# Solid and Liquid Obesogenic Diets Induce Obesity and Counter-Regulatory Changes in Hypothalamic Gene Expression in Juvenile Sprague-Dawley Rats[1,2]

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## Abstract

Contemporary foods and beverages that constitute the diets of adults and children almost certainly contribute to the obesity problem. To develop a model of childhood obesity, we examined the effects of feeding juvenile rats 2 solid diets, either alone or in combination [nonpurified control diet (C), high-energy (HE), or C+HE] with or without the liquid supplement Ensure (EN). Rats were fed C until 4 wk of age and then were assigned to 1 of 6 weight-matched groups that were fed C, HE, C+HE, C+EN, HE+EN, or C+HE+EN for 5 wk. EN accelerated weight gain and increased energy intake and adiposity irrespective of the solid diet consumed. Serum leptin concentrations were increased after the consumption of all diets when compared with C rats, but there was dissociation between leptin levels and adiposity. The type of solid diet had no effect on the expression of a panel of hypothalamic genes except for glutamate-decarboxylase-67. EN decreased mRNA for agouti-related peptide and neuropeptide Y in the arcuate nucleus and DYN in the paraventricular nucleus. Dynorphin and CART mRNA were decreased in the supraoptic retrochiasmatic nucleus. The reduction in orexigenic signaling in the hypothalamus suggests that overconsumption of EN is sensed by the hypothalamus but that any initiated physiological responses fail to compensate effectively and may be negated or overwhelmed by other systems. Providing diets in solid and liquid form, with choice, mimics more closely the human environment. Understanding the interactions between these diets and peripheral and central energy balance systems could be crucial in unraveling the events underlying human obesity and its early development. J. Nutr. 137: 1483–1490, 2007.

## Introduction

Obesity is a complex problem resulting from an imbalance between energy intake and energy expenditure, with genetic, metabolic and behavioral components. Despite a major contribution from genetic susceptibility, the rapid development of the obesity epidemic over the last few decades must reflect substantial changes in environmental factors such as diet. Contemporary Western solid diets are frequently high in fats and sugars, and may be energy dense. These solid dietary components are increasingly consumed in association with sweetened drinks, shakes, and other liquid formulations. For example, evidence suggests that the consumption of sugar-sweetened soft drinks by children more than doubled between 1965 and 1996 (1). The ability of liquid diets to stimulate overconsumption in rats more readily than solid diets has been described (2–4), but the mechanistic basis of this effect, and specifically the interaction of such obesogenic diets with peripheral and hypothalamic energy balance systems, has not been well studied. The current interest in rodent models of diet-induced obesity is a testament to the importance given to the generation of mechanistic information in this area. The trends in soft drink consumption by children are associated with an alarming increase in the prevalence of obesity and co-morbidities in children and adolescents (5–8) and generate dire predictions for the long-term effect of early onset obesity as this generation progresses to adulthood (9). There are few studies of the dietary induction of obesity in juvenile rodents, and dietary interactions with energy balance systems in early life remain largely unexplored. Different types of diets have been used to induce obesity in juvenile rodent models, including high-fat solid diets (10), high-energy (HE)[3] solid diets (moderately high in fat, but

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[3] Abbreviations used: AGRP, agouti-related peptide; ARC, arcuate nucleus; BDNF, brain-derived neurotrophic factor; C, control diet; CART, cocaine- and amphetamine-regulated transcript; cSON, caudal supraoptic nucleus; DYN, dynorphin; EN, Ensure; ENK, enkephalin; GAD67, glutamate decarboxylase 67; HE, high energy; NEFA, nonesterified fatty acids; NPY, neuropeptide Y; OBRb, leptin receptor; POMC, proopiomelanocortin; PVN paraventricular nucleus; SON, supraoptic nucleus; SOR, supraoptic retrochiasmatic nucleus; WAT, white adipose tissue.

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high in sugar or other simple carbohydrates) (11,12), and palatable liquid diets and sweetened drinks (13,14). Of these, HE diets have been frequently used with the Sprague-Dawley rats with diet-induced obesity (12,15–18). Our earlier work on juvenile male Sprague-Dawley rats revealed that the HE diet caused obesity without excess body weight gain and resulted in elevated leptin levels and a reduced expression of 2 orexigenic neuropeptides, agouti related peptide (AGRP) and neuropeptide Y (NPY), in the arcuate nucleus (ARC) of the hypothalamus (12). One interpretation of this obese but normal weight phenotype was that constraints on the amount of HE diet ingested resulted in a relative protein deficiency compared with nonpurified control (C)-fed rats during a time of rapid growth when protein requirements would be high.

To develop a better model of juvenile obesity (i.e., one with both excess weight gain and excess fat deposition) in the current study, we tested different combinations of the diets previously employed with the Sprague-Dawley diet-induced obesity model, namely, pelleted C and HE diets, and the complete, balanced, liquid-diet supplement, chocolate-flavored Ensure (EN). The EN diet, originally used by Levin et al. (15–17), demonstrated that supplementing a solid HE diet with EN caused a sustained overconsumption of energy that resulted in increased body weight and obesity in adult Sprague Dawley rats. Ensure was selected for the current study, in preference to a carbohydrate solution, to prevent further constraint on protein availability in juvenile rats. We also observed previously, in a study of adult rats involving supplementation of HE diet with EN, that, although the expression levels of a number of hypothalamic energy balance genes appeared to be influenced by feeding an obesogenic diet, the responses were similar between the HE diet and HE+EN despite the different effects of these diets on energy intake, body weight gain, and body composition. Furthermore, rats fed these various diets showed markedly different responses when they were transferred back to a C diet (18).

The current study addresses the following hypotheses concerning juvenile obesity in rats: *1*) providing a choice of diets, including EN, will overcome the limitation of the HE diet alone in inducing both excess weight gain and increased body fat in juvenile rats, and *2*) the effect of EN supplementation on the expression of hypothalamic energy balance genes will be apparent when the additional obesogenic effect of EN is assessed against 3 solid diet backgrounds.

## Material and Methods

*Animals.* All procedures were licensed under the Animals (Scientific Procedures) Act of 1986 and received approval from the Rowett Research Institute's Ethical Review Committee. Sixty 3-wk–old, outbred male Sprague-Dawley rats, weighing 57.7 ± 0.19 g (Charles River Laboratories), were housed individually at 21–22°C on a 12:12-h light to dark cycle. All rats ate a nonpurified control diet ad libitum (Purina 5001, PMI Nutrition International) for 1 wk prior to allocating rats to 6 weight-matched groups ($n$ = 10). The control group remained on the nonpurified control diet (112.2 ± 1.79 g) containing 14 kJ/g, with 23% energy as protein, 12% as fat, and 65% as carbohydrate, the latter primarily in the form of complex polysaccharide. A second group was transferred to a HE (112.1 ± 1.83 g) diet consisting of 8% corn oil, 44% sweetened condensed milk, and 48% nonpurified control diet (C11024; Research Diets) containing 18.9 kJ/g, with 15% energy as protein, 33% as fat, and 52% as carbohydrate (of which 30% are starches). A third group was given access to both C and HE diets (C+HE; 112.2 ± 1.66 g) by subdividing the food hopper into 2 compartments. A fourth group was given chocolate-flavored Ensure (EN) in addition to C diet (C+EN; 112.7 ± 2.09 g). Ensure (Ross Products) is a liquid diet that contains 4440 kJ/L with 14% of the metabolizable energy content as protein, 22% as fat, and 64% as carbohydrate, and it was fed to the rats using an additional drinking bottle. The 2 remaining groups were given access to EN in addition to the HE diet (HE+EN; 112.0 ± 1.64 g) or to all 3 diets (C+HE+EN; 112.4 ± 1.57 g). All diets were consumed ad libitum throughout the study. Food intake was measured at lights on and just before lights off, and bodyweight was measured at lights on. Food intake was calculated using a 2 decimal-place balance to weigh back the refusal and spillage for the individual solid diets, and EN intake was determined by weighing back the refused liquid. Water was continually available. Body composition was determined by magnetic resonance imaging (MRI) (Echo MRI Whole Body Composition Analyzer, Echo Medical Systems). Diet manipulation was carried out on d 7 with d 0–6 being the period on C diet, and MRI scanning was performed on d 6, 15, 20, 27, 34, and 41. Body composition data were analyzed as grams of fat or lean tissue and percentage of body fat or lean tissue per gram of body weight. After 5 wk on the respective diets, all rats were anaesthetized with isofluorane and killed by decapitation. Rats were killed during the light phase, between 1200 h and 1600 h, over a 3-d period, and tissues harvested. Rats were killed from each group in turn, to minimize any time-of-day effect. Blood plasma and serum were collected. Brains were removed, immediately frozen on dry ice, and then stored at −80°C for in situ hybridization. Six fat pads (subcutaneous, epididymal, retroperitoneal, mesenteric and omental white adipose tissue, and interscapular brown adipose tissue), 2 hind leg muscles (soleus and gastrocnemius), liver, adrenals, and testes were collected and weighed.

*Circulating hormones and metabolites.* Serum leptin concentrations were measured using a rat-specific radioimmunoassay kit (Linco, RL-83K, Biogenesis). The sensitivity of the assay was reported at 0.5 $\mu$g/L, and the intra-assay CV was 1.7%. Plasma insulin was measured using a rat-specific radioimmunoassay kit (Linco, RI-13K; Biogenesis). The sensitivity of the assay was stated as 17.2 pmol/L, and the intra-assay CV was 10%. Plasma glucose, nonesterified fatty acids (NEFA), and triglyceride concentrations were determined using the fully automated KONE analyzer methods (12,19). The sensitivities of the assays were 0.34 mmol/L, 0.04 mmol/L, and 0.06 mmol/L, respectively.

*Hypothalamic gene expression.* Hypothalamic gene expression for a number of energy balance–related neuropeptides and receptors was quantified using in situ hybridization techniques, described in detail elsewhere (20,21). Riboprobes, complementary to partial fragments of NPY, AGRP, proopiomelanocortin (POMC), cocaine and amphetamine-regulated transcript (CART), leptin receptor (OBRb), brain-derived neurotrophic factor (BDNF) and pro-dynorphin (DYN) were generated from cloned cDNA, as previously described (12,18,22–25). Glutamate decarboxylase 67 (GAD67) and enkephalin (ENK) cDNA fragments were cloned from mouse hypothalamic cDNA. The 392 bp fragment of GAD67 was amplified using primers 5′-CTAAACCATCTCGCAAGCA-ACTA-3′ and 5′-TACAAATGGGAAGAAAATACAAGA-3′ (Genebank NM_008077.2) with 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s. DNA fragments were ligated into pGEMT-easy. The 473 bp fragment of ENK was amplified using primers 5′-CAAGAGGTATGGC-GGYTTCA-3′ and 5′-CAGTTGCTCAYGGGGGATGG -3′ (Genebank NM 017139*)* with 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. DNA fragments were ligated into pCR4Blunt-TOPO cloning vector (Invitrogen), and transformed into One Shot MAX Efficiency DH5$\alpha$-T1 chemically Competent *Escherichia Coli* (Invitrogen), and automated sequencing was performed to verify the sequences of interest.

Hypothalamic coronal 20 $\mu$m sections were collected from the very caudal extent of the arcuate nucleus (ARC) rostrally onto 2 sets of 10 slides. Under this sectioning regime, sections on an individual slide would have been separated by 200 $\mu$m in their brain of origin. Representative sections were quantified for each hypothalamic region of interest. The first set of slides spanned the full extent of the ARC, ~–4.52 to –2.30 mm, relative to Bregma, and according to the atlas of the rat brain (26). The second set of slides continued through to −1.40 mm, relative to Bregma. Sections were fixed, acetylated, and hybridized overnight at 58°C using $^{35}$S-labeled antisense riboprobes (1–1.5 $\times 10^{10}$ dpm/L). Slides were treated with ribonuclease A to remove the unhybridized probe and then desalted



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with a final high stringency wash in 0.1 × saline-sodium citrate at 60°C for 30 min. The slides were air dried and apposed to Biomax MR (Sigma). Autoradiographic images were quantified using the Image-Pro Plus system (Media Cybernetics), determining the intensity and area of the hybridization signal on the basis of set parameters. Integrated intensity was then computed using standard curves generated from $^{14}C$ autoradiographic microscales (Amersham). Image analysis was performed by an observer who was unaware of the respective treatment groups, on 4 or 5 sections spanning the ARC for NPY, AGRP, POMC, CART, DYN, ENK, GAD67, and OBRb. Three or 4 sections, spanning the ventromedial nucleus (VMH), were used to analyze OBRb, DYN and BDNF. Two or 3 sections from the dorsomedial nucleus (DMH) were used to analyze NPY, ENK, and DYN. DYN gene expression was analyzed in the lateral hypothalamus (LH), paraventricular nucleus (PVN), and supraoptic retrochiasmatic nucleus (SOR) using 2 sections, as was CART gene expression in the SOR.

*Statistical analysis.* Values are means ± SEM, and significance was determined at $P \leq 0.05$. Data were analyzed by 2-way ANOVA using GenStat, version 7.2 (Hemel Hempstead) in consultation with Biomathematics and Statistics Scotland, using the solid diet (C, HE, or C+HE) and EN as factors. For simplicity, ANOVA results were presented using the terminology "effect of solid diet" and "effect of EN," and interactions are stated only when significant. All pair-wise comparisons were analyzed by 1-way ANOVA and Student-Newman-Keuls method using SigmaStat for Windows, version 1.0 (Jandel). Regression analysis was used to examine the relation between grams of body fat and circulating leptin for each diet group, and treatments were compared by ANOVA with all pair-wise comparisons analyzed by Bonferroni, using GenStat, version 7.2.

## Results

*In vivo measures.* Body weight gain during the 5-wk study was not affected when rats consumed the solid diet, but it was increased by EN ($P < 0.01$; **Fig. 1***A, B*); EN supplementation of C, HE, or C+HE caused weight differentials of 42.5 g, 9.2 g, and 14.5 g, respectively (Fig. 1*B*). Energy intake on d 37 was also increased by EN ($P < 0.05$; Fig. 1*C*). The C+EN group had a higher body weight gain than all other groups except C+HE+EN. Irrespective of the diet supplied, ~90% of total energy was consumed during the night, and increased diet choice did not increase the percentage of energy consumed during the day (data not shown). The proportional contribution of each individual diet to total energy consumption was heavily influenced by the dietary choice available (**Table 1**). Thus HE was preferred over C when the 2 solid diets were supplied but not when EN was also provided. More than 50% of the total energy intake was consumed as EN when it was supplemented to the C diet, but not when supplemented to the HE diet. When all 3 diets were supplied, energy intake from EN (and C, see above) exceeded that from HE. Analysis of the proportion of total energy intake derived from each macronutrient suggested that the rats were trying to achieve an optimal protein intake (Table 1). Rats given access to combination diets that included C (i.e., C+HE, C+EN, and C+HE+EN) voluntarily selected a diet that contained 17.4–18.3% energy as protein. Rats fed HE alone or combined with EN could achieve a protein intake of only 14–15%, perhaps explaining their preference for HE, with its slightly higher protein content in the combination diet.

Whole-body MRI scans showed that EN had increased body fat content (**Fig. 2***A* ) and percentage of body fat (Fig. 2*B*) at the end of the study ($P < 0.001$). Solid diet did not have effects on these variables but there were significant interactions, reflecting the increased body fat of rats fed the HE diet. Final lean tissue content was not affected by solid diet or EN but there was a significant interaction between them (Fig. 2*C*). The percentage of lean tissue was not affected by solid diet but was decreased by EN ($P < 0.001$) and the interaction was significant (Fig. 2*D*).

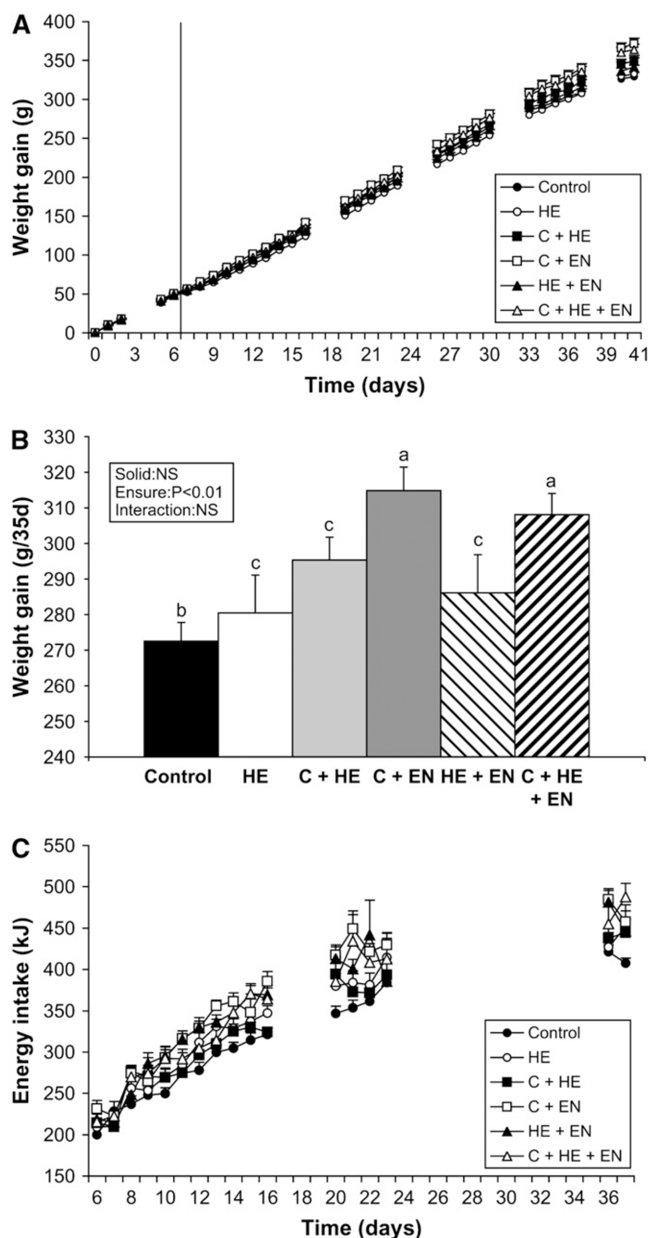


**FIGURE 1** Cumulative body weight gains (*A*), total body weight gains (*B*) and cumulative energy intakes (*C*) in male rats fed diets varying in energy content for 5 wk. Vertical line in (*A*) indicates point that diet treatments began. Values are means ± SEM, $n = 10$. Means without a common letter differ, $P \leq 0.05$. Not significant, $P > 0.05$.

*Blood parameters.* Terminal glucose and insulin concentrations were similar in all groups (**Table 2**) and there was no effect of solid diet or EN. NEFA and triglyceride concentrations were affected by the solid diet, but there was no effect of EN. Serum leptin levels were affected by both the solid diet ($P < 0.01$) and EN ($P < 0.05$), with all diet manipulations increasing circulating levels relative to rats fed C. All 5 obesogenic diets elevated blood NEFA, triglycerides, and leptin, and similar profiles of dietary influence were observed for each of the 3 analytes. The serum



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**TABLE 1** Contribution of each diet to total energy consumption and macronutrient intakes as a proportion of energy intake in male rats offered various combinations of the 3 diets

| Group | Diet | | | Macronutrient | | |
|---|---|---|---|---|---|---|
| | C | HE | EN | Fat | Carbohydrate | Protein |
| | *% of total intake* | | | *% energy* | | |
| C | 100 | — | — | 12 | 65 | 23 |
| HE | — | 100 | — | 33 | 52 | 15 |
| C+HE | 41.8 | 58.2 | — | 24.2 | 57.5 | 18.3 |
| C+EN | 45.1 | — | 54.9 | 17.5 | 64.4 | 18.1 |
| HE+EN | — | 77.4 | 22.6 | 30.5 | 54.7 | 14.8 |
| C+HE+EN | 33.7 | 28.7 | 37.6 | 21.8 | 60.9 | 17.4 |

leptin concentration was positively correlated with body fat (calculated from final MRI scan; $P < 0.001$) for each of the 6 diet groups (**Fig. 3***A*), but there was a significant overall effect of diet ($P < 0.01$) whereby, at a given level of body adiposity, the leptin concentration was lower in the C+EN group than in the HE and C+HE groups. A comparison between rats that did not receive EN and those that did, using simple linear regression (Fig. 3*B*), revealed that EN affected the association between adipose tissue and leptin by reducing the relative serum leptin level by ~1.3 $\mu$g/L at a fixed level of adipose tissue.

*Carcass dissection.* Tissue weights were recorded immediately postmortem (**Table 3**). Pooled dissected white adipose tissue (WAT) and epididymal WAT weight were not affected by solid diet but were increased by EN supplementation ($P < 0.01$), and there were significant interactions ($P < 0.05$), reflecting the obesogenic properties of the HE diet. Pooled dissected WAT weight had the same the rank order as grams of body fat measured by MRI. Retroperitoneal, mesenteric, omental and subcutaneous WAT, and liver were not affected by solid diet, but were increased by EN supplementation ($P < 0.01$). Interscapular brown adipose tissue was affected by the solid diet ($P < 0.01$) and was increased by EN ($P < 0.01$). There was no effect of diet on gastrocnemius or soleus muscles, adrenals, or combined testis weights.

*Hypothalamic gene expression.* Solid diet did not affect NPY or AGRP gene expression in the ARC (**Table 4**), but both mRNA levels were decreased by EN ($P < 0.01$). In addition, there was an interaction between solid diet and EN on AGRP gene expression ($P < 0.05$), reflecting increased adiposity, as indicated by the same statistical results as for body fat content. DYN gene expressions in the PVN, SON, and SOR were not affected by solid diet, but were decreased by EN ($P < 0.001$). CART gene expression in the SOR was similarly affected ($P < 0.05$; **Fig. 4**). Solid diet affected GAD67 gene expression in the ARC ($P < 0.05$), and there was an interaction between solid diet and EN ($P < 0.05$). In addition, there were no effects of dietary manipulation in gene expressions of POMC, CART, OBRb, DYN, or ENK in the ARC; OBRb, BDNF, or DYN in the ventromedial hypothalamus; NPY or ENK in the dorsomedial hypothalamus, and DYN in the lateral hypothalamus.

## Discussion

The main objectives of this study were *1*) to develop a rat model of juvenile obesity characterized by excess weight gain, increased body fat, and preserved or enhanced lean tissue by the provision of diet choice and solid and liquid dietary formulations, and *2*) to establish the effect of supplementation with the complete liquid diet, Ensure (EN), on the expression of hypothalamic energy balance genes in Sprague-Dawley rats. Building on our previous study (12), a juvenile obesity model should incorporate the key characteristics outlined in *1*), above, to be of value in mechanistic investigations. We were successful in identifying more than one dietary manipulation that met the above criteria, whereas other dietary combinations were inappropriate for rats of this age. Furthermore, these studies effectively juxtaposed diets that induce obesity due to obligatory consumption of unbalanced macronutrient combinations with those that allow rats to express dietary preferences and to select a more optimal macronutrient profile while retaining the capacity for palatability-driven overconsumption. Our study demonstrated the clear effects of EN supplementation on hypothalamic gene

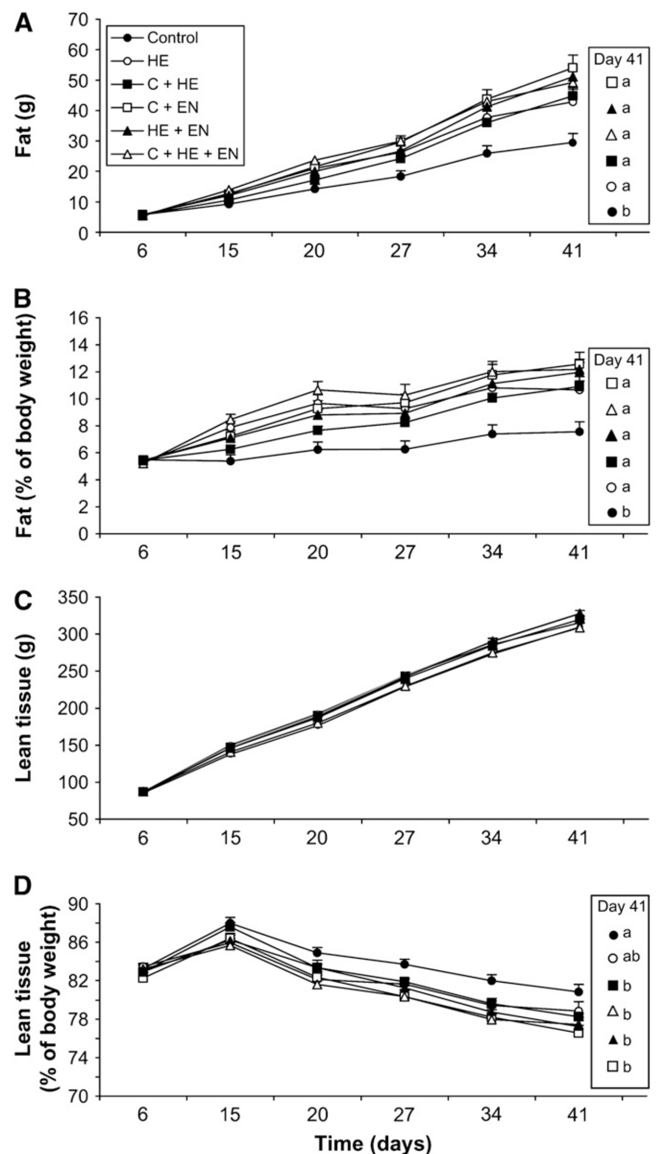


**FIGURE 2** Total body fat (*A*), body fat as a % of body weight (*B*), total body lean tissue (*C*) and body lean tissue as a percentage of body weight (*D*) in male rats fed diets varying in energy content for 5 wk. Values are means ± SEM, $n = 10$. Significant differences between means are indicated in the right hand box, where means without a common letter differ, $P \leq 0.05$.



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**TABLE 2** Plasma glucose, NEFA, triglycerides, insulin, and serum leptin concentrations in male rats fed various combinations of the 3 diets[1]

| | Diet groups | | | | | | 2-Way ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | HE | C + HE | C + EN | HE + EN | C + HE + EN | Solid | Ensure | Interaction |
| Glucose, *mmol/L* | 8.2 ± 0.47 | 9.9 ± 0.32 | 8.0 ± 0.77 | 9.1 ± 0.64 | 8.8 ± 0.59 | 8.7 ± 0.79 | NS[2] | NS | NS |
| NEFA, *mmol/L* | 0.3 ± 0.02[c] | 0.7 ± 0.06[a] | 0.5 ± 0.08[b] | 0.4 ± 0.06[b] | 0.6 ± 0.08[a] | 0.6 ± 0.09[a] | $P < 0.001$ | NS | NS |
| Triglycerides, *mmol/L* | 1.9 ± 0.18[a] | 4.0 ± 0.50[a] | 3.0 ± 0.60[a] | 2.8 ± 0.45[a] | 3.9 ± 0.66[a] | 4.0 ± 0.61[a] | $P < 0.01$ | NS | NS |
| Insulin, *pmol/L* | 279 ± 22.4 | 274 ± 43.1 | 298 ± 27.5 | 279 ± 25.9 | 241 ± 43.1 | 284 ± 27.6 | NS | NS | NS |
| Leptin, *μg/L* | 4.0 ± 0.43[b] | 8.7 ± 1.52[a] | 8.3 ± 0.77[a] | 7.8 ± 1.18[a] | 9.0 ± 1.07[a] | 9.4 ± 1.08[a] | $P < 0.01$ | $P < 0.05$ | NS |

[1] Values are means ± SEM, $n = 10$. Means in a row with superscripts without a common letter differ, $P \leq 0.05$.
[2] Not significant, $P > 0.05$.

expression, supporting the hypothesis that the additional effect on body weight and composition of excess energy in liquid form engaged the same energy balance systems that we previously demonstrated were engaged by a solid high energy (HE) diet (12,18). But again, the induced changes in these signals, which are assumed to translate into changes in peptide concentration, were ineffective in countering the developing obese phenotype.

*Model development.* Our previous investigation of juvenile diet-induced obesity, using 4-wk–old rats fed the solid HE diet, resulted in the unexpected phenotype of reduced body weight but increased adiposity. The outcomes observed in this study, with this diet alone, were similar. Feeding just the HE pellet diet obliges rats to consume an energy-dense diet with a relatively low protein content, and we speculated that the phenotype observed previously (12) was due to an inadequate protein availability to meet the demands of growing rats. When given an appropriate choice, rats selected a diet that contained at least 17% protein, with dietary preferences influenced by the combinations of diets given. In general, the provision of dietary choice is obviously a more realistic manipulation in the context of the human food environment than is provision of a single diet with an imbalanced macronutrient composition. Maximum weight gain and fat and lean tissue growth was achieved by rats in groups that were able to select a diet containing at least 17% protein (with C), and where EN was also available (C+EN and C+HE+EN). Consumption of the HE and HE+EN diets provided <15% of energy from protein, with these groups returning the lowest lean tissue weight by MRI, and supporting our earlier suggestion of a marginal protein deficiency in these juvenile rats. It is also notable that the young rats studied here selected less of their total energy intake from EN than did older rats in earlier studies (18,27). This probably reflects the high protein requirement for supporting rapid lean growth in juvenile rats.

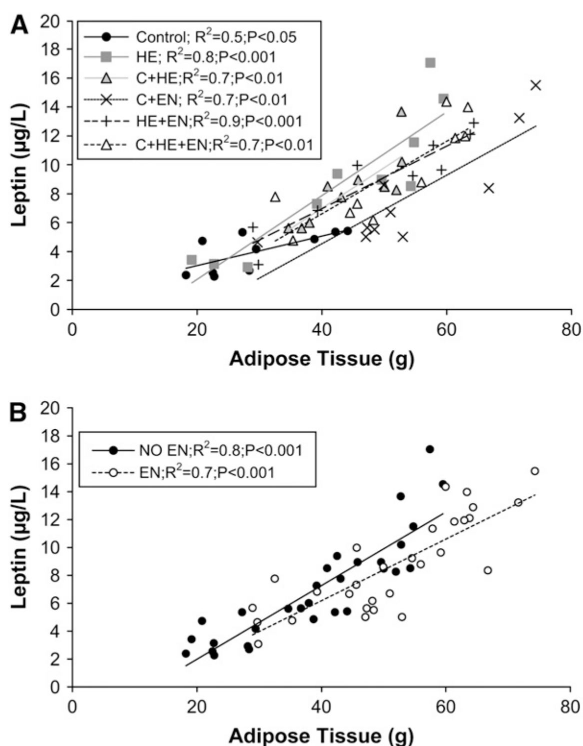


**FIGURE 3** Correlations between serum leptin concentrations and total body fat assessed by MRI scanning on d 41 for rats in each of the 6 diet groups ($n = 10$ each) (*A*) and for groups that did or did not received EN ($n = 30$ each) (*B*). In panel *A*, Control: $y = 0.124x + 0.9466$; HE: $y = 0.289x - 3.7084$; C+HE: $y = 0.282x - 4.3117$; C+EN: $y = 0.2367x - 4.959$; HE+EN: $y = 0.2157x - 1.6732$; C+HE+EN: $y = 0.2508x - 3.4376$. In panel *B*, NO EN: $y = 0.2633x - 3.2947$; EN: $y = 0.2208x - 2.6693$.

Although the consumption of the C+EN and C+HE+EN diets maximized the accumulation of fat and lean tissue, there were clear diet-specific effects on circulating metabolites and hormones that were apparently independent of body composition. Intriguingly, rats fed the C+EN diet had the lowest NEFA, triglycerides, and leptin of the 5 obesogenic-diet groups. Thus, circulating leptin levels were increased by all 5 diets relative to C, consistent with rats fed HE or high fat diets (18,28), and leptin levels within each group correlated with body fat mass, but C+EN rats had the lowest serum leptin concentration at any level of body adiposity, and grouping by EN or no EN revealed an overall depressive effect of EN on serum leptin concentrations. This apparent dissociation between adipose tissue weight and circulating leptin was reported previously in female Wistar rats fed a high-fat diet (29) and was discussed as a possible contributory factor in the weight gain on high-fat diets.

*Hypothalamic gene expression.* An overall effect of solid diet was limited to expression levels in the ARC of GAD67, a rate-limiting enzyme in the production of γ-aminobutyric acid. The complex profile of GAD gene expression following diet manipulation did not occur in any of the other variables measured, and additional studies would be required to unravel the importance of the current findings in diet-induced obesity.

The effects of EN supplementation on NPY and AGRP gene expression in the ARC supported our previous findings in juvenile rats (12). All 5 obesogenic diets lowered the expression of both NPY and AGRP, and mRNA concentrations were maximally decreased by EN supplementation in C or C+HE, the



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**TABLE 3** Tissue weights of male rats fed various combinations of the 3 diets[1]

| | Diet groups | | | | | | 2-Way ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | HE | C + HE | C+ EN | HE + EN | C+ HE+EN | Solid | Ensure | Interaction |
| | | | | *g* | | | | | |
| IBAT[2] | 0.3 ± 0.03$^{c}$ | 0.6 ± 0.04$^{ab}$ | 0.5 ± 0.04$^{b}$ | 0.5 ± 0.05$^{b}$ | 0.7 ± 0.05$^{a}$ | 0.6 ± 0.04$^{ab}$ | $P < 0.001$ | $P < 0.001$ | NS[3] |
| Retroperitoneal | 4.4 ± 0.63$^{b}$ | 6.4 ± 0.85$^{ab}$ | 6.7 ± 0.81$^{ab}$ | 9.0 ± 0.83$^{a}$ | 7.9 ± 0.72$^{a}$ | 8.4 ± 0.64$^{a}$ | NS | $P < 0.001$ | NS |
| Epididymal | 4.0 ± 0.37$^{b}$ | 6.3 ± 0.77$^{a}$ | 6.2 ± 0.44$^{a}$ | 7.2 ± 0.68$^{a}$ | 6.0 ± 0.47$^{a}$ | 7.0 ± 0.60$^{a}$ | NS | $P < 0.01$ | $P < 0.01$ |
| Subcutaneous | 4.7 ± 0.52$^{b}$ | 5.4 ± 0.64$^{ab}$ | 6.2 ± 0.37$^{ab}$ | 7.6 ± 0.82$^{a}$ | 6.5 ± 0.62$^{ab}$ | 7.3 ± 0.68$^{a}$ | NS | $P < 0.01$ | NS |
| Mesenteric | 2.8 ± 0.30$^{b}$ | 3.6 ± 0.39$^{ab}$ | 3.5 ± 2.00$^{ab}$ | 4.2 ± 0.31$^{a}$ | 3.8 ± 0.33$^{ab}$ | 4.0 ± 0.22$^{a}$ | NS | $P < 0.01$ | NS |
| Omental | 0.5 ± 0.07$^{b}$ | 0.8 ± 0.13$^{ab}$ | 0.6 ± 0.05$^{ab}$ | 1.00 ± 0.1$^{a}$ | 0.9 ± 0.09$^{a}$ | 0.9 ± 0.07$^{a}$ | NS | $P < 0.001$ | NS |
| Pooled adipose | 16.5 ± 1.60$^{b}$ | 22.4 ± 2.69$^{ab}$ | 23.2 ± 1.69$^{ab}$ | 28.9 ± 2.53$^{a}$ | 25.0 ± 1.93$^{a}$ | 27.6 ± 1.85$^{a}$ | NS | $P < 0.001$ | $P < 0.05$ |
| Liver | 16.5 ± 0.35$^{b}$ | 16.7 ± 0.79$^{b}$ | 17.2 ± 0.52$^{ab}$ | 19.3 ± 0.58$^{a}$ | 17.7 ± 0.76$^{ab}$ | 18.7 ± 0.36$^{ab}$ | NS | $P < 0.001$ | NS |
| Testes | 3.3 ± 0.06 | 3.4 ± 0.08 | 3.3 ± 0.05 | 3.4 ± 0.09 | 3.4 ± 0.05 | 3.4 ± 0.05 | NS | NS | NS |
| Adrenal | 0.07 ± 0.005 | 0.07 ± 0.005 | 0.08 ± 0.012 | 0.08 ± 0.005 | 0.08 ± 0.004 | 0.07 ± 0.003 | NS | NS | NS |
| Soleus | 0.18 ± 0.01 | 0.16 ± 0.01 | 0.19 ± 0.01 | 0.17 ± 0.01 | 0.17 ± 0.01 | 0.17 ± 0.01 | NS | NS | NS |
| Gastrocnemius | 2.4 ± 0.06 | 2.5 ± 0.10 | 2.5 ± 0.09 | 2.5 ± 0.05 | 2.5 ± 0.37 | 2.5 ± 0.06 | NS | NS | NS |

[1] Values are means ± SEM, $n = 10$. Means in a row with superscripts without a common letter differ, $P \leq 0.05$.
[2] Interscapular brown adipose tissue.
[3] Not significant, $P > 0.05$.

groups with the highest weight gain, fat, and lean tissue mass. The downregulation of orexigenic neuropeptide gene expression is consistent with the hypothesis of counter-regulatory processes attempting to limit excessive adiposity, but failing to do so (12). Overall, the supplementation of solid diets with EN appears to accentuate this downregulation. The precise feedback signals involved in downregulation of orexigenic gene expression remain to be established, however, because body fat was a more accurate predictor of magnitude of effect on gene expression, than absolute leptin levels per se.

Dynorphin mRNA is widely distributed in the hypothalamus of rats (30), and accumulating evidence suggests that consuming palatable diets rich in fat and sucrose increases expression in the ARC (31). Pair-feeding studies indicate that a contributory factor in this effect may be palatability-induced overconsumption; however, data from an earlier study of adult Sprague Dawley rats (18) suggests that palatability alone is not the only regulator of DYN gene expression. Interestingly, EN had a substantial depressive effect on DYN gene expression in the PVN, the SON, and the SOR. The SOR consists of magnocellular neurons and probably represents a caudal extension of the supraoptic nucleus [SON; also known as the caudal supraoptic nucleus (cSON)] (26, 30). Whereas DYN gene expression in the PVN and SON exhibited a similar response to diet as did NPY and AGRP in the ARC, the regulatory changes in DYN gene expression in the SOR, although again similar, were closely paralleled by the profile of the CART gene expression in the same nucleus. Depending on the precise role of CART in energy balance regulation (32,33), this coregulation may suggest an involvement in separate processes. The coregulation of DYN and CART gene expression may be explained by the substantial coexpression of these 2 peptide genes in the SOR/cSON and, indeed, in the SON (30). There is growing evidence that SON, and thus the SOR, are involved in the regulation of feeding and energy balance (33–35). A coordinated variation in the expression of multiple energy-balance genes makes this a fascinating area for further experimental investigation.

In this further development of a model of juvenile obesity, our earlier observation of an obese but normal weight phenotype in rats consuming the HE diet (12) was confirmed, highlighting that this diet alone or in combination with other diets of similar protein content is probably inappropriate for mechanistic investigation in such young rats. Providing rats with a choice of diets allows them to meet their protein requirements; also, palatability-driven overconsumption appears to more reasonably represent the type of dietary exposure of adolescent humans leading to the development of an overweight and obese

**TABLE 4** Hypothalamic neuropeptide or receptor gene expression in male rats offered various combinations of the 3 diets[1]

| | Diet groups | | | | | | 2-Way ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | HE | C + HE | C + EN | HE + EN | C + HE + EN | Solid | Ensure | Interaction |
| | | | | *% of C* | | | | | |
| NPY-ARC | 100 ± 6.5$^{a}$ | 86.9 ± 5.14$^{a}$ | 78.0 ± 5.8$^{a}$ | 70.7 ± 9.9$^{a}$ | 74.8 ± 9.8$^{a}$ | 69.0 ± 5.9$^{a}$ | NS[2] | $P < 0.01$ | NS |
| AGRP | 100 ± 8.2$^{a}$ | 90.3 ± 10.6$^{a}$ | 73.9 ± 6.2$^{ab}$ | 58.2 ± 7.5$^{b}$ | 74.1 ± 7.1$^{ab}$ | 76.0 ± 7.9$^{ab}$ | NS | $P < 0.01$ | $P < 0.05$ |
| DYN-ARC | 100 ± 11.2 | 128.9 ± 21.2 | 147.3 ± 16.2 | 138.5 ± 25.0 | 129.7 ± 26.3 | 112.8 ± 13.5 | NS | NS | NS |
| DYN-PVN | 100 ± 17.2$^{a}$ | 91.4 ± 10.8$^{a}$ | 77.4 ± 6.8$^{a}$ | 60.5 ± 4.8$^{a}$ | 60.2 ± 4.5$^{a}$ | 71.3 ± 9.6$^{a}$ | NS | $P < 0.001$ | NS |
| DYN-SOR | 100 ± 8.9$^{ab}$ | 87.6 ± 19.8$^{ab}$ | 105.5 ± 13.6$^{a}$ | 51.2 ± 15.4$^{ab}$ | 47.0 ± 11.1$^{b}$ | 57.4 ± 10.7$^{ab}$ | NS | $P < 0.001$ | NS |
| DYN-SON | 100 ± 7.1$^{a}$ | 114.5 ± 14.8$^{a}$ | 86.4 ± 5.9$^{a}$ | 69.8 ± 6.4$^{a}$ | 65.3 ± 9.4$^{a}$ | 68.0 ± 8.4$^{a}$ | NS | $P < 0.001$ | NS |
| GAD67-ARC | 100 ± 15.5$^{a}$ | 126.6 ± 11.2$^{a}$ | 68.9 ± 6.4$^{a}$ | 116.4 ± 20.3$^{a}$ | 83.6 ± 12.7$^{a}$ | 83.8 ± 11.5$^{a}$ | $P < 0.05$ | NS | $P < 0.05$ |
| CART-SOR | 100 ± 14.9$^{a}$ | 90.4 ± 7.9$^{a}$ | 93.9 ± 12.1$^{a}$ | 69.6 ± 7.2$^{a}$ | 59.8 ± 9.4$^{a}$ | 90.5 ± 11.6$^{a}$ | NS | $P < 0.05$ | NS |
| OBRb-ARC | 100 ± 8.4 | 86.4 ± 6.9 | 86.9 ± 7.6 | 85.1 ± 14.7 | 75.8 ± 7.0 | 82.7 ± 11.1 | NS | NS | NS |

[1] Values are means ± SEM, $n = 10$. Means in a row with superscripts without a common letter differ, $P \leq 0.05$.
[2] Not significant, $P > 0.05$.



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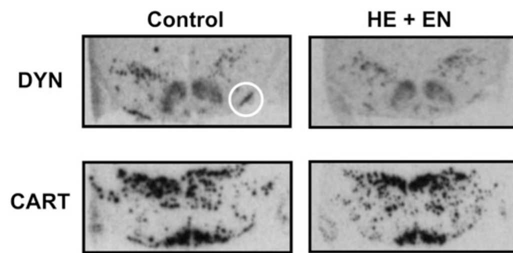


**FIGURE 4** DYN and CART gene expression in adjacent sections of hypothalamus from 2 rats fed either C or HE+EN. White circle identifies gene expression in the SOR/cSON.

phenotype. These diet combinations are not only more realistic as a means of inducing obesity than the imposition of a diet that is unbalanced in terms of its macronutrient content, but are also more controlled than classical "cafeteria-type" diets. The experimental design of 3 solid diet combinations, with or without EN supplementation, successfully demonstrates the effects of EN on hypothalamic gene expression. In general, developing obesity appears to be recognized at the hypothalamic level, although the pathways to obesity differ between diets, possibly because of their macronutrient content (36,37). EN supplementation appears to engage some of the same energy balance systems as the solid obesogenic diet, HE (12), and accentuates these regulatory events. However, despite a 40–50% downregulation of individual gene expression, the induced changes in these signals are apparently ineffective in fully countering the developing obese phenotype. Assuming that substantial downregulation of the activity of orexigenic neuropeptide systems, such as NPY and AGRP, would lead the animal into a state of negative energy balance in the absence of any overriding and counteracting signal, it is necessary to invoke a role for such signals. The engagement of obesogenic diets with the reward systems in the forebrain is likely to be important. Whereas our knowledge of the sensitivity of hypothalamic systems to dietary manipulation and developing obesity is growing, the interaction of endogenous homeostatic and reward systems on different dietary and genetic backgrounds remains poorly described, although the neural pathways themselves have been the subject of detailed investigation (38–41). The neural pathways that permit the dominance of corticolimbic processes over hypothalamic signaling may provide one basis for differential susceptibility to obesity.

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