Effects of Chronic Weight Perturbation on Energy Homeostasis and Brain Structure in Mice

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Running Head: Weight perturbation alters defended body weight

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ABSTRACT

Maintenance of reduced body weight in lean and obese human subjects results in the persistent decrease in energy expenditure (EE) below what can be accounted for by changes in body mass and composition. Genetic and developmental factors may determine a CNS-mediated minimum “threshold” of somatic energy stores below which behavioral and metabolic compensations for weight loss are invoked. A critical question is whether this threshold can be altered by environmental influences, and by what mechanisms such alterations might be achieved.

We examined the bioenergetic, behavioral, and CNS structural responses to weight reduction of diet-induced obese (DIO) and never-obese (CON) C57BL/6J male mice. We found that weight-reduced DIO and CON animals showed reductions in energy expenditure – adjusted for body mass and composition - comparable (-10 -15%) to those seen in human subjects. The proportion of excitatory synapses on ARC POMC neurons was decreased by approximately 50% in both DIO and CON weight-reduced mice.

These data suggest that prolonged maintenance of an elevated body weight (fat) alters energy homeostatic systems to “defend” a higher level of body fat. The synaptic changes could provide a neural substrate for the disproportionate decline in energy expenditure in weight-reduced individuals. This response to chronic weight elevation may also occur in humans. The mouse model described here could help to identify the molecular/cellular mechanisms underlying both the defense mechanisms against sustained weight loss and the upward re-setting of those mechanisms following sustained weight gain.
INTRODUCTION:

Long-term maintenance of even modest reductions in body weight ameliorates or eliminates many of the co-morbidities of obesity (11). The recidivism rate to obesity in formerly-obese individuals is 75-85% (64), reflecting the potent metabolic and environmental pressures opposing long-term maintenance of a reduced body weight. We have previously shown that the maintenance of a 10% or greater reduction in body weight in both lean and obese humans is associated with a decrease in energy expenditure that is 15-20% below what can be accounted for by changes in body mass and body composition. This adaptive thermogenesis does not abate over time (49), and predominantly reflects increased mechanical work efficiency of skeletal muscle, decreased circulating concentrations of bioactive thyroid hormones, and reduced sympathetic autonomic nervous system tone (4, 33, 50, 59).

Leptin is an adipocyte-derived hormone whose circulating plasma concentrations are correlated with fat stores at usual (stable) body weight, but which rapidly decline during food restriction and/or fasting (1). We have proposed that central nervous system (CNS) energy homeostasis mechanisms respond asymmetrically (in a threshold-like mechanism) to changes in circulating plasma leptin concentrations (32). This asymmetry is evident in the demonstrations that reductions in circulating leptin concentrations in weight reduced/food restricted humans induces a strong leptin-reversible metabolic adaptation (energy expenditure reduced beyond expected per unit of metabolic mass) (47), while increases in plasma leptin concentrations as a result of weight gain do not provoke long term changes in energy expenditure (56). Even 10-fold increases in circulating plasma leptin concentrations resulting from exogenous leptin administration do not invoke consistent increases in energy expenditure or decreases in energy intake (17). Regulatory pathways – constituted by specific neurons and their connections – in the
hypothalamus (60) and brainstem (14, 22) provide the neural substrate for the proposed threshold mechanism that uses ambient leptin as a primary afferent signal (32). This threshold – set by genetic and developmental influences on its molecular and anatomic substrates – determines a minimum circulating concentration of leptin (hence body fat) that is “accepted” by the CNS as sufficient to ensure reproductive capacity (1) and survival in circumstances of restricted access to food calories (32). The secular trend towards increasing prevalence of obesity, and its continued resistance to long-term successful therapy (44, 64, 67), suggest that increasing levels of body fatness are being “defended” and that structural/molecular changes in CNS regulatory regions for energy homeostasis may play a critical role in these changes. An important question in this context is whether the “threshold” for minimum adiposity can be reset upward by environmental factors, leading to physiological defense of an acquired increase in fat mass.

In rodents, weight loss due to caloric restriction results in decreased energy expenditure per unit of metabolic mass (“metabolic adaptation”) consistent with the “defense” of body fat stores by CNS-mediated responses to circulating leptin and other signals (e.g. insulin) reflecting the status of somatic energy stores (3, 7, 19, 24, 28, 38-40, 43, 58). Rats selected by breeding to be predisposed to diet-induced obesity defend higher body weights than DIO resistant rats (36), readily regain lost weight following a switch back to ad-libitum food access after a period of hypocaloric feeding (34), and have increased arcuate nucleus (ARC) expression of neuropeptide Y (NPY), a key anabolic neuropeptide released from leptin-sensitive neurons that increases food intake and decreases energy expenditure (35). Inbred mouse strains fed a high fat diet gain different amounts of body fat that correlates with differential expression of genes in key regions of the arcuate nucleus (21, 66).
The aim of the present study was to assess - in a mouse model - the physiological and molecular consequences of maintenance of increased body fat (by high fat diet) and the subsequent adaptations following caloric restriction and maintenance of a reduced body weight. Our hypothesis was that such a chronic elevation in body fat would invoke changes in the structure of the hypothalamus resulting in an upward resetting of the threshold for minimum body fat. To assess the neural substrates for changes in energy expenditure and food intake in these circumstances, we analyzed excitatory and inhibitory synapses onto the cell bodies of POMC neurons in the arcuate nucleus, a cell population that is known to play a role in body weight regulation and whose synapses are leptin-responsive. We hypothesized that prolonged maintenance of an elevated body weight by DIO followed by weight loss would result in mice that were hypometabolic compared to DIO and never-obese mice, indicating that maintenance of an elevated body weight results in long-term upward “re-setting” of a minimum threshold for body fat (32). We anticipated that the ratio of excitatory/total synapse ratios onto the POMC population would be lower in the two weight-reduced groups, a phenotype similar to the decreases characterizing congenitally leptin deficient mice (45).
Materials and Methods

Animals and Diets: 18 week-old C57BL/6J male mice were obtained from Jackson Laboratory (Bar Harbor, ME). Sixteen diet-induced obese (DIO mice – fed a high fat diet starting at 6 weeks of age; Research Diets, Inc. D12492i, 60 kcal% fat), and 16 control diet-fed (CON mice – fed a low fat diet also starting at 6 weeks of age; Research Diets, Inc. D12450Bi, 10 kcal% fat) were used for these studies (Figure 1). Upon receipt, animals were kept in a pathogen-free barrier facility maintained at 22-24 ºC with a 12-h dark-light cycle (lights on at 0700 h). The mice were individually housed in plastic pens with corn-cob based bedding, fed the same diet they had been provided at Jackson Laboratory, and given ad libitum access to food (diet as specified) and water during a 30 day acclimatization period. The cages were equipped with feeding baskets specially designed to minimize food spillage. During this period, body weight and food intake were monitored every 2 to 3 days.

The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

Study Design: After the 30-day acclimatization period, mice in each group (DIO or CON) were paired by body weight (nearest body weight ± 0 -1.7g) and one member of each pair randomized to either an ad-libitum fed group (DIO-AL & CON-AL) or a weight-reduced group (DIO-WR & CON-WR). There were 8 mice in each of the 4 groups. Mice in the weight-reduced groups received 50% of their average ad-libitum daily food intake until their body weight reached 80% of initial value (defined as day 0, Figure 1A) at which time the mice were switched to 80% of their initial daily food intake. Subsequent adjustments in calories provided were made daily for the rest of the experimental period in order to maintain each mouse between 79-81% of initial (pre-caloric restriction) body weight (Figure 1A). Weight-reduced mice (DIO-WR and CON-
WR) had free access to water and were given 1/3 of their individual calculated food ration (± 0.1 g) in the morning (07:45-08:15h) and 2/3 of the food ration in the evening (18:30-19:00). All *ad-libitum* fed mice (DIO-AL and CON-AL) had free access to food and water throughout the day. In a subsequent study, 7 DIO-AL mice and 12 DIO-WR mice were weight perturbed in the same manner as described above and the DIO-WR were subsequently switched to *ad-libitum* access to the high fat diet. Food intake (g) and metabolizable energy intake (kcal/24h) was measured over the first twenty four hours (*Table 3*).

**Body weight, body composition and food intake:** Body weight was measured (± 0.1 g) daily before morning feeding using an Ohaus Scout Pro 200g scale (Nänikon Switzerland, between 07:45-08:15h). For *ad-libitum* fed mice (DIO-AL and CON-AL), body composition (fat mass: FM, fat-free mass: FFM, & extracellular fluid) was measured by time-domain-NMR (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany) (16) before the morning feeding every 2-3 weeks; before and after calorimetry measurements (see below); before start of the weight reduction protocol; and on the day prior to sacrifice. Food intake was recorded daily for the WR mice, and every 2 to 3 days for the AL mice (by weighing specially constructed feeding baskets designed to minimize spillage) during the entire weight perturbation experiment (*Table 1*).

**Energy expenditure by indirect calorimetry:** Energy expenditure was measured with a LabMaster-CaloSys-Calorimetry System (TSE Systems, Bad Homburg, Germany). O2 and CO2 measurements were taken every 14 minutes during a 72 hour period while mice were maintained on their respective weight maintenance feeding schedules. Because of possible initial stress related to transfer to the chambers, only the last 48 hours of measurements were used to calculate total 24-hour energy expenditure (TEE; expressed in kcal/day) and respiratory quotient (RQ =
Resting energy expenditure (REE in kcal/day) was defined as the lowest one hour period of energy expenditure, which coincided with the lowest 1 hour of total ambulatory activity during the 48-hour period and this value was extrapolated to 24 hours. Non-resting energy expenditure (NREE) was calculated as the difference between total energy expenditure (TEE) and REE. Physical activity was measured by an infrared beam system integrated with the LabMaster system. Total activity (beam breaks) in X, Y, and Z axis was stored every 14 minutes. The system is designed to differentiate between fine motor movement (defined as a single X or Y axis beam break), ambulatory movement (defined as the simultaneous breaking of two adjacent X or Y beams), and rearing, defined as the breaking of the Z axis infrared beam.

**Calculations:** Energy expenditure is proportional to body mass and composition [fat-free (FFM) and fat (FM mass)]. We related total energy expenditure (TEE; kcal/day) of DIO-AL and CON-AL mice to both FFM and FM by multiple regression analysis (2, 33). There was no significant effect of diet composition on TEE. We therefore pooled the data from *ad-libitum* fed mice to create a baseline regression equation relating TEE (kcal/24h) to FFM and FM (grams) (TEE = 0.34 * FFM + 0.06 * FM + 5.16, R² = 0.66, p < 0.01). This equation was used to predict TEE for all mice following experimental weight perturbation, as we have done in similar studies of human subjects (33, 56). The residuals (i.e. the difference between measured and predicted values) were calculated for each animal and were tested against the null hypothesis that they were equal to zero. Baseline regression equations relating resting energy expenditure (REE – lowest one hour period of energy expenditure extrapolated to 24h) and non-resting energy expenditure (NREE = TEE - REE) to FFM and FM, predicted REE and NREE values, and residuals were also calculated from data obtained by indirect calorimetry as described above.
(REE = 0.17 * FFM + 0.14 * FM + 4.96, $R^2 = 0.74$; NREE = 0.18 * FFM - 0.08 * FM + 0.20, $R^2 = 0.53$).

**Serum Hormone and Metabolite Profiles:** Before initiation of the weight reduction protocol, and at time of sacrifice, blood glucose (by tail bleed) and circulating leptin, insulin and bioactive thyroid hormone concentrations (by retro-orbital bleed) concentrations were determined after a 4-hour fast (see arrows on Figure 1). Blood for hormone and metabolite assays was allowed to clot for 1 hour at room temperature, spun at 4°C for 10 minutes at 1000g, and serum collected and frozen at -80°C until time of assay. Leptin was assayed using Quantikine ELISA kit (R&D Systems, Minneapolis, USA); insulin using the Mercodia Ultrasensitive Mouse Insulin ELISA (Mercodia AB, Uppsala, Sweden); T3 and T4 using RIA at Hormone Assay & Analytical Services Core at Vanderbilt University (Vanderbilt University, Nashville, TN); TSH by RIA at the National Hormone and Peptide Program (UCLA Medical Center, Torrance, CA). All assays were conducted according to manufacturer’s protocols. HOMA2 (calculator developed by University of Oxford - http://www.dtu.ox.ac.uk/index.php?maindoc=/HOMA/index.php based on (37)) was used to estimate insulin resistance (HOMA IR) and insulin sensitivity (HOMA S).

**Synaptic quantification on POMC neurons:** Animals were deeply anesthetized then transcardially perfused with 50 ml of heparinized saline followed by 200 ml of fixation solution (4% paraformaldehyde 0.195% Picric acid and 0.1% glutaraldehyde in 0.1M phosphate buffer (PB, pH 7.4) and then brains processed for immunolabeling for POMC for subsequent electron microscopic examination. Ultrathin sections were cut on a Leica ultra microtome, collected on Formvar-coated single-slot grids and analyzed with a Tecnai 12 Biotwin (FEI Company) electron microscope. The quantitative and qualitative analysis of synapse number was performed in an
unbiased fashion as described earlier (12, 45). To obtain a complementary measure of axo-
somatic synaptic number, unbiased for possible changes in synaptic size, the dissector technique
was used. On consecutive 90-nm-thick sections we determined the average projected height of
the synapses and used about 30% of this value as the distance between the dissectors. On the
basis of this calculation, the number of axo-somatic synapses was counted in two consecutive
serial sections about 270 nm apart ("reference" and "look-up" sections) of 7 perikarya profiles in
each animal. Synapse characterization was performed at a magnification of 20,000. Symmetric
and asymmetric synapses were counted on all selected neurons only if the pre- and/or
postsynaptic membrane specializations were seen and synaptic vesicles were present in the
presynaptic bouton. Synapses with neither clearly symmetric nor asymmetric membrane
specializations were excluded from the assessment. The plasma membranes of selected cells
were outlined on photomicrographs and their length was measured with the help of Scion image
software (NIH). Plasma membrane length values measured in the individual animals were added
and the total length was corrected to the magnification applied. Synaptic densities were evaluated
according to the formula NV=Q-/Vdis where Q- represented the number of synapses present in
the "reference" section that disappeared in the "look-up" section. Vdis is the dissector volume
(volume of reference) which is the area of the perikaryon profile multiplied by the distance
between the upper faces of the reference and look-up sections, i.e., the data are expressed as
numbers of synaptic contacts per unit volume of perikaryon. The synaptic counts were expressed
as numbers of synapses on a membrane length unit of 100 µm. We analyzed 6 POMC
immunolabelled neurons per animal (DIO-AL n = 6, DIO-WR n = 8, CON-AL n =7, and CON-
WR n = 8)
**Statistical analysis:** Data are expressed as means ± SEM. Statistical analyses were performed using JMP (ver. 7; SAS, North Carolina). Where applicable, 2-way ANOVA’s were conducted using diet (DIO or CON) and treatment (WR or AL) as grouping variables. To determine whether the relationship between circulating leptin and fat mass differed among treatment groups, within group regressions were performed relating leptin to FM (Figure 1C) and then re-analyzed by ANCOVA using group as a covariate for all groups wherein the relationship of leptin to FM was statistically significant, i.e., all groups except CON-WR. To ascertain that circulating leptin concentrations were reduced following weight loss, comparisons of absolute leptin concentrations were made between DIO-AL and DIO-WR and between CON-AL and CON-WR. To ascertain that any metabolic differences between DIO-WR and CON-AL groups were not due to lower circulating leptin concentrations in DIO-WR, a comparison of absolute circulating leptin concentrations was made between DIO-WR and CON-AL. Statistical significance was prospectively defined as $P<0.05$.

**Results**

**Body Weight & Body Composition**

At the start of the weight-reduction phase of the study (day 0, mice aged 22 weeks), DIO mice weighed 54±3% more than *ad-libitum* fed CON mice and had significantly higher fractional body fat (DIO, 29±1%; CON, 5±1% fat) (Figures 1 and 2A). From days 0 to 183 of the weight reduction phase, both *ad-libitum*-fed groups (DIO-AL and CON-AL) gained a significant amount of body mass (Figures 1 and 2A). The increase in mass of both DIO-AL and CON-AL mice was primarily the result of increased fat mass (81±4% of weight increment in DIO-AL and 79±6% CON-AL). At time of sacrifice, DIO-AL body weight was 62±3% higher
than CON-AL body weight; 75±3% of this excess weight was accounted for by increased FM. By design, caloric restriction (from day 0 to day 183) resulted in a 20% decrease in body weight in both DIO-WR and CON-WR groups. DIO-WR mice lost significant amounts of FM and FFM (FM accounted for 65±4% of weight loss), whereas CON-WR mice showed a significant decrease only in FFM (FFM accounted for 87±3% of lost weight). Weight and body composition of DIO-WR and CON-AL mice were not significantly different (Table 1 and Figure 2A and B).

**Energy Expenditure (TEE, REE, and NREE)**

Absolute TEE and REE of DIO-AL-fed mice were significantly higher than in CON-AL (Table 1). While DIO-AL mice were heavier and fatter than CON-AL, the relationships between TEE and REE and body composition (FM and FFM) were not significantly affected by diet composition. Residuals for 24-hour TEE and of REE of WR mice were significantly below predicted (p<0.001; Figure 3) indicating that TEE and REE were reduced beyond what could be attributed to changes in body mass and composition. Residuals were calculated based on actual values minus those predicted based on FFM and FM in all AL mice. However, similar results were obtained regardless of whether residuals were calculated based on FFM alone or FFM & leptin (data not shown). In addition, the absolute values of TEE in DIO-WR mice were significantly lower than in CON-AL mice despite the near-identity of body weight and body composition in these two groups (Table 1). REE of DIO-WR mice was 7.4±2.7% lower than predicted, accounting for 67% of the reduction in total 24-hour TEE (-0.7 kcal/day); in CON-WR mice, REE was 32.8±4.5% lower than predicted. This decrease of REE (-2.5 kcal/day) in CON-WR exceeded the decrease in TEE (-2.2 kcal/day). The difference of 0.3 kcal/day is accounted for by an increase in NREE (0.3 kcal/day) due to increased locomotor activity - probably related
to food seeking behavior - as reflected in the measures of physical activity (see Physical Activity and Figure 4A and 4C).

Non-resting energy expenditure (NREE = TEE - REE) of CON-AL mice was significantly higher than the two DIO groups (Table 1). When adjusted for body mass and composition, residuals of NREE for DIO-WR mice were significantly decreased (-0.3kcal below expected when adjusted for FFM and FM; p<0.05), while NREE residuals were significantly increased in the CON-WR group (+0.3kcal, p<0.05).

**Physical Activity**

Total 24 hour physical activity (ambulatory movement), measured by the TSE infrared movement system, was highest in CON-WR and lowest in DIO-AL (Figure 4A); these were the only groups that were significantly different from one another (p<0.05) in this regard. DIO-WR mice and CON-AL mice had nearly identical 24h total activity (Figure 4A), yet DIO-WR group had significantly lower NREE (2.9±0.1 units vs. 3.4±0.1 respectively; Table 1) indicating that the DIO-WR were expending approximately 15% less energy per unit of movement than CON-AL although some of this decrease in NREE may be attributable to decreased thermic effect of feeding (TEF) (see Discussion). Cumulative ambulatory activity rhythms (sum of every 14 minute measuring period; Figures 4B for DIO & 4C for CON) over 48 hours show higher peaks of movement for WR mice, irrespective of diet, in the 1 hour period prior to AM and PM feeding times (see black bars on bottom of figures). Quantification of ambulatory activity in the 1 hour periods prior to feeding of WR mice showed that WR mice have higher levels of ambulatory activity than AL mice, probably as a result of increased food seeking behavior (see Figure 4D) (41).
Leptin

The key comparison is that of absolute circulating leptin concentrations in CON-AL and DIO-WR mice. If circulating leptin concentrations were significantly reduced in the DIO-WR mice compared to CON-AL mice, then the study would be biased towards our hypothesis: i.e. that DIO-WR mice will be hypometabolic and hypothyroid compared to CON-AL. In fact, the opposite was true and circulating leptin concentrations were significantly higher in DIO-WR mice compared to CON-AL (by t test comparison: Table 2B). There was no effect of diet composition on circulating leptin concentrations since the regression equations relating leptin to fat mass are almost identical between the DIO-WR and CON-AL groups (Figure 1C), and so the inter-group differences in circulating leptin concentrations are attributable to the higher fat mass of DIO-WR.

Overall, leptin concentrations were highly correlated with total fat mass (by NMR) at the start and end of the experiment (respectively, $r = 0.97$ & $0.93$, both $p<0.001$; see Figure 1B; (Data prior to weight stabilization of all 4 groups of animals are not shown). The best fit for the relationship of leptin to fat mass was non-linear ($r = 0.96$, $p < 0.0001$), suggesting that the relationship between leptin and fat mass might differ at extremes of adiposity. To determine whether there were differences among groups in the relationship of leptin to fat mass, regressions relating leptin to FM for each of the four groups were made (see Figure 1C). As reported by others (15, 22), DIO mice showed a disproportionately greater increase in circulating leptin concentrations relative to fat mass (Figure 1C). Regression equations relating leptin to FM were almost identical between CON-AL and DIO-WR indicating no significant effect of diet composition on this relationship. There was no significant correlation of leptin and fat-mass in the CON-WR animals which is similar to what has been reported in studies of leptin in humans.
with extremely low FM (18, 35). The lack of significant difference in absolute circulating leptin concentrations between CON-AL and CON-WR probably reflects the non-linearity of the relationship of leptin to FM in CON-WR animals.

**Other Hormones and Metabolites**

Prior to the start of the weight loss protocols, circulating glucose and insulin concentrations were all significantly higher in DIO mice than CON mice (Table 2). Weight reduction resulted in significant decreases in circulating insulin, T3, and glucose concentrations in DIO-WR compared to DIO-AL mice. T3 concentrations significantly decreased in the CON-WR compared to CON-AL mice. Weight reduction, irrespective of diet, significantly decreased circulating glucose concentrations and increased insulin sensitivity (HOMA2).

**Synapses onto POMC neurons in the arcuate nucleus**

Figure 5A and B are examples of electron microscopy images of POMC cell bodies with either asymmetrical/excitatory synapses (Figure 5A – large white arrows) or symmetrical/inhibitory synapses (Figure 5B – large black arrows). Figure 5C is a magnified section showing both asymmetrical/excitatory (large white arrow) and symmetrical/inhibitory synapses (large black arrow). Small black arrows point to the specialization below the postsynaptic density of the asymmetrical contact (Figure 5A and C). Figure 5D represents consecutive serial sections of the symmetrical contact shown on in Figure 5B. CON-AL mice had the highest excitatory/total synapse ratios of the 4 groups (Figure 5E) indicating a predominance of excitatory synapses over inhibitory ones in these animals during a period (ad-libitum feeding) of relative satiety. In the weight-reduced groups of CON and DIO mice, there
was a similar decrease in this ratio (-52% in DIO-WR and -53% in CON-WR when compared to CON-AL mice; p<0.01). In these groups of demonstrably more hungry animals, inhibitory synapses dominated over excitatory ones. DIO-AL mice also had decreased ratios of excitatory/total synapses (37% below CON-AL), revealing a predominance of inhibitory inputs on POMC perikarya at the time of relative satiety. DIO-WR mice had lower ratios than DIO-AL, although this difference did not quite reach statistical significance (p = 0.13).
Discussion

The major findings of this study are: 1.) As in humans, mice maintaining a reduced body weight (DIO-WR and CON-WR) show decreases in REE and TEE (adjusted for FM and FFM). Most notably, the DIO-WR animals “defend” a higher body weight following an extended period of diet-induced obesity. 2.) There are no significant differences in the relationships in TEE or REE and body weight/composition between CON-AL mice on a chow diet and DIO-AL maintaining an elevated body weight; 3.) Mice maintained at a reduced body weight – regardless of initial weight - have a significantly lower ratio of excitatory/total synapses onto POMC cell bodies than CON-AL fed animals, ratios that are similar to those observed in leptin deficient $Lep^{ob}$ animals (45). These changes are accompanied by increased ad libitum food intake – i.e. increased hunger and food seeking behavior (Table 3 and Figure 4 B, C, & D) that has been documented in weight-reduced humans (52), mice (1), and rats (26, 38, 39, 41). The relative hypometabolism and decreased excitatory input into hypothalamic POMC neurons in DIO-WR mice compared to never-obese animals (CON-AL) with similar body composition and circulating leptin concentrations is consistent with the hypothesis that prolonged maintenance of an elevated body weight results in an upward “resetting” of the leptin threshold.

The magnitude of the decline in energy expenditure (both TEE and REE) following weight loss observed in the DIO-WR compared to the CON-AL in this study is similar to those seen in humans (36). Interestingly, non-resting energy expenditure (NREE) was lower in DIO-WR compared to CON-AL (2.9±0.1SEM vs. 3.4±0.1 kcal/day respectively) although they had similar body weights (Table 1A; 33.3±1.2 vs. 32.3±1.4 g, respectively) and total activity counts (Figure 4A) suggesting that the DIO-WR require less energy to accomplish similar amounts of activity.
(i.e. their skeletal muscles may be more efficient). Such an effect is, in fact, observed in weight-reduced human subjects (58).

In relating rodent data to human studies, it is important to consider the differences in the fractional contributions to energy expenditure, behavioral changes as a result of weight loss, and even definitions of the different components of TEE. TEE of weight-reduced obese humans (who have lost 10% or 20% of their initial body weight) and never-obese humans (who have lost 10% of their initial body weight) - adjusted for body composition - is approximately 15% below that predicted by the losses of fat-free and fat mass (38). In humans, most of this relative decline in energy expenditure is attributable to an increase in skeletal muscle work efficiency (16, 52, 63). In mice, our estimates of energetic cost of locomotion suggest that there may be an increase in activity-related efficiency following weight loss, but its contribution to the overall decline in TEE remains unclear. Unlike humans, the major component of decreased TEE in WR mice is decreased REE rather than NREE.

Comparisons of rodent and human TEF data are complicated by different definitions. In humans, TEF refers specifically to the energy expended during digestion in a sedentary subject (31, 46), while in rodents this term includes postprandial changes in energy expended in physical activity (11), which may in part account for the lower NREE observed in DIO-WR vs. CON-AL mice. TEF in mice accounts for a significantly greater fraction of TEE (>15%) than in humans (<10%) (12, 64). It is possible that changes in TEF, either due to the decreased caloric intake of WR animals or to an actual decline in the fraction of caloric intake utilized in digestion, accounts for some of the observed declines in TEE and NREE. Given the significant decline in circulating concentrations of T3 in WR animals, and the report that hypothyroidism, is associated with a decrease in TEF in rats (27), it is possible that WR animals would expend a lower
percentage of their ingested calories in TEF. However, studies of human subjects (36) and rats (12) who are being maintained at an approximately 10-15% reduced weight have reported no changes in TEF, suggesting that maintenance of a reduced body weight is not associated with a significant decline in TEF expressed as a fraction of caloric intake.

Studies in humans suggest that there is no remission of the relative hypometabolism that accompanies the chronic maintenance of a weight-reduced state (54). Similarly, in rats, maintenance for 16 weeks of a stable lower body weight was accompanied by a persistent hypometabolic phenotype and hyperphagia, and weight regain once ad-libitum feeding was resumed (45). This apparent “irreversibility” of the metabolic and behavioral consequences of sustained weight loss does not seem to occur following sustained weight gain; the data presented here suggest that prolonged elevation of body weight results in an upward “resetting” defended levels of energy stores.

In the present study, long-term (16 weeks) diet-induced obese mice (DIO-AL) mice were not hypermetabolic (adjusted TEE) when compared to CON-AL mice by ANCOVA or multivariate regression. In shorter term overfeeding studies in rats (63) and humans (33), 10-15% increases in adjusted TEE are observed. Our data suggest that over longer periods of time, energy expenditure in weight-gained individuals returns to levels (adjusted for body mass and composition) that are comparable to those of individuals maintaining their usual (pre-gain) body weight. Such an inference is supported by the fact that weight-stable obese and non-obese humans have comparable adjusted energy expenditures (33). Unlike mice that return to their usual body weight after short-term overfeeding and are then eumetabolic compared to their never-obese littermates, long term DIO mice who were weight-reduced (DIO-WR) are hypometabolic compared to both DIO-AL and CON-AL mice, but are metabolically similar to
CON-WR mice (Figure 3). The reduction in energy expenditure in the weight-reduced DIO mice – to levels less than those of age, genotype and body mass/composition-matched CON-AL mice – is consistent with our hypothesis that sustained maintenance of an increased body weight results in an upward resetting of the “threshold” for minimum body fat. It might be argued that the decline in energy expenditure of the DIO-WR is related to CNS effects that are specific to the HFD. However, the same responses are seen in the CON-WR mice being fed a low fat diet, and high fat diets are certainly prevalent among human populations. Nevertheless, it would be interesting to examine the responses of DIO-WR to a lower fat diet in terms of energy intake and expenditure.

Relevant to this issue, others have found that DIO mice “settle” at higher body weights (i.e. increased adiposity) than never-obese animals when switched from an ad-lib HFD to an ad-lib chow diet (61) (personal communication, Dr. Silvia Corvera). Chow-fed formerly DIO rats also resist weight reduction when fed a hypocaloric diet by becoming hypometabolic (like non-DIO weight-reduced rats) (61). Furthermore, mice that are switched from a high fat diet to a low fat diet and then back to the high fat diet readily regain weight to levels similar before the diet switch (29).

Epidemiological observations of the increasing prevalence of obesity in humans (25, 44, 65) and long-term difficulties in sustaining even mild degrees of weight loss, suggest that the threshold for the minimal body weight that is metabolically defended may be elevated via maintenance of greater adiposity for prolonged periods of time and/or at specific time-points during development. In both humans and rodents, weight reduction results in decreased concentrations of circulating leptin, T3, and insulin. In the present study, as expected, we detected significant effects of treatment (AL or WR) on insulin sensitivity as reflected by
HOMA2 (37): CON-WR HOMA2 was 197% higher than CON-AL, and DIO-WR was 241% higher than DIO-AL; p<0.05. Circulating concentrations of leptin are closely proportional to body fat mass in weight-stable mice and humans (55). Leptin’s capacity to reverse metabolic phenotypes seen in both rodents and humans following weight loss and/or during caloric restriction, and its effects on energy homeostatic processes in the brain, renders it a prime candidate as a mediator of metabolic adaptation under conditions of decreased somatic energy stores and/or negative energy balance (1, 48, 54). The higher circulating leptin concentrations relative to fat mass in DIO-AL mice, and the loss of linearity in the relationship of leptin to fat mass in CON-WR mice are consistent with other studies of weight maintenance in rodents following overfeeding (13, 18) and underfeeding (15, 30). A non-linear regression analysis improved the leptin to FM ratio ($r^2 = 0.96$, p < 0.0001) when including all groups because of DIO-AL increased production of leptin per unit fat mass (see slope of linear regression in Figure 1C). The differences in the relationship of leptin to fat mass during weight maintenance following extreme weight loss or gain are less pronounced than the striking decreases or increases in the ratio of leptin to fat mass observed in humans (56) and rodents (6) during dynamic weight loss or gain, respectively. The observation that the relationship between leptin and fat mass was not different between DIO-WR and CON-AL groups in this study (i.e. that they fell on similar regression lines: Figure 1C) is an indication that these animals were, in fact, in similar states of energy balance. Since the regression of leptin on fat mass has a “non-zero Y-axis intercept”, the ratios of leptin to FM, as used by some laboratories, are not appropriate for assessing “leptin sufficiency/insufficiency” as they reflect solely the slope but not the intercept of the relationship between the variables (see Figure 1C for individual group regressions).
Similar considerations dictate our use of multivariate regression to assess energy expenditure related to both FFM and FM as opposed to using ratios of TEE/FFM (23).

Thyroid hormone concentrations in blood correlate with energy expenditure by mechanisms that are not fully understood (8, 9). T3 is increased during overfeeding (51, 62) and reduced during underfeeding and/or weight loss (51). Serum T3 in the CON-WR was decreased by 51% and by 47% in DIO-WR mice compared to their respective AL controls (Table 2). These changes in T3 concentrations following weight loss are similar to those noted in humans (51).

Chronic changes in leptin signaling have been associated with structural changes in the hypothalamus (45). These are plausible neural substrates for the consequent attenuations in energy intake and expenditure (27). Leptin deficient \( Lep^{ob} \) mice had decreased ratios of excitatory/total synapses onto POMC arcuate neurons when compared to wild type mice and exogenous leptin or estrogen normalized this phenotype (12, 45). In the DIO-WR and CON-WR mice we observed ratios of excitatory/total synapses onto POMC neurons that were 52% and 53% below those in ad-lib animals and comparable to those observed in the leptin deficient \( Lep^{ob} \) mice (45). We have previously shown that the excitatory/total synaptic ratio positively correlates with POMC mRNA expression (20, 45). The comparability of these changes in DIO-WR and CON-WR animals supports our inference that the DIO-WR animals are now “defending” a higher level of body fat, and that the reduced excitatory/total synapses onto POMC neurons constitute a “signature” of relative leptin deficiency. Consistent with the reduced excitatory tone in POMC neurons in the weight reduced state, POMC mRNA in the arcuate is reduced in chronically food restricted rats (26), and restored to fed levels by exogenous leptin. It is possible that opposite changes in orexigenic/anabolic NPY/AgRP neurons contributes to the phenotype (36). If these structural differences have functional consequences – a likely possibility given the
physiology of leptin signaling in POMC neurons – the bioenergetics and endocrine profiles of the animals in this study could be accounted for (32). The bioenergetic/neuroendocrine, behavioral, and fMRI responses of weight-reduced humans to low dose exogenous leptin are consistent with this inference (53, 57). The DIO-AL animals had lower excitatory/total synapse ratios than CON-AL, possibly reflecting effects of diet composition, the obese phenotype (i.e. increased leptin levels), or both. Feeding mice a high fat diet reduces apparent arcuate leptin sensitivity as early as 6 days after switching to high fat diet (42). Enriori et al. showed that decreases in leptin responsiveness in the arcuate nucleus following diet-induced obesity could be reversed by decreasing the fat content of the diet (10). These effects may account for the smaller difference in excitatory/total synapse ratios observed between DIO-AL and DIO-WR ratios. There may be a “floor” to this ratio.

Perspectives and Significance

These data suggest that prolonged maintenance of an acquired elevation in body weight induces changes in energy homeostatic systems that lead to “defense” of a body weight higher than that dictated by genetic/developmental status of the animal. Structural changes in the arcuate nucleus (and elsewhere) may play a role in upward resetting of defended body weight (5, 27, 45). Comparable processes, if present in humans, could account for some aspects of the secular trend to increasing obesity that clearly cannot be attributed to intercurrent genetic change. Understanding the neuro-biological predicates of such an acquired upward resetting of the minimum defended level of adiposity “threshold” could provide novel approaches to the prevention and treatment of obesity. For instance, the observations presented here suggest that pharmacological elevation of melanocortinergic tone may be particularly effective in the prevention of weight regain in formerly obese subjects. These studies are also consonant with
studied in humans indicating that the neurobiological responses to maintenance of a reduced body weight do not accommodate over time; i.e. that the physiology of the weight-reduced state persists.
Acknowledgements

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Disclosures

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Bibliography


21. **Huang XF, Han M, South T, and Storlien L.** Altered levels of POMC, AgRP and MC4-R mRNA expression in the hypothalamus and other parts of the limbic system of mice prone or resistant to chronic high-energy diet-induced obesity. *Brain Res* 992: 9-19, 2003.


Figure legends

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**Table Legends**

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<table>
<thead>
<tr>
<th>Table 1.</th>
<th>DIO-AL (n=7)</th>
<th>DIO-WR (n=8)</th>
<th>CON-AL (n=8)</th>
<th>CON-WR (n=8)</th>
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<tr>
<td><strong>Indirect calorimetry measurements (days 132-144)</strong></td>
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<tr>
<td>Body Weight (g)</td>
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<td>33.3±1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.3±1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.7±0.5&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Fat-Free Mass (FFM, g)</td>
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<td>20.0±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Fat Mass (FM, g)</td>
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<td>6.7±0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.4±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Food Intake (g/day)</td>
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<td>3.4±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>MEI (kcal/day)</td>
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<td>9.0±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.85±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
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<table>
<thead>
<tr>
<th>Table 2.</th>
<th>A. Data obtained during initial bleed – day 0 (Figure 1)</th>
<th>B. Data obtained during terminal bleed – days 173-179 (Fig 1)</th>
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<tr>
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<td>DIO (n=15)</td>
<td>CON (n=16)</td>
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<tr>
<td>Leptin (ng/ml)</td>
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<td>4.8 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Insulin (ug/l)</td>
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<td>0.8 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>121.7 ± 4.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>T3 (ng/dl)</td>
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<td>19.9 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>48.2 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>TSH (ng/ml)</td>
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<td>170.2 ± 22.9</td>
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<td>Leptin (ng/ml)</td>
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<td>25.1 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>105 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>T4 (ng/ml)</td>
<td>45.8 ± 4</td>
<td>49.9 ± 2</td>
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<td>TSH (ng/ml)</td>
<td>283.1 ± 30.5</td>
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<td>HOMA2 S</td>
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<td>24.3 ± 4.3&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>HOMA2 IR</td>
<td>11.1 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.9 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
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<th>Metabolizable Energy Intake (kcal/24h)</th>
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<td>DIO-AL (n=7)</td>
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<td>18.5±0.6*</td>
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<td>DIO-WR (n=12)</td>
<td>5.2±0.2</td>
<td>27.0±1.0</td>
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