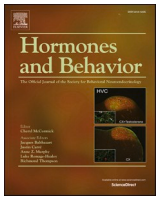
Hormones and Behavior 169 (2025) 105693

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh



The role of ovarian hormone dynamics in metabolic phenotype and gene expression in female mice☆

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ARTICLE INFO

Keywords:
Ovarian hormones
Estrous cycle
Estradiol
Metabolic function
Gene expression
Hypothalamus
Fat tissue

ABSTRACT

Ovarian hormones, particularly estradiol, play an important role in the regulation of metabolic function including in food intake, thermogenesis, activity, fat distribution, and overall weight management. While it is known that weight and food intake follow cyclical patterns across the rodent estrous cycle, the majority of metabolic studies still focus on ovariectomized rodent models and estrogen replacement. Here we provide a comprehensive metabolic profiling of female mice under different ovarian hormone states, from having naturally-cycling ovarian hormone levels to complete ovarian hormone depletion and "estrous cycle-like" estrogen replacement (0.2 or 1 μg estradiol benzoate every 4 days). Every domain of metabolic function that we examined including activity levels, food intake, and body composition was affected by ovariectomy and contributed to >30 % weight gain and nearly two-fold increase in fat mass in ovarian hormone-depleted mice over the 12-week period. By combining physiological and hormone replacement paradigms, we show that cyclical estrogen levels are necessary and sufficient to maintain optimal body weight and fat mass. We show that the hypothalamic expression of genes encoding estrogen receptor alpha (*Esr1)* and neuropeptides involved in feeding behavior (*Agrp*, *Pomc*) changes across the cycle and with ovariectomy, and is partially "rescued" by cyclical estrogen treatment. The drastic fat mass changes following ovariectomy are accompanied by changes in adipose tissue gene expression, including a decreased responsiveness to estrogens due to *Esr1* down-regulation. Our study highlights the importance of understanding the dynamic regulation of metabolic function by ovarian hormones and calls for more naturalistic and higher-resolution approaches to studying the molecular basis of ovarian hormone action.

1. Introduction

Ovarian hormones play an important role in the regulation of food intake, thermogenesis, activity levels, fat distribution, and overall weight management in women and other people with ovaries (Mauvais-Jarvis et al., 2013). Accordingly, cessation of ovarian function during the menopausal transition and following menopause is associated with significant weight gain, redistribution of fat mass, and increased risk for obesity and metabolic syndrome (Greendale et al., 2019; Mauvais-Jarvis et al., 2013). More generally, women are at higher risk for eating disorders and obesity than men are, and gonadal hormones play an important role in this sex difference (Massa and Correa, 2020).

The role of estrogens in the regulation of metabolic function has been studied for decades, most frequently using ovariectomy and estrogen replacement in rodent models. Removal of ovaries leads to increased body weight, accompanied by increased food intake and decreased activity, and these phenotypes can be "rescued" by estrogen treatment (Mauvais-Jarvis et al., 2013). Studies have revealed multiple subregions and cellular populations in the hypothalamus that regulate different domains of metabolic function and are sensitive to estrogens, primarily mediated by estrogen receptor alpha (ERα) (Correa et al., 2015; Krause et al., 2021; Tran et al., 2022; van Veen et al., 2020; Xu et al., 2011).

Within the ventromedial nucleus of the hypothalamus (VMH), either lesion or knockdown of ERα induce increased body weight and adiposity, mediated by increased food intake, lower activity, and decreased thermogenesis (Musatov et al., 2007). Within the arcuate nucleus of the hypothalamus (ARH), energy balance is critically regulated by two neuronal populations producing proopiomelanocortin

☆ This article is part of a special issue entitled: 'Highlights of the 2023 annual meeting of the Society for Behavioral Neuroendocrinology' published in Hormones and Behavior.

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https://doi.org/10.1016/j.yhbeh.2025.105693
Received 10 August 2024; Received in revised form 22 December 2024; Accepted 23 January 2025
Available online 12 February 2025

(POMC) and neuropeptide Y/agouti-related peptide (NPY/AgRP). Activation of POMC neurons decreases food consumption while activation of NPY/AgRP neurons stimulates food intake (Aponte et al., 2011). These populations maintain homeostasis by responding to estrogens (Olofsson et al., 2009) as well as to other circulating signals of energy state such as leptin, ghrelin, and insulin (Vohra et al., 2022).

However, the levels of ovarian hormones cycle during the reproductive period and understanding how fluctuating hormones maintain healthy weight and prevent metabolic syndrome is critical (Rocks et al., 2022a) and cannot be directly examined from hormone replacement studies. Few studies have comprehensively examined the role of the estrous and menstrual cycle in metabolic regulation through the hypothalamus. It has been shown that food intake and body weight show cyclical patterns across the estrous cycle in ovary-intact rats (Asarian and Geary, 2002; Eckel et al., 2000) and mice (Olofsson et al., 2009). Dynamic, ovarian hormone-sensitive changes in hypothalamic gene expression including genes encoding NPY and AgRP have also been reported (Olofsson et al., 2009) but the molecular mechanisms of cyclical hormonal effects are still largely underexplored.

In this study, we uniquely compared the effect of changing ovarian hormones across the estrous cycle and following ovariectomy on body weight, locomotor activity, food intake, and fat mass, as well as on hypothalamic and adipose tissue gene expression in female mice. By combining physiological and hormone replacement paradigms, we show that cyclical estrogen levels are necessary and sufficient to maintain optimal body weight and fat mass. Changes in estrogen levels are also accompanied by changes in the expression of genes that regulate estrogens' action, food intake, and response to circulating metabolic cues. Our study highlights the importance of understanding the dynamic regulation of metabolic function by rhythmic changes in ovarian hormones and calls for more naturalistic approaches to studying the underlying molecular basis of ovarian hormone action.

2. Material and methods

2.1. Animals and study design

For all studies, female C57BL/6J mice were received from Jackson Laboratory and housed at the Fordham University Animal Facility. The first cohort of animals (weeks 8–21; $N = 64$) was utilized for the analysis of body weight, activity, feeding, fat mass, and gene expression across the estrous cycle and following ovariectomy (Fig. 1A). A subset of these animals underwent either ovariectomy ($N = 12$) or sham surgery (ovary-intact) (N = 12) at Jackson Laboratory at 8 weeks of age. After one-week recovery, surgically treated mice were shipped together with ovary-intact regular cycling females ($N = 40$) and arrived at Fordham University at 9 weeks of age. After two weeks of habituation, the sham and regular cycling mice underwent daily estrous cycle tracking from week 11–13 to predict estrous cycle phase for behavioral testing. Vaginal smear cytology of ovariectomized (OVX) animals was followed for one week to confirm that ovariectomy was successful. From week 13–14, animals were behaviorally tested in the open field. Regular cycling and sham females were tested during either the proestrus or early diestrus phase (see *Estrous cycle determination*), along with the OVX animals. From week 14–16, the estrous cycle was continually monitored and animals were singly housed to track daily food intake. Additionally, daily weights were tracked from week 11–16. From week 16–21, animals were returned to group housing. Weekly weights were tracked throughout the study from week 9–21. Before animals were sacrificed, the estrous cycle was tracked again for one week in order to sacrifice regular cycling animals in either proestrus or early diestrus phases. Animals were sacrificed via cervical dislocation at 21 weeks of age. For each animal, the brain was extracted, and the hypothalamus was dissected on ice and then flash frozen in liquid nitrogen. Tissue was stored at −80 °C before further processing for gene expression analysis. In this cohort, a subset of animal trunks ($N = 23$) were preserved for body composition analysis and the remaining animals were used to extract adipose tissue for gene expression analysis.

A second cohort of animals (weeks 6–15; $N = 40$) was used for the estradiol replacement experiments and went through the assessment of body weight, activity, fat mass, and hypothalamic gene expression (Fig. 1B). Female mice were received at 6 weeks of age and habituated for 2 weeks. In this cohort, ovariectomy and sham surgeries were also performed at 8 weeks of age but they were done in house in the Fordham University Animal Facility. Estradiol benzoate (EB) replacement began 1 week following the ovariectomy, at 9 weeks of age, and continued every four days for six weeks (Week 9–15) to mimic the rhythm of endogenous estrogens. OVX animals were grouped to receive: corn oil vehicle [subcutaneously (s.c.), $N = 8$], 0.2 μg EB in corn oil (s.c., N = 8), or 1 μg EB in corn oil (s.c., N = 8), and were tracked alongside non-

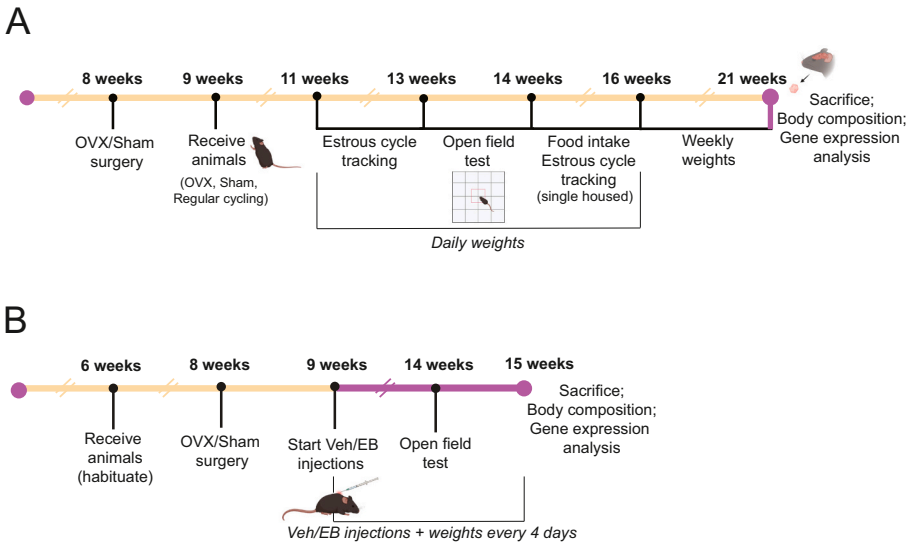

Fig. 1. Study design. Two mouse cohorts were investigated including measurements of body weight, activity, food intake, and body composition, followed by the gene expression analysis in the hypothalamus and/or fat tissue. A. The first cohort included ovariectomized (OVX) animals that were tested along with cycling "sham" and regular mice, from 9 to 21 weeks of age. B. The second cohort analyzed the effect of estrogen replacement on metabolic phenotypes in OVX mice treated with two doses, 0.2 and 1 μg, of estradiol benzoate (EB) every 4 days, along "sham", regular cycling, and vehicle-treated OVX mice. Created with Biorender.com.

treated, ovary-intact animals that either underwent sham surgery (N = 8) or were regular (N = 8). Absence of the estrous cycle in the OVX females was confirmed by vaginal smear cytology. The estrous cycle of the regular and sham ovary-intact females was also briefly tracked to ensure proper cycling; however, in this cohort the estrous cycle was not tracked for testing days. In addition, the analysis of uterine weight was used to further confirm the effectiveness of the surgery and estrogen treatment. For the duration of the study, weights of all groups were obtained from week 9–15 every 4 days when vehicle or EB injections were administered and on the final day of the study. From week 14–15 animals were behaviorally tested in the open field. Animals received the last treatment (T11) at week 14.5 and were sacrificed at 15 weeks of age. The brain was extracted, and the hypothalamus was dissected for gene expression analysis. A subset of animals was used for body composition analysis ($N = 30$) and the remaining animals were used for the analysis of uterine weight ($N = 10$).

All mice were housed in same-group cages ($N = 4$ per cage) in a room set to 21 °C ambient temperature and 30–70 % humidity and were kept on a 12:12 h light:dark cycle (lights on at 8 a.m.) with ad libitum access to food (Envigo 7012) and water. All animal procedures were approved by the Institutional Animal Care and Use Committee at Fordham University.

2.2. Ovariectomy surgery

In the second cohort, animals underwent bilateral ovariectomy or sham surgery at 8 weeks of age in house at the Fordham University Animal Facility. In short, animals were anesthetized with monitored inhalation of isoflurane. Aseptic technique was maintained throughout the procedure. Ovariectomy was performed intra-abdominally by exposing the ovaries via a bilateral skin incision. Each ovary was visualized and excised at the tip of the uterine horn. Sham ovariectomy followed the same procedure, exposing and manipulating the ovaries; however, the ovaries remained intact. After surgery, animals were returned to their original cages and observed daily for 3–4 days to monitor weights, incision healing and were administered carprofen for 2 days. Nutra-Gel food packs (Bioserv, S4798) were placed in cages to limit hind leg mobility during the post-operative period. The success of the ovariectomy was verified by: 1) loss of estrous cycle using vaginal smear cytology; and 2) by absence of the ovaries and atrophy of the uterine tubes by post-mortem examination.

2.3. Estrous stage determination

Estrous cycle stage was determined using vaginal smear cytology, as described previously (Jaric et al., 2019). Briefly, vaginal smears were collected by filling a disposable transfer pipette with 100 µl of distilled water and collecting vaginal cells via lavage at the vaginal opening. Vaginal smears were applied to a microscope slide and once dried, stained with 0.1 % crystal violet in distilled water for 1 min, then washed and placed at room temperature to dry prior to examination with light microscopy. Cycle stage was determined by examining relative quantities of nucleated epithelial cells, cornified epithelial cells, and leukocytes. Diestrus is characterized by the presence of polymorphonuclear leukocytes and nucleated cells with limited cornified epithelial cells. Proestrus is characterized by the presence of mostly nucleated epithelial cells and some cornified cells. In Estrus, mostly cornified epithelial cells are seen. Metestrus is characterized by the presence of cornified epithelial cells, polymorphonuclear leukocytes and some nucleated epithelial cells. In general, we tracked the estrous cycle in order to predict the estrous cycle stage of animals for the analysis of weight, activity levels, food intake, and body composition, as well as for tissue collection. For activity, food intake, body composition and tissue collection, we focused on the proestrus and early diestrus phases, which mimic human follicular and luteal phases, respectively. Using steroid hormone analysis, we previously confirmed that proestrus represents the high estradiol-low progesterone phase and early diestrus represents the low estradiol-high progesterone phase (Jaric et al., 2019), as expected. Following sacrifice, cycle stage predictions were confirmed by collecting and analyzing vaginal smears post-mortem.

2.4. Open field

Animals were behaviorally tested using the open field at 13–14 weeks of age in cohort 1 and 14–15 weeks of age in cohort 2. The open field apparatus was used to quantify the total distance traveled as a measure of overall locomotor activity. Each animal was placed in a square chamber acrylic box (40 × 40 × 35 cm) with clear walls and a gray base plate (Stoelting). The animal was placed in the lower left corner of the apparatus and allowed to freely explore the open field for 10 min. The data was collected using the ANY-maze software (Stoelting). For regular cycling animals, animals were selected for testing based on daily cycle stage predictions targeting the proestrus and early diestrus phase. Following the behavioral test, estrous cycle phase predictions were confirmed by vaginal smear cytology.

2.5. Weights

In the first cohort, animals were weighed weekly from week 9–21. These weekly weights were used to construct the growth curve of the 3 groups – OVX, Sham and regular cycling animals. Daily weights measured from week 14–16 were used to construct the growth curve of cycling animals which included 3 consecutive cycles. For this analysis, only animals with regular 4–5 day estrous cycles were included. In each consecutive estrous cycle period, the weight data included all 4 estrous cycle stages for each individual animal and were expressed relative to the weight of the animals in the diestrus phase of their first cycle (D1). For a comparison, OVX animals were also weighed daily for the duration of 3 average cycles, which is typically 12 days. In the second cohort of animals, from week 9–15, weights from treatment days were used to construct growth curves of the 5 groups - OVX-Veh, OVX-0.2 µg EB, OVX-1 µg EB, Sham and regular cycling animals.

2.6. Food intake

The first cohort of animals was single-housed from 14 to 16 weeks of age for food intake quantification. Singly housed animals were provided with additional nesting material and a plastic dome (InnoDome; Bioserv, S3174) for additional cage enrichment. For the duration of these two weeks, animals were weighed and their estrous cycle was tracked daily. In addition, food was weighed daily in each animal cage. Food intake was first analyzed using the entirety of intake data (total in grams) from week 14–16. OVX animals ($N = 12$) were compared to a pooled group of sham and regular cycling females ($N = 52$). To quantify food intake relative to the estrous cycle phase (gram/day), we used intake data from the second week of single housing (week 15–16). Data from the second week was used to ensure that following the transition from group housing, ovary-intact animals continued to cycle regularly as well as to be able to determine the cycle stage accurately. Pooled Sham and regular cycling animals identified in early diestrus or proestrus during this time period were used to analyze 24-h phase-specific intake. Sham and regular cycling animals were randomly assigned to include data (intake gram/day) from either the early diestrus ($N = 26$) or proestrus (N = 26) phases and compared to the average daily gram/day intake of OVX ($N = 12$) animals in the final week.

2.7. Body composition

After animals were sacrificed, trunks of a subset of animals in both cohorts (Cohort 1: total $N = 23$; OVX, $N = 6$; Sham Diestrus, $N = 3$; Regular Diestrus, $N = 5$; Sham Proestrus, $N = 3$; Regular Proestrus, $N = 6$; Cohort 2: total $N = 30$; OVX-Veh, $N = 6$; OVX-0.2 µg EB, $N = 6$; OVX-1

μg EB, $N = 6$; Sham, $N = 6$; and regular cycling animals, $N = 6$) were transported to the Albert Einstein College of Medicine Animal Physiology Core for body composition testing. The EchoMRI Whole Body Composition Analyzer (EchoMRI) was used to quantify fat mass. Individual animal trunks were placed into a tubular animal holder and into the MRI machine, and were then scanned to determine lean, fat, and water mass (grams). Total fat measurements were analyzed relative to animal weight at time of sacrifice.

2.8. RNA isolation and gene expression analysis

In the first cohort of animals, gene expression analysis was performed on the hypothalamus (total $N = 36$; OVX, $N = 12$; Proestrus, $N = 12$; Diestrus, $N = 12$) and white adipose tissue excised from the perigonadal region (total $N = 24$; OVX, $N = 8$; Proestrus, $N = 8$; Diestrus, $N = 8$). Following confirmation that Sham and regular cycling groups had no significant differences across phenotypes, animals from those two groups were pooled to increase total number of animals for analysis. In the second cohort of animals, gene expression analysis was performed on the hypothalamus (total $N = 40$; OVX-Veh, $N = 8$; OVX-0.2 μg EB, $N = 8$; OVX-1 μg EB, $N = 8$; Sham (ovary-intact), $N = 8$; Regular ovary-intact, $N = 8$). Hypothalamic RNA was purified using the QIAGEN Allprep DNA/RNA Mini Kit (QIAGEN, Cat. # 80204). Adipose tissue RNA was first homogenized in Trizol, then purified using the Qiagen Allprep DNA/RNA Mini Kit. cDNA was generated using 300 ng of RNA and the SuperScript III First Strand Synthesis System (Invitrogen, Cat. #1808051). qPCR reactions were performed using the Fast SYBR Green Master Mix (Applied Biosystems, Cat. #4385612). Expression levels of the genes of interest were quantified using the QuantStudio3 Real Time PCR system with QuantStudio software. Primer sequences used to assess the mRNA levels of Estrogen Receptor alpha (*Esr1)*, Estrogen receptor beta (*Esr2*), G protein-coupled estrogen receptor 1 (*Gper1*), Agouti-related peptide (*Agrp)*, Pro-opiomelanocortin (*Pomc)*, Leptin receptor (*Lepr*), Growth hormone secretagogue receptor (*Ghsr)*, and Cyclophilin A *(Ppia)* are provided in Supplementary Table 1. Relative mRNA expression was calculated using the standard $2^{-\Delta\Delta CT}$ method with OVX or vehicle control OVX samples as a reference sample and *Ppia* as an endogenous reference gene.

2.9. Statistical analysis

Weekly weights and EB treatment day weights were analyzed using two-way repeated measures analysis of variance (ANOVA) with group and time as factors with the Tukey post hoc test. The estrous cycle-dependent weights of cohort 1 were analyzed using one-way repeated measures ANOVA with post hoc Tukey test. The OVX 12-day weights of cohort 1 were analyzed using the non-parametric Friedman one-way repeated measure analysis of variance with Dunn's post hoc test. Total food intake of cohort 1 (OVX vs. cycling females) was analyzed using Welch's *t*-test. Activity, phase-specific intake (OVX vs. proestrus/early diestrus), fat mass, gene expression and uterine weight were analyzed using one-way ANOVA with post hoc Tukey test. Statistical analysis was completed in GraphPad Prism and R software. $P < 0.05$ was considered significant.

3. Results

3.1. The effect of the estrous cycle and ovariectomy on body weight

We first compared body weight patterns of OVX mice, Sham mice, and regular cycling female mice over the 12-week period, starting from 9 weeks until 21 weeks of age, by taking weekly weights into account (Figs. 1A, 2A, Supplementary Table 2). We found significant effects of week ($F_{(12, 732)} = 197.3$, $P < 0.001$), group ($F_{(2, 61)} = 29.54$, $P < 0.001$), and week by group interaction ($F_{(24, 732)} = 31.86$, $P < 0.001$) on weight gain across this period. OVX animals were significantly heavier than

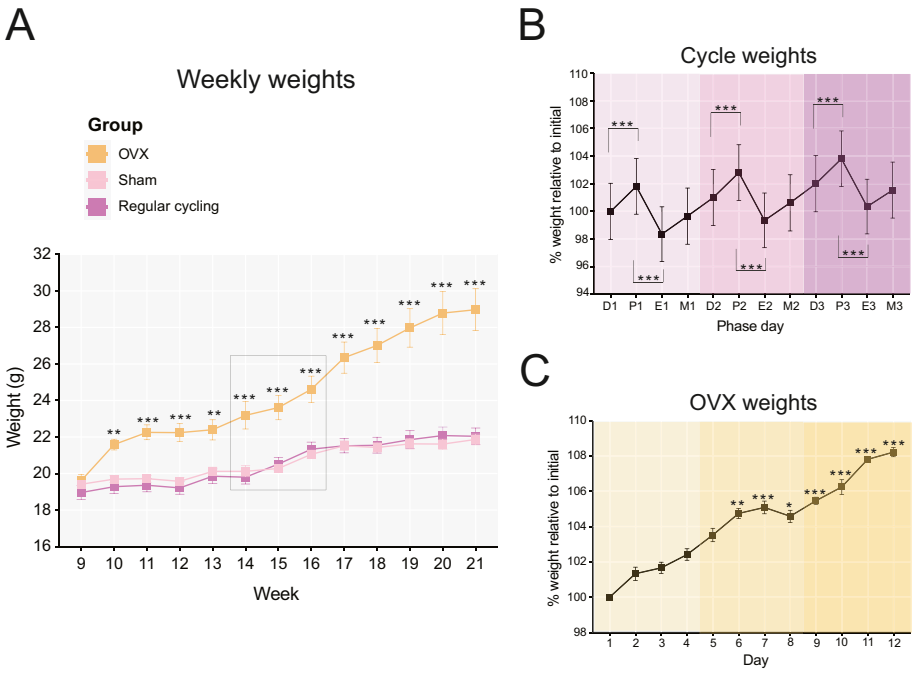

Fig. 2. Ovarian hormones and estrous cycle regulate weight changes in female mice. A. Growth curve analysis of ovariectomized (OVX), Sham, and regular cycling animals ($N = 64$) is presented for weeks 9–21. For simplicity, significance is shown for post hoc weight comparison of OVX and Sham groups across time only (two-way repeated measures ANOVA with the Tukey's post hoc); the gray rectangle highlights weeks 14–16 that were the focus of the daily analyses of weight shown in B and C. B. There is a cyclical pattern of weights in ovary-intact animals undergoing the estrous cycle across 3 consecutive cycles (1–3) depicted with different shades of purple background ($N = 40$); D, Diestrus; P, Proestrus; E, Estrus; M, Metestrus (one-way repeated measures ANOVA with the Tukey's post hoc test; for simplicity, only significance for D-P and P-E comparisons was shown in each cycle); C. Ovarian hormone-depleted OVX animals show consistent weight increase ($N = 12$) across 12 days, corresponding to 3 consecutive estrous cycles (Friedman one-way repeated measure analysis with Dunn's post hoc); ** $P < 0.01$; *** $P < 0.001$.

regular cycling ($P < 0.01$; Tukey's post hoc test) and Sham ($P < 0.01$) female mice as early as at 10 weeks of age (Fig. 2A). This significant difference was maintained across the entire period, from week 10 to 21 (Fig. 2A). At the final point of measurement, at 21 weeks, OVX animals were significantly heavier than regular cycling ($P < 0.001$) and Sham surgery ($P < 0.001$) counterparts, reaching, on average, close to 32 % or 7 g increase in weight in OVX mice (28.98 g ± 1.147) compared to Sham (22.04 g ± 0.452) and regular (21.88 ± 0.291) cycling female mice. Importantly, we found no effect of surgery on weight, as there were no significant weight differences between Sham and regular cycling animals at any point of the study.

We next wanted to explore the effect of the estrous cycle on weight in cycling females and how this could be interrupted following the ovariectomy. We focused on three consecutive estrous cycles from weeks 14–16, when we had daily weight measurements and the estrous cycle stage assignments into the following groups: diestrus, proestrus, estrus, and metestrus (Fig. 1A). Similar to what was reported previously (Olofsson et al., 2009), we observed a cyclic weight pattern in ovary-intact female mice and found a significant effect of estrous cycle day ($F_{(11,132)} = 96.07$, $P < 0.001$). When we focused on weight within each cycle, the lowest point was observed during estrus phase ($P < 0.001$) whereas the highest point was observed during proestrus phase ($P < 0.001$) (Fig. 2B, Supplementary Table 3). In contrast to cycling females, OVX mice showed a steady increase in weight ($\chi^2(11) = 114.7$, $P < 0.001$; Friedman one-way repeated measure) during the period of 12 days corresponding to three cycles in ovary-intact mice (weeks 14–16), beginning at day 6 ($P < 0.01$; Dunn's post hoc) and continuing until day 12 ($P < 0.001$) (Fig. 2C, Supplementary Table 4). These data demonstrate that ovarian hormones dynamically regulate weight across the estrous cycle, and that this cyclicity is essential for the body weight maintenance, which is lost with the ovarian hormone depletion.

3.2. The effect of the estrous cycle and ovariectomy on activity levels

To evaluate the effects of ovarian hormones on overall activity levels, we analyzed total distance traveled across groups, including OVX mice and diestrus and proestrus stages in Sham and regular cycling mice (Fig. 3). We found a significant effect of group ($F_{(4,44)} = 27.85$, $P < 0.001$; one-way ANOVA), which was driven by OVX animals that traveled significantly less compared to Sham ($P < 0.001$) and regular ($P < 0.001$) diestrus animals, as well as compared to Sham ($P < 0.001$) and regular ($P < 0.001$) proestrus animals (Fig. 3). There was no significant difference in distance traveled between diestrus and proestrus groups either among Sham or regular cycling mice (Fig. 3).

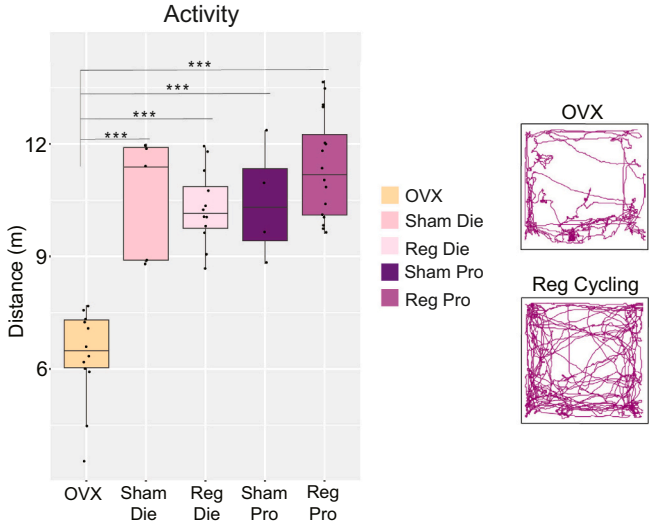

Fig. 3. Ovarian hormones regulate activity in female mice. Behavioral testing in open field reveals ovarian hormone-dependent activity (distance traveled, left, N = 40) of ovariectomized (OVX), Sham and Regular (Reg) cycling groups; representative track plots of OVX (top right) and regular cycling (bottom right) animals in the open field are shown; Box plots (box, 1st–3rd quartile; horizontal line, median; whiskers, min/max); (One-way ANOVA with the Tukey's post hoc test); ***$P < 0.001$. Die, diestrus; Pro, proestrus.

3.3. The effect of the estrous cycle and ovariectomy on feeding behavior

To determine the effects of ovarian hormones on feeding behavior, we compared individual food intake across groups. Interestingly, overall, OVX animals and cycling animals showed no significant difference in food intake (Fig. 4A). However, once cycling animals were segregated into proestrus and diestrus groups (Fig. 4A), we found a significant effect of group on feeding behavior ($F_{(2,\ 61)} = 26.40$, $P < 0.001$; one-way ANOVA). With a post hoc test, we found that both OVX mice ($P < 0.001$) and diestrus mice ($P < 0.01$) had increased food intake compared to high-estrogenic proestrus animals (Fig. 4A), while no significant differences were found between OVX and diestrus animals (Fig. 4A). Thus, these results are consistent with our cyclical weight data (Fig. 2B) and imply that a decreased food intake during proestrus (Fig. 4A) may be driving, in part, decreased body weight found in the succeeding estrus phase of the cycle (Fig. 2B).

3.4. The effect of the estrous cycle and ovariectomy on fat mass

At 21 weeks of age, we compared fat mass of OVX, Sham and regular diestrus and proestrus animals (Fig. 4B) and found an overall effect of group on fat mass ($F_{(4,18)} = 24.58$, $P < 0.001$; one-way ANOVA). OVX mice were found to have dramatically higher fat mass (42.17 % ± 1.973 %) compared to diestrus Sham (22 % ± 2.168 %, $P < 0.001$), diestrus regular (24.67 % ± 1.202 %; $P < 0.001$), proestrus Sham (24.5 % ± 1.204 %, $P < 0.001$) and proestrus regular (26.33 % ±1.202; $P < 0.001$) female mice (Fig. 4B).

3.5. The effect of the estrous cycle and ovariectomy on hypothalamic gene expression

To explore the molecular basis of the observed metabolic phenotypes, we next examined the hypothalamic expression of several genes known to regulate metabolic function and are regulated by ovarian hormones. We focused on early diestrus and proestrus animals and tested them along with OVX mice. There was an effect of group on the expression of *Esr1,* gene encoding ERα, ($F_{(2,32)} = 19.66$, $P < 0.001$; one-way ANOVA), with proestrus mice having a higher *Esr1* expression than both cycling low-estrogenic diestrus females ($P < 0.01$) and ovarian hormone-depleted OVX mice ($P < 0.001$; Fig. 5A, left). This effect was specific to the ERα receptor gene, as we found no differences in the expression of genes encoding ERβ (*Esr2*) or G protein-coupled estrogen receptor (*Gper1*) among the three groups (Supplementary Fig. 1). However, the expression of genes encoding orexigenic Agouti-related neuropeptide (*Agrp*) and anorexigenic proopiomelanocortin (*Pomc*) also varied with the ovarian hormone status. There was the main effect of group on the expression of *Agrp* ($F_{(2,33)} = 19.66$, $P < 0.001$; one-way ANOVA). We found increased *Agrp* mRNA levels in diestrus compared to proestrus ($P < 0.01$); however, the expression of this gene was significantly down-regulated in OVX females compared to both estrous cycle stages, diestrus ($P < 0.001$) and proestrus ($P < 0.05$; Fig. 5A, middle). We also found an effect of group on the expression of *Pomc* ($F_{(2,33)} = 8.198$, $P < 0.01$; one-way ANOVA). *Pomc* expression was higher in proestrus females compared to both OVX ($P < 0.01$) and diestrus ($P < 0.05$) females (Fig. 5A, right). There was no significant difference between diestrus and OVX mice in the expression of *Pomc* (Fig. 5A, right).

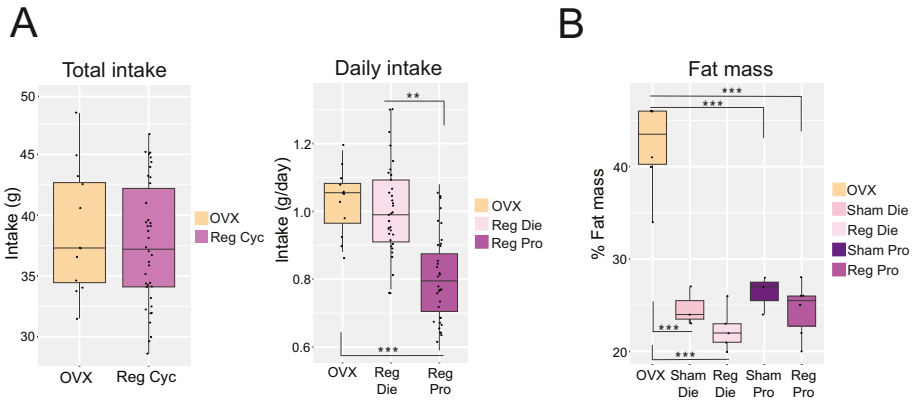

Fig. 4. Ovarian hormones and estrous cycle regulate feeding and body composition. A. Food intake of ovariectomized (OVX) and regular cycling (Reg Cyc) animals show no significant differences (left, N = 64, Welch's two sample *t*-test) before accounting for the estrous cycle (right, N = 64, one-way ANOVA with the Tukey's post hoc test). B. % Fat mass relative to weight of OVX, Sham, and regular cycling animals at 21 weeks reveals significant fat mass increase in OVX animals; Box plots (box, 1st–3rd quartile; horizontal line, median; whiskers, min/max); (One-way ANOVA with the Tukey's post hoc test); ** P < 0.01; *** P < 0.001; Die, diestrus; Pro, proestrus.

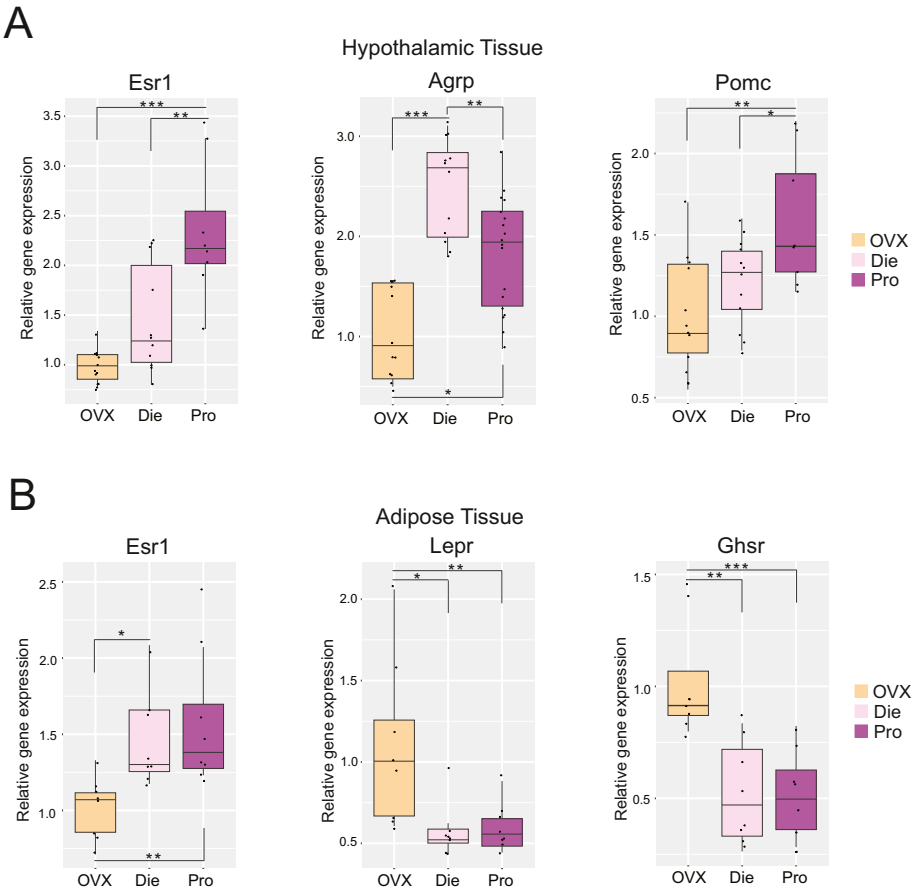

Fig. 5. Ovarian hormone-dependent gene expression changes in the female mouse hypothalamus and adipose tissue. A. Gene expression analysis of *Esr1* (estrogen receptor alpha, left), *Agrp* (Agouti related peptide, middle) and *Pomc* (pro-opiomelanocortin, right) in the hypothalamus of ovariectomized (OVX), diestrus (Die), and proestrus (Pro) female mice. B. Adipose gene expression analysis of *Esr1* (left), *Lepr* (leptin receptor, middle) and *Ghsr* (growth hormone secretagogue receptor, right) of OVX, Die, and Pro female mice; Box plots (box, 1st–3rd quartile; horizontal line, median; whiskers, min/max); (One-way ANOVA with the Tukey's post hoc test); *$P < 0.05$; **P < 0.01; ***P < 0.001.

3.6. The effect of the estrous cycle and ovariectomy on gene expression in adipose tissue

To explore the molecular basis for the significant fat mass changes that we observed (Fig. 4B), we analyzed gene expression in white adipose tissue that has been shown to exhibit endocrine functions. While a typical example is leptin, an adipocyte-secreted hormone with a critical role in energy balance (Trayhurn and Beattie, 2001), estrogens are also synthesized by the adipose tissue (Hetemäki et al., 2021). We first examined the expression of *Esr1* in adipose tissue and found a significant

effect of group ($F_{(2,21)} = 6.001$, $P < 0.01$; one-way ANOVA), with both cycling groups - proestrus ($P < 0.01$) and diestrus ($P < 0.05$) – showing higher *Esr1* expression compared to OVX animals (Fig. 5B, left). Interestingly, we found no estrous cycle-dependent difference in adipose tissue *Esr1* expression in the proestrus-diestrus comparison (Fig. 5B, left), which was in contrast to what we found in the hypothalamus (Fig. 5A, left). Further, we found a significant effect of group on *Lepr* expression ($F_{(2,21)} = 7.054$, $P < 0.01$; one-way ANOVA), which was significantly higher in OVX mice compared to both proestrus ($P < 0.01$) and diestrus ($P < 0.05$) females (Fig. 5B, middle). Finally, there was a significant effect of group on the expression of *Ghsr*, gene encoding ghrelin receptor ($F_{(2,21)} = 12.17$, $P < 0.001$, one-way ANOVA), which is more highly expressed in metabolically dysregulated OVX mice compared to both diestrus ($P < 0.01$) and proestrus ($P < 0.001$) females (Fig. 5B, right).

3.7. Estradiol treatment rescues metabolic phenotype in OVX mice in a dose-dependent manner

Our previous results confirmed the role of ovarian hormones in the range of metabolic phenotypes and gene expression in the hypothalamus and fat tissue (Figs. 1–5). We next wanted to examine the role of estradiol in these phenotypes and test the hypothesis that ovariectomy-induced metabolic dysfunction can be rescued, in part, by the "cyclical" 4–day estradiol benzoate (EB) treatment, mimicking estrogen fluctuations across the estrous cycle (Fig. 1B). To assess the efficiency of the estradiol treatment, we first checked uterine weight across groups and found a significant effect of group ($F_{(4,\ 5)} = 16.80$, $P < 0.01$) on this measure (Supplementary Fig. 2). As expected, OVX-Veh animals showed a significantly lower uterine weight compared to Sham ($P < 0.01$) and regular ($P < 0.01$) cycling females. The EB treatment showed a dose-

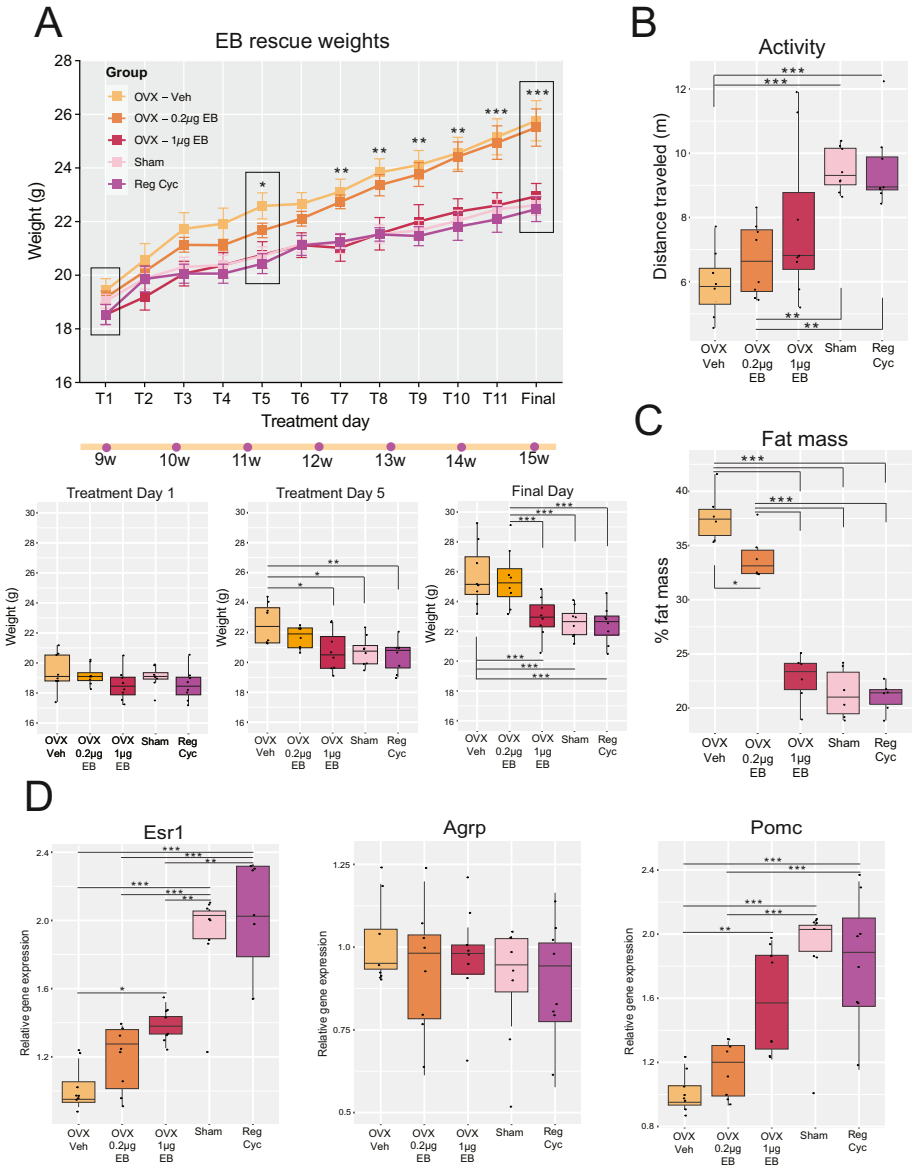

Fig. 6. Estradiol benzoate replacement ameliorates metabolic dysfunction following ovarian hormone depletion. A. Body weight (N = 40) was examined in OVX animals treated with either vehicle (OVX-Veh), 0.2 μg EB (OVX-0.2 μg EB), or 1 μg EB (OVX-1 μg EB), as well as in Sham and regular cycling (Reg Cyc) female mice. For simplicity, significance is shown for post hoc weight comparison of OVX-Veh and OVX-1 μg across time only; T, treatment day; Final, final day of the study; (Two-way repeated measures ANOVA with the Tukey's post hoc) (top panel). The highlighted parts of the body weight curve are shown separately to emphasize the comparison of groups on Treatment Days 1 and 5 as well as on Final day; one-way ANOVA with the Tukey's post hoc (bottom plots). B. Activity levels (distance traveled, N = 40); C. % Fat mass ($N = 30$); and D. Hypothalamic gene expression of *Esr1*, *Agrp*, and *Pomc* (N = 40) are also shown across the groups. Box plots (box, 1st–3rd quartile; horizontal line, median; whiskers, min/max); (One-way ANOVA with the Tukey's post hoc test); *P < 0.05; **P < 0.01; ***P < 0.001.

dependent effect, with 1 μg ($P < 0.05$) but not 0.2 μg EB treatment being sufficient to rescue the effect of ovariectomy on uterine weight (Supplementary Fig. 2). We then tested the effect of EB replacement on body weight, activity levels, fat mass, and hypothalamic gene expression, finding similar dose-dependent effects (Fig. 6).

We first found a significant effect of group ($F_{(4, 35)} = 6.242$, $P < 0.001$), dose day ($F_{(11, 385)} = 183.2$, $P < 0.001$), and group by dose day interaction ($F_{(44, 385)} = 3.401$, $P < 0.001$) on weight in our second animal cohort (Fig. 6A, top panel). Starting with the treatment day 5 (T5), OVX-Veh animals had a significantly higher weight (22.59 g ± 0.48 g) than OVX-1.0 μg EB (20.76 g ± 0.49 g, $P < 0.05$), Sham (20.73 g ± 0.34 g, $P < 0.05$) and regular cycling (20.44 g ± 0.37 g, $P < 0.01$) animals and no significant difference when compared to OVX-0.2 μg EB animals (21.68 g ± 0.27 g; Fig. 6A, bottom panel). Interestingly, the effect of EB treatment was dose-dependent; while 0.2 μg EB was not sufficient to rescue the OVX weight phenotype, OVX-1 μg EB animals were not significantly different from either Sham or regular cycling animals. At the completion of the study (Final Day), following 11 treatment days, OVX-Veh animals had a significantly higher weight (25.76 g ± 0.75 g) than OVX-1 μg EB (22.95 g ± 0.47 g, $P < 0.001$), Sham (22.61 g ± 0.36 g, $P < 0.001$) and regular cycling (22.46 g ± 0.46 g, $P < 0.001$) animals but not OVX-0.2 μg EB animals (25.51 g ± 0.70 g; Fig. 6A). Similarly, we found no significant differences between OVX-1 μg EB, Sham and regular cycling animals (Fig. 6A, Supplementary Table 5).

We next assessed animal activity levels (Fig. 1B, 6B). We found a significant effect of group on total distance traveled in the open field ($F_{(4, 35)} = 10.04$, $P < 0.001$). We confirmed our previous results (Fig. 3), by showing that OVX-Veh animals traveled significantly less than Sham ($P < 0.001$) and regular ($P < 0.001$) cycling animals. OVX-0.2 μg EB animals also demonstrated significantly less activity relative to Sham ($P < 0.01$) and regular cycling ($P < 0.01$) counterparts. In contrast, the activity of OVX-1 μg EB animals, was not significantly different either from Sham and regular cycling mice, or from OVX-Veh and OVX-0.2 μg EB groups. These data indicate that 1 μg EB replacement in OVX females mitigated decreases in activity associated with depleted endogenous ovarian hormones.

Given the significant differences in fat mass demonstrated by the prior animal cohort, we next examined fat mass differences across all OVX groups and cycling animals. We again found a significant effect of group on fat mass ($F_{(4, 25)} = 82.81$, $P < 0.001$; Fig. 6C). As previously demonstrated in OVX animals (Fig. 4B), OVX-Veh animals had significantly greater fat mass relative to Sham ($P < 0.001$) and regular ($P < 0.001$) cycling mice (Fig. 6C). Fat mass of OVX-0.2 μg EB animals was significantly different compared to OVX-Veh animals ($P < 0.05$); however, it differed from OVX-1 μg EB ($P < 0.001$), Sham ($P < 0.001$), and regular ($P < 0.001$) cycling counterparts. When examining the OVX-1 μg EB group, these animals did not exhibit different fat mass compared to Sham and regular cycling animals; however, their fat mass was significantly different from OVX-Veh ($P < 0.001$) and OVX-0.2 μg EB ($P < 0.001$) groups (Fig. 6C). These results further suggest that 1 μg EB is sufficient to rescue body weight changes, ameliorate decreases in activity, and halt significant accumulation in fat mass observed in OVX female mice.

Finally, we tested the effect of estrogen replacement on hypothalamic gene expression of previously described genes of interest – *Esr1*, *Esr2*, *Gper1*, *Agrp* and *Pomc* (Fig. 6D, Supplementary Fig. 3). *Esr1* gene expression (Fig. 6D, left panel) demonstrated a significant difference between groups ($F_{(4,35} = 20.41$, $P < 0.001$; one-way ANOVA). Regular cycling and sham females had a significantly higher *Esr1* expression compared to OVX-1 μg EB ($P < 0.01$), OVX-0.2 μg EB ($P < 0.001$), and OVX-Veh ($P < 0.001$) animals. However, OVX-1 μg EB animals had significantly higher *Esr1* expression than OVX-Veh ($P < 0.05$) animals, demonstrating that 1 μg EB dose attenuates the down-regulation of *Esr1* gene expression in OVX animals. However, consistent with our previous data (Supplementary Fig. 1), we found no significant effect of group on *Esr2* and *Gper1* expression in our second animal cohort (Supplementary Fig. 3). In addition, we found no significant difference in *Agrp* expression across the groups (Fig. 6D, middle panel). Finally, we looked at *Pomc* expression (Fig. 6D, right panel) which demonstrated a significant effect of group ($F_{(4, 35)} = 13.56$, $P < 0.001$). We found no significant difference when regular cycling and sham animals were compared to the OVX-1 μg EB group. However, regular and sham cycling animals exhibited significantly higher expression than OVX-Veh ($P < 0.001$) and OVX-0.2 μg EB ($P < 0.001$) counterparts. OVX-1 μg EB animals demonstrated significantly higher expression than OVX-Veh animals ($P < 0.01$). These findings show that 1 μg EB can partially rescue gene expression changes in OVX animals, likely contributing to the "rescue" of their metabolic phenotype.

4. Discussion

Ovarian hormones coordinately regulate reproductive and metabolic function, and this interplay seems to be critical for the reproductive fitness in mammalian females. In addition, estrogens are potent modulators of mood, emotion, reward processing, and cognition, and metabolic symptoms often accompany psychiatric disorders, particularly in women (Galea et al., 2017; Kundakovic and Rocks, 2022). Therefore, understanding how ovarian hormones affect metabolic function has broad implications for neuroscience and neuropsychiatric disorders.

Here we provide a comprehensive metabolic profiling of female mice under different ovarian hormone states, from having naturally-cycling ovarian hormone levels to complete ovarian hormone depletion and estrogen replacement. In general, we confirm that ovariectomy leads to a significant weight gain (Asarian and Geary, 2002; Santollo et al., 2021; Tarttelin and Gorski, 1973). Every aspect of metabolic function that we examined including activity levels, food intake, and fat composition was affected by ovariectomy and contributed to weight gain and metabolic dysregulation in OVX mice. Here we also confirmed the cyclic weight and feeding pattern across the estrous cycle (Asarian and Geary, 2002; Blaustein and Wade, 1976; Olofsson et al., 2009). However, we also demonstrate that a decreased food intake coinciding with a surge of estrogen during proestrus is driving, in part, decreased body weight found in the succeeding estrus phase of the cycle. The ovarian hormone-driven cyclical changes in weight seem to be critical for the maintenance of the healthy weight, as OVX animals lacking this pattern keep gaining weight and end up with significantly increased body weight and adiposity over the period of 12 weeks.

Importantly, we show that a repeated, 4-day cycle estradiol regimen, mimicking estrogen rhythmic changes in a typical mouse estrous cycle, is sufficient to maintain normal body weight trajectories in OVX mice. While a previous study of cyclical estradiol treatment showed the "rescue" at the level of food intake and total body weight in rats (Asarian and Geary, 2002), we extend these observations to mice as well as to the activity levels, fat distribution, and hypothalamic gene expression. There are two important findings regarding the effect of estrogen replacement that we observed. First, the effect was dose-dependent and, while 2 μg EB dose was sufficient to rescue the metabolic phenotype in OVX rats (Asarian and Geary, 2002), a rough equivalent of this dose in mice (0.2 μg) was insufficient and the phenotype was rescued by the 1 μg EB dose only. It is not clear why mice require a relatively higher EB dose than rats for the "metabolic rescue". However, the *acute* 1 μg EB treatment was previously shown to be effective in mouse metabolic studies (Krause et al., 2021), and here we show that 1 μg (but not 0.2 μg) EB treatment rescues ovariectomy-induced uterine atrophy, further implying that 1 μg EB dose is required for optimal estrous cycle-like estrogen replacement in mice. Second, changes in hypothalamic gene expression follow the dose-dependent effects of EB treatment, confirming that altered gene expression induced by estradiol plays a critical role in the examined metabolic phenotypes.

We explored the expression of genes encoding estrogen receptors (*Esr1, Esr2*, and *Gper1*) and neuropeptides that affect food intake (*Agrp* and *Pomc)* in the hypothalamus. While *Esr2* and *Gper1* showed no

changes with the ovarian hormone status, *Esr1* showed highest expression during proestrus, the high-estrogenic phase of the cycle, and was down-regulated with a drop in estrogen in diestrus and with the ovariectomy. The 1 μg-dose estrogen regimen increased *Esr1* levels in OVX mice, implying that the induction of ERα expression plays a role in beneficial metabolic effects of estradiol, both in ovary-intact and OVX mice.

We also found that the hypothalamic expression of *Pomc,* encoding the neuropeptide that signals satiety and reduces food intake (Vohra et al., 2022), is highest during proestrus, consistent with the anorexigenic effect of estradiol (Massa and Correa, 2020). It is known that estradiol exerts this effect, in part, by increasing POMC neuronal activity (Stincic et al., 2018a). In terms of *Pomc* expression, however, previous studies showed no change with the cycle (Olofsson et al., 2009) or either an increase (Pelletier et al., 2007) or a decrease (Yang et al., 2017) in *Pomc* expression with acute EB treatment in OVX mice. Here we show that both proestrus and the effective 1 μg EB treatment in OVX mice are associated with the increased *Pomc* expression in the hypothalamus, supporting the role of estrogen-driven *Pomc* gene expression in feeding and metabolic phenotype.

We also show that the hypothalamic expression of *Agrp*, which signals hunger and promotes food intake, is higher in diestrus than in proestrus, which is consistent with the previous study (Olofsson et al., 2009) and with the higher food intake we observed in the low-estrogenic phase of the cycle. Unexpectedly, *Agrp* expression was lower in OVX animals than in that of both ovary-intact groups, and this findings may be consistent with an earlier observation that feeding in OVX animals increases initially but goes back to "near-normal" levels about one month following ovariectomy (Asarian and Geary, 2006; Clegg et al., 2007). Thus, hypothalamic downregulation of *Agrp* may be part of the compensatory mechanism in OVX mice. While not shown with our chronic EB treatment, *Agrp* expression was increased in the ARH of OVX mice with a shorter estradiol treatment (Yang et al., 2017), further suggesting that this gene is sensitive to dynamic changes in estrogen levels.

Importantly, with regards to mechanisms underlying estrogenic regulation of POMC and NPY/AgRP neuronal populations in the hypothalamus, it is known that POMC neurons express *Esr1* (Frank et al., 2014) and it is likely that ERα receptors mediate, directly or indirectly, the effects of estradiol on *Pomc* gene expression. In fact, reduced POMC levels have been reported in *Esr1* knockout mice (Hirosawa et al., 2008). However, while NPY/AgRP neurons mostly lack *Esr1* expression, estrogens can still modulate these neurons and their *Agrp* expression via membrane estrogen receptors or alternate pathways (Frank et al., 2014; Stincic et al., 2018b), highlighting the complexity of the estrogenic regulation of metabolic function within the hypothalamus.

It is also interesting to note the contribution of fat tissue changes to the changes in overall metabolic phenotype in our OVX female mice. First, the increase in fat mass is very dramatic; the percent of fat tissue in OVX mice nearly doubles compared to ovary-intact animals across the 13-week period. Interestingly, we note changes in gene expression in adipose tissue that could help explain the adiposity phenotype. While a recent study revealed a significant correlation between gene expression in the hypothalamus and adipose tissue as a function of estrogen state (Massa et al., 2023), here we show that one of the primary mechanisms through which ovariectomy leads to adiposity may involve altered expression of ERα in fat tissue. In adipose tissue, we do not find changes in *Esr1* expression across the cycle but we demonstrate a decreased responsivity of fat cells to estrogen after ovariectomy due to down-regulated *Esr1* expression. In stark contrast with this, we observed significant increases in the expression of genes encoding receptors for leptin and ghrelin. Interestingly, while leptin and ghrelin receptors in the hypothalamus are known to regulate appetite (Picó et al., 2022) and feeding (Hucik et al., 2021), here we show that their overexpression in adipose tissue may promote increase in fat mass and adiposity in metabolically dysregulated OVX mice. Importantly, cyclic estrogen supplementation is also able to rescue the adiposity phenotype, keeping fat tissue in OVX mice at the same level as in ovary-intact animals.

In sum, in this study we provide evidence that ovarian hormones dynamically change gene expression in the hypothalamus and fat tissue, providing in part a mechanism that underlies changes in metabolic phenotypes. While estrogen receptors can regulate metabolically-relevant gene expression directly and indirectly, through nuclear and membrane-bound receptor types (Marrocco and McEwen, 2016; Srivastava et al., 2011; Yang et al., 2016), the specific estrogen-sensitive cellular populations and underlying mechanisms remain poorly understood. We previously demonstrated that cyclical ovarian hormones dynamically change chromatin accessibility (Jaric et al., 2019) and 3D chromatin organization (Rocks et al., 2022b) in brain regions such as the ventral hippocampus (Jaric et al., 2019; Rocks et al., 2022b) and the nucleus accumbens (Rocks et al., 2023), and it is very plausible that similar mechanisms operate in the hypothalamus which is rich in estrogen receptors (Frank et al., 2014). The challenge with the hypothalamus is its heterogeneity and single cell gene expression and chromatin accessibility assays are now available (Hao et al., 2021; Kundakovic and Tickerhoof, 2024; Steuernagel et al., 2022) to help reveal specific cellular populations and genes that change with the physiological cycle and following ovariectomy. It is our hope that these approaches will reveal new downstream estrogen targets that will help design new treatments for metabolic disorders and obesity in women and other people with ovaries.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2025.105693.

CRediT authorship contribution statement

Laila Ouldibbat: Writing – original draft, Visualization, Investigation, Formal analysis. **Devin Rocks:** Writing – review & editing, Investigation, Formal analysis. **Branden Sampson:** Investigation. **Marija Kundakovic:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Acknowledgement

This work was supported by the National Institutes of Health award R01MH123523 and the Fordham University Office of Research Faculty Research Grant (to M.K.).

Data availability

Data will be made available on request.

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