2010

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Running Head: Early overeating, feeding and vagal afferents

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ABSTRACT

BIDDINGER, J.E., AND FOX, E.A. Early postnatal overnutrition disrupts satiety without altering vagal gastrointestinal afferents. PHYSIOL BEHAV 00(0) 000-000, 2010. Early postnatal overnutrition results in a predisposition to develop obesity due in part to hypothalamic and sympathetic dysfunction. Potential involvement of another major regulatory system component - the vagus nerve - has not been examined. Moreover, feeding disturbances have rarely been investigated prior to development of obesity when confounds due to obesity are minimized. To examine these issues, litters were culled on the day of birth to create small litters (SL; overnutrition), or normal-size litters (NL; normal nutrition). Body weight, fat pad weight, meal patterns, and vagal sensory duodenal innervation were compared between SL and NL adult mice prior to development of obesity. Meal patterns were studied 18 hour/day for 3 weeks using a balanced diet. Then vagal mechanoreceptors were labeled using anterograde transport of wheatgerm agglutinin-horseradish peroxidase injected into the nodose ganglion and their density and morphology were examined. Between postnatal day 1 and weaning, body weight of SL mice was greater than for NL mice. By young adulthood it was similar in both groups, whereas SL fat pad weight was greater in males, suggesting postnatal overnutrition produced a predisposition to obesity. SL mice exhibited increased food intake, decreased satiety ratio, and increased first meal rate (following mild food deprivation) compared to NL mice, suggesting postnatal overnutrition disrupted satiety. The density and structure of intestinal IGLEs appeared similar in SL and NL mice. Thus, although a vagal role cannot be excluded, our meal parameter and anatomical findings provided no evidence for significant postnatal overnutrition effects on vagal gastrointestinal afferents.

Keywords: satiety, gastrointestinal tract, ingestive behavior, intraganglionic laminar endings, adipose tissue, meal pattern, meal microstructure

1. INTRODUCTION

Exposure to early postnatal overnutrition in both humans and animals can result in a predisposition to develop obesity and metabolic disorders in adulthood [1-4]. This is especially concerning because the prevalence of obesity in childhood has tripled over the past thirty years [5] and the incidence of overweight children has also increased [6]. These children are at risk to become obese adults and to suffer from weight-related health problems in adulthood [2].

Early postnatal overnutrition appears to have widespread consequences on an animal's physiology that could contribute to this predisposition. Alterations to two neural systems that have been well characterized include the central nervous system, in particular the hypothalamus [7] and the sympathetic nervous system [8-9]. Additionally, a subset of taste pathways exhibit altered structure and sensitivity following perinatal undernutrition [10-11]. Surprisingly, to our knowledge, the potential effects of postnatal overnutrition on vagal sensory GI innervation have not been explored. This pathway carries the bulk of negative feedback signals – chemical, hormonal and mechanical – from the gut to the brain that contribute to satiation, or meal termination [12-13]. Since postnatal overeating would lead to excessive stimulation of vagal GI sensory receptors, and these receptors are still developing postnatally [14-16], it is possible they would develop decreased sensitivity to GI stimuli through reduced development of their structural or functional components. The result would be decreased vagal signaling to the brain during a meal and thus increased meal size - the alteration most often observed in obese animals and people [17-18].

Very few studies have examined feeding behavior in detail using meal patterns and microstructure prior to development of obesity. Obesity has been found to be due largely to overeating [19], and therefore, examining changes in feeding behavior that

contribute to this overeating could be valuable in narrowing down underlying mechanisms, and possibly neural systems involved [18, 20-21]. Moreover, if it were possible to examine the effects of postnatal overnutrition on feeding behavior in mice with relatively normal body weights it would be advantageous because the confounding effects produced by obesity or metabolic syndrome would be minimized. Importantly, primary effects of postnatal overnutrition could be identified, which may include those underlying the subsequent development of disease. For instance, increased meal size is often associated with obesity [17-18], and rats subjected to postnatal overnutrition exhibited increased meal size at 9 months of age [22]. However, it is not possible to know from these studies whether increased meal size was a cause of obesity, or if it was a result of obesity or weight gain. The pattern of body weight changes in mice that experienced postnatal overnutrition suggest testing mice with normal or near normal body weight may be possible at young adult ages. Postnatal overnutrition is associated with greater-than-normal body weight between birth and weaning, an effect that extends for varying periods of time beyond weaning. In some studies that have maintained these mice on a balanced diet after weaning, by young adulthood their body weight returned to normal, or close to normal prior to developing obesity or the metabolic syndrome [23-24].

To begin to provide information on how the effects of postnatal overnutrition on feeding regulatory neurohormonal systems alter feeding behavior, and possibly vagal afferent development and function, we investigated food intake, including meal patterns and microstructure in young adult mice that had been exposed to early postnatal overnutrition. Microstructural analysis can help pinpoint whether changes in meal parameters are due to altered negative feedback, which is mainly vagally-mediated vs. altered palatability or motivation, which is mainly CNS in origin [25]. To further address possible vagal sensory involvement, we examined the density and structure of intraganglionic laminar endings (IGLEs) that innervate the duodenum. IGLEs, the main

class of vagal tension receptor in the duodenum [26-28], are thought to play a role in GI motility and negative feedback signaling to the brain that stops food ingestion [29]. We focused on duodenal innervation because it would be stimulated by postnatal overeating during consumption as food emptied from the stomach into the duodenum, and it is an important contributor of satiation signals generated during a meal. Moreover, effects of IGLE loss from the small intestine were detectable by meal pattern analysis [30].

2. METHODS

2.1 Animals. Female nulliparous C57BL/6 mice were impregnated at Harlan Laboratories, Inc. (Indianapolis, IN) and arrived in our laboratory when embryos were 15 days of age. Dams were housed individually in standard mouse polycarbonate cages 11 in x 7 in x 5 in with aspen bedding and fitted with wire lids that held water bottles and food pellets and maintained on a standard chow diet (Laboratory Rodent Diet 5001; 28% protein, 60% carbohydrate, and 12% fat, and 3.34 kcal/g caloric density; Purina Nutrition, St. Louis, MO) and a 12 hr light/12 hr dark cycle at 23℃. At postnatal day zero (P0), the day of birth, newborn litters were randomly assigned to sizes of 7 pups (normal litter size, NL) or 3 pups (small litter size, SL) and then culled to this size. The 7 pup litter size was used as the control group, as this is the approximate average, naturally-occurring number of mouse pups per litter for this strain of mouse according to the primary breeders and the supplier of the mice used in the present study [31]. Mouse pups were not disturbed between culling and weaning at P22, and after weaning they were maintained on standard chow in the same type of cage. Mice were weighed daily during the 22 days of meal pattern experiments at 3-4 months of age. Both males and females were used. All procedures were conducted in accordance with Principles of Laboratory Animal Care and the American Association for Accreditation of Laboratory

Animal Care Guidelines and are approved by the Purdue University Animal Care and Use Committee.

2.2 Body weight. Reducing litter size in rodents has been a reliable, reproducible means for producing postnatal overnutrition and the consequent predisposition to obesity and diabetes [3, 24, 32-36]. To characterize the weight gain associated with presumptive overeating of mice raised in small litters, extra female and male C57/BL6 offspring from the timed pregnancies described above were mated in our laboratory and a second set of NL and SL groups were created at P0 in the same manner as described above. These mice were weighed at the same time each day from birth until weaning. A total of six litters were used for this experiment; three NL (6-7 pups per litter) and three SL (3 pups per litter).

2.3 Adult fat pad weight. In previous studies, increases in fat pad weight and in some instances involving increased fat cell number, have been observed prior to development of obesity [37, 38]. Therefore, we also examined fat pad weight. At the time of perfusion for WGA-HRP nerve tracing (see below), when the mice were 6-8 months old, mice were weighed and the subcutaneous, retroperitoneal, and gonadal fat pads on both the left and right sides of the animal were removed after fixative perfusion and weighed immediately. Fat pads were collected from 12 animals per group (NL: males n = 6, females n = 6; SL: males n = 8, females n = 4).

2.4 Meal Pattern Analysis. Meal patterns and microstructure were examined in NL (n = 18; males n = 10, females n = 8) and SL (n = 16, males n = 9, females n = 7) mice. Meal pattern data were collected from both male and female NL and SL mice at 3-4 months of age as previously described [30]. Briefly, all mice were adapted to the

experiment room and cages, and food pellets (10 pellets on each of 3 separate occasions; 20 mg dustless precision pellets; 22% protein, 66% carbohydrate, 12% fat, and 3.623 kcal/g caloric density; Bio-Serv, Frenchtown, NJ) for at least one week before the experiment began. Automated pellet dispensers (Coulbourn Instruments, Inc., Whitehall, PA) detected removal of a single pellet and immediately replaced it. The time and occurrence of each pellet removal was registered by a computer running Graphic State software (v. 2.0, Coulbourn Instruments, Inc). Food intake patterns were monitored for three weeks 18 hours each day (12 hr dark; 6 hr light) to provide time for weighing the animal and for cage and system maintenance. Additionally, the 6 hour fast during the light phase provides a mild deprivation that ensures all animals start eating at each daily run (and dark) onset as required for microstructural analysis [25]. One NL female was dropped from the analysis due to hoarding of pellets (final NL female n = 7).

2.5 Labeling of duodenal IGLEs. Anterograde transport of the nerve tracer wheatgerm agglutinin-horseradish peroxidase (WGA-HRP) injected into the nodose ganglion was used to label the vagal afferent nerve fibers and their terminal endings as previously described [27, 39]. Mice from the NL and SL groups were injected and processed together in pairs to ensure any inadvertent changes in the procedure or reagents that could occur over time did not contribute to group differences. Briefly, mice were anesthetized using ketamine hydrochloride (75 mg/kg; VetaKet Laboratories, Shenandoah, IA) and xylazine (50 mg/kg; Anased Lloyd Laboratories, Decatur, IL) mixture administered intraperitoneally (i.p.). The left nodose ganglion was surgically exposed and WGA-HRP (0.5 μL, 4%; PL-1026, Vector Laboratories, Burlingame, CA) pressure-injected (PicoSpritzer III; General Valve Operation, Fairfield, NJ; 55 pounds per square inch; 5 msec) into the ganglion using a glass micropipette (inner tip diameter, 25 μm). The incision was closed with sutures, mice were administered buprenorphone

hydrochloride i.p. (0.1 mg/kg; Bedford Labs, Bedford, OH) for pain and placed on a heating pad to recover.

2.6 Tissue Processing. Twenty-four hr after tracer injection, animals were injected i.p. with a lethal dose of sodium methohexital (120 mg/kg; Monarch Pharmaceuticals, Bristol, TN) and the thoracic organs exposed. Twenty U heparin sodium and 0.02 mg propranolol hydrochloride were injected into the left ventricle. Mice were then perfused transcardially with 0.9% saline at 37℃ for 5-10 min until the liver cleared followed by cold 3% paraformaldehyde/0.75% gluteraldehyde in 0.1M phosphate buffered saline at pH 7.4 and 4℃ for 30 min. Immediately prior to perfusion with fixative the stomach and duodenum were expanded by infusion of 0.9% saline at 35℃ into the stomach. Care was taken to produce the same degree of expansion in all mice of both SL and NL groups. The anterior 8 cm of duodenum were divided into two 4 cm lengths and whole mounts of each prepared by separating the outer muscular layers from the inner submucosa/mucosa using sharp dissection. Wholemounts of the muscle layers that contained WGA-HRP-labeled vagal afferents were processed with tetramethyl benzidine (TMB) according to the protocol of Mesulam [40], and then mounted, flattened with weights, dried, cleared in xylene, and coverslipped as described [39], and examined with dark-field illumination (Leica DM 5000 microscope, Wetzlar Germany).

2.7 Quantification of IGLE Density. In order to quantify the IGLEs present in the duodenum of the small intestine, all IGLEs identified that met the counting criteria were included in the analysis. In order for a nerve ending to qualify as an IGLE, it had to have met criteria as previously described [27, 39, 41] and have a diameter of at least 40 µm. IGLE diameter was measured by overlaying an image of a stage micrometer on an image of labeled IGLEs taken at the same magnification. An IGLE consists of a group of

nerve terminal puncta in close association with each other that lie within a plane on the surface of all or a portion of a myenteric ganglion. Each individual cluster of terminal puncta (IGLE “leaf”) was counted as one IGLE. IGLE density was calculated as the number of IGLEs in the 8 cm duodenum length divided by its area (calculated by multiplying the length and width of the flattened wholemount). The entire duodenum sample from a mouse was excluded from quantitative analysis if any of the peroxidase reaction artifacts that can occur [40] obscured HRP-labeled vagal sensory elements in a large portion of the tissue. Samples were also excluded if there were any incomplete injections indicated by the absence of labeled vagal sensory elements in a sufficiently large region of tissue. The specific criterion used to determine if absence of labeling warranted exclusion was that IGLE densities were less than 2 SD’s from the mean density of all samples in a group. All counting was done blind to experimental conditions of litter size and sex. Of the 34 animals tested for meal patterns, 3 died during nodose injections and 8 failed to meet all of the criteria, which permitted analysis of 23 duodenums (SL: total n = 11, males n = 6, females n = 5; NL: total n = 12, males n = 8, females n = 4).

2.8 Statistical Analysis and Representation of Data. All data are represented as means $\pm$ SEMs. All datasets were first checked for normality using Shapiro-Wilks test and homogeneity of variance using Bartlett’s or Levene’s tests. Significance of differences for each meal pattern parameter that fit a normal distribution and exhibited homogeneity of variance between NL and SL mice and males and females were determined using two-way repeated measures analysis of variance (ANOVA) with litter size and sex as independent variables and the meal parameter as the dependent variable (Statistica, v5.0, StatSoft, Tulsa, OK). Data from males and females were combined when there were no significant effects or interactions of sex. Significance of

differences for each meal pattern parameter that did not fit a normal distribution, or did not exhibit homogeneity of variance were determined using Kruskal-Wallis ANOVA. Minute-to-minute changes in intake rate during the first 30 min of testing each day were fit to the Weibull function ($y = A\exp[(-Bt)^C]$; [25]) using Tongue Twister (v1.45, Tallahassee, FL). The A parameter describes the initial intake rate, which characterizes the motivation of the animal to eat, or the palatability of the food. The B parameter describes the decay of the intake rate, which characterizes the post-ingestive negative feedback effects of food intake that are thought to be dominated by vagal sensory signals. The C parameter is determined by the overall shape of the curve, which estimates the duration the initial rate is maintained and is influenced by postingestive factors. Significance of differences in IGLE density between the litter size groups was determined using one-way ANOVA. Post-hoc comparisons were conducted using Tukey's test. For all analyses the significance level was $p < 0.05$. Graphical representations of data were constructed using GraphPad software (Prism Version 4.0). Photoshop software (v6.0 Adobe Systems, Mountain View, CA) was used to apply scale bars and text, adjust brightness and contrast and organize final layouts.

3. RESULTS

3.1 Body weight. The day that the litters were culled, P0, there was no significant difference in the body weights of the NL and SL pups. By P1 SL mouse pups showed a 29% increase in body weight compared to NL pups (NL: 1.37 ± 0.039 g; SL: 1.77 ± 0.12 g, $p < 0.01$; Fig.1A,) that grew to 42% by weaning (NL: 8.21 ±0.27 g; SL: 11.70 ± 0.64 g, $p < 0.05$; Fig.1A). Body weights for each group were also measured over the course of meal pattern testing and the group averages are plotted in Fig. 1B. There were no significant differences in body weight between NL and SL mice on either the first day of

testing, or the last day. However, each male group weighed significantly more than their respective female group at each of these time points ($p < 0.05$).

3.2 Adult fat pad weight. As shown in Fig.1C, at 6-8 mo of age when fat pads were removed, body weights of SL and NL males were the same, as were body weights of SL and NL females, although the body weights of each male group were increased compared to those of the corresponding female group (SL: 28%, NL: 23%; $p < 0.05$). Combined weights of the subcutaneous, retroperitoneal, and gonadal fat pads were greatest in SL males (1.29 ± 0.21 g), intermediate in SL females (0.64 ± 0.23 g) and lowest in NL males (0.49 ± 0.07 g) and females (0.44 ± 0.07 g), but only the differences between SL males and NL males or females were significant (195% increases, $p < 0.05$, Fig. 1D). Comparisons of individual fat pads showed similar trends that were significant for SL males vs. NL males or females for gonadal and retroperitoneal fat pads ($p < 0.05$, Fig. 1E).

3.3 Meal Pattern Analysis. Meal parameters stabilized by the end of wk 1. Therefore, data from days 8 – 22 were analyzed. Meal parameters quantified are shown for NL and SL mice in Fig. 2 A-H. Meal pattern parameter means and standard errors of NL males, NL females, SL males, and SL females are shown in Table I. There were no significant differences between sexes in the same litter size groups, or interactions between litter size and sex (for those parameters that could be assessed with parametric statistics), using two-way repeated measures ANOVA, as shown in Table II. Therefore, data in the text and in Fig. 2 are presented as litter size groups with NL males and females combined into one group and SL males and females combined into another group. All meal pattern parameters met both the normality and homogeneity of variance assumptions for parametric statistics except for meal size ($W = 0.913$, $p=0.01$), IMI ($W=$

0.917, p= 0.015), average intake rate of the first meal (W= 0.898, p=0.0047) and average intake rate of all meals (W= 0.819, p=0.00008). These parameters were tested using the non-parametric Kruskal-Wallis ANOVA.

NL animals ate an average of 2.87 ± 0.12 grams of a balanced solid diet each day with an average meal size of 0.36 ± 0.02 grams, an average meal duration of 29.54 ± 3.04 min, at an average rate of 0.012 ± 0.0021 g/min. Moreover, they consumed an average of 8.53 ± 0.51 meals per day with an average IMI of 71.62 ± 4.61 minutes and they exhibited a satiety ratio of 206.36 ± 12.59 min/g (satiety ratio is the ratio of meal size to the following intermeal interval). Compared with NL animals, SL mice exhibited a small, but reliable increase in daily food intake compared to NL animals (11%, 3.23 ± 0.15 grams, F (1,29)= 6.79, $p < 0.01$; Fig. 2A). Interestingly, small trends in several meal parameters of SL mice appeared to account for their increase in daily food intake, but because of the distributed nature of these changes, and the small magnitude of the increase in food intake, none of them were significant. Meal size increased (8%, 0.39 ± 0.024 grams; Fig. 2B), meal duration increased (18%, 35.88 ± 4.18 min, Fig.2C), meal number increased (5%, 8.99 ± 0.55 meals per day; Fig.2D), and concomitantly IMI decreased compared to NL mice (10% Fig.2E, NL: 71.62 ± 4.61 minutes; SL: 64.91 ± 4.37 minutes). However, SL mice did show a significant decrease in satiety ratio as compared to NL mice (20%, 172.27 ± 10.14 min/g, F (1,29)= 4.67, $p < 0.05$; Fig.2F), suggesting the small increase in intake of the SL group resulted because a given amount of food consumed produced less satiety than in NL mice. Interestingly, although there were no group differences in intake rate averaged over all meals (NL and SL: 0.012 ± 0.0021g/min; Fig.2G), SL animals displayed a 27% increase in average rate during the first meal (first 30 minutes of daily meal pattern collection; SL: 0.021 ± 0.002 g/min, NL: 0.017 ± 0.0014 g/min; H (1, N=33)= 5.83, $p < 0.05$; Fig.2H). This suggests the SL mice were more sensitive than NL mice to the mild food deprivation that preceded

this meal (fast occurred during the light phase and lasted for 6 hour prior to dark onset and food access).

3.4 Microstructure analysis. To identify a possible source of the rate difference during the first meal, the first 30 min rate curve was fit to the Weibull function. Unfortunately, none of the Weibull parameters exhibited group differences, although the intake profiles suggested that the similar initial rates were maintained slightly longer in the SL group (not shown), consistent with a trend for an increase in C, shape factor, for SL mice compared to NL mice (Table III).

3.5 Analysis of duodenal IGLEs. Duodenal IGLEs were small compared with gastric IGLEs, they were ovoid or elongated perpendicular to the organ's long axis, often interconnected by small fiber bundles and distributed relatively evenly throughout the muscle wall in the typical pattern of the myenteric plexus (Fig. 3). There were no differences in IGLE density in the duodenum between SL and NL animals (SL: 1388 ± 165/cm^2; NL: 1306 ± 108/cm^2; Fig.3). Given that fat pad changes were only significant in males, we compared IGLE density between groups separately for males and females, but there were still no differences (not shown). Also, on the basis of qualitative inspection of all samples there did not appear to be differences in the morphology of IGLEs in the duodenum between SL and NL groups (Fig.4).

4. DISCUSSION

In the present study mice 3-4 months of age that experienced early postnatal overnutrition, but had not yet become obese exhibited a small, but reliable increase in daily food intake, an increase in intake rate during their first daily meal, and a decrease

in satiety ratio compared to mice that experienced normal postnatal nutrition. The increased daily food intake of SL mice resulted from a combination of small non-significant changes distributed over several meal parameters. Additionally, early postnatal overnutrition had no effect on either the density or morphology of IGLEs that innervate the muscle wall of the duodenum.

4.1. Body weight and fat pad weight

The use of divergent litter sizes is a well-established method in rodents to manipulate the amount of mother's milk consumed by nursing offspring [3,24,32-36]. The differences in growth for SL and NL mice prior to weaning in the present investigation correspond well with those observed in previous studies [3,32,34,36]. SL animals exhibited greater fat pad weight as compared to NL mice at 6-8 months of age even though their body weights were similar. This finding is consistent with previous studies that found increased fat pad weight, cell number, or cell size prior to development of significant body weight differences [1,38,42], and it suggests the underlying predisposition to develop obesity was present. Interestingly, this increased fat pad weight was significant for SL males vs. NL males and females, but not for SL females. This sex difference is similar to previous findings, which had been interpreted to suggest that females were partially resistant to the effects of altered early nutrition [4].

4.2. Feeding behavior: early signs of predisposition to develop obesity

No striking changes in the feeding behaviors assayed were observed in response to early postnatal overnutrition. However, a reliable pattern of related behavior changes that were modest in magnitude was observed. These included increased food intake, increased rate of food intake during the first daily meal, and a decreased satiety ratio. The decreased satiety ratio suggested the increased food intake was due to a reduced

effectiveness of food to induce satiety. This deficit may also have contributed to the increased sensitivity to mild food deprivation observed in SL mice, which was expressed as an increased rate of intake during the first meal of each day. Importantly, the increased food intake, increased first meal rate and satiety ratio effects, as well as the increased fat pad weight observed in SL males all occurred without differences in body weight between SL and NL mice, suggesting they may represent primary effects of the impact of postnatal overnutrition on development or function of neurohormonal systems regulating body weight.

The degree of satiety produced by a meal influences the length of the delay until initiation of the next meal. Food is still present in the upper GI tract after a meal ends, and therefore may contribute to satiety through vagal afferent activation. Additionally, the hormones released upon nutrient stimulation of the GI tract, as well as absorption of nutrients from the GI tract and their utilization by peripheral tissues and the brain make major contributions to satiety, and are thus sites that should be considered for examination of the primary effects of early postnatal overnutrition.

One factor that may have contributed to the small non-significant group differences on most primary meal parameters observed is that an undernutrition group (large litter size) was not included for comparison with normal and overnutrition groups. Studies reporting large differences on some measures between animals raised in different litter sizes often compared animals from small litters with animals from large litters rather than normal size litters [1, 37]. For example, Faust et al. [37] found a 28% increase in body weight in rats that experienced early postnatal overnutrition compared to rats that received undernutrition, whereas, we found a 10% increase in body weight when comparing effects of early postnatal overnutrition with those of normal nutrition (normal litter size). Another factor that could have minimized meal parameter differences was that we combined data from the dark (all 12 hours) and light (first 6 hours) phases

for these analyses. Most feeding occurs in the dark phase and previously we have observed some group differences in meal parameters to be larger in this phase than in both phases combined [43].

4.3. Vagal GI Afferents

The meal pattern, microstructural and neuroanatomical analyses employed in the present study did not reveal any potential deficits in either development or function of vagal GI afferents. The only indicator of potential vagal involvement that approached significance was the Weibull parameter, C, or shape factor, which if significant would have suggested that vagal sensory signaling of negative feedback was reduced by postnatal overnutrition. Nevertheless, it remains possible that development of vagal GI afferents or efferents that were not examined in the present study was altered, including those that innervate the stomach wall and the duodenal mucosa. Such partial loss of vagal function might not be detected under the conditions we employed, in particular, if remaining receptors compensated. Lacking this, the present results suggest that development and function of vagal GI afferents, or at minimum, duodenal IGLEs - possibly as a consequence of their critical role in regulation of food intake - are resistant to the effects of nutritional overstimulation at early postnatal ages, including the blunting of the neonatal leptin surge it produces [44].

One factor that may have contributed to the lack of a difference in IGLE density among SL and NL mice was the lack of an undernutrition group as described above for meal parameters. Factors that could have masked an actual effect of overnutrition on IGLE density were altered length, diameter or thickness of the outer muscle wall of the small intestine due to overnutrition. This could have differentially affected the IGLE density determinations in the NL and SL groups and could have negated a group difference. However, we determined that there were no differences in duodenum

diameter between the groups, and in a subset of the animals in which the small intestine length had been measured we found no group difference (not shown). We did not measure thickness of the muscle wall. However, IGLEs lie in a plane immediately above or below the myenteric plexus, which is single tissue layer. Therefore, a change in thickness of the muscle layers above and below the myenteric plexus layer, including IGLEs should not impact the numbers of IGLEs counted per unit area. In contrast, counts of elements within the muscle layers could be affected.

Given the conclusion that early postnatal overnutrition did not alter vagal afferents, it is most probable that the effects we observed on feeding behavior are early symptoms of the well characterized effects of postnatal overnutrition on hypothalamic function. Changes that occur with aging and availability of a palatable, high energy diet may be necessary for the predisposition to develop obesity to become fully expressed. It will therefore be important to determine: (1) whether the pattern of effects we observed can be replicated in the same and different mouse strains, (2) whether the altered satiety observed would be detected using satiety sequence methodology, (3) whether the magnitude of these effects correlates with degree of obesity and metabolic disorders that ultimately develop, (4) how the meal pattern and microstructural changes progress as obesity develops, and (5) whether a similar pattern of changes occurs in response to other manipulations of perinatal nutrition that predispose animals to develop obesity and the metabolic syndrome. Determining the mechanisms underlying early symptoms common to different mouse strains and nutritional manipulations will aid precise delineation of the primary effects of hypothalamic, sympathetic and hormonal alterations caused by abnormal perinatal nutrition that lead to obesities and the metabolic syndrome of developmental origin.

ACKNOWLEDGEMENTS

The authors would like to thank Amanda Sharp for assistance with meal pattern data collection, Terry Powley and Kim Kinzig for helpful comments on an earlier version of this manuscript, and Tom Karam for animal care. Preliminary reports of the present findings were presented in abstract form at the annual meeting of the Society for Neuroscience (Biddinger, Sharp and Fox, 2009). This manuscript was based in part on a Master's thesis submitted by Jessica Biddinger in partial fulfillment of the requirements of the Master's degree. This work was supported by National Institutes of Health R01 grant NS046716.

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FIGURES LEGENDS

Figure 1. SL pups gained more weight than NL pups, suggesting they experienced overnutrition, and had more fat as adults, despite no difference in their body weight. (A) Body weights of NL or SL mouse pups measured daily from P0 until weaning. SL mice began to diverge and weigh significantly more than NL mice beginning at P1 (open squares, NL; filled squares, SL). (B) Body weight during collection of meal pattern data shown at the start of the meal pattern analysis, body weight was not significantly different between NL and SL males, or between NL and SL females. (C) At 6-8 months of age, when fat pads were removed, body weights of both NL and SL males were higher than females. There was no difference within sex. (D) Total fat pad weights of SL males at 6-8 months of age, the age of the animals at nodose injection, were higher than NL males and females. Fat pad weights from SL females were not different from SL males, or either sex from NL. (E) Gonadal, and retroperitoneal fat pads from SL males weighed more than the respective fat pads from the other groups. * denotes $p < 0.05$.

Figure 2. At 3-4 months of age, SL mice displayed greater total daily intake than NL mice, and small trends in several meal parameters of SL mice appeared to account for this increase in daily food intake. (A) Total daily food intake by SL mice was slightly, but significantly higher than NL mice (open bars, NL; filled bars, SL). (B) SL mice showed a trend towards larger meal size than NL mice. (C) SL mice showed a trend towards longer meal durations than NL mice. (D) SL mice trended toward consuming more meals than NL mice. (E) SL mice showed a trend towards a decreased IMI as compared with NL mice. (F) Satiety ratio was significantly decreased in SL mice compared to NL mice. (G) Average food intake rate throughout the 18 hr of daily meal pattern collection was

similar in mice raised in both SL and NL mice. (H) Average food intake rate during the first daily meal was higher in SL mice as compared with NL mice.

Figure 3. NL and SL mice showed similar average IGLE densities. IGLEs were labeled by anterograde WGA-HRP transport after injection into the left nodose ganglion followed by staining with TMB at 6-8 months of age.

Figure 4. Animals from both litter sizes qualitatively showed similar IGLE morphology. Examples are shown from an NL male (A), an SL male (B), an NL female (C), and an SL female (D). Arrows denote IGLEs. All IGLEs shown are from the 4-8 cm segment of the duodenum. All images are photomontages of equal magnification (total magnification, 100X). Scale bar is 200µm.

TABLES

Table I. Means ± SEM of meal pattern parameters for male and female mice of NL and SL groups.

	NL		SL	
Meal Pattern Parameter	Male	Female	Male	Female
Total Intake, g	2.85 ± 0.12	2.89 ± 0.14	3.37 ± 0.12	3.06 ± 0.14
Meal Size, g	0.36 ± 0.02	0.35 ± 0.03	0.38 g ± 0.02	0.39 g ± 0.03
Meal Duration, min	31.38 ± 5.40	26.91 ± 6.46	32.99 ± 5.69	39.59 ± 6.46
Meal Number, meals	8.53 ± 0.59	8.51 ± 0.71	9.52 ± 0.63	8.30 ± 0.71
Intermeal Interval, min	71.18 ± 3.89	72.24 ± 4.68	64.14 ± 4.11	65.89 ± 4.66
Satiety Ratio, min/g	203.28 ± 15.94	206.37 ± 22.60	174.72 ± 12.46	169.12 ± 11.07
Average Rate, g/min	0.014 ± 0.002	0.016 ± 0.003	0.015 ± 0.003	0.010 ± 0.003
1st 30 min Rate, g/min	0.009 ± 0.0006	0.009 ± 0.0007	0.011 ± 0.0006	0.011 ± 0.0007

Table II. Statistics for litter size and sex comparisons and litter size x sex interactions.

Meal Pattern Parameter	Litter Size	Sex	Litter Size x Sex
Total Intake†	F (1,29)= 6.79, p= 0.014*	F(1,29)= 0.967, p= 0.33	F(1,29)= 1.95, p= 0.17
Average Meal Duration†	F (1,29)= 1.41, p= 0.24	F (1,29)= 0.0314, p= 0.86	F (1,29)= 0.847, p= 0.37
Meal Number†	F (1,29)= 0.349, p= 0.56	F (1,29)= 0.858, p= 0.36	F (1,29)= 0.824, p= 0.37
Satiety Ratio†	F (1,29)= 4.67, p= 0.039*	F (1,29)= 0.00040, p= 0.98	F (1,29)= 0.226, p= 0.64
Meal Size¥	H (1, N=33)= 1.68, p= 0.19	H (1, N=33)= 0.16, p= 0.69	
Average Intermeal Interval¥	H (1, N=33)= 2.18, p= 0.14	H (1, N=33)= 0.0013, p= 0.97	
Average Rate¥	H (1, N=33)= 1.27, p= 0.26	H (1, N=33)= 0.017, p= 0.90	
Average 1st 30 minute Rate¥	H (1, N=33)= 5.83, p= 0.01*	H (1, N=33)= 0.58, p= 0.46	

* Indicates significance. Meal pattern parameters that did not violate normal distribution assumptions were analyzed for significance using repeated measures ANOVA are denoted by †. Non-parametric meal pattern parameters were analyzed using the Kruskal-Wallis test and are denoted by ¥.

Table III. Weibull parameters derived from the first meal of daily meal pattern collection.

Weibull parameter	NL	SL	P - value
Initial (A)	21.67 ± 2.29	20.71 ± 2.8	0.577
Decay (B)	2.93 ± 0.74	3.93 ± 0.97	0.565
Shape (C)	2.41 ± 1.12	0.74 ± 0.35	0.134

Intake rates were calculated for each animal daily and averaged over days 8-22 of behavioral testing for mice raised in either NL's or SL's and the resulting curves were fit with the Weibull function. P-values of < 0.05 were taken as statistically significant and denoted by *.