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Estradiol and body weight during temporally targeted food restriction: Central pathways and peripheral metabolic factors

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ABSTRACT

We used temporally-targeted food restriction (TTFR), in which ovariectomized rats had chow only for 2 h/day, to test the hypothesis that estradiol benzoate (EB) suppresses feeding and decreases body weight during brief (4 day) TTFR, as it does during ad libitum feeding. All rats lost weight during TTFR, but the loss was greater with EB treatment. However, OIL and EB-treated rats ate comparable amounts of chow during TTFR. We next investigated central nervous system pathways and peripheral hormonal and metabolic changes that accompany the effects of TTFR to determine the mechanism for this effect. Immunolabeling for fos in the nucleus of the solitary tract, the terminal site of vagal afferents from the gastrointestinal tract, was increased when rats on TTFR had access to chow for 1 h on the test day, indicating neuronal activation associated with consumption of the meal. However, fos immunolabeling was not affected by EB treatment, nor were numbers of the α subtype of estrogen receptors. TTFR had the expected effects on carbohydrate and lipid metabolites and metabolic hormones, with only slight differences in plasma glucose, triglycerides, and free fatty acids attributable to EB treatment. Interestingly, plasma corticosterone levels were greater in EB-treated rats on TTFR, and increased further after eating. Given that corticosterone affects metabolism, these findings suggest that elevated corticosterone may explain the persistence of EB-induced differences in body weight during TTFR despite the lack of effect on food intake.

1. Introduction

Research into the controls of food intake and body weight is increasingly important as the number of overweight and obese Americans continues to rise (Flegal et al., 2012a, 2012b). Body weight increases substantially in women after menopause (Carr, 2003; Lovejoy and Sainsbury, 2009), an observation that suggests a role for ovarian hormones in body weight regulation. Unfortunately, the strategy many women employ to lose weight—decreasing the amount of calories and/or the number of meals consumed—has limited success, particularly over the long-term (Meckling et al., 2002; Rossner, 1998; Stunkard et al., 1955). Better understanding of the peripheral mechanisms and central neural pathways by which ovarian hormones affect body weight during periods of food restriction, therefore, has the potential to provide critical insights into maintaining healthy body weight.

Research using laboratory rats has been useful in advancing our knowledge on the controls of eating and body weight, especially in regard to sex differences. It has been known for approximately 100 years that ovarian hormones reduce feeding and body weight, and more recent studies have provided a wealth of information about the role of the ovarian hormone, estradiol, in modulating feeding and body weight (Asarian and Geary, 2002, 2006, 2007; Butera et al., 2010; Eckel, 2011; Geary and Asarian, 1999; Krause et al., 2006; Wade, 1975). However, these investigations typically were conducted in conditions when chow was freely available (Asarian and Geary, 2006; Butera et al., 2010; Eckel and Geary, 1999; Geary and Asarian, 1999), circumstances that stand in striking contrast to the voluntary food restriction employed by many people in weight loss programs (Eshghinia and Mohammadzadeh, 2013; Meckling et al., 2002; Rossner, 1998). Indeed, many obese dieters opt to skip breakfast and/or lunch, consuming only a single large meal each evening (Beaudoin and Mayer, 1953; Dole et al., 1954; Stunkard et al., 1955), and little research in female rats has examined the controls of eating and body weight under conditions of such temporally-targeted food restriction (TTFR).

In 1962, Hollifield and Parson employed a TTFR protocol with male rats (Hollifield and Parson, 1962a, 1962b) during which rats were given chow for only 2 h each day for seven days. After seven days of TTFR, these rats consumed large amounts of chow (60% of daily ad libitum

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intake) during comparatively brief daily access. Interestingly, this large influx of nutrients was not stored as glycogen but rather, was converted to lipids. The authors proposed that the shift in fuel usage—in which newly synthesized lipids were catabolized as the major energy source, thereby limiting glucose utilization to the small amounts required by the central nervous system (CNS)—was attributable to adaptation to TTFR. Clearly, such a shift in fuel utilization has profound implications for obesity and weight management during obesity, but it remains to be determined whether a similar shift occurs in females. Moreover, to our knowledge, the effect of TTR on central pathways associated with feeding and body weight has not been investigated in females. Accordingly, the goal of these studies was to assess the effect of TTFR on eating, body weight, and metabolism in female rats. In particular, we sought to determine whether estradiol-induced modulation of feeding and body weight persists during TTFR, and the central and peripheral factors associated with such changes.

2. Materials and methods

2.1. General methods

2.1.1. Animals, surgeries, and hormone treatments

Adult female Sprague-Dawley rats (Charles River), three months of age, were housed in individual cages in a temperature controlled (22 ± 2 °C) room on a 12:12 light:dark cycle (lights on 07:00). All rats were given ad libitum access to water and chow (Harlan rodent diet #2018) except as described. Experimental protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Oklahoma State University - Center for Health Sciences Institutional Animal Care and Use Committee. After one week to acclimate to the colony room, rats were bilaterally ovariectomized (OVX) under pentobarbital anesthesia (50 mg/kg i.p.; Sigma-Aldrich) using a midventral incision and then allowed to recover for 7–10 days. After recovery, a regimen of 0.1 ml subcutaneous injections with 17-β estradiol-3-benzoate (EB; 10 μg/0.1 ml sesame oil; Fisher Scientific), or sesame oil vehicle (OIL; 0.1 ml) was initiated, with injections given on two consecutive days at weekly intervals for the 3-week duration of the experiment. All rats were sacrificed 48 h after the second OIL or EB injection (i.e., Day 4) of Week 3. We and others have used this 4-day protocol in tests of behavioral and neural effects of estradiol (Eckel and Geary, 1999; Geary and Asarian, 1999; Asarian and Geary, 2002; Eckel et al., 2002; Asarian and Geary, 2006; Krause et al., 2006; Santollo and Eckel, 2008; Jones and Curtis, 2009; Butera et al., 2010; Graves et al., 2011; Rivera et al., 2012), as it mimics the pattern of fluctuations of estradiol that occurs in reproductively intact rats (see also, Curtis, 2015).

2.1.2. Feeding protocol

During Week 1 and Week 2, rats were weighed each day at 10:00, and chow and water were removed from the cages. Pre-measured chow was returned to the cages from 13:00 to 15:00, along with water in graduated cylinders fitted with drinking tubes. Water intake was measured after 2 h and uneaten chow was collected and weighed to determine intake during this period. For the first two weeks (Week 1 and Week 2), pre-weighed chow and water were returned at 15:00, rats had ad libitum access to chow and water overnight, and intakes of both were determined the following morning.

After these two weeks to adapt to the targeted intake period, some groups of OIL- and EB-treated rats underwent TTR on Week 3. These rats were weighed on Day 0 and food was removed from the cages. Thereafter, access to chow was restricted to the targeted period on Day 1-Day 4, though rats continued to have ad libitum access to water each night. Other groups of OIL- or EB-treated OVX rats were maintained on ad libitum access to chow (Ad lib) for the duration of the experiment and, thus, served as untreated controls.

During Week 3, all rats were weighed and received OIL or EB injections on Day 1 and Day 2, as described, and were sacrificed on Day 4. On Day 4, some OVX rats on TTFR were permitted to eat for 1 h (Fed) and then sacrificed 1 h later; chow intake during this 1-h intake test was measured. To control for non-specific effects of TTFR, other OVX rats on TTFR were not permitted to eat on Day 4 (Fasted) prior to sacrifice. Chow intake by Ad lib fed rats also was measured during 1-h intake tests prior to sacrifice on Day 4. In some of these rats, stomachs with contents were removed, blotted and weighed, then incised and contents removed. Empty stomachs were rinsed with saline, blotted and weighed again. Stomach contents were expressed as difference in weight (g). In other rats, uteri were removed and stripped of fat; a 10 mm section was cut at the level of the uterine bifurcation, blotted dry, and weighed.

The 4-day hormone protocol and experimental timeline are delineated in (Fig. 1), which also shows body weights throughout the experiment for purposes of illustration.

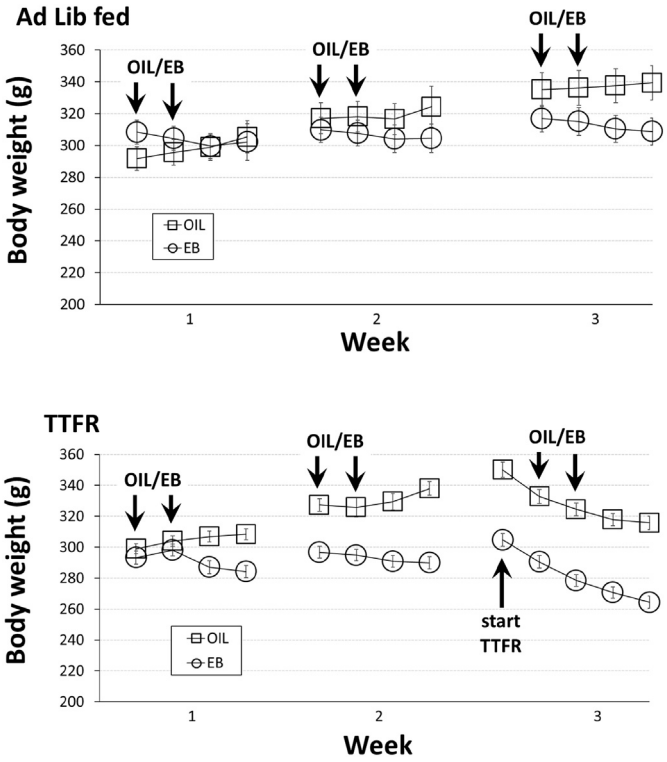


Fig. 1. Body weights and experimental timeline. To illustrate the experimental timeline and effects of hormone treatment and temporally targeted food restriction (TTFR), body weight is shown for all ovariectomized rats treated with Oil vehicle (OIL; squares) or estradiol benzoate (EB; circles) that were tested in these experiments. Rats were injected s.c. with OIL or EB on Day 1 and 2 each week (down arrows) for 3 weeks. During Week 1 and Week 2, pre-weighed chow and water were available for a targeted period during the light phase; rats had ad libitum access to chow and water overnight. In some OVX rats (Ad Lib fed; OIL, n = 8; EB, n = 9), ad libitum feeding procedures described for Week 1 and Week 2 continued during Week 3. Other OVX rats (OIL, n = 25; EB, n = 24). underwent temporally-targeted food restriction (TTFR). For these rats, TTFR was initiated on Day 0 of Week 3 by removal of food (up arrow); thereafter, rats were given food only for the targeted period each day. TTFR continued until rats were sacrificed on Day 4. During TTFR, rats continued to have ad libitum access to water each night.

2.1.3. Statistics

All data are presented as means ± standard error of the means.

Food intake on Day 4 of Week 3 was analyzed by 2-way analysis of variance (ANOVA) with hormone (OIL, EB) and feeding condition (Ad lib, Fed) as factors. Uterine weight and weight of stomach contents were normalized to 100 g body weight and analyzed by 3-way ANOVA with hormone (OIL, EB) and feeding condition (Ad lib, Fasted, Fed) as

factors. Percent change in body weight during the first and last day of TTFR also was calculated and analyzed by 2-way repeated measures (rm) ANOVA with hormone (OIL, EB) and day as factors, repeated for day. Pair-wise comparisons of significant main effects or interactions were evaluated using Fisher's LSD test. Effect sizes were estimated as partial η^2 ($_p\eta^2$).

2.2. Central neural pathways

2.2.1. Perfusion, brain extraction, and sectioning

On Day 4 of Week 3, subsets of OVX rats that were OIL- or EB-treated and fed as described (OIL: Ad lib – n = 6; TTFR Fasted – n = 3; TTFR Fed – n = 8; EB: Ad lib – n = 5; TTFR Fasted – n = 4; TTFR Fed – n = 8) were deeply anesthetized with pentobarbital (25 mg/rat, i.p.) and then perfused transcardially with 0.15 M NaCl (~300 ml) followed by phosphate buffered 4% paraformaldehyde (~300 ml). Brains were removed, stored in paraformaldehyde overnight at 4 °C, and then transferred to 30% sucrose for cryoprotection. Brains were stored at 4 °C until the hindbrain blocks were cut into 40-μm sections in a 1-to-3 series using a cryostat (Leica). Sections were stored at −20 °C in a cryoprotectant solution (1% polyvinylpyrrolidone, 30% ethylene glycol, 30% sucrose in sodium phosphate; pH 7.2), until processed for immunolabeling. Given that vagal afferents from the gastrointestinal tract terminate in the hindbrain nucleus of the solitarty tract (NTS; Contreras et al., 1982; Olson et al., 1993; Thammacharoen et al., 2008), we limited our analyses to the NTS in this study.

2.2.2. fos immunolabeling and quantification

One series of free-floating hindbrain sections was rinsed of cryoprotectant, incubated in 0.5% H_2O_2, washed with 0.05 M Tris NaCl, and incubated in 10% NGS (mixed in 0.5% Tween 20 with 0.05 M Tris NaCl). Sections then were transferred to the primary antibody (Santa Cruz SC-52, rabbit anti-c-Fos) diluted 1:30,000 in 2% NGS and incubated at room temperature for 1 h before overnight incubation (~18 h) at 4 °C.

The following morning, sections were brought to room temperature, rinsed three times for 5 min with 2% NGS and then incubated for 2 h in the secondary antibody (Vector biotinylated goat anti-rabbit IgG) diluted 1:300 in 2% normal goat serum (NGS). Sections then were rinsed two times with 2% NGS, followed by three rinses with 0.05 M Tris NaCl. Labeling was amplified with avidin-biotin (Vector, Vectastain ABC kit) for 90 min and sections then were rinsed. Labeling was visualized with nickel-intensified diaminobenzidine (Vector DAB kit) to produce a blue-black nuclear reaction product. The reaction was terminated by multiple rinses with 0.05 M Tris NaCl.

Labeled sections were ordered and mounted on gelatin-coated slides, allowed to dry and then dehydrated in an series of ethanols (70%, 90% and 100%), defatted in xylenes, and coverslips were attached with Cytoseal (Thermo Scientific). Slides were examined under brightfield illumination using a Nikon Eclipse 80i microscope that also was fitted with FITC and rhodamine filters and NIS Elements software. Sections containing the NTS were identified using the atlas of Paxinos and Watson (Paxinos and Watson, 1998). The NTS was outlined caudal to calamus scriptorius (cNTS; −14.30 to −14.60 mm relative to Bregma); and at the middle level subadjacent to the area postrema (midNTS; −13.68 to −14.08 mm relative to Bregma). Fos-positive cells within the cNTS and midNTS were quantified in 2–3 representative sections from each area, matched between animals. Average numbers of fos-positive cells were calculated for the cNTS and for the midNTS for each animal; group means then were determined for each feeding condition.

2.2.3. ERα immunohistochemistry and quantification

Another series of free-floating hindbrain sections was rinsed of cryoprotectant by six 5-min washes with 0.05 M Tris NaCl and then incubated in 0.5% H_2O_2 in 0.05 M Tris NaCl for 30 min at room temperature. After six 5- min washes with 0.05 M Tris NaCl, sections were incubated in 10% NGS (mixed in 0.5% Triton-X with 0.05 M Tris NaCl) for 1 h at room temperature. Sections were then transferred to the primary antibody (Millipore, rabbit anti-estrogen receptor alpha (ERα)) diluted 1:10,000 in 2% NGS for 1 h at room temperature, followed by 70 h incubation at 4 °C.

Sections were brought to room temperature, rinsed three times for 5 min each with 2% NGS and then incubated for ~4 h in the secondary antibody, (Jackson Immunoresearch goat anti-rabbit IgG - Cy2) diluted 1:300 in 2% NGS. Sections then were rinsed two times with 2% NGS, followed by three rinses with 0.05 M Tris NaCl.

Labeled sections were ordered and mounted on gelatin-coated slides, allowed to dry, then dehydrated and defatted, and coverslips were attached as described. Slides were examined using a Nikon Eclipse 80i microscope, and sections containing the cNTS and midNTS were identified and outlined as described. ERα-labeled neurons were counted in 2–4 sections at each level that were matched between animals. Average numbers of ERα-labeled cells were calculated for each animal at each level and group means for each level were determined for each feeding condition. In this analysis, we focused on OIL-and EB-treated rats that were Ad lib fed or were on TTFR and fasted prior to sacrifice.

2.2.4. Statistics

All data are presented as means ± standard error of the means. Numbers of rats from each group included in fos and ERα immunolabeling are shown in the respective figure legends.

Numbers of fos-positive cells in the cNTS and midNTS were analyzed using separate 2-way ANOVAs with hormone (OIL, EB) and feeding condition (Ad lib, Fasted, Fed) as factors. Numbers of ERα-positive cells in the cNTS and midNTS were analyzed using separate 2-way ANOVAs with hormone (OIL, EB) and feeding condition (Ad lib, Fasted) as factors. Pair-wise comparisons of significant main effects or interactions were evaluated using Fisher's LSD test; effect sizes were estimated as partial η^2 ($_p\eta^2$) and η^2.

2.3. Peripheral and metabolic factors

2.3.1. Tissue collection

On Day 4 of Week 3, other subsets of OVX rats that were OIL- or EB-treated and fed as described (OIL: Ad lib – n = 6; TTFR Fasted – n = 6; TTFR Fed – n = 6; EB: Ad lib – n = 6; TTFR Fasted – n = 6; TTFR Fed – n = 6) were rendered unconscious with CO_2 and decapitated. Trunk blood was collected into Lithium-EDTA tubes and centrifuged to obtain plasma, which was then frozen in aliquots for later analysis of hormones and metabolites.

Abdominal fat and liver samples were collected and quick frozen for later analysis of glycogen, triglycerides (TGS) and free fatty acids (FFAs). Adrenals were removed and weighed.

2.3.2. Plasma hormone analyses

Leptin, insulin, and corticosterone were measured by ELISA according to the manufacturers' instructions.

Plasma leptin levels were measured spectrophotometrically via a sandwich ELISA (Millipore), with increased absorbency at 450 nm, corrected from the absorbency at 590 nm after acidification of formed products. The sample concentration was based on interpolation from a reference curve generated in the same assay with standards of known concentrations of rat leptin.

Plasma insulin levels also were measured spectrophotometrically via a sandwich ELISA (Millipore), with increased absorbency at 450 nm, corrected from the absorbency at 590 nm after acidification of formed products. The sample concentration was based on interpolation from a reference curve generated in the same assay with standards of known concentrations of rat insulin.

Plasma corticosterone levels were measured colorimetrically via ELISA (Enzo) after 1:60 dilution. Optical density (measured as intensity

of bound antibody at 405 nm) was used to calculate the concentration of corticosterone based on a reference curve generated from standards of known concentrations of rat corticosterone.

2.3.3. Plasma metabolite analyses (colorimetric)

Glucose, glycogen, free fatty acids (FFAs) and triglycerides (TGs) were measured with colorimetric kits according to the manufacturers' instructions.

Plasma glucose levels were measured in one microliter of undiluted plasma sample via colorimetric assay (BioVision) read at 570 nm. Sample concentrations were calculated from the standard curve.

Plasma glycogen levels were measured via colorimetric assay (Abcam) at 450 nm.

Plasma FFAs were measured via fluorimetric assay (Cayman Chemical). Resorufin fluorescence was measured with a 530–540 nm excitation wavelength and a 585–595 nm emission wavelength, with sample concentrations determined from a standard curve.

Plasma TGs were measured via spectrophotometric assay (Abcam) from the oxidation reaction product of glycerol, converted from FFAs, read at 570 nm.

2.3.4. Liver and abdominal adipose metabolites

Frozen liver or adipose tissue was placed into 2 ml microcentrifuge tubes, weighed and homogenized on ice at 5 mg/ml in 4% Nonidet P-40 using a sonic homogenizer, then centrifuged at 14000 xg for 5 min at 4° C.

For liver and adipose levels of TGs, supernatant was removed, diluted ten-fold in deionized water, and frozen at -20° C until analyzed using methods described above.

For liver levels of glycogen, supernatant was removed and analyzed the same day using methods described above.

2.3.5. Statistics

All data are presented as means ± standard error of the means. Numbers of rats from each group included in fos and ERα immunolabeling are shown in the respective figure legends.

Metabolite and hormone data were analyzed by two-way ANOVA with hormone (OIL, EB) and feeding condition (Ad lib, Fasted, Fed) as factors. Adrenal and uterine weight, and weight of stomach contents also were analyzed by two-way ANOVA with hormone (OIL, EB) and feeding condition (Ad lib, Fasted, Fed) as factors. Pair-wise comparisons of significant main effects or interactions were evaluated using Fisher's LSD test; Effect sizes were estimated as partial η^2 ($_p\eta^2$) and η^2.

3. Results

To illustrate the effects of EB-treatment and TTFR, the time course of daily body weights of OVX rats that were fed Ad lib throughout the experiment or were on TTFR on Week 3 are shown in Fig. 1.

The change in body weight in OIL- and EB-treated rats on the first and last day of TTFR on Week 3 is shown in Fig. 2. Two-way ANOVA for these data revealed main effects of day [$F_{(1,47)} = 582.72$, $p < 0.001$; $_p\eta^2 = 0.925$; $\eta^2 = 0.620$] and of hormone treatment [$F_{(1,47)} = 10.75$, $p < 0.01$; $_p\eta^2 = 0.186$; $\eta^2 = 0.057$]. There also was an interaction between hormone treatment and day [$F(1,47) = 22.45$, $p < 0.001$; $_p\eta^2 = 0.323$; $\eta^2 = 0.024$], and pairwise comparisons indicated that both OIL- and EB-treated rats lost significantly more weight on the last day than on the first day (both ps < 0.001). However, on the last day of the protocol, EB-treated rats lost significantly more weight than did OIL-treated rats ($p < 0.0010$).

As shown in Table 1, food intake on Day 4 of Week 3 depended on whether rats were fed Ad lib or were on TTFR. Two-way ANOVA revealed a main effect of feeding condition [$F_{(1,41)} = 105.31$, $p < 0.001$; $_p\eta^2 = 0.720$; $\eta^2 = 0.716$], with food intake by rats on TTFR greater than that by Ad lib fed rats, independent of hormone treatment. There

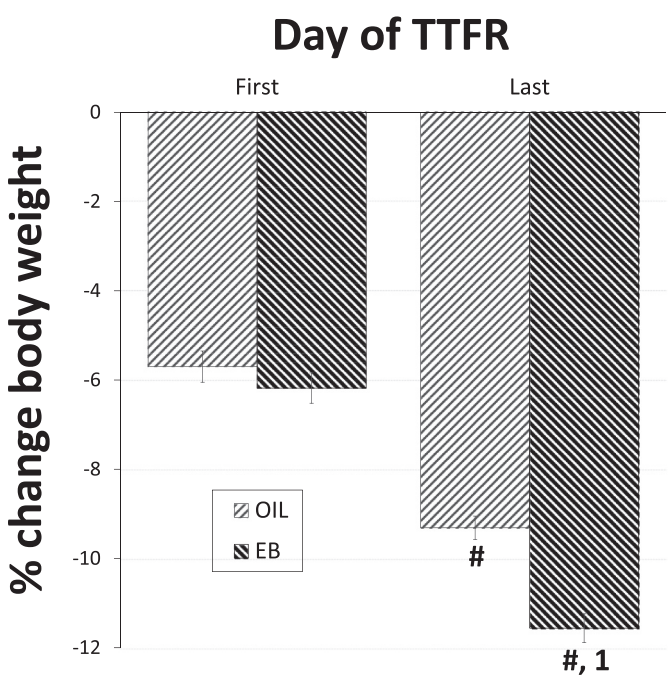


Fig. 2. Change in body weight during temporally targeted food restriction (TTFR). Percent change in body weight during TTFR for all OIL- (n = 25) and EB- (n = 24) treated ovariectomized rats tested in these experiments.
= significantly less than first day; 1 = significantly different from OIL on corresponding day.

was no effect of hormone treatment and no interaction between hormone treatment and feeding condition.

The weight of stomach contents also is shown in Table 1, and here, too, there was a main effect of feeding condition [$F_{(2,35)} = 75.65$, $p < 0.001$; $_p\eta^2 = 0.812$; $\eta^2 = 0.811$]. Pairwise comparisons revealed that, independent of hormone treatment, stomach weights were significantly greater in rats on TTFR that were Fed prior to sacrifice compared to both Ad lib fed rats and rats on TTFR that were Fasted prior to sacrifice (both ps < 0.001). Stomach weights in Ad lib fed rats were significantly greater than those in rats on TTFR that were Fasted prior to sacrifice ($p < 0.05$). There was no effect of hormone treatment and no interaction between hormone treatment and feeding condition.

Table 1 also shows uterine weights, which clearly were greater in EB-treated rats [$F(1,28) = 166.06$, $p < 0.001$; $_p\eta^2 = 0.856$; $\eta^2 = 0.841$]. There was no effect of feeding condition and no interaction between hormone treatment and feeding condition.

3.1. Central neural pathways

Fig. 3 shows fos immunolabeling in the NTS. In both the cNTS (Fig. 3A, *bottom*) and midNTS (Fig. 3B, *bottom*), few cells were labeled for fos in Ad lib fed rats, which ate little or nothing (0–0.14 g) in the intake test prior to sacrifice, or in rats on TTFR that were Fasted prior to sacrifice. In contrast, fos immunolabeling was elevated in both levels of the NTS of rats on TTFR that were Fed prior to sacrifice. Two-way ANOVAs revealed main effects of feeding condition in both the cNTS [$F_{(2,20)} = 5.46$, $p < 0.05$; $_p\eta^2 = 0.353$; $\eta^2 = 0.348$] and midNTS [$F_{(2,21)} = 13.63$, $p < 0.001$; $_p\eta^2 = 0.565$; $\eta^2 = 0.556$], with no effect of hormone treatment or interactions for either level. For the cNTS, pairwise comparisons of the main effect of feeding condition showed that, regardless of hormone treatment, numbers of fos-positive cells were significantly greater in rats on TTFR that were Fed than in Ad lib fed rats ($p < 0.05$) and in rats on TTFR that were Fasted ($p < 0.01$) groups, which were not different from each other. Similarly, in the midNTS, pairwise comparisons of the main effect of feeding condition

Table 1
Food intake, weight of stomach contents, and uterine weight on the final day of the experiment. Ovariectomized rats were given s.c. injections of OIL vehicle (OIL) or estradiol benzoate (EB) twice weekly for three weeks and fed ad libitum (Ad lib) throughout, or were subjected to temporally-targeted food restriction (TTFR) during the third week. Some rats on TTFR were fasted prior to sacrifice on Day 4 (Fasted); others were fed and sacrificed 1 h later (Fed). Numbers in parentheses indicate numbers of rats. 1 = significantly greater than OIL; a = significantly greater than Ad lib; b = significantly greater than Fasted.

	OIL	EB	
1-hr food intake (g/100 g body weight)			
Ad lib	0.24 ± 0.13 (8)	0.13 ± 0.07 (9)	
Fasted	-----	-----	
Fed[a]	3.08 ± 0.32 (14)	2.75 ± 0.24 (14)	
Stomach contents (g/100 g body weight)			
Ad lib[b]	1.29 ± 0.13 (6)	1.39 ± 0.15 (7)	
Fasted	0.66 ± 0.12 (7)	0.55 ± 0.06 (7)	
Fed[a,b]	4.12 ± 0.51 (7)	3.94 ± 0.43 (7)	
Uterine weight (mg/100 g body weight)			
Ad lib	7.01 ± 1.24 (5)	34.23 ± 3.52 (6)	1
Fasted	9.80 ± 0.55 (6)	39.86 ± 4.36 (6)	
Fed	8.36 ± 0.62 (6)	34.71 ± 1.96 (6)	

showed that numbers of fos-positive were significantly greater in rats on TTFR that were Fed than in rats that were fed Ad lib or were on TTFR and Fasted (both ps < 0.001), with no differences between Ad lib fed and Fasted rats.

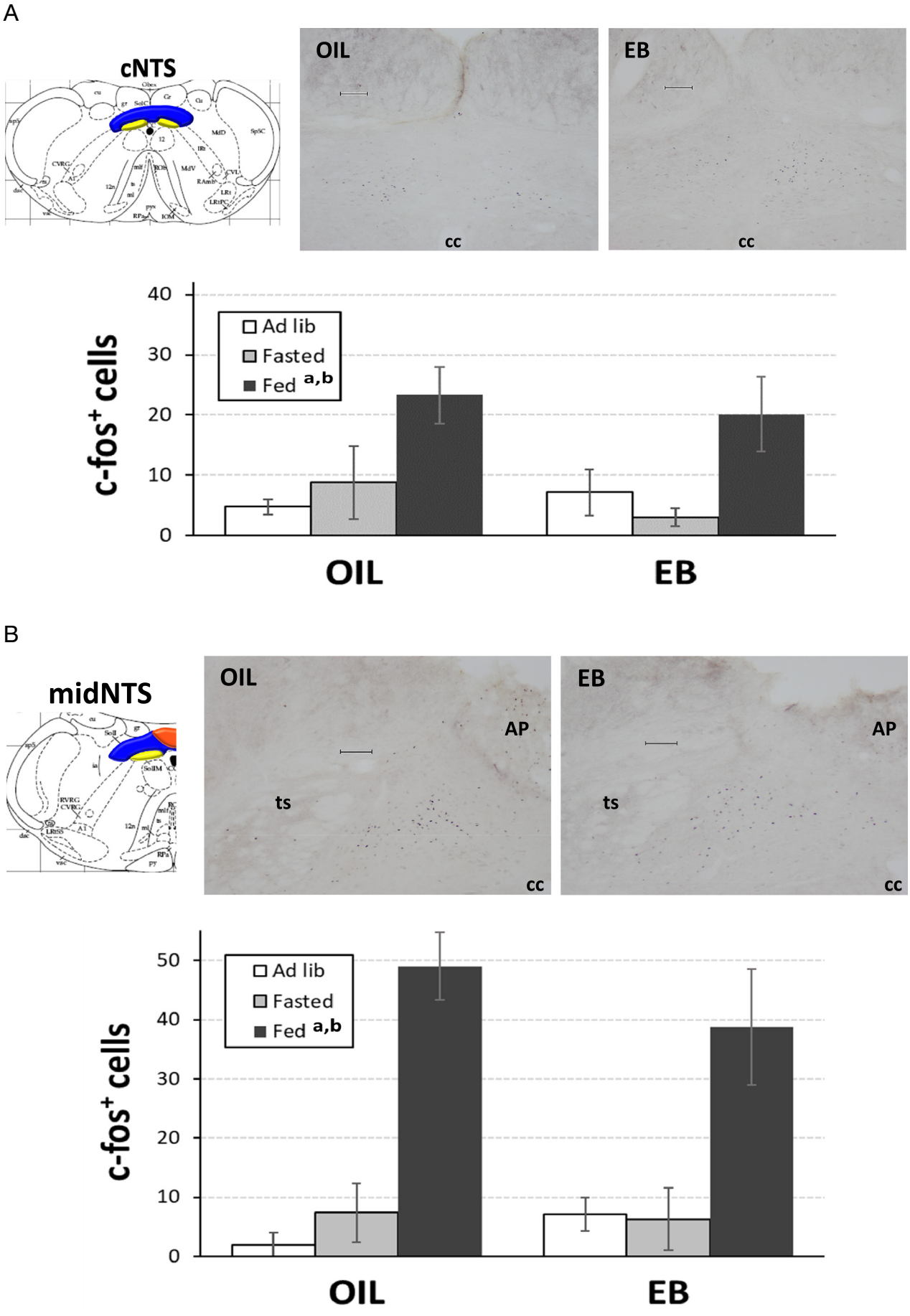
Fig. 4 shows ERα immunolabeling in the cNTS and midNTS. Neither feeding condition, hormone treatment, nor the interaction altered numbers of ERα-positive cells in the cNTS. There was a main effect of hormone treatment on ERα immunolabeling in the midNTS [$F_{(1,7)} = 12.02$, $p < 0.05$; ${}_p\eta^2 = 0.632$; $\eta^2 = 0.588$]; however, the number of ERα-positive cells was very low, overall.

3.2. Peripheral and metabolic factors

Fig. 5 shows plasma glucose and hepatic glycogen levels, along with plasma insulin levels. Two-way ANOVA revealed a main effect of hormone treatment on plasma glucose levels [$F_{(1,30)} = 14.22$, $p < 0.001$; ${}_p\eta^2 = 0.322$; $\eta^2 = 0.292$] with plasma glucose in EB-treated rats significantly less than those in OIL-treated rats, independent of feeding condition. There was no effect of feeding condition, nor was there an interaction between hormone treatment and feeding condition. In contrast, there was a main effect of feeding condition on hepatic glycogen levels [$F_{(2,18)} = 56.03$, $p < 0.001$; ${}_p\eta^2 = 0.862$; $\eta^2 = 0.839$] and pairwise comparisons showed that, independent of hormone treatment, liver glycogen was significantly greater in Ad lib fed rats compared to that in rats on TTFR that were Fed or Fasted (both ps < 0.001). In addition, glycogen was greater in rats on TTFR that were Fed than in those that were Fasted ($p < 0.01$). There was no hormone treatment effect or interaction. Two-way ANOVA demonstrated a main effect of feeding condition on plasma insulin levels [$F_{(2,28)} = 11.43$, $p < 0.001$; ${}_p\eta^2 = 0.450$; $\eta^2 = 0.428$], with pairwise comparisons showing that levels of insulin in rats on TTFR that were Fasted were significantly less compared to both Ad lib fed rats and rats on TTFR that were Fed (ps < 0.001). No hormone treatment effect or interaction between hormone treatment and feeding condition was observed.

Fig. 6 shows plasma TG, FFA, and leptin levels. Two-way ANOVA revealed a main effect of hormone treatment on plasma TG levels [$F_{(1,29)} = 18.89$, $p < 0.001$; ${}_p\eta^2 = 0.394$; $\eta^2 = 0.222$] with plasma TGs in EB-treated rats significantly greater than those in OIL-treated rats. There also was a main effect of feeding condition [$F_{(2,29)} = 16.11$, $p < 0.001$; ${}_p\eta^2 = 0.526$; $\eta^2 = 0.379$] and pairwise comparisons showed that plasma TGs in rats on TTFR that were Fasted were significantly less than those in rats that were Ad lib fed or that were on TTFR and Fed (ps < 0.001), which were not different from each other. There was no interaction between hormone treatment and feeding condition on plasma TG levels. Similar patterns also were observed for plasma FFA levels, with main effects of both hormone treatment [$F_{(1,30)} = 10.15$, $p < 0.01$; ${}_p\eta^2 = 0.253$; $\eta^2 = 0.165$] and feeding condition [$F_{(2,30)} = 9.35$, $p < 0.001$; ${}_p\eta^2 = 0.384$; $\eta^2 = 0.304$], but no interaction. Here, too, plasma FFAs were greater in EB-treated rats, and in rats on TTFR that were Fasted ($p < 0.05$, 0.001). Two-way ANOVA also revealed a main effect of feeding condition on plasma leptin levels [$F_{(2,29)} = 9.02$, $p < 0.001$; ${}_p\eta^2 = 0.383$; $\eta^2 = 0.357$], with pairwise comparisons showing that rats on TTFR that were fasted had significantly less leptin than did rats that were Ad lib fed ($p < 0.001$), as well as rats on TTFR that were Fed ($p < 0.05$). Interestingly, there was no difference between the latter two conditions, nor was there a hormone treatment effect or an interaction. Finally, as shown in Table 2, there were no main effects or interactions for either adipose or hepatic TG levels.

Adrenal weights and plasma corticosterone levels are shown in Fig. 7. Two-way ANOVA revealed a main effect of hormone treatment on adrenal weights [$F_{(1,27)} = 18.09$, $p < 0.001$; ${}_p\eta^2 = 0.856$; $\eta^2 = 0.397$], with EB-treated rats having greater adrenal weights than did OIL-treated rats. There were also was a main effect of feeding condition [$F_{(2,27)} = 13.01$, $p < 0.001$; ${}_p\eta^2 = 0.856$; $\eta^2 = 0.571$] and pairwise comparisons revealed that adrenal weights in rats that were fed Ad lib were significantly less than those in rats on TTFR, whether they were Fed or Fasted (both ps < 0.001), which were not different from each other. There was no interaction between hormone treatment and feeding condition. Similarly, 2-way ANOVA revealed that plasma corticosterone levels were affected by main effects of feeding condition [$F_{(2,22)} = 15.05$, $p < 0.001$; ${}_p\eta^2 = 0.578$; $\eta^2 = 0.233$] and hormone treatment [$F_{(1,22)} = 56.91$, $p < 0.001$; ${}_p\eta^2 = 0.721$; $\eta^2 = 0.441$]. However, there also was an interaction between feeding condition and hormone treatment [$F_{(2,22)} = 10.02$, $p < 0.001$; ${}_p\eta^2 = 0.477$; $\eta^2 = 0.155$). Pairwise comparisons of this interaction revealed no differences in the corticosterone levels in rats that were fed Ad lib, but plasma corticosterone was significantly greater in EB-treated rats on



(caption on next page)

Fig. 3. fos immunolabeling in the nucleus of the solitary tract (NTS) of ovariectomized rats treated with Oil vehicle (OIL) or estradiol benzoate (EB). *A: caudal NTS (cNTS).* Schematic of cNTS (*top left*, adapted from [Paxinos and Watson, 1998]) and representative photomicrographs of fos immunolabeling (dark nuclear staining) in the cNTS of OIL-treated (*top middle*) and EB-treated (*top right*) rats on temporally-targeted food restriction (TTFR) that were allowed to eat (Fed) prior to sacrifice on Day 4. *Bottom* panel shows mean numbers of fos^+ cells in OIL- and EB-treated rats that were fed ad libitum (Ad lib; white bars; OIL, n = 2; EB, n = 3), or Fasted (gray bars; OIL, n = 3; EB, n = 4) or Fed (black bars; OIL, n = 7; EB, n = 7) prior to sacrifice after TTFR. *B: middle NTS (midNTS).* Schematic hemisection of midNTS (*top left*, adapted from [Paxinos and Watson, 1998]) and representative photomicrographs of fos immunolabeling in hemisections of the midNTS of OIL-treated (*top middle*) and EB-treated (*top right*) rats on TTFR that were Fed prior to sacrifice. *Bottom* panel shows mean numbers of fos^+ cells in OIL- and EB-treated rats that were Ad lib fed (OIL, n = 2; EB, n = 3), or Fasted (OIL, n = 3; EB, n = 4) or Fed (OIL, n = 7; EB, n = 8) prior to sacrifice after TTFR. Blue shading - NTS; yellow shading - dorsal motor nucleus of the vagus; red shading - Area Postrema (AP). ts - tractus solitarius; cc - central canal; scale bars - 100 μm. a = significantly greater than Ad lib; b = significantly greater than Fasted.

TTFR than in OIL- treated rats on TTFR, both Fed and Fasted (both ps < 0.001). Moreover, plasma corticosterone in EB-treated rats on TTFR that were Fed was significantly greater than that in EB-treated rats that were fed Ad lib ($p < 0.001$), and corticosterone in EB-treated rats on TTFR that were Fasted were significantly greater than those in EB-treated rats that were Ad lib fed ($p < 0.001$) or were on TTFR and fasted ($p < 0.05$). There were no differences in corticosterone levels in OIL-treated rats between feeding conditions.

4. Discussion

Over the last 40 years, the role of estrogens in feeding and body weight regulation has become the subject of intensive investigation. From seminal observations of feeding behaviors in rats by Wade and colleagues in the mid-1970s (Blaustein and Wade, 1976; Wade, 1975) to more recent studies employing sophisticated techniques to explore the molecular bases of the actions of estrogens in the control of ingestive behaviors (Eckel et al., 2000; Musatov et al., 2007), work in this area has led to better understanding of the control of feeding and body weight during ad libitum feeding conditions. Indeed, investigators are beginning to dissect out specific CNS areas, neurotransmitter systems, and estrogen receptor subtypes (Eckel et al., 1998; Hrupka et al., 2002; Rivera et al., 2012; Santollo and Eckel, 2008; Santollo et al., 2011, 2013), that will allow manipulations for even more finely-honed approaches to understanding the mechanism(s) of the actions of estrogens, especially as it pertains to the CNS. Certainly, there are conflicting findings about the location of central estrogen receptors that control feeding and body weight (Hrupka et al., 2002; Santollo et al., 2011), and the relative influence of specific central neurotransmitter systems remains to be determined (Eckel et al., 1998; Rivera et al., 2012; Sakurazawa et al., 2013; Santollo and Eckel, 2008; Santollo et al., 2011, 2013). Nonetheless, remarkable progress has occurred, particularly in regard to the control of feeding during conditions of ad libitum access.

To our knowledge, this is the first comprehensive investigation focused on the influence of estradiol on chow intake and body weight of OVX rats during TTFR. Given the propensity of overweight and obese individuals to eat only one large meal at the end of the day, when energy levels are low (Beaudoin and Mayer, 1953; Dole et al., 1954; Stunkard et al., 1955), and the fact that females–especially Black and Hispanic females (Lovejoy and Sainsbury, 2009; Flegal et al., 2012a, 2012b)–suffer disproportionately from obesity, the goals of this study, therefore, were to examine the effects of estradiol during TTFR and to test CNS and peripheral mechanisms that may underlie these effects. Better understanding of interactions between estradiol and inhibitory signals during TTFR has the potential to provide important insights for weight control in humans.

4.1. Estradiol effects on body weight and uterine weight

In this study, EB-treated rats lost weight during ad libitum feeding, while OIL-treated rats gained weight throughout (Fig. 1). While shown for purposes of illustration, it should be noted that these findings that are consistent with previous work from our lab and others (Geary and Asarian, 1999; Asarian and Geary, 2002; Asarian and Geary, 2006; Krause et al., 2006; Jones and Curtis, 2009; Butera et al., 2010; Graves et al., 2011; Eckel, 2011). In addition, uteri assessed after EB-treatment were hypertrophied compared to those in OIL-treated rats (Table 1), as we have reported previously (Graves et al., 2011; Curtis, 2015). This hypertrophy occurs in conjunction with increased circulating estradiol (Curtis, 2015); thus, together, these measures demonstrate the efficacy of the EB protocol.

4.2. Estradiol effects on chow intake and body weight during TTFR

During ad libitum access to chow, the intake that occurred overnight appeared to be satiating, as neither EB- nor OIL-treated group ate appreciable amounts of chow during intake tests conducted during the day (Table 1). Our TTFR protocol was intended to mimic eating patterns of some obese and overweight people who often eat only one large evening meal. Accordingly, we restricted the time rats were permitted to eat, rather than restricting the amount of chow provided. As a result, both EB-treated and OIL-treated rats consumed substantial and similar amounts of chow in intake tests during TTFR. These findings were unexpected, given numerous reports that EB decreases food intake during ad libitum feeding (Asarian and Geary, 2002, 2006; Butera et al., 2010; Eckel, 2011; Geary and Asarian, 1999; Krause et al., 2006), and suggest that the effect of estradiol in the control of eating is altered during TTFR. Thus, one might predict changes in body weight would parallel the effects on eating. However, even taking into account that EB-treated rats weighed less than OIL-treated rats at the end of the prior ad libitum feeding period, and despite substantial body weight loss in both groups during TTFR (Fig. 2), EB-treated rats lost more weight than did OIL-treated rats.

4.3. Mechanisms underlying estradiol effects during TTFR

4.3.1. Estradiol effects on central pathways

During ad libitum feeding conditions, the EB-induced decrease in chow intake is primarily a function of decreased meal size (Blaustein and Wade, 1976; Geary and Asarian, 1999) due, in part, to enhanced sensitivity to gastric distension and/or the gut hormone, CCK (Asarian and Geary, 2007; Cummings and Overduin, 2007; Eckel and Geary, 1999, 2001; Eckel et al., 2002; Moran, 2006). In the present study, both OIL- and EB-treated rats consumed substantial amounts of chow during TTFR. In fact, amounts of chow consumed in these 1-h tests were approximately half the amounts typically consumed by rats during 24 h of ad libitum access to chow (Hubert et al., 2000), and far in excess of what would typically be consumed in a single meal during ad libitum feeding (Asarian and Geary, 2002; Blaustein and Wade, 1976). These intakes likely generated considerable gastric distension and, thereby, considerable activation of stretch receptors that project to the NTS via the gastric vagus (Contreras et al., 1982; Dockray and Burdyga, 2011; Kentish et al., 2012). Gastric distension–even more moderate distension associated with a meal of modest size—contributes to the cessation of feeding (Moran, 2006; Eckel, 2011; Maniscalco et al., 2013). Thus, the observation that both OIL- and EB-treated rats ate well in excess of the volumes reported in those studies suggests that both groups were less sensitive to inhibitory signals related to gastric distension during TTFR.

Input from gastric vagal afferent fibers signaling gastric distension (Contreras et al., 1982; Dockray and Burdyga, 2011; Kentish et al.,

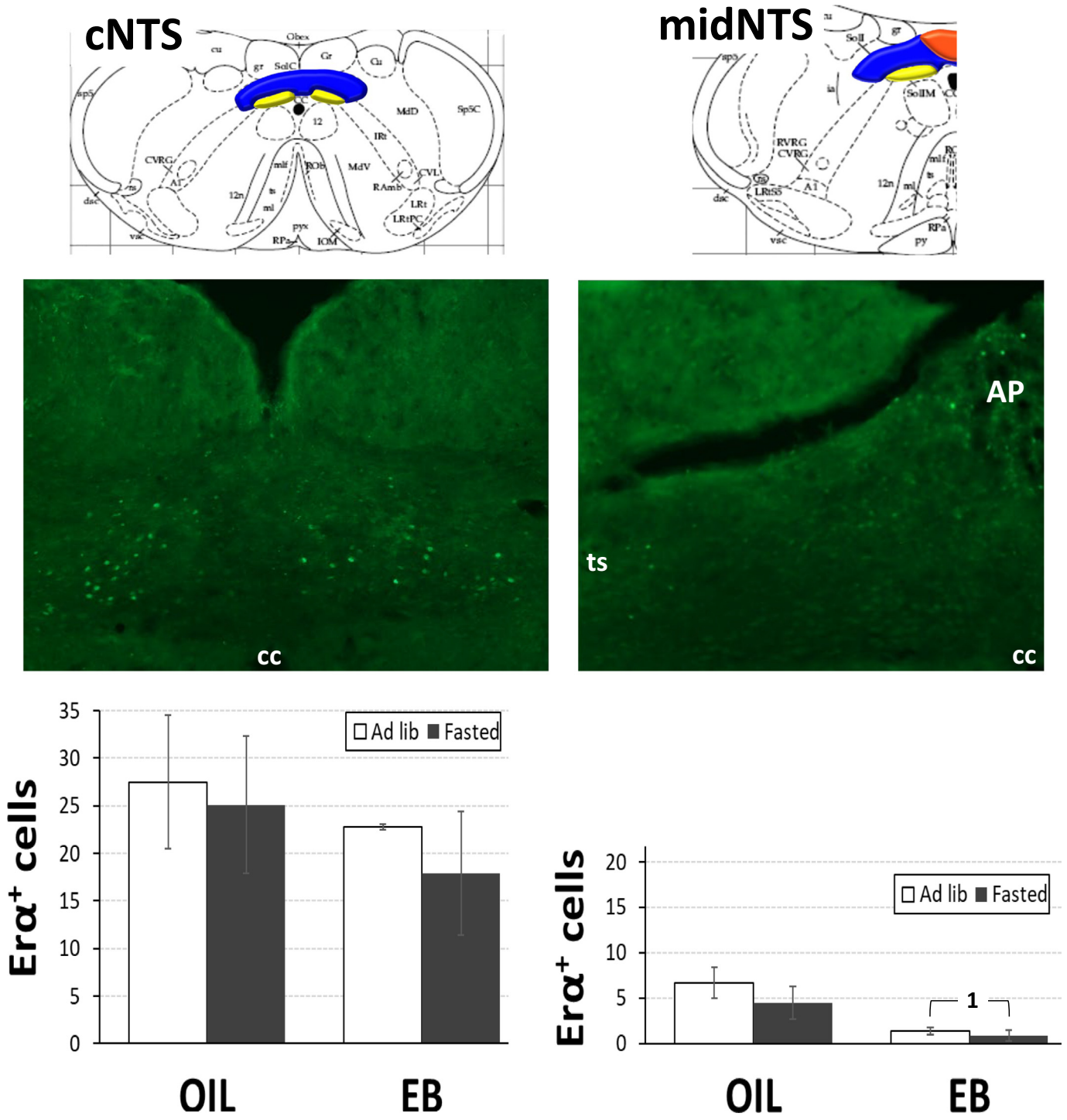


Fig. 4. ERα immunolabeling in the nucleus of the solitary tract of ovariectomized rats treated with Oil vehicle (OIL) or estradiol benzoate (EB). *Left column: caudal NTS (cNTS)*. Schematic of cNTS (*top*; adapted from [Paxinos and Watson, 1998]), representative photomicrograph of ERα immunolableing (green nuclear staining) in the cNTS (*middle*), and mean numbers of ERα$^+$ cells in the cNTS (*bottom*) of OIL- and EB-treated rats that were fed ad libitum (Ad lib; white bars; OIL n = 2, EB, n = 2) or were Fasted (black bars; OIL, n = 4, EB, n = 3) prior to sacrifice on Day 4 of temporally targeted food restriction (TTFR). *Right column: middle NTS (midNTS)*. Schematic hemisection of midNTS (*top*; adapted from [Paxinos and Watson, 1998]), representative photomicrograph of ERα immunolabeling in a hemisection of the midNTS (*middle*), and mean numbers of ERα$^+$ cells in the midNTS (*bottom*) of OIL- and EB-treated rats that were Ad lib fed (OIL, n = 2; EB, n = 2) or were Fasted (OIL, n = 3; EB, n = 4) prior to sacrifice after TTFR. Blue shading - NTS; yellow shading - dorsal motor nucleus of the vagus; red shading - Area Postrema (AP); ts - tractus solitarius; cc - central canal; scale bars - 100 μm. 1 = significantly less than OIL.

2012) produces neuronal activation in the NTS (Olson et al., 1993; Ceccatelli et al., 1989; Dockray and Burdyga, 2011; Eckel and Geary, 2001; Eckel et al., 2002; Geary and Asarian, 1999). Accordingly, we opted to employ immunohistochemical labeling of the fos protein as a strategy to assess neuronal activation in the NTS in response to consumption of chow during TTFR (Fig. 3). There was little fos immunolabeling in the NTS of EB- or OIL-treated rats that had ad libitum access to chow and therefore ate little during the intake test prior to sacrifice. Similarly, fos immunolabeling in the NTS of rats on TTFR that were Fasted was sparse (Eckel and Geary, 2001), an important finding

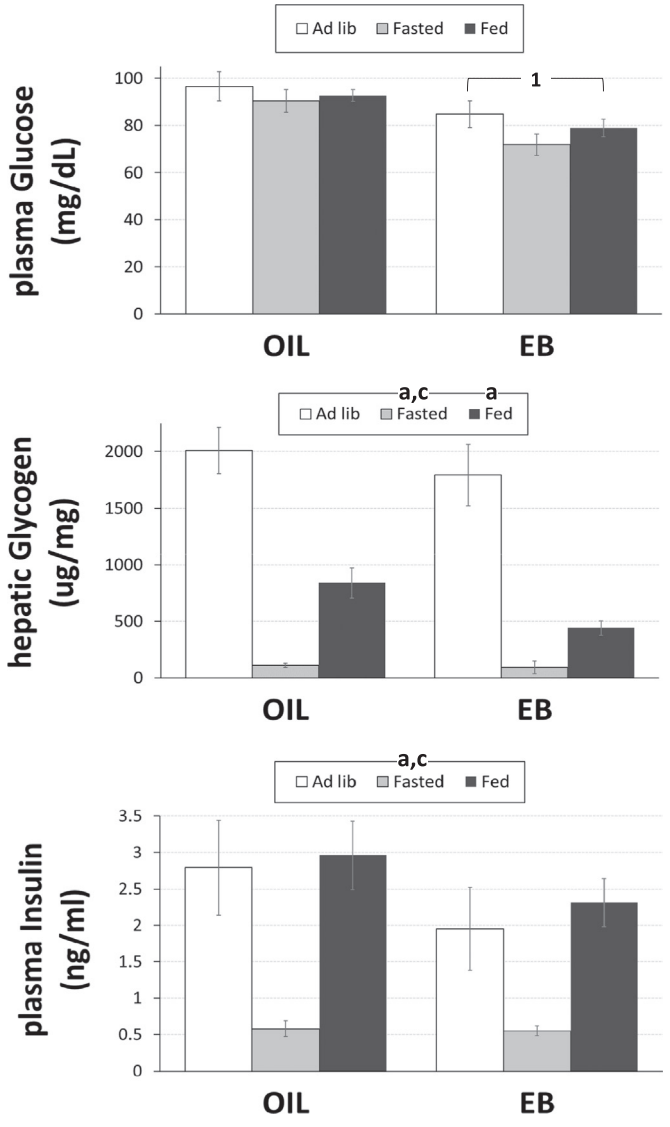


Fig. 5. Carbohydrate metabolites and hormones in ovariectomized rats treated with Oil vehicle (OIL) or estradiol benzoate (EB). *Top*: plasma glucose levels in OIL- and EB-treated rats that were fed ad libitum (Ad lib; white bars; OIL, n = 6; EB, n = 6), or were Fasted (gray bars; OIL, n = 6; EB, n = 6) or Fed (black bars; OIL, n = 6; EB, n = 6) prior to sacrifice on Day 4 of temporally targeted food restriction (TTFR). *Middle:* hepatic glycogen levels in OIL- and EB-treated rats that were Ad lib fed (OIL, n = 4; EB, n = 4), or were Fasted (OIL, n = 2; EB, n = 3) or Fed (OIL, n = 5; EB, n = 6) prior to sacrifice after TTFR. *Bottom:* plasma insulin levels in OIL- and EB-treated rats that were Ad lib fed (OIL, n = 6; EB, n = 6), or were Fasted (OIL, n = 5; EB, n = 5) or Fed (OIL, n = 6; EB, n = 6) prior to sacrifice after TTFR. a = significantly less than Ad lib; c = significantly less than Fed.

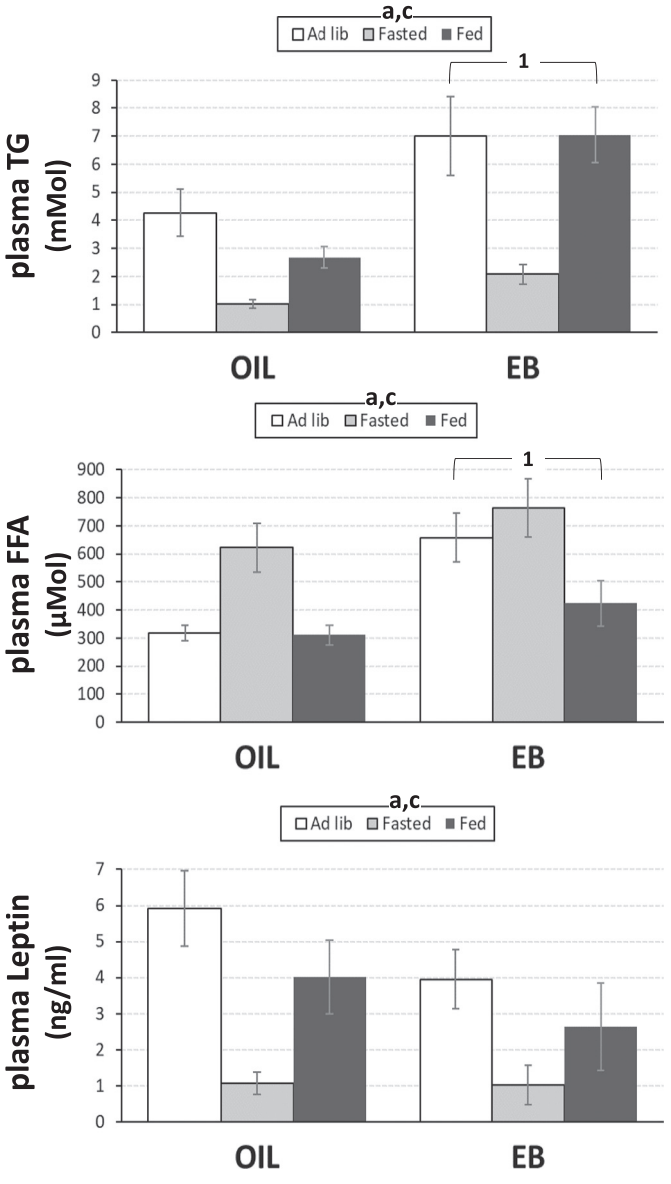


Fig. 6. Lipid metabolites and hormones in ovariectomized rats treated with Oil vehicle (OIL) or estradiol benzoate (EB). *Top*: plasma triglyceride (TG) levels in OIL- and EB-treated rats that were fed ad libitum (Ad lib; white bars; OIL, n = 6; EB, n = 5), or were Fasted (gray bars; OIL, n = 6; EB, n = 6) or Fed (black bars; OIL, n = 6; EB, n = 6) prior to sacrifice on Day 4 of temporally targeted food restriction (TTFR). *Middle:* plasma free fatty acid (FFA) levels in OIL- and EB-treated rats that were Ad lib fed (OIL, n = 6; EB, n = 6), or were Fasted (OIL, n = 6; EB, n = 6) or Fed (OIL, n = 6; EB, n = 6) prior to sacrifice after TTFR. *Bottom:* plasma leptin levels in OIL- and EB-treated rats that were Ad lib fed (OIL, n = 6; EB, n = 6), or were Fasted (OIL, n = 5; EB, n = 6) or Fed (OIL, n = 6; EB, n = 6) prior to sacrifice after TTFR. a = significantly less than Ad lib; c = significantly less than Fed.

which suggests that the TTFR itself did not elicit neuronal activation in the NTS. In contrast, fos immunolabeling in the NTS of rats on TTFR that were Fed before sacrifice was elevated, with no difference between hormone treatments. This finding further supports the idea that EB- and OIL-treated rats are less sensitive to inhibitory gastric distension signaling during TTFR.

In contrast to these findings, Eckel and colleagues (Eckel and Geary, 2001) reported that, when meal size was controlled after previous ad libitum feeding so that EB- and OIL-treated rats ate the same amount, there was *greater* fos labeling in in the NTS of EB-treated rats. It should be noted that while EB- and OIL-treated rats also consumed similar amounts of food during TTFR, we restricted the time rats were permitted to eat, rather than restricting the amount of chow provided. This strategy produced very large intakes in a comparatively short period of time, suggesting that fos immunolabeling in the NTS of EB- and OIL-treated rats may be attributable to activation in response to maximal input from gastric vagal afferents. Nonetheless, extreme gastric stretch due to consumption of large amounts of chow would increase vagal signaling to the CNS and, ultimately, would be sufficient to terminate feeding (Daly et al., 2011; Kentish et al., 2012). We cannot rule out the possibility that such large intakes produced maximal fos activation in the NTS of both OIL- and EB-treated rats. However, both the sensitivity (Kentish et al., 2012) and the phenotype of gastric vagal afferents (Dockray and Burdyga, 2011) depend on feeding condition and the

Table 2
Hepatic and adipose triglyceride (TG) levels in ovariectomized rats treated with OIL vehicle (OIL) or estradiol benzoate (EB). Rats were fed ad libitum throughout (Ad lib) or were or were subjected to temporally-targeted food restriction (TTFR) during the third week. Some rats on TTFR were fasted prior to sacrifice on Day 4 (Fasted); others were fed and sacrificed 2 h later (Fed). Numbers in parentheses indicate numbers of rats.

	OIL	EB
Hepatic TG (mg/ng tissue)		
Ad lib	85.1 ± 5.6 (3)	82.5 ± 19.0 (5)
Fasted	111.0 ± 11.8 (5)	79.9 ± 13.5 (5)
Fed	100.3 ± 30.5 (4)	68.1 ± 12.0 (6)
Adipose TG (mg/ng tissue)		
Ad lib	490.8 ± 99.0 (4)	525.8 ± 116.6 (4)
Fasted	460.6 ± 51.5 (6)	406.4 ± 81.6 (5)
Fed	500.3 ± 49.0 (4)	400.4 ± 84.7 (4)

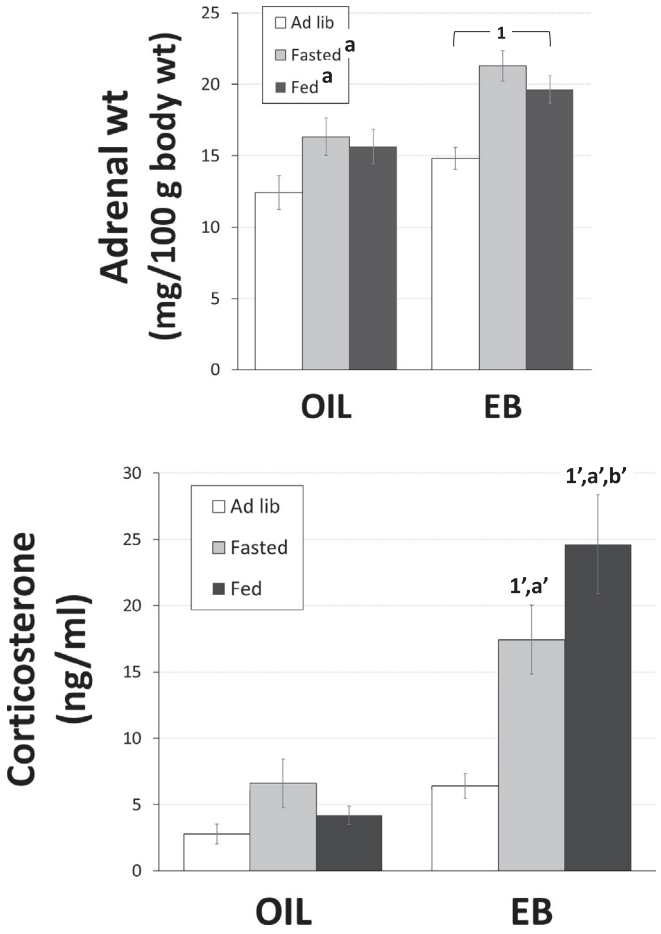


Fig. 7. Adrenal weights and corticosterone levels in ovariectomized rats treated with Oil vehicle (OIL) or estradiol benzoate (EB). *Top*: adrenal weights in OIL- and EB-treated rats that were fed ad libitum (Ad lib; white bars; OIL, n = 6; EB, n = 6), or were Fasted (gray bars; OIL, n = 5; EB, n = 5) or Fed (black bars; OIL, n = 5; EB, n = 6) prior to sacrifice on Day 4 of temporally targeted food restriction (TTFR). *Bottom:* plasma corticosterone levels in OIL- and EB-treated rats that were Ad lib fed (OIL, n = 4; EB, n = 6), or were Fasted (OIL, n = 5; EB, n = 4) or Fed (OIL, n = 5; EB, n = 5) prior to sacrifice after TTFR. a = significantly greater than Ad lib; 1′ = significantly less than corresponding OIL group; a' = significantly greater corresponding Ad lib fed group; b' = significantly greater than corresponding Fasted group.

amounts of food consumed, raising the intriguing possibility that despite similar numbers of fos-positive neurons in the NTS of OIL- and EB-treated rats on TTFR, different populations may be activated by large eating bouts, depending on specific afferents that are activated.

Estradiol effects on feeding under ad libitum feeding conditions are mediated in part by estradiol binding to the α subtype of estrogen receptor in the NTS (Thammacharoen et al., 2008). We saw no differences in ERα immunolabeling in the cNTS as a result of feeding condition or hormone treatment (Fig. 4), whereas numbers of ERα positive cells in the midNTS were greater in EB-treated rats. Thus, there was no differential effect of TTFR on ERα numbers in the NTS. Although ERα and fos labeling were not done in the same tissue sections because the primary antibodies were raised in the same animal, we found less ERα labeling than fos labeling in both the cNTS and midNTS. This is particularly notable in the midNTS, in which scant ERα labeling was observed. This observation that more neurons were activated by eating during TTFR than there were neurons containing ERα suggests that ERα in the NTS play a minor role control of feeding during TTFR. However, it should be noted that ERα *numbers* may not reflect affinity of the receptors to bind estradiol. Thus, we cannot rule out a role for ERα in the control of feeding during TTFR, particularly given the broad distribution of ERα throughout the CNS.

Together, these findings suggest that, unlike EB effects during ad libitum conditions, during TTFR, neither food intake nor the detection of meal-induced inhibitory signals at the level of the NTS is affected by EB. However, although neural activation was not quantitatively different, different types of neurons may be activated. If this is the case, the effect is unlikely to be attributable to ERα-bearing neurons in the NTS which were not affected by TTFR. These interpretations rest on the assumption that the ingested chow is emptied from the stomach at comparable rates. In other words, although food intake and fos immunolabeling in the NTS were similar between OIL- and EB-treated rats during TTFR, it is possible that gastric emptying was different. If so, the degree of gastric distension may not have been comparable, and consistent with this idea, estradiol decreases emptying of a modest gastric load (Chen et al., 1995). However, we found no differences between EB- and OIL-treated rats in the weight of stomach contents with TTFR (Table 2), suggesting there are no differences in gastric distension signals or processing of food through the stomach during TTFR. Nonetheless, even during TTFR, EB effects on body weight persisted.

4.3.2. Estradiol effects on metabolism

Food restriction and dieting are known to reduce metabolism and metabolic rate (Benton and Young, 2017), whereas estradiol has been reported to increase metabolism and metabolic rate (Wade and Gray, 1979), as well as locomotor activity (e.g., Eckel et al., 2000). However, most of these latter studies have been conducted during ad libitum feeding conditions. If estradiol effects on metabolism persist during TTFR, it could contribute to estradiol-mediated differences in body weight we observed during TTFR. Accordingly, we investigated effects of EB treatment on carbohydrate and fat metabolism, focusing on hormones and primary metabolites associated with carbohydrate and fat metabolism.

4.3.2.1. Carbohydrate metabolism. Circulating levels of insulin, the major regulator of glucose levels in carbohydrate metabolism, were not different in OIL-treated and EB-treated rats (Fig. 5), but were significantly different between feeding conditions. Specifically, insulin levels in OIL-treated and EB-treated rats that were fed Ad lib approximated physiological values, as also has been reported in other studies (Eringa et al., 2002), with a trend toward lower levels in EB-treated rats. Insulin levels in rats on TTFR that were Fasted were reduced as expected, given the decrease in glucose influx during TTFR with fasting. In contrast, insulin was restored to ad libitum levels within the short (1 h) time of refeeding in both OIL- and EB-treated rats, presumably because of increased glucose influx. Thus, the hormonal regulator of glucose homeostasis, insulin, was not affected by EB-treatment during TTFR. We also measured plasma glucose in OIL- and EB-treated rats, and found that glucose levels were significantly less in

EB-treated rats, independent of feeding condition. This decrease in plasma glucose with EB treatment has been previously reported (Mandour et al., 1977); however, glucose was not decreased to the point of hypoglycemia, likely owing to the tight regulation of this is critical metabolite. Nonetheless, it is possible that the lower glucose levels in EB-treated rats reflects an overall increase in metabolic rate that persists during TTFR. An alternative explanation for these results is that there is impaired glycogenesis in EB-treated rats that leads to differences in glucose storage as glycogen; however, there were no EB-mediated differences in liver glycogen. Certainly, glycogen was reduced in both EB- and OIL-treated rats on TTFR that were not permitted to eat prior to sacrifice, and utilization of these stores would be expected to maintain plasma glucose levels during TTFR. Interestingly, hepatic glycogen levels began to increase within 1 h after eating in both EB- and OIL-treated rats, and though the increase in EB-treated rats was somewhat blunted, the difference was not significant.

Thus, differences in carbohydrate metabolism do not appear to be an important factor in the reduced body weight of EB-treated rats during TTFR. Nonetheless, it should be noted that all measurements were taken 1 h after rats were fed; consequently, additional studies will be necessary to determine if the slight difference in liver glycogen, in conjunction with the lower overall plasma glucose levels, indicate a meaningful difference in carbohydrate metabolism in EB-treated rats during TTFR that may contribute to differences in body weight.

4.3.2.2. Lipid metabolism. Leptin is released from adipocytes and is proportional to body adiposity. Despite decreased body weight in EB-treated rats during both ad libitum conditions and TTFR, EB did not affect circulating leptin (Fig. 6). There was a tendency toward reduced plasma leptin in EB-treated rats that were fed ad libitum, but these levels were within the physiological range previously reported (Watanobe et al., 1999). Leptin levels in rats on TTFR that were Fasted prior to sacrifice were profoundly reduced, presumably because of the decreased adiposity in fasted animals but, interestingly, leptin levels were restored nearly to ad libitum levels within a short time after eating. This effect may be attributable to the intraluminal source of leptin (Bado et al., 1998; Cinti et al., 2000; Sobhani et al., 2000) and suggests that in addition to leptin from adipose tissue which serves as a long-term adiposity signal, a rapidly-occurring increase in leptin originating from the GI tract may play a role in feeding and/or body weight regulation. In any case, neither the intra-luminal nor the adipose leptin was affected by estradiol in these studies.

Previous studies of women and rats (Spellacy et al., 1973; Kim and Kalkhoff, 1975) report that estradiol elevates plasma TGs after fasting, suggesting a role for estradiol in fat metabolism. We found that plasma TGs in EB-treated rats were greater in all feeding conditions compared to OIL-treated rats. In addition, plasma TGs were slightly less in rats on TTFR that were Fasted prior to sacrifice, compared to both ad libitum fed rats and rats on TTFR that were Fed prior to sacrifice. These data suggest that TGs are being utilized for energy during fasted conditions in both EB- and OIL-treated rats; however, plasma TGs increased within 1 h after eating during TTFR, an effect that was particularly pronounced in EB-treated rats. Neither adipose nor hepatic TGs were affected by TTFR, suggesting that these sources of energy are not utilized during TTFR. Nonetheless, adipose TGs in EB-treated rats on TTFR tended to be less than those in EB-treated rats that were fed Ad lib, which may suggest a slower rate of uptake TGs into adipose tissue. This idea, which is consistent with increased plasma TGs in EB-treated rats, may be due to the slight decrement in insulin levels with EB treatment that likely reduces lipolysis. Alternatively, lipolysis and mobilization of FFAs may be accelerated in EB-treated rats during TTFR. Consistent with this idea, plasma FFA were elevated in EB-treated rats, independent of feeding condition. In addition, plasma FFA increased in rats on TTFR that were fasted prior to sacrifice, an effect that was more pronounced in OIL-treated rats, and may derive from the breakdown of TGs in circulation and, possibly in adipose, as well.

4.3.3. Corticosterone and adrenals

Both OIL- and EB-treated rats on TTFR had increased adrenal weights (Fig. 7), observations that are suggestive of activation of the hypothalamus-pituitary-adrenal (HPA) axis. However, although circulating corticosterone levels were comparable in OIL- and EB-treated rats that were fed ad libitum, corticosterone increased after TTFR only in EB-treated rats, an effect that was enhanced by eating. It is possible that interoceptive signals associated with fasting or the rapid consumption of large amounts of food activate the HPA axis (see Maniscalco et al., for review) in EB-treated but not Oil-treated rats. Consistent with this idea, estradiol is associated with enhanced corticosterone release in response to a variety of stressors (Chang et al., 2002; White-Welkley et al., 1995), as well as with hypertrophy of the adrenals (Fig. 7; Saruhan and Ozdemir, 2005). Alternatively, although typically thought of as a stress hormone that is involved in 'fight or flight' responses, corticosterone also affects carbohydrate, fat and protein metabolism, functions that are particularly important in the face of TTFR. Thus, in EB-treated rats, increased corticosterone may be responsible for differences in lipid metabolism that contribute to the persistently decreased body weight in EB-treated rats on TTFR. Further studies will be necessary to address this idea, as well as to determine whether these effects persist during access to high-fat or high-sugar chow that more closely approximates Western diets.

5. Summary

In summary, EB treatment causes reduced body weight that persists during TTFR despite an increase in food intake to levels not different to those consumed by OIL-treated rats. This separation of estradiol effects on eating behavior from those on body weight contrasts with previous reports of effects observed during ad libitum feeding (Asarian and Geary, 2006; Blaustein and Wade, 1976; Bonavera et al., 1994; Butera et al., 2010). Moreover, TTFR also appears to reverse estradiol effects on gastric emptying (Chen et al., 1995) and CNS processing of signals from the gastrointestinal tract (Clegg et al., 2006). Nonetheless, we observed slight differences in a number of peripheral metabolic hormones and metabolites that, in conjunction with estradiol effects on locomotor activity and basal metabolic rate (Wade and Gray, 1979; Eckel et al., 2000) may explain the persistent body weight reduction. In this regard, a study of male rats on a TTFR protocol (Hollifield and Parson, 1962a, 1962b) is instructive. The authors report that the large load of nutrients taken in during TTFR were not stored as glycogen, but that newly synthesized lipids were presumably used as fuels, a shift that was proposed to be due to adapting to TTFR. This adaptation involved catabolizing fat as the major energy source and limiting glucose utilization to the small amounts required by the CNS (Hollifield and Parson, 1962a, 1962b). These findings suggest that EB-treated rats may use a similar strategy during TTFR, but with slight differences that prevent better maintenance of body weight during conditions of limited fuel availability. Thus, estradiol-induced enhancement of corticosterone release during TTFR may increase basal metabolic rate (Damjanovic et al., 2009) resulting in greater and more efficient energy utilization, and thus, decreased body weight.

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References

Asarian, L., Geary, N., 2002. Cyclic estradiol treatment normalizes body weight and restores physiological patterns of spontaneous feeding and sexual receptivity in ovariectomized rats. Horm. Behav. 42, 461–471.

Asarian, L., Geary, N., 2006. Modulation of appetite by gonadal steroid hormones. Philos Trans Royal Soc London, Biol Sci 361, 12511263.

Asarian, L., Geary, N., 2007. Estradiol enhances cholecystokinin-dependent lipid-induced satiation and activates estrogen receptor-alpha-expressing cells in the nucleus tractus solitarius of ovariectomized rats. Endocrinol 148, 5656–5666.

Bado, A., Levasseur, S., Attoub, S., Kermorgant, S., Laigneau, J.P., Bortoluzzi, M.N., Moizo, L., Lehy, T., Guerre-Millo, M., Le Marchand-Brustel, Y., Lewin, M.J., 1998. The stomach is a source of leptin. Nature 394, 790–793.

Beaudoin, R., Mayer, J., 1953. Food intakes of obese and non-obese women. J AmerDiet Assoc 29, 29–33.

Benton, D., Young, H.A., 2017. Reducing calorie intake may not help you lose body weight. Perspect. Psychol. Sci. 12, 703–714.

Blaustein, J.D., Wade, G.N., 1976. Ovarian influences on the meal patterns of female rats. Physiol. Behav. 17, 201–208.

Bonavera, J.J., Dube, M.G., Kalra, P.S., Kalra, S.P., 1994. Anorectic effects of estrogen may be mediated by decreased neuropeptide-Y release in the hypothalamic paraventricular nucleus. Endocrinol 134, 2367–2370.

Butera, P.C., Wojcik, D.M., Clough, S.J., 2010. Effects of estradiol on food intake and meal patterns for diets that differ in flavor and fat content. Physiol. Behav. 99, 142–145.

Carr, M.C., 2003. The emergence of the metabolic syndrome with menopause. J. Clin. Endocrinol. Metab. 88, 2404–2411.

Ceccatelli, S., Villar, M.J., Goldstein, M., Hokfelt, T., 1989. Expression of c-Fos immunoreactivity in transmitter-characterized neurons after stress. Proc Nat Acad Sci 86, 9569–9573.

Chang, L.L., Kau, M.M., Wun, W.S., Ho, L.T., Wang, P.S., 2002. Effects of fasting on corticosterone production by zona fasiculata-reticularis cells in ovariectomized rats. J. Investig. Med. 50, 86–94.

Chen, T.S., Doong, M.L., Chang, F.Y., Lee, S.D., Wang, P.S., 1995. Effects of sex steroid hormones on gastric emptying and gastrointestinal transit in rats. Amer J Physio 268, G171–G176.

Cinti, S., Matteis, R.D., Pico, C., Ceresi, E., Obrador, A., Maffeis, C., Oliver, J., Palou, A., 2000. Secretory granules of endocrine and chief cells of human stomach mucosa contain leptin. Internat J Obesity Metab Disorders 24, 789–793.

Clegg, D.J., Brown, L.M., Woods, S.C., Benoit, S.C., 2006. Gonadal hormones determine sensitivity to central leptin and insulin. Diabetes 55, 978–987.

Contreras, R.J., Beckstead, R.M., Norgren, R., 1982. The central projections of the trigeminal, facial, glossopharyngeal and vagus nerves: an autoradiographic study in the rat. J Auton Nervous Sys 6, 303–322.

Cummings, D.E., Overduin, J., 2007. Gastrointestinal regulation of food intake. J. Clin. Invest. 117, 13–23.

Curtis, K.S., 2015. Estradiol and osmolality: behavioral responses and central pathways. Physiol. Behav. 152, 422–430.

Daly, D.M., Park, S.J., Valinsky, W.C., Beyak, M.J., 2011. Impaired intestinal afferent nerve satiety signalling and vagal afferent excitability in diet induced obesity in the mouse. J. Physiol. 589, 2857–2870.

Damjanovic, S.S., Stojic, R.V., Lalic, N.M., Jotic, A.Z., Macut, D.P., Ognjanovic, S.I., Petakov, M.S., Popovic, B.M., 2009. Relationship between basal metabolic rate and cortisol secretion throughout pregnancy. Endocrine 35, 262–268.

Dockray, G.J., Burdyga, G., 2011. Plasticity in vagal afferent neurones during feeding and fasting: mechanisms and significance. Acta Physiol. 201, 313–321.

Dole, V.P., Schwartz, I.L., Thaysen, J.H., Thorn, N.A., Silver, L., 1954. Treatment of obesity with a low protein calorically unrestricted diet. Amer J Clin Nutr 2, 381–391.

Eckel, L.A., 2011. The ovarian hormone estradiol plays a crucial role in the control of food intake in females. Physiol. Behav. 104, 517–524.

Eckel, L.A., Geary, N., 1999. Endogenous cholecystokinin's satiating action increases during estrus in female rats. Peptides 20, 451–456.

Eckel, L.A., Geary, N., 2001. Estradiol treatment increases feeding-induced c-Fos expression in the brains of ovariectomized rats. Amer J Physiol Regu, Integrat Comp Physiol 281, R738–R746.

Eckel, L.A., Langhans, W., Kahler, A., Campfield, L.A., Smith, F.J., Geary, N., 1998. Chronic administration of OB protein decreases food intake by selectively reducing meal size in female rats. Amer J Physiol 275, R186–R193.

Eckel, L.A., Houpt, T.A., Geary, N., 2000. Spontaneous meal patterns in female rats with and without access to running wheels. Physiol. Behav. 70, 397–405.

Eckel, L.A., Houpt, T.A., Geary, N., 2002. Estradiol treatment increases CCK-induced c-Fos expression in the brains of ovariectomized rats. Amer J Physiol Regu, Integrat Comp Physiol 283, R1378–R1385.

Eringa, E.C., Stehouwer, C.D., Merlijn, T., Westerhof, N., Sipkema, P., 2002. Physiological concentrations of insulin induce endothelin-mediated vasoconstriction during inhibition of NOS or PI3-kinase in skeletal muscle arterioles. Cardiovasc. Res. 56, 464–471.

Eshghinia, S., Mohammadzadeh, F., 2013. The effects of modified alternate-day fasting diet on weight loss and CAD risk factors in overweight and obese women. J Diabetes Metab Disorders 12, 4.

Flegal, K.M., Carroll, M.D., Kit, B.K., Ogden, C.L., 2012a. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. JAMA 307, 491–497.

Flegal, K.M., Carroll, M.D., Ogden, C.L., 2012b. Effects of trimming weight-for-height data on growth-chart percentiles. Amer J Clin Nutr 96, 1051–1055.

Geary, N., Asarian, L., 1999. Cyclic estradiol treatment normalizes body weight and test meal size in ovariectomized rats. Physiol. Behav. 67, 141–147.

Graves, N.S., Hayes, H., Fan, L., Curtis, K.S., 2011. Time course of behavioral, physiological, and morphological changes after estradiol treatment of ovariectomized rats. Physiol. Behav. 103, 261–267.

Hollifield, G., Parson, W., 1962a. Metabolic adaptations to a "stuff and starve" feeding program. I. Studies of adipose tissue and liver glycogen in rats limited to a short daily feeding period. J. Clin. Invest. 41, 245–249.

Hollifield, G., Parson, W., 1962b. Metabolic adaptations to a "stuff and starve" feeding program. II. Obesity and the persistence of adaptive changes in adipose tissue and liver occurring in rats limited to a short daily feeding period. J. Clin. Invest. 41, 250–253.

Hrupka, B.J., Smith, G.P., Geary, N., 2002. Hypothalamic implants of dilute estradiol fail to reduce feeding in ovariectomized rats. Physiol. Behav. 77, 233–241.

Hubert, M.F., Laroque, P., Gillet, J.P., Keenan, K.P., 2000. The effects of diet, ad libitum feeding, and moderate and severe dietary restriction on body weight, survival, clinical pathology parameters, and cause of death in control Sprague-Dawley rats. Toxicol. Sci. 58, 195–207.

Jones, A.B., Curtis, K.S., 2009. Differential effects of estradiol on drinking by ovariectomized rats in response to hypertonic NaCl or isoproterenol: implications for hyper- vs. hypo-osmotic stimuli for water intake. Physiol. Behav. 98, 421–426.

Kentish, S., Li, H., Philp, L.K., O'Donnell, T.A., Isaacs, N.J., Young, R.L., Wittert, G.A., Blackshaw, L.A., Page, A.J., 2012. Diet-induced adaptation of vagal afferent function. J. Physiol. 590, 209–221.

Kim, H.J., Kalkhoff, R.K., 1975. Sex steroid influence on triglyceride metabolism. J. Clin. Invest. 56, 888–896.

Krause, E.G., Curtis, K.S., Stincic, T.L., Markle, J.P., Contreras, R.J., 2006. Oestrogen and weight loss decrease isoproterenol- induced Fos immunoreactivity and angiotensin type 1 mRNA in the subfornical organ of female rats. J. Physiol. 573, 251–262.

Lovejoy, J.C., Sainsbury, A., 2009. Sex differences in obesity and the regulation of energy homeostasis. Obes. Rev. 10, 154–167.

Mandour, T., Kissebah, A.H., Wynn, V., 1977. Mechanism of oestrogen and progesterone effects on lipid and carbohydrate metabolism: alteration in the insulin: glucagon molar ratio and hepatic enzyme activity. European J Clin Invest 7, 181–187.

Maniscalco, J.W., Kreisler, A.D., Rinaman, L., 2013. Satiation and stress-induced hypophagia: examining the role of hindbrain neurons expressing prolactin-releasing peptide or glucagon-like peptide 1. Front. Neurosci. https://doi.org/10.3389/fnins.2012.00199.

Meckling, K.A., Gauthier, M., Grubb, R., Sanford, J., 2002. Effects of a hypocaloric, low-carbohydrate diet on weight loss, blood lipids, blood pressure, glucose tolerance, and body composition in free-living overweight women. Canadian J Physiol Pharmacol 80, 1095–1105.

Moran, T.H., 2006. Gut peptide signaling in the controls of food intake. Obesity 14 (Suppl. 5), 250S–253S.

Musatov, S., Chen, W., Pfaff, D.W., Mobbs, C.V., Yang, X.J., Clegg, D.J., Kaplitt, M.G., Ogawa, S., 2007. Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to to metabolic syndrome. Proc Nat Acad Sci 104, 2501–2506.

Olson, B.R., Freilino, M., Hoffman, G.E., Stricker, E.M., Sved, A.F., Verbalis, J.G., 1993. c-Fos expression in rat brain and brainstem nuclei in response to treatments that alter food intake and gastric motility. Molec Cell Neurosci 4, 93–106.

Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates. Academic, New York.

Rivera, H.M., Santollo, J., Nikonova, L.V., Eckel, L.A., 2012. Estradiol increases the anorexia associated with increased 5- HT(2C) receptor activation in ovariectomized rats. Physiol. Behav. 105, 188–194.

Rossner, S., 1998. Effects of 46 weeks of very-low-calorie-diet treatment on weight loss and cardiac function–a case report. Obesity Res 6, 462–463.

Sakurazawa, N., Mano-Otagiri, A., Nemoto, T., Shibasaki, T., 2013. Effects of intracerebroventricular ghrelin on food intake and Fos expression in the arcuate nucleus of the hypothalamus in female rats vary with estrous cycle phase. Neurosci. Lett. 541, 204–208.

Santollo, J., Eckel, L.A., 2008. Estradiol decreases the orexigenic effect of neuropeptide Y, but not agouti-related protein, in ovariectomized rats. Behav. Brain Res. 191, 173–177.

Santollo, J., Torregrossa, A.M., Eckel, L.A., 2011. Estradiol acts in the medial preoptic area, arcuate nucleus, and dorsal raphe nucleus to reduce food intake in ovariectomized rats. Horm. Behav. 60, 86–93.

Santollo, J., Marshall, A., Daniels, D., 2013. Activation of membrane-associated estrogen receptors decreases food and water intake in ovariectomized rats. Endocrinol 154, 320–329.

Saruhan, B.G., Ozdemir, N., 2005. Effect of ovariectomy and of estrogen treatment on the adrenal gland and body weight in rats. Saudi Med J 26, 1705–1709.

Sobhani, I., Bado, A., Vissuzaine, C., Buyse, M., Kermorgant, S., Laigneau, J.P., Attoub, S., Lehy, T., Henin, D., Mignon, M., Lewin, M.J., 2000. Leptin secretion and leptin receptor in the human stomach. Gut 47, 178–183.

Spellacy, W.N., Buhi, W.N., Birk, S.A., Cabal, R., 1973. The effects of estrogens, progestogen, oral contraceptives, and intrauterine devices on fasting triglycerides and insulin levels. Ferti Steril 24, 178–184.

Stunkard, A.J., Grace, W.J., Wolff, H.G., 1955. The night-eating syndrome; a pattern of food intake among certain obese patients. Amer J Med 19, 78–86.

Thammacharoen, S., Lutz, T.A., Geary, N., Asarian, L., 2008. Hindbrain administration of estradiol inhibits feeding and activates estrogen receptor-alpha-expressing cells in the nucleus tractus solitarius of ovariectomized rats. Endocrinol 149, 1609–1617.

Wade, G.N., 1975. Some effects of ovarian hormones on food intake and body weight in female rats. J Comp Physiol Psychol 88, 183–193.

Wade, G.N., Gray, J.M., 1979. Gonadal effects on food intake and adiposity: a metabolic hypothesis. Physiol. Behav. 22, 583–593.

Watanobe, H., Suda, T., Wikberg, J.E., Schioth, H.B., 1999. Evidence that physiological levels of circulating leptin exert a stimulatory effect on luteinizing hormone and prolactin surges in rats. Biochem. Biophys. Res. Commun. 263, 162–165.

White-Welkley, J.E., Bunnell, B.N., Mougey, E.H., Meyerhoff, J.L., Dishman, R.K., 1995. Treadmill exercise training and estradiol differentially modulate hypothalamic-pituitary-adrenal cortical responses to acute running and immobilization. Physiol. Behav. 57, 533–540.