Adult-onset obesity induced by early life overnutrition could be reversed by moderate caloric restriction

Hung-Wen Liu, Malathi Srinivasan, Saleh Mahmood, Dominic J. Smiraglia, and Mulchand S. Patel

Department of Exercise and Nutrition Sciences, School of Public Health and Health Professions, University at Buffalo, Buffalo, New York; Department of Biochemistry, School of Medicine and Biomedical Sciences, University at Buffalo, Buffalo, New York; and Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, New York

Submitted 21 May 2013; accepted in final form 26 July 2013

Adult-onset obesity induced by early life overnutrition could be reversed by moderate caloric restriction. Am J Physiol Endocrinol Metab 305: E785–E794, 2013. First published July 30, 2013; doi:10.1152/ajpendo.00280.2013.—Overnutrition during the suckling period (small litter, SL) results in the development of adult-onset obesity. Our aim was to investigate whether two levels of caloric restriction (CR) in the early postweaning period can reverse obese phenotype in SL rats. The normal litter (NL) had 12 pups/dam and SL had 3 male pups/dam from the postnatal day 3 until day 21. After weaning, rats consumed lab chow as indicated: 1) NL and SL groups were on ad libitum regimen up to day 140, 2) another SL group was pair-fed (SL/PF) to NL (~14% reduction), 3) SL/PF/AL group was pair-fed up to day 94 and then switched to ad libitum feeding, 4) SL/CR group received 24% reduction (moderate CR) in food intake compared with SL, and 5) SL/CR/AL group was on 24% CR up to day 94 and then switched to ad libitum feeding. Pair-feeding reduced body weight gains and serum insulin and leptin levels compared with SL rats, but these parameters were restored to SL levels in the SL/PF/AL rats after switching to ad libitum feeding. Interestingly, the moderate CR normalized these parameters in SL/CR and SL/CR/AL rats compared with NL. The expression of neuropeptide Y, proopiomelanocortin, and leptin receptor returned to control levels in hypothalami from SL/CR and SL/CR/AL rats. These results indicate that appropriate manipulation of energy intake during the early postweaning period could lead to longer-lasting effects on the regulation of body weight homeostasis via reversal of the early preweaning programming effects on the hypothalamic appetite regulation mechanism.

early overnutrition; metabolic programming; caloric restriction; hypothalamic appetite regulation; DNA methylation

THE HIGH INCIDENCE OF OBESITY, particularly in Western societies, is a major concern from both public health and economic standpoints. Although genetics, sedentary life style, and increased consumption of calorie-dense foods are considered to be the major causes underlying the current obesity epidemic (38), increasing evidence from epidemiological data and results from animal models suggest that altered nutritional experiences during early periods of development (in utero and/or immediate postnatal period) via the phenomenon of developmental programming also contribute to the onset of metabolic disorders in later life (1, 2, 23, 26). The maturation of organs such as pancreatic islets and the hypothalamic neuronal system in rodents is completed only in the immediate postnatal period (12, 14). Hence, altered nutrient exposure in rodents during this period alone can function as independent cues for the onset of developmental programming. Although these immediate effects enable the organism to survive the nutritional insult, in the long run they are counterproductive. For example, it has been shown that cross-fostering of normal rat offspring by a diabetic or protein-restricted dam resulted in metabolic disorders in adulthood (7, 16). Extensive studies from this laboratory have shown that artificial rearing of rat neonates on a high-carbohydrate milk formula resulted in chronic hyperinsulinemia and adult-onset obesity (23). A well-established model for metabolic programming effects due to altered nutritional experience only during the suckling period is the small litter (SL) rat model (21, 28, 31). Overnourishment induced by the reduction of litter size for newborn pups (SL) resulted in increased levels of serum insulin and leptin and increased body weight gains during the suckling period (28). These effects persisted in the postweaning period with the manifestation of obesity and other metabolic disorders in adulthood.

The hypothalamus acts as a primary site for regulation of body weight homeostasis by controlling various processes such as energy intake, storage, metabolism, and energy expenditure. There are two populations of neurons in the arcuate nucleus (ARC) that produce orexigenic [e.g., neuropeptide Y (NPY)] and anorexigenic [e.g., proopiomelanocortin (POMC)] neuropeptides, respectively (35). These neurons also express receptors for insulin and leptin, which are the peripheral signals of satiety (5). Since SL rats developed hyperphagia, several studies have focused on alterations in hypothalamic energy homeostasis in this rat model. Electrophysiological studies performed on coronal slices obtained from brains of SL and age-matched normal litter (NL) rats revealed significant changes in the response to various stimuli/inhibitors. Some of these observations include 1) leptin and insulin resistance in the ARC (9, 10); 2) inhibition of neurons in the paraventricular nuclei and the ventromedial nucleus by NPY, agouti-related protein, corticotrophin-releasing factor, and dopamine-favoring feeding and reduced energy expenditure (8, 19); and 3) increase in the number of NPY neurons in the ARC and a decrease in cholecystokinin-positive neurons (29, 30).

Caloric restriction (CR; restriction of energy intake without malnutrition) has been shown to reduce body weight gain and improve health outcomes in obese animal models. CR effectively reduced inflammation and oxidative stress and normalized plasma levels of insulin and leptin (15) in rats consuming a chow diet. Importantly, in diet-induced obesity (DIO)-prone rats, CR restored the levels of OB-Rb protein and mRNA levels as well as the level of phosSTAT3 protein in the hypothalamus (42), indicating that CR may have a potential impact on appetite regulation. Hyperphagia and adult-onset obesity are characteristics of the SL rat model (21, 28, 31).
METHODS

Animal protocols. Animal protocols employed in this study were approved by the Institutional Animal Care and Use Committee of University at Buffalo (protocol no. BCH06064N). Untimed pregnant Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). They were housed individually under controlled conditions of temperature (23°C) and a 12:12-h light-dark cycle with ad libitum access to a standard rodent laboratory chow (Harlan Teklad, South Easton, MA).

SL model. One day after birth, the litter size was adjusted to 12 pups per dam, and only the natural litter size between 9 and 14 pups per dam was used in this study. On postnatal day 3 (d3), pups were assigned randomly to normal litter (NL) or small litter (SL). The NL group litter size was adjusted to 12 male pups per dam to provide a similar level of nourishment, while the SL group litter size was adjusted to three male pups per dam (for overnourishment). SL pups were nursed by their natural dams. NL pups from several mothers were pooled and randomly assigned to dams. For studies on adult animals, male pups were weaned on d21 and were housed individually. Unless otherwise indicated, after being weaned from the mother, NL and SL rats had ad libitum access to a standard rodent laboratory chow and water.

Body weight and food intake. To determine the immediate effects of overnourishment due to litter size reduction, body weights of NL (n = 26) and SL (n = 34) pups were measured from d3 to d24. For studies on adult rats, body weight and food intake were measured on a weekly basis for all groups.

Mild CR (pair-feeding regimen, equalizing food intake to that of NL rats). The pair-feeding regimen was initiated from d24 and continued until d140. Based on the quantity of rodent chow consumed by NL rats during the postweaning period, the daily food supply was given to one group of SL rats (SL/PF) during the entire postweaning period unless otherwise indicated (Fig. 1). On d94, one-half of the SL/PF rats continued receiving CR by pair-feeding, while the remaining SL/PF rats were allowed ad libitum feeding of rodent chow and are referred to as SL/PF/AL rats (Fig. 1).

Moderate CR intervention. Based on the food intake of SL rats, the daily food supply was initially reduced by 10% for one group SL rats (SL/CR) from d24 to d44 to slow down their weight gain in the immediate postweaning period. Around puberty (starting from d45) (17), SL/CR rats started receiving a moderate CR of 24% in total daily food intake compared with the SL group until d140. On d94, one-half of the SL/CR rats continued receiving moderate CR, while the remaining SL/CR rats were switched over to ad libitum feeding of rodent chow and are referred to as SL/CR/AL rats (Fig. 1).

Tissue collection. On d140, all groups of rats were anesthetized by intraperitoneal injection of ketamine (75 mg/kg body wt) and xylazine (10 mg/kg body wt) followed by decapitation between 9:30 and 11:00 AM. Trunk blood for insulin and leptin measurements was collected from random-fed rats in nonheparinized tubes. Serum was separated by centrifugation at 3,000 rpm for 15 min and stored at −20°C. The brain was frozen on dry ice, and the hypothalamus-enriched region was dissected out from the frozen brain based on the protocol described by Reyes et al. (32).

Serum insulin and leptin levels. Serum insulin and leptin levels were measured using radioimmunoassay kits according to the manufacturer’s instructions (Millipore, Billerica, MA).

Real-time PCR. Hypothalamic total RNA was isolated from all groups using the TRIzol-chloroform procedure (GIBCO-BRL, Rockville, MD). Total RNA concentration was quantified by NanoDrop meter, and 1 μg total RNA was reverse transcribed into cDNAs using the iScript cDNA kit (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. mRNA levels of Npy, Pomic, insulin receptor (Insr), Lepr, signal transducer and activator of transcription-3 (Stat3), and suppressor of cytokine signaling-3 (Socs3) in hypothalamus were quantified using the MyiQ Real-Time System (Bio-Rad). Primer sequences for hypothalamic mRNAs levels are shown in Table 1. Real-time PCR was performed using a SYBR Green supermix kit (Bio-Rad). The PCR reaction included the following components: each primer at a concentration of 10 μM, diluted cDNA template, and iQ SYBR Green supermix and running 40 cycles. Each cDNA sample was run in triplicate, and 18s primers as an internal control were included in each run to correct sample-to-sample variation and to normalize mRNA levels. The relative mRNA level was calculated according to the comparative ΔΔCt method (20).

DNA methylation analysis. Tail genomic DNA from a normal adult male rat was amplified using Taq polymerase (Fermentas, Pittsburg, PA). By using genomic DNA, the target regions of two specific genes were amplified using the primer pairs (Table 2). The PCR products from amplified genomic DNA fragments were purified with a QiAquick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into the vector pC2.1-TOPO (Invitrogen, Grand Island, NY). Transformants were assayed for the presence of recombinant inserts by the blue/white colony phenotype. The plasmid DNA was extracted with a QIAprep Spin Miniprep Kit (Qiagen). Plasmid DNAs containing the promoter inserts were artificially methylated with SssI (CpG) methylase (New England Biolabs, Ipswich, MA) and S-adenosylmethionine according to the manufacturer’s instructions. The methylated plasmid DNA was considered as 100% methylated DNA. In order to get 0, 25, 50, 75, and 100% methylated DNA, known ratios of completely methylated DNA (100%) and unmethylated DNA were mixed. Each DNA sample was separately spiked with 750 ng of human genomic DNA at a one genome equivalent level. All DNA samples were then

Since we observed an obese phenotype in SL pups during the suckling period that persisted in the postweaning period, we postulated that CR from the time of weaning might have a positive impact on the development of obesity in adult life via reversal of the early programming effects. For this purpose, we employed two levels of CR on SL rats from the time of weaning to the end of the experiment (postnatal day d140). Additionally, we investigated the effects of caloric restriction at two different levels on body weight gains for a period of 10 wk followed by the effects of ad libitum feeding for about 7 wk on body weight gains and hormonal profile. The effects of the above-mentioned feeding regimens on the hypothalamic energy circuitry were determined. Our results indicate that only the moderate CR resulted in permanent reversal of the programmed effects for hyperphagia in the SL rats.

![Fig. 1](http://ajpendo.physiology.org/)

**Fig. 1.** Experimental groups are depicted schematically. SL, small litter; NL, normal litter; CR, caloric restriction; PF, pair-feeding; AL, ad libitum feeding. Timing and degree of CR is indicated. PF (referred to as mild CR) to the level of food intake of NL rats on a daily basis; CR, referred to as moderate CR by ∼24% of the SL rats.
bislufite treated using the sodium bisulfite method with an EZ DNA Methylation kit (Zymo Research, Orange, CA) and followed by MALDI-TOF-MS analysis as described previously (22).

Hypothalamic genomic DNA (750 ng) was bisulfite treated as described above. The sequence primers of Npy and Pomc genes (Table 2) were designed using Methprimer. Each reverse primer had a T7 promoter tag for in vitro transcription, and the forward primer was tagged with a 10mer to balance TM. Approximately 10 ng of bisulfite-treated DNA was amplified using HotStar Taq Polymerase (Qiagen) in a 5-µl reaction volume using PCR primers at a 200 nM final concentration. PCR conditions for sequence primers were as follows: 95°C for 15-min hot start, followed by denaturing at 94°C for 20 s, annealing at 60°C for 30 s, extension at 72°C for 1 min for 45 cycles, and final incubation at 72°C for 3 min. After shrimp alkaline phosphatase treatment, 2 µl of the PCR products was used as a template for in vitro transcription, and RNase A was desalted and spotted on a 384-pad SpectroCHIP (Sequenom, San Diego, CA) using a MassARRAY nanodispenser followed by spectral acquisition on a MassARRAY Analyzer Compact MALDI-TOF-MS. MALDI-TOF-MS analysis was described earlier (22). The average methylation was calculated as mean value of CpGs methylation value and expressed as percent methylation.

Statistical analysis. Results are expressed as means ± SE. When only the SL and NL groups of rats were involved, statistical analysis of the differences between the two groups was determined using Student’s t-test (SigmaPlot 12.0). For multiple comparisons, a one-way ANOVA was used to determine the statistical significance of the differences among the groups. ANOVA was followed by post hoc assessment by Student-Newman-Keuls method correction for multiple comparisons (SigmaPlot 12.0). A P value < 0.05 was considered to be statistically significant.

RESULTS

Effects of postweaning pair-feeding (mild CR) and moderate CR on body weight and food intake in rats overnourished during the suckling period. After litter size reduction to three pups per dam on d3, SL male pups immediately gained more body weight and were 20% heavier than age-matched NL male pups on d24 (Fig. 2A). The 24-day-old normal litter (NL) had body weight 70 ± 1.5 g (n = 26). On the day 24, the SL group (n = 34) was subdivided in two subgroups: SL (d24, body wt 86 ± 1.9 g, n = 11) and SL/PF (d24, body wt 83 ± 1.5 g, n = 23). The SL/PF group was pair-fed to the NL starting on d24, and their body weights were monitored 1 wk later on d31 (the first data point in Fig. 2B). By that time, the SL/PF rats had body weights (117 ± 1.2 g, n = 23), which were significantly different from those of the 31-day-old SL group (145 ± 3 g, n = 11), but their body weights were not significantly different from those of the 31-day-old NL group (117 ± 2.7 g, n = 10; Fig. 2B, d31). SL rats continued gaining more body weight on the ad libitum feeding regimen on rodent laboratory chow during the postweaning period and were 18% heavier than age-matched NL rats on d140. The increased body weight was associated with an increase in food consumption; therefore, the accumulative food intake of SL rats during the entire postweaning period was 116% compared with NL rats (Fig. 2C). To investigate the long-term effects of mild CR by pair-feeding regimen from the start of the postweaning period (d24) on metabolic programming, a group of SL rats were pair-fed (SL/PF) from d24 to d140. The growth of SL/PF rats was similar to that of the age-matched NL rats during the entire postweaning period (Fig. 2B). To investigate the effect of unlimited access to food following a period of pair-feeding, a subgroup of SL/PF rats was allowed to consume food ad libitum (SL/PF/AL) from d94 to d140. The body weight of SL/PF/AL rats was significantly heavier than that of age-matched NL and SL/PF rats starting from d101 onward, and there was no significant difference between SL/PF/AL and SL rats from the d122 onward, indicating a catch-up in growth of these rats. The food intake of SL/PF/AL rats increased markedly from d94 to d101, reaching that of SL rats. The weekly food intake from d101 to d140 showed that SL/PF/AL rats consumed more rodent chow than age-matched NL rats, and there was no significant difference in food consumption between the SL/PF/AL and SL rats during that period (Fig. 2C).

To slow down the body weight gains, SL/CR rats consumed 10% less food per day compared with SL rats from d24 to d44. During that period, the body weights of SL/CR rats were significantly higher than those of age-matched NL rats and were significantly lower than those of SL rats (Fig. 2D). To investigate the effect of moderate CR from the early postweaning period to adulthood, a group of SL rats was subjected to a higher level of CR (SL/CR) from d45. From d45 to d140, the total amount of food given to SL/CR rats was, on average, 24% less than that consumed by SL rats. Due to further reduction of daily food intake, SL/CR rats gained less body weight than age-matched NL as well as SL rats from d66 onward. Also, NL

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequences (5’→3’)</th>
<th>Primer Locations (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Npy</td>
<td>F: CCAGAGATCGTCCCGACCTACTGCA</td>
<td>−377 / −358</td>
</tr>
<tr>
<td></td>
<td>R: AGATCTGAGGATGTGCTCA</td>
<td>+179 / +178</td>
</tr>
<tr>
<td></td>
<td>−398 / −379</td>
<td>+173 / +192</td>
</tr>
<tr>
<td>Pomc</td>
<td>F: TCTCGAGTCCGACCTTCTC</td>
<td>−242 to +103</td>
</tr>
<tr>
<td></td>
<td>R: CCAATCAATCTCTGACCTCA</td>
<td>No. of CpG sites</td>
</tr>
</tbody>
</table>

Table 2. Primers for DNA methylation analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequences (5’→3’)</th>
<th>Primer Locations (bp)</th>
<th>No. of CpG sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Npy</td>
<td>F: AAGAGGACCTGGGAAATG</td>
<td>−298 to +43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CCAATCAATCTCTGACCTCA</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Pomc</td>
<td>F: TCTCGAGTCCGACCTTCTC</td>
<td>−242 to +103</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CCAATCAATCTCTGACCTCA</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>
and SL rats were ~11% and ~31% heavier than SL/CR rats on d140, respectively (Fig. 2D). To investigate whether moderate CR from the early postweaning period to adulthood is able to permanently reverse obese phenotype in SL rats, a group of SL/CR rats was switched over to ad libitum feeding (SL/CR/AL) on d94. On the ad libitum feeding regimen, SL/CR/AL rats gained weight slowly but did not reach the body weights of age-matched NL rats on d140 (Fig. 2D). Although the weekly food consumption in SL/CR/AL rats varied from d94 to d140, the accumulative food intake during that period in those rats was not significantly different from that of NL rats, suggesting that long-term moderate CR from the early postweaning period to adulthood had a desirable effect on eating behavior in SL/CR/AL rats (Fig. 2E).

Effects of postweaning pair-feeding (mild CR) and moderate CR on serum insulin and leptin levels in rats overnourished during the suckling period. The levels of serum insulin and leptin were significantly increased in SL rats compared with
NL rats on d140 (Fig. 3, A and B). The reduction of weight gain due to pair-feeding of SL/PF rats was reflected in a significant reduction of the levels of serum insulin and leptin in SL/PF rats compared with SL rats and were similar to the levels observed in NL rats (Fig. 3, A and B). After switching from the pair-feeding regimen to ad libitum feeding, significant increases in the levels of serum insulin and leptin were observed in SL/PF/AL rats compared with NL and SL/PF rats, and these parameters were similar to the levels of SL rats (Fig. 3, A and B).

Moderate CR resulting in a greater reduction in body weight gain in SL/CR rats was accompanied by a significant reduction of serum insulin and leptin levels compared with SL rats and normalization of the serum leptin levels to the levels of NL rats (Fig. 3, C and D). Furthermore, a significant reduction of serum insulin levels was observed in SL/CR rats compared with NL rats. A similar trend was observed in SL/CR/AL rats, such that these rats had lower serum insulin and leptin levels compared with SL rats and had similar serum hormone levels compared with NL and SL/CR rats (Fig. 3, C and D).

**Effects of pair-feeding (mild CR) and moderate CR on hypothalamic mRNA expression.** On d140, mRNA was examined in the hypothalamus from NL, SL, SL/PF, and SL/PF/AL rats. A 78% increase in Npy mRNA and a 41% decrease in Pomc mRNA expression were observed in the hypothalami from SL rats compared with NL rats (Fig. 4, A and B), supporting a hyperphagic behavior observed for the SL rats (Fig. 2C). Although pair-feeding normalized serum insulin and leptin levels in SL/PF rats to the corresponding levels of NL rats (Fig. 3, A and B), the mild CR (~14%) failed to restore the Npy and Pomc mRNA expression in SL/PF rats to the levels of NL rats (Fig. 4, A and B). Also, compared with SL rats the similar trends of Npy and Pomc mRNA expression were observed in SL/PF/AL rats, which could explain the SL/PF/AL rats consuming more rodent chow when they were fed ad libitum. Insr mRNA expression was not affected by any dietary intervention among the four groups (Fig. 4C). In the present study, Lepr mRNA expression was negatively associated with the serum leptin levels such that obese SL rats with higher serum leptin levels showed a 61% decrease in hypothalamic Lepr mRNA expression compared with NL rats (Fig. 4D). Lepr mRNA expression in SL/PF rats was similar to the level in NL rats and was significantly higher than in SL rats. Lepr mRNA expression was significantly lower in SL/PF/AL rats than in NL and SL/PF rats, and no significant difference was observed between SL/PF/AL and SL rats (Fig. 4D). No significant changes were observed in mRNA levels of the leptin signaling pathway such as Stat3 and Scos3 among the four groups (Fig. 4, E and F).

As shown in Fig. 2E, the hyperphagic behavior observed in SL rats was directly associated with an increase in Npy and a decrease in Pomc mRNA expression (Fig. 4, G and H). For SL/CR rats, moderate CR for 49 days (from d45 to d94) restored Npy and Pomc mRNA expression to the levels observed in NL rats (Fig. 4, G and H). The Npy and Pomc mRNA expression observed in SL/CR/AL rats was similar to that in SL/CR and NL rats, which was consistent with their food intake data during the ad libitum period (Fig. 2E). In contrast, Insr mRNA expression was not affected by any dietary intervention (Fig. 4F). To evaluate the effects of moderate CR (~24% reduction) on the hypothalamic leptin signaling pathway, quantitative PCR was also performed for 140-day-old NL, SL, SL/CR, and SL/CR/AL rats. Obese SL rats with higher serum leptin levels showed lower Lepr mRNA expression than NL rats (Fig. 4J). For SL/CR rats, moderate CR for 49 days (from d45 to d94) restored Lepr mRNA expression to the levels observed in NL rats, and a similar change was also observed in SL/CR/AL rats (Fig. 4J). Interestingly, Stat3 and Scos3 mRNA expression was not affected by any dietary intervention (Fig. 4, K and L).

![Graphs showing serum insulin and leptin levels](image-url)
DNA methylation status of *Npy* and *Pomc* genes in the hypotalamus. Based on the above observations, DNA methylation analysis of specific CpG dinucleotides in the promoter region of *Npy* and *Pomc* genes was performed to investigate whether moderate CR contributed to the altered methylation status of these genes in these rats. Putative transcription factor binding sites in the promoter region were identified by TRNASFAC, v. 8.3. A segment of the *Npy* promoter encompassing /H11001 to /H11002 bp including 24 CpG dinucleotides is shown in Fig. 5, top. Percent methylation of the CpG dinucleotides in the promoter region from purified genomic DNA from the four groups of rats is shown in Fig. 5, bottom. A significant reduction in methylation at CpG positions 20 and 22–23 in SL rats was observed compared with the corresponding position in the age-matched NL and SL/CR rats, respectively (Fig. 5). Furthermore, a significant increase in methylation at CpG position 15 in SL rats was observed compared with the corresponding position in the age-matched NL rats. For *Pomc* promoter methylation analysis in the hypothalamus from these four groups of rats, we focused on a 341-bp promoter region that ranged from +143 to −298 bp harboring 23 CpG dinucleotides (Fig. 6). The degree of methylation ranged from 2.3% (CpG 16) to 30% (CpG 17–18) in adult rats. There was no significant difference in the level of methylation in any CpG dinucleotide among the four groups of rats (Fig. 6).

**DISCUSSION**

Altered nutritional experiences during early developmental periods of life are recognized as important factors in the etiology of the obesity epidemic currently prevalent, particularly in Western societies (4, 39). In the search for interventions for ameliorating the long-term consequences of excessive body weight, calorie restriction has emerged as an effective option. Results from the present study revealed that the two levels of CR imposed on male SL rats during the postweaning period had distinct effects on the metabolic phenotype of these rats. The mild CR (14% reduction by pair-feeding) for SL/PF rats resulted in suppression of the programmed effects only while it was being implemented. The catch-up growth achieved by SL/PF/AL rats upon free access to rodent chow suggests that the SL rats may not be amenable to a permanent reversal of the obese phenotype by the mild CR regimen implemented in this study. Interestingly, the moderate CR (24% reduction) had a longer lasting beneficial effect on the suppression of the development of the obese phenotype in the SL/CR/AL rats. On the basis of the normalization of daily food consumption and body weight trajectory of SL/CR/AL rats, we speculate that they would be protected against the weight gain observed in SL rats even if SL/CR/AL rats were to continue on ad libitum regimen for an extended period.

**Effects of postweaning pair-feeding regimen (mild CR) on reversal of obese phenotype.** Pair-feeding (mild CR) of SL rats from the time of weaning resulted in normalization of body weight, food intake, and serum hormonal levels in SL/PF rats, culminating in an NL-like metabolic phenotype in these rats. Although PF did have a positive impact on the metabolic profile in SL/PF rats, these effects were effective only as long as the PF regimen was imposed; when SL/PF rats were given
ad libitum access to rodent chow, the SL phenotype was rapidly regained by these rats. Such an observation indicates that the apparent beneficial effects of PF were most likely due to the reduced availability of dietary calories. A similar observation was recently reported in an obese rat model in which metabolic programming was induced by feeding a milk formula high in carbohydrate-derived calories (HC) during the suckling period (37). In the high-fat diet-induced obesity-prone rats, although a 15% reduction in daily calorie intake starting from d28 to d70 improved metabolic parameters in these rats, resumption of ad libitum feeding for an additional 7 wk resulted in reversal of these parameters to obese levels (24).

The results from this study (Fig. 4) as well as from earlier studies (21, 31) indicate aberrations in the hypothalamic energy regulation mechanism in SL rats, evident by an increase in the mRNA levels of the orexigenic neuropeptide Npy and a decrease in the mRNA levels of the anorexigenic neuropeptide Pomc. The observation that there were no differences in the mRNA levels of Npy and Pomc in the hypothalamus of SL, SL/CR, and SL/CR/AL rats indicates that the PF regimen (mild CR) had no effect on the programmed effects for hyperphagia and development of obesity in these rats. This conclusion is supported by regaining of the SL phenotype by SL/PF/AL rats upon switching from the PF regimen to ad libitum feeding. A similar observation of increased Npy and decreased Pomc in mRNA levels was observed in the hypothalamus of HC, HC/PF, and HC/PF/AL rats, another example of nutritional programming due to an increased intake of carbohydrate-derived calories in the suckling period (37).

Leptin signaling in the hypothalamus plays a vital role in the regulation of body weight homeostasis (33). A state of leptin resistance in the hypothalamus of SL rats is indicated by reduced mRNA levels of Lepr in the present study and previous reports (21, 31). Decreased Stat3 and no change in Socs3 in mRNA levels were also observed in a previous study (31); however, no difference was observed in these two genes between NL and SL rats in the present study. PF normalized Lepr mRNA levels in the SL/PF rats, which paralleled the reduced levels of serum leptin levels in these rats. Upon resumption of ad libitum feeding, reduction in Lepr mRNA expression was observed in SL/PF/AL rats, representing a negative correlation with the higher serum leptin levels and the SL phenotype in these rats. In the present study, no changes were observed in the mRNA levels of Stat3 and Socs3 in the NL, SL, SL/PF, and SL/PF/AL rats. Similar results on Lepr mRNA levels were observed in the HC, HC/PF, and HC/PF/AL rats (37). In summary, our results indicate that the developmental programming effects predisposing the SL rats for adult-onset obesity could not be permanently reversed by the PF regimen imposed in this study.
Effects of postweaning moderate CR on reversal of obese phenotype. On the basis of the observations from the mild CR experiment, we investigated whether moderate CR would result in a permanent reversal of the early programmed effects in the SL rat. Imposition of moderate CR (24% reduction in food intake compared with SL rats) in a group of SL/CR rats resulted in progressive weight loss such that by postnatal d 140 their body weights and serum insulin levels were significantly lower than those of age-matched NL as well as SL rats. SL/CR/AL rats did not exhibit the SL phenotype at the time when the experiment was concluded on d 140. In the period from d 94 to d 140, SL/CR/AL rats fed ad libitum demonstrated a moderate catch-up growth and by d 140 nearly matched the body weight of NL rats. The observation of normalization of food intake in SL/CR/AL rats upon ad libitum feeding to the level of that consumed by NL rats suggests that the slower pace of catch-up growth could be attributed to lack of hyperphagic behavior in SL/CR/AL rats. Whether there was a permanent reversal of programming effects for hyperphagia and obese phenotype in SL/CR/AL rats needs to be further investigated by extending this dietary treatment for another month or two beyond the d 140 (the end period of the present study).

Using the SL rat model, Bassett and Craig (3) also demonstrated less weight gain and suppression of hyperphagia in a group of SL/CR rats after discontinued CR (imposition of ~30% reduction in total calorie intake from d 23 to d 55). In a genetic obese rat model, ~25–30% reduction in total daily calorie intake from weaning to d 45 prevented body weight gain by suppressing hyperphagic behavior after discontinued CR (34). The results from the present study indicate that the moderate CR had a beneficial effect on preventing the development of obesity in SL/CR/AL rats in later life. Thus, appropriate manipulation of energy intake starting from the early postweaning period leads to longer lasting effects on the subsequent regulation of energy homeostasis into adulthood by reversing early-life metabolic programming effects.

SL/CR rats demonstrated a considerably greater loss of body weight (even significantly less than age-matched NL rats) in response to the moderate CR regimen imposed on them. In keeping with this observation, no differences in the mRNA levels of Npy, Pomc, and Lepr were observed in the hypothalamus of NL, SL/CR, and SL/CR/AL rats. These results suggest that the moderate CR imposed from the early postweaning period normalized the central molecular mechanisms underlying the development of obesity in the SL rats.

DNA methylation and histone tail modifications are important epigenetic mechanisms that regulate transcription of genes. Recent evidence indicates that these processes could be
involved in the long-term memory of early programming effects due to altered nutritional exposure during early periods in life (13, 25). The results from this study suggest that the moderate CR imposed on SL rats appears to have reversed the programming effects on the expression of Npy and Pomc genes in the hypothalamus of SL/CR and SL/CR/AL rats. In the case of Npy gene, although the overall degree of methylation was low (0.2 to 6.2%), the DNA methylation status tended to decrease in CpG dinucleotides 20 (P < 0.05) and 22–23 (P = 0.156) and to increase in CpG dinucleotides 15 (P < 0.05) in SL rats compared with NL rats. Transcription factors like AP-1, AP-2, and NGFI within the NGF response element may have binding affinities in the vicinity of CpG 15 dinucleotides in the proximal promoter region of the Npy gene. A potential binding site for E2F within GSG-3 element is located within the CpG 20 dinucleotides in the Npy gene. These transcription factors have been shown to be involved in regulation of Npy transcriptional activity (18, 41). Our results suggest that changes in the methylation status of specific CpG dinucleotides could possibly modify the ability of specific transcription factors to bind to the promoter and thereby contribute to the restoration of the expression of Npy, as observed in the hypothalamus of SL/CR and SL/CR/AL rats. In another study on SL rats, no changes were observed either in the expression or in the methylation pattern of the CpG dinucleotides in the proximal promoter region of Npy in 21-day-old rats (27). In yet another study, it was shown that, although postnatal CR induced altered expression of hypothalamic neuropeptide genes such as Npy, Agpr, and Pomc, such changes did not correlate with changes in the DNA methylation pattern in the promoter region of these genes (36).

Although a decrease in the mRNA levels of Pomc in SL rats and its normalization in SL/CR and SL/CR/AL rats compared with NL rats was observed in the hypothalami from these three groups of rats, there were no significant differences in the DNA methylation pattern of the CpG dinucleotides in the proximal promoter region of this gene. However, other studies using different animal models have suggested that methylation status is involved in regulation of gene expression. For example, hypermethylation of CpG dinucleotides upstream of the Sp-1 binding site and in the NF-kB binding site and hypomethylation of the CpG dinucleotide located in the glucocorticoid response elements (a negative regulator of Pomc expression) were observed in the hypothalami of 21-day-old SL rats, although there were no changes in the expression of this gene (27). The observed differences in the results between our study and the above-cited study could be due to differences in the rat strain used. Feeding a low-protein diet to pregnant rats to induce fetal programming resulted in a mismatched correlation between Pomc gene expression and CpG methylation status in the offspring (6). In high-fat diet-fed obese mice, altered expression of Pomc and Lepr in the hypothalamus was shown not to be correlated with DNA methylation in the proximal promoter region of these two genes because of no changes in methylation status between control and DIO mice (11). In summary, our results indicate that the developmental programming effects predisposing the SL rats to adult-onset obesity could be reversed by the moderate CR regimen imposed in this study, and this outcome may not involve altered DNA methylation patterns of specific neuropeptide genes.

**Conclusion and relevance.** Our results show that a mild CR induced by pair-feeding (14% reduction) in SL rats was not effective in reversing the nutritionally programmed effects for the development of adult-onset obesity in these rats. However, a more stringent CR regimen (increased from 14% for PF to 24% for CR) imposed on these rats resulted in beneficial effects. SL/CR rats demonstrated greater loss in body weight and could not catch up to the body weight of SL rats after resumption of ad libitum feeding for about 7 wk. The moderate CR protocol normalized the expression levels of key modulators of central appetite regulation in the hypothalamus.

Based on our results, the antiobesity response demonstrated by SL/CR/AL rats was due to a reversal of the central molecular mechanisms driving hyperphagic behavior. It is possible that if these rats were allowed to continue for a longer period on the ad libitum feeding regimen they would eventually catch up with the NL rats, but most likely could not catch up with the SL rats. This, however, needs to be experimentally tested. It can be speculated that a shorter duration for moderate CR (shorter than from d45 to d94) could be equally effective in achieving long-term benefits in erasing postnatal life metabolic programming. An equally intriguing possibility that remains to be tested is the beneficial effects of an intermediate level of CR (~20%) on reverting to early life metabolic programming.

The outcomes of this study may have relevance for overfeeding in infancy. Since development of organs continues in the immediate postnatal period, both the quality and quantity of nutrition provided to newborns could alter the trajectory of their growth in later life and would likely be difficult to reverse to a normal metabolic phenotype by interventions implemented in adult life. Results on the SL model from this study imply that there is a possibility to reset altered set points in target organs, especially in the hypothalamus, due to nutritional programming effects occurring in the preweaning period by manipulating early postweaning energy intake. The neural pathways regulating energy homeostasis are not completely developed in rodents until the third postnatal week (nearly the end of the suckling period) (40). Hence, overnourishment of rat pups during the suckling period, as reported here, is particularly relevant for its impact on neuronal regulation on energy balance. Since the neural circuits involved in regulation of energy balance develop primarily prenatally in humans, non-human primates, pigs, and other species (40), our findings in the rat should be extrapolated to these species with some caution. It would, however, be of interest to investigate the effects of an overnourishment/caloric restriction paradigm on metabolic programming in animals (such as pigs) showing precocious neural development.

**ACKNOWLEDGMENTS**

We thank Drs. Todd C. Rideout and David L. Williamson IV of the Department of Exercise and Nutrition Sciences, University of Buffalo, for constructive comments on the manuscript.

**GRANTS**

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-61518 (M. S. Patel).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS

Author contributions: H.-W.L. and S.M. performed experiments; H.-W.L. and S.M. prepared figures; H.-W.L. and M.S. drafted manuscript; H.-W.L., M.S., S.M., and M.S.P. edited and revised manuscript; H.-W.L., M.S., S.M., D.J.S., and M.S.P. approved final version of manuscript; M.S. and M.S.P. conception and design of research; D.J.S. interpreted results of methylation experiments.

REFERENCES