

Clegg et al., Estradiol decreases ghrelin-induced food intake

**Title: Estradiol-dependent decrease in the orexigenic potency of ghrelin in female rats**

Received for publication 4 January 2006 and accepted in revised form 16 January 2007

**Abbreviated title:** Estradiol decreases ghrelin-induced eating

**Authors:** Deborah J. Clegg<sup>1</sup>, Lynda M. Brown<sup>1</sup>, Christopher J. Kemp<sup>1</sup>, April D. Strader<sup>2</sup>, Stephen C. Benoit<sup>1</sup>, Stephen C. Woods<sup>1</sup>, Michela Mangiaracina<sup>3</sup>, and Nori Geary<sup>3,4</sup>

**Affiliations:** <sup>1</sup>Department of Psychiatry, University of Cincinnati, PO Box 670559, Cincinnati, OH 45267-0559; <sup>2</sup>Department of Physiology Life Sciences, University of Southern Illinois School of Medicine, Carbondale, IL 62901; <sup>3</sup>Department of Psychiatry, Weill Medical College of Cornell University, New York, NY 1002; <sup>4</sup>Institute for Animal Science, ETH Zürich, 8603 Schwerzenbach, Switzerland.

**Corresponding author:**

Deborah J. Clegg  
Department of Psychiatry  
University of Cincinnati  
PO Box 670559  
Cincinnati, OH 45267-0559, USA  
Email: Debbie.clegg@uc.edu

**To whom reprint requests should be addressed:** Dr. D. Clegg, address above.

**Key words:** NPY, AgRP, food intake, obesity, meal patterns, hunger, sex differences

**Abstract**

Ghrelin, the only known orexigenic gut hormone, is secreted mainly from the stomach, increases with fasting and prior to meal initiation in humans and rats, and increases food intake after central or peripheral administration. To investigate sex differences in ghrelin's action, we assessed the effects of exogenous ghrelin in intact male and female rats, in ovariectomized (OVX) and estradiol (E2)-treated female rats, as well as the effects of OVX on plasma ghrelin and hypothalamic orexigenic neuropeptide expression in rats and on food intake and weight gain in transgenic mice lacking the ghrelin receptor (*Ghsr* <sup>-/-</sup> mice). Male and OVX female rats were significantly more sensitive than intact female rats to the orexigenic effects of both centrally (3<sup>rd</sup>-ventricular, i3vt, 0.01, 0.1, 1.0 nmol) and systemically (ip, 3, 6, 9 nmol) administered ghrelin. This difference is likely to be estradiol-dependent because E2 attenuated the orexigenic action of ghrelin in OVX female and male rats. Furthermore, OVX increased food intake and body weight in wild type mice, but not in *Ghsr* <sup>-/-</sup> mice, suggesting that OVX increases food intake by releasing ghrelin from a tonic inhibitory effect of estradiol. In addition, following OVX, there was an increase in plasma ghrelin that was temporally associated with increased food intake, body weight and hypothalamic NPY and AgRP mRNA expression. Collectively, these data suggest that estradiol inhibits the orexigenic action of ghrelin in females, that weight gain associated with OVX is ghrelin-mediated, and that this endocrine interaction may account for an important sex differences in food intake and the regulation of body weight.

The 28-amino acid peptide ghrelin was discovered in 1999 (1) to be the endogenous ligand of the growth hormone secretagogue receptor (GHSR, (2)). The biological functions of ghrelin have been the subject of intensive research (1; 3-5). One apparent physiological role of ghrelin is in the control of eating and energy balance (1; 3-7). Systemic ghrelin administration stimulates eating in rats and humans (1; 5; 8-11). Additionally, when administered directly into hypothalamus (as well as other brain sites; see ((12) for a review), ghrelin also stimulates eating, perhaps via GHSR located in this part of the brain (13). Ghrelin's orexigenic action can be reduced by antagonism of endogenous ghrelin signaling by administration of ghrelin antibodies either peripherally or centrally (9; 14; 15), GHSR antagonists (16), or GHSR antisense mRNA (17). Furthermore, transgenic GHSR-null mice (*Ghsr* <sup>-/-</sup> mice) that are fed a high-fat diet eat less and gain less weight than control mice (18; 19).

An important question that has received little attention is whether there are sex differences in ghrelin's orexigenic effect. Prominent sex differences, predominately mediated by estradiol (E2), have been reported in the effects of several other endocrine signals that alter eating, including cholecystokinin (CCK), pancreatic glucagon, leptin and insulin, all of which inhibit eating (6; 20-24). Therefore, we investigated the estrogenic influences and mechanisms of the acute eating-stimulatory effect of ghrelin in rats and in *Ghsr* <sup>-/-</sup> mice. We first determined whether the eating-stimulatory potency of intraperitoneal (ip) or intra-3<sup>rd</sup>-ventricular (i3vt) ghrelin differs in intact male and

female rats and if it is affected by ovariectomy (OVX) in females or E2 treatment in OVX female or intact male rats. We also assessed whether ip or i3vt ghrelin-induced eating varies during the ovarian cycle in intact female rats. Then, to investigate the role of ghrelin in OVX-induced hyperphagia and weight gain, we then assessed the effects of OVX on plasma ghrelin levels and hypothalamic expression of the orexigenic neuropeptides NPY and AgRP, both of which have been reported to increase in male rats after ghrelin administration (3; 5; 9; 25-27). Finally, to investigate whether ghrelin plays a necessary role in the normal, E2-mediated ovarian inhibition of eating and body weight (22; 28), we compared the effects of OVX in *Ghsr* <sup>-/-</sup> and wild-type mice. The collective results revealed substantial and physiologically relevant sex differences in the acute eating-stimulatory effect of ghrelin that are mediated in large part by an activational effect of E2.

## Materials and Methods

### *Animals and Surgery.*

The spontaneous feeding experiment was done at Weill Medical College of Cornell University using female Long-Evans rats (60-65 days old Charles River, Wilmington, MA), and all of the other experiments were conducted at the University of Cincinnati using male and female Long Evans rats (age-matched, 60-65 days old, Harlan, Harlan, IN). Additionally, breeding pairs of transgenic *Ghsr* <sup>-/-</sup> and wild-type mice were obtained from Dr. Joel Elmquist (Harvard Medical School, Boston, MA), and adult female offspring were tested at the University of Cincinnati. Zigman et al. (19) have

previously described the generation and phenotype of these mice. Except as noted, all animals were individually housed in Plexiglas tubs with sterile bedding with *ad libitum* access to pelleted chow (Purina, St. Louis, MO) and tap water and maintained on a 12:12-h light-dark cycle in a temperature-controlled, AAALAC-accredited vivarium. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati or of Weill Medical College of Cornell University.

For the rats, surgeries were performed at least 7 days after arrival in the laboratory. Rats were anesthetized with 1 ml/kg ip injections of a mixture of 70 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 2 mg/kg xylazine (Lloyd Laboratories, Shenandoah, IA). For i3vt cannulation, rats were placed in a stereotaxic instrument with the skull held horizontally. The sagittal sinus was displaced laterally, and a 21-gauge stainless-steel guide cannula (Plastics One, Roanoke, VA) was lowered directly on the midline, 2.2 mm posterior to bregma, and 7.5 mm ventral to the dura and fixed to the skull with anchor screws and dental acrylic. Obdurators that extended 0.5 mm beyond the cannula tips were inserted. When rats regained their pre-operative body weights following surgery, placement of i3vt cannulas was confirmed functionally by verifying that i3vt injection of 10 ng angiotensin II (American Peptides, Sunnyvale California) in 1  $\mu$ l normal saline in water-replete rats elicited an intake of at least 5 ml water within in 60 min, as has been reported many times (e.g., (29; 30). Animals that did not meet this criterion were not used.

OVX was done via either midline laparotomies or bilateral flank incisions. Ligatures were placed between the oviduct and the tip of the uterine horn bilaterally, the fatty tissue between the ovary and the kidney was cut with a thermocautery, and the ovaries were cut free and removed distal to the ligature. Mice were ovariectomized using the same approach under aerosolized isoflurane (Abbott Laboratories, North Chicago, IL). Intact female control rats received a sham OVX in which the ovaries were exposed and briefly manipulated, but left intact.

#### *Sex differences in the effects of ip and i3vt ghrelin on eating in rats.*

To determine whether the feeding response to ip ghrelin differs in male and female rats, 10 male (250-275 g) and 20 age-matched female (60-65 day old, 220-225 g) rats were used. Half of the females were OVX and half were sham-operated. The OVX animals were allowed to recover for 4 weeks after the procedure; at this time, as has been reported previously (24; 31), food intakes of OVX and intact rats were similar, but the OVX rats were significantly heavier. Rat ghrelin (3, 6, or 9 nmol, Phoenix Pharmaceuticals, Belmont, CA) in 1 ml/kg 0.15 M saline or the saline vehicle alone was ip injected 6 h prior to the onset of the dark, and food intake was measured 30, 60, 90 and 120 min and 24 h later. Ghrelin doses were selected on the basis of prior reports in male rats (9-11; 32; 34), and animals received the different doses in a counterbalanced design, with 4-5 d between tests to ensure that food intake and body weight recovered to baseline levels. Vaginal smears were taken from the intact female rats each day in order to determine the phase of the estrus cycle (35).

The effects of i3vt ghrelin were tested in separate groups of male, intact female and OVX rats, using the same general procedure, except i3vt doses of 0.01, 0.1, or 1.0 nmol ghrelin in 1  $\mu$ l saline or the saline vehicle alone were used. These doses have previously been reported to stimulate eating in male rats (34).

*Estrogenic modulation of ip and i3vt ghrelin's eating-stimulatory effects in rats*

Male and OVX female rats were treated with E2 to determine whether ghrelin's orexigenic action is modulated by an activational effect of estradiol. Twenty OVX and 10 sham-operated (intact) females received i3vt cannulas. Ten of the OVX rats were maintained on a chronic cyclic E2 treatment regimen, in which 2  $\mu$ g of E2 benzoate (Sigma, St. Louis, MO) in 100  $\mu$ l sesame oil (Sigma) or 100  $\mu$ l sesame oil as the control, was subcutaneously (sc) injected in the middle of the light phase each fourth day; this regimen reproduces near physiological cyclic changes in plasma estradiol and is sufficient to normalize spontaneous feeding patterns, total food intake and body weight in OVX rats (36). The effects of an ip dose of 6 nmol ghrelin and an i3vt dose of 0.1 nmol ghrelin were then assessed as above (these were the lowest doses to reliably increase food intake in that experiment). In rats receiving E2 treatment, ghrelin was tested on the second day after E2 injections, which models the day of estrus in intact rats (36).

We also tested whether ghrelin's eating-stimulatory effect varies in synchrony either with the cyclic exogenous E2 treatment regimen in OVX rats or with the ovarian cycle in intact rats. Twenty OVX rats were maintained on the same chronic cyclic regimen of E2

treatment and, over a period of several weeks, the effect of i3vt ghrelin or saline on food intake was assessed on each of the 4 cycle days. Twenty intact females were similarly tested on each day of their estrus cycle. A dose of 0.1 nmol ghrelin was used in each test.

Finally, 10 males were sc injected with 2  $\mu$ g of E2 and 10 with the sesame oil vehicle and were administered i3vt ghrelin (0.01 nmol) or saline two days later. This dose of estradiol in males brings their plasma estradiol to levels comparable to those of intact female rats (unpublished data). Food intake was measured 1 and 2 h later.

*Estrogenic modulation of ip ghrelin's effects on spontaneous meal patterns in rats*

To determine how ip ghrelin affects spontaneous feeding patterns, eight oil-treated OVX rats and eight OVX rats that received cyclic E2 treatment were maintained in computerized cages instrumented for the measurement of spontaneous feeding patterns (ENV-23, Med Associates, St. Albans, VT). Meal patterns were determined by photocell detection of the time of removal of 45-mg chow pellets (Research Diets, New Brunswick, NJ) from a feeding trough that was automatically replenished with a single pellet each time one was removed. Ghrelin (6 nmol, Phoenix Pharmaceuticals, Belmont, CA) in 1 ml/kg 0.15 M saline or the saline vehicle alone was ip injected 6 h prior to the onset of the dark on the second day after E2 injections. Data were analyzed with TongueTwister software (37), with spontaneous meals defined as feeding bouts of at least 2 pellets separated from other bouts by at least 15 min (36).

*Food intake, plasma ghrelin and hypothalamic orexigenic gene expression following OVX in rats*

Forty OVX female and 40 sham-operated female rats had food intake and body weight measured daily. At weekly intervals after surgery, 8 animals from each group were anesthetized with CO<sub>2</sub> and decapitated, and trunk blood and brains were collected (see methods below). Animals had their food removed 12 h prior to sacrifice, the animals were sacrificed 4 h prior to the onset of the dark phase, a time prior to the endogenous peak in plasma ghrelin levels (38). The plasma was stored at -80° C until analyzed for ghrelin using a human ghrelin RIA kit (Phoenix Pharmaceuticals, Belmont, CA).

*Effects of OVX on food intake and body weight in Ghnr -/- mice.*

The genotypes of offspring of *Ghnr* -/- and wild-type mice were verified by PCR using DNA isolated from mouse tails. Primers 5'-CCTGGCAGACAGAGCACCTG-3' and 5'-CAGGTCAGTCAAGTCTGTCTC-3' amplified an 842-bp band from the wild-type allele, whereas primers 5'-CCTGGCAGACAGAGCACCTG-3' and 5'-GCAGCGCATCGCCTTCTATC-3' amplified a 630-bp band from the knockout allele (19). Sixteen female mice of each genotype were observed from weaning through puberty, which occurred at similar ages in both groups (age at vaginal opening, 45 ± 2 days for both wild type and *Ghnr* -/-). Both groups of mice cycled normally after puberty. OVX was done at least two weeks after vaginal opening. As reported previously (19), there were no differences in body weight between in *Ghnr* -/- and wild-type mice at this age. Completeness of ovariectomy was confirmed by measuring uterine

weight, which was at least 20± 0.5 % lower in all OVX mice than in intact mice.

*Hypothalamic orexigenic gene expression*

Following sacrifice, brains were rapidly removed and placed in RNAlater (Ambion; Austin, TX) and stored at 4° C overnight. The hypothalamus was dissected using the tuber cinereum as the ventral landmark for cuts to remove the frontal lobe and lateral and posterior portions of the brain. The cortex was peeled away from the remaining ventral brain piece containing the hypothalamus, which was then homogenized in 1 ml Tri Reagent (MRC; Cincinnati, OH). After bromochloropropane (MRC) addition and centrifugation, the RNA was recovered from the aqueous phase by isopropanol precipitation and treated with the DNA-free DNase Treatment and Removal Reagents (Ambion) to remove any contaminating genomic DNA. cDNA was synthesized from 5 µg total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad CA) for RT-PCR. The cDNA was then diluted 1:3 in nuclease-free water and stored at -20° C. The integrity of the cDNA was confirmed by conventional RT-PCR amplification of L32, the housekeeping gene. In addition, a control reaction for each RNA sample was performed with no reverse transcriptase enzyme added.

*Quantitative real-time PCR*

Each primer set was optimized such that the correlation coefficient was 0.99-1.0 and the PCR efficiency was 90-100%. Real-time PCR was performed in triplicate using an iCycler and the iQ SYBR Green Supermix (BioRad; Hercules, CA) with 2-step amplification (95° C for 10 sec, annealing temperature for 30 sec) for 40 cycles. L32 was amplified from every sample for use as an

endogenous control. For the data analysis, the average threshold cycle (CT) of each set of triplicates was calculated. Then, to normalize the data, the  $\Delta$ CT was calculated for each sample by subtracting the average CT of L32 from the average CT of the gene of interest. For relative quantification, the  $\Delta$ CT was averaged for the defined control group and was then subtracted from the  $\Delta$ CT of each experimental sample to generate the  $\Delta\Delta$ CT. The  $\Delta\Delta$ CT was then used to calculate the approximate fold difference,  $2^{\Delta\Delta$ CT (for primer sequences and annealing temperatures, see Table 1).

### *Statistical Analyses*

Food intake and meal pattern data were analyzed by two-way ANOVA, with drug dose a within-subjects factor and “sex status” (i.e., intact or estradiol-treated male, estradiol-treated or untreated OVX, or intact female) as a between-subjects factor. Because in all experiments the effects of ghrelin on cumulative food intake were clearest in the 1-h data, but comparable in all respects up to 2 h post-injection, and were gone by 24 h, only 1-h data are presented below. In the spontaneous feeding tests, latency to the first meal and intermeal interval data were logarithmically transformed for normality. Hypothalamic NPY and AgRP mRNA expression, relative to L32, were calculated as percents of male baseline average and then analyzed by one- or two-way ANOVA. The bases of significant main effects and interactions were assessed by post-hoc Bonferroni tests. Minimum significance was  $\alpha < 0.05$ . Data are expressed as mean  $\pm$  SEM throughout.

## **Results**

### *Sex differences in the effects of ip and i3vt ghrelin on eating in rats*

Male and OVX female rats increased food intake in response to ip ghrelin, but intact females did not. Doses of 3, 6 and 9 nmol ghrelin each significantly increased 1-h food intake in OVX females, but were not effective in intact females (Figure 1A). The 6 and 9 nmol doses increased 1-h food intake in males. The lowest dose of i3vt ghrelin (0.01 nmol) increased 1-h food intake in males and OVX females, but not in intact females (Figure 1B). The 0.1 and 1.0 nmol ghrelin doses increased food intake in all three groups by amounts that were statistically distinguishable.

### *Estrogenic modulation of eating induced by ip and i3vt ghrelin in rats*

As in the first experiment, oil-treated OVX rats increased food intake after receiving ip ghrelin (Figure 2A) or i3vt ghrelin (Figure 2B), whereas E2-treated OVX rats were unresponsive. When ghrelin was administered i3vt to OVX rats receiving E2 on specific days simulating the normal estrus cycle, ghrelin significantly increased food intake on Days 1 and 2 of the treatment cycle, which simulate diestrus (Figure 2C). In contrast, i3vt ghrelin had no effect on food intake in the same rats on Days 3 and 4 of the treatment cycle, which model proestrus and estrus, respectively. Similarly, in intact females, i3vt ghrelin increased food intake when the rats were in diestrus, but had no effect during proestrus or estrus (Figure 2D). Finally, whereas untreated males increased food intake in response to i3vt ghrelin, estradiol-treated males did not (Figure 3).

Consistent with previous reports (22; 31; 39), when tested several weeks post-OVX, estradiol-treated rats ate smaller but more frequent spontaneous meals than oil-treated controls, and total

daily food intake did not differ between groups. Ghrelin (6.0 nmol, ip) significantly increased food intake in ovariectomized (OVX) rats by decreasing the latency to onset of the next spontaneous meal after injection, and there was a non-significant decrease in the duration of the following intermeal interval, with no increase in meal size (Table 2). Ghrelin had no detectable effect on food intake or meal patterns in estradiol-treated rats.

*Food intake, plasma ghrelin levels and hypothalamic orexigenic gene expression following ovariectomy*

Again as expected from previous data (22; 31; 39), OVX caused a permanent increase in body weight, which was significant beginning Week 2 after OVX (Figure 4A), and a transient increase in food intake, which was significant during Weeks 2-4 after OVX (Figure 4B). The period of hyperphagia and rapid weight gain was associated with increased plasma ghrelin during Weeks 2-4 after OVX (Figure 5), increased hypothalamic NPY mRNA during Week 2 after OVX (Figure 6A), and increased hypothalamic AgRP mRNA during Weeks 1-3 after OVX (Figure 6B).

*Effects of OVX on food intake and body weight in Ghrelin<sup>-/-</sup> mice.*

As expected (22; 28), in wild type mice OVX caused a permanent increase in body weight gain, which was significant beginning d 8 after OVX (Figure 7A), and a transient increase in food intake, which was significant on d 1-14 after OVX (Figure 7B). In contrast, in the *Ghrelin<sup>-/-</sup>* mice OVX had no effect on food intake or body weight gain.

**Discussion**

Gonadal hormones potently control eating in female animals, mainly

via activational effects of E2 (22; 28; 31). In rats and mice, E2 exerts a tonic inhibitory effect on meal size and daily food intake throughout the ovarian cycle and a phasic or cyclic inhibitory effect during the peri-ovulatory phase (21; 22; 28; 40), and abrogation of these effects leads to hyperphagia and obesity. A similar peri-ovulatory decrease in daily food intake occurs in women, and there may be increases in eating after menopause that parallel the tonic effect of E2 in animals (41). The present data demonstrate that these estrogenic effects on eating in rats and mice involve substantial E2-mediated sex differences in the acute eating-stimulatory effect of the putative hunger signal ghrelin.

We first found that the potency of exogenous ghrelin to acutely stimulate feeding is less in intact female rats than in male rats or OVX females (Figure 1). That is, whereas, ip or i3vt ghrelin administration led to reliable increases in feeding in intact male rats (as has been reported several times before (9-11; 32-34)), and OVX females, the threshold for a significant effect by either route of administration was at least a log unit more in intact females than in males or OVX females. Furthermore, when OVX rats were treated with E2, moderate ip or i3vt doses of ghrelin no longer stimulated eating (Figure 2A and 2B). These data indicate that E2 affects the ghrelin signaling pathway so as to substantially reduce ghrelin's orexigenic potency. Thus, careful attention to sex differences and gonadal hormone status should be included in development of any ghrelin-based clinical control of eating. Finally, E2 also reduced the eating-stimulatory effect of i3vt ghrelin in male rats (Figure 3), indicating that the estrogenic effect

exists in both sexes, another point of potential therapeutic relevance.

We also demonstrate that ghrelin's eating-stimulatory effect varies across different phases of the ovarian cycle in intact rats. Although i3vt ghrelin had no reliable overall effect when cycle day was not taken into account (Figure 1), when cycle day was considered, i3vt ghrelin increased eating during diestrus 1 and diestrus 2, but not during proestrus or estrus (the latter being the peri-ovulatory phase in this species; see Figure 2D). Furthermore, cyclic E2 treatment in OVX rats showed that E2 mediates this effect of ghrelin as well. That is, in E2-treated OVX rats, ghrelin increases eating on the days that modeled diestrus in intact rats, but not on the days that modeled proestrus and estrus (Figure 2C). Our data therefore suggest that the cyclic variation in eating in rats and mice, in which spontaneous food intake is maximal during diestrus and minimal during estrus, and the analogous peri-ovulatory decrease in eating in women may be due to changes in estrogenic tone on ghrelin's eating-stimulatory action.

The feeding-inhibitory effect of E2 in OVX rats, as well as the peri-ovulatory decrease in food intake during the ovarian cycle in rats, is manifest purely as a decrease of meal size (36; 39). However, we observed that the decrease in the acute feeding-stimulatory effect of ip ghrelin in E2-treated OVX rats is expressed as an increase in the latency to eat, with no change in meal size (Table 2). Consistent with this, Faulconbridge et al. (42) also observed that i3vt ghrelin reduced the latency of male rats to eat with no change of meal size. In a test of male mice under conditions when ghrelin led to more prolonged stimulation of feeding than in

these experiments, however, ip ghrelin selectively increased meal size without affecting meal frequency (43). This issue warrants further investigation.

In rats and mice, food intake increases for about a month after OVX and then returns to near-normal levels, so that the initial hyperphagia-induced weight gain is maintained permanently (31; 39). We therefore investigated the effects of OVX on basal plasma ghrelin levels and on hypothalamic orexigenic neuropeptide signaling, which has been related to ghrelin in male rats (3; 5; 9; 25-27). There were two important findings. First, plasma ghrelin levels were increased during the period of OVX-induced hyperphagia and rapid weight gain (Figures 4 and 5). These data extend a previous report of brief increases in ghrelin secretion following OVX and normalization with E2 treatment in peri-pubertal rats (52). Taken together, these data indicate that, in addition to decreasing ghrelin-induced orexigenic signaling as described above, E2 may downregulate ghrelin's orexigenic effect by reducing ghrelin secretion. Consistent with this association between plasma ghrelin levels and eating after OVX in rats, ghrelin levels also increase in post menopausal women (44).

Second, we demonstrate that OVX leads to increases in hypothalamic AgRP and NPY mRNA expression (weeks 1-3 after OVX, Figure 6). Although OVX and E2 treatment have previously been reported to regulate hypothalamic NPY mRNA (reviewed in (22)) this is the first report of increased AgRP mRNA after OVX. Furthermore, the close temporal association between increased hypothalamic AgRP mRNA signaling and hyperphagia after OVX suggest that

AgRP might be an important mediator of OVX-induced eating. Our data do not disclose, however, whether hypothalamic orexigenic neuropeptide signaling is normally tonically inhibited via a direct, hypothalamic action of E2 that is independent of ghrelin or as an indirect consequence of E2-downregulation of ghrelin secretion. The latter possibility is consistent with reports that ghrelin regulates hypothalamic orexigenic neuropeptide signaling in male rats (3; 5; 9; 25-27).

In order to assess the importance of ghrelin signaling in OVX-induced hyperphagia and obesity, we tested the effect of OVX in *Ghsr*<sup>-/-</sup> mice lacking the GHSR. These mice, which before surgery were similar to wild type mice in body weights and food intake, showed no increase in food intake or body weight gain OVX (Figure 7). We interpret this to indicate that E2 tonically inhibits endogenous ghrelin signaling in mice and that release from this inhibition is necessary for OVX to increase food intake and body weight. Presumably the same estrogenic inhibition of ghrelin signaling reduces the eating-stimulatory effect of exogenous ghrelin described above. This mechanism is also likely to contribute to other sex differences in eating and weight regulation previously reported in *Ghsr*<sup>-/-</sup> mice; for example, Zigman et al. (19) reported that on a high-fat diet female *Ghsr*<sup>-/-</sup> mice increased adiposity less than males, and that on a low-fat diet female *Ghsr*<sup>-/-</sup> mice ate less and increased body weight less than males. Thus, ghrelin-signaling appears a necessary component of the estrogenic control of eating and weight regulation and the sex differences that they cause.

Finally, because the site of the GHSR mediating eating remains unclear (12; 25; 26; 45), our data may reflect E2-mediated sex differences in the actions of ghrelin on its receptors in one or more of the central and peripheral sites. Within the brain, GHSR in the Arc, PVN, ventral tegmental area, and dorsal vagal complex have been implicated in ghrelin's eating-stimulatory effect in males (13; 32; 42; 45-48). Because ip and i3vt ghrelin may reach all of these sites and all of them contain estrogen receptors, each is a potential mediator of the sex difference observed here. We previously reported (24) that microinjections of E2 into the 3<sup>rd</sup> ventricle increase the eating-inhibitory effect of exogenous leptin. Because ghrelin and leptin appear to act through the same Arc NPY/AgRP neurons to control eating, and because these same neurons express ER $\alpha$  (reviewed in (50)), it is possible that our results reflect a direct estrogenic action on Arc neurons, but this remains to be proven. In apparent contrast, Currie et al. (49) failed to find any sex difference in the effects of direct Arc or PVN microinjections of 15, 30 or 60 pmol ghrelin. However, because they did not monitor ovarian cycling, and it is possible that their female rats were acyclic; i.e., in a constant diestrus-like state, in which case, as discussed above, one might expect an artificial increase in ghrelin-induced eating. It is also possible that an estrogenic action on NTS neurons contributes to the effects we observed. This is because Thammacharoen et al. (51) reported that exogenous E2 acts in ER $\alpha$ -expressing neurons in the NTS to inhibit eating, and ghrelin microinjection into the NTS stimulates eating in male rats (42).

In conclusion, the present findings increase our understanding of the

mechanisms underlying the estrogenic regulation of eating and body weight in rats and mice and provide a potential basis for better understanding the control of these functions in women, in whom changing stages of reproductive function, including puberty, the ovarian cycle, and menopause, are associated with marked

alterations in vulnerability to eating disorders and obesity.

#### ACKNOWLEDGEMENTS

This research was supported by research grants DK 17844 and DK 54523 from the National Institutes of Health .

## REFERENCES

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402:656-660., 1999
2. Howard AD, Feighner SD, Cully DF, Arena JP, Liberators PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevich M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LH: A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 273:974-977, 1996
3. Kojima M, Kangawa K: Ghrelin: structure and function. *Physiol Rev* 85:495-522, 2005
4. Ueno H, Yamaguchi H, Kangawa K, Nakazato M: Ghrelin: a gastric peptide that regulates food intake and energy homeostasis. *Regul Pept* 126:11-19, 2005
5. van der Lely AJ, Tschöp M, Heiman ML, Ghigo E: Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocr Rev* 25:426-457, 2004
6. Geary N: Endocrine controls of eating: CCK, leptin, and ghrelin. *Physiol Behav* 81:719-733, 2004
7. Williams DL, Cummings DE: Regulation of ghrelin in physiologic and pathophysiological states. *J Nutr* 135:1320-1325, 2005
8. Inui A: Ghrelin: an orexigenic and somatotrophic signal from the stomach. *Nature Reviews Neuroscience* 2:551-560, 2001
9. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S: A role for ghrelin in the central regulation of feeding. *Nature* 409:194-198., 2001
10. Tschöp M, Smiley DL, Heiman ML: Ghrelin induces adiposity in rodents. *Nature* 407:908-913., 2000
11. Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR: Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 50:2540-2547., 2001
12. Arnold M, Mura A, Langhans W, Geary N: Gut vagal afferents are not necessary for the eating-stimulatory effect of intraperitoneally injected ghrelin in the rat. *J Neurosci* 26:11052-11060, 2006
13. Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL: The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37:649-661, 2003
14. Bagnasco M, Tulipano G, Melis MR, Argiolas A, Cocchi D, Muller EE: Endogenous ghrelin is an orexigenic peptide acting in the arcuate nucleus in response to fasting. *Regul Pept* 111:161-167, 2003
15. Murakami N, Hayashida T, Kuroiwa T, Nakahara K, Ida T, Mondal MS, Nakazato M, Kojima M, Kangawa K: Role for central ghrelin in food intake and secretion profile of stomach ghrelin in rats. *J Endocrinol* 174:283-288, 2002
16. Asakawa A, Inui A, Kaga T, Katsuura G, Fujimiya M, Fujino MA, Kasuga M: Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. *Gut* 52:947-952, 2003

17. Shuto Y, Shibasaki T, Otagiri A, Kuriyama H, Ohata H, Tamura H, Kamegai J, Sugihara H, Oikawa S, Wakabayashi I: Hypothalamic growth hormone secretagogue receptor regulates growth hormone secretion, feeding, and adiposity. *J Clin Invest* 109:1429-1436, 2002
18. Wortley KE, del Rincon JP, Murray JD, Garcia K, Iida K, Thorner MO, Sleeman MW: Absence of ghrelin protects against early-onset obesity. *J Clin Invest* 115:3573-3578, 2005
19. Zigman JM, Nakano Y, Coppari R, Balthasar N, Marcus JN, Lee CE, Jones JE, Deysher AE, Waxman AR, White RD, Williams TD, Lachey JL, Seeley RJ, Lowell BB, Elmquist JK: Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J Clin Invest* 115:3564-3572, 2005
20. Geary N, Asarian L, Sheahan J, Langhans W: Estradiol-mediated increases in the anorexia induced by intraperitoneal injection of bacterial lipopolysaccharide in female rats. *Physiol Behav* 82:251-261, 2004
21. Geary N: Estradiol, CCK and satiation. *Peptides* 22:1251-1263, 2001
22. Geary N: The estrogenic inhibition of eating. In *Handbook of Behavioral Neuroscience* Stricker E, Woods SC, Ed. New York, Kluwer Academic Press, 2004, p. 305-343
23. Clegg DJ, Riedy CA, Smith KA, Benoit SC, Woods SC: Differential sensitivity to central leptin and insulin in male and female rats. *Diabetes* 52:682-687, 2003
24. Clegg DJ, Brown, L.M, Woods, S.C., Benoit, S.C.: Gonadal hormones determine Sensitivity to central leptin and insulin. *Diabetes* in press, 2006
25. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I: Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. *Endocrinology* 141:4797-4800., 2000
26. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I: Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. *Diabetes* 50:2438-2443., 2001
27. Tschop M, Statnick MA, Suter TM, Heiman ML: GH-releasing peptide-2 increases fat mass in mice lacking NPY: indication for a crucial mediating role of hypothalamic agouti-related protein. *Endocrinology* 143:558-568, 2002
28. Asarian L, Geary N: Modulation of appetite by gonadal steroid hormones. *Philos Trans R Soc Lond B Biol Sci* 361:1251-1263, 2006
29. Lotter EC, McKay LD, Mangiapane ML, Simpson JB, Vogel KW, Porte D, Jr., Woods SC: Intraventricular angiotensin elicits drinking in the baboon. *Proc Soc Exp Biol Med* 163:48-51, 1980
30. Fitzsimons JT: Angiotensin, thirst, and sodium appetite. *Physiol Rev* 78:583-686, 1998
31. Wade GN: Gonadal hormones and behavioral regulation of body weight. *Physiol Behav* 8:523-534, 1972
32. Horvath TL, Diano S, Sotonyi P, Heiman M, Tschop M: Minireview: ghrelin and the regulation of energy balance--a hypothalamic perspective. *Endocrinology* 142:4163-4169., 2001
33. Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillon WS, Ghatei MA, Bloom SR: Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab* 86:5992., 2001

34. Davidson TL, Kanoski SE, Tracy AL, Walls EK, Clegg D, Benoit SC: The interoceptive cue properties of ghrelin generalize to cues produced by food deprivation. *Peptides* 26:1602-1610, 2005
35. Becker JB, Arnold AP, Berkley KJ, Blaustein JD, Eckel LA, Hampson E, Herman JP, Marts S, Sadee W, Steiner M, Taylor J, Young E: Strategies and methods for research on sex differences in brain and behavior. *Endocrinology* 146:1650-1673, 2005
36. Asarian L, Geary N: Cyclic estradiol treatment normalizes body weight and restores physiological patterns of spontaneous feeding and sexual receptivity in ovariectomized rats. *Horm Behav* 42:461-471, 2002
37. Houpt TA, Frankmann SP: TongueTwister: an integrated program for analyzing lickometer data. *Physiology and Behavior* 60:1277-1283, 1996
38. Drazen DL, Vahl TP, D'Alessio DA, Seeley RJ, Woods SC: Effects of a fixed meal pattern on ghrelin secretion: evidence for a learned response independent of nutrient status. *Endocrinology* 147:23-30, 2006
39. Blaustein JD, Wade GN: Ovarian influences on the meal patterns of female rats. *Physiol Behav* 17:201-208, 1976
40. Drewett RF: Sexual behaviour and sexual motivation in the female rat. *Nature* 242:476-477, 1973
41. Geary NaJL: *Sex differences in energy metabolism, obesity and eating behavior*. New York, Oxford University Press, in press
42. Faulconbridge LF, Cummings DE, Kaplan JM, Grill HJ: Hyperphagic effects of brainstem ghrelin administration. *Diabetes* 52:2260-2265, 2003
43. Azzara AV: Peripheral Ghrelin Administration Increases Food Intake, Meal Size, and Progressive-ratio responding for Food. In *Society for the study of ingestive behaviors (SSIB)* Pittsburgh, PA, Appetite, 2005, p. 332
44. Kellokoski E, Poykko SM, Karjalainen AH, Ukkola O, Heikkinen J, Kesaniemi YA, Horkko S: Estrogen replacement therapy increases plasma ghrelin levels. *J Clin Endocrinol Metab* 90:2954-2963, 2005
45. Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K: Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuro peptide Y/Y1 receptor pathway. *Diabetes* 50:227-232, 2001
46. Horvath TL, Diano S, Tschop M: Ghrelin in hypothalamic regulation of energy balance. *Curr Top Med Chem* 3:921-927, 2003
47. Willesen MG, Kristensen P, Romer J: Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology* 70:306-316, 1999
48. Naleid AM, Grace MK, Cummings DE, Levine AS: Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. *Peptides* 26:2274-2279, 2005
49. Currie PJ, Mirza A, Fuld R, Park D, Vasselli JR: Ghrelin is an orexigenic and metabolic signaling peptide in the arcuate and paraventricular nuclei. *Am J Physiol Regul Integr Comp Physiol* 289:R353-R358, 2005
50. Grove KL, Cowley MA: Is ghrelin a signal for the development of metabolic systems? *J Clin Invest* 115:3393-3397, 2005

51. Thammacharoen S, T.A. Lutz, N. Geary and L. Asarian: Hindbrain estradiol implants inhibit feeding and increase NTS c-Fos immunoreactivity in female rats. *Appetite* 46:387, 2006

52. Matsubara M, Sakata I, Wada R, Yamazaki M, Inoue K, Sakai T: Estrogen modulates ghrelin expression in the female rat stomach. *Peptides* 25:289-297, 2004

## FIGURE LEGENDS

**Figure 1. Ghrelin increases food intake in males and ovariectomized females, but not in intact female rats.** A. Intact males (Male; n = 10), ovariectomized females (OVX; n = 10), and intact females (Intact; n = 10) were administered ghrelin peripherally, (3, 6, 9 nmol ghrelin and vehicle alone (saline, in similar volume)) on different days in counterbalanced order. B. Intact males (Male; n = 10), ovariectomized females (OVX; n = 10), intact females (Intact; n = 10), were administered ghrelin centrally (0.01, 0.1, 1.0 nmol ghrelin /1  $\mu$ l) and vehicle alone (saline, 1  $\mu$ l) on different days in counterbalanced order. Data are expressed as mean (+ SEM) percent of vehicle baseline. Repeated measures ANOVA revealed that ghrelin significantly increased 1-hr food intake (A, B) in intact males, ovariectomized females, but not in intact females. <sup>a</sup> P < 0.05 compared to vehicle, <sup>b</sup> compared to vehicle and to 0.01 nmol ghrelin.

**Figure 2. Estradiol decreases hyperphagia elicited by ip and i3vt ghrelin.** A. Vehicle-treated ovariectomized females (OVX + V; n = 10) and OVX females injected sc with 2  $\mu$ g 17  $\beta$ -estradiol benzoate (OVX + E; n = 10) were administered ghrelin ip (6 nmol) and vehicle (saline in the same volume) on separate days and tested on the second day after injections. B. OVX + V (n = 13) and OVX + E (n = 10) were administered i3vt ghrelin (0.1 nmol/1  $\mu$ l) and vehicle (saline, 1  $\mu$ l). C. OVX + V (n = 13) and OVX + E (n = 14) were administered i3vt ghrelin (0.1 nmol/1  $\mu$ l) and vehicle (saline, 1  $\mu$ l) on different days mimicking the estrus cycle. Day 1 simulates diestrus 1, Day 2 simulates diestrus 2, Day 3 simulates proestrus, and Day 4 simulates estrus. D. Intact females were tracked across their estrus cycles (E = estrus, P = proestrus, D1 = diestrus day 1, D2 = diestrus day 2) and were administered i3vt ghrelin (0.1 nmol/1  $\mu$ l) and vehicle (saline, 1  $\mu$ l) on different days of the estrus cycle. Data represent 8 animals per cycle day. Data are expressed as mean (+ SEM). <sup>a</sup> P < 0.05 compared to vehicle.

**Figure 3. I3vt ghrelin increases food intake males, but not males administered estradiol.** A. Control males (Male; n = 10) and males injected sc with 2  $\mu$ g 17  $\beta$ -estradiol benzoate (2 days earlier to mimic plasma estradiol levels of females in estrus) (Male + E; n = 10) were administered i3vt ghrelin (0.01 nmol/1  $\mu$ l saline) and vehicle (1  $\mu$ l saline) on separate days. Data are expressed as mean (+ SEM) percent of vehicle baseline. <sup>a</sup> P < 0.05 compared to vehicle.

**Figure 4. Ovariectomy increases food intake and body weight.** Ovariectomized (OVX; n = 14) and intact females (Intact; n = 15) had body weights (A) and food intake (B) assessed weekly for 5 weeks. Surgeries were performed midway through the first week as indicated. Data are expressed as mean ( $\pm$  SEM). <sup>a</sup> P < 0.05 compared to intact females.

Figure 5. **Ovariectomy transiently increases plasma ghrelin.** Ovariectomized (OVX; n = 8/week) and intact females (Intact; n = 8/week) were sacrificed each week following surgery. Animals were sacrificed in the middle of the light phase following 24-hr food deprivation. Data are expressed as mean ( $\pm$  SEM). <sup>a</sup> P < 0.05 compared to intact females.

Figure 6. **Ovariectomy transiently increases orexigenic gene expression.** A. Ovariectomized females (OVX; n = 8/week) and intact females (Intact; n = 8/week) were sacrificed each week following surgeries. (A) NPY mRNA and (B) AgRP mRNA. Data are expressed as mean ( $\pm$  SEM) percent of intact female values at Week 0. <sup>a</sup> P < 0.05 compared to intact baseline.

Figure 7. **Ovariectomy increases food intake and body weight in wild type, but not *Ghsr*<sup>-/-</sup> female mice.** Ovariectomized wild type (WT OVX; n = 8), intact wild type females (WT; n = 8), ovariectomized *Ghsr*<sup>-/-</sup> (*Ghsr*<sup>-/-</sup> OVX; n = 8), intact *Ghsr*<sup>-/-</sup> females (*Ghsr*<sup>-/-</sup>; n = 8) had change in body weight (% change) (A) and food intake (B) assessed daily for 25 days. Surgeries were performed on the first day. Data are expressed as mean ( $\pm$  SEM). <sup>a</sup> P < 0.05 compared to WT females.

**TABLE 1**

<b>Gene</b>	<b>Primer sequences</b>	<b>T° C</b>
L32	Forward: 5'-CAG ACG CAC CAT CGA AGT TA-3'	61.2
	Reverse: 5'-AGC CAC AAA GGA CGT GTT TC-3'	
AgRP	Forward: 5'-TTC CCA GAG TTC TCA GGT CTA-3'	55
	Reverse 5'-ATC TAG CAC CTC TGC CAA A-3'	
NPY	Forward: 5'-CTC TGC GAC ACT ACA TCA A-3'	61.2
	Reverse 5'-GGG GCA TTT TCT GTG CTT T-3'	

Primer sequences for each gene are presented with the annealing temperature (T° C). RNA isolated from the dissected basomedial hypothalamus and used for cDNA synthesis. Quantitative PCR was then used to compare hypothalamic gene expression compared to the L32 housekeeping gene.

**TABLE 2.** Effects of IP injection of 6.0 nmol ghrelin on spontaneous feeding parameters in estradiol-treated and vehicle-treated ovariectomized rats.

	Vehicle-treated		Estradiol-treated	
	Saline (n=8)	Ghrelin (n=8)	Saline (n=8)	Ghrelin (n=8)
<b>M<sub>1</sub> Latency (min)</b>	28.8 ± 15.0	1.8 ± 1.2*	16.2 ± 8.4	19.2 ± 5.3
<b>M<sub>1</sub> Size (g)</b>	1.6 ± 0.3	1.2 ± 0.2	0.6 ± 0.2 <sup>+</sup>	0.7 ± 0.2
<b>M<sub>1-2</sub> IMI (min)</b>	158 ± 20	115 ± 30	60 ± 17 <sup>+</sup>	52 ± 14
<b>M<sub>2</sub> Size (g)</b>	2.0 ± 0.7	1.8 ± 0.7	0.9 ± 0.3 <sup>+</sup>	1.0 ± 0.4
<b>2-h Food Intake (g)</b>	1.6 ± 0.4	2.5 ± 0.4*	1.5 ± 0.3	1.4 ± 0.2
<b>2-h Meal #</b>	1.0 ± 0.3	1.6 ± 0.2	2.0 ± 0.4 <sup>+</sup>	2.0 ± 0.3

M<sub>1</sub> Latency is the latency to the onset of the first nocturnal meal; M<sub>1</sub> Size and M<sub>2</sub> Size are the sizes of the first and second nocturnal meals, respectively; M<sub>1-2</sub> IMI is the interval between these meals; 2-h Food Intake (g) is the cumulative food intake during the first 2 h of the dark; and 2-h Meal # is the number of meals begun in these 2 h. ANOVA revealed a significant interaction (E2 x ghrelin) effect for M<sub>1</sub> Latency,  $F_{1,26} = 4.92$ , a significant main effect of ghrelin for 2-h Food Intake,  $F_{1,26} = 3.57$ , and significant main effects of E2 for M<sub>1</sub> Size,  $F_{1,26} = 10.54$ , M<sub>1-2</sub> IMI,  $F_{1,26} = 12.38$ , M<sub>2</sub> Size,  $F_{1,26} = 8.35$ , and 2-h Meal #,  $F_{1,26} = 6.87$ . \*Significant difference between ghrelin and saline injection values in vehicle-treated rats, Bonferroni test,  $P < 0.05$ ; <sup>+</sup>Significant difference between saline values in vehicle-treated and in estradiol-treated rats, Bonferroni test,  $P < 0.05$ .

Figure 1

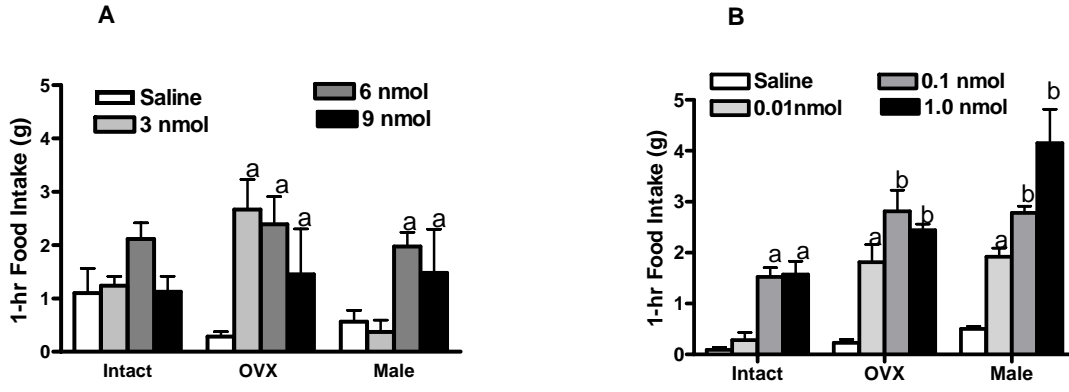


Figure 2

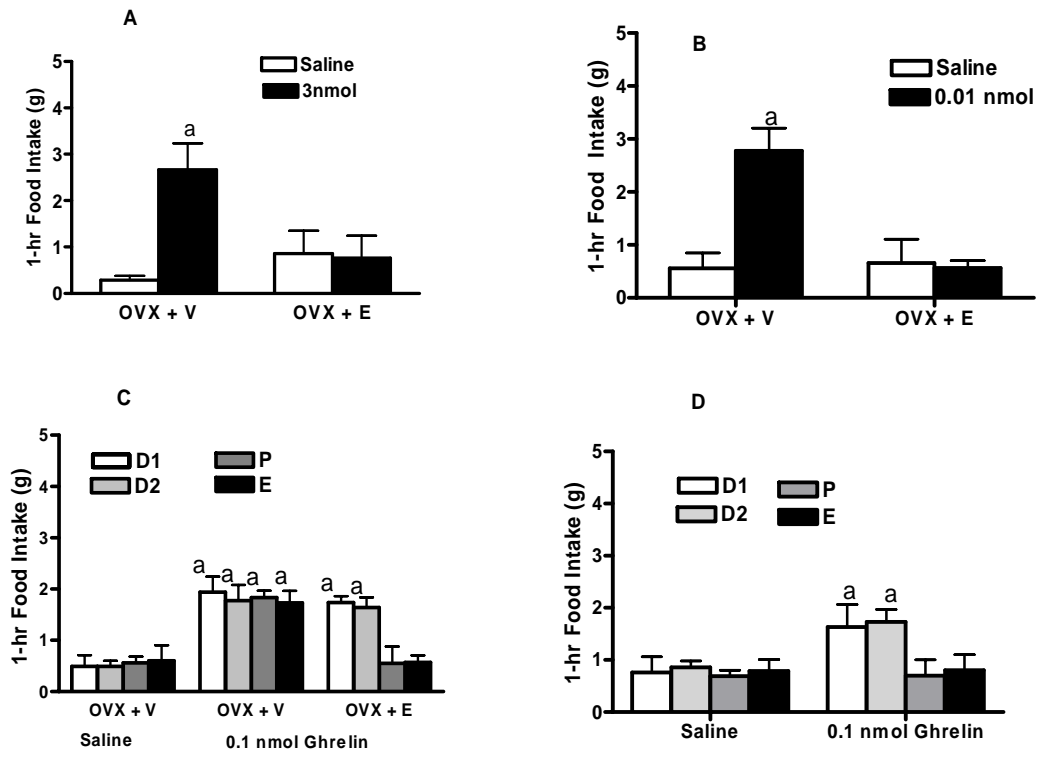


Figure 3

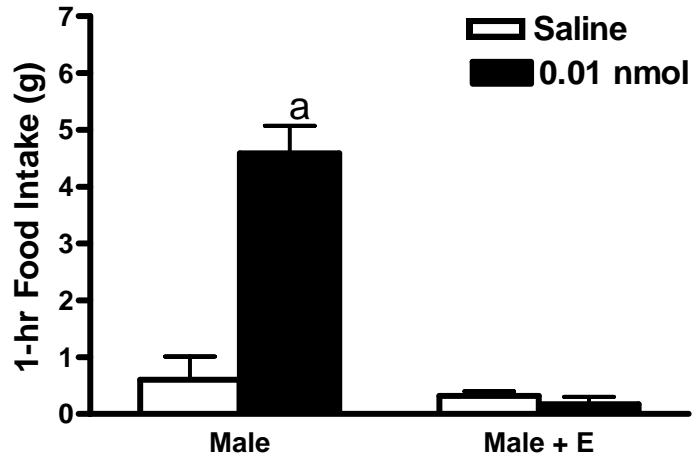


Figure 4

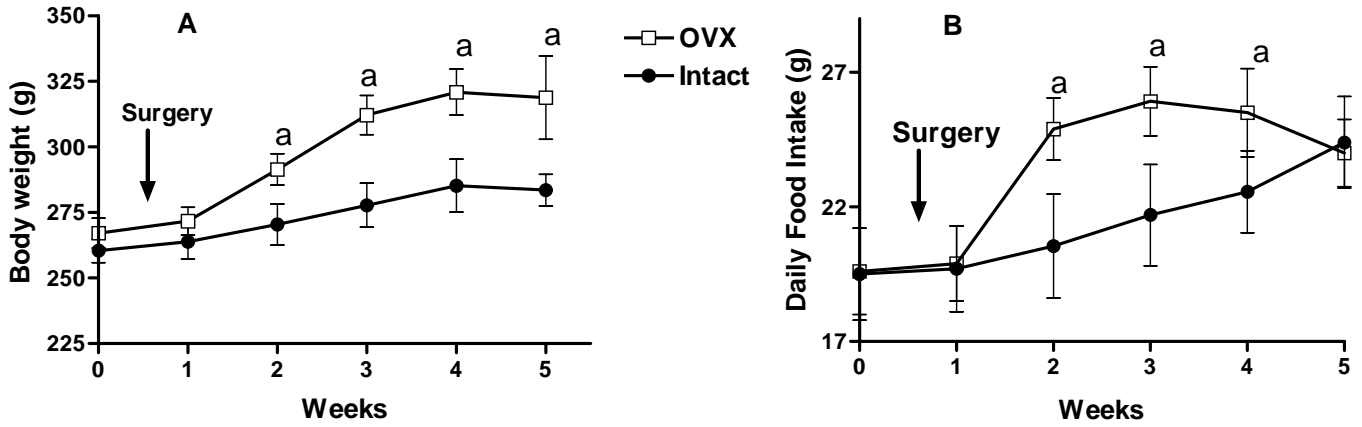


Figure 5

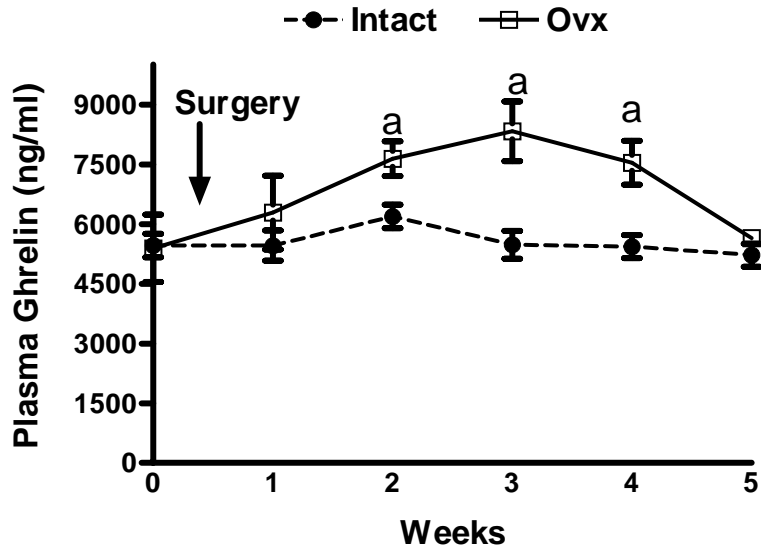


Figure 6

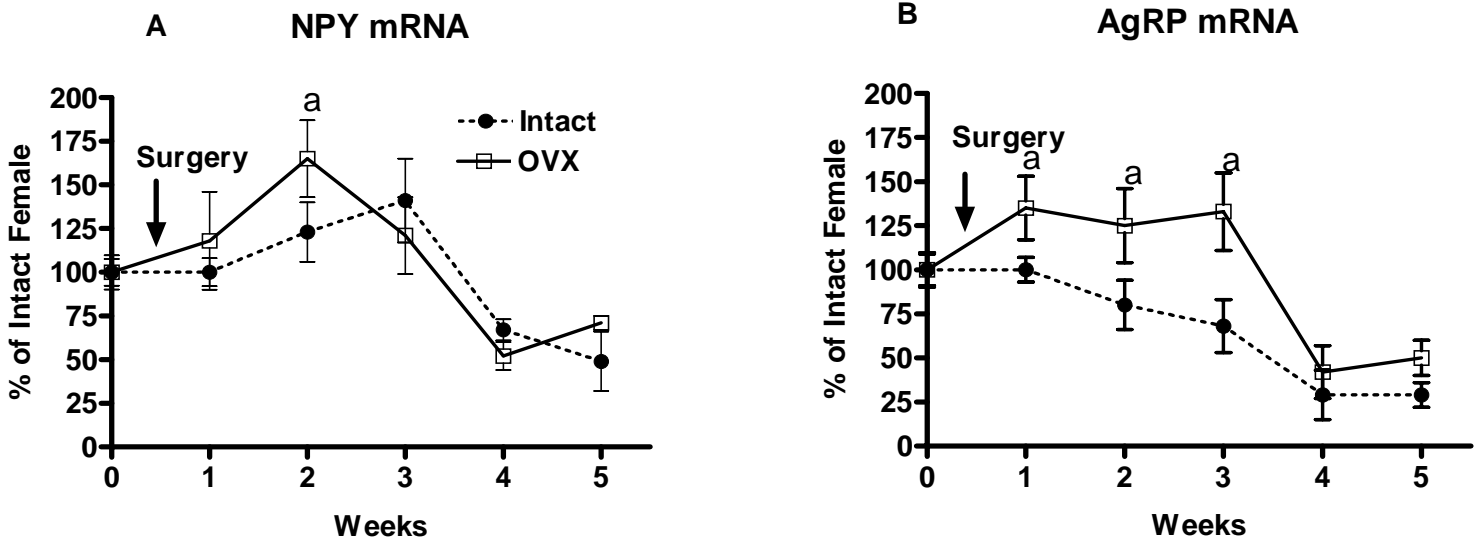


Figure 7

