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The roles of leptin receptors on POMC neurons in the regulation of sex-specific energy homeostasis

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Abstract

Leptin regulates energy homeostasis and reproduction. One key population of leptin receptors (Lepr) are found on proopiomelanocortin (POMC) neurons in the hypothalamic arcuate nucleus, and evidence links the action of gonadal estrogens to these same POMC neurons. To determine whether Lepr on POMC neurons are critical for reproductive capacity or for sex-specific energy and glucose homeostasis, we studied Cre/loxP mice lacking Lepr specifically on POMC neurons (*Pomc-Cre, Lepr^{fllox/fllox}* mice) and their controls with normal Lepr (*Lepr^{fllox/fllox}* mice). *Pomc-Cre, Lepr^{fllox/fllox}* mice maintained normal reproductive capacity and accumulated more body fat than their same-sex controls. Ovariectomy (OVX) was performed to investigate the effects of the estrogens and Lepr on POMC neurons on body fat accumulation and glucose tolerance. OVX *Pomc-Cre, Lepr^{fllox/fllox}* females accumulated more fat than OVX *Lepr^{fllox/fllox}* females did. *Pomc-Cre, Lepr^{fllox/fllox}* males were glucose intolerant and insulin insensitive compared with control males. In contrast, control and *Pomc-Cre, Lepr^{fllox/fllox}* females had similar glucose tolerance before and after OVX. Therefore leptin's action on POMC neurons reduces body fat accumulation, but is not critical for regulation of reproduction. The sex difference in leptin signaling on POMC neurons on glucose tolerance appears independent of ovarian hormones.

Keywords

reproduction; body fat distribution; ovariectomy; glucose tolerance

Introduction

Energy balance and glucose homeostasis are regulated in part by a complex calculus of peripheral signals that provides inputs to the CNS (1). Leptin, a 16 kDa peptide hormone encoded by the obese gene, is primarily synthesized and secreted from white adipose tissue (2) and circulates at levels proportional to total adiposity. Leptin crosses the blood brain barrier, enters several brain areas including the arcuate nucleus of the hypothalamus, and binds to specific receptors located on two populations of arcuate neurons (3). Specifically, leptin inhibits the activity of neurons that express neuropeptide Y and agouti-related peptide,

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and increases the activity of proopiomelanocortin (POMC) neurons. Leptin thus influences several downstream targets of the arcuate including those that regulate reproduction, energy balance, and glucose homeostasis. Lack of leptin signaling due to mutation of either leptin (as occurs in *ob/ob* mice) or leptin receptors (*Lepr*; as occurs in *db/db* mice) indicates a state of energy deficit to the brain and triggers complex neuroendocrine changes which result in the development of morbid obesity, insulin resistance and impaired glucose homeostasis (4–5). Leptin also influences reproductive function and fertility. *db/db* mice and Zucker *fa/fa* rats with aberrant *Lepr* are hypogonadal and reproductively infertile (6–7), and experimentally-induced expression of *Lepr* in the brain of *db/db* mice restores their fertility (8), implying that central *Lepr* is critical in the regulation of reproduction.

Like leptin, the class of gonadal steroid estrogens also exerts a potent influence on energy balance, adiposity and glucose homeostasis (9). Visceral fat varies inversely with plasma estradiol. As the circulating level of estradiol decreases, visceral adiposity increases (9). In humans, pre-menopausal women are more insulin-sensitive than age-matched men (10), and the reduction in estrogens during menopause is associated with increased visceral adiposity and the development of glucose intolerance and insulin resistance (11). Hormonal replacement therapy reduces the incidence of type 2 diabetes in postmenopausal women (12). Similarly, OVX rodents become obese, preferentially gain visceral fat (13), and have impaired insulin sensitivity and glucose metabolism (14); and both effects are reversed by replacement of estrogens (13–14).

Estrogens interact with leptin to mediate its inhibition of feeding behavior (15). Estrogens cross the blood-brain barrier, bind to estrogen receptors (ER) throughout the brain including several hypothalamic nuclei, and reduce food intake and body weight. Neurons in the arcuate nucleus express both ER and the long form of *Lepr* (16). Estrogens reportedly influence *Lepr* expression. OVX causes a marked reduction in expression of the long form of *Lepr* in the hypothalamus, and estradiol replacement restores its expression (17–18). Additionally, when delivered locally into the cerebral ventricles, estradiol decreases food intake through its actions on the same POMC neurons that are responsible for leptin's anorectic responses (19). Thus, there is a closely coupled interaction between leptin and estrogens on POMC neurons in the regulation of behavioral and neuroendocrine mechanisms of energy homeostasis (9).

We hypothesized that (1) since leptin signaling on POMC neurons is critical for fertility, reproduction would be compromised in the absence of *Lepr* on POMC neurons; (2) because of close interaction of estrogen and *Lepr* on POMC neurons in regulating energy homeostasis, sex-specific body fat distribution and glucose homeostasis would be affected in the absence of *Lepr* on POMC neurons. In the present series of experiments, we utilized mice that express leptin receptors normally throughout the body, lacking them only on POMC-expressing cells (i.e. *Pomc*-Cre, *Lepr^{flox/flox}* mice), and their controls *Lepr^{flox/flox}* mice to determine whether or not leptin signaling on POMC neurons is necessary for reproduction and sex-specific regulation in energy homeostasis.

Methods

Animals

Mice lacking *Lepr* on POMC neurons (*Pomc*-Cre, *Lepr^{flox/flox}* mice) were obtained by crossing *Pomc*-Cre mice on an FVB background (provided by Dr. Bradford B. Lowell, Beth Israel Deaconess Medical Center, Boston, MA) with *Lepr^{flox/flox}* (129-C57Bl6/J × FVB N2) mice (provided by Dr. Streamson C. Chua, Jr., Albert Einstein University, New York, NY). All mice were bred in the colony at the University of Cincinnati using harem breeding design, i.e., one male was housed and bred with two or three females, and breeding records

were kept. Mice were maintained on pelleted chow and had ad libitum access to water throughout. After weaning at 3 weeks of age, mice were housed individually in micro-isolator cages in pathogen-free, temperature- and humidity-controlled rooms with a 12/12-h light/dark cycle with lights on at 0500 and off at 1700 h. RT-PCR analysis was used to genotype the mice. All animal procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Assessment of reproduction

To assess reproduction, litter size at birth and litter size at weaning were recorded, the onset of puberty was determined, and estrous cycles were monitored in female mice. Specifically, the number of offspring at one birth by one female dam was recorded as litter size. Offspring was weaned at 3 weeks of age and the survival rate from birth to weaning was calculated by the ratio of number of litters weaned to litter size (number of litters weaned/litter size $\times$ 100%). The onset of puberty of female mice was indicated by day of vaginal opening, and estrous cyclicity was monitored by daily vaginal lavage. Vaginal smears were performed between 1000 and 1100 h each day during the middle of the light cycle and were stained with a DipQuick staining kit (Jorgensen Laboratories Inc., Loveland, CO) for the determination of the estrous cycle phase (20).

Ovariectomy (OVX) and sham surgeries

OVX and sham surgeries were performed in 8-wk old mice. Mice were anesthetized with avertin (2-2-2 tribromoethanol dissolved in tert-amyl alcohol, Sigma-Aldrich Co; diluted to 20 mg/ml with saline; 400 mg/kg ip) and ovariectomy (OVX) was performed by making bilateral skin and muscle incisions on the dorsolateral flank of the animal, paralleling the spinal column, such that the ovary could be rapidly visualized and removed without disturbing the uterus, oviduct, or parametrial adipose tissue. In the sham procedure, a similar incision was made and the ovary visualized, but no tissue was removed. After all surgeries, the muscle was sutured with sterile absorbable vicryl sutures, and the skin was closed with sterile wound clips. Surgical success was confirmed by monitoring the estrous phase of females and at the end of the study by measuring plasma estradiol.

Estradiol radioimmunoassay assay

Sham-operated cycling female mice at proestrous phase, OVX females, or sham-operated male mice were sacrificed 8 weeks after surgeries. At termination of study trunk blood samples were obtained between 1000 and 1200 h during the middle of the light cycle. Blood samples were centrifuged and plasma was stored at -20°C until assay. Circulating estradiol was measured by radioimmunoassay (Diagnostic Systems Laboratories Inc., Webster, TX, DSL-39100). The intra- and inter-assay coefficients of variation are 3.9–4.1%, and the sensitivity is 1.5 pg/mL.

Quantitative PCR (Q-PCR) analyses of hypothalamic ER α gene expression

There is a closely coupled interaction between leptin and estrogens via the Lepr and ER α in the hypothalamus (see Introduction). To investigate whether or not lack of Lepr would affect the mRNA level of ER α , total RNA was isolated from hypothalami of sham-operated female mice at proestrous when ER α gene expression is highest (21–22), as well as from hypothalami of OVX female mice and sham-operated males. Following DNAase treatment (Ambion, Austin, TX), cDNA was synthesized using an iScript kit (BioRad, Hercules, CA). The mouse ER α (GenBank Accession number: AU041214) forward primer is 5'-TCCTTCATGAGGGCTGTAGG, and the reverse primer is 5'-CCTCAAGCTGCCTTTACTGC. L-32 was used as an endogenous control to indicate relative quantification of gene expression from every sample. The mouse L32 Q-PCR

forward primer is 5'-GCC AGG AGA CGA CAA AAA, and the reverse primer is 5'-AATCCTCTTGCCCTGATCCT. Q-PCR was performed using a BioRad iCycler and the iQ SYBRGreen Supermix (BioRad, Hercules, CA) with 2-step amplification with 95° C for 10 sec, and annealing temperature of 61.2° C for 30 sec for 40 cycles. Relative expression of ER α was determined using manufacturer's instructions (Applied Biosystems, Foster City, CA).

Body composition and body fat distribution

A mouse-specific nuclear magnetic resonance (NMR) Echo MRI whole body composition analyzer (EchoMedical Systems, Houston, TX) was used to assess body fat and lean mass in conscious mice, providing longitudinal data (23) before and 8 weeks after surgeries. After the mice were sacrificed, the amounts of subcutaneous and non-subcutaneous carcass fat were assessed separately by dividing the carcass into two portions and using the pelting method (13). In this procedure, the skin with any attached fat, and fat on the outer surface of any skeletal muscle, was removed from the carcass. This pelt portion and the remaining carcass portion including all muscle, skeleton, organs, intramyocellular and internal fat were then assessed separately in the NMR to determine subcutaneous fat and non-subcutaneous carcass fat from the pelt portion and carcass portion respectively.

Intraperitoneal glucose tolerance tests (ipGTT)

Glucose tolerance as assessed by ipGTT in mice does not vary over the stages of the estrous cycle (24). ipGTTs were therefore conducted prior to and exactly 3 weeks following surgeries regardless of the phase of the estrous cycle. Mice were fasted for 16 h and blood samples were obtained from the tip of the tail vein. After a baseline blood sample was taken (0 min), 1.5 g/kg body weight of 20% D-glucose (Phoenix Pharmaceutical Inc., St. Joseph, MO) was injected intraperitoneally. Subsequent blood samples were taken at 15, 30, 45, 60 and 120 min after glucose administration. Glucose was measured on duplicate samples using FreeStyleTM glucometers and test strips (FreeStyle, Alameda, CA). An additional blood sample was taken from the tail vein 13–15 min after the glucose administration for measurement of plasma insulin using the mouse insulin enzyme-linked immunosorbent assay (ELISA) kits (Crystal Chem Inc., Downers Grove, IL). The coefficients of variation of intra-assay and inter-assay were 5.5% and 6.1%, respectively. To evaluate glucose tolerance, calculations of the area under the glucose curves (AUC) were made based on the glucose baseline levels at 0 min.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Comparisons among multiple groups were made using appropriate analysis of variance (ANOVA; SigmaStat 3.1, San Rafael, CA). Specifically, one-way ANOVA was used for comparisons for plasma estradiol concentrations and hypothalamic ER α expressions among all groups. Two-way ANOVA was used for the analysis of terminal fat distribution and body fat change (sex-procedure $\times$ genotype, 3 $\times$ 2). Three-way ANOVA was used for the analysis of body mass, total body fat, AUC, glucose and insulin measurements (sex-procedure $\times$ genotype $\times$ time point, 3 $\times$ 2 $\times$ 2). Post-hoc tests of individual groups were made using Tukey's tests (SigmaStat 3.1). Significance was set at $P < 0.05$. Exact probabilities and test values were omitted for simplicity and clarity of the presentation of the results.

Results

Reproductive function, circulating estradiol levels, and hypothalamic ER α mRNA level

From our two-year record for 32 dams, similar numbers of litters were born and weaned by *Pomc-Cre*, *Lepr^{flox/flox}* and *Lepr^{flox/flox}* females. The average litter size was 3.8 ± 0.3 for *Lepr^{flox/flox}* mice and 4.0 ± 0.3 for *Pomc-Cre Lepr^{flox/flox}*. The average survival rate from birth to weaning was $98.41 \pm 0.82\%$ for *Lepr^{flox/flox}* mice and $99.07 \pm 0.76\%$ for *Pomc-Cre Lepr^{flox/flox}*. In addition, female *Pomc-Cre, Lepr^{flox/flox}* mice had a similar onset of puberty compared to *Lepr^{flox/flox}* females. Specifically, the age for onset of puberty varied between 29 and 36 days for female *Lepr^{flox/flox}* mice, and between 29 and 41 days for female *Pomc-Cre Lepr^{flox/flox}* mice. The mean age at puberty onset did not differ between the two groups (*Lepr^{flox/flox}*: 33.3 ± 0.6 days; *Pomc-Cre Lepr^{flox/flox}* mice: 35.2 ± 1.1 days). After reaching adulthood, the duration of estrous cycles was comparable for the two genotypes (*Lepr^{flox/flox}*: 6.1 ± 0.2 days; *Pomc-Cre Lepr^{flox/flox}* mice: 6.2 ± 0.3 days).

Plasma estradiol levels were also similar between sham-operated cycling *Lepr^{flox/flox}* (n = 9) and *Pomc-Cre, Lepr^{flox/flox}* females (n = 9) at proestrous phase (Fig. 1A). OVX *Pomc-Cre, Lepr^{flox/flox}* females (n = 9) and OVX *Lepr^{flox/flox}* females (n = 12) did not cycle and had significantly lower circulating estradiol level than intact females. In addition, OVX females had similar estradiol levels as *Pomc-Cre, Lepr^{flox/flox}* (n = 9) and *Lepr^{flox/flox}* (n = 8) males (Fig. 1A). Hypothalamic ER α mRNA levels were similar between *Pomc-Cre, Lepr^{flox/flox}* and control males, and were both comparable to the ER α mRNA levels of OVX *Pomc-Cre, Lepr^{flox/flox}* and OVX control females (Fig. 1B). In contrast, hypothalamic ER α mRNA level was significantly lower in sham-operated *Pomc-Cre, Lepr^{flox/flox}* females than sham-operated *Lepr^{flox/flox}* females at proestrous phase, when estrogen receptors are most activated (21–22); and both levels were significantly higher than those of males and OVX females (Fig. 1B).

Effects of ovariectomy on total body fat and body fat distribution

Age-matched male *Lepr^{flox/flox}* and *Pomc-Cre, Lepr^{flox/flox}* mice were heavier than their same genotype female counterparts before surgeries, total body fat mass however was comparable between sexes within the same genotype (Table 1). Male *Lepr^{flox/flox}* mice did not significantly change their body weight but significantly increased their body fat following sham surgeries (Table 1). Sham-operated female *Lepr^{flox/flox}* mice did not significantly change their body weight or body fat whereas OVX *Lepr^{flox/flox}* females significantly increased body weight and body fat (Table 1). As a result, sham-operated male or OVX female *Lepr^{flox/flox}* mice gained significantly more total body fat than sham-operated *Lepr^{flox/flox}* females did (Fig. 2A).

Before sham surgeries *Pomc-Cre, Lepr^{flox/flox}* males were heavier and had greater adiposity than *Lepr^{flox/flox}* males. *Pomc-Cre, Lepr^{flox/flox}* males accumulated more adiposity than *Lepr^{flox/flox}* males across the 8-week post-surgery period when the total fat is considered (Table 1). In addition, sham-operated *Pomc-Cre, Lepr^{flox/flox}* males had greater carcass and subcutaneous fat than *Lepr^{flox/flox}* males (Fig. 2B and 2C). Both female *Lepr^{flox/flox}* and *Pomc-Cre, Lepr^{flox/flox}* mice gained weight and body fat following OVX (Table 1). Specifically, body fat of female *Lepr^{flox/flox}* mice was increased by $49.5 \pm 10.8\%$ from 2.07 ± 0.15 g to 3.04 ± 0.20 g (Table 1). The average body fat gain of female *Pomc-Cre, Lepr^{flox/flox}* mice was $91.4 \pm 16.2\%$ from 2.80 ± 0.33 g to 5.33 ± 0.67 g (Table 1). OVX *Pomc-Cre, Lepr^{flox/flox}* females accumulated a significantly greater percentage of body fat than OVX *Lepr^{flox/flox}* females did (Fig. 2A).

When fat distribution was examined, sham-operated *Lepr^{flox/flox}* females had significantly less non-subcutaneous carcass fat and subcutaneous fat than sham-operated *Lepr^{flox/flox}*

males (Fig. 2B and 2C). Interestingly, such sex difference was not detected between sham-operated male and female *Pomc-Cre, Lepr^{flox/flox}* mice (Fig. 2B and 2C). Carcass and subcutaneous fat of OVX *Lepr^{flox/flox}* females were significantly greater than those of sham-operated *Lepr^{flox/flox}* females; in contrast, subcutaneous fat, but not carcass fat, of OVX *Pomc-Cre, Lepr^{flox/flox}* females was significantly greater than that of sham-operated *Pomc-Cre, Lepr^{flox/flox}* females (Fig. 2B and 2C). OVX abolished sex difference in fat distribution by increasing both subcutaneous and carcass fat in *Lepr^{flox/flox}* females and OVX *Lepr^{flox/flox}* females had similar body fat distribution as *Lepr^{flox/flox}* males. *Pomc-Cre, Lepr^{flox/flox}* females had greater adiposity than control females, and they further accumulated more subcutaneous but not carcass adiposity following OVX (Fig. 2B and 2C).

Sham-operated female *Lepr^{flox/flox}* mice had significantly greater percentages of carcass fat and less percentages of subcutaneous fat than sham-operated male and OVX female *Lepr^{flox/flox}* mice (Fig. 2D and 2E). Sham-operated male *Pomc-Cre, Lepr^{flox/flox}* and *Lepr^{flox/flox}* mice had similar percentages of carcass and subcutaneous fat, whereas sham-operated female *Pomc-Cre, Lepr^{flox/flox}* mice had a significantly lower percentage of carcass fat and greater percentage of subcutaneous fat than *Lepr^{flox/flox}* females (Fig. 2D and 2E). Similar to the male groups, OVX female *Pomc-Cre, Lepr^{flox/flox}* and *Lepr^{flox/flox}* mice had comparable percentages of carcass and subcutaneous fat (Fig. 2D and 2E).

Effects of ovariectomy on glucose tolerance

Before sham operation, *Pomc-Cre, Lepr^{flox/flox}* males had higher glucose levels during glucose tolerance tests and a greater area under the glucose clearance curves than *Lepr^{flox/flox}* males, indicating that *Pomc-Cre, Lepr^{flox/flox}* males were glucose intolerant compared to *Lepr^{flox/flox}* males (Fig. 3A; Table 2). In addition, pre-surgical male *Pomc-Cre, Lepr^{flox/flox}* mice had higher 15-min insulin levels than *Lepr^{flox/flox}* males following glucose administration (Table 2), suggesting that *Pomc-Cre, Lepr^{flox/flox}* males were insulin-insensitive compared to *Lepr^{flox/flox}* males. In contrast, pre-sham and pre-OVX female *Pomc-Cre, Lepr^{flox/flox}* mice had similar glucose and 15-min insulin levels as female *Lepr^{flox/flox}* mice (Fig. 3C and 3E; Table 2). Thus, although female *Pomc-Cre, Lepr^{flox/flox}* mice had more carcass fat than *Lepr^{flox/flox}* females (Fig. 2B), they had similar glucose tolerance as *Lepr^{flox/flox}* females. It is possible that the normal reproductive function and circulating estrogen levels of female *Pomc-Cre, Lepr^{flox/flox}* mice protect them from developing glucose intolerance.

We assessed whether or not removal of ovarian hormones using OVX would lead to glucose intolerance in female *Pomc-Cre, Lepr^{flox/flox}* mice. All OVX female mice stayed in diestrus phase one week following OVX surgeries, and ipGTTs were performed 3 weeks after surgeries. Similar to what was seen from pre-surgical ipGTT, sham-operated male *Pomc-Cre, Lepr^{flox/flox}* mice had higher glucose and 15-min insulin levels than sham-operated *Lepr^{flox/flox}* males (Fig. 3B; Table 2). Both sham-operated *Pomc-Cre, Lepr^{flox/flox}* and *Lepr^{flox/flox}* female groups had similar areas under the glucose curves and 15-min insulin levels compared to pre-surgical measurements (Fig. 3D; Table 2). In contrast, female *Lepr^{flox/flox}* mice, but not *Pomc-Cre, Lepr^{flox/flox}* females, significantly increased the area under the glucose curves after OVX (Table 2), indicating development of glucose intolerance in female *Lepr^{flox/flox}* mice following OVX. The two OVX female groups had comparable glucose levels (Fig. 3F), however, OVX female *Pomc-Cre, Lepr^{flox/flox}* mice had significantly higher 15-min insulin levels than OVX *Lepr^{flox/flox}* females (Table 2).

Discussion

Leptin and its central signaling have many roles beyond its lipostatic function. A wide range of data links leptin to both the regulation of energy homeostasis and reproduction. One key

population of *Lepr* is found on POMC neurons in the arcuate nucleus, and evidence links the anorexigenic effects of estrogen as being mediated through these POMC neurons as well (19). *Pomc-Cre, Lepr^{flox/flox}* mice lacking leptin receptors specifically on POMC neurons and control *Lepr^{flox/flox}* mice with normal leptin receptors were used in the current studies to determine whether or not leptin receptors on POMC neurons are critical for reproductive capacity and/or for estrogens' influence on adiposity and glucose homeostasis. Our data suggest that lack of *Lepr* on POMC neurons has no obvious influence on reproductive capacity. ER α has been suggested to be the main nuclear estrogen receptor that mediates estradiol's catabolic effect in both sexes (25). We found that the hypothalamic ER α mRNA level was lower in sham-operated female *Pomc-Cre, Lepr^{flox/flox}* mice compared to *Lepr^{flox/flox}* females, and it was comparable between male *Pomc-Cre, Lepr^{flox/flox}* and *Lepr^{flox/flox}* mice, suggesting that central leptin signaling dysfunction due to lack of *Lepr* on POMC neurons modulates hypothalamic ER α gene expression in cycling females. It is not clear how lacking leptin signaling on POMC neurons would result in decreased hypothalamic ER α gene expression, and is worth further investigation.

Pomc-Cre, Lepr^{flox/flox} males had greater adiposity and were glucose intolerant and insulin insensitive compared with control males. The impaired glucose homeostasis in the male *Pomc-Cre, Lepr^{flox/flox}* mice was likely a secondary effect of visceral obesity. In contrast, control and *Pomc-Cre, Lepr^{flox/flox}* females had similar glucose tolerance although *Pomc-Cre, Lepr^{flox/flox}* females had greater carcass adiposity than *Lepr^{flox/flox}* females. We further investigated the interaction between estrogens and *Lepr* on POMC neurons on body fat accumulation and glucose tolerance using OVX mice. OVX *Pomc-Cre, Lepr^{flox/flox}* and *Lepr^{flox/flox}* females did not cycle and had significantly lower circulating estradiol levels than intact females, indicating successful OVX surgeries. OVX increased adiposity in both *Lepr^{flox/flox}* and *Pomc-Cre, Lepr^{flox/flox}* females. Specifically, OVX increased both subcutaneous and carcass adiposity in female *Lepr^{flox/flox}* mice, whereas majority of the increased adiposity was allocated in the subcutaneous compartment of *Pomc-Cre, Lepr^{flox/flox}* females (Fig. 2B and 2C). The fat distribution data were consistent with the finding that OVX significantly impaired glucose tolerance in *Lepr^{flox/flox}* females but only had a trend to do so in *Pomc-Cre, Lepr^{flox/flox}* females (Table 2). The two OVX female groups had comparable glucose levels during ipGTT, suggesting that the sex difference in leptin-POMC signaling on glucose tolerance was independent of ovarian hormones. OVX *Pomc-Cre, Lepr^{flox/flox}* females secreted significantly more insulin than OVX *Lepr^{flox/flox}* females during the ipGTT (Table 2); thus, although OVX *Pomc-Cre, Lepr^{flox/flox}* did not develop glucose intolerance, they were insulin resistant compared to OVX *Lepr^{flox/flox}* females.

The long form of *Lepr* has been reported to be expressed in several peripheral tissues. Several lines of evidence suggest that it is the central *Lepr* that is critical in the regulation of energy and glucose homeostasis. First, mice with deletion of *Lepr* in the brain are obese and insulin resistant, whereas mice with deletion of *Lepr* in the liver are normal (26), suggesting that direct leptin signaling in the liver alone does not affect energy balance and glucose homeostasis. Furthermore, removal of the signaling domain of the leptin receptor in most peripheral tissues including liver, adipose tissues, and small intestine, but not in the brain, causes no changes in energy balance or glucose homeostasis (27). Second, a neuron-specific *Lepr* transgene (28) or expressing *Lepr* exclusively in POMC neurons (29) completely rescues the obesity, diabetes, and infertility in *db/db* mice, supporting the hypothesis that leptin's effects on energy balance, glucose homeostasis, and fertility are centrally mediated. *Pomc-Cre, Lepr^{flox/flox}* mice, with *Lepr* removed only from the POMC neurons, exhibit an obesity phenotype in both sexes and a diabetes phenotype in males. Taken together, these data suggest that *Lepr* signaling in the CNS, other than in peripheral leptin receptor-

expressing cells, plays a critical role in the regulation of energy balance and glucose homeostasis.

Leptin has significant effects on many aspects of reproduction, including an association with the onset of puberty as well as fertility for males and females. Male and female leptin-deficient obese mice are infertile (30) and adding back leptin reverses the infertility and resulted in sexual maturity (31). Balthasar et al. previously reported that “*Pomc-Cre, Lepr^{flox/flox}* are fertile and able to lactate” (32). Whether or not the leptin receptors on POMC neurons are critical in the regulation of reproductive capacity and influence processes such as puberty, sex hormone levels, and estrous cycle has been inconclusive until the current study. We compared their reproductive function, onset of puberty, vaginal cytology, and circulating estradiol levels of female control *Lepr^{flox/flox}* and *Pomc-Cre, Lepr^{flox/flox}* mice. Our findings are in agreement with the previous observation that “*Pomc-Cre, Lepr^{flox/flox}* mice are fertile and able to lactate” (32). In addition, we found that *Pomc-Cre, Lepr^{flox/flox}* mice displayed a similar onset of puberty and had comparable reproductive capacity, as indicated by number of litters produced and reproductive success rate, and also comparable circulating estradiol levels as control *Lepr^{flox/flox}* mice.

The long form of *Lepr* is expressed in many extra-arcuate regions, including ventromedial hypothalamus, dorsomedial hypothalamus, premammillary ventral nucleus, lateral hypothalamus, ventral tegmental area, and nucleus of the solitary tract (33–35). Although significant attention has been focused on the arcuate nucleus in terms of the regulation of energy and glucose homeostasis, other leptin receptor expressing neurons may have key roles in the regulation of reproduction. The dorsomedial hypothalamus and premammillary ventral nucleus innervate areas related to reproductive control including the anteroventral periventricular nucleus and the medial preoptic area (36). The fact that reproductive function was not altered in the *Pomc-Cre, Lepr^{flox/flox}* mice suggests that *Lepr* in leptin target neurons other than arcuate POMC neurons may play an important role in mediating leptin’s effect on reproduction. Indeed, recent studies indicated that *Lepr* expressing neurons on hypothalamic premammillary nucleus regulate luteinizing hormone secretion (37) and project to the rostral hypothalamus to directly innervate GnRH neurons (38) to regulate reproduction.

Visceral fat varies inversely with circulating estrogens; as levels of estrogens decrease, visceral adiposity increases. An increase in visceral adiposity is often associated with a reduction in estrogens in females, but this was not the case in these female *Pomc-Cre, Lepr^{flox/flox}* mice which had similar circulating estradiol level as their controls (Fig. 1A) but accumulated carcass adiposity (Fig. 2B). There are several possibilities for this. First, the increased visceral adiposity could be due to reduced central and peripheral estrogen action, as indicated by reduced hypothalamic ER α gene expression. Second, the combination of estrogen and functional leptin signaling may be required for sex-specific fat distribution. Sex differences in total body fat and fat distribution were detected in sham-operated male and female control *Lepr^{flox/flox}* mice but not in *Pomc-Cre, Lepr^{flox/flox}* mice with interrupted central leptin signaling; such sex differences between male and female *Lepr^{flox/flox}* mice were abolished by estrogen deficiency via OVX (Fig. 2). Lack of either estrogen or functional leptin signaling would consequently lead to visceral obesity in females. It is possible that although circulating estradiol is maintained at normal levels in *Pomc-Cre, Lepr^{flox/flox}* females, it fails to enhance leptin’s ability to stimulate sympathetic activity of visceral fat, the most highly innervated fat depot, in the absence of leptin POMC signaling, and thus leads to accumulation of visceral adiposity.

There is a complex interaction between estrogens and leptin signaling in the regulation of energy balance and adiposity. Estrogen may regulate circulating leptin level and *ob* gene

expression. A significant increase in circulating leptin level has been observed following the weight gain associated with OVX in female rats (13, 17, 39). These results are in contrast with one study that observed no change in plasma leptin concentration in OVX females, despite observing that the OVX rats weighed significantly more than sham-operated females (18). Estradiol treatment increased *ob* mRNA levels in white adipose tissue of OVX rats (40–41). Using ovary-intact instead of OVX rats, Rocha et al. reported that estradiol treatment reduced body fat mass but did not change plasma leptin concentration or *ob* gene expression in white adipose tissue (42). These varying results indicate that the animal models (ovary-intact vs. OVX) and assay time may be critical for studying the physiological roles of estrogen and leptin in regulating energy balance.

Whether or not estrogens modulate leptin concentration or *ob* gene expression does not exclude the estrogen interacting with hypothalamic leptin signaling as a mechanism to regulate energy balance. Previous studies investigated leptin sensitivity using either peripheral or central leptin administration in estrogen-treated or estrogen-deficient OVX rodents. OVX did not alter subcutaneously administered leptin's ability to reduce food intake or body fat in rats (43) or mice (44), suggesting that estrogen might not directly mediate leptin's effect. On the contrary, the ability of central leptin administration to reduce food intake was greater in ovary-intact females than in males (13, 45) or in OVX females (13, 39). Conversely, administration of estradiol to OVX females restored their central leptin sensitivity and changed their body fat distribution to mirror that of intact females (13, 45). Additionally, altering the sex hormone milieu in males with estradiol administration increased sensitivity to central leptin administration and increased subcutaneous fat deposition (13, 45). Estrogens influences hypothalamic *Lepr* expression. OVX caused a significant (17–18) or a 50% but statistically non-significant (39) reduction in expression of the long form of *Lepr* in the hypothalamus, and estradiol replacement restored its expression (17–18). Since only *Lepr* mRNA expression has been measured, it is unknown how estrogen may impact the *Lepr* protein or signaling. The differences in leptin sensitivity caused by the presence or absence of estrogen may occur downstream of *Lepr* transcription and translation.

In summary, we have provided evidence that *Lepr* in POMC neurons, although important for leptin's effect on the regulation of body fat accumulation and glucose homeostasis, is not required for leptin's effect on reproduction. In addition, the sex difference in leptin signaling in POMC neurons on glucose homeostasis appears independent of ovarian hormones.

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Fig. 1A

Fig. 1B

Figure 1.

Circulating estradiol levels (A) and hypothalamic ER α mRNA levels (B) of sham-operated (sham) or ovariectomized (OVX) mice.

* denotes significant difference from sham female *Lepr^{flox/flox}* mice ($P < 0.05$).

† denotes significant difference from sham female *Pomc-Cre, Lepr^{flox/flox}* mice ($P < 0.05$).

Figure 2.

Total body fat change (A), carcass (B) and subcutaneous (C) fat, carcass (D) and subcutaneous (E) fat % of male and female mice before and after surgeries. * denotes difference between genotypes within same sex procedure (e.g., *Lepr^{flax/flax}* vs. *Pomc-Cre, Lepr^{flax/flax}* within Female Sham). † denotes difference between sex procedure within genotype (e.g., Male Sham vs. Female Sham vs. Female OVX within *Lepr^{flax/flax}*). $P < 0.05$.

Figure 3. ipGTT of pre-sham (A) and post-sham (B) males, pre-sham (C) and post-sham (D) females, and pre-OVX (E) and post-OVX females (F). * denotes significant difference between genotypes ($P < 0.05$).

Table 1

Body weight and total body fat of sham-operated females, ovariectomised (OVX) females, and sham-operated males.

	Before Surgeries			After Surgeries		
	Sham Male (n = 8)	Sham Female (n = 9)	OVX Female (n = 12)	Sham Male (n = 8)	Sham Female (n = 9)	OVX Female (n = 12)
<i>Lepr^{fllox/flox}</i>						
Body Mass (g)	26.72 ± 1.17	22.01 ± 0.83	23.71 ± 0.35	29.26 ± 1.03	20.99 ± 0.56 [†]	27.33 ± 0.54 [#]
Total Fat (g)	1.59 ± 0.12	2.18 ± 0.31	2.07 ± 0.15	2.48 ± 0.14 [#]	1.90 ± 0.28 [†]	3.04 ± 0.20 [#]
	Before Surgeries			After Surgeries		
<i>Pomc-Cre, Lepr^{fllox/flox}</i>						
Body Mass (g)	30.98 ± 0.86 [*]	23.76 ± 0.57	25.98 ± 0.81	33.58 ± 0.77 ^{**}	24.09 ± 0.46 ^{*†}	30.55 ± 1.20 ^{**}
Total Fat (g)	3.11 ± 0.27 [*]	2.70 ± 0.30	2.80 ± 0.33	5.25 ± 0.58 ^{**}	3.84 ± 0.26 ^{*†#}	5.33 ± 0.67 ^{**}

* denotes significant difference between genotypes within sex-procedure (e.g., *Lepr^{fllox/flox}* vs. *Pomc-Cre, Lepr^{fllox/flox}* within Sham Male).

[†] denotes significant difference between sex-procedures within genotype (e.g., Sham Female vs. OVX Female within *Pomc-Cre, Lepr^{fllox/flox}*).

[#] denotes significant difference before and after surgeries within same sex-procedure and genotype (e.g., before vs. after for OVX Female *Pomc-Cre, Lepr^{fllox/flox}*).

P < 0.05

Table 2
Glucose tolerance tests of pre- and post-surgical male or female *Lept^{flox/flox}* and *Pomc-Cre, Lept^{flox/flox}* mice.

	Baseline glucose (mg/dL)	Area under the glucose curve <i>Lept^{flox/flox}</i> mice	15 min Insulin (ng/ml)	Baseline glucose (mg/dL)	Area under the glucose curve <i>Pomc-Cre, Lept^{flox/flox}</i> mice	15 min Insulin (ng/ml)
Pre-surgical						
Sham Male	116.29 ± 4.54	2021.79 ± 460.76	8.29 ± 2.02	122.78 ± 6.71	4348.33 ± 495.47 ^{*†}	19.88 ± 5.19 *
Sham Female	96.34 ± 3.58	2638.59 ± 298.69	11.03 ± 1.97	96.15 ± 7.29	2817.75 ± 292.78	10.98 ± 2.84
OVX Female	92.50 ± 2.62	2485.96 ± 349.89	12.89 ± 2.51	101.50 ± 9.04	2913.75 ± 408.34	15.67 ± 4.48
Post-surgical		<i>Lept^{flox/flox}</i> mice			<i>Pomc-Cre, Lept^{flox/flox}</i> mice	
Sham Male	101.00 ± 6.63	3949.82 ± 900.16	8.13 ± 1.96	125.61 ± 8.93 *	5622.92 ± 516.03 [†]	14.74 ± 3.76 *
Sham Female	102.25 ± 9.39	2611.88 ± 363.76	10.86 ± 2.13	101.75 ± 7.83	2944.69 ± 354.99	12.36 ± 3.04
OVX Female	101.12 ± 4.44	3521.83 ± 229.52 #	9.57 ± 1.67	104.86 ± 7.28	3343.39 ± 390.67	19.97 ± 3.15 *

* denotes significant difference between genotypes within sex-procedure (e.g., *Lept^{flox/flox}* vs. *Pomc-Cre, Lept^{flox/flox}* within Sham Male).
[†] denotes significant difference between sex-procedures within genotype (e.g., Male Sham vs. Female Sham vs. Female OVX within *Pomc-Cre, Lept^{flox/flox}*).
denotes significant difference before and after surgeries within same sex-procedure and genotype (e.g., before vs. after for OVX Female *Lept^{flox/flox}*).

P < 0.05