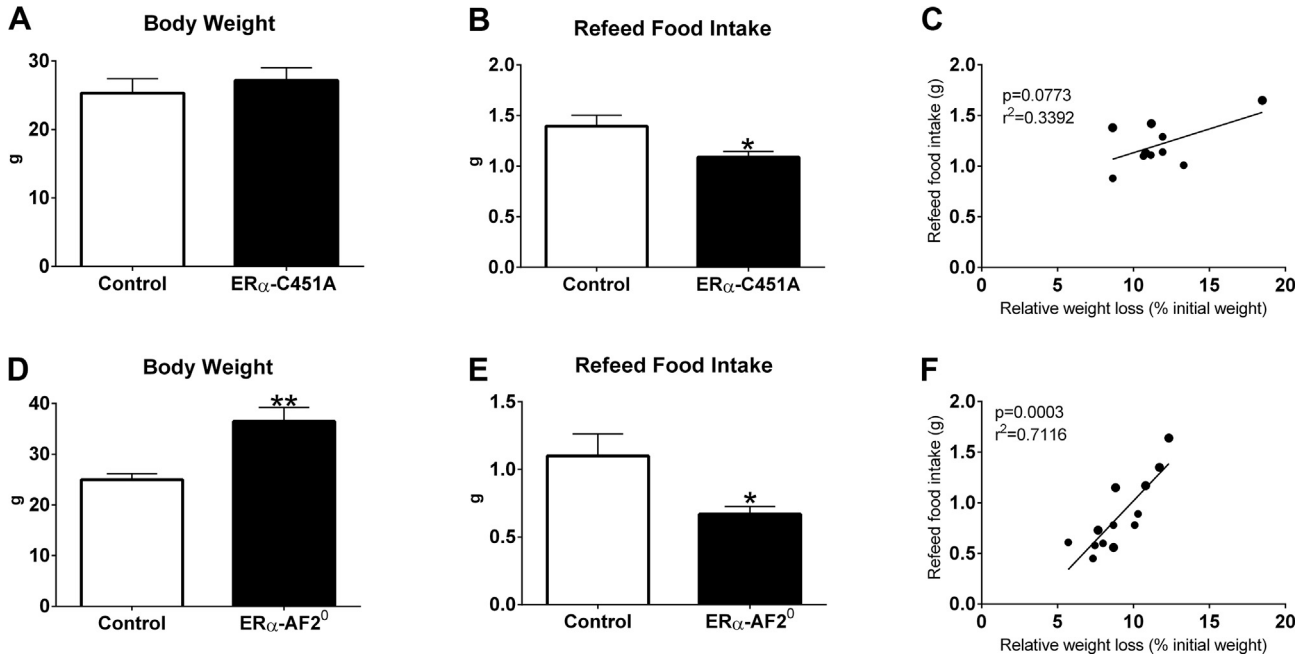

Figure 2: Membrane-bound ERα activity is required for acute adaptive refeeding after starvation. (A) Body weight of the control and ERα-C541A mice before fasting. (B) Two-hour fasting-induced refeeding of the control and ERα-C541A mice. N = 4 or 6 mice per group. (C) Correlation of the relative body weight loss and 2-hour fasting-induced refeeding in the control and ERα-C451A mice. (D) Body weight of the control and ERα-AF20 and mice. (E) Two-hour fasting-induced refeeding of the control and ERα-AF2^0 mice. N = 6 or 7 mice per group. Data are presented as mean ± SEM. *p < 0.05 and **p < 0.01 in the two-sided t-tests. (F) Correlation of the relative body weight loss and 2-hour fasting-induced refeeding in the control and ERα-AF2^0 mice.

The ERα-AF2^0 mutant mice were significantly heavier than their age-matched controls (Figure 2D). After overnight fasting, these ERα-AF2^0 mutant mice showed significantly reduced 2-hour refeeding compared to the control mice (Figure 2E). However, we found that the fasting-induced relative body weight loss was positively correlated with the 2-hour refeeding response in these mice (Figure 2F; $p = 0.0003$ and $r^2 = 0.7116$). These data implied the possibility that the reduced refeeding response in the heavier ERα-AF2^0 mutant mice might have been secondary to the relatively smaller body weight loss after overnight fasting, but not caused by the ERα-AF2^0 mutation per se.

To further ascertain the potential confounding effects of different fasting-induced weight loss, we tested the effects of ICV injection of 2-DG in satiated mice, which induced a transient central glucopenia and mimicked fasting without actual weight loss. As expected, ICV 2-DG induced a significant increase in 2-hour food intake in the female control mice compared to the saline group (Figure 3A). Interestingly, this 2-DG-induced feeding was significantly attenuated in the ERα-C451A mutant mice (Figure 3A). Importantly, 2-DG evoked comparable feeding behavior in the ERα-AF2^0 mutant mice and their control littermates (Figure 3B), although the ERα-AF2^0 mutant mice were

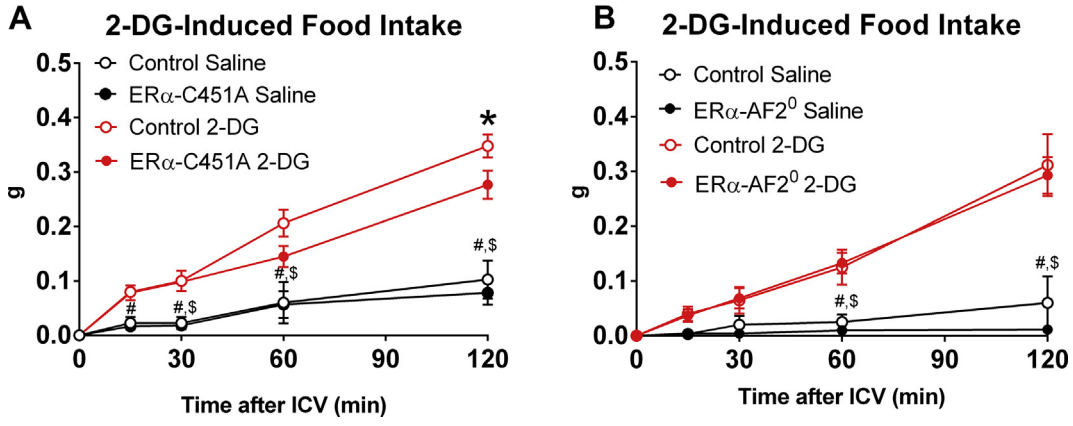

Figure 3: Membrane-bound ERα activity is required for feeding induced by central glucopenia. (A) Food intake in the satiated control and ERα-C541A mice after ICV injection of saline or 2-DG. Data are presented as mean ± SEM. N = 4 or 6 mice per group. *p < 0.05 between the control 2-DG and ERα-C451A 2-DG groups; $p < 0.05 between the control saline and control 2-DG groups; #p < 0.05 between the ERα-C451A saline and ERα-C451A 2-DG groups in two-way ANOVA followed by post hoc Tukey's multiple comparisons. (B) Food intake in the satiated control and ERα-AF2^0 mice after ICV injection of saline or 2-DG. Data are presented as mean ± SEM. N = 6 or 7 mice per group. $, p < 0.05 between the control saline and control 2-DG groups; #p < 0.05 between the ERα-AF2^0 saline and ERα-AF2^0 2-DG groups in two-way ANOVA followed by post hoc Tukey's multiple comparisons.

significantly heavier than the controls (Figure 2D). Collectively, these results further support that the effect of E2 on acute refeeding was mediated by membrane-bound ERα activity.

3.3. Membrane-bound ERα is required for neuronal responsiveness to an ERα agonist

Our previous study showed that ERα in the ARH and vlVMH is important for body weight balance in female mice fed ad libitum [17]. In the present study, we tested whether ERα-C451A and/or ERα-AF2^0 mutations affected the neural activity of these ERα^{ARH} and ERα^{vlVMH} neurons (labeled by the ZsGreen fluorescence protein) from the female control, ERα-C451A, and ERα-AF2^0 mutant mice (Figure 4A—B). First, we found that the ERα-C451A mutation caused a significant reduction in the baseline firing rate of the ERα^{ARH} neurons without changing the same parameter in the ERα^{vlVMH} neurons, while the ERα-AF2^0 mutation did not affect the baseline firing rate of either neuron types (Supplemental Figure 2A—B). Then we examined the effects of a selective ERα agonist, propyl pyrazole triol (PPT) [44], on the neural activities of the ERα^{ARH} and ERα^{vlVMH} neurons. In the female control mice, PPT puff rapidly increased the firing rate of the ERα^{ARH} neurons, but these PPT-induced responses were significantly blunted in the ERα^{ARH} neurons from the female ERα-C451A mice (Figure 4C,D, and 4F and Supplemental Figure 2C). Interestingly, the ERα-AF2^0 mutation did not affect the PPT-induced activation in the ERα^{ARH} neurons (Figure 4E—F and Supplemental Figure 2C). Similarly, the ERα^{vlVMH} neurons from the female control mice were rapidly activated by PPT puff, but these effects were significantly attenuated by the ERα-C451A mutation (Figure 4G and Supplemental Figure 2D). Notably, the ERα-AF2^0 mutation modestly but significantly attenuated the PPT-induced increases in the firing rate in the ERα^{vlVMH} neurons (Figure 4G and Supplemental Figure 2D). Thus, these results validated that the ERα-C451A mutation impaired the membrane-bound ERα activity and

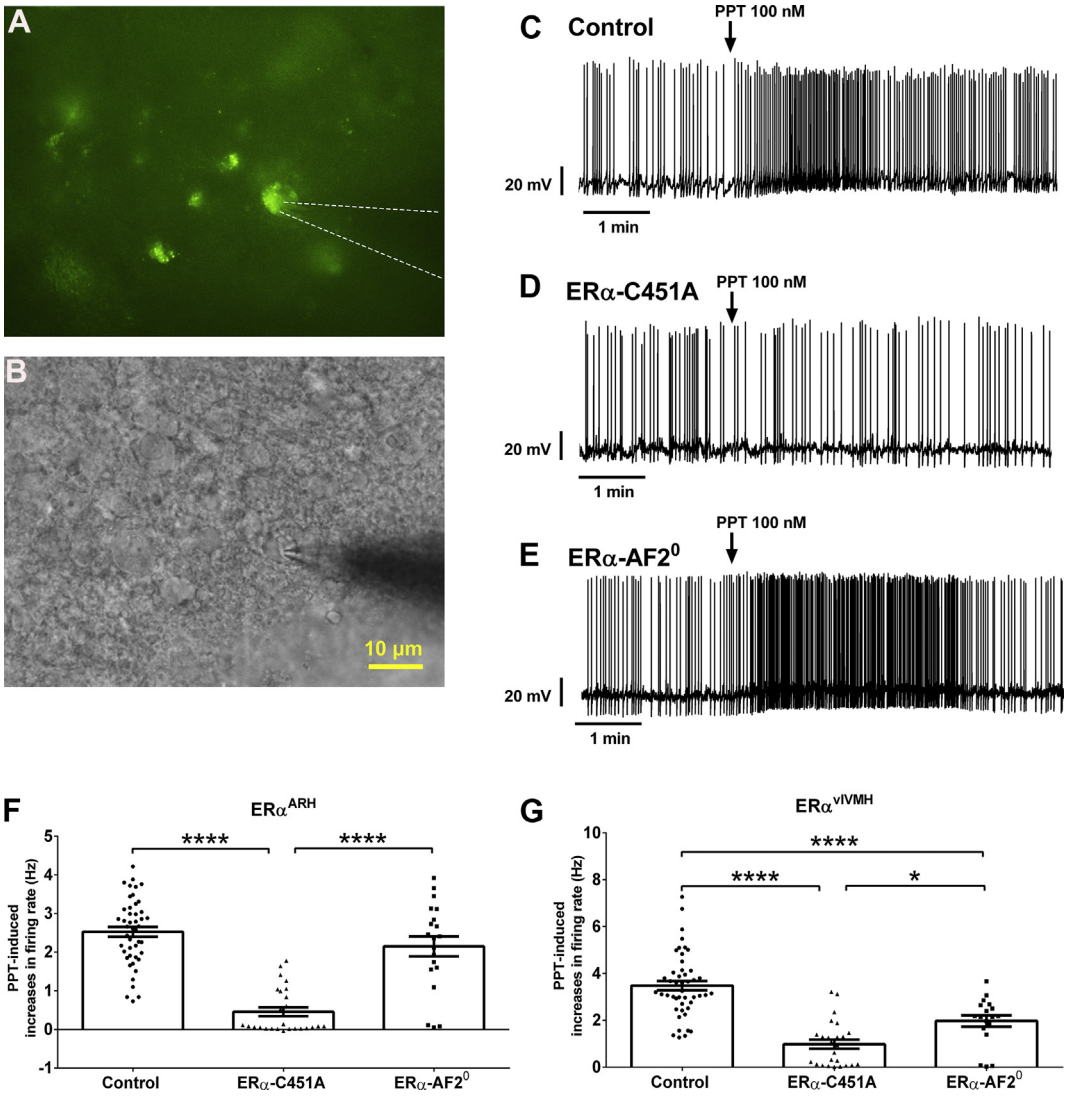

Figure 4: Membrane-bound ERα is required for the neuronal responsiveness to PPT. ZsGreen fluorescent (A) and brightfield illumination (B) of a recorded ERα-positive neuron. Scale bar = 10 μm. The shadow in (B) corresponding to the dashed lines in (A) is the recording pipette. (C) Representative traces of an ERα^{ARH} neuron from a control mouse that was activated by PPT. (D) Representative traces of an ERα^{ARH} neuron from an ERα-C451A mouse that was not responsive to PPT. (E) Representative traces of an ERα^{ARH} neuron from an ERα-AF2^0 mouse that was activated by PPT. (F) PPT-induced increases in the firing rate of the ERα^{ARH} neurons. Data are presented as mean ± SEM with individual data points. N = 20—48 neurons per group. (G) PPT-induced increases in the firing rate of the ERα^{vlVMH} neurons. Data are presented as mean ± SEM with individual data points. N = 18—50 neurons per group. *p < 0.05, ***p < 0.001, and ****p < 0.0001 in one-way ANOVA followed by post hoc Tukey's multiple comparisons.

rendered the attenuated acute responsiveness to the ERα agonist in the ERα^{ARH} and ERα^{vlVMH} neurons. However, compared to the ERα-C451A mutation, the deficiency in ERα transcriptional activity (rendered by the ERα-AF2^{0} mutation) only marginally affected the rapid responsiveness of the hypothalamic ERα neurons to the ERα agonist.

3.4. Membrane-bound ERα activity is required for glucose-sensing functions

Acute refeeding is partially evoked by hypoglycemia during starvation, which regulates activities of the glucose-sensing neurons in the brain to stimulate appetite [21,25–27]. Both the ARH and vlVMH contain abundant glucose-sensing neurons and are implicated in the regulation of food intake and glucose balance [20,22–25,28]. In this study, we further examined the glucose-sensing functions of the ERα^{ARH} and ERα^{vlVMH} neurons in response to hypoglycemia. In the female control mice, the ERα^{ARH} neurons were composed of approximately 40.28% GE neurons, 37.5% GI neurons, and 22.22% non-glucose-sensing neurons (Figure 5A-D and Supplemental Figure 3A). This composition was not significantly altered in either the ERα-C451A mutant mice or ERα-AF2^{0} mutant mice (Figure 5D, $p = 0.6462$ between the control vs ERα-C451A and $p = 0.1710$ between the control vs ERα-AF2^{0} in $\chi 2$ tests), indicating that neither of these mutations altered the glucose-sensing nature of the ERα^{ARH} neurons. We further analyzed the magnitudes of glucose-sensing responses and found that the GE-ERα^{ARH} neurons from the controls, ERα-C451A, and ERα-AF2^{0} mice showed comparable changes in the firing rate in response to hypoglycemia (Figure 5E and Supplemental Figure 3A). Interestingly, the GI-ERα^{ARH} neurons from the ERα-C451A mutant mice demonstrated significantly attenuated responses in the firing rate compared to the responses in the controls (Figure 5F and Supplemental Figure 3A). Importantly, there was no significant difference in the responses of the GI-ERα^{ARH} neurons between the control and ERα-AF2^{0} mice (Figure 5F).

Consistent with our previous report [29], all of the tested ERα^{vlVMH} neurons from the female control mice were glucose-sensing, with 57.6% being GE neurons and 42.4% being GI neurons (Figure 5G and Supplemental Figure 3B). We further demonstrated that all of the tested ERα^{vlVMH} neurons from the ERα-C451A and ERα-AF2^{0} mice were also glucose-sensing with similar GE/GI compositions (Figure 5G). The magnitudes of the glucose-sensing responses of both the GE- and GI-ERα^{vlVMH} neurons were significantly attenuated in the ERα-C451A mutant female mice compared to those from the controls (Figure 5H-I and Supplemental Figure 3B). However, the magnitudes of the glucose-sensing responses of both the GE- and GI-ERα^{vlVMH} neurons in the ERα-AF2^{0} mice were largely comparable to those from the controls (Figure 5H-I and Supplemental Figure 3B). Together, these results suggested that membrane-bound ERα activity was required to maintain the glucose-sensing functions of the GI-, GE-ERα^{vlVMH}, and GI-ERα^{ARH} neurons, while the transcriptional activity of ERα only had a minor role.

4. DISCUSSION

In the current study, we demonstrated an unexpected role of E2 in maintaining acute adaptive refeeding behavior in hungry female animals. At the mechanistic level, these estrogenic effects on acute refeeding required the membrane-bound ERα activity. We further showed that two hypothalamic ERα neural populations, ERα^{ARH} and ERα^{vlVMH} neurons, required membrane-bound ERα activity to maintain their acute responsiveness to either ERα agonist or hypoglycemia. However, the transcriptional activity of ERα only played a minor role in acute refeeding response and hypothalamic ERα neuron activities. Together, our study indicates that endogenous E2 and membrane-bound ERα activity in female animals are required to maintain acute adaptive refeeding behavior in response to starvation.

A prior study reported that the administration of E2 at a high dose (450 μg within 24 h) in gonad-intact female mice inhibited fasting-induced refeeding [46]. The discrepancy between this finding and our observations from ovariectomized mice receiving a much lower dose of E2 (2 μg every 4 days) may likely result from different E2 levels in these studies. In the present study, we first assessed the functions of endogenous E2 by comparing gonad-intact vs ovariectomized mice to reveal reduced fasting-induced refeeding caused by the depletion of endogenous ovarian hormones (including E2), and we further demonstrated that replacement of E2 in ovariectomized mice can rescue this phenotype. Importantly, we observed that E2 supplement in ovariectomized mice rescued the phenotypes to similar levels of gonad-intact female mice, suggesting that our E2 treatment mimicked the physiological effects of endogenous E2, but not the pharmacological effects of high E2. In addition, the physiological functions of endogenous E2 are further supported by our observations in the ERα-C451A mutant mice, in which we did not perform any pharmacological manipulations of the E2 levels. Notably, the ERα-C451A mutation does not significantly alter serum E2 levels [38]. Thus, the reduced fasting-induced refeeding and 2-DG-induced feeding observed in the ERα-C451A mice were likely attributed to impaired membrane-bound ERα activity, but not to altered E2 levels.

We and others previously demonstrated that E2 inhibits overeating in animals fed ad libitum with either regular chow or a high-fat diet [1,3–5,47–49]. In this study, for the first time, we found that E2 and ERα signals are required to maintain normal acute refeeding in hungry female mice. These findings suggest that the role of E2 in feeding regulation depends on the internal energy state of the animal and/or environmental food availability. On the one hand, when animals have unlimited access to food and never experience prolonged hunger, E2 functions to inhibit overeating and therefore prevents excess weight gain. On the other hand, after prolonged food deprivation in animals, E2 is required to trigger efficient and rapid refeeding when food becomes available again, which is a critical adaptation for animals to survive in an environment with scarce food. While these estrogenic effects on feeding appear to be paradoxical, they both reduce the deviation of the energy balance by inhibiting eating in a satiated state and promoting eating when hungry, and therefore contribute to the stability of energy homeostasis. Interestingly, similar bidirectional effects of E2 also exist in the regulation of thermogenesis, another important component of energy balance. We and others demonstrated that E2 robustly enhances thermogenesis in female animals housed at room temperature [17–19]. However, recent evidence indicates that in female mice chronically exposed to cold (6 °C), the E2-ERα signal functions to inhibit thermogenic browning of white adipose tissue [50], an adaptive response probably to better conserve energy storage and enhance survival in cold environments. Thus, emerging evidence suggests that estrogenic actions on energy homeostasis are more complicated than originally thought and are likely dependent on the internal energy state of animals as well as on external environmental challenges. Future studies are warranted to further investigate estrogenic actions on energy balance under various conditions (satiated or fasted, and cold or warm). Of note, other estrogen receptors, ERβ [51] and GPR30 [52–54], are also implicated in the regulation of energy balance at least in mice fed ad libitum. The roles of these estrogen receptors in adaptive acute feeding after starvation remain to be examined.

The function of E2 can be mediated by the transcriptional activity of ERα to regulate gene expression [36]. ERα regulates gene

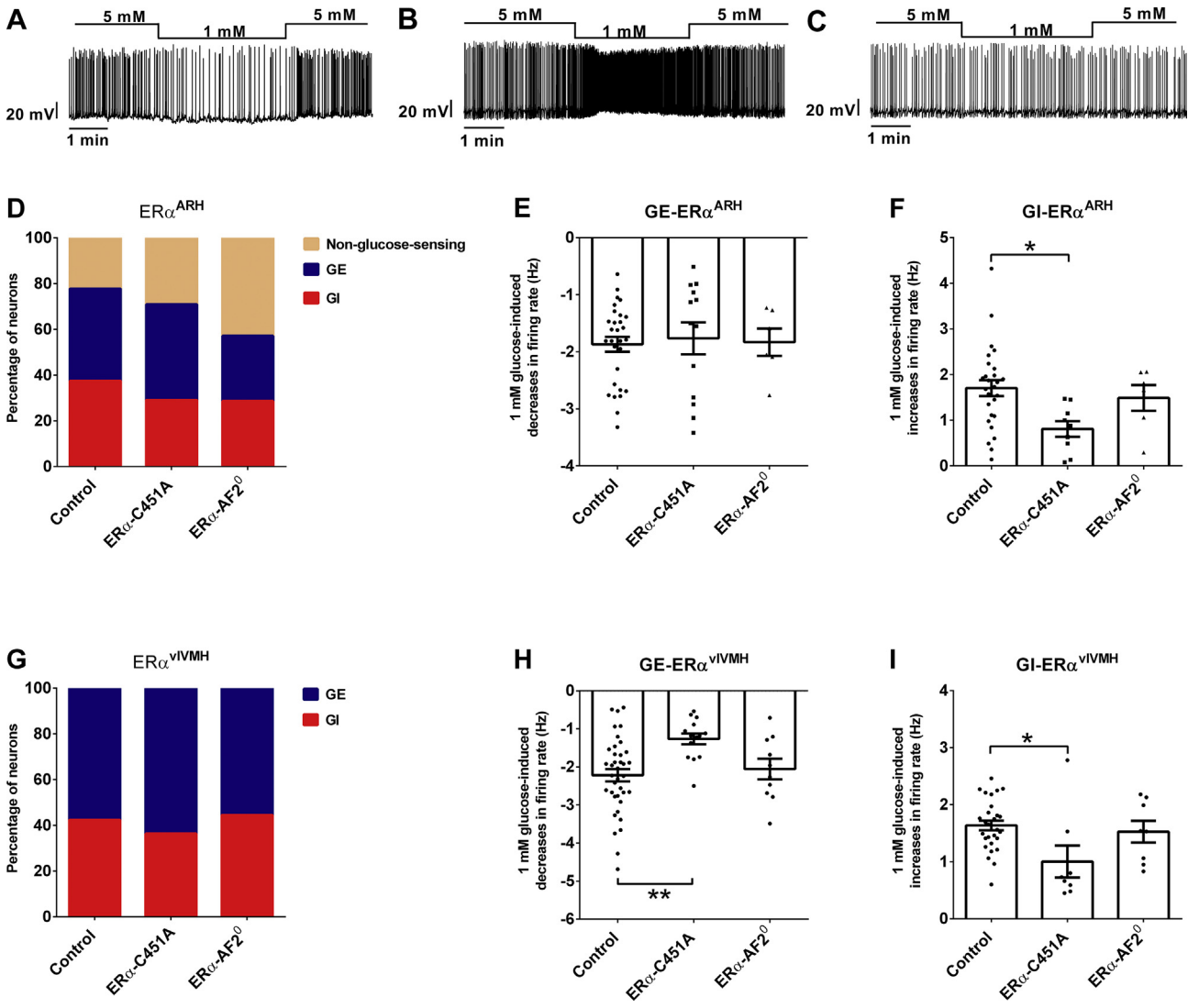

Figure 5: Membrane-bound ERα activity is required for glucose-sensing functions. Representative traces of a GE neuron (A), GI neuron (B), and non-glucose-sensing neuron (C). (D) Percentage of GE, GI, and non-glucose-sensing neurons within the ERα^{ARH} populations among the control, ERα-C451A, and ERα-AF2^{0} mice, respectively. N = 35–49 neurons in each group. (E) Hypoglycemia-induced decreases in the firing rate of the GE-ERα^{ARH} neurons. (F) Hypoglycemia-induced increases in the firing rate of the GI-ERα^{ARH} neurons. (G) Percentage of GE and GI neurons within the ERα^{vlVMH} populations among the control, ERα-C451A, and ERα-AF2^{0} mice respectively. N = 44–62 neurons in each group. (H) Hypoglycemia-induced decreases in the firing rate in the GE-ERα^{vlVMH} neurons. (I) Hypoglycemia-induced increases in the firing rate in the GI-ERα^{vlVMH} neurons. Data are presented as mean ± SEM. N = 6–38 neurons per group. *p < 0.05 and **p < 0.01 in one-way ANOVA followed by post hoc Tukey's multiple comparisons.

transcription through two activation functions, ERα-AF1 and ERα-AF2. In particular, ERα-AF2^{0} mutant mice display obesity when fed ad libitum [35], indicating that the transcriptional activity of ERα is required to prevent body weight gain in animals with unlimited access to food. We consistently showed that female ERα-AF2^{0} mutant mice were significantly heavier than their control littermates when fed ad libitum. These heavier ERα-AF2^{0} mutant mice also displayed impaired refeeding response after overnight fasting. However, we noted that the refeeding responses in these mice were positively correlated to the relative weight loss induced by fasting, which may have confounded the fasting-induced refeeding response. Indeed, acute feeding induced by central glucopenia was intact in the ERα-AF2^{0} mutant mice. Thus, we suggest that the loss of ERα transcriptional activity per se does not directly affect the acute refeeding response in hungry mice. Of note, the serum E2 levels were higher in the ERα-AF2^{0} mice [35], and therefore we could not fully exclude the possibility that reduced feeding observed in the ERα-AF2^{0} mice may also have resulted, at least partly, from the excess levels of E2.

In addition to regulating gene expression as a classic nuclear receptor, a subset of intracellular ERα molecules is concentrated on the cytomembrane and can initiate rapid signaling pathways [36]. The point mutation ERα-C451A has been demonstrated to specifically disrupt membrane-bound ERα activity [37,38]. In this study, we found that this mutation impaired acute feeding responses induced either by overnight fasting or the central glucopenia. Thus, these results indicate that membrane-bound ERα activity is required for acute adaptive refeeding in response to starvation. The transgenic mouse models used in the present study were useful to determine the respective roles of nuclear vs extranuclear/membrane actions of ERα, especially in vascular pathophysiology. Indeed, the key role of ERα membrane-initiated steroid signaling was demonstrated in two endothelial actions of estrogens (increase in nitric oxide production and acceleration of

reendothelialization) [38], whereas other vascular effects were all dependent on nuclear ERα, including the prevention of atheroma, angiotensin II-induced hypertension [55], and neointimal hyperplasia after artery injury [56].

Our findings demonstrated an additional important role of membrane ERα in hypothalamic ERα neural populations, namely ERα^{ARH} and ERα^{vlVMH} neurons, in maintaining their acute responsiveness to either the ERα agonist or hypoglycemia. The role of membrane-bound ERα was also described to be important in the brain, especially for the organization of the circuits underlying sexually differentiated responses of the male brain [57]. In addition to the impaired fasting-induced refeeding, the ERα-C451A mutation also causes severe deficits in the glucose-sensing capability of ERα^{ARH} and ERα^{vlVMH} neurons. Because glucose-sensing neurons in the ARH and vlVMH are reported to regulate eating in response to hypoglycemia [25,58], we suggest that the reduced acute adaptive refeeding seen in the ERα-C451A mutant mice was at least partly attributed to the impaired glucose-sensing functions of the ERα^{ARH} and ERα^{vlVMH} neurons. The neurochemical identities of the ERα^{ARH} and ERα^{vlVMH} neurons were not specifically investigated in the current study. Within the vlVMH, ERα-expressing neurons partially overlap with those expressing steroidogenic factor-1 [17] and/or vesicular glutamate transporter-2 [59]. We recently identified a chloride ion channel, anoctamin 4, as a GI-ERα^{vlVMH} marker, and Abcc8, a subunit of the ATP-sensitive potassium channel, as a GE-ERα^{vlVMH} marker [[29]]. These two ion channels are important for mediating the glucose-sensing functions of GI- and GE-ERα^{vlVMH} neurons, respectively [[29]]. Within the ARH, ERα-expressing neurons partially overlap with those expressing pro-opiomelanocortin [17,41], kisspeptin [60], or tyrosine hydroxylase [61]. A few neuropeptide Y (NPY) neurons in the ARH co-express ERα [62], which may reduce the inhibitory synaptic inputs to these neurons [63], although others failed to observe co-expression of ERα and NPY within the ARH [46]. The specific roles of ERα in these subsets of hypothalamic neurons in adaptive acute feeding remained to be examined.

Another interesting question is how membrane-bound ERα regulates the glucose-sensing functions of hypothalamic neurons. ERα is known to activate a number of rapid signaling pathways in hypothalamic neurons, namely mTOR [64] and PI3K [65–67]. E2 has also been reported to inhibit hypothalamic AMPK, which mediates estrogenic actions on energy balance [18,68], although others reported that E2 increases AMPK protein levels and phosphorylation in the vlVMH [69]. The effects of membrane-bound ERα on these rapid signals and their functional relevance in hypothalamic glucose-sensing and adaptive acute feeding in hungry animals warrant future investigations.

Of note, the ERα-C451A mutation exists in the whole body in these mice. Thus, we could not exclude the possibility that the loss of membrane-bound ERα activity in the peripheral tissues may also contribute to reduced acute adaptive refeeding. Nevertheless, central glucopenia-induced feeding is also impaired in ERα-C451A mutant mice, further highlighting the roles of brain membrane-bound ERα in the regulation of feeding in hungry female animals. In addition to the ARH and vlVMH, ERα is also abundantly expressed in multiple brain regions that are implicated in the regulation of feeding behavior, including the lateral hypothalamus, nucleus of the solitary tract, and dorsal Raphe nuclei [70]. The ERα neurons in these brain regions may also contribute to acute refeeding, which needs to be further investigated.

5. CONCLUSIONS

In conclusion, we found a new role of E2 in feeding control. In addition to the anorexic actions in female animals fed ad libitum, E2 is also required to maintain normal acute refeeding in response to starvation. This estrogenic effect requires membrane-bound ERα activity. We further provided evidence that membrane-bound ERα activity is required for ERα^{ARH} and ERα^{vlVMH} neurons to maintain their normal glucose-sensing capability, which may play a key role in promoting feeding in hungry mice. These results extend the understanding about estrogenic actions on feeding regulations, which could be bidirectional depending on the internal energy state and contribute to the maintenance of energy homeostasis in face of various environmental challenges. Although E2 levels and the expression of ERα in the hypothalamus may not undergo dynamic changes during the fasting–feeding transition in normal females [71], our finding that E2 and ERα signals are required for female animals to properly respond to hunger provided new mechanistic insights on the association of abnormal feeding behavior with irregular estrogen levels in certain medical conditions that disproportionally affect women, such as anorexia nervosa [72–74], and therefore may facilitate the development of suitable therapeutic strategies for these conditions.

AUTHOR CONTRIBUTIONS

KY and YH participated in the experimental design and most of the procedures, data acquisition, and analyses, and wrote the manuscript. IH, ZP, YY, PX, XC, HL, NQ, HL, YH, MY, CL, TY, and JW assisted with the production of the study mice and the experimental procedures. JFA, PG, and FL provided the ERα-C451A and ERα-AF2^{0} mutant mouse lines and contributed to the study design, data interpretation, and manuscript writing. CW and YX were involved in the study design and writing the manuscript. They had full access to all of the study data and take responsibility for the data integrity and the accuracy of the data analysis.

ACKNOWLEDGMENTS

The investigators involved in this study acknowledge funding support from the American Heart Association (19CDA34660335 to CW), the National Institutes of Health (NIH K01 DK119471 to CW), US Department of Agriculture/Agricultural Research Service (CRIS3092-5-001-059 to YX), and INSERM and ANR (BENEFIT and NOSE4).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2020.101053.

REFERENCES

[1] Drewett, R.F., 1973. Sexual behaviour and sexual motivation in the female rat. Nature 242(5398):476–477.

[2] Schwartz, S.M., Wade, G.N., 1981. Effects of estradiol and progesterone on food intake, body weight, and carcass adiposity in weanling rats. American Journal of Physiology 240(5):E499–E503.

[3] Geary, N., Asarian, L., Korach, K.S., Pfaff, D.W., Ogawa, S., 2001. Deficits in E2-dependent control of feeding, weight gain, and cholecystokinin satiation in ER-alpha null mice. Endocrinology 142(11):4751–4757.

[4] Wallen, W.J., Belanger, M.P., Wittnich, C., 2001. Sex hormones and the selective estrogen receptor modulator tamoxifen modulate weekly body weights

and food intakes in adolescent and adult rats. Journal of Nutrition 131(9): 2351–2357.

[5] Roesch, D.M., 2006. Effects of selective estrogen receptor agonists on food intake and body weight gain in rats. Physiology and Behavior 87(1):39–44.

[6] Rogers, N.H., Perfield 2nd, J.W., Strissel, K.J., Obin, M.S., Greenberg, A.S., 2009. Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. Endocrinology 150(5):2161–2168.

[7] Santollo, J., Wiley, M.D., Eckel, L.A., 2007. Acute activation of ER alpha decreases food intake, meal size, and body weight in ovariectomized rats. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 293(6):R2194–R2201.

[8] Wegorzewska, I.N., Walters, K., Weiser, M.J., Cruthirds, D.F., Ewell, E., Larco, D.O., et al., 2008. Postovariectomy weight gain in female rats is reversed by estrogen receptor alpha agonist, propylpyrazoletriol. American Journal of Obstetrics and Gynecology 199(1):67 e61–65.

[9] Hamilton, D.J., Minze, L.J., Kumar, T., Cao, T.N., Lyon, C.J., Geiger, P.C., et al., 2016. Estrogen receptor alpha activation enhances mitochondrial function and systemic metabolism in high-fat-fed ovariectomized mice. Physics Reports 4(17).

[10] Vidal, O., Lindberg, M., Savendahl, L., Lubahn, D.B., Ritzen, E.M., Gustafsson, J.A., et al., 1999. Disproportional body growth in female estrogen receptor-alpha-inactivated mice. Biochemical and Biophysical Research Communications 265(2):569–571.

[11] Heine, P.A., Taylor, J.A., Iwamoto, G.A., Lubahn, D.B., Cooke, P.S., 2000. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. Proceedings of the National Academy of Sciences of the United States of America 97(23):12729–12734.

[12] Okura, T., Koda, M., Ando, F., Niino, N., Ohta, S., Shimokata, H., 2003. Association of polymorphisms in the estrogen receptor alpha gene with body fat distribution. International Journal of Obesity and Related Metabolic Disorders 27(9):1020–1027.

[13] Ribas, V., Nguyen, M.T., Henstridge, D.C., Nguyen, A.K., Beaven, S.W., Watt, M.J., et al., 2010. Impaired oxidative metabolism and inflammation are associated with insulin resistance in ERalpha-deficient mice. American Journal of Physiology. Endocrinology and Metabolism 298(2):E304–E319.

[14] Manrique, C., Lastra, G., Habibi, J., Mugerfeld, I., Garro, M., Sowers, J.R., 2012. Loss of estrogen receptor alpha signaling leads to insulin resistance and obesity in young and adult female mice. Cardiorenal Medicine 2(3):200–210.

[15] Antonson, P., Omoto, Y., Humire, P., Gustafsson, J.A., 2012. Generation of ERalpha-floxed and knockout mice using the Cre/LoxP system. Biochemical and Biophysical Research Communications 424(4):710–716.

[16] Musatov, S., Chen, W., Pfaff, D.W., Mobbs, C.V., Yang, X.J., Clegg, D.J., et al., 2007. Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. Proceedings of the National Academy of Sciences of the United States of America 104(7):2501–2506.

[17] Xu, Y., Nedungadi, T.P., Zhu, L., Sobhani, N., Irani, B.G., Davis, K.E., et al., 2011. Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. Cell Metabolism 14(4):453–465.

[18] Martinez de Morentin, P.B., Gonzalez-Garcia, I., Martins, L., Lage, R., Fernandez-Mallo, D., Martinez-Sanchez, N., et al., 2014. Estradiol regulates brown adipose tissue thermogenesis via hypothalamic AMPK. Cell Metabolism 20(1):41–53.

[19] Correa, S.M., Newstrom, D.W., Warne, J.P., Flandin, P., Cheung, C.C., Lin-Moore, A.T., et al., 2015. An estrogen-responsive module in the ventromedial hypothalamus selectively drives sex-specific activity in females. Cell Reports 10(1):62–74.

[20] Silver, I.A., Erecinska, M., 1998. Glucose-induced intracellular ion changes in sugar-sensitive hypothalamic neurons. Journal of Neurophysiology 79(4): 1733–1745.

[21] Levin, B.E., Dunn-Meynell, A.A., Routh, V.H., 1999. Brain glucose sensing and body energy homeostasis: role in obesity and diabetes. American Journal of Physiology 276(5):R1223–R1231.

[22] Song, Z., Levin, B.E., McArdle, J.J., Bakhos, N., Routh, V.H., 2001. Convergence of pre- and postsynaptic influences on glucosensing neurons in the ventromedial hypothalamic nucleus. Diabetes 50(12):2673–2681.

[23] Dunn-Meynell, A.A., Routh, V.H., Kang, L., Gaspers, L., Levin, B.E., 2002. Glucokinase is the likely mediator of glucosensing in both glucose-excited and glucose-inhibited central neurons. Diabetes 51(7):2056–2065.

[24] Santiago, A.M., Clegg, D.J., Routh, V.H., 2016. Estrogens modulate ventrolateral ventromedial hypothalamic glucose-inhibited neurons. Molecular Metabolism 5(10):823–833.

[25] Hirschberg, P.R., Sarkar, P., Teegala, S.B., Routh, V.H., 2020. Ventromedial hypothalamus glucose-inhibited neurones: a role in glucose and energy homeostasis? Journal of Neuroendocrinology 32(1):e12773.

[26] Levin, B.E., Routh, V.H., Kang, L., Sanders, N.M., Dunn-Meynell, A.A., 2004. Neuronal glucosensing: what do we know after 50 years? Diabetes 53(10): 2521–2528.

[27] Park, Y.B., Choi, Y.J., Park, S.Y., Kim, J.Y., Kim, S.H., Song, D.K., et al., 2011. ATP-sensitive potassium channel-deficient mice show hyperphagia but are resistant to obesity. Diabetes and Metabolism Journal 35(3):219–225.

[28] Karnani, M., Burdakov, D., 2011. Multiple hypothalamic circuits sense and regulate glucose levels. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 300(1):R47–R55.

[29] He, Y., Xu, P., Wang, C., Xia, Y., Yu, M., Yang, Y., et al., 2020. Estrogen receptor-α expressing neurons in the ventrolateral VMH regulate glucose balance. Nature Communications 11(1):2165.

[30] Billon-Gales, A., Fontaine, C., Filipe, C., Douin-Echinard, V., Fouque, M.J., Flouriot, G., et al., 2009. The transactivating function 1 of estrogen receptor alpha is dispensable for the vasculoprotective actions of 17beta-estradiol. Proceedings of the National Academy of Sciences of the United States of America 106(6):2053–2058.

[31] Abot, A., Fontaine, C., Raymond-Letron, I., Flouriot, G., Adlanmerini, M., Buscato, M., et al., 2013. The AF-1 activation function of estrogen receptor alpha is necessary and sufficient for uterine epithelial cell proliferation in vivo. Endocrinology 154(6):2222–2233.

[32] Billon-Gales, A., Krust, A., Fontaine, C., Abot, A., Flouriot, G., Toutain, C., et al., 2011. Activation function 2 (AF2) of estrogen receptor-alpha is required for the atheroprotective action of estradiol but not to accelerate endothelial healing. Proceedings of the National Academy of Sciences of the United States of America 108(32):13311–13316.

[33] Arao, Y., Hamilton, K.J., Ray, M.K., Scott, G., Mishina, Y., Korach, K.S., 2011. Estrogen receptor alpha AF-2 mutation results in antagonist reversal and reveals tissue selective function of estrogen receptor modulators. Proceedings of the National Academy of Sciences of the United States of America 108(36): 14986–14991.

[34] Borjesson, A.E., Windahl, S.H., Lagerquist, M.K., Engdahl, C., Frenkel, B., Moverare-Skrtic, S., et al., 2011. Roles of transactivating functions 1 and 2 of estrogen receptor-alpha in bone. Proceedings of the National Academy of Sciences of the United States of America 108(15):6288–6293.

[35] Handgraaf, S., Riant, E., Fabre, A., Waget, A., Burcelin, R., Liere, P., et al., 2013. Prevention of obesity and insulin resistance by estrogens requires ERalpha activation function-2 (ERalphaAF-2), whereas ERalphaAF-1 is dispensable. Diabetes 62(12):4098–4108.

[36] Roepke, T.A., Qiu, J., Bosch, M.A., Ronnekleiv, O.K., Kelly, M.J., 2009. Cross-talk between membrane-initiated and nuclear-initiated oestrogen signalling in the hypothalamus. Journal of Neuroendocrinology 21(4):263–270.

[37] Pedram, A., Razandi, M., Lewis, M., Hammes, S., Levin, E.R., 2014. Membrane-localized estrogen receptor alpha is required for normal organ development and function. Developmental Cell 29(4):482–490.

[38] Adlanmerini, M., Solinhac, R., Abot, A., Fabre, A., Raymond-Letron, I., Guihot, A.L., et al., 2014. Mutation of the palmitoylation site of estrogen receptor alpha in vivo reveals tissue-specific roles for membrane versus nuclear actions. Proceedings of the National Academy of Sciences of the United States of America 111(2):E283–E290.

[39] Saito, K., He, Y., Yan, X., Yang, Y., Wang, C., Xu, P., et al., 2016. Visualizing estrogen receptor-alpha-expressing neurons using a new ERalpha-ZsGreen reporter mouse line. Metabolism 65(4):522–532.

[40] Zhu, L., Yang, Y., Xu, P., Zou, F., Yan, X., Liao, L., et al., 2013. Steroid receptor coactivator-1 mediates estrogenic actions to prevent body weight gain in female mice. Endocrinology 154(1):150–158.

[41] Wang, C., He, Y., Xu, P., Yang, Y., Saito, K., Xia, Y., et al., 2018. TAp63 contributes to sexual dimorphism in POMC neuron functions and energy homeostasis. Nature Communications 9(1):1544.

[42] Yan, C., He, Y., Xu, Y., Shu, G., Wang, C., Yang, Y., et al., 2016. Apolipoprotein A-IV inhibits AgRP/NPY neurons and activates pro-opiomelanocortin neurons in the arcuate nucleus. Neuroendocrinology 103(5):476–488.

[43] Duerrschmid, C., He, Y., Wang, C., Li, C., Bournat, J.C., Romere, C., et al., 2017. Asprosin is a centrally acting orexigenic hormone. Natura Medicine 23(12):1444–1453.

[44] Stauffer, S.R., Coletta, C.J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., et al., 2000. Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. Journal of Medicinal Chemistry 43(26):4934–4947.

[45] Kong, D., Vong, L., Parton, L.E., Ye, C., Tong, Q., Hu, X., et al., 2010. Glucose stimulation of hypothalamic MCH neurons involves K(ATP) channels, is modulated by UCP2, and regulates peripheral glucose homeostasis. Cell Metabolism 12(5):545–552.

[46] Olofsson, L.E., Pierce, A.A., Xu, A.W., 2009. Functional requirement of AgRP and NPY neurons in ovarian cycle-dependent regulation of food intake. Proceedings of the National Academy of Sciences of the United States of America 106(37):15932–15937.

[47] Blaustein, J.D., Wade, G.N., 1976. Ovarian influences on the meal patterns of female rats. Physiology and Behavior 17(2):201–208.

[48] Rogers, N.H., Perfield 2nd, J.W., Strissel, K.J., Obin, M.S., Greenberg, A.S., 2009. Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. Endocrinology.

[49] Zhu, L., Xu, P., Cao, X., Yang, Y., Hinton Jr., A.O., Xia, Y., et al., 2015. The ERalpha-PI3K cascade in pro-opiomelanocortin progenitor neurons regulates feeding and glucose balance in female mice. Endocrinology en20151660.

[50] Lapid, K., Lim, A., Berglund, E.D., Lu, Y., 2019. Estrogen receptor inhibition enhances cold-induced adipocyte beiging and glucose tolerance. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy 12:1419–1436.

[51] Foryst-Ludwig, A., Clemenz, M., Hohmann, S., Hartge, M., Sprang, C., Frost, N., et al., 2008. Metabolic actions of estrogen receptor beta (ERbeta) are mediated by a negative cross-talk with PPARgamma. PLoS Genetics 4(6):e1000108.

[52] Haas, E., Bhattacharya, I., Brailoiu, E., Damjanovic, M., Brailoiu, G.C., Gao, X., et al., 2009. Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. Circulation Research 104(3):288–291.

[53] Sharma, G., Hu, C., Brigman, J.L., Zhu, G., Hathaway, H.J., Prossnitz, E.R., 2013. GPER deficiency in male mice results in insulin resistance, dyslipidemia, and a proinflammatory state. Endocrinology 154(11):4136–4145.

[54] Davis, K.E., Carstens, E.J., Irani, B.G., Gent, L.M., Hahner, L.M., Clegg, D.J., 2014. Sexually dimorphic role of G protein-coupled estrogen receptor (GPER) in modulating energy homeostasis. Hormones and Behavior 66(1):196–207.

[55] Guivarc'h, E., Buscato, M., Guihot, A.L., Favre, J., Vessieres, E., Grimaud, L., et al., 2018. Predominant role of nuclear versus membrane estrogen receptor alpha in arterial protection: implications for estrogen receptor alpha modulation in cardiovascular prevention/safety. Journal of the American Heart Association 7(13).

[56] Smirnova, N.F., Fontaine, C., Buscato, M., Lupieri, A., Vinel, A., Valera, M.C., et al., 2015. The activation function-1 of estrogen receptor alpha prevents arterial neointima development through a direct effect on smooth muscle cells. Circulation Research 117(9):770–778.

[57] Khbouz, B., de Bournonville, C., Court, L., Taziaux, M., Corona, R., Arnal, J.F., et al., 2020. Role for the membrane estrogen receptor alpha in the sexual differentiation of the brain. European Journal of Neuroscience 52(1):2627–2645.

[58] Dunn-Meynell, A.A., Sanders, N.M., Compton, D., Becker, T.C., Eiki, J., Zhang, B.B., et al., 2009. Relationship among brain and blood glucose levels and spontaneous and glucoprivic feeding. Journal of Neuroscience 29(21):7015–7022.

[59] Wu, M.V., Tollkuhn, J., 2017. Estrogen receptor alpha is required in GABAergic, but not glutamatergic, neurons to masculinize behavior. Hormones and Behavior 95:3–12.

[60] Mayer, C., Acosta-Martinez, M., Dubois, S.L., Wolfe, A., Radovick, S., Boehm, U., et al., 2010. Timing and completion of puberty in female mice depend on estrogen receptor alpha-signaling in kisspeptin neurons. Proceedings of the National Academy of Sciences of the United States of America 107(52):22693–22698.

[61] Steyn, F.J., Anderson, G.M., Grattan, D.R., 2007. Expression of ovarian steroid hormone receptors in tuberoinfundibular dopaminergic neurones during pregnancy and lactation. Journal of Neuroendocrinology 19(10):788–793.

[62] Roepke, T.A., Qiu, J., Smith, A.W., Ronnekleiv, O.K., Kelly, M.J., 2011. Fasting and 17beta-estradiol differentially modulate the M-current in neuropeptide Y neurons. Journal of Neuroscience 31(33):11825–11835.

[63] Smith, A.W., Bosch, M.A., Wagner, E.J., Ronnekleiv, O.K., Kelly, M.J., 2013. The membrane estrogen receptor ligand STX rapidly enhances GABAergic signaling in NPY/AgRP neurons: role in mediating the anorexigenic effects of 17β-estradiol,. American Journal of Physiology. Endocrinology and Metabolism 305(5):632–640.

[64] Gonzalez-Garcia, I., Martinez de Morentin, P.B., Estevez-Salguero, A., Contreras, C., Romero-Pico, A., Ferno, J., et al., 2018. mTOR signaling in the arcuate nucleus of the hypothalamus mediates the anorectic action of estradiol. Journal of Endocrinology 238(3):177–186.

[65] Zhu, L., Xu, P., Cao, X., Yang, Y., Hinton Jr., A.O., Xia, Y., et al., 2015. The ERalpha-PI3K cascade in proopiomelanocortin progenitor neurons regulates feeding and glucose balance in female mice. Endocrinology 156(12):4474–4491.

[66] Saito, K., He, Y., Yang, Y., Zhu, L., Wang, C., Xu, P., et al., 2016. PI3K in the ventromedial hypothalamic nucleus mediates estrogenic actions on energy expenditure in female mice. Scientific Reports 6:23459.

[67] Park, C.J., Zhao, Z., Glidewell-Kenney, C., Lazic, M., Chambon, P., Krust, A., et al., 2011. Genetic rescue of nonclassical ERalpha signaling normalizes energy balance in obese Eralpha-null mutant mice. Journal of Clinical Investigation 121(2):604–612.

[68] Ueda, K., Takimoto, E., Lu, Q., Liu, P., Fukuma, N., Adachi, Y., et al., 2018. Membrane-initiated estrogen receptor signaling mediates metabolic homeostasis via central activation of protein phosphatase 2A. Diabetes 67(8):1524–1537.

[69] Tamrakar, P., Briski, K.P., 2015. Estradiol regulation of hypothalamic astrocyte adenosine 5'-monophosphate-activated protein kinase activity: role of hindbrain catecholamine signaling. Brain Research Bulletin 110:47–53.

[70] Merchenthaler, I., Lane, M.V., Numan, S., Dellovade, T.L., 2004. Distribution of estrogen receptor alpha and beta in the mouse central nervous system: in vivo autoradiographic and immunocytochemical analyses. Journal of Comparative Neurology 473(2):270–291.

[71] Iwasa, T., Matsuzaki, T., Mayila, Y., Yano, K., Irahara, M., 2017. Developmental changes in hypothalamic SF-1, POMC, and ERalpha mRNA expression and their sensitivity to fasting in male and female rats. Endocrine Journal 64(12):1157–1163.

[72] Golden, N.H., Shenker, I.R., 1992. Amenorrhrea in anorexia nervosa: etiology and implications. Adolescent Medicine 3(3):503–518.

[73] Ohwada, R., Hotta, M., Sato, K., Shibasaki, T., Takano, K., 2007. The relationship between serum levels of estradiol and osteoprotegerin in patients with anorexia nervosa. Endocrine Journal 54(6):953–959.

[74] Traboulsi, S., Itani, L., Tannir, H., Kreidieh, D., El Masri, D., El Ghoch, M., 2019. Is body fat percentage a good predictor of menstrual recovery in females with anorexia nervosa after weight restoration? A systematic review and exploratory and selective meta-analysis. J Popul Ther Clin Pharmacol 26(2): e25–e37.

