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Estradiol and leptin have separate but additive anorexigenic effects and differentially target fat mass in rats

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Abstract

We recently showed that male compared to female rats exhibit lower hypophagia and body weight (BW) loss following central leptin delivery, suggesting a role for estradiol in leptin responsiveness. Addressing this, we delivered *Ob* (Leptin) or GFP (Control) gene into the brain of male rats that were simultaneously treated with estradiol or Vehicle. In a reciprocal approach, we compared estradiol-deficient (Ovx) to intact females (Sham) that received Leptin or Control vector. Changes in food intake (FI), BW, and body composition were examined. In males, estradiol and Leptin resulted in lower cumulative food intake (15%) and endpoint body weight (5%) but rats receiving dual treatment (Estradiol-Leptin) ate 28% less and weighed 22% less than Vehicle-Control. Changes in FI were unique to each treatment, with a rapid decrease in Vehicle-Leptin followed by gradual renormalization. In contrast, hypophagia in Estradiol-Control was of lower amplitude and sporadic. Leptin selectively targeted fat mass and endpoint abdominal fat mass was 65–80% lower compared to their respective Control groups. In females, both Leptin groups had lower body weight (endpoint values 20% lower than Control groups) with the highest extent in Sham animals (endpoint value was 28% less in Sham-Leptin than in Sham-Control). OvX rats rapidly started regaining their lost BW reminiscent of the pattern in males. Leptin rapidly and robustly reduced fat mass with endpoint values 30–35% less than Control treated animals. It appears that Leptin and

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Author contribution

IC, HZT, DM, and PJS conceived and designed the study. SMG, HZT, and IC collected the data. IC, JFY, CFC, DM, CSC, NT, and PJS analyzed and interpreted the data. IC, JFY, DM, and PJS wrote and edited the manuscript.

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Declaration of interest

None to declare.

estradiol decreased FI and BW through different mechanisms, with the pattern of Estradiol-Leptin reminiscent of that observed in females and with the pattern of Ovx-Leptin, reminiscent of that observed in males. Estrogen status did not influence initial fat mass loss by Leptin. It can be concluded that estradiol modulates long-term response to central leptin overexpression but its actions on energy homeostasis are additive and independent of those of leptin.

Keywords

Male; Female; Estradiol; Leptin; Weight loss

Introduction

The female hormone environment has been shown to prevent weight gain and the adverse outcomes of obesity, even in males [1]. Those health benefits are to some extent mediated by estrogens which activate catabolic and anorexigenic signals in the brain, similar to those recruited by the anti-obesity hormone leptin [2]. Administration of the female hormone estradiol mitigated the obese phenotype of leptin-deficient animals and rewired defective hypothalamic feeding circuits such that estrogens have been termed leptin mimetics [2]. Given the intertwined signaling pathways and physiological functions, a crosstalk network between these two hormones has been proposed [3].

Important evidence supports the theory that the functions of estrogens and leptin are coupled. For instance, leptin is essential for the secretion of gonadotropin-releasing hormone from the pituitary and, hereby, has an indirect effect on secretion of estrogens [4]. Reciprocally, estrogens activate leptin secretion in adipose tissue and have a role in the expression of the long form of leptin receptor in the hypothalamus [5,6]. Beyond those permissive effects, estrogens have been shown to enhance central leptin signaling. Indeed, the brain of males is relatively less sensitive to the catabolic action of leptin than the brain of females [7]. However, feminization of the male hormone milieu through administration of 17 β -estradiol enhances acute central leptin sensitivity [8]. Similarly, in females, estrogen deficiency by Ovariectomy decreases central leptin sensitivity and hormone replacement therapy can reestablish brain leptin response [8]. We recently reported that male compared with female rats are less responsive to long-term central leptin overexpression, as assessed by decreased food intake and delta body weight. Our findings suggested that the exacerbated response in females was at least partly due to the higher level of estradiol, but this interpretation remains speculative. The present studies further examine whether estradiol enhances long term leptin responses or if the actions of estrogen are independent of leptin. To this end, we conducted two additional experiments manipulating the hormonal milieu of rats that chronically overexpressed leptin in the brain. First, we attempted to recapitulate the female leptin response in males by daily administration of estradiol in rats that were centrally delivered leptin gene. We then utilized a reciprocal approach in which we examined the response to central leptin overexpression in ovariectomized (estradiol-deficient) compared with intact female rats.

Subjects and methods

Animals and groups.

For Study 1, three-month old Sprague Dawley rats ($n = 32$), were obtained from Taconic (Germantown, NY). For Study 2, (Fisher 344 $\times$ Brown Norway)F1 rats ($n = 32$) were obtained from the National Institute on Aging (NIA) Colony at Charles River Laboratories (Wilmington, MA). The hybrid rats are an ideal strain for these studies because they display more stable body weight at adulthood [9]. However, these animals are bred at the NIA with limited distribution. It was not possible to obtain sufficient animals for both experiments, hence the use of two different strains. Animals were housed individually on a 12-h light and 12-h dark cycle. All rats were allowed at least one week to acclimate to their new environment and daily handling before beginning any experiment. During this initial acclimation period, in Study 1, animals were given daily injections of saline to mimic stress induced during the experiment. We found in the past that acclimation to daily injections is useful to prevent stress-induced weight loss, which constitutes an important artefact in our experiments. All rats from these two experiments were fed a standard rodent chow (18% kcal from fat, no sucrose, 3.1 kcal/g, Envigo Teklad Global diet 2918). Health status, body weight, and food intake were monitored daily throughout the study. All experimental protocols were approved by the University of Florida's Animal Care and Use Committee, and in compliance with the eighth edition of "Guide for the Care and Use of Laboratory Animals". The day before the actual experiment started, rats were pseudo-randomized into two weight-matched groups that were centrally delivered a vector to overexpress either a green fluorescent protein (Control), or Leptin. For Study 1, animals from each of these two groups were then assigned to received daily injections of either Estradiol (25 μ g/kg diluted in sesame oil; S.C.; 0.5 mL/kg) or Vehicle (sesame oil; 0.5 mL/kg). We selected daily rather than cyclic injection because estradiol is quickly metabolized. Oil was chosen over ethanol to promote slower but more constant release of estradiol [10].

Therefore, there were four male groups: Vehicle-Control, Vehicle-Leptin, Estradiol-Control, and Estradiol-Leptin. All treatments, daily injections and gene delivery started on experimental day 0. For Study 2, half of the animals from Control and Leptin group were assigned to either Ovariectomy (Ovx), or Sham operation (Sham). There were also four groups of females: Ovx-Control, Ovx-Leptin, Sham-Control, and Sham-Leptin. In the manuscript, Sham groups are often referred as groups with "higher estrogen levels" and Ovx animals are referred as groups with "lower estrogen levels". In figures, data for Ovx groups are presented on the left side for clarity. The typical presentation order (Sham-Control on the left side) was reversed to better align experimental conditions, left columns represent groups of animals with lower estrogen levels and right columns comprise groups of animals with high estrogen level.

Surgeries

For both studies, on day 0, rats were anaesthetized with isoflurane (2–3%) and administered the analgesics: buprenorphine (0.025 mg/kg; SC) and Carprofen (5 mg/kg; SC) every 24 hours for 72 hours starting immediately prior the surgery. Each rat received 1 μ L of *rAAV1* (2.3×10^{13} vg/mL) into the third ventricle using the following coordinates: 1.3 mm anterior

to Bregma, 0.0 mm from midline to depth of 9.6 mm ventral from surface of skull, at an angle of 20° [11]. These coordinates were validated by injecting a bromothymol blue dye. The pTR(2)ObW construct encoding leptin transgene was packaged as previously described [12]. The viral vectors were packaged by the University of Florida vector core (Gainesville, FL, USA). In females, we attempted to minimize stress associated with each surgical procedure (i.e. anesthesia, pain medication) and performed both surgical procedures simultaneously. Therefore, bilateral Ovariectomy or Sham operation right after gene delivery [13].

Determination of body composition using time-domain nuclear magnetic resonance

Body composition was assessed at day 0, 7, 14, and 25 (or 28) using time-domain nuclear magnetic resonance (TD-NMR; MiniSpec, Bruker Optics, The Woodlands, TX). The MiniSpec quantifies whole body fat mass, lean mass, and free fluid. TD-NMR fat mass is not limited to adipose tissue but also detects ectopic fat since it analyzes signals from protons in the entire rat. Therefore, it is expected that fat mass values measured by TD-NMR exceed fat mass measured in adipose tissues collected at the end of the experiment. The manufacturer software calculates values reported in grams. All scans in these experiments were acquired in conscious animals. The final values represent the mean of two scans for each subject, at each time point.

Tissue Collection, Harvesting and, Preparation

On day 25 (Study 1) or day 28 (Study 2), rats were euthanized between 10 AM and 12 PM by exsanguination (intra-cardiac blood collection) under anesthesia (Isoflurane; 5%). The hypothalamus was dissected from the whole brain by a medial incision to the piriform lobes, caudal to the optic chiasm and anterior to the cerebral crus to a depth of 2.5 mm and immediately snap frozen in liquid nitrogen, then samples were stored at -80 °C until analyses were performed. Four abdominal fat depots were collected and weighed: mesenteric, perirenal, epididymal/parametrial, and retroperitoneal.

Reverse transcription polymerase chain reaction

Total RNA was isolated from the hypothalamus using TRI reagent (Sigma Aldrich, St. Louis MO). Total RNA (2 µg) was reverse-transcribed into complementary DNA using high-capacity complementary DNA reverse transcription kits (Applied Biosystems; Waltham MA). Leptin expression was determined with SYBR Green Supermix using primer sets designed to amplify leptin transgene from the vector (forward 5' GGCAACGTGCTGGTTATTGT 3' and 5' ATATCCATCACA CTGGCGGC 3'). *Gapdh* (forward 5' TCTCTGCTCCTCCCTGTTCT 3' and reverse 5' TACGGCCAAATCCGTTTCA 3') was used to normalize. The IQ (Bio-Rad, Hercules CA) was employed to detect the amplification threshold and programmed with an initial step of 3 min at 95°C, followed by forty cycles for 5 s at 95°C and 15 s at 60°C. All reactions were run in duplicate, and the average of threshold cycle (C_T) was used in our calculations. The relative quantification of the target genes was calculated with the

C_T method. The C_T values of the target genes were normalized ($C_T = C_{T \text{ target}} - C_{T \text{ Gapdh}}$) and compared with a calibrator ($C_T = C_{T \text{ Sample}} - C_{T \text{ Calibrator}}$). Relative expression (RQ) was calculated using the IQ software (Bio-Rad; Hercules CA).

Western analyses

The hypothalami were sonicated in 270–300 μ L homogenization buffer (Tris-HCL 10 mM; pH 6.9, and 2% SDS in the presence of phosphatase/protease inhibitors; Thermo Scientific, Rockford, IL). Protein lysates were separated on a 4–20% SDS-PAGE gel and transferred to 0.2 μ m nitrocellulose membranes. Immunoreactivity was detected with ECL prime (GE Healthcare, Piscataway NJ), scanned with a ChemiDoc XRS+ (BioRad, Hercules, CA) and quantified using ImageJ software. All values, were normalized to the mean of the Control-Vehicle group. Phospho-STAT3 (P-STAT3) content was determined by comparing the signals obtained using antibodies specific to (Phospho STAT3 Tyrosine 705; Catalog # 9145; Cell Signaling, Danvers, MA, Dilution of 1: 1000 in 5% BSA) relative to the signals recorded with beta-actin antibody (Catalog # 4970; Cell Signaling, Danvers, MA, Dilution of 1: 4000 in 5% Milk) or total STAT3 (Catalog # 30835; Cell Signaling, Danvers, MA, Dilution of 1: 1000 in 5% BSA).

Serum leptin, estradiol, and testosterone

Enzyme immunoassays were used to measure serum hormone levels. Serum was separated from blood drawn in fed animals and it should be noted that estradiol was not administered on the day of euthanasia. Therefore, serum analyses were performed ~24h after the last estradiol injection. Leptin kit used (Millipore, Waltham, MA) has sensitivity to detect levels between 0.04 and 40 ng/mL with an intra-assay variability of ~ 2%. Estradiol kit can detect levels between 1.4– 200 pg/mL with intra-assay variability of <9% (ALPCO, Salem, NH). Testosterone kit has sensitivity to detect serum levels of 0.1–25 ng/mL with intra-assay variability of <9% (ALPCO, Salem, NH).

Statistical analyses

Results are expressed as means and standard errors. For endpoint analyses, differences between means were tested for statistical significance ($P < 0.05$) using a regular Two-way ANOVA with Leptin and Estrogen status (Estradiol treatment for males, and Ovx for females) as main factors, and Tukey's multiple comparison tests were applied in the event of a significant interaction ($P < 0.05$). For longitudinal analyses, we employed repeated measures (mixed model) ANOVA using time and groups as main factors and performed a Tukey's posttest in the case of significance.

Results

Leptin transgene was delivered in a way that favored leptin receptor binding throughout the brain. We utilized rAAV serotype one to readily infect cells adjacent to the 3rd ventricle and because this serotype is less specific to neurons. Our leptin vector construct contains a secretory sequence directing the transgene product to be discharged into the 3rd ventricle to activate leptin receptors throughout the brain. Transgene expression was ascertained by qPCR using a sense primer against the vector that is not present in leptin gene and an antisense primer against native leptin gene. We confirmed the presence of leptin transgene in the hypothalamus of animals from Study 1 and 2. In Study 1, leptin transgene expression relative to beta-actin in Leptin treated animals was 3,800-fold that of Control animals ($P < 0.001$). Similarly, in Study 2, leptin transgene expression relative to beta-actin in Leptin

treated animals was 5,800-fold that of Control animals ($P < 0.001$). Data were reported in previous publications [14,15].

Study 1: Estradiol administration into Male rats

Estradiol and Leptin effects are independent but additive—Initially, there was the expected surgery-related decrease in food intake followed by partial recovery (Fig. 1A, days 1–4). At days 4–5 after administration of the vectors, at a time when the anorexic response to AAV-Leptin is first expected to surface, food consumption was diminished in both Vehicle-Leptin and Estradiol-Leptin compared with Vehicle-Control and Estradiol-Control. Over time, the anorexia waned with a gradual renormalization typical of leptin-induced leptin resistance in male rats [16] (Fig. 1A). However, the anorexic response was more sustained in Estradiol-Leptin compared with Vehicle-Leptin as evidenced by the significant reduction of cumulative intake for the 25-day period (Fig. 1B). Estradiol is an established anorexigenic molecule known to stimulate a subset of neurons that are also activated by leptin, the POMC neurons [2]. It was expected that male rats with low (normal) estrogen levels would eat significantly more than those with high estrogen levels. Indeed, the Estradiol-Vehicle group had diminished food intake compared with Vehicle-Control; however, the pattern was distinctly different from that induced by Leptin. The decrease in food intake was of lower amplitude and sporadic (Fig. 1A). Moreover, separate t-test analyses revealed that, early in the study, food intake was lower in Vehicle-Leptin than in Estradiol-Control (cumulative food intake days 5–10, Fig. 1A: $P < 0.01$); in contrast, near the end of the study the Estradiol-Control rats consumed less food than Vehicle-Leptin rats (cumulative food intake days 20–25, Fig. 1A: $P < 0.05$), demonstrating that these two treatments have distinct and independent anorexigenic effects. However, there was no difference in overall cumulative food consumption between these two groups (Vehicle-Leptin vs Estradiol-Control: Fig. 1B; $P > 0.05$). The combined effect of high estrogen and Leptin was more than that each effect alone (Estradiol-Leptin vs Estradiol-Control/Vehicle-Leptin: Fig. 1A; $P_s < 0.001$) but did not exceed the summation of the effect of each treatment alone, hence the effects were not potentiating, but simply additive.

Estradiol and leptin have distinct effects on body composition as reflected by significant differences in lean to fat mass ratio between the two single treatment groups—Leptin vector also had significant impact on body weight and body composition. At the initiation of the study, body weights of the four male groups were similar (average of 410 ± 2.3 g). Leptin induced weight loss (–5% of initial body weight) and diminished body weight gain as evidenced by differences in body weight. Endpoint body weight was 13% less in Leptin-treated males than in their corresponding Control groups (Vehicle-Leptin vs Vehicle-Control, and Estradiol-Leptin vs Estradiol-Control: Fig. 1C; $P_s < 0.001$). Similarly, fat mass was diminished by Leptin from day 7 until the end of the study and endpoint fat mass was 22–25% lower in Leptin groups than in Control groups (Vehicle-Leptin vs Vehicle-Control, and Estradiol-Leptin vs Estradiol-Control: Fig. 2A; $P_s < 0.001$). The overall effect of Leptin on fat mass was reflected by the lower endpoint abdominal fat mass (retroperitoneal, perirenal, epididymal, and mesenteric fat pad mass) which was 68–82% lower in Leptin groups than in Control groups and was also reflected by the 50–70% lower serum leptin levels (Vehicle-Leptin vs Vehicle-Control, and Estradiol-

Leptin vs Estradiol-Control: Fig. 3A and B; $P_s < 0.001$). Starting day 14, lean mass was significantly lower in Leptin groups than in respective control groups and was 15–17% lower at endpoint (Vehicle-Leptin vs Vehicle-Control, and Estradiol-Leptin vs Estradiol-Control: Fig. 2B; $P_s < 0.05$). Estradiol is also known to have significant effect on body weight through its anorexigenic potential and activation of the sympathetic nervous system. As expected, Estradiol treatment decreased body weight or prevented weight gain (Vehicle-Control vs Estradiol-Control or Vehicle-Leptin vs Estradiol-Leptin: Fig. 1A; $P_s < 0.001$). Estradiol treatment alone did not reduce initial fat mass beyond 7 days of treatment but prevented fat mass gain or regain observed in Vehicle treated groups. Endpoint fat mass was 13–18% less in Estradiol treated than in Vehicle treated (Vehicle-Control vs Estradiol-Control or Vehicle-Leptin vs Estradiol-Leptin: Fig. 2A; $P_s < 0.001$).

Male rats that received dual treatment (Estradiol-Leptin) lost significantly more body weight than all other groups (Fig. 1C; $P_s < 0.001$). On the other hand, fat mass loss in the groups with high estrogen and Leptin was mostly due to Leptin, at least in the first three weeks. From day 0 to 21, there was no additional fat mass loss in the Leptin group that received estradiol than in the group that did not (Estradiol-Leptin vs Vehicle-Leptin: Fig. 2A; $P_s > 0.05$). The presence of both anorexigenic conditions resulted in a more sustained fat mass loss. At day 25, Estradiol-Leptin males had maintained most of their fat mass loss while Vehicle-Leptin males essentially regained it (Vehicle-Leptin vs Estradiol-Leptin: Fig. 2A; $P < 0.001$). This was also reflected by the 50% lower endpoint abdominal fat mass in the Estradiol-Leptin, compared to Vehicle-Leptin (Fig. 3A; $P < 0.001$). Although Estradiol-Leptin group had significantly lower fat mass than Vehicle-Leptin, there was no difference in serum leptin between these two groups (Estradiol-Leptin vs Vehicle-Leptin: Fig. 3A; $P > 0.05$). The lost lean body mass appeared to be mainly due to estradiol. At the end of the study, lean body mass was 12–25% less than all other groups (Estradiol-Leptin vs Estradiol-Control/Vehicle-Leptin/Vehicle-Control: Fig. 2B; $P_s < 0.001$). Due to the time between the last estradiol injection and blood draw, we did not observe a significant increase in serum estradiol (Vehicle 2.72 ± 0.87 pg/mL vs Estradiol 6.78 ± 4.38 pg/mL; $P > 0.05$). It appears that delay in the blood draw was sufficient time to clear the exogenous estradiol from the blood stream. The other potential reasons for this lack of effect is the choice of vehicle (sesame oil), which minimizes release kinetics of estradiol and lowers peak amplitude compared to ethanol [10]. Regardless, male rats in Estradiol groups displayed significantly lower serum testosterone levels (Vehicle-Control vs Estradiol-Control and Vehicle-Leptin vs Estradiol-Leptin: Fig. 3A $P_s < 0.001$). This outcome is expected given that estradiol provides strong feedback inhibition on the LH/FSH axis resulting in suppression of testosterone secretion [17,18].

Estradiol increases the proportion of activated STAT3 but not the absolute amount of P-STAT3—Phosphorylation of STAT3 (P-STAT3) was examined in the hypothalamus at death. No exogenous leptin was administered, thus detected P-STAT3 was due to centrally overexpressed leptin. As expected, P-STAT3: STAT3 ratio was greater in both leptin groups compared with respective controls (Fig. 4A $P_s < 0.001$). Estradiol treated animal also exhibited a significantly higher P-STAT3: STAT3 ratio (Vehicle-Control vs Estradiol-Control and Vehicle-Leptin vs Estradiol-Leptin: Fig. 4A $P < 0.01$) with the highest

P-STAT3 relative abundance in the Leptin-Estradiol group, suggesting that estradiol may alter leptin signaling. However, when normalized to Beta-Actin, P-STAT3 was not affected by estradiol, although P-STAT3 was higher in both Leptin groups compared with Control groups (Fig. 4B $P_s < 0.001$). These differential results are apparently due to a relative increase in total STAT3 with leptin treatment and a decrease in total STAT3 with estradiol treatment when normalized to Beta-Actin (Fig. 4C $P_s < 0.01$). This indicates a proportional increase in P-STAT3, but not an absolute increase in P-STAT3 with estradiol treatment.

Study 2: Ovariectomized versus ovary intact females

Leptin induced anorexia, regardless of their estrogen status—In Study 1, leptin induced anorexia in all male rats regardless whether they were Vehicle-treated (i.e., low estrogen levels) or Estradiol-treated. In Study 2, at days 0–7 post central vector delivery, animals treated with AAV-Leptin underwent the typical initial hypophagic phase and food consumption was diminished in both Leptin groups whether estrogen levels were low (Ovx) or normal (Sham). Indeed, both Leptin groups ate significantly less food than Control groups (Ovx-Leptin vs Oxv-Control, and Sham-Leptin vs Sham-Control: Fig. 5A; $P_s < 0.001$). Although the initial hypophagia in response to leptin faded over time, Leptin-treated animals consumed significantly less food after the expected hypophagic phase (Ovx-Leptin vs Oxv-Control, and Sham-Leptin vs Sham-Control: Fig. 5A; $P_s < 0.001$). Similar to estradiol treatment in males, the presence of endogenous female hormones in Sham lowered food intake but this effect appeared to be distinct from Leptin. In this sense, during Leptin-hypophagic response (day 0–7), Oxv did not significantly affect food intake (Ovx-Leptin vs Sham-Leptin, and Oxv-Control vs Sham-Control: Fig. 5A; $P > 0.05$). Beyond the initial Leptin hypophagic phase (day 8–28), Oxv animals consumed more food than Sham animals (Ovx-Control vs Sham-Control, and Oxv-Leptin vs Sham-Leptin: Fig. 5A and B; $P_s < 0.001$). Like males, animals with dual anorexigenic condition (Sham [gonadally-intact/higher estrogen] + Leptin) ate significantly less food than groups with single anorexigenic condition (Sham-Leptin vs Oxv-Leptin or Sham-Control; Fig. 5B; $P_s < 0.001$). However, there was no difference in cumulative (Day 0–28) food consumption between the two groups with single anorexigenic condition (Sham-Control vs Oxv-Leptin: $P_s > 0.05$, data not shown). As observed in males, the combined effect of higher estrogen and leptin (Sham-Leptin) was greater than that of each effect alone (Sham-Control or Oxv-Leptin) but were simply additive.

Ovariectomy did not prevent females from losing body weight in response to leptin.—As anticipated, Leptin-treated rats lost a significant amount of body weight (Sham-Control vs Sham-Leptin, Oxv-Control vs Oxv-Leptin: Fig. 5C; $P_s < 0.001$). As observed in males, high estrogen status resulted in higher body weight loss (Sham-Control vs Oxv-Control and Sham-Leptin vs Oxv-Leptin: Fig. 5C; $P_s < 0.001$). However, despite consuming the same amount of food, the Oxv-Leptin group lost significantly more body weight than Sham-Control and endpoint body weight was 8% less in Oxv-Leptin and Sham-Control (Fig. 5C: $P < 0.001$). Fat mass was drastically reduced in both Leptin groups and endpoint fat mass measured by TD-NMR was 33–35% lower than in Control groups (Sham-Control vs Sham-Leptin, Oxv-Control vs Oxv-Leptin: Fig. 6A; $P_s < 0.001$). Fat mass was unchanged by estrogen status at day 14. At day 28, fat mass was 13% higher in Oxv-Control compared

to Sham-Control (Fig. 6A; $P < 0.05$); however, this difference was not reflected in abdominal fat mass (sum of retroperitoneal, perirenal, parametrial, and mesenteric fat pads) at death (Fig. 7A). Endpoint lean mass was 15–18% lower in Leptin compared to Control groups (Sham-Control vs Sham-Leptin, Ovx-Control vs Ovx-Leptin: Fig. 6B; $P_s < 0.001$). Like males, animal with lower estrogen levels (Ovx) had higher lean mass and endpoint values were 14–24% higher in Ovx than Sham rats (Sham-Control vs Ovx-Control and Sham-Leptin vs Ovx-Leptin: Fig. 6B; $P_s < 0.05$). The Leptin vector completely depleted abdominal fat stores and reduced serum leptin levels by 67–68%, (Ovx-Leptin vs Ovx-Control, and Sham-Leptin vs Sham-Control: Fig. 7A and B; $P_s < 0.001$), but there was no effect of ovariectomy. Ovariectomy resulted in dramatically lower uterus weight ($P_s < 0.001$), confirming success of the surgical procedure (Fig. 7C).

Discussion

Fundamental aspects of metabolic homeostasis are sex-biased and have important implications for the treatment of obesity-related health conditions. For instance, a combination of glucose lowering agents have greater therapeutic potential in females than males [19]. Another concrete example is the sexual dimorphism in the anorexigenic response to Methylphenidate, a drug that increases dopamine in the brain. There is a superior satiation effect in males compared with females [20]. Given that male and female feeding and metabolic circuitries are wired differently [21], further understanding sexual dimorphisms in energy homeostasis may help design better therapeutic interventions to combat obesity in both sexes.

The adipocyte-derived hormone leptin may constitute a primary source of sex differences in energy homeostasis. Leptin regulates both aspect of the energy balance, intake and expenditure. Cumulative evidence indicate that leptin acts more effectively in females than in males. Indeed, acute central leptin treatment induces stronger anorexia in females than in males [22]. In line with these findings, we recently demonstrated that females display greater and more persistent physiological responses to central leptin gene delivery [14]. Several sex differences in the hypothalamic feeding circuits controlled by leptin have been reported. For instance, the *Mc4r* gene, an important anorexigenic signaling event downstream to the leptin receptor, is expressed at a lower level in the brain of males compared to females [23]. Therefore, it is not surprising that administration of the MC4R antagonist HS014 has a stronger obesogenic effect in males than in females [24]. The orexigenic gene *Npy*, suppressed by leptin, is expressed at a higher level in males than in females rats and testosterone increases the concentration of NPY in the arcuate nucleus, potentially constituting an important underlying mechanism for larger appetite in males [25]. Expression levels of two orexigenic molecules controlled by leptin, NPY and AgRP are inversely correlated with estrogen levels throughout the estrous cycle [26]. Therefore, estradiol levels appear to play an important role in the regulation of appetite.

In males, estradiol and leptin have separate but cumulative effects on food intake and body weight

We hypothesized that the enhanced response to central leptin gene delivery in female rats was due to the hormonal milieu, more specifically, to the higher level of estradiol. To further explore this possibility, we compared Vehicle-treated versus Estradiol-treated males that were centrally delivered via AAV to overexpress either leptin or a control gene. As expected, Estradiol-Leptin male rats had the greatest and most sustained body weight loss, reminiscent of the female response to chronic overexpression of leptin in the brain [14]. In this experiment, estradiol treatment effectively suppressed circulating testosterone to values that were consistent with that of intact females, which limited potential interactions among testosterone and estradiol as a confounding variable in data interpretation [27]. However, our treatment did not completely replicate physiological condition in females as males received daily injections of estradiol dissolved in vehicle that reduces release kinetics, while female estradiol concentrations vary throughout the estrous cycle. This may have affected the outcomes. Future studies will be necessary to determine whether constant variations in serum estradiol levels similar to female estrous cycle would affect the final outcomes. Estradiol and Leptin effects on body weight loss were not potentiating but mostly additive. In fact, Estradiol and Leptin decreased body weight and cumulative food intake to the same extent but their anorexigenic and catabolic effects appeared to be working independently of one another. In line with this interpretation, changes in food intake were unique to each treatment, with a rapid decrease in Vehicle-Leptin followed by gradual renormalization typical of leptin-induced leptin resistance in male rats [28]. In contrast, decrease in food intake in the Estradiol-Control group was of lower amplitude but sustained over the 25 days. This finding is in agreement with a previous report in which chronic but not acute estradiol treatment suppressed feeding in ovariectomized sheep [29]. The long-term suppression of food intake by estradiol may be explained by near-complete suppression of testosterone since it is a potent orexigenic hormone [30]. Testosterone induces the expression of orexigenic NPY and represses the expression of anorexigenic proopiomelanocortin in the ARC [30]. It is unclear whether estradiol treatment influenced leptin signaling in our study. On the one hand, estradiol treated groups displayed higher proportion of activated STAT3 (P-STAT3: STAT3 ratio) compared to vehicle treated, suggesting that leptin signaling was enhanced. This interpretation is supported by previous reports indicating that estradiol increases phosphorylation of STAT3 [31,32]. However, when P-STAT3 levels were determined relative to Beta-Actin expression, there was no effect of estradiol, suggesting that estradiol did not affect leptin signaling. Assessment of leptin STAT3 phosphorylation signaling following an acute leptin injection in estradiol treated male rats may help resolve this issue.

In males, Leptin but not estradiol appears to reduce body weight primarily through fat mass loss

Other evidence supporting the conclusions that estradiol and leptin act independently were the fat mass changes over time. Within the first 14 days post vector delivery, estradiol alone minimally reduced fat mass loss while leptin robustly depleted fat stores, independent of estrogen status. The effect of estradiol on fat mass surfaced toward the end of the study when it could be observed estradiol treatment clearly mitigated fat mass gain or regain in

Control and Leptin groups, respectively. Treating the males with estradiol did enhance and prolong Leptin-mediated fat mass loss but did not completely recapitulate the female phenotype. In our previous study, females receiving the same treatment maintained a fat mass loss of 22% of their initial fat mass [14] while males in the present study essentially regained their lost fat mass. Environment variables could have contributed to the inter-experiment differences; however, given the similarities of the animals (age, weight, and strain) and treatment (same vector batch, dose, and duration of the experiment), it is likely that other anabolic factors in males and/or other catabolic factors in females account for the differences. For example, male animals in the present study exhibited a near-complete suppression of circulating testosterone in response to estradiol administration, which was expected given that estradiol produces potent feedback inhibition on the LH/FSH axis and suppresses testosterone secretion in males [17,33]. This effect may, at least partially, explain the adiposity differences among studies because testosterone and estradiol collectively lower fat mass in males and inhibit a variety of lipogenic-related genes in visceral fat [34]. On the other hand, the effect of each treatment on lean mass was less obvious. Both single treatment groups maintained a steady level of lean mass throughout the study, while both estradiol treated groups ended the experiment with slightly lower absolute lean mass than baseline. The lower lean mass in estradiol treated groups may be explained by muscle wasting. Indeed, gastrocnemius muscle mass was 10% lower in Estradiol groups than in Vehicle treated groups ($P < 0.01$; data not shown) most likely due to low testosterone levels. In agreement with this hypothesis, testosterone deficiency by gonadectomy reduces muscle mass [35].

In females, estrogen status and leptin also have separate but cumulative effects on food intake and body weight

The second experiment also points toward separate actions for estrogen and leptin. In this experiment, successful Ovx surgery was confirmed by evaluating uterine weights, which were relatively normal in Sham females and dramatically lower after Ovx. These results were expected and indicate the presence of estradiol in Sham females and near-absence of estradiol in response to Ovx [36]. Like males, the effect of estrogen was additive to the effect of Leptin, therefore Sham-Leptin females lost the highest amount of weight. As observed in the male experiment, there was a rapid and robust decrease in daily food intake by Leptin early in the study (day 0–7). During that same period, estrogen status did not affect appetite. However, beyond leptin hypophagic phase (day 8–28), higher levels of female gonadal hormones were associated with lower food intake, supporting the conclusions drawn from the male study that leptin and estradiol have distinct effects on food intake. Leptin and estrogen status also appeared to differentially affect body weight. As expected, Ovx animals did not lose body weight to the same extent as Sham animals in response to Leptin, reminiscent of the pattern observed in males. However, Sham-Leptin animals lost more body weight than Ovx-Leptin animals; although, differences in body weight were due to lean mass loss, not fat mass loss. These data support the interpretation that female hormones contribute to enhanced body weight loss independently of leptin.

In females, Leptin but not estradiol appears to reduce body mass primarily through fat mass loss

Among the most striking observations from this study, were that estradiol and leptin independently decrease body weight by targeting different body compartments. In this regard, the female study is consistent with the male study and indicates that Leptin primarily targets fat mass. In both studies, estrogen status did not affect the initial fat mass response. Differences in Leptin-induced body weight loss in OvX versus Sham were not due to fat mass. Instead, endpoint body weight of these two groups differed because of lean mass. Consistent with males with low estrogen levels, OvX animals tended to preserve their lean mass better than did Sham animals. It can be explained at least partly by differences in cumulative food intake. Ovariectomy is an extensively used approach to evaluate the contribution of female hormones in biological processes but it has some limitations such as the sudden depletion of estrogen levels. Such a rapid change certainly triggered several physiological compensations that are difficult to account for or duplicate in control rats. Nevertheless, the present study does not support a major role of estrogen in the female leptin response and it appears that estradiol and leptin have independent effect in the regulation of energy homeostasis.

Our findings indicate that estradiol and leptin have independent modes of actions to maintain energy homeostasis. Feminizing the male hormonal milieu by daily treatment of estradiol in male rats enhanced and prolonged leptin responses reminiscent of the pattern observed in females. In comparison, estrogen withdrawal in females reduced and shortened leptin response reminiscent of the pattern in males. Although female gonadal hormones clearly play a role in long-term leptin response, hormonal status did not affect the initial rapid and robust fat mass loss in response to leptin. Taken together, these data suggest that estradiol may contribute to the sexual dimorphism in long-term response to leptin, however, its actions are additive and independent of that of leptin and do not seem to primarily affect fat mass.

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Fig. 1. Daily and cumulative food intake and body weight in Study 1

A. Daily food intake was reduced by Estradiol and/or Leptin. There was a significant main effect of Leptin from day 5 to 14 († Vehicle-Control vs Vehicle Leptin, Estradiol-Control vs Estradiol-Leptin: $P_s < 0.05$). The effect of estradiol was sporadic, but could be observed throughout the duration of the study (* Vehicle-Control vs Estradiol-Control, Vehicle-Leptin vs Estradiol-Leptin: $P_s < 0.05$). There were significant differences between the two single-treatment groups from day 2 to 9: Vehicle-Leptin vs Estradiol-Control: $P_s < 0.05$ (not labelled). **B.** Cumulative food intake followed the same pattern as daily food intake with two

significant main effects: Leptin and Estradiol (††† Vehicle-Control vs Vehicle Leptin, ††† Estradiol-Control vs Estradiol-Leptin, *** Vehicle-Control vs Estradiol-Control, *** Vehicle-Leptin vs Estradiol-Leptin: $P_s < 0.001$). There was no difference between the two single-treatment groups: Vehicle-Leptin vs Estradiol-Control: $P > 0.05$. C. Body weights were reduced by Leptin and/or Estradiol. At the initiation of the study, body weights of the four groups were similar (Vehicle-Control: 407.5 ± 6.2 , Vehicle-Leptin: 410.8 ± 5.6 , Estradiol-Control: 409.2 ± 5.4 , and Estradiol-Leptin: 403.4 ± 7.6). Body weight patterns followed changes in food intake with significant differences between Leptin and Control groups as well as between Estradiol and Vehicle groups (Vehicle-Control vs Vehicle-Leptin, Estradiol-Control vs Estradiol-Leptin, Vehicle-Control vs Estradiol-Control, Vehicle-Leptin vs Estradiol-Leptin: $P_s < 0.05$). * Estradiol effect (day 11–25; $P_s < 0.05$), † Leptin effect (day 9–25; $P_s < 0.05$). Values are means $\pm$ standard errors.

Fig. 2. Absolute fat mass, and lean mass in Study 1

A. There was a significant loss in fat mass by Leptin and starting day 7, Leptin-treated groups displayed significantly lower fat mass than corresponding Control groups (Vehicle-Leptin vs Vehicle-Control and Estradiol-Leptin vs Estradiol-Control: P s < 0.001). Estradiol treatment decreased fat mass toward the end of the experiment and absolute fat mass was significantly lower in Estradiol treated groups at day 25 (Estradiol-Control vs Vehicle-Control and Estradiol-Leptin vs Vehicle-Leptin: P s < 0.001). Noteworthy, there was a significant difference in absolute fat mass between the two single-treatment groups at day 7,

14, and 21 (Estradiol-Control vs Vehicle-Leptin: $P_s < 0.01$). Absolute values at days 0, 7, 14, and 25 are displayed. *** Estradiol effect ($P < 0.001$), * ($P < 0.05$); ††† Leptin effect ($P < 0.001$). **B.** Lean mass was diminished by Estradiol at day 7 (Estradiol-Control vs Vehicle-Control: $P < 0.001$). At that time point, lean mass was not changed by Leptin. The Estradiol effect size on lean mass was increasingly higher over time. Starting day 21, there was a significant effect of Leptin (Vehicle-Leptin vs Vehicle-Control and Estradiol-Leptin vs Estradiol-Control: $P_s < 0.05$). At day 25 only, there was a slight but significant difference in delta lean body mass between the two single-treatment groups (Estradiol-Control vs Vehicle-Leptin: $P < 0.05$). Values represent individual change (%) in initial values recorded the day of vector delivery. Values at days 0, 7, 14, and 25 are displayed. *** Estradiol effect ($P < 0.001$); ††† Leptin effect ($P < 0.001$), † ($P < 0.05$).

Fig. 3. Endpoint fat mass, and serum leptin levels in Study 1

A. Intra-abdominal fat mass (sum of epididymal, retroperitoneal, perirenal, and mesenteric fat depot weights) was decreased by Leptin ($P < 0.001$) and Estradiol ($P < 0.05$). **B.** Serum leptin levels were reduced in Estradiol-Control compared to Vehicle-Control ($P < 0.05$), but there was no difference between Estradiol-Leptin and Vehicle-Leptin ($P > 0.05$). The effect of Leptin was observed in both Vehicle and Estradiol-Vehicle treated males (Vehicle-Leptin vs Vehicle-Control and Estradiol-Leptin vs Estradiol-Control: $P < 0.001$). Values are mean $\pm$ standard errors. **C.** Serum testosterone was significantly lower in both Estradiol groups ($P < 0.001$).

< 0.001)*** Estradiol effect ($P < 0.001$), * ($P < 0.05$); ††† Leptin effect ($P < 0.001$), † ($P < 0.05$).

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Fig. 4. Hypothalamic P-STAT3 levels in Study 1

A. When normalized to total STAT3, relative expression of P-STAT3 was significantly increased in Leptin groups compared to Control groups ($P < 0.001$) and in Estradiol compared to Vehicle groups ($P < 0.01$). Values represent densitometry of P-STAT3 analysis divided by densitometry obtained from total STAT3 analysis. **B.** When normalized to Beta-Actin, relative expression of P-STAT3 was significantly increased in Leptin groups compared to Control groups ($P < 0.001$); however, there was no difference between Estradiol and Vehicle treated groups. Values represent densitometry of P-STAT3 analysis

divided by densitometry obtained from Beta-Actin analysis. **C.** Total STAT3 protein levels were normalized to beta-actin. Leptin treated groups exhibited significantly higher STAT3 levels compared to Control groups ($P < 0.01$). In contrast, Estradiol groups displayed significantly less STAT3 than Vehicle treated groups ($P < 0.01$). Values are mean $\pm$ standard errors. ** Estradiol effect ($P < 0.01$) ††† Leptin effect ($P < 0.001$). †† ($P < 0.01$)

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Fig. 5. Food intake during (day 0–7) or after (day 8–28) the initial hypophagic response to Leptin, and changes in body weight in Study 2

A. During the expected initial hypophagic phase, there was a main effect of Leptin (Sham-Control vs Sham-Leptin, Ovx-Control vs Ovx-Leptin, $P_s < 0.001$); however, there was no main effect of Ovx: $P > 0.05$. **B.** Cumulative food intake after the expected initial hypophagic phase induced by Leptin, between day 8 and 28. Although the initial hypophagia in response to leptin faded, there was still a main Leptin effect (Sham-Control vs Sham-Leptin, Ovx-Control vs Ovx-Leptin, $P_s < 0.001$) and the Ovx effect also surfaced (Sham-Control vs Ovx-

Control, Sham-Leptin vs Ovx-Leptin: $P_s < 0.001$). There was no difference between Ovx-Leptin and Sham-Control: $P > 0.05$. **C.** Body weight was significantly changed by Leptin and Estrogen status (Sham-Control vs Sham-Leptin, Ovx-Control vs Ovx-Leptin, Sham-Control vs Ovx-Control, Sham-Leptin vs Ovx-Leptin: $P_s < 0.001$). Despite consuming the same amount of food, the Ovx-Leptin group lost significantly more body weight than Sham-Control: $P < 0.001$. Values represent individual change (%) in initial values (body weight) recorded the day of vector delivery. *** Ovx effect ($P < 0.001$), ††† Leptin effect ($P < 0.001$). Vehicle-Control ($n = 6$), Vehicle-Leptin ($n = 7$), Estradiol-Control ($n = 7$), Estradiol-Leptin ($n = 9$)

Fig. 6. Absolute fat mass, and lean mass in Study 2

A. Fat mass was drastically decreased by Leptin and both Leptin-treated groups lost about 30% of their initial fat mass 14 days after vector delivery (Sham-Control vs Sham-Leptin, OvX-Control vs OvX-Leptin: P s < 0.001) and the magnitude of this effect was maintained until the end of the experiment (P s < 0.001). There was no effect of estrogen status on fat mass at day 14. However, at day 28, Sham-Control animals had slightly lower fat mass gain than OvX-Control rats (P < 0.05). Values represent individual change (%) in initial values recorded the day of vector delivery. Values at days 0, 14, and 28 are displayed. * OvX effect

($P < 0.05$); ††† Leptin effect ($P < 0.001$). **B.** Lean mass was significantly decreased by Leptin at day 14 and 28 (Sham-Control vs Sham-Leptin, Ovx-Control vs Ovx-Leptin: $P_s < 0.01$). At day 14, the Ovx-Control group gained more lean mass than Sham-Control ($P < 0.05$) but there was no significant difference between Ovx-Leptin and Sham-Leptin. At day 28, both Ovx groups gained or regained lean mass (Ovx-Control vs Sham-Control or Ovx-Leptin vs Sham-Leptin: $P_s < 0.001$). Values represent individual change (%) in initial values recorded the day of vector delivery. Values at days 0, 14, and 28 are displayed. *** Ovx effect ($P < 0.001$), * ($P < 0.05$); ††† Leptin effect ($P < 0.001$), †† ($P < 0.01$).

Fig. 7. Endpoint fat mass, and serum leptin levels in Study 2

A. Abdominal fat mass at endpoint was completely depleted in both Leptin groups (Sham-Control vs Sham-Leptin, OvX-Control vs OvX-Leptin: $P_s < 0.001$). There was no effect of Estrogen status on endpoint fat mass. **B.** Serum leptin levels reflected fat mass depletion and were significantly reduced in Leptin groups (Sham-Control vs Sham-Leptin, OvX-Control vs OvX-Leptin: $P_s < 0.001$). Estrogen status did not affect serum leptin levels. **C.** Uterus weights at endpoint was significantly lower in OvX groups (Sham-Control vs OvX-Control, Sham-

Leptin vs OvX-Leptin: $P_s < 0.001$). Uterus weights were lower in Sham-Leptin compared to Sham-Control ($P < 0.01$).

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