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Research Article

PI3K in the VMH Attenuates Diet-Induced Obesity and Participates in the Effects of E2 on Energy Expenditure in Mice

Aline Alves de Jesus,[1] Raoni Conceição Dos-Santos,[2] Isabelle Rodrigues-Santos,[1] Hellen Veida-Silva,[3] Milene Mantovani Mata,[1] Rafaella Eduarda Volpi,[1] Gabriel Henrique Marques Gonçalves,[3] Luiz Carlos Navegantes,[1] Carol Fuzeti Elias,[4] José Antunes-Rodrigues,[1] and Lucila Leico Kagohara Elias[1]

[1]Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP 14049-900, Brazil
[2]Department of Cell and Molecular Biology, Tulane University, New Orleans, LA 70118, USA
[3]Division of Endocrinology and Metabolism, Department of Internal Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, SP 14049-900, Brazil
[4]Department of Molecular & Integrative Physiology, University of Michigan, Ann Arbor, MI 48109, USA

Correspondence: Lucila Leico Kagohara Elias, PhD, Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900—Campus da USP, Ribeirão Preto, SP 14049-900, Brazil. Email: llelias@fmrp.usp.br; or Aline Alves de Jesus, PhD, Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900—Campus da USP, Ribeirão Preto, SP 14049-900, Brazil. Email: alves.alinejesus@gmail.com.

Abstract

Obesity is associated with the development of several illnesses, such as diabetes mellitus, cancer, and cardiovascular diseases. Elucidating the mechanisms of body weight control is important for the development of effective therapeutic strategies against obesity. In response to the action of hormones such as leptin and 17β-estradiol (E2), the ventromedial hypothalamus (VMH) plays an essential role in protection against diet-induced obesity (DIO) through the regulation of food intake and energy expenditure. However, little is known about the intracellular mechanisms involved in these effects. To assess the role of phosphoinositide 3-kinase (PI3K) signaling in neurons that express steroidogenic factor 1 (SF1) in the VMH in energy homeostasis, we used Cre–lox technology to generate male and female mice with specific disruption of the catalytic subunit P110α in SF1 neurons in the VMH. We demonstrated that the conditional knockout of P110α in SF1 neurons in the VMH affects body weight, energy expenditure, and thermogenesis in animals fed a high-fat diet. In addition, we demonstrated that female mice with genetic disruption of PI3K activity in VMH neurons exhibited greater weight gain than their male counterparts. Furthermore, inhibition of PI3K activity in the VMH partially blocked the effects of E2 on body weight regulation, stimulation of energy expenditure, and thermogenesis in female ovariectomized mice. Collectively, our results indicate that PI3K activity in VMH neurons plays a relevant role in protecting against DIO and contributes to the effects of estradiol on energy expenditure in females.

Key Words: VMH, SF1, PI3K, diet-induced obesity, 17β estradiol, energy expenditure

Abbreviations: ARC, arcuate nucleus; BAT, brown adipose tissue; DIO, diet-induced obesity; E2, 17β-estradiol; Erα, estrogen receptor-α; HFD, high-fat diet; KO, knockout; OVX, ovariectomy; PI3K, phosphoinositide-3-kinase; PBS, phosphate-buffered saline; SEM, standard error of the mean; SF-1, steroidogenic factor 1; SNS, sympathetic nervous system; vlVMH, ventrolateral subdivision of the ventromedial hypothalamic nucleus; VMH, ventromedial nucleus of the hypothalamus.

Obesity is characterized by an increase in body weight, especially due to the accumulation of fat mass in adipocytes. Obesity is often caused by a long-term positive energy balance, which occurs due to excess food intake and decreased energy expenditure and is an important risk factor for the development of comorbidities, such as cardiovascular diseases [1], type 2 diabetes mellitus, cancer [2], and musculoskeletal disorders [3]. Body weight is regulated through physiological adjustments that require the integration of various peripheral and central signals, whereas the hypothalamus is the major regulator of energy homeostasis by promoting the control of food intake and energy expenditure [4].

The ventromedial nucleus of the hypothalamus (VMH) plays an important role in the control of body weight by modulating energy expenditure and regulating food intake [5, 6]. The VMH is also characterized by the expression of steroidogenic factor 1 (SF1), which is an essential factor for the development and function of this hypothalamic region and the regulation of energy homeostasis, as demonstrated in animal models in which SF1 is deleted [7-9]. The VMH receives different hormonal inputs involved in the regulation of energy homeostasis. Leptin and 17β-estradiol (E2) activate VMH neurons. Selective deletion of the leptin receptor (LepR) and estrogen receptor-α (ERα) in SF1 neurons exacerbates

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diet-induced obesity (DIO) [5, 6, 10]. However, little is known about the intracellular mechanisms by which VMH neurons regulate energy homeostasis.

Xu and collaborators previously demonstrated that male mice with specific disruption of the catalytic subunit P110α of the enzyme phosphoinositide 3-kinase (PI3K) in the VMH develop obesity and metabolic disturbances when fed a high-fat diet (HFD) [10]. A similar phenotype was also observed in animals with deletion of P110ß in VMH neurons [11]. These findings indicate that the PI3K intracellular signaling pathway in the VMH is required for the regulation of body weight under conditions of excessive energy intake, as occurs with exposure to HFD. However, despite sex differences in the control of the body, the specific role of PI3K subunits in the VMH in explaining this sex difference has not been well established.

Produced by the ovaries, E2 in the central nervous system plays an essential role in maintaining energy balance and body weight control, exerting protective effects against weight gain in both animals and humans [12, 13]. ERα expressed in proopiomelanocortin neurons in the hypothalamic arcuate nucleus (ARC) regulates food intake [14, 15], while neurons in the ventrolateral subdivision of the ventromedial hypothalamic nucleus (vlVMH) modulate 2 components of energy expenditure, spontaneous physical activity and thermogenesis in females [14, 16]. However, the molecular mechanisms by which VMH neurons regulate energy homeostasis through estradiol/ERα signaling have not been fully elucidated. To fill this gap, we investigated the role of PI3K in SF1 neurons of the VMH in terms of body weight control in male and female mice fed a HFD and the participation of PI3K in the effects of E2 on energy homeostasis.

Materials and Methods

Animal Care

All the experimental procedures were approved by the Institutional Animal Care and Use Committee of Ribeirão Preto Medical School (CEUA-FMRP number 076/2019). Mice were housed at 22 to 24 °C under a 12 hours light/12 hours dark cycle. The animals were fed a standard chow diet or a HFD (Research Diets, 5.24 kcal/g, 60% kcal from fat; New Brunswick, NJ) with ad libitum access to water and chow. To generate animals with specific knockout (KO) of the P110α subunit in the SF1 neurons of the VMH (SF1-cre;P110α$^{flox/flox}$), male mice that were homozygous for the floxed P110α allele and heterozygous for the Sf-1-Cre transgene were crossed with female mice homozygous for the floxed p110α allele. Littermate mice homozygous for the floxed P110α allele (P110α$^{flox/flox}$) served as controls (Ctr). For body weight and food intake recordings, animals were individually caged and fed a regular diet or HFD, and body weight and food intake were registered weekly.

Metabolic Cage Studies

Mice were individually housed at room temperature (22-24 °C) under an alternating 12 hours light/12 hours dark cycle. After 2 days of adaptation, oxygen consumption (VO2), carbon dioxide production (VCO2), and locomotor activity (XTOT) were measured for 24 hours using a Comprehensive Laboratory Monitoring System (CLAMS, Columbus Instruments, OH). For standard regular diet experiments, we used body weight–matched animals at 17 weeks of age. For the HFD experiments, we subjected body weight–matched animals at 17 weeks of age to a HFD for 1 week and assessed energy expenditure as described above. The energy expenditure was obtained based on the following formula: $EE = [VO2 \times (3.815 + 1.232 \times RER)]$ [17].

Ovariectomy and 17β-estradiol Treatment

After 3 consecutive estrous cycles, 16-week-old female SF1-cre;P110α$^{flox/flox}$ and P110α$^{flox/flox}$ mice were anesthetized with inhaled isoflurane and subjected to bilateral ovariectomy (OVX). Half of these mice received subcutaneous implantations of pellets releasing 17β-estradiol (0.18 mg for 60 days OVX + E; Innovative Research of America) following ovariectomy. The animals were fed with chow diet and had the body weight and food intake monitored daily.

Calorimetry Assessment: Tail and Brown Adipose Tissue Temperature

After 10 days post OVX-surgery, between 8:00 AM and 9:00 AM, a subset of female mice (n = 6 per treatment and genotype) underwent analyses of thermograph and indirect calorimetry. The tail and brown adipose tissue (BAT) temperatures of the free-moving mice were assessed through infrared images using a thermogenic camera (FLIR E6; FLIR Systems, Inc., Wilsonville, OR, USA). Images were analyzed using FLIR Tools software. All images were obtained at a distance of 1 m between the researcher and the animals. Subsequently, the animals were habituated to the metabolic chambers. After an adaptation period of 2 days, measurements of VO2 consumption, VCO2 production, energy expenditure, and locomotor activity were taken over 1 day. At the end of the experiment, the animals were anesthetized and euthanized to collect BAT for the analysis of thermogenic markers.

Blood Sample Collection

The animals were previously subjected to isoflurane inhalation. Subsequently, the mice were decapitated for blood collection. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure plasma leptin (R&D Systems, Minneapolis, MN), and an in-house radioimmunoassay was used for testosterone and corticosterone measurements, as previously described by Borges et al [18]. Plasma and tissue noradrenaline concentrations were measured using high-performance liquid chromatography (HPLC) as previously described by Garofalo et al [19].

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

BAT samples were frozen under RNAse-free conditions for total RNA extraction. The procedure was performed using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The quantity and purity of the mRNA were verified via a spectrophotometer (SpectraMax® i3x Multi-Mode Microplate Reader). Absorbance ratios of 260/280 and 260/230 nm were used to determine the purity DNase I treatment (Invitrogen) was performed using 1 µg of total RNA extracted to avoid DNA contamination. A commercial high-capacity cDNA reverse transcription kit (Applied Biosystems®) was used to synthesize complementary DNA (cDNA) from 500 ng of RNA. Quantitative real-time polymerase chain

reaction (qPCR) was performed in triplicate using the following Taqman® assays (Applied Biosystems®): *Ucp1* (Mm01244861_m1), *Ppargc1a* (Mm01208835_m1), *Ppara* (Mm00440939_m1), *Cidea* (Mm00432554_m1) and ACTB (actin beta-4352341E) as endogenous control genes in the StepOne Plus system (Applied Biosystems®). The threshold cycle (Ct) was used to calculate the relative expression of the target gene using the ΔΔCt method.

Immunofluorescence

The animals were anesthetized and subjected to stereotaxic surgery for implantation of a cannula in the lateral ventricle (anteroposterior: −0.5 mm; lateral: −1.0 mm; vertical: −2.5 mm), following the coordinates of the atlas of Paxinos and Franklin (2008). After 7 days of recovery, the animals were fasted for 12 hours and subsequently received ICV injection of insulin (4.4 mU/2 μL) [20]. Thirty minutes after the central injection of insulin, the animals were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight) and subjected to cardiac perfusion for the brain collection. Transcardiac perfusion was initiated with the infusion of 50 mL of PBS solution (0.1M), followed by the infusion of 100 mL of 4% paraformaldehyde in PBS (pH 7.2). The tissue was post-fixed in 4% paraformaldehyde for 60 minutes and, after this period, stored in a 30% sucrose solution at 4 °C.

Twenty-five micrometer coronal brain sections were used for the *P*-AKT labeling. Sections were blocked in 0.01M PBS containing 10% normal horse serum, 0.1% Triton X-100 and 0.04% NaN3 for 2 hours at room temperature. Briefly, sections were incubated with the anti-phospho-AKT (Thr308) primary antibody [1:1000] (rabbit anti-pAKT, Cell Signaling # 2965. RRID: AB_2255933) for 24 hours at 4 °C. After rinsing, the sections were incubated with a biotinylated donkey anti-rabbit secondary antibody (Alexa Fluor 594 [1:500] 1:200; ab150076. RRID: AB_2782993) for 1 hour at room temperature. Images were obtained with a Leica TCS SP5 confocal microscope system equipped with a 488-nm (argon-krypton) laser. For each group, all images were obtained at identical acquisition settings.

Statistical Analysis

The data obtained are expressed as the mean ± standard error of the mean (SEM) and were analyzed using GraphPad Prism Software version 8.02. For two-group comparisons, two-tailed Student *t* tests were used. The analyses were also performed by two-way analysis of variance (ANOVA), followed by the Sidak or Tukey post hoc test. The significance level was set at $P < 0.05$.

Results

Generation of VMH Neurons Lacking the Catalytic Subunit P110α of PI3K

To generate animals with deletion of the catalytic subunit P110α of PI3K specifically in SF1 neurons, we sequentially crossed SF1-cre mice with P110α^{flox} animals to generate SF1-cre;P110α$^{flox/flox}$ mice. For the control animals, we used P110α$^{flox/flox}$ littermates. The deletion of P110α in SF1 neurons in the VMH was confirmed by immunohistochemistry for phosphorylated AKT (pAKT), considered the pathway recruited and activated through the action of PI3K. We found that central insulin stimulation in P110α$^{flox/flox}$ animals promoted AKT phosphorylation in the VMH and adjacent regions, such as the ARC. On the other hand, in SF1-cre; P110α$^{flox/flox}$ mice, there was a reduction in AKT phosphorylation in the VMH (Fig. 1A).

SF1 is also expressed in other regions, such as the pituitary, adrenal gland, and gonads, and is important for the regulation of metabolism [21]. Thus, we analyzed the effect of P110α deletion on the weight of the adrenal gland, testis, seminal vesicle, ovary, and uterus. The deletion of P110α in SF-1 neurons promoted a reduction in testis weight (Fig. 1B, $P < .001$). However, no change in seminal vesicle weight was observed (Fig. 1C), but we found similar values of plasma testosterone between SF1-cre, P110α$^{flox/flox}$, and P110α$^{flox/flox}$ mice at 20 weeks of age (Fig. 1D), indicating that gonadal activity was preserved. There were no differences in uterine or ovarian weight between the experimental groups (Fig. 1G-1H). No differences in adrenal weight or plasma corticosterone concentrations were observed between SF1-cre, P110α$^{flox/flox}$, and P110α$^{flox/flox}$ in the male (Fig. 1E-1F) and female (Fig. 1I-1J) groups. However, as a limitation of this study, histological analysis of the adrenal gland to fully characterize the adrenal phenotype was not performed.

Deletion of the P110α Catalytic Subunit in SF1 Neurons of the VMH Impairs Energy Homeostasis in Animals Fed a HFD in a Sex-Specific Pattern

The reduction in PI3K activity in SF1 neurons did not affect body weight or food intake in male mice (Supplementary Fig. S1A-S1C [22]) fed a regular chow diet. There was also no difference between genotypes in terms of body weight in female mice (Supplementary Fig. S1D-S1E [22]). In contrast, compared with control mice, chow-fed female SF1-p110α-KO mice exhibited modest but significant increases in food intake (Supplementary Fig. S1F [22]; $P < .05$). Both male and female SF1-cre;P110α$^{flox/flox}$ mice fed a regular chow diet presented similar liver weight, white adipose tissue, BAT weight, and plasma leptin levels compared to those of the respective P110α$^{flox/flox}$ group (Supplementary Fig. S2A-S2D [22]).

Compared with P110α$^{flox/flox}$ control animals, SF1-cre; P110α$^{flox/flox}$ male and female mice fed a HFD presented a marked increase in body weight (Fig. 2A-2B; Fig. 2D-2E; $P < .05$). However, no difference was observed in food intake (Fig. 2C and 2F). Interestingly, there was a gender difference in the body weight gain induced by HFD in SF1-cre; P110α$^{flox/flox}$ mice, with it being higher in females than males (30% vs 24%), compared to respective control P110α$^{flox/flox}$ mice treated with HFD. In addition, in contrast to males (Supplementary Fig. S2E-S2F [22]), females with reduced PI3K activity in the VMH showed an increase in liver weight, retroperitoneal white adipose tissue weight (Supplementary Fig. S2G [22]; $P < .01$ and $P < .05$, respectively) and plasma leptin concentration (Supplementary Fig. S2H [22]; $P < .001$).

Deletion of the P110α Catalytic Subunit in SF1 Neurons in the VMH Reduces Energy Expenditure and Thermogenesis in Male and Female Mice Fed a HFD

After 1 week of HFD, the SF1-cre;P110α$^{flox/flox}$ male mice exhibited decreased indirect caloric activity during the light and

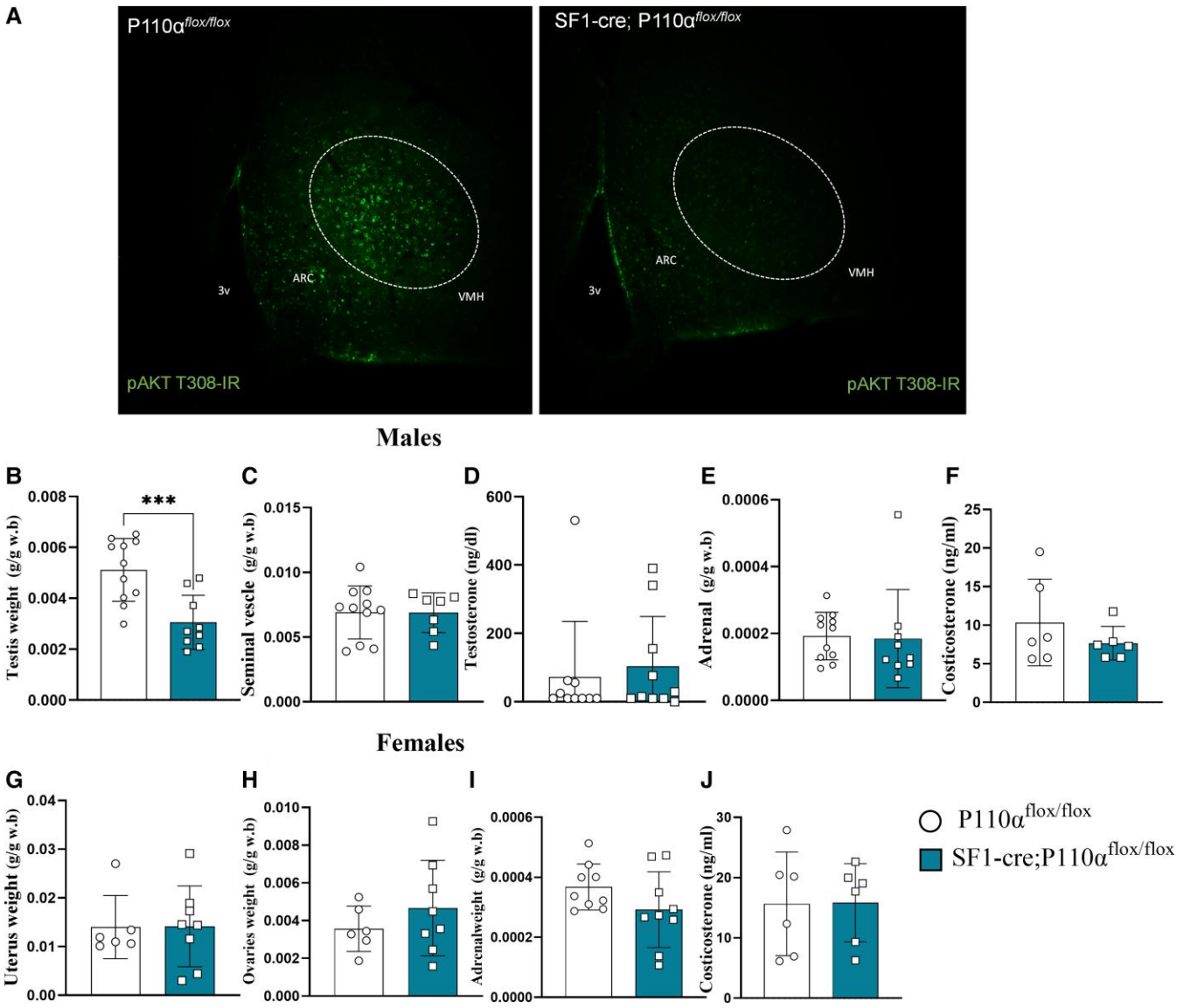

Figure 1. Validation of deletion of catalytic subunit P110α of PI3K in VMH neurons. (A) Representative images of phosphorylated AKT (pAKT) in the medio-basal hypothalamus 30 minutes after insulin injection (intracerebroventricular 4.4 mU/2 μL) in SF1-cre;P110α$^{flox/flox}$ and P110α$^{flox/flox}$ mice. Testis weight (B; n = 10); seminal vesicle weight (C; n = 10); plasma testosterone levels (D; n = 10/group); adrenal weight (male: E; n = 7 plasma corticosterone levels (male: F; n = 6-7); uterine (G; n = 6-7/group) and ovary weight of females in estrus (H; n = 6-7/group); adrenal weight (I; n = 8-11/group); plasma corticosterone levels (female: J; n = 6). Tissue weights in ratio to total body weight. SF1-cre;P110α$^{flox/flox}$ and P110α$^{flox/flox}$ mice treated with regular diet at 20 weeks of age. Values are shown as mean ± SEM and the two-tailed Student *t* test was used for two-group comparisons; *P**** < .0001 between groups.

dark cycle (Fig. 3A-3D, $P < .05$) compared to the P110α$^{flox/flox}$ mice with paired body weights. No significant differences were observed between the experimental groups regarding locomotor activity (Fig. 3E). In SF1-cre;P110α$^{flox/flox}$ females, we observed reduced oxygen consumption and energy expenditure during the dark cycle (Fig. 3F-3G and Fig. 3I; $P < .001$ and $P < .05$). No differences were observed in VCO2 production or locomotor activity in females (Fig. 3H and 3J).

Using thermography, we also analyzed the tail temperature of males and females with a reduction in PI3K activity in SF1 neurons, and no difference was found (Fig. 4A-4B) compared with that of the respective controls. However, compared with P110α$^{flox/flox}$ mice, both female and male SF1-cre;P110α$^{flox/flox}$ mice fed a HFD presented a reduction in the skin temperature around the BAT (Fig. 4C, $P < .001$ and $P < .05$). Furthermore, we analyzed the mRNA expression of thermogenesis markers in BAT. There was a reduction in the expression of the uncoupling protein 1 (UCP-1) gene in the SF1-cre; P110α$^{flox/flox}$ male mice, but there was no difference in the expression of other thermogenesis markers, such as *Cidea, Ppara, and Ppargc1a* (Fig. 4D, $P < .001$). Female mice presented a reduction in the expression of *Ucp1* and *Cidea* in BAT, and the expression of *Ppara* and *Ppargc1a* was similar between the genotypes in females (Fig. 4E $P < .05$). Taken together, these results demonstrate that deletion of the catalytic subunit P110α in SF1 neurons reduces BAT thermogenesis.

Deletion of the P110α Catalytic Subunit in SF1 Neurons in the VMH Partially Reduces the Effects of 17β-Estradiol on Body Weight Control in OVX Females

Since the deletion of PI3K in SF-1 neurons increased the weight gain in response to HFD in females, we hypothesized

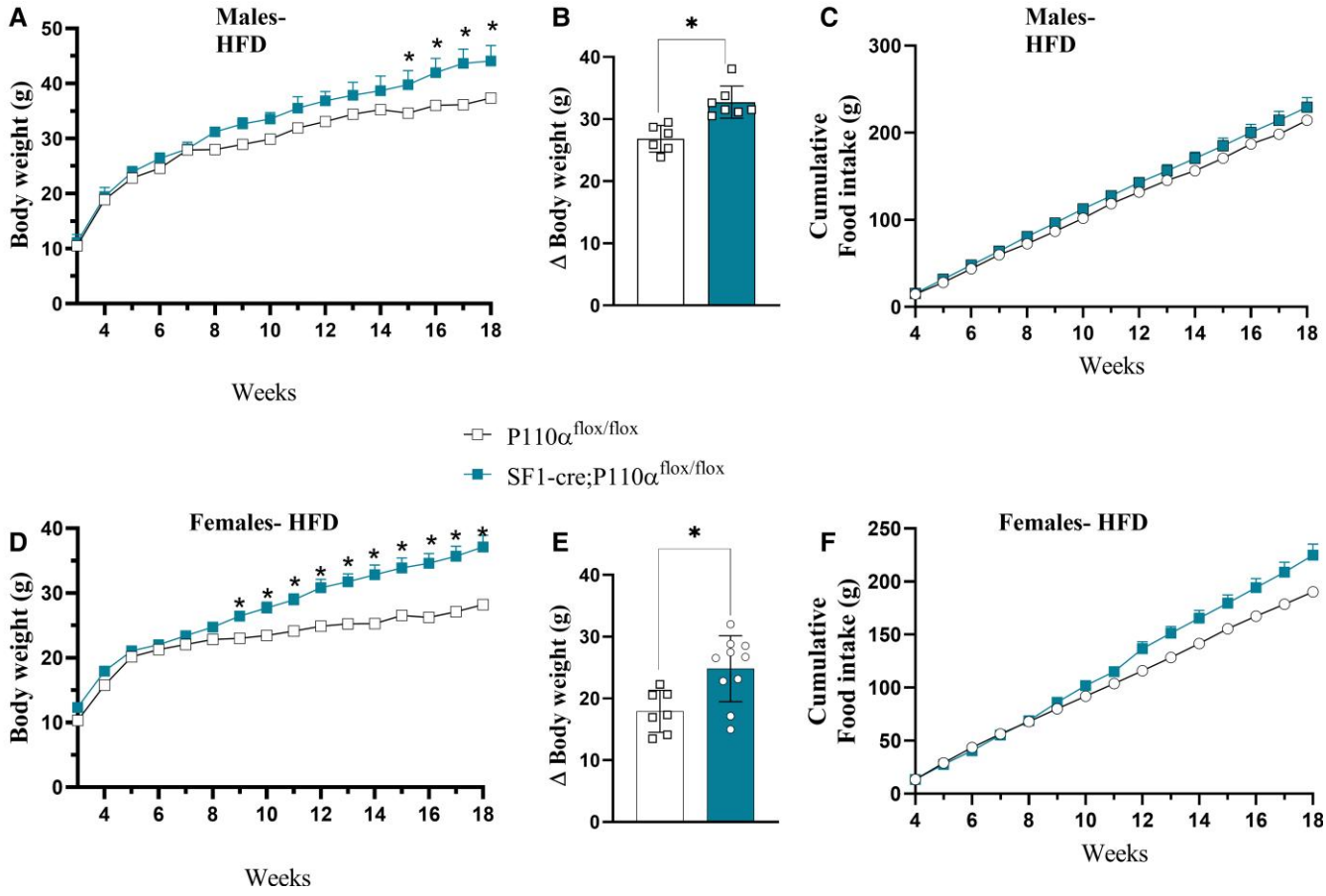

Figure 2. Deletion of P110α catalytic subunit in SF1 neurons of the VMH impairs energy homeostasis in animals fed a HFD in a sex-specific pattern. Male body weight (A; n = 6/group), body weight delta (B; n = 6/group), cumulative food intake (C; n = 6/group); female body weight (D; n = 7-9/group), body weight delta (E; n = 7-9/group), cumulative food intake (F; n = 7-9/group). Data in A, C, D and F panels are shown as means ± SEM and differences between groups were determined by two-way ANOVA followed by Sidak post hoc test; $*P < .05$.

that PI3K could mediate the effects of E2 on body weight balance. To investigate this interaction, ovariectomized female mice of both genotypes were studied with or without E2 treatment. In both $P110\alpha^{flox/flox}$ and SF1-cre; $P110\alpha^{flox/flox}$ mice, OVX + E2 treatment significantly reduced body weight compared to that of the respective OVX mice. However, the body weight–lowering effects of estradiol treatment were significantly lower in SF1-cre;$P110\alpha^{flox/flox}$ mice than in $P110\alpha^{flox/flox}$ mice (Fig. 5A-5B; $P < .05$). Furthermore, we found no difference in food intake between the experimental groups (Fig. 5C).

To confirm the efficacy of the E2 treatment used in the experiments, the uteri were weighed as an index of the circulating E2 concentration. Uterine weight was greater in both $P110\alpha^{flox/flox}$ and SF1-cre;$P110\alpha^{flox/flox}$ OVX animals that received chronic treatment than in untreated animals (Supplementary Fig. S3A [22]; $P < .001$). To characterize metabolic phenotypes, we also analyzed adiposity. We observed no difference in liver weight between the groups (Supplementary Fig. S3B [22]). On the other hand, E2 treatment was able to reduce adiposity only in the $P110\alpha^{flox/flox}$ group (Supplementary Fig. S3C [22]; $P < .001$), indicating a reduced effect of E2 treatment in SF1-cre;$P110\alpha^{flox/flox}$ mice. No difference in the weight of inguinal fat tissue or BAT was found between the experimental groups (Supplementary Fig. S3D-S3E [22]).

Indirect calorimetry demonstrated that energy expenditure (Fig. 6A-6E), VO2 (Supplementary Fig. S4A-S4E [22]) and VCO2 (Supplementary Fig. S5A-S5E [22]) throughout the 24-hour period were increased in $P110\alpha^{flox/flox}$ OVX mice that received E2 treatment. However, estradiol treatment did not promote an increase in indirect calorimetry in SF1-cre;$P110\alpha^{flox/flox}$ mice. No difference in locomotor activity was observed between the groups (Supplementary Fig. S6A-S6E [22]).

Deletion of the P110α Catalytic Subunit in SF1 Neurons in the VMH Reduces the Effects of 17β-Estradiol on Thermogenesis in OVX Females

Activation of VMH SF1 neurons promotes sympathetic modulation in BAT, promoting increased heat production through thermogenesis [23]. Thus, we analyzed the effect of P110α deletion in VMH neurons on BAT thermogenesis. We observed that, compared with those in the OVX group, the skin temperature around the BAT was greater in $P110\alpha^{flox/flox}$ OVX mice treated with E2. However, remarkably, this response was not observed in SF1-cre;$P110\alpha^{flox/flox}$ mice that received estradiol replacement (Fig. 7A-7B, $P < .05$). Furthermore, there was no difference in tail temperature between the experimental groups (Fig. 7C). To investigate the participation of sympathetic activity in thermogenesis, we

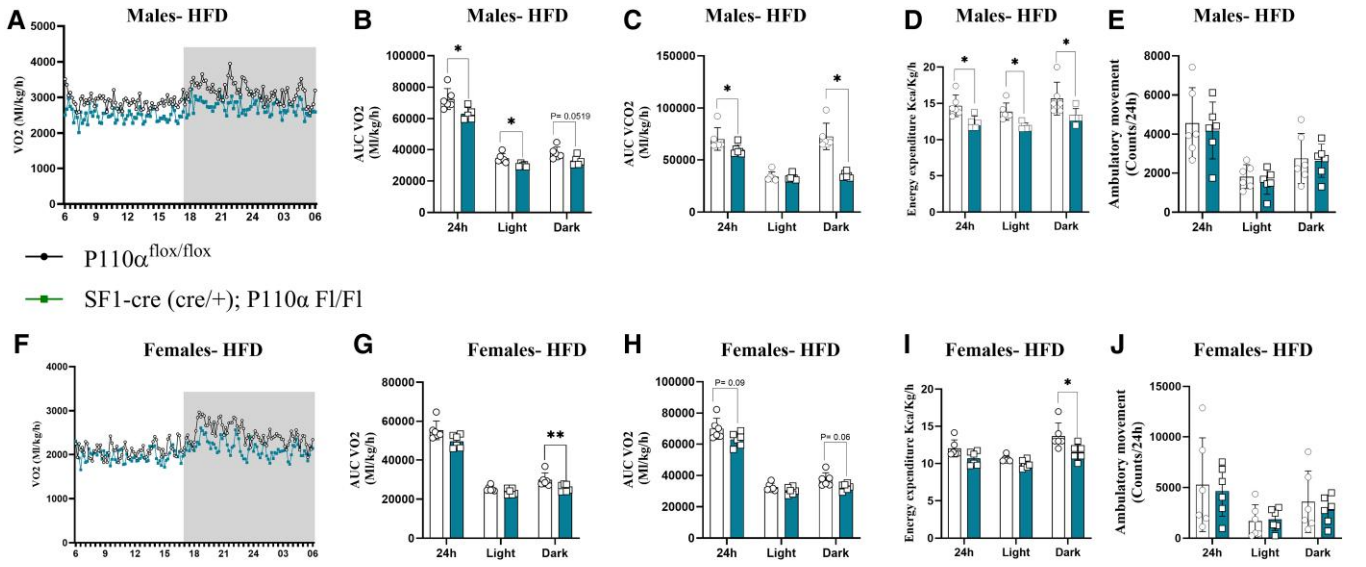

Figure 3. Deletion of P110α catalytic in SF1 neurons of the VMH decreases energy expenditure in male and female mice fed with a HFD. Oxygen consumption (VO2) over a 24 hours period (A; n = 6/group); area under the curve (AUC) of VO2 (B; n = 6-7/group); AUC of carbon dioxide production (VCO2) average over a 24-hour period (C; n = 6-7/group); ambulatory movement (D; n = 6/group); energy expenditure (E; n = 6-7/group) in male mice. VO2 over a 24 hours period (F; n = 6/group); under the curve (AUC) of VO2 (G; n = 6-7/group); AUC of carbon dioxide production (VCO2) average over a 24 hours period (H; n = 6-7/group); ambulatory movement (I; n = 6/group); energy expenditure (J; n = 6-7/group) in female mice. Data shown as mean ± SEM and the two-tailed Student *t* test was used; * $P < .05$.

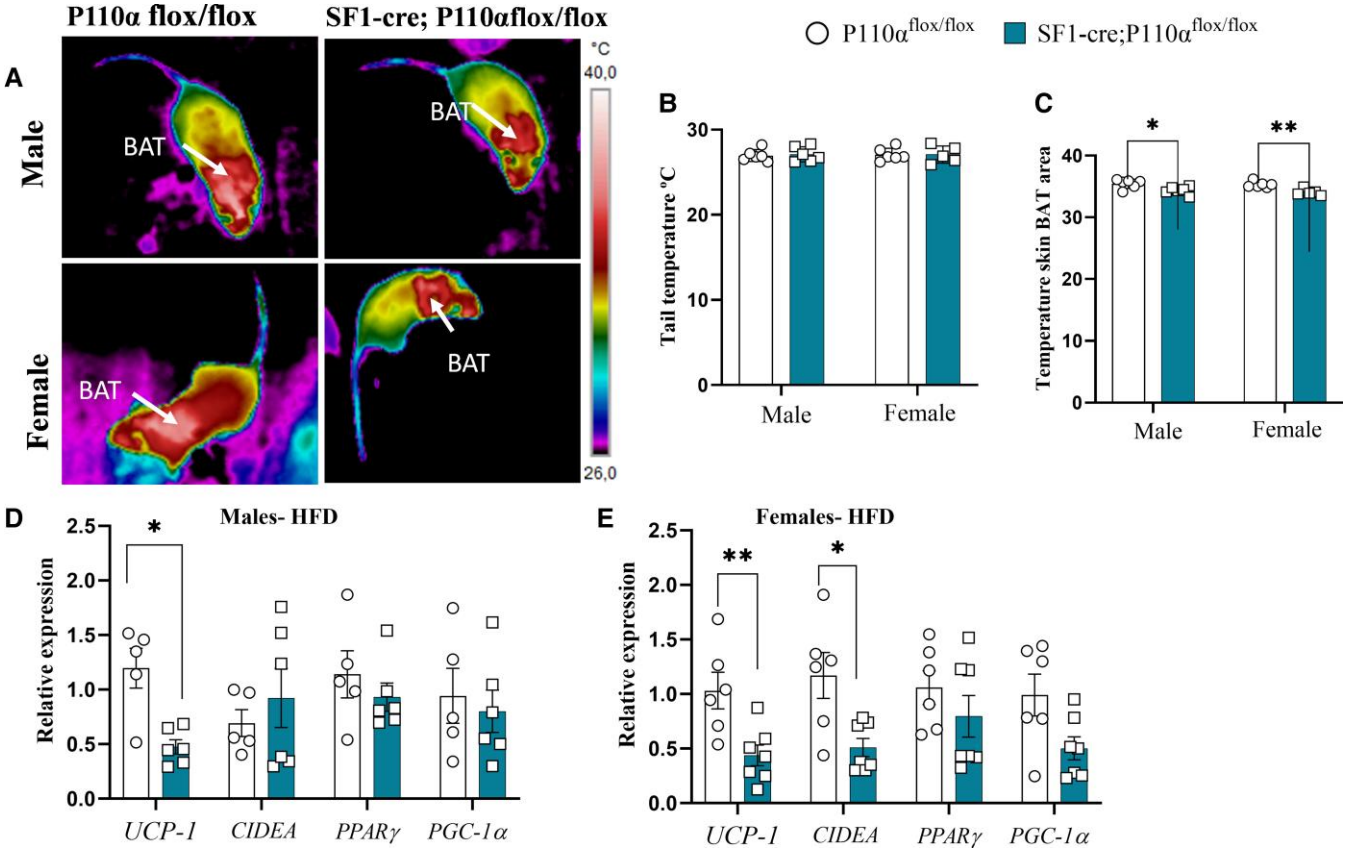

Figure 4. Deletion of the P110α catalytic subunit in SF1 neurons in the VMH reduces BAT temperature and thermogenic markers in male and female mice fed a HFD. Thermographic images of SF1-cre;P110α$^{flox/flox}$ and P110α$^{flox/flox}$ male and female mice (A); Tail temperature (B; n = 6/group) and skin temperature in the BAT area at the dark circle (C; n = 6/group). mRNA relative expression of *Ucp1, Cidea, Ppara, Ppargc1a* in the BAT of male (D; n = 5-7/group) and female SF1-cre;P110α$^{flox/flox}$ and P110α$^{flox/flox}$ mice (E; n = 6-7/group). Data shown as mean ± SEM and the two-tailed Student *t* test was used; ** $P < .001$ and * $P < .05$ between groups.

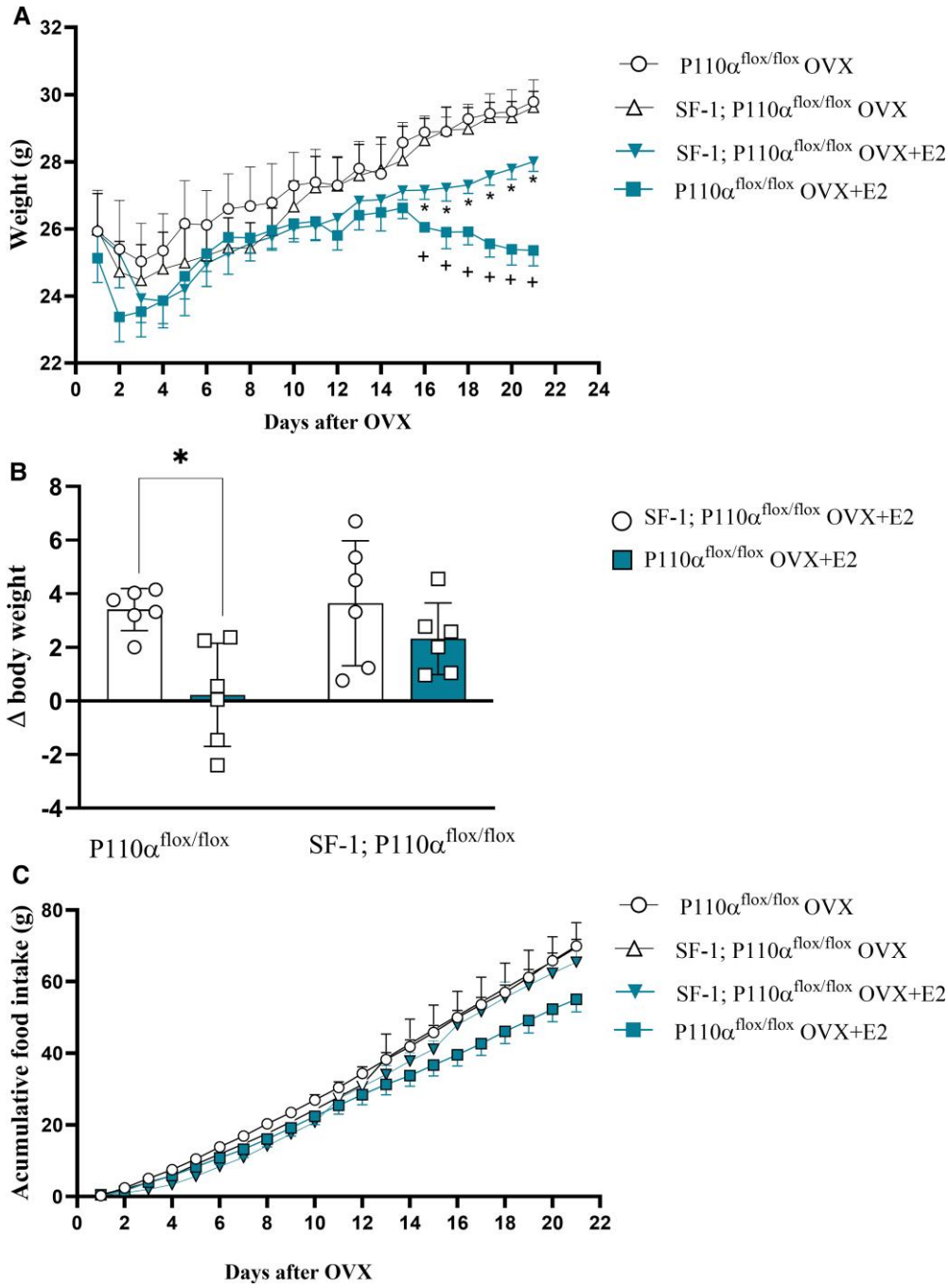

Figure 5. Deletion of the P110α catalytic subunit in SF1 neurons in the VMH partially reduces the effects of 17β-estradiol on body weight control in OVX females. Body weight (A; n = 6/group); body weight delta (B; n = 6/group) and cumulative food intake (C; n = 6/group) in OVX control or treated with 17β-estradiol in SF1-cre;P110α$^{flox/flox}$ and P110α$^{flox/flox}$ female mice fed with regular diet. Data shown as mean ± SEM. *P** < .05 between groups determined by two-way ANOVA followed by Sidak post hoc test.

analyzed plasma and tissue concentrations of noradrenaline. There was no change in the circulating noradrenaline concentration (Fig. 7D). In the BAT, ovariectomized P110α$^{flox/flox}$ mice that received hormone replacement with E2 showed an increase in noradrenaline concentrations. In contrast, in mice with reduced PI3K activity in the VMH, E2 was not able to increase sympathetic activity in the BAT (Fig. 7E; $P < .05$). There was no difference between the groups in terms of noradrenaline concentrations in inguinal white adipose tissue (Fig. 7F).

We analyzed the gene expression of thermogenesis markers such as UCP1, CIDEA, PPARγ, and PGC1α. The results showed that, in the P110α$^{flox/flox}$ OVX + E2 female mice, there was an increase in the expression of the *Ucp1* and *Cidea* genes in the BAT compared to that in the P110α$^{flox/flox}$ group (Fig. 8A-8B; $P < .05$). On the other hand, this response was not observed in mice with reduced PI3K activity treated with E2 (Fig. 8A-8B). No difference was observed in the relative expression of *Ppara* or *Ppargc1* between the experimental groups (Fig. 8C-8D).

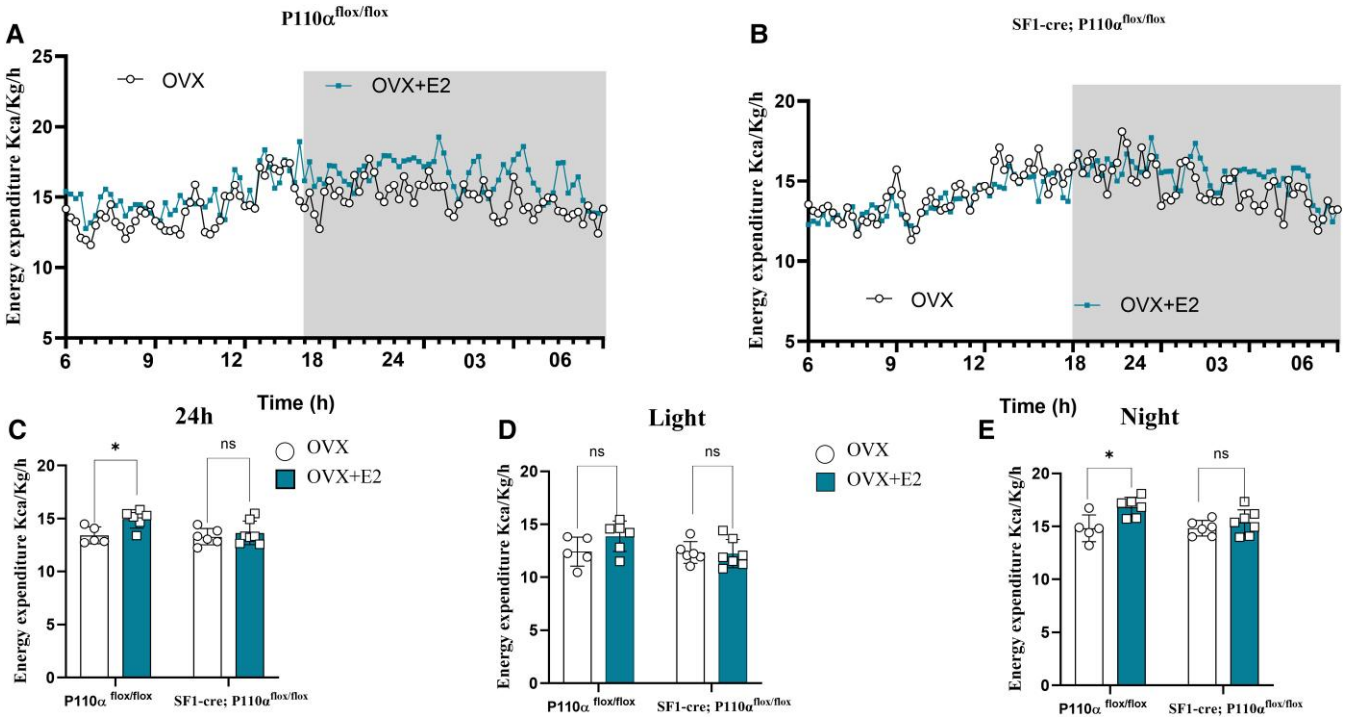

Figure 6. Deletion of the P110α catalytic subunit in SF1 neurons in the VMH attenuates the effects of 17β-estradiol in increasing energy expenditure. Energy expenditure in OVX or OVX + E-treated P110α$^{flox/flox}$ (A) and SF1-cre;P110α$^{flox/flox}$ female mice (B; n = 6/group). Energy expenditure during the 24-hour period (C; n = 6/group), the light cycle (D; n = 6/group) or the dark cycle (E; n = 6/group) in all 4 groups. Data are presented as mean ± SEM. *$P < .05$ between OVX and OVX + E the two-tailed Student *t* test was used.

Discussion

The VMH plays an important role in protecting against diet-induced obesity (DIO) by affecting food intake and energy expenditure [5, 6, 10, 24]. However, the intracellular mechanisms involved in these actions are still poorly understood. Here, we assessed the effect of specific deletion of the catalytic subunit P110α of PI3K in SF1 neurons of the VMH on body weight, food intake, energy expenditure, and BAT thermogenesis in male and female mice. We found that a decrease in PI3K activity in the VMH causes impairment of energy homeostasis in animals fed a HFD in a sex-specific pattern.

The dysregulation of energy balance observed in animals with P110α deletion in SF1 neurons when subjected to a HFD demonstrated the role of the VMH in controlling body weight in the context of a positive energy balance. Different experimental models with genetically modified animals resulted in similar effects. Deletion of the leptin receptor (LepR) in SF1 neurons of the VMH, for example, causes an increase in the body weight of animals only under HFD conditions [5, 6]. The same response was observed with the deletion of molecules such as ERα and sirtuin 1 (SIRT1) or STAT3, specifically in SF1 neurons of the VMH [14, 24, 25]. All these findings emphasize the role of SF1 neurons in promoting protective effects on body weight gain in situations of increased caloric consumption. In this context, our results reinforce the important role of the PI3K signaling pathway in the protective effects of the VMH against DIO. Furthermore, the present study indicated that PI3K mediates protection against DIO via VMH neurons by increasing energy expenditure.

Xu et al demonstrated for the first time that the deletion of P110α in SF1 neurons promotes increased body weight and reduced metabolism in male mice fed a HFD. Furthermore, they showed that the effect of leptin in reducing food intake was significantly blunted in male P110α knockout mice [10]. Considering these findings, we can hypothesize that the reduction in PI3K activity in SF1 VMH neurons contributes to the reduced effect of leptin on energy homeostasis.

SF1 knockout mice develop obesity mainly because they have a reduction in energy expenditure [9]. In the present study, we showed that the body weight gain of male and female mice lacking P110α in the VMH is not attributed to an increase in food intake but rather to a reduction in energy expenditure, similar to what has been observed in SF1 knockout mice. We found a decrease in energy expenditure but no change in locomotor activity in animals with a reduction in PI3K activity in the VMH, so we hypothesize that thermogenesis could be reduced.

The results shown here revealed that in both sexes, deletion of P110α in SF-1 neurons reduced the skin temperature around the BAT and decreased *Ucp1* mRNA expression, indicating a reduction in thermogenesis. Previous reports have indicated that sympathetic nervous system (SNS) input is necessary to maintain the thermogenic capacity of BAT [26]. Moreover, the activation of VMH SF1 neurons promotes sympathetic modulation of BAT thermogenesis [22]. Disruption of SNS signaling promotes the whitening of BAT accompanied by a reduction in mitochondrial activity and the accumulation of lipid droplets [27]. In fact, DIO mice exhibit impaired SNS activity and BAT whitening. In addition, previous studies have shown that lesions in the VMH cause mitochondrial dysfunction and reduce fatty acid oxidation [28, 29], indicating that an intact VMH is important for maintaining BAT function. Our results showed that impairment of

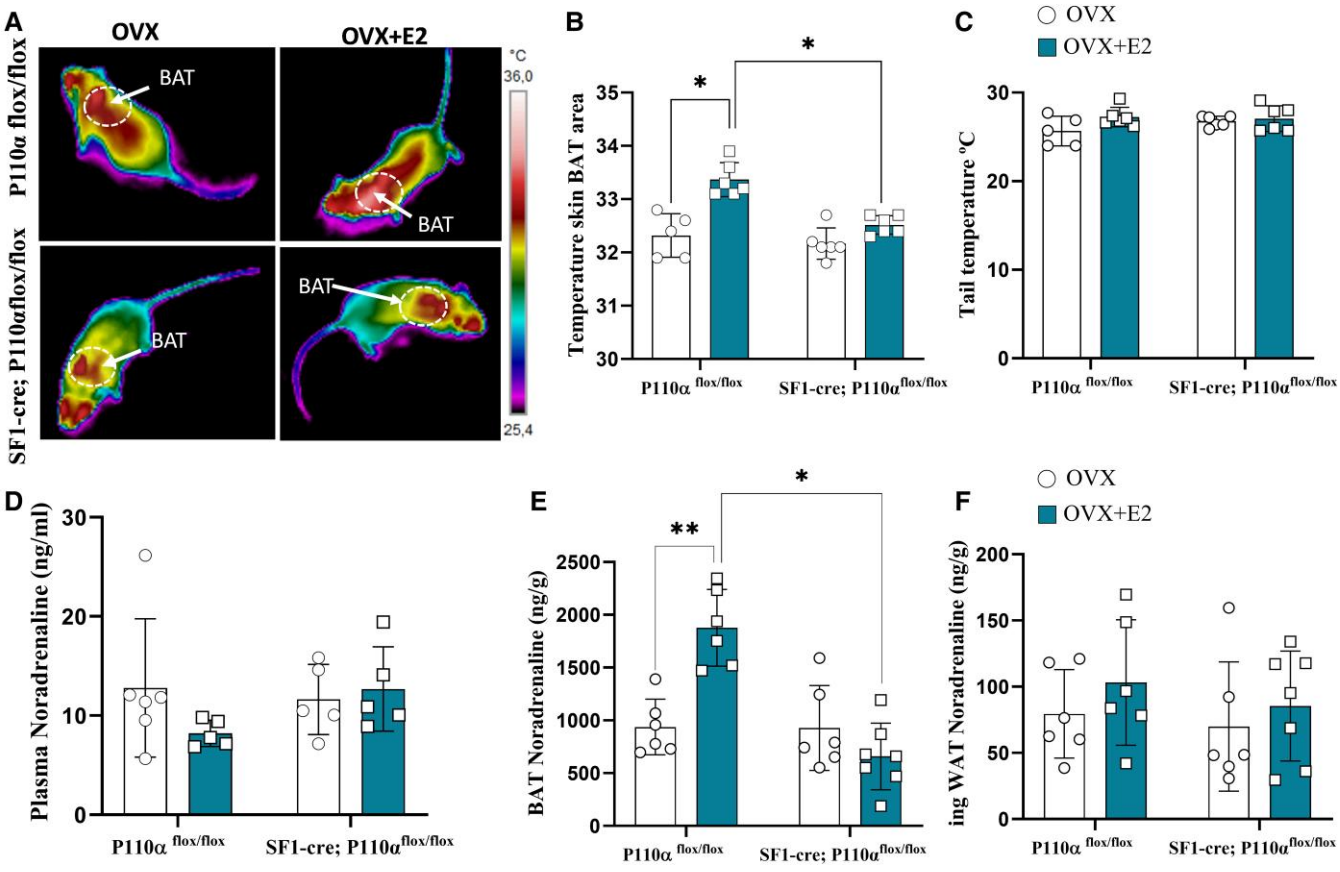

Figure 7. Deletion of the P110α catalytic subunit in SF1 neurons in the VMH attenuates the effects of 17β-estradiol on sympathetic activity and thermogenesis in the BAT. Thermographic images of SF1-cre;P110α$^{flox/flox}$ and P110α$^{flox/flox}$ OVX mice with or without treatment with estradiol (n = 6/group) (A); Temperature skin BAT area (B) and tail temperature (C). Plasma noradrenaline levels (D; n = 6/group), noradrenaline concentration in the BAT (E; n = 6/group) and inguinal white adipose tissue (F; iWAT, n = 6/group). Data shown as mean ± SEM. *$P < .05$ between groups determined by two-way ANOVA followed by Tukey post hoc test.

PI3K activity in VMH neurons reduces BAT temperature and the expression of thermogenesis markers in HFD-fed males and females, indicating that P110α in the VMH might be a critical component of SNS inputs to BAT and thermogenesis.

Our results demonstrate the importance of the PI3K pathway in the VMH in attenuating DIO and corroborate the findings of previous studies carried out by Xu et al [10]. Furthermore, our findings show for the first time that, compared with male mice, female mice fed a HFD exhibit a greater increase in body weight and adiposity and a greater reduction in PI3K activity in the VMH. We previously showed that, under an obesogenic diet, a reduction in STAT3 signaling, considered to be one of the main pathways recruited by leptin in the VMH, promotes greater body weight gain in female mice than in male mice [24]. Taken together, these data suggest that females challenged with a HFD become more susceptible to metabolic disturbances when the activity of specific intracellular signaling pathways, such as STAT3 and PI3K, in the VMH is impaired.

Sex differences in energy homeostasis are well established. Here, we found that the impairment of body weight and adiposity in SF1-cre; P110α$^{flox/flox}$ mice was more prominent in female mice fed HFD. A lack of estradiol after menopause is associated with an increased likelihood of developing obesity and type 2 diabetes [30]. In experimental animals, a decrease in the circulating level of E2 after ovariectomy leads to the development of hyperphagia, obesity, and hyperglycemia, which are reversed by estradiol replacement [31, 32]. In addition, the VMH is considered one of the main sites of action of ovarian hormones in energy homeostasis since the deletion of ERα in SF1 neurons increases body weight in females fed a HFD [14].

The signaling pathway involved in the actions of E2 on energy homeostasis is not fully understood. However, a study carried out by Malyala et al [33] demonstrated through in situ hybridization that E2 increases the mRNA expression of the catalytic subunit of PI3K in the VMH. Furthermore, a single subcutaneous injection dose of E2 significantly increases AKT phosphorylation in the VMH but not in the ARC [34]. Our results showed that a reduction in PI3K activity in the VMH partially reduces the effect of E2 on reducing body weight and adiposity and increasing energy expenditure in ovariectomized female mice. Our results reinforce the findings of Saito and collaborators, who similarly demonstrated that a reduction in PI3K activity in SF1 neurons partially reduces the effect of E2 on the control of energy homeostasis [35].

Stimulation of ERα neurons in the vlVMH region via chemogenetics promotes increased BAT activity and heat production in male and female mice, suggesting that E2 acts via the VMH to increase energy expenditure [36]. Our results demonstrate for the first time that without preserved PI3K activity in SF1 neurons, E2 treatment is ineffective at increasing sympathetic activity and BAT temperature. These data are reinforced by the findings of a recent study by Ye et al [37], which revealed a pathway downstream of ERα vlVMH to 5-HT neurons in the dorsal raphe nucleus that increases BAT

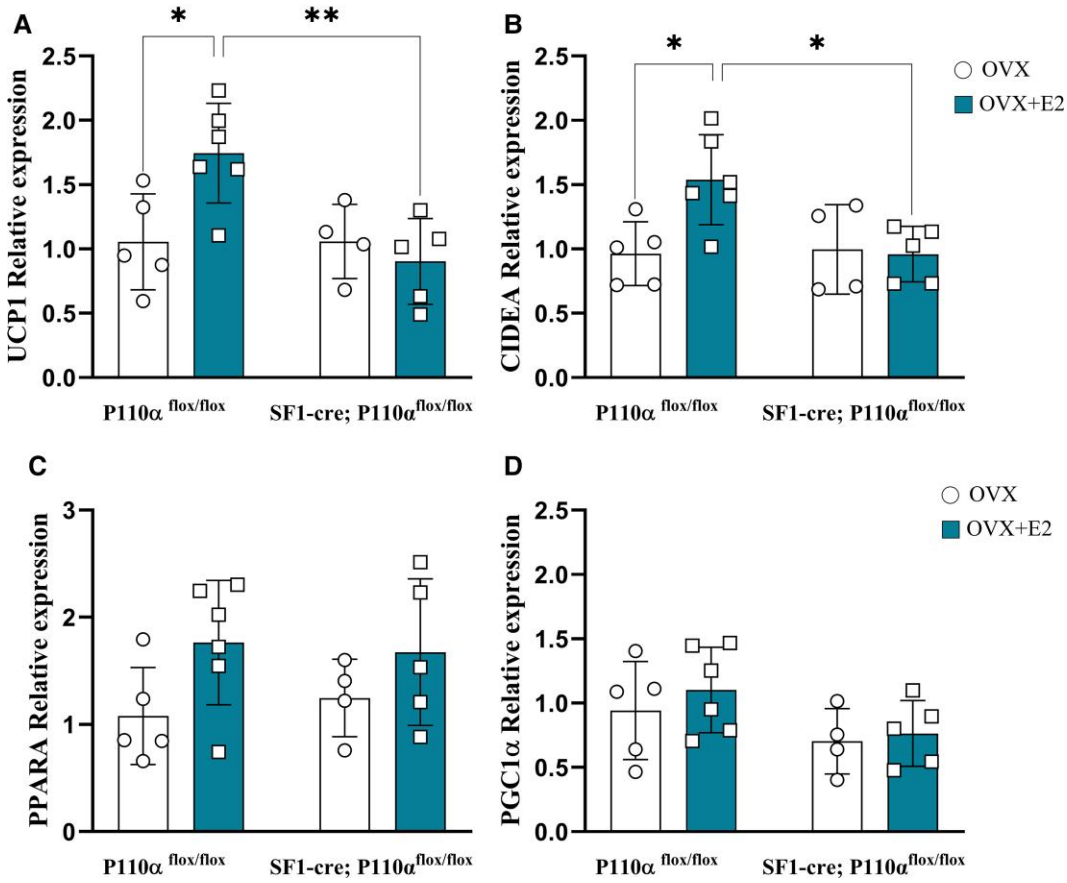

Figure 8. Deletion of the P110α catalytic subunit in VMH SF1 neurons reduces the effects of 17β-estradiol on gene expression of thermogenesis markers in OVX females. Relative mRNA expression of *Ucp1* (A; n = 5-6/group), *Cidea* (B; n = 5-6/group), *Ppara* (C; n = 5-6/group), and *Ppargc1a* (D; n = 5-6/group) in the BAT of female P110α$^{flox/flox}$ and SF1-cre;P110α$^{flox/flox}$ mice with or without hormone replacement with E2. Data shown as mean ± SEM. *$P < .05$ between groups, determined by two-way ANOVA followed by Sidak post hoc test.

thermogenesis. Considering these findings from the literature, we can hypothesize that PI3K signaling might be recruited during the ERα-mediated activation of high-order hypothalamic circuitry involved in the control of energy expenditure.

In summary, our study showed that the PI3K pathway plays a crucial role in protection against DIO via VMH neurons. Our results highlight that loss of the catalytic subunit P110α of PI3K in VMH neurons reduces BAT temperature and energy expenditure in HFD-fed male and female mice, indicating that this signaling pathway in the VMH is a critical component of sympathetic activity inputs to BAT and thermogenesis. Furthermore, our findings demonstrated that the PI3K-mediated pathway in SF-1 neurons is required for the effects of estradiol on energy homeostasis in female ovariectomized mice.

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Author Contributions

Conceptualization: A.A.J., C.E.F., J.A.R., and L.L.K.E. Design and work methodology: A.A.J., R.C.S., I.R.S., M.M.M., G.G.H.M., C.E.F., J.A.R., and L.L.K.E. Data collection: A.A.J., R.C.S., I.R.S., H.V.S., R.E.V., and L.L.K.E. Data acquisition: A.A.J., R.C.S., I.R.S., H.V.S., M.M.M., R.E.V., L.C.N., and L.L.K.E. Data analysis and interpretation: A.A.J., R.C.S., I.R.S., M.M.M., R.E.V., G.G.H.M., L.C.N., C.E.F., J.A.R., and L.L.K.E. Funding acquisition, project administration, resources and supervision: J.A.R. and L.L.K.E. Writing: A.A.J., R.C.S., and L.L.K.E. All authors reviewed the manuscript.

Disclosures

The authors have nothing to disclose.

Data Availability

The data presented in this study is available from the corresponding author on reasonable request.

Statement of Ethics

All procedures were approved by the Committee for Animal Care and Use (CEUA-FMRP number 076/2019), of School of Medicine of Ribeirão Preto, University of São Paulo.

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