Early postnatal overnutrition increases adipose tissue accrual in response to a sucrose-enriched diet

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Submitted 5 December 2011; accepted in final form 10 April 2012


Numerous studies have shown that an individual’s propensity to gain weight during adulthood can be affected by prenatal and neonatal changes in nutrition (23, 42, 57). Moreover, it is now clear that it is not only the amount but also the type of energy consumed that determines weight gain and body composition during both early development and adulthood (1, 23, 40, 54). Thus, poor nutrition and overnutrition during maturational periods and adulthood are likely to synergize in causation of the exponential rise in obesity observed in many populations.

The response to a high-fat diet in adulthood is clearly affected by early developmental events (6, 16, 18, 23, 58). For example, Glavas et al. (23) demonstrated that the adverse responses to a high-fat diet in adulthood are augmented in mice that had increased food availability during lactation due to a reduction in litter size. Reduction in litter size is a relatively noninvasive experimental manipulation that results in increased consumption due to increased maternal milk availability. This induces increased weight gain and food intake, not only during nursing but also after being weaned, in addition to modifying the response to dietary challenges in adulthood (6, 7, 17, 19, 20, 23, 44, 57). Current dietary habits include not only a high intake of fat but also increased carbohydrate consumption. However, how early nutritional status affects the response to a diet rich in sugars is less well studied.

Increased sugar consumption has been associated with the current rise in obesity and its secondary complications in humans (8, 15, 27). This rise in sugar consumption is due partially to the increased intake of sugar-sweetened beverages, often containing high-fructose corn syrup, which is composed of 55% fructose and 45% glucose, or sucrose (table sugar), which is 50% fructose and 50% glucose. It has been reported that, in contrast to glucose, fructose does not stimulate insulin or leptin production, resulting in differing metabolic responses (15, 27, 53), with sucrose having intermediate effects on some, but not all, metabolic parameters (53). Thus, not only does the increase in sugar intake constitute an added caloric burden, but the individual’s overall metabolism is differentially affected depending on the type of carbohydrate ingested. These differential metabolic effects may help to explain the diverse weight gain responses reported after increased sugar consumption, because some studies describe increased weight gain (38, 46, 56), whereas others suggest the opposite response (9, 20, 22, 28, 36). These different outcomes may be due to the type of subjects studied as well as the different sugars analyzed, the duration of increased sugar intake, or the age at which this intake commences. Because the proliferative potential of adipocytes is affected by age and the prenatal/neonatal nutritional status (5, 33, 63), the effects on this tissue may vary depending upon when during postnatal development a dietary change is introduced.

In the studies reported here, our aim was to analyze how neonatal overnutrition due to a reduction in litter size affects
the weight gain and fat accrual response to a diet rich in sucrose in later life. To determine whether the duration and timing of sucrose intake affects this response, sucrose was introduced to the diet either at the moment rats were weaned or in adulthood.

MATERIALS AND METHODS

Animals. All experiments were designed according to the European Union laws for animal care, and the studies were approved by the local institutional ethics committee. Wistar rats were employed for all experiments. On the day of birth, litters were adjusted to four (L4) or 12 (L12) pups per dam, with equal numbers of males and females in each litter. A total of 36 litters were used with four to eight different litters of each experimental group. Cross-fostering of all litters was employed to increase the genetic variability in each litter. Mean initial body weight (BW) did not differ between litters. After being weaned on postnatal day (PND) 21, rats were placed two per cage under constant conditions of temperature (20–22°C), humidity (50 ± 1%), and light (lights on from 0730 to 1930). Only males were used for these experiments. In each parameter analyzed, rats from three to four different litters of each experimental group were used, with equal representation of each litter in each analysis. Rats were weighed weekly, and 24-h food intake was determined once/wk from PND 21 until the time of euthanization. Food intake was measured by placing a known quantity of food in each cage and weighing the remaining amount 24 h later and dividing by the number of rats in the cage.

All rats were euthanized by decapitation at 80 days of age between 900 and 1100 after a 12-h fast. Trunk blood was collected in cooled tubes, allowed to clot, and centrifuged, and the serum was stored at −80°C until hormone levels were measured. The brains, soleus muscle, and visceral and subcutaneous adipose tissue were removed, weighed, rapidly frozen on dry ice, and stored at −80°C until they were processed.

Experimental design 1. Male rats raised in litters of four or 12 pups were allowed to eat normal rat chow (Panlab, Barcelona, Spain) and drink fresh tap water ad libitum until PND 65. At this time, each group was divided into two groups, those that continued to receive fresh tap water (Ct) or those receiving a solution of 33% sucrose (S) in tap water. All rats continued to receive normal rat chow and were allowed to eat and drink ad libitum for 2 wk (2W) until they were euthanized, as described above. This resulted in the following groups: L4Ct (n = 8), L4S2W (n = 8), L12Ct (n = 12), and L12S2W (n = 12).

Experimental design 2. Male rats raised in litters of four or 12 pups were employed. On PND 21, when they were weaned, one half of each group received rat chow and were allowed to eat and drink ad libitum for 2 mo (2M) until they were euthanized at 80 days of age, as described above. This resulted in the following groups: L4Ct (n = 16), L4S2M (n = 12), L12Ct (n = 24), and L12S2M (n = 24).

In both experimental designs, liquid intake was measured daily once sucrose was introduced into the diet.

Oral glucose tolerance test. In both experiments an oral glucose tolerance test (OGTT) was performed on PND 73. The day before they underwent the OGGT, the rats were weighed and fasted overnight. The following morning, glucose was measured by extracting blood by subclavicular venepuncture and measured with a glucometer (ACU-Chek; Roche Diagnostics). The rats were allowed to recover for 1 h from the venipuncture before receiving a bolus of glucose (1 mg/kg BW) by introduction of a probe into the esophagus. Glycemia was determined 20, 60, and 120 min after the bolus of glucose was administered. The area under the curve (AUC) for changes in glycemia was calculated by the following formula: 0.25 × (fasting value) + 0.5 × (half-hour value) + 0.75 × (1-h value) + 0.5 × (2-h value) (25, 45) and by the trapezoidal method, which gave the same results.

Corticosterone and total and acylated ghrelin RIA. Total and acylated ghrelin were measured by RIA, following the manufacturer’s instructions (Linco Research, St. Charles, MO). The sensitivity of the method was 93 pg/ml for both assays, and the intra- and interassay coefficients of variation were 6.4 and 16.3%, respectively, for total ghrelin and 7.4 and 13.4%, respectively, for acylated ghrelin, respectively. To avoid decyacetylation, an aliquot of serum was rapidly frozen and used only for this determination. This has been shown previously to prevent degradation (29).

Serum corticosterone levels were measured by RIA, following the manufacturer’s instructions (MP Biomedicals, Orangeburg, NY). The assay sensitivity was 7.7 ng/ml, and the intra- and interassay variations were 7.3 and 6.9%, respectively. Samples were run in duplicate and within the same RIA for all determinations.

Leptin, insulin, and adiponectin ELISAs. Serum levels of leptin, insulin, and adiponectin were measured by ELISA, following the manufacturer’s instructions (Linco Research). The sensitivities of the methods for leptin, insulin, and adiponectin were 0.04, 0.2, and 0.16 ng/ml, respectively. Absorbance in each well was measured using a Tecxan (Grödig, Austria) Infinite M200, and serum hormone concentrations were calculated from the standard curve. For all hormones, samples were run in duplicate and within the same assay. The intra-assay variation was 2.2% for leptin, 1.9% for insulin, and 1.3% for adiponectin.

Determination of serum cholesterol, triglycerides, free fatty acids, glyceral, and total protein. Serum glyceral levels were determined by using a free glycerol determination kit (Sigma Aldrich, St. Louis, MO). This assay involves coupled enzyme reactions, resulting in the production of quinoneimine dye that shows an absorbance maximum at 540 nm. The increase in absorbance is directly proportional to the free glyceral concentration in the sample. Total lipids, triglycerides, cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were measured in plasma samples by using commercial enzymatic colorimetric assay kits purchased from SpinReact (Sant Esteve de Bas, Gerona, Spain) and free nonesterified fatty acids (FFA) with a kit from Wako Chemicals (Neuss, Germany).

All assays were performed according to the manufacturer’s instructions.

Quantitative real-time PCR. The hypothalami were isolated on ice using the following boundaries: an anterior cut made at the level of the optic chiasm, a posterior coronal cut anterior to the mammillary bodies, two sagittal cuts parallel to the lateral ventricles, and a dorsal horizontal section at the level of the anterior commissure. RNA was extracted from each hypothalamus (n = 4–6/group) according to the Tri-Reagent protocol. Quantitative real-time PCR was performed on cDNA prepared from 1 µg of total RNA isolated from hypothalamic samples. Assay-on-demand kits with Universal PCR Master Mix (Applied Biosystems, Foster City, CA) were used according to the manufacturer’s protocol and analyzed by using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Neuropeptide Y (NPY; Rn01410145), proopiomelanocortin (POMC; Rn00595020), agouti-related protein (AgRP; Rn01431703), cocaine- and amphetamine-related transcript (CART; Rn00567382), orexin (Hcrt; Rn00565995), and leptin receptor (Lepr; Rn01433205) gene expression was assessed in each hypothalamic sample. Values were normalized to the reference gene phosphoglycerate kinase 1 (Pgk1; Rn00821429). The ΔΔCT method was used for relative quantification according to manufacturer’s guidelines. Statistics were performed using the ΔΔCT values.

Analysis of epididymal adipocyte morphology. Crystallization images (10 µm) of epididymal fat were fixed with 10% formol, stained with hematoxylin and eosin, and visualized by using a light microscope. Images were captured with a digital camera, and Image-Pro Plus software (version 5.0; Media Cybernetics, Silver Spring, MD) was used to measure the mean adipocyte circumference. Six random images were captured from four different sections from each animal. Six cells were analyzed on each section (144 adipocytes/rat). The mean adipocyte perimeter was then calculated for each animal (n = 4).
and used to determine the mean circumference for each experimental group.

Statistical analysis. All data are presented as means ± SE. When only two groups were compared, a two-tailed Student t-test was employed. A two-way ANOVA was used to determine the effect and interaction of litter size and sucrose on each variable analyzed. A two-way ANOVA with repeated measurements was used when multiple measurements in the same animal were taken (weight, food intake, OGTT). When significant interactions were found, a one-way ANOVA was then performed to determine where significant differences existed. Scheffe’s F-test was used for posthoc analyses. Linear correlations were performed by using Pearson’s Product Moment Correlation. The results were considered statistically significant at P < 0.05.

RESULTS

Effect of neonatal overnutrition and short-term sucrose consumption on food and liquid intake and weight gain. The mean daily chow intake from the time that the rats were weaned (PND 21) until sucrose was introduced into the diet (PND 65) was greater (P < 0.001) in rats from L4 (24.3 ± 1.0 g) than in those from L12 (21.3 ± 0.3 g). Rats from L12 weighed less than those from L4 before the diet change, with no difference between the rats of the same litter size that were to receive sucrose or tap water for the next 2 wk [L12Ct 240.8 ± 4.6 g, L12S2W 239.9 ± 4.2 g, L4Ct 252.8 ± 6.3 g, L4S2W 251.4 ± 3.6 g; F(1,38): 1.108; P < 0.01].

The mean daily food intake during the 2 wk of diet change was affected by sucrose intake [F(1,19): 15.989, P < 0.001], with no interaction between litter size and diet. Rats given the sucrose solution ate significantly less chow than their controls from the same size litter [F(1,19): 7.095, P < 0.004; Fig. 1A].

There was an interaction between litter size and the sucrose-enriched diet on the mean amount of liquid ingested [F(1,19): 11.975, P < 0.005]. L12Ct rats drank more than L4Ct rats. L4 rats drinking sucrose ingested significantly more liquid than their controls [F(3,19): 3.892, P < 0.03; Fig. 1B].

There was an effect of diet on total kcal intake/day [F(1,19): 6.020, P < 0.03]. L4 rats ingesting sucrose consumed more kcal than their controls, whereas L4Ct rats consumed fewer calories than L12Ct rats [F(3,19): 11.293, P < 0.0001; Fig. 1C]. The total kcal consumption/day from sucrose was higher in rats from L4 (36.3 ± 1.4 kcal/day) than those from L12 [32.2 ± 1.2 kcal/day, F(1,19): 439.866, P < 0.0001].

During 2 wk of a sucrose-enriched diet, weight gain was affected by both litter size [F(1,38): 10.759, P < 0.003] and sucrose intake [F(1,38): 7.198, P < 0.02]. Control L4 rats gained more weight during this period of the study than control L12 rats [F(3,38): 6.283, P < 0.01; Fig. 1D]. Sucrose intake reduced weight gain in rats from L12, whereas this decrease was not significant in L4 rats.

There was an effect of both diet [F(1,30): 28.416, P < 0.0001] and litter size [F(1,30): 17.362, P < 0.0002] on metabolic

Fig. 1. Mean chow intake/day (A), liquid intake/day (B), total kcal/day (C), weight gain (D) and metabolic efficiency (g gained/kcal ingested; E) during 2 wk (2W) of a sucrose (S)-enriched diet (33% sucrose solution instead of water), and the final weight at the end of the study (80 days of age; F) in male rats that were raised in litters of 12 (L12) or 4 pups (L4) from the day of birth. L12Ct, rats from litters of 12 pups receiving control diet (n = 12); L4Ct, rats from litters of 4 pups receiving control diet (n = 8); L12S2W, rats from litters of 12 pups receiving a S-enriched diet for 2 wk (n = 12); L4S2W, rats from litters of 4 pups receiving a S-enriched diet for 2 wk (n = 8).

#One-way ANOVA, P < 0.0001; *1-way ANOVA, P < 0.05.
efficiency (grams gained/kcal consumed). Control L4 rats had a higher metabolic efficiency during the period of diet change compared with L12 controls [$F_{(3,30)}$: 13.119, $P < 0.0001$; Fig. 1E]. Sucrose intake decreased metabolic efficiency in both groups.

There was an effect of litter size [$F_{(1,23)}$: 18.387, $P < 0.0001$] and sucrose [$F_{(1,23)}$: 15.601, $P < 0.0001$] on BW at the time of euthanization. On a normal diet L12 rats weighed less than L4 rats, and sucrose intake decreased BW in L4 rats [$F_{(3,23)}$: 12.372, $P < 0.05$; Fig. 1F].

**Effect of neonatal overnutrition and short-term sucrose consumption on adipose tissue and muscle mass.** Sucrose intake, but not litter size, affected the amount of subcutaneous fat normalized to BW (g/100 g BW) [$F_{(1,24)}$: 35.042, $P < 0.0001$]. Rats on a sucrose-enriched diet had more subcutaneous fat regardless of litter size [$F_{(3,24)}$: 12.488, $P < 0.0001$; Fig. 2A]. Similar results were found for absolute weight of fat pads [L12Ct 1.09 ± 0.10 g, L12S2W 1.55 ± 0.08 g, L4Ct 1.13 ± 0.07 g, L4S2W 1.70 ± 0.09 g; $F_{(3,24)}$: 3.778, $P < 0.0001$].

Visceral fat mass normalized to BW was affected by sucrose intake [$F_{(1,24)}$: 6.897, $P < 0.02$] but not litter size. Relative visceral fat mass was increased by sucrose intake in L4 but not in L12 rats [$F_{(3,24)}$: 4.537, $P < 0.01$; Fig. 2B]. However, there was an effect of both litter size [$F_{(1,24)}$: 18.341, $P < 0.0003$] and sucrose intake [$F_{(1,24)}$: 14.085, $P < 0.001$] on the absolute weight of visceral fat pads [L12Ct 2.7 ± 0.2 g, L12S2W 3.3 ± 0.2 g, L4Ct 3.4 ± 0.1 g, L4S2W 4.3 ± 0.2 g; $F_{(3,24)}$: 2.414, $P < 0.0001$], with L4 rats having significantly more visceral fat than L12 rats.

Both neonatal overnutrition [$F_{(1,15)}$: 144.979, $P < 0.0001$] and sucrose intake [$F_{(1,15)}$: 44.848, $P < 0.0001$] induced hypertrophy of visceral adipocytes, with an interaction between these two factors [$F_{(1,15)}$: 21.685, $P < 0.0001$; Fig. 2, C–F]. Mean adipocyte perimeter was greater in L4 compared with L12 controls, with sucrose intake augmenting this parameter in L4 but not L12 rats [$F_{(3,15)}$: 70.504, $P < 0.0001$; Fig. 2G].

As an index of changes in lean mass, the soleus muscle was weighed. There was no effect of either neonatal nutrition or sucrose intake on this parameter (L12Ct 0.50 ± 0.02 g, L12S2W 0.47 ± 0.01 g, L4Ct 0.51 ± 0.01 g, L4S2W 0.50 ± 0.01 g/100 g BW).

**Effect of neonatal overnutrition and short-term sucrose consumption on glycemia, circulating hormone levels, and serum biochemical parameters.** On the day of the OGTT, basal glucose levels were significantly higher in L4 compared with those from L12 regardless of which diet they received [L12Ct 70.0 ± 4.9, L12S2W 81.1 ± 6.8, L4Ct 95.6 ± 2.4, and L4S2W 95.9 ± 4.2 mg/dl; $F_{(3,26)}$: 5.570, $P < 0.001$]. However, there was no difference in the AUC for glycemia during the OGTT (L12Ct 283.4 ± 11.8, L12S2W 285.4 ± 17.1, L4Ct 292.4 ± 22.7, L4S2W 312.6 ± 19.2 mg·dl$^{-1}$·120 min$^{-1}$).

The effect of neonatal overnutrition and short-term sucrose intake on circulating hormone levels and biochemical parameters at the moment of euthanization is shown in Table 1. There was no significant effect of either sucrose intake or litter size on fasting insulin levels. Both litter size [$F_{(1,28)}$: 7.459, $P < 0.01$] and sucrose intake [$F_{(1,28)}$: 13.177, $P < 0.0001$] affected leptin levels, with no interaction between these factors. Sucrose intake increased leptin levels regardless of litter size, with this increase being significantly greater in L4 rats. There was a significant positive correlation between circulating leptin levels and mean visceral adipocyte perimeter ($r = 0.70, P < 0.003$).

Sucrose intake increased adiponectin levels [$F_{(1,29)}$: 27.228, $P < 0.0001$] regardless of litter size. Total ghrelin levels were modified by litter size [$F_{(1,23)}$: 28.138, $P < 0.0001$], with no effect of sucrose, whereas acetylated ghrelin levels were unaffected. L4 rats had reduced total ghrelin levels compared with L12. This resulted in the percent acylation being modified by litter size [$F_{(1,23)}$: 10.200, $P < 0.005$], with rats from L4 having...
a higher percentage of acylation when ingesting a high-sucrose diet.

Corticosterone levels were affected by sucrose intake [F(1,199) = 9.095, P < 0.008], with sucrose intake reducing corticosterone levels in L12 but not in L4 rats.

Sucrose intake affected both total cholesterol [F(1,123) = 13.651, P < 0.001] and LDL cholesterol levels [F(1,23) = 16.950, P < 0.001], with the circulating levels of both parameters being weaned and the introduction of sucrose into the diet, rats on the sucrose-enriched diet already weighed less than their controls, and this difference persisted throughout the study (Fig. 4A). Throughout the study, L4 rats ingesting sucrose weighed significantly more than L12 rats ingesting sucrose. However, the difference between L4 and L12 controls observed at weaning was no longer significant 3–5 wk after being weaned, becoming significant once again on the 6th wk after being weaned.

The mean daily chow intake from the time the rats were weaned until PND 80 was affected by litter size [F(1,137) = 23.049, P < 0.0001] and sucrose intake [F(1,137) = 233.700, P < 0.0001], being greater in L4 than in L12 rats regardless of whether they were fed a normal or sucrose-enriched diet [F(1,137) = 103.716, P < 0.0001; Fig. 4B]. Sucrose reduced chow intake in both groups.

The daily liquid intake from the time the rats were weaned until euthanization was affected by diet [F(1,137) = 21.249, P < 0.0001]. Rats on the sucrose-enriched diet ingested less liquid compared with their controls [F(1,137) = 8.155, P < 0.0001; Fig. 4C].

Both litter size [F(1,137) = 16.577, P < 0.0001] and diet [F(1,137) = 75.880, P < 0.0001] affected the mean number of kilocalories consumed. L4 rats consumed more than L12 rats, and all rats on a sucrose-enriched diet had a higher kilocalorie intake than their controls [F(1,137) = 29.967, P < 0.0001; Fig. 4D].

The metabolic efficiency from weaning until euthanization at 80 days of age was affected by litter size [F(1,73) = 6.087, P < 0.02] and sucrose intake [F(1,73) = 162.937, P < 0.0001]. Rats consuming sucrose gained fewer grams per kilocalories consumed [F(1,73) = 64.343, P < 0.0001; Fig. 4E].

Body weight at the end of the study was affected by litter size [F(1,73) = 17.063, P < 0.0001] and sucrose intake [F(1,73) = 9.095, P < 0.008], with sucrose intake reducing corticosterone levels [F(1,199) = 9.095, P < 0.008].
38.898, \( P < 0.001 \), with L4 rats weighing more than L12 rats on the same type of diet. Sucrose intake decreased BW regardless of litter size [\( F_{(3,73)}: 22.821, P < 0.0001 \); Fig. 4F].

**Effect of neonatal overnutrition and long-term sucrose consumption on adipose tissue.** Long-term sucrose intake affected the amount of subcutaneous fat/100 g BW [\( F_{(1,51)}: 24.305, P < 0.0001 \)], with an interaction between litter size and sucrose intake [\( F_{(1,51)}: 5.365, P < 0.03 \)]. In L4 rats, the sucrose-enriched diet resulted in more subcutaneous fat [\( F_{(3,51)}: 8.634, P < 0.0001 \); Fig. 5A]. Similar results were found in the absolute weight of subcutaneous fat pads [L12Ct 1.65 ± 0.16, L12S2M 1.88 ± 0.07, L4Ct 1.59 ± 0.14, L4S2M 2.33 ± 0.12 g; \( F_{(3,51)}: 4.574, P < 0.0001 \)].

The amount of visceral fat relative to BW was affected by litter size [\( F_{(1,51)}: 9.280, P < 0.005 \)] and sucrose intake [\( F_{(1,51)}: 70.09, P < 0.0001 \)]. Sucrose induced the accumulation of visceral fat in rats of both litter sizes, with this effect being greater in those with neonatal overnutrition [\( F_{(3,51)}: 26.469, P < 0.0001 \); Fig. 5B]. The absolute weight of visceral fat pads was significantly greater in rats with neonatal overnutrition and increased in response to sucrose intake regardless of litter size [L12Ct 3.2 ± 0.2, L12S2M 4.4 ± 0.3, L4Ct 3.8 ± 0.1, L4S2M 6.2 ± 0.6 g; \( F_{(3,51)}: 16.183, P < 0.0001 \)].

Both neonatal overnutrition [\( F_{(1,15)}: 244.514, P < 0.0001 \)] and sucrose intake [\( F_{(1,15)}: 18.001, P < 0.002 \)] induced hypertrophy of visceral adipocytes, with an interaction between these factors [\( F_{(1,15)}: 32.424, P < 0.0001 \); Fig. 5, C–F]. Mean adipocyte perimeter was greater in L4 compared with L12 controls, with sucrose intake augmenting this parameter in L12 but not L4 rats [\( F_{(3,15)}: 98.313, P < 0.0001 \); Fig. 5G].

There was a significant effect of both litter size [\( F_{(1,51)}: 9.547, P < 0.003 \)] and sucrose intake [\( F_{(1,51)}: 42.417, P < 0.0001 \)] on relative muscle weight. Rats with neonatal overnutrition had increased relative muscle mass, whereas sucrose intake decreased muscle mass regardless of neonatal nutrition [L12Ct 0.49 ± 0.02, L12S2M 0.39 ± 0.02, L4Ct 0.59 ± 0.02, L4S2M 0.42 ± 0.02 g/100 g BW; \( F_{(3,51)}: 17.321, P < 0.0001 \)].
Effect of neonatal overnutrition and long-term sucrose consumption on glycemia and serum hormone levels and biochemical parameters. One week before termination of the study, rats were submitted to an OGTT. Basal glucose levels were not different between groups (L12Ct 130.0 ± 15.3, L12S2M 122.6 ± 6.2, L4Ct 132.4 ± 4.9, L4S2M 118.1 ± 9.6 mg/dl). There was an effect of long-term sucrose intake on glycemia levels over time in the OGTT \(F(1,31) = 10.296, P < 0.003; \) Fig. 6], with rats ingesting sucrose having reduced glycemia levels and AUC compared with their controls [L12Ct 376.1 ± 22.5, L12S2M 337.9 ± 12.9, L4Ct 402.1 ± 19.1, L4S2M 324.4 ± 14.1 AUC; \(F(3,31); 4.078, P < 0.02\). At 120 min after the glucose bolus, L4 rats maintained elevated glycemia levels compared with L12 and L4S2M rats [L12 184.1 ± 9.1, L12S2M 174.0 ± 7.8, L4 227.0 ± 15.1, L4S2M 158.5 ± 7.6 mg/dl; \(F(3,31); 8.020, P < 0.0005\)].

Serum hormone levels and biochemical parameters for experiment 2 are shown in Table 2. Circulating insulin levels
Long-term sucrose intake increased serum adiponectin levels regardless of litter size \([F_{(1,22)}: 30.500, P < 0.0001]\). There was no effect of litter size or long-term sucrose intake on corticosterone levels.

Total ghrelin levels were affected by litter size \([F_{(1,26)}: 8.606, P < 0.007]\), with L4 rats having reduced levels compared with L12 \([F_{(3,26)}: 3.141, P < 0.04]\). Acylated ghrelin levels were affected by litter size \([F_{(1,26)}: 5.636, P < 0.02]\) and sucrose \([F_{(1,26)}: 8.012, P < 0.009]\), with sucrose increasing acylated ghrelin levels in L12 \([F_{(3,26)}: 4.671, P < 0.01]\) but not L4 rats. However, there was no significant difference between groups in the percentage of acylated ghrelin.

The levels of cholesterol were modulated by sucrose intake \([F_{(1,24)}: 6.724, P < 0.02]\), inducing a reduction in total cholesterol levels. Serum LDL levels were modulated by sucrose intake \([F_{(1,24)}: 9.673, P < 0.006]\), resulting in a reduction in rats from L4. There was an interaction between litter size and sucrose intake on HDL levels \([F_{(1,24)}: 5.685, P < 0.03]\), with all groups having higher levels compared with L12.

Triglyceride levels were modified by litter size \([F_{(1,24)}: 4.733, P < 0.05]\) and sucrose intake \([F_{(1,24)}: 5.358, P < 0.04]\), with an interaction between these factors \([F_{(1,24)}: 30.479, P < 0.0001]\). Triglycerides were decreased in rats with neonatal overnutrition compared with those from normal size litters. In L12 rats, sucrose intake decreased triglyceride levels but increased them in L4 rats. Serum total protein, total lipid, FFA, or glyceral levels were not affected by neonatal nutrition or sucrose intake.

**Effect of neonatal overnutrition and long-term sucrose intake on hypothalamic mRNA levels for metabolic neuropeptides and the LepR.** There was no difference between experimental groups in the mRNA levels of Pgc1 (L12Ct 100 ± 6.7, L12S2M 133 ± 16.3, L4Ct 118 ± 14.9, L4S2M 122 ± 16.8% control). The hypothalamic mRNA levels of NPY were modulated by long-term sucrose intake regardless of litter size \([F_{(1,22)}: 7.200, P < 0.02]\). Rats ingesting sucrose had higher NPY mRNA levels than their controls \([F_{(1,22)}: 3.439, P < 0.05]\; Fig. 7A). There was an interaction between neonatal overnu-
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Table 2. Serum hormone and biochemical data in L12 and L4 rats that were allowed to eat rat chow and drink either water (L12Ct and L4Ct) or a 33% sucrose solution (L12S2M and L4S2M) ad libitum from the time they were weaned (postnatal day 21) until euthanization at 80 days of age.

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<th>L12</th>
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<td>Water (L12Ct)</td>
<td>Sucrose (L12S2M)</td>
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<td>Insulin, ng/ml</td>
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<td>Total ghrelin, pg/ml</td>
<td>1,801.7 ± 209.5</td>
<td>1,840.0 ± 262.9</td>
<td></td>
</tr>
<tr>
<td>Acylated ghrelin, pg/ml</td>
<td>227.7 ± 14.5</td>
<td>328.8 ± 32.5*</td>
<td></td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>114.9 ± 14.4</td>
<td>139.9 ± 8.4</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>59.1 ± 2.8</td>
<td>51.1 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>12.6 ± 1.5</td>
<td>18.9 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>32.3 ± 1.5</td>
<td>26.5 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>95.3 ± 6.2</td>
<td>71.7 ± 7.3*</td>
<td></td>
</tr>
<tr>
<td>Total lipids</td>
<td>542.9 ± 25.2</td>
<td>653.5 ± 70.9</td>
<td></td>
</tr>
<tr>
<td>FFA</td>
<td>1.41 ± 0.12</td>
<td>1.17 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Glyceroil</td>
<td>356.1 ± 22.5</td>
<td>420.1 ± 77.5</td>
<td></td>
</tr>
<tr>
<td>Total protein</td>
<td>5.9 ± 0.1</td>
<td>5.8 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

|                     | Water (L4Ct)         | Sucrose (L4S2M)      |             |
| Insulin, ng/ml      | 0.81 ± 0.04          | 2.02 ± 0.16*         | P < 0.0001  |
| Leptin, ng/ml       | 4.34 ± 0.60*         | 5.29 ± 0.79*         | P < 0.002   |
| Adiponectin, ng/ml  | 37.4 ± 5.7           | 71.9 ± 8.1b          | NS          |
| Total ghrelin, pg/ml| 1,045.9 ± 37.1*      | 1,402.7 ± 167.5      | P < 0.05    |
| Acylated ghrelin, pg/ml| 182.9 ± 22.2       | 240.5 ± 34.1*        | P < 0.01    |
| Corticosterone, ng/ml| 136.9 ± 18.9        | 132.9 ± 15.0         | NS          |
| Total cholesterol   | 55.3 ± 2.4           | 51.5 ± 1.9           | NS          |
| HDL                 | 21.6 ± 2.3           | 18.5 ± 3.2           | NS          |
| LDL                 | 29.6 ± 2.4           | 22.5 ± 1.5b          | NS          |
| Triglycerides       | 70.6 ± 9.3           | 128.3 ± 6.2*         | P < 0.0001  |
| Total lipids        | 642.1 ± 34.0         | 592.0 ± 37.7         | NS          |
| FFA                 | 1.14 ± 0.09          | 1.10 ± 0.07          | NS          |
| Glyceroil           | 356.7 ± 37.3         | 379.3 ± 65.2         | NS          |
| Total protein       | 5.8 ± 0.2            | 5.5 ± 0.3            | NS          |

Values are means ± SE; n = 6–7/group. *Significantly different from L12Ct by Scheffe F-test post hoc analysis. **Significantly different from L4Ct by Scheffe F-test post hoc analysis. ***Significantly different from L12S2M by Scheffe F-test post hoc analysis.

Metabolic efficiency was also affected by neonatal overnutrition. Not only was the cumulative intake increased in rats from small litters, but they gained more weight per gram of food ingested, suggesting a possible decrease in energy expen-

trition and long-term sucrose intake on AgRP mRNA levels $[F(1,22); 5.367, P < 0.04]$, with sucrose increasing AgRP levels in L12 but not L4 rats (Fig. 7B). There was no significant effect of either sucrose or litter size on POMC (Fig. 7C), CART (Fig. 7D), or orexin (Fig. 7E) mRNA levels.

Hypothalamic levels of LepR mRNA were modified by sucrose intake $[F(1,22); 3.588, P < 0.03]$, with rats from L12 ingesting sucrose having lower levels than their controls $[F(3,22); 3.473, P < 0.05]$; Fig. 7F).

DISCUSSION

Neonatal overnutrition increased BW at weaning and in adulthood, as described previously (23, 57). This greater adult weight was not associated with modifications in the percentage of body fat, but the absolute weight of visceral fat pads was greater, and this was coincident with hypertrophy of visceral adipocytes and increased circulating leptin levels. We cannot rule out a difference in body length as a contributing factor to the increased BW. Although the increase in lean mass was not significant, it is clear that neonatal overnutrition alters body weight and composition even on a standard diet.

In contrast to some previous reports (44), neonatally overnourished rats were not hyperinsulinemic. Because the rats were young adults at the end of the study, it is possible that insulin resistance could develop at a later age. Indeed, the delayed return of glycemia to baseline in L4 rats during the OGTT suggests that glucose metabolism is altered. However, other authors using a similar experimental design report that fasting insulin levels are not modulated even at later ages (23, 57). Likewise, circulating lipid and adiponectin levels were not affected.

Although neonatally overnourished rats weighed more than controls at weaning, 3 wk later there was no weight difference, with a significant difference returning at ~6 wk postweaning. We have shown previously that weight gain is not affected by neonatal overnutrition in the period immediately following weaning (17), with increased weight gain being resumed post-pubertally. This is similar to that reported by others (47, 57).

Whether this delay in accelerated weight gain is related to an effect of neonatal overnutrition on pubertal changes remains to be determined.

The greater BW at study termination was associated with an increase in mean cumulative food intake. However, neonatally overnourished rats did not eat more than controls at all time points. During the last 2 wk of the first study, rats from L4 ingested fewer kilocalories than those from L12, although they gained more weight and had an increased metabolic efficiency. This is in contrast to that observed in the second experiment, where before euthanization L4C continued to ingest more chow than L12 rats and had no significant difference in metabolic efficiency. There were no obvious differences between the controls in these two experiments. The rats were from the same animal facility, were handled in the same manner, and were of the same age, with the experiments being started within a time frame of only 2 wk difference. The above discrepancy may stem from differences in various basal parameters between the control groups of the two experiments of as yet unknown causes. Notwithstanding, neonatal overnutrition was shown to increase both visceral fat pads and leptin levels in both experiments, and hence, this effect was regardless of control baseline values.

In both experiments, neonatal overnutrition decreased total ghrelin levels, with no effect on acylated ghrelin levels, which resulted in an increase in the percentage of acylated ghrelin. Whether the higher proportion of acylated ghrelin, with no change in absolute levels of this isoform, is involved in the increase in food intake remains to be determined. It has been shown previously that weight gain is accompanied by a decrease in serum ghrelin levels (60), and because the active form of ghrelin induces food intake and decreases metabolism (12), this reduction in the percentage of acylated ghrelin could be a compensatory mechanism to reduce food consumption.

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diture, as reported previously (61). However, these changes in food intake and metabolism were not coincident with changes in the mRNA levels of hypothalamic neuropeptides or leptin receptor. Other studies have suggested that early changes in nutrition may not affect basal neuropeptide expression or content (57), but they may modify hypothalamic structural organization and the responsiveness of specific neuronal populations to metabolic changes (42, 44). Early overfeeding hypermethylates the hypothalamic POMC promoter, reducing its response to leptin and insulin (42) and the insulin receptor promoter (44). Thus, neonatal overnutrition modifies the response of the hypothalamus to metabolic signals, which undoubtedly plays an important role in their increased food intake and weight gain. In addition, these modifications most likely affect their response to further dietary challenges.

Circulating glycerol levels were unchanged in neonatally overfed rats, indicating that they have the same rate of triglyceride degradation compared with controls. The increased fat mass and adipocyte size does not necessarily imply a decrease in lipolysis, but it could also be the result of increased lipogenesis or decreased sensitivity of adipocytes to lipolytic signals such as leptin or corticosterone.

The effect of a diet rich in carbohydrates on weight gain is not clear from the literature since it has been reported to increase (38, 46), decrease (9, 22, 28, 36), or have no effect (2, 13, 21, 39, 49, 50, 52, 62) on BW. These contrasting outcomes could be due to differences in species, sex, type of carbohydrate, the age that the diet was introduced, or the length of exposure to the diet. We have reported recently that a diet rich in sucrose for 2 wk during adulthood either decreases or has no affect on weight gain, with the outcomes depending on sex and the neonatal nutritional status (17). Here we show that although weight may not increase, fat accumulation does, and the distribution of fat accumulation depends on the neonatal nutritional status. Moreover, sucrose induced hypertrophy of visceral adipocytes only in neonatally overnourished rats, which was associated with a greater increase in circulating leptin levels. The fact that the percentage of calories

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**Fig. 7. Relative hypothalamic mRNA levels of NPY (A), POMC (B), AgRP (C), CART (D), orexin (E), and LepR (F) in L12 and L4 male rats receiving either a control diet (regular rat chow and water ad libitum) or rat chow plus a 33% solution of S for 2M. L12Ct, n = 6; L4Ct, n = 5; L12S2W, n = 6; L4S2W, n = 4. *One-way ANOVA, P < 0.05.**
derived from sucrose was greater in neonatally overnourished rats could be involved in this differential response in fat accumulation.

A sucrose-enriched diet from the time of weaning increased the mean total caloric intake in all rats, with neonatally overnourished rats ingesting more calories from both chow and sucrose than control rats. Sucrose intake reduced metabolic efficiency and weight gain, resulting in a significantly lower BW in all rats after only 1 wk. These rats ingested ~30% less rat chow, which could have induced protein insufficiency during their development. Serum protein levels were not affected by long-term sucrose intake, which suggests that they were not suffering from malnutrition. Although this diet may have also reduced body length, which unfortunately was not measured, it clearly increased body fat. Both subcutaneous and visceral fat pads were increased, with this increase being greater in neonatally overnourished rats. Again, this may be related to their greater sucrose intake.

In contrast to short-term sucrose intake, longer exposure to this diet produced hypertrophy of visceral adipocytes and visceral fat mass in control rats. In neonatally overnourished rats the inverse was observed with short-term but not long-term sucrose intake inducing hypertrophy of visceral adipocytes. In these rats, long-term sucrose intake stimulated visceral fat accrual but not adipocyte hypertrophy, suggesting that they may have a greater number of adipocytes. Because both neonatal overnutrition and sucrose were introduced at an age when adipose tissue is still developing, the acquisition of adipocytes may have been affected. Indeed, overnutrition during early life is associated with an increase in the number of fat cells (3, 43). Adipocyte hypertrophy is associated with increased expression of factors involved in insulin resistance and development of type 2 diabetes (30, 37, 59) as well as circulating leptin levels (59). Indeed, we found a significant correlation between serum leptin levels and adipocyte size in both studies. It is interesting to note that, although neonatally overnourished rats did not have an increase in percent body fat compared with controls, visceral adipocytes were hypertrophied and circulating leptin levels significantly elevated. Long-term sucrose intake by these rats increased body fat significantly but did not modify leptin levels or the mean visceral adipocyte perimeter. Thus, it is possible that, in addition to the amount and distribution of adipose tissue, adipocyte morphology may be important in determining metabolic outcome.

Chronically increased corticosterone stimulates adiposity (32), and the experimental paradigm used in these studies could have been stressful because rats were forced to drink sucrose, with no alternative liquid being available. However, corticosterone levels were reduced with short-term sucrose intake, which could be a consequence of the pleasurable hedonic effect of sucrose (4, 14, 48). Interestingly, rats exposed to sucrose from the time of weaning had no change in circulating corticosterone levels, which may suggest that they became habituated to this stimulus.

Adiponectin levels were increased by both long-term and short-term sucrose intake. This is in accord with previous studies showing that high-carbohydrate diets increase circulating adiponectin levels (31, 55) and that both fructose and glucose increase adiponectin production by adipocytes in vitro (10). Adiponectin is reported to protect against metabolic syndrome, be anti-inflammatory (64), improve insulin sensitivity, and decrease hepatic glucose output (41). Thus, its induction by a high-sucrose diet may be involved in maintaining glucose homeostasis. The rise in adiponectin may also participate in the increase in total caloric intake (35).

Insulin levels were increased by long-term sucrose intake with no change in glycemia, suggesting the possible development of insulin resistance. Acylated ghrelin levels were also increased by long-term sucrose intake. Because both insulin (26) and acylated ghrelin have direct effects on adipocytes to stimulate glucose uptake and increase adipocyte size, the increased percentage of acylated ghrelin and insulin levels could also be related to the higher efficiency in fat mass accrual (11, 24). Whether this greater percentage of acylated ghrelin is involved in the increased total energy intake remains to be determined.

Excess carbohydrate intake is associated with increased serum triglyceride levels (20). However, triglycerides were elevated only in neonatally overnourished rats exposed to a long-term sucrose-enriched diet. Ghezzi et al. (20) reported previously that early nutrition also affects the triglyceride increase in response to a high-fructose diet.

Short-term sucrose intake had no effect on NPY, POMC, or CART but increased orexin mRNA levels in L4 rats. Because orexin stimulates food intake, this rise could be related to their greater increase in caloric intake. Leptin receptor mRNA levels also rose with short-term sucrose intake, with this rise being greater in L4 rats. In contrast, long-term sucrose intake had no effect on orexin but stimulated NPY in the hypothalamus regardless of preweaning nutrition. Ghrelin stimulates NPY neurons to mediate part of its orexigenic effects (34); thus, the rise in acylated ghrelin could underlie the changes in the expression of this neuropeptide. Moreover, NPY not only promotes increased energy consumption but can increase fat mass independent of increased food intake (51).

The mRNA levels of AgRP and the leptin receptor were increased by long-term sucrose intake, but only in rats from normal-sized litters. Although there was some variability in the results, there was no indication that this peptide would increase in L4 rats with sucrose even with a larger number of animals to reduce the error. This differential response at the hypothalamic level could be involved in the differences in long-term systemic outcomes, such as more fat mass or higher leptin levels in neonatally overnourished rats. Indeed, an increase in leptin receptor levels could augment the response of the hypothalamus to this hormone, resulting in better maintenance of weight in those from L12 compared with L4 in both experiments.

In conclusion, it is clear that neonatal overnutrition has long-term metabolic effects that are modulated during aging and become more exaggerated in response to dietary challenges. High-carbohydrate diets may not increase net weight gain, but body composition is affected dramatically with a rise in fat mass accumulation and changes in circulating factors such as leptin that could induce further metabolic complications. The adverse effects on metabolism are augmented when sucrose intake is long term. With the experimental protocol used in these studies, it is impossible to determine whether these differences were due to the duration of sucrose intake, early introduction of sucrose intake, or a combination of both. No matter what the predominant mechanism might be, our data clearly demonstrate that poor nutritional conditions during early (postnatal) maturation and later in life synergize to
 promote body weight and metabolic disturbances that are likely to contribute to the dramatic increase in obesity in many populations.

ACKNOWLEDGMENTS
We thank Francisca Díaz and Sandra Canelles for their excellent technical support.

GRANTS
This work was funded by grants from Fondos de Investigación Sanitaria (PI07/0035), Ministerio de Ciencia e Innovación (BFU2011-27492), Centro de Investigacion Biomedica en Red Fisiopatología de Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, and Fundación de Endocrinología y Nutrición. M. Granado was supported by the Juan de la Cierva program, C. García-Cáceres was supported by a predoctoral fellowship from the Ministerio de Educación y Ciencia (FPU AP2006/02761), E. Fuente-Martin was supported by a predoctoral fellowship from Fundos de Investigación Sanitaria (FB07/0035), and J. A. Chowen was supported by the biomedical investigation program of the Consejería de Sanidad and Consumo de la Comunidad de Madrid.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
E.F.-M., M.T.-S., L.M.F., J.A., and J.A.C. interpreted the results of the experiments; E.F.-M., M.G., M.T.-S., L.M.F., J.A., and J.A.C. analyzed the data; E.F.-M., C.G.-C., M.G., M.T.-S., L.M.F., J.A., and J.A.C. approved the final version of the manuscript; E.F.-M. performed the experiments; E.F.-M., C.G.-C., and M.A.S.-G. contributed to the study design.

REFERENCES


