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Decreased gastric mechanodetection, but preserved gastric emptying, in CCK-1 receptor deficient OLETF rats

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Abstract

Obese CCK-1 receptor lacking Otsuka Long Evans Tokushima fatty (OLETF) rats are hyperphagic relative to control, non-mutant Long Evans Tokushima Otsuka (LETO) rats. This study sought to assess whether overeating observed in OLETF rats is associated with changes in gastric emptying rates or detection of gastric volume. We performed experiments in both 12 wk and 29 wk old OLETF and LETO rats in order to address possible alterations in gastric functions during development of increased body weight and blood glucose abnormalities in OLETF rats. Gastric emptying of a 5-g solid chow test meal was not significantly different between strains at either 1, 2, or 4 hrs post meal. When tested with *ad libitum* access to chow, there were no significant differences in gastric emptying between strains at any time period despite OLETF consuming significantly more chow than LETO rats. Similar to solid food, 5-min gastric emptying of a 5 ml isosmotic and hyperosmotic saline or glucose load was not significantly different between strains. When the stomach was distended with a 15 ml semi-solid chow load there was no significance difference in emptying at either 1 or 2 hrs. No significant differences in gastric emptying were detected between 12 and 29 wk old rats, under any conditions. Both young and old OLETF rats, however, reduced sham intake significantly less compared to LETO during a brief period of gastric distension by 5 ml or 10 ml balloon inflation. Finally, OLETF rats showed decreased Fos expression in the nucleus of the solitary tract relative to LETO rats following 8 ml gastric distension. These findings demonstrate that OLETF rats do not express deficits in controlling gastric emptying rates, however, they exhibit decreased behavioral and vagal responsiveness to gastric distension that may contribute to increased meal size in these animals.

Keywords

CCK; hyperphagia; CCK-1 receptor; c-Fos; gastric distension; gastric emptying; sucrose; sham feeding

INTRODUCTION

The Otsuka Long-Evans Tokushima Fatty Rat (OLETF) is an outbred mutant strain of the Long Evans Tokushima Otsuka rat (LETO), which lacks CCK-1 receptor expression entirely due to a spontaneous 6.8 kB mutation spanning the promoter and first two exon regions of the CCK-1 receptor gene (45). In addition to the use of these animals as a model of insulin

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resistance, due to their natural manifestation of hyperglycemia and Non-Insulin Dependent Diabetes Mellitus (NIDDM) relative to age-matched LETO rats (16), OLETF rats are also currently under investigation as a model of obesity. OLETF rats exhibit an increased rate of weight gain relative to controls across their life span, with a marked elevation in body weight seen as early as 2-4 postnatal day (2, 26, 42). It is also known that OLETF rats are hyperphagic via increased meal size. This behavior has been attributed not only to deficits in CCK-related satiation mechanisms (26), but also to intestinal nutrient satiation signaling (5, 7, 42) and more recently, enhanced oral responsiveness to palatable stimuli (7, 13).

Gastric emptying is one mechanism through which CCK functions to reduce food intake (28). In non-mutant animals, CCK-1 receptor antagonists have been shown to attenuate inhibition of gastric emptying by both exogenous CCK administration (12, 27), as well as gastric nutrient loads (25, 48). The mechanism for both of these effects appears to be largely mediated through CCK-1 receptor activation of vagal afferents (37, 39). In addition, Schwartz et al. (38) observed enhanced and amplified vagal afferent activity due to the presence of a gastric load when CCK was simultaneously administered, implying that CCK and its receptors contribute to mechanoreception of gastric contents. In this regard, the CCK-1 receptor deficient OLETF rat offers an ideal model to study gastric functions known to be mediated by CCK-1 receptors and at the same time may contribute to the understanding of increased meal-size in this strain.

Therefore, in the present study, we investigated whether in addition to defects in intestinal nutrient and CCK satiation signaling deficits, OLETF rats also express impairments in CCK-mediated satiation via defective gastric contributions to meal termination. Accordingly, in the first series of experiments we assessed gastric emptying rates of solid and liquid foods in OLETF and LETO rats using various feeding conditions and gastric load manipulations.

Diminished detection of gastric volume, due to a lack of gastric vagal afferent CCK-1 receptor activation (38), may also lead to increased meal size in the OLETF rat. To examine this possibility, the second series of experiments examined the effects of stomach distension on sham feeding and gastric emptying. Finally, gastric distension has been shown to excite specific regions of the dorsal medulla controlling for meal size via vagal activation (38). Quantification of the immediate-early gene product Fos has been used as an indicator of neuronal activation stemming from vagal afferent transmission of such signaling (10, 21, 49). Thus, the final experiment examined whether or not OLETF and LETO rats differ in neuronal responsiveness to gastric distension by assessing Fos expression in select areas of the hindbrain. To control for possible confounding gastroparetic effects resultant from NIDDM development (17) in OLETF rats, experiments were performed in two age groups (12 and 29 wks) representing non-diabetic and pre-diabetic OLETF animals unless otherwise indicated.

METHODS

Subjects

Male OLETF and LETO rats were obtained as a generous gift of the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. Twelve (age1: 423 ± 12.0 g and 315 ± 6.0 g for OLETF and LETO rats, respectively) and twenty-nine (age2: 557 ± 17.0 g and 450 ± 6.4 g for OLETF and LETO rats, respectively) wk old rats were used in these experiments. Separate groups of rats were used for each set of experiments within both age groups tested, unless otherwise indicated. All animals were individually housed in mesh-floored, stainless-steel hanging cages in a temperature-controlled vivarium while maintained on a constant 12:12-h light-dark cycle (lights on at 0600). Rats were handled daily for a minimum of one week prior to the onset of experimental procedures. Tap water and pelleted rat chow (Purina

5001) were available ad libitum throughout the experiments. All protocols used were approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

Procedures

Gastric Fistula Implantation: Rats designated for solid food emptying studies were fasted overnight and anesthetized prior to surgery via intramuscular injection with 1.0 ml/kg of a mixture of ketamine HCl (100.0 mg/ml), xylazine (20.0 mg/ml), and acepromazine maleate (10.0 mg/ml), obtained from Burns Veterinary Supply, Rockville Centre, NY and surgically implanted with chronic gastric fistulae according to Yox and Ritter (50). The inner flange of a gastric fistula [stainless steel; 13 mm length; 6 mm (ID); 8 mm (OD)] was inserted through the ventral wall of the non-glandular portion of the stomach near the greater curvature and subsequently secured with a purse string suture. A piece of Marlex mesh was centered to adhere flush with the outer flange of the fistula. The non-flanged end of the fistula was then externalized through a left paramedian abdominal incision. A removable stainless steel screw inserted into the fistula blocked access to the stomach lumen between experiments. The peritoneum and abdominal muscles were simultaneously sealed with absorbable sutures post-implantation. The abdominal skin incision was closed using wound clips, which were removed 7 days post-surgery. Rats were allowed a minimum of one week to recover from fistula implantation surgery before experimentation.

Gastric emptying of a 5 g solid chow meal in OLETF and LETO rats: Due to the known hyperphagic phenotype of the OLETF rat, this experiment controlled for increased meal size by limiting the size of the test meal to a known amount readily consumed by both OLETF and LETO rats within one hour. Overnight (16 hrs) fasted OLETF and LETO rats (n=6-8 per strain, at both age1 and age2) were presented with one, pre-weighed, 5 g pellet of rat chow for consumption. All rats had no food left in the cage before assessment of emptying. Collected spillage was weighed in order to accurately measure how much of the 5 g meal was consumed. Gastric emptying of the chow consumed was measured at 1, 2, or 4 hrs post-presentation. Before the tests, the stainless steel screws occluding the gastric fistulae were removed, and stomach contents were rinsed with warm tap water by continual flushing until the solution withdrawn was clear of ingested chow particles. Both recovered gastric contents and samples of pelleted chow were dried according to procedures previously established in order to isolate dry matter (DM) content (3). DM emptied (%) was calculated by the following equation: DM emptied (%)= [1 − (dry matter of stomach contents (g)) / (dry matter of food ingested (g))] * 100 (3). Experiments were conducted a minimum of two occasions, every other day.

Gastric emptying of solid chow in OLETF and LETO rats allowed ad-libitum access to food: The rates of gastric emptying within a meal (i.e., when gastric contents accumulate as a result of ongoing ingestion) have been previously shown to greatly exceed the rate of nutrient emptying following meal termination (15, 47). Thus, in the hyperphagic OLETF rat, the greater magnitude of gastric fill due to increased meal size may result in increased emptying relative to LETO controls. To investigate this effect, we next allowed animals free access to chow, in contrast to the previous experiment when they were fed with a restricted amount, for either 1, 2 or 4 hrs. After these periods, gastric emptying was measured. This design examined whether increased food consumption by OLETF rats would increase evacuation of gastric contents. Rats used in the previous experiment (n=6-8 per strain, at both age1 and age2) were also used in this study.

Gastric emptying of liquid solutions in OLETF and LETO rats: Since OLETF rats overconsume not only solid but also liquid foods, the following study examined gastric

emptying of both isosmotic and hyperosmotic nutritive and non-nutritive liquid loads. To do so, we measured gastric emptying of known liquid loads [isosmotic 0.9% saline (150mmol/L), isosmotic 5.5% glucose (308mmol/L), hyperosmotic 2.0% saline (347mmol/L), and hyperosmotic 12.5% glucose (694mmol/L)] in OLETF and LETO rats. Each of the four solutions was tested twice in all rats. During testing, 16-hr overnight fasted rats (n= 6-8 per strain, at both age1 and age2) received a 5 ml volume of load containing 0.006% phenol-red instilled into the rat's stomach via oral gavage. Following a 5 min emptying period, the remaining gastric contents were withdrawn, and the stomach rinsed repeatedly with water until withdraws were void of any visible phenol indicator. Gastric emptying was determined by dye-dilution spectrophotometry from absorption at 550nm as previously described by our laboratory (14). Five minute gastric emptying was determined from the following formula: Liquid emptied (%) = [1 − (phenol red recovered from stomach) / (phenol red in instilled load)] * 100 (3).

Gastric Emptying of semi-solid chow load in OLETF and LETO rats: For each of the prior solid chow gastric emptying experiments, rats freely ingested their gastric "load" prior to measurement of emptying rates. Based on previous reports by Kaplan et al. (15) showing that the rate of oral delivery changes the rate of gastric emptying, we elected to test the rate of gastric emptying following direct gastric infusion of a semi-solid chow emulsion, in the absence of oral stimulation. A relatively large load of a semi-solid chow mixture was chosen in order to examine gastric emptying of a load with a high degree of gastric distension, in addition to nutrient content. Specifically, a 15 ml load of semi-solid (25%, wt/v) chow mixture homogenized in distilled water was directly instilled into the stomach through the gastric fistula. The remaining gastric contents were removed at 1 or 2 hrs following instillation of the gastric load and emptying was determined using the DM method as described above.

Sham feeding of sucrose in response to gastric distension in OLETF and LETO rats: This experiment assessed whether or not OLETF and LETO rats differ in their detection of gastric volume in the absence of post-gastric feedback. To do this, we compared sham feeding of naïve OLETF and LETO rats (n=8 per strain at age1; n=6 per strain at age2) in response to volumetric distension by an intragastric balloon. Following a 16-hr fast, the stainless steel screws occluding the gastric fistulae were removed, and stomach contents were lavaged with warm tap water to ensure minimal gastric volume and distension upon start of sham feeding. Rats were placed into Plexiglas sham feeding boxes and acclimated to the sham feeding procedure by presenting them with 0.3M (10.26%) sucrose for 90 min over several sessions until stable baseline intake was reached (approximately 3-4 sessions on consecutive days). Subsequently, on the tests, the effects of gastric distension on intake were evaluated. Different degrees of gastric distension were administered using an 8-fr Foley catheter (Bardex, Bard inc., Covington, Ga) with an inflatable tip. Before presentation of sucrose, the inflatable end of the catheter was fed through a drainage tube attached to the gastric fistula and advanced 0.5-0.7cm into the lumen of the stomach. The catheter was held in place by a rubber band attached to the external end which prevented movement from the original insertion position. Five minutes later, rats were presented with burettes filled with 0.3M sucrose solution. Ten min post-presentation of sucrose, the catheter was inflated (~20 sec inflation time) with either 5ml or 10ml warmed 0.9% saline for a period of 20 min. After the catheter was deflated at 30 min (~20 sec deflation time), rats were allowed to sham feed sucrose for an additional 60 min in order to detect any compensatory changes in sham intake when the effects of distension were removed. Thus, rats had access to 0.3M sucrose for a total of 90 min. Each distension load was given a minimum of two experimental days, and always bracketed by a non-distension experimental day when rats did not receive any load during sham feeding. These non-distension days served to assess possible baseline intake

shifts due to distension on the previous experimental day. Sham intake was measured to the nearest 0.1 ml every 5 min. In all sham feeding tests gastric drainage was collected in plastic graduated cylinders placed beneath the cages and the volume recorded at experiment termination. In the event that the volume of fluid ingested was greater than the volume of gastric drainage, or if gastric drainage did not occur within 15 s of the start of sham feeding, the data from that subject were discarded on the basis that the gastric fistula was not properly placed or functioning (50).

Analysis of c-Fos expression in the hindbrain of OLETF and LETO rats following gastric distension: A separate group of OLETF and LETO rats were used for analysis of Fos expression in response to gastric distension. Overnight (16hr) food-deprived OLETF and LETO rats were removed from their home cages and placed in Plexiglass sham-feeding boxes as described in the preceding experiment. Twenty min following attachment of the drainage tube, an 8-Fr Foley catheter was inflated with warm tap water as in the previous experiment. Eight rats (n=4 per strain) had their stomachs distended with 8 ml of warmed water for a period of 90 min, while 6 rats (n=3 per strain) underwent all procedures as above except that no inflation of the catheter occurred (sham distension). These methods have been previously described by van de Wall and colleagues with slight modifications (46).

Ninety minutes following the onset of gastric distension, all animals were deeply anesthetized and intracardially perfused using a 0.1M phosphate buffer solution, followed by 4% paraformaldehyde in 0.1M phosphate buffer, (pH 7.4). Whole brains were then removed, subsequently stored for 4 hrs in 4% paraformaldehyde, and finally transferred to 20% sucrose solution for overnight storage. Thirty-μm cryostat cut sections were processed for Fos-like immunohistochemistry (Fos-LI) as previously described (6). Stained brain sections were inspected microscopically and counts of all Fos-LI nuclei were made. The counts were done manually by an individual blinded to the treatments. Fos-LI nuclei were counted bilaterally, in the dorsal vagal complex (DVC) that comprised the nucleus of the solitary tract (NTS), area postrema (AP), area subpostrema (AsP), and dorsal motor nucleus of the vagus (DMV), at six levels of the dorsal hindbrain (−14.30mm, −14.08mm,−13.80mm, −13.68mm, −13.30mm and −13.24mm from bregma) corresponding to plate levels 76-71 according to the stereotaxic atlas of Paxinos and Watson (31). At minimum, three sections per each brain level were analyzed for each rat. The presented data are the average number of Fos-LI cells within or across plate level for each rat and treatment condition.

Determination of stomach weights: OLETF and LETO rats used in experiments at age2 (n=16 per strain) were sacrificed and stomachs harvested after study completion. Briefly, the stomach was exposed via a midline celiotomy, ligated at the pylorus and cardia, resected, and weighed. The resected stomachs were then incised and scraped clean of any food particles. The empty stomachs were blotted to remove excess liquid and weighed (14).

Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT): An oral glucose and insulin tolerance test were performed in a subset of rats (n=7 per strain) within each age group after experimentation. For the OGTT, after a 16hr fast, an oral glucose load (2g/kg) was delivered to each rat orally via latex gavage. For ITT, human regular insulin (0.75 U/kg body weight: Humalin R; Eli Lilly Japan K.K., Kobe, Japan) was administered intraperitoneally (IP) to all rats. For both tests, blood glucose was measured before gavage or pre-injection and at 30, 60, 90, and 120 min post-glucose loading or insulin injection by a standard glucometer (LifeScan, One-Touch Basic). Animals were classified as diabetic if the peak level of plasma glucose was ≥300 mg/dL and a peak glucose level at 120 min > 200 mg/dL (16).

Statistical Analysis—For solid gastric emptying experiments, gastric emptying was analyzed using two way repeated measures analyses of variance (rmANOVA) with strain and time as main factors. Food intake was analyzed using one or two way rmANOVAs where applicable. Liquid gastric emptying was examined using two way rmANOVAs with strain and gastric load as main factors. Gastric emptying of solid and semi-solid chow is presented as percentage dry matter (DM) emptied from the stomach.

For sham feeding/gastric distension experiments, separate two way rmANOVAs were used to calculate effects of distension treatments on individual 5-min intake bins in both OLETF and LETO rats using distension volume and time as main effects. Quantification of Fos-LI nuclei was analyzed by two-way ANOVA with distension treatment (gastric distension vs. sham) and strain as main factors. Blood glucose in OLETF and LETO rats following an OGTT or ITT test were compared using planned t-tests. For all experiments, ANOVA results were subsequently analyzed by Tukey's honestly significant difference (HSD) post-hoc tests when appropriate. All data were expressed as means + SEM. Differences were considered statistically significant if $P<0.05$. Statistical analyses were computed with PC-SAS (version 8.02, SAS Institute, Carey, NC).

RESULTS

Gastric emptying of a 5 g solid chow meal in OLETF and LETO rats

Both OLETF and LETO within the two age groups consumed the entire 5 g chow meal presented, barring spillage. No strain differences in intake were noted at age1 (4.4 ± 0.1 g and 4.3 ± 0.1 g, for OLETF and LETO rats, respectively; $F(1,15)=0.7$, $P=0.274$) or age2 (4.7 ± 0.1 g and 4.7 ± 0.1 g, for OLETF and LETO rats, respectively; $F(1,15)=0.1$, $P=0.988$). ANOVA results at age1 showed no significant strain x time interaction [$F(2,30)=1.2$, $P=0.251$]. Gastric emptying increased across time [$F(2,30)=25.0$; $P<0.001$], however this was not significantly different between OLETF and LETO rats [$F(1,15)=0.8$, $P=0.877$] at either 1hr (40.2 ± 5.8 % and 41.1 ± 4.1 %; respectively), 2 hrs (55.2 ± 3.0 % and 60.2 ± 2.2 %; respectively), or 4 hrs (66.5 ± 2.1 % and 68.3 ± 2.5 %; respectively), post-presentation of a 5 g chow meal. Similarly at age2, no significant interaction effect for strain x time was observed [$F(2,30)=0.5$, $P=0.783$], while a significant main effect for time was noted [$F(2,30)=33.5$,$P<0.001$]. At age2, gastric emptying was again not significantly different between OLETF and LETO rats [$F(1,15)=0.6$, $P=0.854$] at either 1hr (41.6 ± 3.2 % and 41.8 ± 4.5 %; respectively), 2 hrs (53.4 ± 2.3 % and 50.5 ± 3.0 %; respectively), or 4 hrs (68.7 ± 1.9 % and 68.1 ± 1.8 %; respectively), post-presentation of a 5 g chow meal.

Gastric emptying of solid chow in OLETF and LETO rats allowed ad-libitum access to food

As shown in Figure 1A, when given *ad libitum* access to chow for 1 hr after an overnight fast at age1, OLETF rats consumed more food than LETO rats ($F(1,15)=19.0$, $P<0.001$). Gastric emptying ANOVA analyses revealed no significant strain x time interaction [$F(2,30)=0.4$, $P=0.905$], while a main effect for time [$F(2,30)=42.3$,$P<0.001$], but not strain [$F(1,15)=0.3$, $P=0.922$], was shown.

Figure 1B shows results from rats at age2 following *ad libitum* access to chow for 1 hr after an overnight fast. As expected, OLETF rats consumed more food than LETO rats ($F(1,15)=16.3$, $P<0.01$). Gastric emptying of chow showed a main effect of time [$F(2,30)=51.7$; $P<0.001$] but not strain [$F(1,15)=0.2$, $P=0.979$. No interaction between strain x time on gastric emptying after 1hr *ad libitum* feeding was observed at age2 [$F(2,30)=0.4$, $P=0.644$].

When rats were given a larger window of *ad libitum* access to chow at age1, OLETF rats consumed significantly more chow than LETO rats [F(1,15)=13.8, P<0.001] across time [F(2,30)=33.9, P<0.001], however no strain x time interaction was noted [F(2,30)=0.6, P=0.734]. Post-hoc results show increased chow intake in OLETF rats at 1hr (P<0.001), 2 hrs (P<0.05) and 4 hrs (P<0.01) access periods (Figure 2A). At age1, no main effect for strain [F(1,15=0.2, P=0.812] on gastric emptying was observed (Figure 2A) although a significant time effect was shown [F(2,30)=20.1, P<0.001]. No significant strain x time interaction [F(2,30)=0.2, P<0.838] was evident for *ad libitum* chow gastric emptying at age1.

At age2, ANOVA results again showed significant main effects for both strain [F(1,15)=26.9, P<0.001] and time [F(2,30)= 31.8, P<0.001] on chow intake following *ad libitum* access periods, although no strain x time interaction [F(2,30)=0.2, P=0.799]. Post-hoc results showed that OLETF rats consumed more chow than control LETO rats at 1hr, 2 hrs, and 4 hrs (all P's<0.01) access periods (Figure 2B). Figure 2B also illustrates that no significant main effect of strain [F1,15)=0.2, P=0.824] was observed for gastric emptying at age2, however a main effect for time on gastric empting was shown [F(2,30)=18.3, P<0.001].

Gastric emptying of liquid loads in OLETF and LETO rats—No main effects of strain [age1: F(1,15)=0.1, P=0.996; age2: F(1,15)=0.2, P=0.945] or gastric load [age1: F(1,15)=0.4, P=0.639; age2: F(1,15)=0.4, P=0.688] were noted in 5ml emptying of liquid loads among either age group tested. Specifically, gastric emptying of a 5 ml load of isosmotic saline (age1: 76.2 ± 2.0 % and 72.2 ± 3.7 %; for OLETF and LETO rats, respectively: age2: 59.1 ± 2.0 % and 62.5 ± 2.8 %; for OLETF and LETO rats, respectively), hyperosmotic saline (age1: 59.3 ± 3.9 % and 62.2 ± 4.6 %; for OLETF and LETO rats, respectively: age2: 49.2 ± 2.0 % and 50.5 ± 4.4 %; for OLETF and LETO rats, respectively), isosmotic glucose (age1: 59.5 ± 3.6 % and 56.3 ± 2.8 %;for OLETF and LETO rats, respectively: age2: 50.7 ± 3.3 % and 52.7 ± 4.1 %; for OLETF and LETO rats, respectively), or hyperosmotic glucose (age1: 48.2 ± 5.3 % and 44.5 ± 3.3 %; for OLETF and LETO rats, respectively: age2: 44.2 ± 2.7 % and 42.4 ± 4.6 %; for OLETF and LETO rats, respectively) were no different between strains.

Gastric emptying of semi-solid chow in OLETF and LETO rats—No significant main effect of strain [F(1,10)=1.1, P=0.179] in gastric emptying of a 15 ml load of 25% chow mixture load was noted at age 2 between OLETF and LETO rats at either 1hr (35.7 ± 4.2 % and 32.6 ± 2.4 %; for OLETF and LETO rats, respectively) or 2 hrs (52.9 ± 3.4 % and 46.1 ± 3.1 %, for OLETF and LETO rats, respectively).

Sham feeding of sucrose in response to gastric distension in OLETF and LETO rats—Figure 3 depicts 20 min gastric distension effects on sham intake of 0.3M sucrose within five-min bins over a 90 min sham feeding session in OLETF or LETO rats at age1 or age2. Figure 3A shows results of gastric distension in OLETF rats at age1. Two way ANOVA results showed significant main effects for gastric distension [F(2,21)=12.3, P<0.001] and time [F(17,357)= 8.8, P<0.01], as well as a significant distension x time interaction [F(34,357)=3.54, P<0.01]. Post-hoc analyses of these results show the effects on sham intake to be confined to time periods where distension occurred. Specifically, no response to 5 ml distension was noted in OLETF rats at age1, while significant suppressions in sham intake relative to baseline were noted during 10 ml distension conditions at 15 min , 20 min, and 25 min time points (P<0.01 for all three time points).

Figure 3B shows age1 results in LETO rats receiving 20 min gastric distension. Two way ANOVAs in LETO rats at age1 reveal significant main effects for both gastric distension

[F(2,21)=24.1, P<0.001] and time [F(17,357)= 14.3, P<0.001], and also a significant distension x time interaction [F(34,357)=7.7, P<0.001]. Post-hoc analyses of these results depicted in Figure3B show significant reductions in sham intake only within the 20 min distension period. However unlike in OLETF rats, LETO rats showed significant suppression in intake in response to both 5 ml and 10 ml distension volumes. In particular, intake reductions were noted at 15 min (P<0.05 and P<0.001, for 5 ml and 10ml distension, respectively), 20 min (P<0.01 and P<0.001, for 5 ml and 10 ml distension, respectively), and 25 min (P<0.05 and P<0.01, for 5 ml and 10 ml distension, respectively), time points.

Gastric distension effects on sham intake in OLETF rats at age2 are shown in Figure 3C. Similar to results in age1, OLETF rats at age2 showed significant main effects of distension [F(2,15)=15.7, P<0.001] and time [F(17,255)= 14.3, P<0.001], and in addition, a significant distension x time interaction [F(34,255)=2.6, P<0.01]. Intake at the 15 min time point was significantly reduced in both 5 ml (P<0.05) and 10 ml (P<0.01) distension conditions, while intake at 20 min was significantly reduced (P<0.01) in the 10 ml distension condition only.

Figure 3D illustrates effects of gastric distension on sham intake in LETO rats at age2. LETO rats at age2 showed significant main effects of distension [F(2,15)=26.8, P<0.001] and time [F(17,255)= 45.2, P<0.001], and a significant distension x time interaction [F(34,255)=11.9, P<0.001]. Unlike in OLETF rats, post-hoc analyses in LETO rats showed intake reductions during distension at three time points during distention:15 min (P<0.05 and P<0.001, for 5 ml and 10ml distension, respectively), 20 min (P<0.01 and P<0.01, for 5 ml and 10ml distension, respectively), and 25 min (P<0.05 and P<0.01, for 5 ml and 10 ml distension, respectively).

Analysis of c-Fos protein expression in the hindbrain of OLETF and LETO rats due to gastric distension—Analyses of c-Fos expression in the hindbrain of OLETF and LETO rats revealed significant differences within the NTS region of the dorsal vagal complex. Specifically, a strain main effect was noted for both sham [F(1,4)=22.1; P<0.01] and 8 ml distension [F(1,6)=6.94; P<0.05] conditions, indicating decreased average of NTS Fos-LI in OLETF rats relative to LETO rats (Table 1). Post-hoc analyses within NTS plate level showed significant differences between OLETF and LETO rats. OLETF rats distended with an 8ml gastric balloon showed a significant decrease in NTS Fos expression at −13.24mm (P<0.05) and −13.68mm (P<0.01) levels compared to LETO controls. In non-distended sham conditions, OLETF rats showed a decreased Fos expression at −13.30mm (P<0.05) and −13.68mm (P<0.05) levels relative to LETO rats.

There were no significant differences in Fos expression between OLETF and LETO rats within either treatment group at the −14.30mm (P=0.986), −14.08mm (P=0.955), or −13.80mm (P=0.810) plate levels. Additionally, we did not observe any significant differences in Fos expression in any other area of the dorsal vagal complex including the dorsal motor nucleus, the area postrema, or area subpostrema, between OLETF and LETO rats at any plate level examined (all P's>0.05).

Oral Glucose (OGTT) & Insulin (ITT) Tolerance Tests—As shown in Table 2, at both age1 and age2, OLETF rats showed increased blood glucose levels relative to LETO rats after glucose challenge. At age1, significant increases were noted in OLETF rats at 30 min and 60 min (P<0.001 for both time points) compared to LETO rats, with highest blood glucose peak at 30 min (173 ± 5.1 vs. 110 ± 5.5 mg/dl in OLETF and LETO rats, respectively). At age2, all time points measured post-glucose challenge were significantly higher in OLETF rats (P<0.01 for all time points), with highest blood glucose peaks occurring at 60 min (253 ± 30.0 vs. 111 ± 5.9 mg/dl in OLETF and LETO rats, respectively).

After acute insulin injection at age1, OLETF rats show an attenuated decrease in blood glucose 120 minutes post-insulin injection compared to LETO animals (70 ± 3.5 vs. 51 ± 1.5 mg/dl in OLETF and LETO rats, respectively; $P<0.05$) (Table 2). At age2, OLETF rats showed significantly higher blood glucose at 90 min post-insulin injection when compared to LETO rats (53 ± 4.1 vs.35 ± 2.1 mg/dl in OLETF and LETO rats, respectively; $P<0.01$), as well as a non-significant trend of an overall attenuation of decreased blood glucose due to insulin relative to LETO rats.

Determination of stomach weight—Following OGTT and ITT administration at age2, animals were sacrificed and stomachs resected. OLETF rat stomachs were significantly heavier than LETO stomachs [$F(1, 30)= 35.4$; $P<0.01$: 2.6 ± 0.1 g vs. 2.0 ± 0.1 g for OLETF and LETO rats, respectively). However when corrected for body weight (BW: 665.1 ± 16.0 g vs. 547 ± 11.5 g for OLETF and LETO rats), the relative stomach size of OLETF and LETO rats did not differ (stomach weight: 0.39 ± 0.02 vs. 0.37 ± 0.02 g/100 g BW for OLETF and LETO rats, respectively; $P=0.968$).

DISCUSSION

The present findings indicate that OLETF rats do not show deficits in gastric emptying of either solid or liquid loads relative to LETO rats, regardless of age and progression of blood glucose impairments. Specifically, when OLETF rats consumed either a chow meal of equal or greater size within the same allotted meal period, gastric emptying was equal to that of LETO controls. Similarly, applying gastric distension via intragastric instillation of a relatively high volume load did not produce differential gastric emptying between strains. OLETF and LETO rats were also shown to have equal gastric emptying of both isosmotic and hyperosmotic nutritive and non-nutritive gastric loads across both age groups tested. In contrast, when the volumetric effects of gastric mechanodetection were isolated by inflation of an intragastric balloon, we observed reduced feeding responses within OLETF rats relative to LETO in both young and older animals. Furthermore, after gastric distension alone, OLETF rats show marked decreases in Fos expression compared to LETO rats in select regions of the hindbrain known to facilitate the vagal response to changes in gastric volume.

Our gastric emptying data appear to contrast with the expected outcome of accelerated gastric emptying in a CCK-1 receptor deficient animal. An increased rate of emptying would be predicted according to the known role of exogenous CCK to inhibit gastric emptying of solid and liquid nutrients (8, 14, 28, 40) via CCK-1 receptors, as well as from studies observing heightened gastric emptying of liquid nutrients by acute CCK-1 receptor blockade in normal rats (25, 34).

Data by other laboratories have shown decreased duodenal lipid-induced gastric acid secretion (43), and increased susceptibility to gastric mucosal lesions in the OLETF rat (30). More relevant to the current work, however, is that OLETF rats displayed delayed gastric emptying of a methylcellulose load in comparison to LETO rats (44). In that report, the authors showed no differential strain reduction in gastric emptying due to acute administration of corticotropin-releasing factor and the muscarinic receptor antagonist atropine. A closer inspection of the magnitude of these effects, however, shows a much larger, and almost complete, abolishment of gastric emptying in the OLETF rat from atropine (44). It is not clear from the analysis reported whether this increased degree of suppression was significantly higher in OLETF rats. Such a scenario would be suggestive of decreased parasympathetic control of gastric emptying in the OLETF, and may explain these observations.

Ohta et al. extended these findings by showing that OLETF rats exhibit delayed gastric emptying in response to a caloric liquid gastric load (29). It was also theorized that sympathetic nervous function may be enhanced in the OLETF compared to LETO animals as indicated by decreased responsiveness to reserpine-induced gastric emptying acceleration (29). Nonetheless, no studies to date have directly examined the possibility of altered gastric parasympathetic or sympathetic innervation in the OLETF rat.

In the present study, gastric emptying of both caloric and non-caloric, as well as hyperosmotic and isosmotic, gastric loads were no different between OLETF and LETO rats, indicating that fluid emptying in the OLETF rat is also intact. It is worth noting that gastric emptying of a non-nutrient liquid load in CCK-2 receptor knock-out mice is enhanced (23), although the precise mechanism behind this phenomenon is not known. Furthermore, gastric CCK-2 receptor mRNA in OLETF rat has been shown to be up-regulated relative to LETO rats (22). Therefore, the possibility that putative deficits in gastric emptying in the OLETF rat due to the lack of CCK-1 receptor are compensated for by enhanced CCK-2 receptor activation cannot be discounted.

When a large liquid chow mixture was directly instilled into the stomach, we did not detect any differences in gastric emptying amongst OLETF and LETO rats. It has previously been reported that OLETF rats tend to have higher wet weights of stomachs at 12-14 weeks of age (29), which parallels their increased body weight. We have extended these findings by observing slightly increased dry stomach weights in OLETF rats at ~35 weeks of age (age of sacrifice after age2), however when correcting for body weights of the animals at this age, this difference becomes negligible. Nonetheless, despite this apparent increased raw gastric size, our relatively large 15 ml load was not able to produce any distinguishable difference in gastric emptying between OLETF and LETO rats, suggesting that OLETF rats are able to maintain normal gastric emptying rates of a caloric load even when given a volume of distension likely close to the maximum gastric capacity under ad libitum feeding.

Additionally, Schwartz et al. (42) have shown that liquid nutritive gastric preloads are largely equal in their ability to suppress subsequent food intake, whereas duodenal preloads have distinctively diminished satiating effects in OLETF compared to LETO rats. Our data compliment these results by revealing that gastric emptying of a nutrient load in OLETF and LETO rats show no apparent differences, regardless of amount of chow consumed, present in the stomach, or administered in liquid or solid phase. Gastric emptying of a nutrient load is distinguished by a period of increased emptying rate during periods of gastric fill, such as within a meal or resultant from gastric nutrient infusion, while emptying subsequent to meal termination is maintained at a slower, constant tempo (15). While this work does not address potential deficits in initial rates of emptying, our results show clearly that gastric emptying in OLETF rats after meal termination is unchanged relative to LETO rats.

Isolating the volumetric component of distension via intragastric balloon inflation allowed us to examine feeding responsiveness to a fixed volume of gastric distension in OLETF and LETO rats. Our results showed clear differences in intake patterns within OLETF and LETO rats during periods of gastric distension. Specifically, OLETF rats showed no response to 5 ml distension at age1 and reduced intake at only one time point during distension at age2, while LETO rats reduced intake from baseline at multiple time points during 5ml distension at both ages. When distended with a 10 ml gastric volume, responses were similar in OLETF and LETO rats at age1, however the magnitude of intake attenuation in response to distension appears to be greater in LETO relative to OLETF rats. Likewise, at age2, effects of 10 ml distension persisted for a longer period in LETO than OLETF rats. These results suggest that OLETF rats have decreased sensitivity to gastric volumes during feeding, and thus may require a relatively greater degree of volumetric distension in order to reduce food

intake compared to LETO rats. This observation parallels our recent finding showing that OLETF rats exhibit diminished responsiveness to intestinal nutrient infusion in a similar sham feeding design (7). In this context, prior reports have identified the vagal signal induced by stomach distension to be largely a function of mechano-specific receptors (19), in contrast to duodenal vagal afferents which respond to both mechano-, and chemo-sensation (41). In general, of the two main classes of vagal afferent endings, the intraganglionic laminar endings (IGLEs) and intramuscular arrays (IMAs), IMAs have been shown to primarily mediate signals of stretch and length change within the stomach, while IGLEs function more in response to more direct muscular contraction (32). Of particular relevance to our findings are the recent reports that knock-out animals lacking the Neurotrophin-4 (NT-4) gene exhibit deficient intestinal IGLE innervation which result in short term satiation deficits (9). In contrast, NT-4 knock-in mutants have been shown to be hypersensitive to CCK-induced satiation mediated through CCK-1 receptor activation (4). Thus, it is possible that diminished IGLE responsiveness due to a congenital lack of the CCK-1 receptor in the OLETF rat may contribute to increased food intake in these animals. Given the current sham feeding design, however, it is unlikely that duodenal IGLEs play a significant role as no intestinal nutrient feedback was elicited. Nonetheless, when considering the functional differentiations in gastric and duodenal vagal innervation in an intact animal, one possible explanation for our sham feeding results is that gastric vagal mechano-sensitivity is diminished in the OLETF rat, which would explain an attenuated decrease in intake due to stomach distension. Alternatively, a simpler explanation may be that the same degree of balloon inflation may not translate to the same degree of distension detection, due to increased stomach size in the OLETF rat.

However, our gastric emptying results would not support the latter explanation because both small and large amounts of gastric nutrient volumes did not produce variable gastric emptying in any of our experiments at either age tested.

The suppressive effects of gastric distension on food intake have been shown to be largely mediated by activation of both gastric and hepatic branches of the vagus nerve [see for review (33)]. Our final experiment addressed whether diminished feeding response to gastric distension in OLETF rats could be explained, at least partially, through decreased vagal activation of distension signals. Gastric distension induces Fos within the NTS region of the DVC (18, 46), an area established as the primary termination site for vagal afferent input from the stomach (49). Furthermore, distension-induced Fos expression in the NTS is abolished via vagotomy (20, 24). The current results of decreased DVC Fos expression within the NTS suggest that OLETF rats exhibit diminished vagal signaling induced by gastric distension relative to control LETO rats. This is in agreement with previous data from our laboratory (6) as well as others (11) showing a decreased Fos expression in the enteric plexus, nodose ganglia and hindbrain of OLETF compared to LETO rats. In addition, levels of Fos neurons in LETO rats are comparable to Fos counts observed following by other laboratories using a similar degree of gastric distension (36).

Although CCK-1 receptors have been shown to participate in vagal responses following stomach distention, the precise degree of this participation has not been clear. Recently, van De Wall et al (46) reported that lorglumide did not diminish expression of Fos induced by 2 ml distension; however, it completely reversed the enhancement of distension-induced Fos expression by CCK. This suggests that lorglumide specifically reduces the response of vagal afferents to distension. The contributions by specific vagal afferent fiber populations in mediating gastric distension effects may vary according to the distension volume. It is possible that the relatively large 8 ml volume of gastric distension used in our study may result in involvement of CCK-1 receptor activity not captured when testing smaller distension volumes. Alternatively, the effect may be independent of CCK-1 receptors, and

due to a direct effect of reduced vagal transmission of distension signals. A detailed analysis of multiple levels of gastric distension in the OLETF rat would be necessary to test this hypothesis. It is also worth mentioning that our Fos results do not indicate whether the observed decreased neuronal response is a contributor, or an artifact, of spontaneously increased meal size in the OLETF rat. Analysis of gastric distention-induced Fos expression using OLETF rats previously pair-fed to LETO controls in order to limit meal size may be useful in answering this question.

Recent work by Reidelberger et al. have provided interesting data focusing on peripheral vs. central feeding effects of blood brain barrier -permeable and non-permeable CCK-1 receptor antagonists (35). From these data, it appears likely that blockade of central or vagal CCK-1 receptors may be involved in separate, co-operative processes that lead to an overall increase in food intake in non-mutant animals. Indeed, the current Fos results would support peripherally associated CCK-1 receptor deficits in distension signaling. However, there are also reports that cells expressing gastric distension-induced Fos in the NTS activate projections that extend beyond the hindbrain, to forebrain structures such the paraventricular and supraoptic nuclei of the hypothalamus (21). In this context, alterations in neuropeptide Y signaling within the dorsomedial and arcuate nucleus of the hypothalamus have been previously implicated in hyperphagia in the OLETF rat (1). Thus, it is possible that beyond vagal deficits described here, select hypothalamic nuclei implicated in gastric distension controls may be an additional contributor to aberrant feeding behavior in the OLETF rat.

It may also be argued that our findings of slightly decreased NTS Fos expression in sham treated OLETF compared to LETO rats may be indicative of a decreased responsiveness in general and not limited to gastric volume stimuli. This explanation is unlikely for two reasons. First, the actual raw total NTS Fos counts for the sham treatment is between 10 and 20 fold less than in distension treatments. While statistically different between strains, the small number of Fos positive nuclei is rather indicative of a slightly lower background in OLETF rats. This may be attributed to a lower activity of OLETF rats during testing as these animals have been shown to be hypoactive (Li et al. Phys&Behav, 2002). Second, the noted differential strain effects under gastric distension are of such exceedingly high magnitude relative to sham conditions, that any slight baseline differences of a few Fos counts under sham conditions have little impact on the significance of distension effects between strains. This suggests that we did indeed observe specific differential effects of NTS Fos expression attributed to the gastric distension treatment, and not a generalized lower degree of Fos expression in OLETF compared to LETO rats.

To summarize, our findings reveal that OLETF rats, despite showing increased food intake, do not express deficits in their ability to control gastric emptying across multiple levels of gastric capacitance. Nevertheless, OLETF rats do show diminished feeding responses and neuronal activation induced by gastric distension. Thus, it is unlikely that hyperphagia in these animals involves deficient gastric emptying; however, decreased gastric mechanosensation and detection of gastric volume, in combination with previously described satiation defects, may facilitate overconsumption. The present findings seem to be discordant with work using acute CCK-1 receptor antagonism, and may suggest gastric and neuronal alterations in the OLETF not directly specific to CCK-1 receptor deficiency as possible mechanisms.

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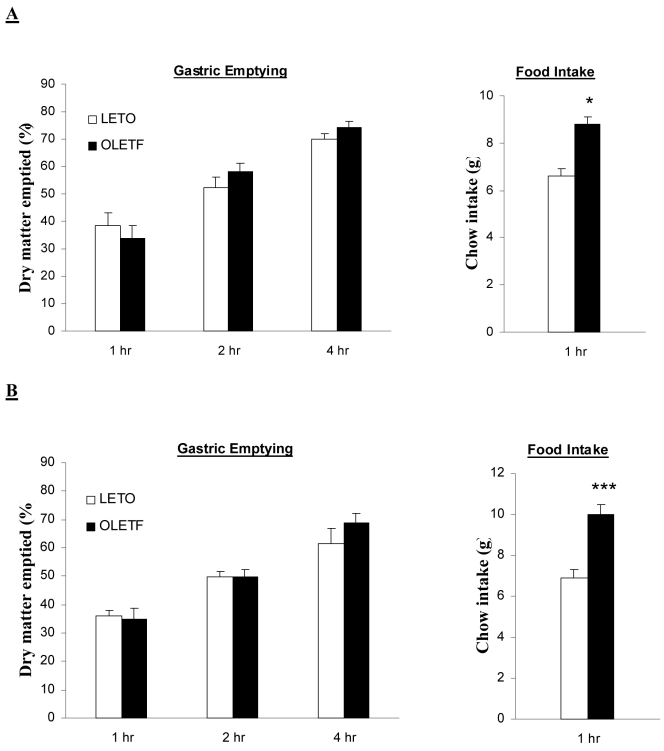

Fig. 1.

Gastric emptying of solid chow in OLETF and LETO rats allowed 1 hr *ad libitum* access to food. Sixteen hour food deprived OLETF and LETO rats were given 1 hr to consume solid rat chow *ad libitum*. Gastric emptying was not different between strain at 1, 2, or 4 hr after presentation of food at age1 (**A, left panel**) or age2 (**B, left panel**), however, OLETF rats consumed significantly more chow than LETO rats at both age1 (**A, right panel**) and age2 (**C, right panel**). *P<0.01, *** P<0.001, between strains

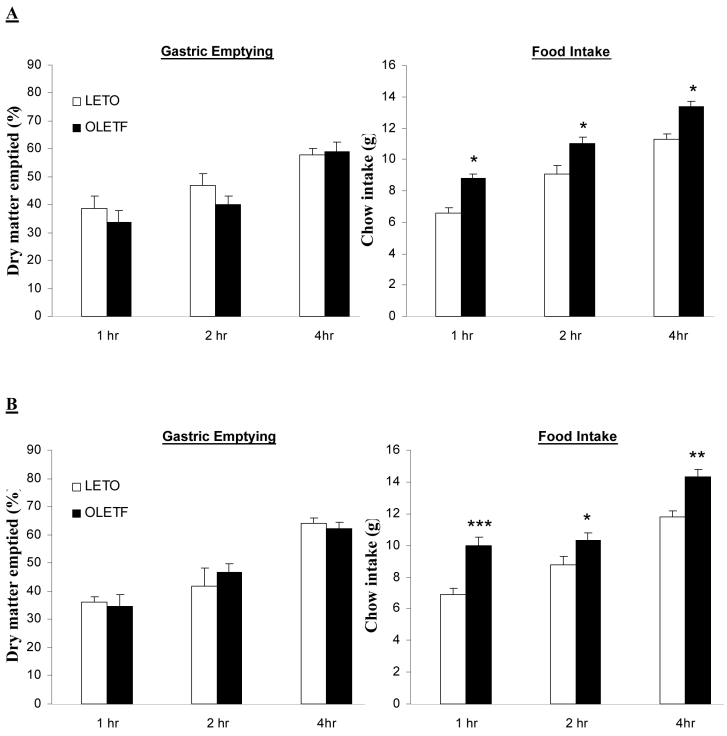

Fig. 2.

Gastric emptying in OLETF and LETO rats allowed variable *ad libitum* solid chow access. Sixteen hour food deprived OLETF and LETO rats were allowed ad libitum access to rat chow for either 1 hr, 2 or 4 hrs. Gastric emptying was not different between strain immediately following the termination of the chow access period, regardless of duration at both age1 (**A, left panel**) and age2 (**B, left panel**), however, OLETF rats consumed significantly more chow than LETO rats during all periods of ad libitum access at age1(**A, right panel**) and age2 (**B, right panel**).

*P<0.05, ** P<0.01, *** P<0.001, between strains

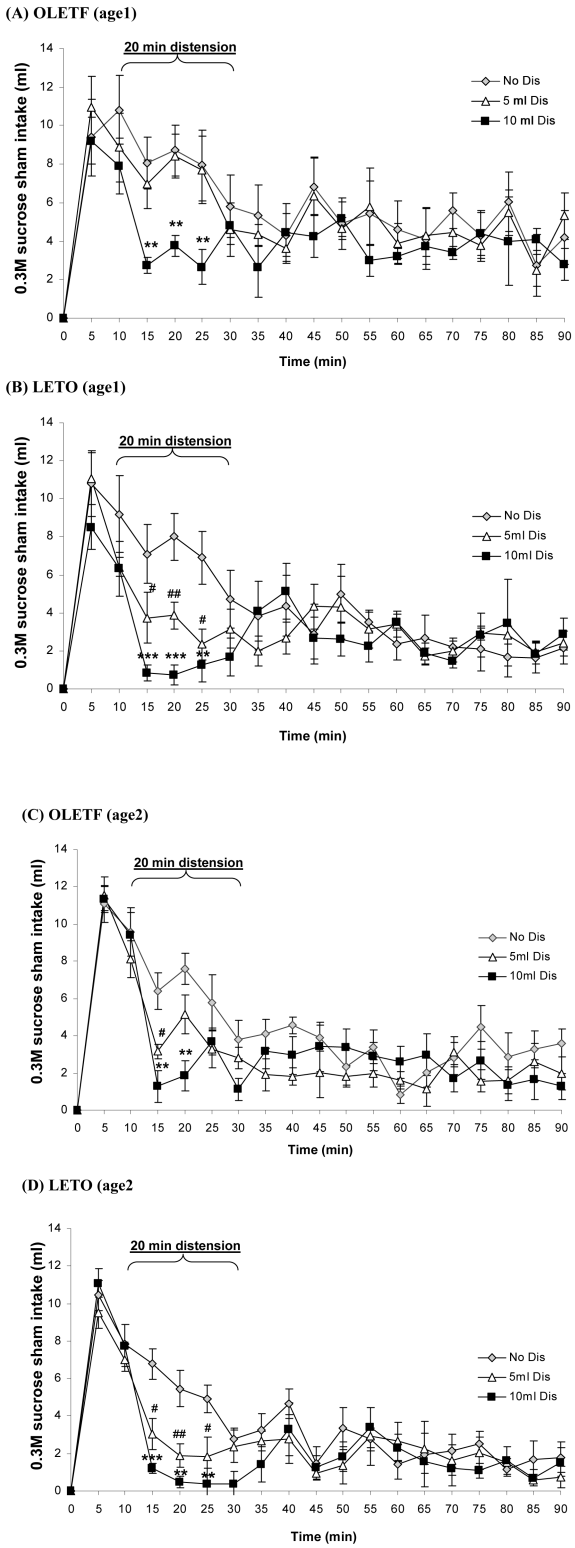

Fig. 3.

Twenty min gastric distension effects on sham intake of 0.3M sucrose within five-min bins over a 90 min sham feeding session in OLETF or LETO rats at age1 or age2. OLETF rats showed significantly attenuated sham intake at age1 during 10 ml distension relative to baseline, no distension conditions at 15, 20, and 25 min time points. No effect on sham intake due to 5ml distension was observed in OLETF rats at age1 (**A**). In contrast, sham intake in LETO rats was significantly smaller after both 5 and 10 ml distension relative to baseline intake at 15, 20, and 25 min time points at age1 (**B**). At age2, sham intake in OLETF rats was significantly decreased during 5 ml distension when compared to baseline intake only at 15 min, while intake during 10 ml distension was decreased at 15, 20, and 25 min compared to non distension intake (**C**). In LETO rats at age2, sham intake was significantly lower than under baseline conditions at 15, 20, and 15 min during both 5 and 10ml distension conditions (**D**). $^{\#}P<0.05$, $^{\#\#}P<0.01$, between no distension and 5 ml distension intake; **P<0.01, ***P<0.01, between no distention and 10 ml distension intake

Table 1

Counts of Fos-LI nuclei within the NTS of 8ml gastric distended or sham distended OLETF and LETO rats

	NTS FOS-LI nuclei			
Plate level	**OLETF Distension**	**LETO Distension**	**OLETF Sham**	**LETO Sham**
−14.30 mm	42.7 ± 4.5	44.1 ± 2.7	4.6 ± 0.7	6.5 ± 2.1
−14.08 mm	108.2 ± 14.3	101.9 ± 5.9	3.0 ± 0.9	5.4 ± 1.4
−13.80 mm	119.5 ± 8.5	132.4 ± 16.5	3.6 ± 1.8	6.8 ± 2.3
−13.68 mm	121.2 ± 22.9	216.2 ± 17.5**	6.9 ± 1.7	12.8 ± 0.7*
−13.30 mm	156.0 ± 28.4	162.3 ± 14.2	5.6 ± 0.9	14.3 ± 3.7*
−13.24 mm	57.7 ± 8.2	102.5 ± 15.7*	3.9 ± 1.6	5.6 ± 0.9
average	129.7 ± 15.9	164.4 ± 8.7*	4.8 ± 1.6	10.9 ± 0.4**

Quantification of Fos-LI in the hindbrain of OLETF and LETO rats. OLETF rats showed decreased average NTS Fos-LI nuclei for both sham and 8 ml distension conditions relative to LETO rats. Post-hoc analyses within NTS plate level showed that OLETF rats distended with an 8 ml gastric balloon showed decreased NTS Fos expression at −13.24mm and −13.68mm levels compared to LETO controls, while in non-distended sham animals, OLETF rats showed decreased Fos expression at −13.30mm and −13.68mm levels.

* P<0.05,

** P<0.01 between OLETF and LETO rats within distension or sham distension conditions.

Table 2

Blood glucose levels (mg/dl) following OGTT or ITT in OLETF and LETO rats at age1 and age2

	Blood glucose levels (mg/dl)			
Test	**OLETF age1**	**LETO age1**	**OLETF age2**	**LETO age2**
OGTT				
Baseline	86.7 ± 2.2	88.2 ± 2.9	81.5 ± 5.5	82.2 ± 1.5
30 min	173.0 ± 5.1	110.0 ± 5.5***	194.3 ± 20.6	110.2 ± 5.8***
60 min	161.3 ± 4.8	105.6 ± 2.4***	253.2 ± 30.0	111.9 ± 5.9***
90 min	127.3 ± 15.5	99.0 ± 2.1	207.3 ± 20.3	101.8 ± 4.8***
120 min	92. 8 ± 2.3	88.8 ± 2.0	140.0 ± 11.5	91.8 ± 3.8***
ITT				
Baseline	84.4 ± 3.2	79.7 ± 2.4	83.3 ± 2.3	77.3 ± 1.3
30 min	72.3 ± 3.0	72.3 ± 2.0	69.4 ± 4.4	59.5 ± 6.4
60 min	63.5 ± 3.5	63.4 ± 6.0	55.9 ± 5.0	48.8 ± 4.4
90 min	67.6 ± 4.3	57.7 ± 1.7	53.4 ± 4.9	35.0 ± 1.8**
120 min	70.0 ± 5.2	51.0 ± 1.5*	46.3 ± 4.5	39.7 ± 3.7

An oral glucose and insulin tolerance test (OGTT and ITT, respectively) were performed within each age group after experimentation. For OGTT, a glucose load (2g/kg) was orally delivered after a 16hr fast. For ITT, all rats received intraperitoneal (IP) injection of insulin. For both tests, blood glucose was measured before glucose loading or pre-injection and at 30, 60, 90, and 120 min post-loading or injection.

* P<0.05,

** P<0.01,

*** P<0.001, strain differences in blood glucose levels within each age group tested.