Original Article

Gonadal Hormones Determine Sensitivity to Central Leptin and Insulin

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Males have proportionally more visceral fat and are more likely to develop complications associated with obesity than females, and the male brain is relatively more sensitive to the catabolic action of insulin and less sensitive to that of leptin than the female brain. To understand the underlying mechanism, we manipulated estrogen through ovariectomy (OVX) and estradiol administration. Rats with relatively high systemic estrogen (intact females and OVX females and males administered estrogen subcutaneously) were significantly more sensitive to leptin's anorexic action in the brain (i3vt), as well as significantly less sensitive to insulin's i3vt action, than intact males. Administering estradiol directly into the brain of our females increased i3vt leptin sensitivity while decreasing i3vt insulin sensitivity and changed the body fat distribution of our females to resemble that of intact females. These data indicate that estrogen acts within the brain to increase leptin sensitivity, decrease insulin sensitivity, and favor subcutaneous over visceral fat. *Diabetes* 55:978–987, 2006

The regulation of body adiposity occurs through coordinated actions of peripheral and central mechanisms. The lipostatic theory of energy regulation, proposed more than a half century ago, holds that circulating factors, generated in proportion to body fat, signal the brain and influence energy intake and expenditure (1). The discovery of leptin and its receptors provided a molecular basis for this theory (2–6). Leptin is secreted from white adipocytes in direct proportion to fat content and has diverse actions throughout the body, including providing an important signal to the brain. Administration of leptin directly into the brain decreases food intake and increases energy expenditure and, when prolonged, leads to a reduction of body weight (3,7–15).

Insulin is also secreted in direct proportion to white fat (16), and like leptin, insulin also elicits a net catabolic response via the brain (17–24). Leptin and insulin each stimulate specific receptors in the hypothalamic arcuate nucleus (5,6,15,25–29); the two activate common intracellular signaling pathways (5,24,30–32), and the catabolic action of each is mediated by the central melanocortin system (30,31,33,34).

We previously reported that the brain of male rats is relatively more sensitive to the catabolic action of insulin, whereas the brain of female rats is relatively more sensitive to the catabolic action of leptin (35,36); analogous data have recently been reported for humans (37). Consistent with this, leptin is a better correlate of body fat in females (38–40), and insulin is a better correlate of body fat in males. Body fat is differentially distributed in males and females (38,41–46). Males carry relatively more fat viscerally whereas females carry more fat subcutaneously, although the mechanisms underlying these sex differences are not known. Like leptin and insulin, the gonadal steroid estrogen reduces food intake and body weight via a direct action within the hypothalamus (47–51). Because females have higher estrogen levels than males, we hypothesized that estrogen exerts its catabolic action within the brain by enhancing leptin sensitivity, and that estrogen action in the brain also alters the distribution of white fat to favor the subcutaneous fat deposition.

RESEARCH DESIGN AND METHODS

Adult male (250–275 g) and age-matched female (220–225 g) Long-Evans rats (Harlan, IN) were individually housed in Plexiglas tubs and maintained on a 12:12-h light-dark cycle (lights out at 1400) in a temperature-controlled, Association for Assessment and Accreditation of Laboratory Animal Care International–accredited vivarium; all procedures were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. The rats were maintained on ad libitum pelleted rat chow and tap water unless otherwise noted. Seven days after arrival in the laboratory, rats were anesthetized with 1.0 ml/kg ketamine/xylazine (10:6.5 vol/vol) and were implanted with 21-gauge stainless steel guide cannulas (Plastics One, Roanoke, VA) in the skull with the tip aimed at the third cerebral ventricle (i3vt). Bregma and lambda were positioned at the same vertical coordinate, and the sagittal sinus was carefully displaced laterally as the guide cannula was lowered directly on the midline, 2.2 mm posterior to bregma, to a point 7.5 mm ventral to dura. Guide cannulas were fixed to the skull with anchor screws and dental acrylic. The guide cannulas were fitted with removable obturators that extended 0.5 mm beyond the tip (52). When rats regained their preoperative body weights following surgery, placement of i3vt cannulas was confirmed by administration of 10 ng angiotensin II in 1 μl normal saline while the animals were water replete. Animals that did not drink at least 5 ml of water within 60 min were not used. These methods are routine in our lab (30,31,35,53–56).

Ovariectomy procedure. Ovariectomy (OVX) or sham surgery was performed in anesthetized female rats by making bilateral dorsal abdominal incisions through the skin, such that the ovary and oviduct could be rapidly removed. In the sham operation, the ovary and oviduct were visualized before the incisions were sutured. The success of the OVX procedure was confirmed at the end of the study by measuring uterine weights and plasma estradiol. Animals began the experiments 4 weeks following surgery except where otherwise noted.

Castration procedure. Castrations (or sham surgeries) were performed on anesthetized males. A 1.0-cm median incision was made through the skin at the posterior tip of the scrotum, a ligature was placed around each vas deferens and associated blood vessels, and the testes were removed. For sham

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ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; i3vt, third cerebral ventricle; OB-Rb, long-form leptin receptor; OVX, ovariectomy; NMR, nuclear magnetic resonance; VMN, ventromedial nucleus.

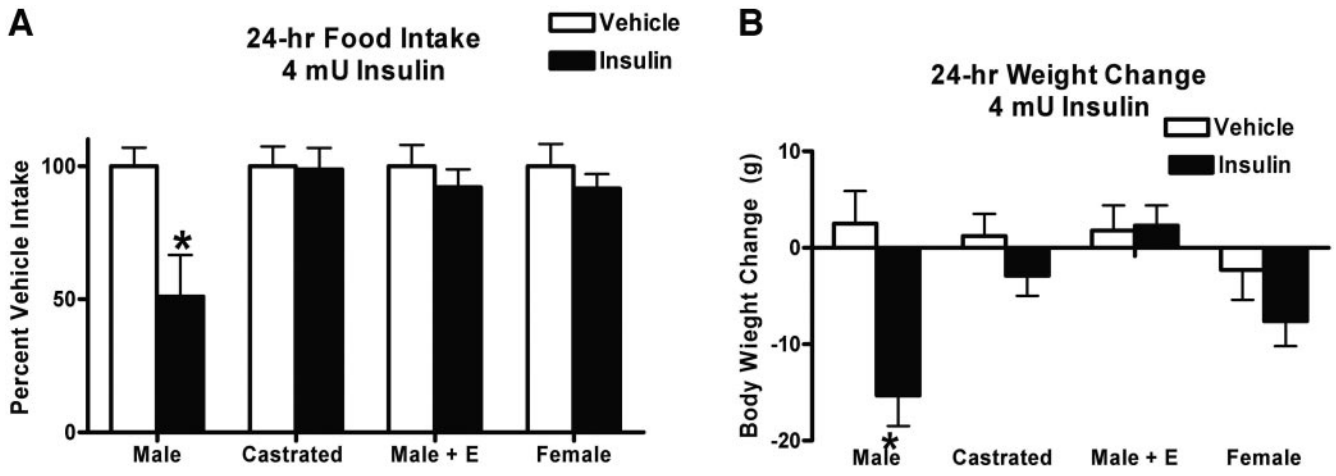

FIG. 1. i3vt insulin reduces food intake and body weight in intact males but not females, castrated males, or males administered 17 β-estradiol. *A*: Intact males (male; $n = 10$), castrated males (castrated; $n = 10$), intact males injected with 17 β-estradiol (male + E; $n = 10$), and intact females (female; $n = 18$) were administered central insulin (4 mU/1 μl saline) and vehicle alone (1 μl saline) on different days in counterbalanced order. Estradiol administration occurred every 4 days for 1 month. Data are expressed as mean (±SE) percent of vehicle baseline. ANOVA revealed that insulin significantly reduced 24-h food intake (*A*) and body weight (*B*) in intact males but not in castrated males, intact males injected with 17 β-estradiol, or females. *$P < 0.05$ compared with vehicle baseline.

surgeries, the testes were visualized and the wound closed. Animals began the experiments once presurgical body weight was achieved.

Estradiol replacement. Subgroups of male and OVX rats received intrascapular subcutaneous injections of 2.0 mg 17 β-estradiol-3-benzoate (Sigma Chemical, St. Louis, MO) in 100 μl sesame oil every 4 days between 0900 and 0930 h for 1 month to simulate estrus cycles; control injections were 100 μl sesame oil (Sigma Chemical). Dosing began 1 week after the surgeries. The dose of 2.0 mg estradiol in 100 μl reportedly produces plasma estradiol levels similar to peak levels occurring during the ovarian cycle in intact rats (57) and, when administered over an extended period of time, normalizes body weight and daily food intake of OVX rats (57). For central estradiol administration, a smaller volume (1 μl) of the same concentration of estradiol (2 mg/100 μl oil or 2 μg centrally) was injected i3vt every 4th day for 1 month. Control animals were injected with the same volume of the vehicle sesame oil.

Protocols. Intact and gonadectomized male and female rats receiving subcutaneous sesame oil and intact males and OVX females administered subcutaneous 17 β-estradiol were used. On a test day, rats had their food removed 4 h before the onset of the dark and were given a bolus i3vt injection of insulin (Iletin II Regular pork insulin; Eli Lilly, Indianapolis, IN) (1 or 4 mU/1 μl) or vehicle (saline, 1 μl). The 4-mU dose was previously found to reduce food intake and body weight in intact male rats (35). All rats received both injections in a counterbalanced design, with subsequent injections occurring after complete recovery of food intake and body weight to baseline levels (generally 5 days). Food was returned at the onset of dark and intake measured over the subsequent 1, 4, and 24 h. After at least 7 days rest, the same rats were then administered leptin (1.0 or 3.5 μg/1 μl Human Leptin; CalBiochem, San Diego, CA) or vehicle (saline, 1 μl) in the same counterbalanced paradigm on separate days.

Plasma analyses. Rats were fasted overnight and killed by decapitation. Trunk blood was collected and the plasma isolated by centrifugation and stored at −80°C until analyzed by radioimmunoassay for plasma leptin using a rat leptin radioimmunoassay kit (Linco Research, St. Charles, MO). This assay is able to detect leptin in 100 μl samples of plasma with intra- and interassay coefficients of variation of 4.6–5.7% for leptin. Estradiol was measured by specific radioimmunoassay, with intra- and interassay coefficients of variation of 8.0–9.7% (Quest Diagnostics, Nichols Institute Diagnostics, San Juan Capistrano, CA). For plasma estradiol measures, blood samples were collected in the middle of light phase on the 2nd day after an estradiol injection.

Body fat determination. Body fat was estimated in two ways. During an ongoing experiment, body fat was assessed by nuclear magnetic resonance (NMR) (EchoMRI; EchoMedical Systems, Houston TX). Unanesthetized rats were placed in a restraint tube and inserted into the NMR. This method provides estimates of total lean tissue, fat tissue, and water. We validated the NMR results by ether extraction at the end of the experiments. For the validation of the carcass analysis by NMR, and to estimate fat distribution as well as quantity, the carcass was separated into two portions. In the process, all of the skin was removed from the carcass, including the fat attached to the skin. The skin and attached fat were then analyzed separately from the rest of the carcass, which contained the muscle, skeleton, organs, and visceral fat. The two portions were wrapped in individual plastic bags and frozen at −80°C. For the analysis, the sections were placed in individual 1,200-ml freeze-dry flasks and dried to constant weight in a high-capacity lyophilizer (Labconco, Kansas City, MO). Each carcass (and flask) was reweighed daily until there was less than 1.0 g of change in 24 h (5–7 days, typically). The difference in weight before and after being lyophilized was recorded as total water content. Each dried portion (skin or rest of carcass) was then placed into a protective cotton sack and repeatedly flushed with 8–10 l of boiling petroleum ether for at least 8 h in a high-capacity Soxlet apparatus. The portions were then removed, dried thoroughly of ether, and reweighed. Weight loss after being in the Soxlet was recorded as lipid weight (58). Note that this provided an estimate of subcutaneous fat (within and attached to the skin) as well as total nonsubcutaneous fat, which is comprised of visceral and organ fat. The total fat content (subcutaneous plus nonsubcutaneous) could then be compared with the value provided by the NMR analysis.

Hypothalamic gene expression. RNA was reverse transcribed in preparation for quantitative real-time PCR. Briefly, RNA was isolated from whole hypothalami using Tri-Reagent (MRC, Cincinnati, OH) according to manufacturer instructions. DNA contamination is eliminated using a removable DNase system (DNAfree; Ambion, Austin, TX). The absence of DNA contamination is confirmed by amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (250 ng RNA/well) with and without a preceding reverse transcriptase step (2 min at 50°C, 30 min at 60°C). Completion of 40 amplification cycles (i.e., 5 min at 95°C × 1; 20 s at 94°C, 60 s at 62°C × 40) without detecting a product in the non–reverse transcriptase wells indicates that the RNA samples are not contaminated. Gene expression is analyzed using the Taqman real-time PCR system (Perkin Elmer, Applied Biosystems, Foster City, CA). Standard curves consisted of pooled RNA from each treatment group in singleplex GAPDH reactions. The cycle number at which the fluorescence exceeds the threshold of detection for GAPDH is subtracted from the gene of interest (DCT). The average DCT for each experimental group is derived from the average DCT of each rat in that group. The percent change in gene expression, relative to the reference group, is defined as 100 *2-DDCT, where DDCT equals the group DCT minus the DCT of the reference group. Since the threshold cycle is inversely proportional to the log of the initial copy number, the more template that is initially present the lower the cycle number where the fluorescence exceeds the threshold. Insulin receptors and the long form of the leptin receptor were measured. Quantitative PCR Primer sequences: L32 (housekeeping gene): forward: 5′-CAT CGT AGA AAG AGC AGC AC-3′; reverse: 5′-GCA CAC AAG CCA TCT ATT CAT-3′; insulin receptor: forward: 5′-TGG CGC TGT GTA AAC TTC AG-3′; reverse: 5′-GGG ATG CAC TTG TTG TTG TG-3′; leptin receptor (long/signaling form, OB-Rb): forward: 5′-GGA AAC ATT CCC CAC TGA GA-3′; reverse: 5′-CAC AGA TTT TTC CCC GTG AT-3′.

Data analysis. Food intake and body weight data were analyzed by multifactor ANOVA (Statistica 6.0; Statsoft, Tulsa OK) with drugs as a within-subject factor. Sex, surgical treatment, and time were included as between-subject factors, depending on the specific experiment. Single-factor between-

TABLE 1
Twenty-four–hour food intake (g) after i3vt insulin or leptin

Sex	*n*	Vehicle	1 mU insulin	4 mU insulin
Male	10	28.8 ± 1.04	25.0 ± 1.85*	21.0 ± 2.91*
Female	18	20.4 ± 1.53	20.6 ± 0.98	20.2 ± 1.10
OVX	12	20.2 ± 0.57	18.4 ± 0.90	21.4 ± 0.50
Castrated male	10	25.4 ± 1.03	24.9 ± 1.73	23.9 ± 1.98
Intact Male + 17 β-estradiol	10	24.7 ± 0.97	24.2 ± 1.24	22.9 ± 0.97
Sex	***n***	**Vehicle**	**1.0 μg leptin**	**3.5 μg leptin**
Male	10	30.4 ± 1.23	29.5 ± 1.39	27.0 ± 0.85
Female	18	22.2 ± 1.70	16.8 ± 1.84†	15.0 ± 2.70†
OVX	12	20.3 ± 0.59	18.4 ± 0.70	17.2 ± 1.98
Castrated male	10	26.7 ± 0.79	20.9 ± 1.2†	18.7 ± 1.22†
Intact male + 17 β-estradiol	10	27.1 ± 1.45	21.3 ± 0.98†	17.0 ± 0.83†
OVX + PE	10	23.4 ± 2.13	17.4 ± 1.67†	16.7 ± 1.98†
OVX + CE	10	23.9 ± 1.98	17.5 ± 2.01†	15.9 ± 1.23†

Data are mean ± SE. ANOVA revealed that insulin (*) and leptin (†) significantly reduced food intake ($P < 0.05$) compared with vehicle. OVX + CE, ovariectomized females with central 17 β-estradiol; OVX + PE, ovariectomized females with peripheral 17 β-estradiol.

subject ANOVAs were used for gene expression, subcutaneous fat, visceral fat, and plasma hormone data. Least significant difference post hoc comparisons were used to assess the bases of reliable interactions and main effects. Significance was set at $P < 0.05$ (two tailed).

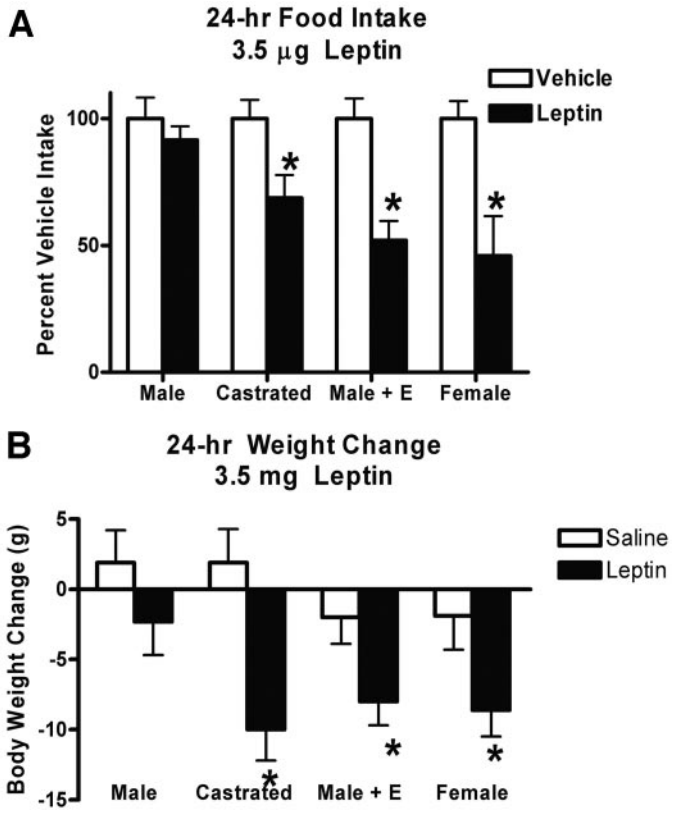

FIG. 2. i3vt leptin reduces food intake and body weight in intact females, castrated males, and 17 β-estradiol–treated males but has little effect in intact males. Intact males (male; $n = 10$), castrated males (castrated; $n = 10$), intact males injected with 17 β-estradiol (male + E; $n = 10$), and intact female (female; $n = 18$) Long-Evans rats were administered leptin (3.5 μg/1 μl saline) or vehicle alone (1 μl saline) on different days in counterbalanced order. Estradiol administration occurred every 4 days for 1 month. Data are expressed as mean (±SE) percent of vehicle baseline. ANOVA revealed that leptin significantly reduced 24-h food intake (*A*) and body weight (*B*) in castrated males, intact males injected with 17 β-estradiol, and females. *$P < 0.05$ compared with vehicle baseline.

RESULTS

i3vt insulin reduces food intake and body weight of intact males, but not intact females, castrated males, or males administered estradiol. Intact males administered i3vt insulin (1 and 4 mU on separate days in a counterbalanced order) ate significantly less food than when administered i3vt saline (Fig. 1*A*; Table 1). They also ate significantly less food than intact females, castrated males, or intact males peripherally administered 17 β-estradiol following the same amount of i3vt insulin (Fig. 1*A*; Table 1). Only the 24-h data are listed, but the differences were also present at all other time points assessed. Consistent with these data, intact males lost significantly more body weight over the 24 h following the injections than rats in the other groups (Fig. 1*B*) (all $P < 0.05$).

i3vt leptin reduces food intake and body weight in intact females, castrated males, and males implanted with estradiol but has little effect in intact males. Over the course of 24 h, i3vt leptin (1.0 and 3.5 μg/1 μl on separate days in a counterbalanced order) significantly reduced food intake in the intact females, castrated males, and intact males administered 17 β-estradiol (Fig. 2*A*; Table 1). Following the leptin administration, all groups had a reduction of food intake over the first 4 h, but this reduction was short lived and no longer apparent by 24 h in the intact males (Fig. 2*A*; Table 1). Animals that had reduced food intake over 24 h also lost significant body weight over the same interval (Fig. 2*B*) (all $P < 0.05$).

Ovariectomy reduces sensitivity to i3vt leptin independent of body weight. Intact but not OVX female rats had reduced food intake following i3vt leptin (Fig. 3*A*). Once again, the anorexia persisted in the intact females, resulting in a significant reduction in body weight (Fig. 3*B*). Because females gain weight following OVX, an additional cohort of OVX animals was pair fed the number of calories consumed by the intact females immediately after the OVX and maintained on that schedule for 1 month following the surgery. The pair-fed OVX females' body weight matched that of the intact females (data not shown). The animals were then injected i3vt with 3.5 μg leptin/1 μl (or saline, 1 μl, as control). Intact females had a significant reduction in food intake and body weight following i3vt leptin, whereas pair-fed OVX and ad libi-

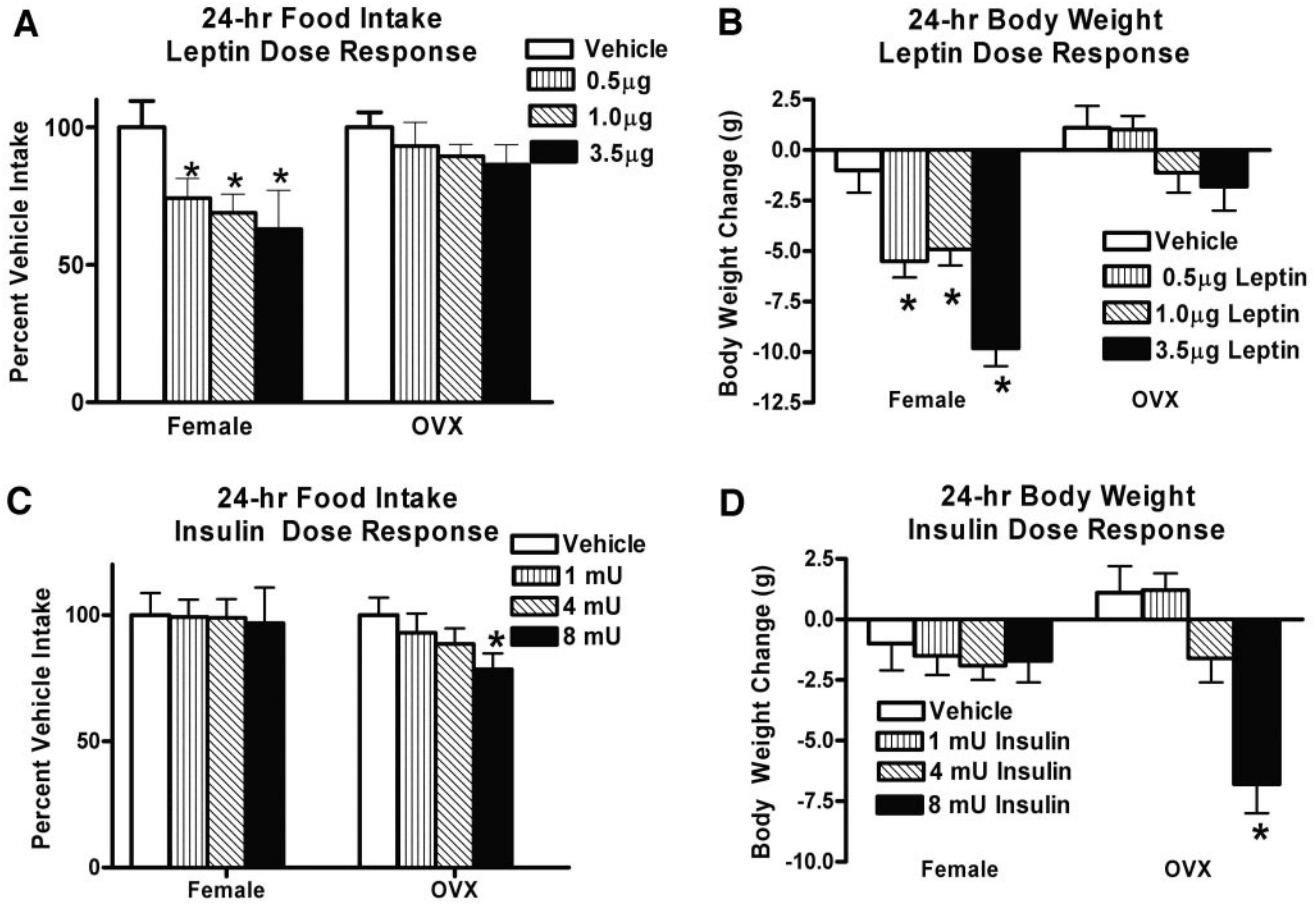

FIG. 3. ***A*** **and** ***B*****: OVX decreases the anorexic effects of i3vt leptin. Intact, sham-operated females (female;** $n = 18$**) and ovariectomized females (OVX;** $n = 20$**) were injected i3vt with leptin (0.5, 1.0, and 3.5 μg/1 μl saline) or vehicle (1 μl saline) i3vt on different days in counterbalanced order. Data are expressed as mean (±SE) percent of vehicle baseline. ANOVA confirmed that leptin significantly reduced food intake (*** A **) and body weight (** B **) relative to vehicle baseline in sham-operated but not OVX rats. *** $P < 0.05$ **relative to vehicle baseline.** C **and** D**: OVX enhances the anorexic effects of i3vt insulin. Intact, sham-operated females (female;** $n = 18$**) and ovariectomized females (OVX;** $n = 20$**) received i3vt insulin (1, 4, and 8 mU/1 μl saline) or vehicle (1 μl saline) on different days in counterbalanced order. Data are expressed as mean (± SE) percent of vehicle baseline. ANOVA revealed that 8 mU insulin significantly reduced 24-h food intake (** C **) and body weight (** D **) only in OVX rats. *** $P < 0.05$ **relative to vehicle baseline.**

tum–fed OVX animals were resistant to the anorexigenic effects of leptin (Fig. 4*A* and *B*).

OVX increases sensitivity to i3vt insulin. OVX but not intact female rats reduced their food intake following i3vt insulin (Fig. 3*C*). The anorexia persisted over 24 h resulting in the OVX, but not the intact females, having a significant reduction in body weight (Fig. 3*D*).

Peripheral estradiol replacement increases sensitivity to i3vt leptin. Plasma estradiol levels were restored to the level of intact females following subcutaneous 17 β-estradiol (Table 2); estradiol levels were assessed following the injections on the day that most closely represents proestrus, the day in which plasma estradiol levels are highest. Peripheral 17 β-estradiol injections significantly reduced body weight of the OVX females to the level of intact females (Table 2). OVX rats receiving subcutaneous 17 β-estradiol ate less food in response to i3vt leptin than control oil-injected OVX females (Table 1), and the magnitude of the responses was comparable to that of intact females. Male rats receiving subcutaneous 17 β-estradiol had enhanced sensitivity to i3vt leptin (Fig. 2*A* and *B*; Table 1). Thus, when systemic estrogen is present (intact females and males or OVX females administered subcutaneous estrogen), leptin is catabolic in the brain, whereas when estrogen is low (OVX females and intact males), leptin is relatively ineffective. Conversely, estrogen reduces the sensitivity of the brain to the anorexigenic action of insulin.

Central estradiol replacement increases sensitivity to i3vt leptin. The dose of estradiol administered into the brain (2 μg) did not cause changes in vaginal cytology and did not increase plasma estradiol (estradiol levels were assayed following central injections on the day that most closely represents proestrus [Table 2]), implying that it had little or no systemic effect (the same dose, when administered peripherally instead of i3vt, also did not influence food intake, body fat distribution, vaginal cytology, or plasma estradiol levels; data not shown). Following 1 month of i3vt estradiol, body weight was significantly reduced in OVX females relative to vehicle-injected OVX controls (Table 2). Additionally, OVX females receiving central estradiol were more sensitive to i3vt leptin than vehicle-treated controls, their level of leptin sensitivity matching that of intact cycling females (Table 1).

Plasma analysis. Intact females, castrated males, and males implanted with 17 β-estradiol had significantly higher plasma leptin than intact males (Table 2).

Body fat distribution. Total fat content determined by ether extraction correlated significantly with fat content estimated by the NMR over a range of 5–125 g of fat ($r =$

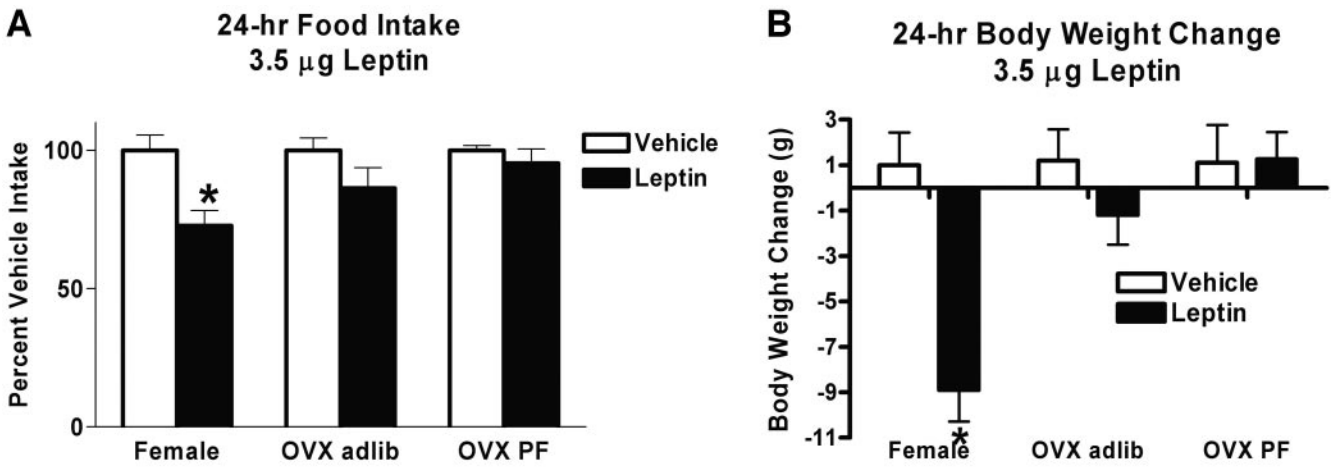

FIG. 4. Pair feeding does not increase the anorexic effects of i3vt leptin in OVX rats. Intact, sham-operated females (female; $n = 18$), ad libitum ovariectomized females (OVX adlib; $n = 20$), and pair-fed ovariectomized females (OVX PF; $n = 10$) were injected with leptin i3vt (3.5 μg/1 μl saline) or saline (1 μl) i3vt on different days. Data are expressed as mean (±SE) percent of vehicle baseline. ANOVA confirmed that leptin significantly reduced food intake (*A*) and body weight (*B*) in the intact females but neither group of OVX rats. *$P < 0.05$ relative to vehicle baseline.

+0.98, $P < 0.01$). Using the NMR, females ($n = 8$) have significantly less visceral and significantly more subcutaneous fat than males ($n = 10$; Fig. 5). Castration ($n = 8$) or the addition of subcutaneous estrogen ($n = 10$) to males increased the amount of subcutaneous fat (Fig. 6). OVX significantly increased visceral fat with no change of subcutaneous fat, resulting in a reduced subcutaneous/visceral ratio (Fig. 7).

Peripheral or central estrogen administration changes body fat distribution. Males that received systemic estradiol had significantly more subcutaneous fat than control males (Fig. 6). OVX females that received peripheral estradiol had increased subcutaneous fat relative to oil-treated control OVX females (Fig. 7). OVX females that received i3vt estradiol had body fat distribution similar to that of intact females (Fig. 7). These data suggest that estradiol signaling through hypothalamic receptors determines body fat distribution.

Hypothalamic gene expression. To determine the cause of the differential sensitivity of the brains to insulin and leptin, we assessed message levels for hypothalamic OB-Rb and insulin receptor. There was an inverse relationship between receptor message and behavioral sensitivity to both leptin and insulin (Table 3). Females had relatively higher insulin receptor mRNA, whereas males had higher leptin receptor mRNA. OVX increased the expression of the leptin receptor message and the expression of the insulin receptor message, and the addition of estradiol to the male rats increased both insulin receptor and OB-Rb message.

DISCUSSION

We previously determined that males and females respond differentially to centrally administered leptin and insulin (35,36). We now report that estrogen status in females alters sensitivity to centrally administered leptin and changes body fat distribution. Specifically, we determined that peripheral or central administration of 17 β-estradiol to OVX females restores their central leptin sensitivity and changes their body fat distribution to be more like that of intact females. Additionally, we found that altering the sex hormone milieu in males by the addition of 17 β-estradiol increases sensitivity to central leptin, decreases sensitivity to central insulin, and increases subcutaneous fat deposition. An important implication from these findings is that gonadal steroids mediate body fat distribution and interact with the integrated adiposity message conveyed to the brain by leptin and insulin, resulting in differential sensitivity to these signals in males and females.

Leptin provides a powerful catabolic signal to the brain resulting in inhibition of food intake (3,5–7,9,10,15,27, 59,60). Leptin levels are higher in females, even before puberty, compared with males, and this is independent of differences in body composition (61–63). After puberty, estrogen and testosterone further modulate leptin synthesis and secretion via sex steroid receptor–dependent transcriptional mechanisms (64). Leptin is secreted from adipose tissue in direct proportion to fat content, and it penetrates the blood-brain barrier to interact with leptin receptors in the hypothalamus and brainstem (2,3,6,11, 13,15,65,66). Although several splice variants of the leptin receptor are known, OB-Rb is the critical variant for regulating energy balance (67). Estrogen receptors are also expressed in the brain, including hypothalamic regions that regulate food intake and body weight (68–75). Consistent with this, OB-Rb expression has been colocalized with the estrogen receptor (specifically ERα) in the arcuate (76), and estrogen has been reported to regulate the expression of OB-Rb mRNA in the arcuate (77), possibly via an estrogen-responsive element on the leptin receptor gene (78), thus providing a potential mechanism by which estrogen may enhance leptin sensitivity. The extensive hypothalamic colocalization of these two receptors suggests a closely coupled interaction in the regulation of behavioral and neuroendocrine mechanisms. That is, our data suggest that when estrogen levels are low, central leptin sensitivity is reduced, as occurs in OVX females (consistent with the findings of Ainslie et al. [79]) and intact males. Conversely, when estrogen levels are relatively high, as occurs in intact females and OVX females and males administered estradiol, leptin sensitivity is high (35,36).

In all species examined so far, mRNA encoding the predominant signaling isoform of the leptin receptor has been localized within the hypothalamus, and these receptors colocalize with several neuropeptides thought to be

TABLE 2
Plasma leptin, estradiol, body weight, and total adipose measurements

Sex	*n*	Plasma leptin (ng/ml)	Plsama estradiol (pg/ml)	Body weight (g)	Total body fat (g)
Male	10	2.52 ± 0.22	32.8 ± 2.1	320.7 ± 9.8	28.9 ± 2.5
Female	18	4.42 ± 0.83*	70.7 ± 4.7*	268.5 ± 9.9*	35.4 ± 1.1*
OVX	12	6.15 ± 0.64*†	32.5 ± 2.0†	310.6 ± 9.7†	42.8 ± 2.1*†
Castrated male	10	3.75 ± 0.38*	36.5 ± 5.6†	278.8 ± 8.9*	39.2 ± 2.1*
Intact male + 17 β-estradiol	10	3.65 ± 0.79*	78.7 ± 4.9*	269.9 ± 8.2*	32.9 ± 3.1*
OVX + PE	10	4.12 ± 0.96*	68.7 ± 7.0*	272.1 ± 8.9*	36.6 ± 2.2*
OVX + CE	10	4.01 ± 1.34*	33.7 ± 4.0†	268.7 ± 6.9*	38.9 ± 3.1*

Data are mean ± SE. OVX + CE, ovariectomized females with central 17 β-estradiol; OVX + PE, ovariectomized females with peripheral 17 β-estradiol. *$P < 0.05$. ANOVA revealed significant differences from intact male rats; †indicates significant differences from intact females.

important for controlling both food intake and reproduction. Leptin either activates or inhibits these neurons (80–83). Thus leptin is ideally situated to serve as a signal linking metabolic status and brain function. Diano et al. (76) reported colocalization of leptin and estrogen receptors by light and electron microscopic immunolabeling. We therefore predicted that animals with higher estrogen levels would have higher plasma leptin levels and higher hypothalamic OB-Rb expression. In fact, we found an inverse relationship between plasma leptin and hypothalamic OB-Rb mRNA that was not correlated with estrogen status. Additionally, we found that OVX animals had more hypothalamic OB-Rb mRNA expression compared with intact females, consistent with the findings of Bennett et al. (84) but in contrast to those of Ainslie et al. (79), who observed no differences in hypothalamic OB-Rb expression between sham and ovariectomized rats, and Kimura et al. (85), who reported decreased hypothalamic expression of OB-Rb mRNA in OVX rats compared with sham-operated females. We analyzed hypothalamic expression of OB-Rb utilizing RT-PCR, whereas Kimura et al. analyzed OB-Rb mRNA via Northern blots. Additionally, Kimura et al. found no change in plasma leptin levels in OVX females despite their findings that the OVX rats weighed significantly more than the sham-operated females (85); this in turn is in contrast to the findings we report here as well as those of Ainslie et al. (79), who found a significant increase in plasma leptin following the weight gain associated with OVX. What might be concluded from these varying results is that the time at which leptin or OB-Rb is assayed is critical with respect to understanding the physiological role that estrogen and leptin play to regulate body weight. Additionally, only OB-Rb mRNA expression has been measured, such that how estrogen may impact OB-Rb protein or signaling through this receptor has not yet been evaluated. Finally, our data may differ from other reports due to the fact that we assayed whole hypothalamus for OB-Rb. That is, there may have been significant changes in arcuate neurons that were masked by our assay of the whole hypothalamus.

Our findings imply that differences in leptin sensitivity based on the presence or absence of estrogen must occur downstream of OB-Rb gene expression and transcription of the receptor protein. Consistent with our findings that females are more sensitive to the anorexigeinc effects of leptin, but in opposition of our findings that they have fewer hypothalamic OB-Rb, we have found that following i3vt leptin, females have both more c-Fos and more pSTAT3 immunoreactivity in the arcuate than males (preliminary unpublished data). These data suggest that despite having fewer whole hypothalamic OB-Rbs, there is increased leptin signaling in the arcuate of females.

Insulin receptors are distributed in discrete brain areas including the hypothalamus (86–88). Hypothalamic insulin receptors are thought to mediate food intake and body weight regulation via similar mechanisms by which leptin regulates food intake and body weight (5,6,24,28,30). A gonadal influence on hypothalamic insulin receptors has not been previously reported, although a relationship between testosterone levels and insulin sensitivity has been established. Testosterone regulates peripheral insulin sensitivity (89,90), and it has been previously demonstrated that changes in testosterone levels directly affect insulin sensitivity in adipose tissue. Our hypothesis therefore was that lack of testosterone would diminish central sensitivity to insulin in male rats. We found that whereas castration diminished sensitivity to centrally administered insulin, it also increased hypothalamic insulin receptor

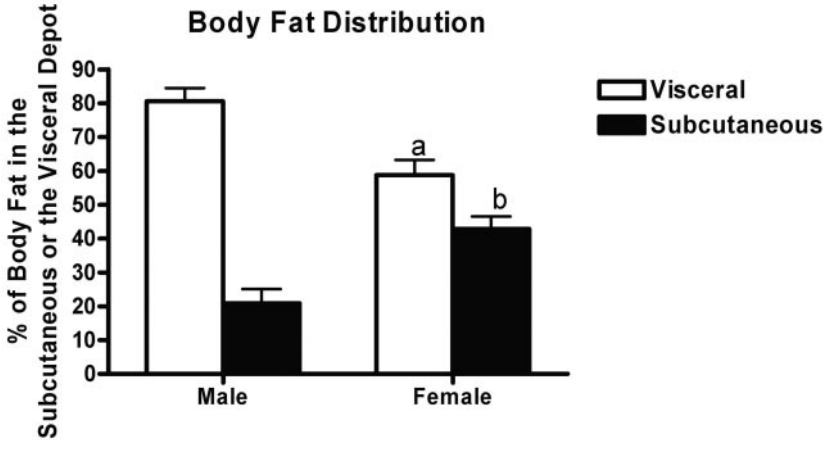

FIG. 5. Male rats have proportionally more visceral fat than females, and females have more subcutaneous fat as measured by NMR. Females ($n = 10$) had a higher percentage of total fat stored in the subcutaneous depot than weight-matched male rats ($n = 10$). Data are depicted as mean ± SE percent fat located in each of the different depots. *a*: $P < 0.05$ relative to male visceral fat; *b*: $P < 0.05$ relative to male subcutaneous fat.

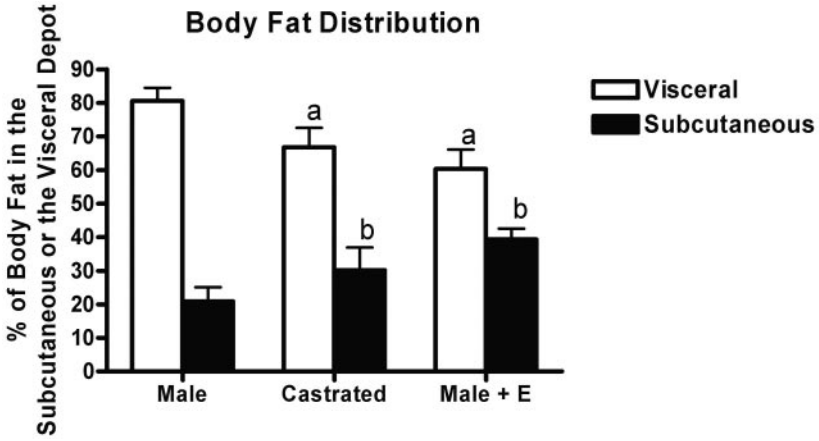

FIG. 6. Male rats have more visceral fat, as measured by NMR, than castrated or males treated with peripheral 17 β-estradiol. Intact males (male; $n = 10$) had more fat in the visceral depot than did the castrated males (castrated; $n = 9$) or males treated with peripheral 17 β-estradiol (male + E; $n = 10$). Castrated males and 17 β-estradiol–treated males had significantly more subcutaneous fat than intact males. Estradiol administration occurred every 4 days for 1 month. Data are expressed as mean ± SE percent of total fat located in each of the different depots. *a*: $P < 0.05$ relative to intact male visceral fat; *b*: $P < 0.05$ relative to intact male subcutaneous fat.

expression. Additionally, we found that the addition of 17 β-estradiol to intact male rats reduced sensitivity to centrally administered insulin and increased hypothalamic insulin receptor expression. These results are consistent with previous findings suggesting a correlation between testosterone and insulin (89,90). We found no affect of estrogen on the hypothalamic expression of the insulin receptor.

It is not known whether androgens can act directly on the brain to influence body weight and food intake, although it is known that testosterone can act on nonneuronal tissues to alter body weight and composition (91–93). Consistent with this, we have found that removal of testosterone affects body fat distribution, central insulin sensitivity, and expression of the hypothalamic insulin receptor. In contrast to effects of androgens, the literature supports a hypothalamic role of estrogen in mediating food intake and body weight. We found that although brain estrogen influences body fat distribution and central sensitivity to leptin, its actions are not mediated via the hypothalamic expression of the leptin receptor and are perhaps downstream of the leptin receptor. Testosterone, on the other hand, did not directly influence hypothalamic leptin receptor expression; however, the lack of testosterone did increase sensitivity to centrally administered leptin. It is therefore possible that a threshold amount of estrogen is necessary to enhance central sensitivity to leptin. Our findings that addition of estrogen to intact males increased their sensitivity to centrally administered leptin supports this possibility.

The coexpression of ERα and OB-Rb in the arcuate suggests that estrogen may act locally there to reduce food intake and body weight. Estrogens are produced in the ovary and testes, as well as in adipocytes, and circulating estrogens are increased in proportion to total body fat (45,94,95). Estrogens may consequently provide an adiposity signal reflecting both the quantity and distribution of body fat, and they could act at one or both of the two known estrogen receptors (ERs) (ERα or ERβ). Both receptors are expressed in female and male adipose tissue (45,62,96,97) and brain (68–72,74,75). When we administered a small dose of estradiol directly into the i3vt, there was an increase of leptin sensitivity that cannot be attributed to leakage of estrogen out of the CSF since there were no changes of plasma estrogen and no vaginal cytological changes. Furthermore, the dose was two orders of magnitude less than effective doses administered systemically. Hence, we conclude that estrogen has a local action in the brain to increase central leptin sensitivity and to favor distribution of fat subcutaneously. Precisely how this occurs remains to be investigated, and we hypothesize that estrogen may act to alter the sympathetic output to specific fat depots.

In the hypothalamus, ERα is expressed in the ventromedial nucleus (VMN), the medial preoptic area, and the paraventricular nucleus as well as the arcuate (68–72). The present data do not allow determination as to which population(s) of brain estrogen receptors is important for determining leptin sensitivity and body fat distribution. ERβs are located in the some of the same hypothalamic areas as ERα (98,99). However, ERβ expression is significantly lower than that of ERα in the hypothalamus, and ERα but not ERβ reportedly has a major influence on energy homeostasis (47). Consistent with this, male and female mice with a targeted deletion of ERα are obese, whereas ERβ knockout mice are not, thus linking estrogen signaling with body weight regulation (47). In humans, polymorphisms in the ERα gene have been associated with increased visceral fat as indicated by increased waist-to-hip ratios in premenopausal women (100–102). The polymorphism is not associated with visceral adiposity in postmenopausal women or in men. Thus, polymorphisms of the human ERα gene that may impair estrogen signaling are associated with increased visceral adiposity and its attendant health risks.

Young women, whether lean or obese, carry more fat in the subcutaneous depots than in the visceral depots (103), relative to the distribution in males, and we have found that normal female rats have a similar profile relative to males. Our data support a role for estrogen in mediating this pattern of fat distribution. Following OVX, reductions in estradiol resulted in fat accumulation in the visceral compartment, similar to what has been reported in women when they go through menopause (45,46,62). Further, when OVX females were administered exogenous estradiol systemically, body fat distribution reverted back toward the female pattern, and when males were administered systemic estradiol, subcutaneous fat deposition increased.

Because microinjections of small amounts of estradiol into the brain reduce food intake (48,51,104) and increase sensitivity to leptin's catabolic action, we also assessed body fat distribution following the administration of a very low dose of estradiol into the i3vt. Following 1 month of every-4th-day administration of i3vt estradiol, food intake, body weight, and plasma leptin were decreased and the brain was more leptin sensitive. Importantly, fat distribution also became more female like. Thus, our data support a critical role for hypothalamic estrogen receptors medi-

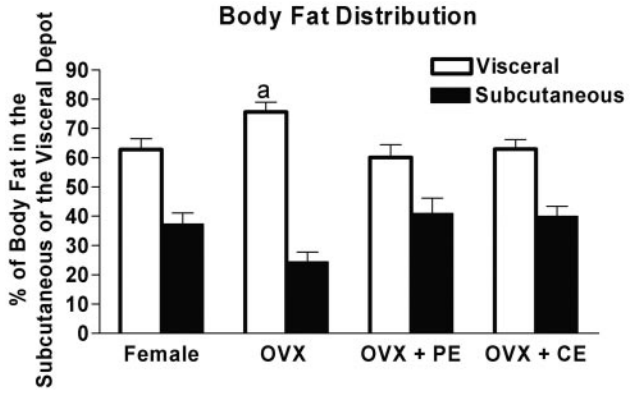

FIG. 7. Ovariectomized females have more visceral fat than intact females, and intact females have more subcutaneous fat as measured by NMR. Peripheral or central administration of 17 β-estradiol restores body fat distribution to that of intact females. Intact females (female; $n = 10$) had a higher percentage of fat in the subcutaneous depot than ovariectomized females (OVX; $n = 10$). Ovariectomized females peripherally administered 17 β-estradiol (OVX + PE; $n = 20$) or centrally administered 17 β-estradiol (OVX + CE; $n = 20$) had a higher percentage of fat in the subcutaneous depot than ovariectomized females (OVX; $n = 20$). Estradiol was administered every 4 days for 1 month. Data are expressed as mean ± SE percent of total fat located in each of the different depots. *a*: $P < 0.05$ relative to female visceral fat.

ating estradiol's affects on modulating food intake, sensitivity to adiposity signals, and body fat distribution.

Hormones secreted in proportion to body fat provide an important regulatory signal to the brain. The present findings indicate that altering the endocrine milieu through manipulating gonadal steroids influences sensitivity to leptin and insulin as well as body fat distribution. This implies that the relative amount of androgens and estrogens is a key determinant of the brain's sensitivity to the catabolic actions of insulin and leptin, with proportionally more estrogen favoring leptin sensitivity and proportionally less estrogen favoring insulin sensitivity. Finally, our data suggest that estrogen's direct actions in the brain determine body fat distribution.

The present data as well as other recent observations from our own and other labs allow us to hypothesize that the ovarian steroid estrogen, acting at ERα in distinct regions of the ventral hypothalamus, is responsible for normal body weight regulation. The ventromedial hypothalamus contains two key nuclei: the arcuate, which contains populations of neurons that regulate food intake and body weight, and the VMN, which alone has not been found to have a prominent role in the regulation of food intake and body weight but does influence energy expenditure. When portions of both the VMN and the arcuate are destroyed in a prototypical ventromedial hypothalamus lesion, animals, and especially female animals, eat more, burn less energy, and become obese (105–107). We hypothesize that the lesion is more effective in females than males because both the arcuate and the VMN densely express ERα. Reduced estrogen signaling in otherwise intact animals results in increased body weight, as evidenced by the effects of ovariectomy. Further, animals devoid of ERα are obese. Collectively, these findings implicate ERα in the regulation of energy balance in females. We therefore hypothesize that estrogen signaling through critical hypothalamic regions where ERαs are located, enhance leptin sensitivity.

TABLE 3
Hypothalamic insulin receptor and OB-Rb mRNA

Sex	*n*	Insulin receptor mRNA	OB-Rb mRNA
Male	10	100.0 ± 4.0	100.0 ± 6.9
Female	10	167.7 ± 8.5*	72.98 ± 1.4*
OVX	18	193.4 ± 11.9*†	80.29 ± 3.9*
Castrated male	10	129.8 ± 6.4*†	91.75 ± 5.4†
Intact male + 17 β-estradiol	10	131.0 ± 7.8*†	92.47 ± 8.6†

Data are mean ± SE. Whole hypothalamus was extracted and assayed for insulin receptor and OB-RB mRNA. *ANOVA revealed significant differences ($P < 0.05$) from intact male rats; †represents significant differences from intact females.

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