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ORIGINAL PAPER

High-fat diet-induced changes in body mass and hypothalamic gene expression in wild-type and leptin-deficient mice

Kristy L. Townsend · Magen M. Lorenzi · Eric P. Widmaier

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Abstract We tested whether diet-induced obesity results from increased energy consumption, is associated with changes in expression of genes involved in leptin signal transduction, and is altered by hyperleptinemia. C57BL/6 mice were fed a low-fat diet (LFD) or high-fat diet (HFD) for up to 15 weeks. HFD mice weighed significantly more than LFD controls by 3 weeks, despite consuming less energy. HFD mice had significantly greater leptin, insulin, and glucose levels than LFD mice, suggesting leptin and insulin resistance. Adiponectin levels declined with age but were unaffected by diet. HFD was associated with altered hypothalamic expression of genes whose products regulate the activity or nuclear translocation of STAT3, an important mediator of leptin actions. Expression of two isoforms of the leptin receptor decreased at 15 weeks in hypothalami of HFD mice in a tissue-specific manner. The type of fat (saturated versus unsaturated) did not influence weight gain on an HFD, but animals on LFD gained significantly more weight and adiposity if the dietary fat consisted mostly of saturated fats; this occurred despite no difference in energy consumption or absorption. Replacement of leptin to leptin-deficient *ob*/*ob* mice decreased hypothalamic leptin receptor expression and did not prevent HFD-induced weight gain. It is concluded that (1) increased energy consumption is not required for HFD-induced obesity in C57BL/6 mice, (2) HFD results in weight gain partly by modulating hypothalamic leptin-signaling pathways, (3) saturated fats induce weight gain even when total fat content of the diet is low, and (4) the effects of HFD are manifest in the presence or absence of circulating leptin.

K. L. Townsend · M. M. Lorenzi · E. P. Widmaier (✉)
Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215, USA
e-mail: widmaier@bu.edu

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Introduction

Chronic consumption of a high-fat diet (HFD) is a contributing factor to the advent of obesity in humans and in animal models. When the obesity-prone strain of C57BL/6 mice are fed an HFD, they gain weight as a consequence of increased adiposity [1]. Whether increased energy consumption is part of the mechanism by which HFD induces these changes is unresolved. In mice and rats, for example, it has been reported that HFD is [2, 3] or is not [1, 4–6] associated with an increase in caloric intake. Moreover, the relative contributions of saturated and unsaturated fats to HFD-induced weight gain are unclear. Thus, these fundamental questions regarding the mechanisms of HFD-induced obesity remain to be answered.

Notwithstanding changes in energy consumption, the mechanisms whereby HFD results in obesity may involve aberrations in the central control of appetite and metabolism. For example, the anorexigenic hormone leptin acts on cells within the hypothalamus to inhibit appetite and increase metabolic rate. A deficiency of leptin, or a resistance of the hypothalamus to the actions of leptin, results in obesity in rodents [7, 8] and in humans [9, 10]. HFD mice develop hypothalamic resistance to leptin after several weeks on the diet and become hyperleptinemic [1, 11]. The cause of leptin resistance is unknown, but could arise by several possible mechanisms, including changes in intracellular leptin signaling [11, 12] and cytosolic inhibitors of leptin action [13]. Leptin exerts its central and systemic actions via at least three signaling pathways, including the JAK2/STAT3, IRS2/PI3K, and MAPK/ERK pathways [7].

Its effects on appetite and metabolism are mediated by receptors located in the hypothalamus that activate the JAK2/STAT3 pathway (reviewed in Ref. [14]), although extra-hypothalamic sites have also been implicated [15]. Decreases in mRNA and protein of the long form of the leptin receptor, OBRb, have been reported in hypothalami of rodents fed an HFD [16, 17], although simultaneous quantitative assessments of OBRa (short form of the receptor) and OBRb mRNA remain to be done.

STAT3 is a cytosolic transcription factor that enters the nucleus upon becoming activated by phosphorylation in response to the binding of leptin to OBRb. The events mediating translocation of STAT3 from the cytosol through the nuclear envelope and its subsequent binding to promoter regions appear to be controlled by several other cytosolic proteins, including STAT3-interacting protein 1 (StIP1) [18] and two members of the family of importins, or karyopherins [19, 20]. Whether expression of these key genes is altered by chronic consumption of an HFD is currently unknown and has never been examined. Here, we hypothesize that dietary fat may be associated with changes in gene expression that regulate the expression and action of the leptin receptor and its intracellular signaling and regulatory molecules in the hypothalamus.

It has been suggested that hyperleptinemia itself contributes to leptin resistance in animal models of obesity, possibly due to such events as ligand-induced down-regulation of the leptin receptor (ObR) [21–24] or to leptin-induced generation of intracellular inhibitory signals [13]. One way to address the question of how circulating leptin and HFD interact over the long term in the development of obesity is to examine the effects of HFD in leptin-deficient mice with or without leptin replacement. *ob*/*ob* mice do not produce active leptin protein because of a mutation in the leptin gene [25]. These mice are hyperphagic and insulin-resistant [25], with decreased metabolic rates compared to wild-type C57BL/6 mice of the same genetic background [26]. Recently, it has been demonstrated that *ob*/*ob* mice have an altered hypothalamic circuitry [27, 28] that may be the result of leptin-deficiency during development [27]. Notwithstanding, sufficient neuronal plasticity exists in the adult *ob*/*ob* mouse hypothalamus such that the mice become sensitive to administration of leptin with very little latency [28]. Thus, leptin treatment reverses the obese phenotype of *ob*/*ob* mice and restores body mass and insulin-sensitivity to normal [25].

Because *ob*/*ob* mice lack circulating leptin, hyperleptinemia does not occur in these animals as they become obese. We anticipated that leptin replacement in obese *ob*/*ob* mice would cause weight loss and improve the metabolic profiles of the mice, as expected. However, we hypothesized that HFD would cause additional weight gain in these animals and that the weight gain would not be prevented when circulating leptin was clamped at physiological or supraphysiological levels. If true, this would suggest that the chronic hyperleptinemia associated with obesity in mice is not a prerequisite for development of the obese phenotype in animals consuming HFDs.

In the present study, therefore, we sought to determine (1) whether HFD-induced obesity was associated with increased energy consumption and (2) with changes in hypothalamic expression of genes associated with leptin signaling and control of appetite and metabolism; (3) the relative contributions to weight gain of saturated and unsaturated fats in mice consuming diets that were either high or low in total fat content; and (4) whether HFD could induce weight gain in obese mice in which circulating leptin levels were clamped. We hypothesized that saturated fats would result in increased adiposity and body weight, without altering energy consumption, possibly by increasing the absorption efficiency of ingested calories. We further hypothesized that leptin-deficient mice would gain weight on an HFD with or without leptin-replacement.

Results

After several weeks on the diets, body weights of wild-type HFD mice were significantly greater than low-fat diet (LFD) controls (Fig. 1a). Although the weights appeared to diverge by 3 weeks, an unexplained drop in body weight in this experiment at 4 weeks in the HFD group resulted in the first statistically significant increase above LFD animals at 5 weeks. HFD was associated with continued weight gain throughout the remainder of the study period, while body weights of LFD mice increased only slightly during this time. Energy consumption by HFD mice, calculated from measurements of 24-h food intake per cage of four mice, was not significantly greater than in LFD mice (Fig. 1b). In fact, at week 3 and again over the last few weeks of the study, HFD mice consumed significantly less energy than did LFD mice.

To more precisely determine the latency of the effects of HFD on body weight, and to gain additional timepoints for gene expression analyses, additional animals were established on LFD or HFD for 2 days or 3 weeks and then euthanized to obtain blood, liver, and hypothalami. The first significant increase in body weight in the HFD animals occurred at 12 days (Fig. 2).

To verify the anticipated endocrine and metabolic phenotype of HFD mice, plasma was obtained at 2 days, 3 weeks, and 15 weeks after changing diets. HFD resulted in significantly greater plasma levels of leptin, insulin, and glucose compared to LFD controls (Table 1). The differences in leptin and insulin were first apparent at 3 weeks; glucose was only determined at 15 weeks. Plasma levels of

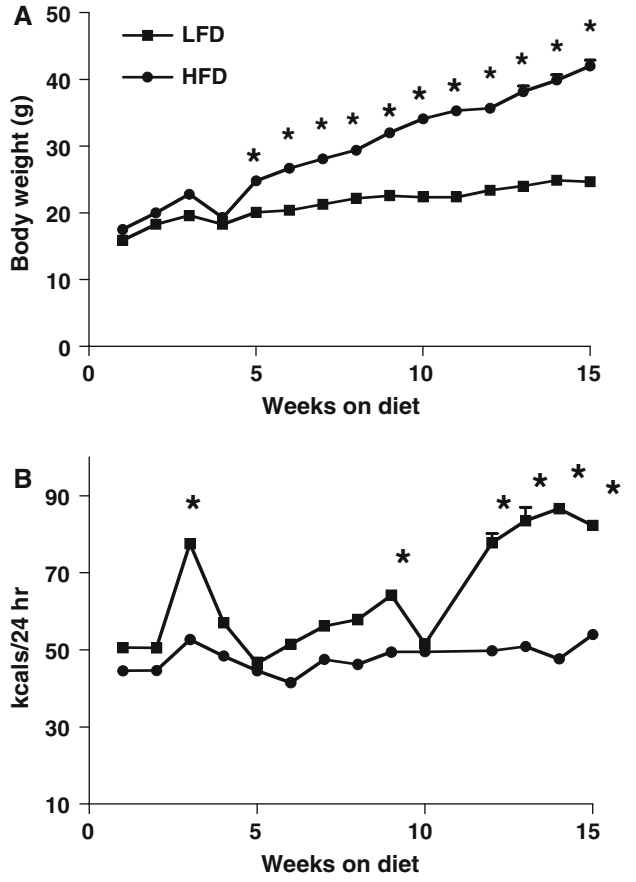

Fig. 1 Effects of consuming a high-fat diet (HFD) or low-fat diet (LFD) on body weight (**a**) and daily caloric intake (**b**) in female mice. The times at which the two groups differed significantly from each other are shown by asterisks (at least $P < 0.001$). Values are means and SEM (many error bars are obscured by the data symbols). For body weights, $N = 48$ and 32 for HFD and LFD, respectively. For caloric intake, values represent the caloric intake per 24 h of 12 (HFD) and 8 (LFD) cages with four animals per cage

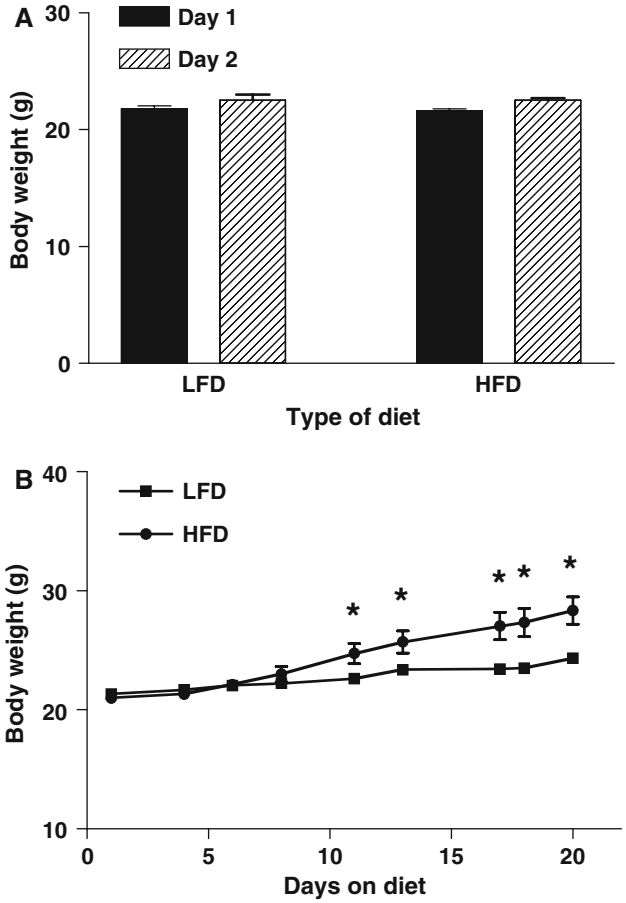

Fig. 2 Effects of short-term HFD on body weights of female mice. Values represent the means/SEM of eight animals per group. Groups of mice were sacrificed after either (**a**) 2 days or (**b**) 3 weeks for collection of blood and tissues. Body weights for those periods are shown here. The asterisks represent significant differences (at least $P < 0.0.05$) between HFD and LFD

total adiponectin (all forms), an adipokine implicated in obesity, energy homeostasis, appetite control, and insulin sensitivity, decreased significantly with age but were unaffected by diet (Table 1).

Despite the lack of effect of diet on plasma levels of adiponectin, adiponectin release, as determined by its accumulation in the medium of incubated fat tissue, was significantly greater from omental fat of HFD mice than from LFD mice at 15 weeks (Fig. 3). There was no significant effect of diet on release from subcutaneous fat (Fig. 3).

Primers were developed to quantify expression of several genes whose products are known or believed to play a role in mediating the actions of leptin and STAT3, or to be otherwise associated with appetite and metabolism. Real-time PCR analysis of hypothalami from the mice in Fig. 1 revealed significant upregulation in HFD mice of several genes with known or putative roles in STAT3 signaling, including StIP1, importin $\alpha 2$ (KPNA2), importin $\beta 1$ (KPNB1), and PIAS3 isoform 2 (Table 2). In addition, the phosphorylated form of STAT3 was determined by ELISA in extracts of hypothalami from HFD and LFD mice. There was no difference in the pSTAT3 (tyr 705)/A280 ratio between groups (LFD: 2.2 ± 0.4; HFD: 2.3 ± 0.4, arbitrary units).

By contrast, HFD was associated with first an increase and then a significant decrease in hypothalamic levels of OBRb mRNA (Table 2). The change in OBRb expression was tissue-specific, as it did not occur in the liver (Table 2). OBRa mRNA was significantly increased in hypothalami of HFD mice at 3 weeks, but was not detectable in hypothalami of mice from either group at 15 weeks (Table 2). As with OBRb, the changes in OBRa were specific; in liver, the relative amount of OBRa mRNA increased in HFD mice at both 3 and 15 weeks (Table 2).

Several other genes of interest were examined because of their known or putative roles in leptin signaling and

Table 1 Endocrine and glucose levels in plasma of female wild-type mice fed either a low-fat diet (LFD) or high-fat diet (HFD)

Diet and duration	Leptin (ng/ml)	Insulin (ng/ml)	Adiponectin (μg/mL)	Glucose (mg/dL)
2-days LFD	5.1 ± 1.9 (8)	2.3 ± 0.6 (8)	16.8 ± 3.8** (8)	ND
2-days HFD	8.4 ± 2.2 (8)	1.1 ± 0.3 (8)	15.0 ± 1.5** (8)	ND
3-weeks LFD	2.6 ± 0.3 (8)	0.5 ± 0.2 (8)	9.0 ± 1.5 (8)	ND
3-weeks HFD	36.0 ± 3.6* (8)	2.5 ± 0.9* (8)	5.9 ± 0.9 (8)	ND
15-weeks LFD	18.5 ± 3.4 (32)	1.3 ± 0.4 (19)	1.4 ± 0.1 (12)	133.1 ± 13.9 (12)
15-weeks HFD	116.3 ± 1.6* (48)	2.7 ± 0.5* (11)	2.2 ± 0.2 (22)	208.5 ± 12.7* (22)

Note: Values are means and SEM with the number of animals in each group in parentheses. Leptin was determined in all animals at 15 weeks; sufficient plasma was only available to determine insulin, adiponectin and glucose in a subset of animals

ND, not determined

* $P < 0.05$ vs LFD for that timepoint; ** $P < 0.05$ vs 3-week and 15-week LFD and HFD

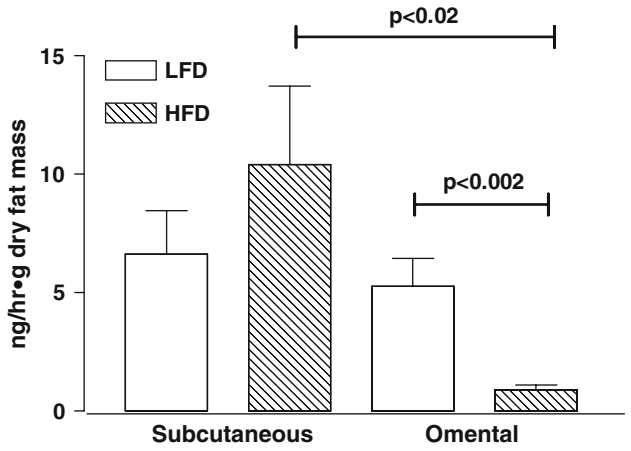

Fig. 3 Accumulation of adiponectin in medium from subcutaneous or omental fat in vitro. Fat samples were collected from HFD or LFD mice after 15 weeks on the respective diets, and incubated for 1 h at 37°C in 1 ml Krebs buffer supplemented with glucose, protease inhibitors, and BSA. After filtering the samples to remove fat, the medium was subjected to adiponectin radioimmunoassay. Each value is the mean and SEM of seven animals. Significant differences between groups are indicated by the brackets

obesity and were found not to change in hypothalami as a function of diet. These genes included SHP2, PDE3b, TNFα, POMCα, SOCS3, CJUN, SREBP1, TTR, and the truncated form of STAT3 (STAT3β) (data not shown).

In order to determine the relative contributions of saturated and unsaturated fats to weight gain, animals were fed diets that were either high or low in total fat content, and with either a high or low ratio of saturated to unsaturated fats (Fig. 4). Mice consuming an LFD that consisted primarily of unsaturated fats maintained the lowest body weights. Animals fed an LFD but one that consisted primarily of saturated fats gained significantly more weight than did animals fed the same percentage fat but primarily unsaturated fats. Regardless of fat composition, diets containing a high fat content resulted in significant weight gain.

The differences in body weight of the mice in Fig. 4 were not reflected in similar differences in energy consumption. There was no overall effect of diet on energy consumption, and mice that were fed the low fat/unsaturated diet did not consume significantly fewer calories than did animals in all other groups (Table 3). The body mass indices (BMIs) of mice were highest in the two high-fat groups, intermediate in the low fat/saturated group, and lowest in the low fat/unsaturated group (Table 3). Adiposity was estimated using a fat index consisting of the combined weights of the inguinal and perirenal fat pads, normalized to residual body mass, as shown in Table 3. Adiposity showed a similar significant trend across the four dietary groups as BMI. Plasma leptin concentrations followed a similar pattern (Table 3). There was no difference between diets in energy absorption over two 24 h periods, as estimated by the difference in energy intake and energy excretion (Table 3).

In order to determine whether HFD can induce weight gain in already obese *ob*/*ob* mice with or without leptin replacement, *ob*/*ob* mice were implanted with saline- or leptin-filled miniosmotic pumps. Changes in body weights of the mice on each diet (LFD or HFD) and in each treatment group (saline or either 6 or 12 μg/day leptin) are depicted in Fig. 5. As expected, control (saline-treated) mice gained weight throughout the duration of the study regardless of diet composition. Leptin administration resulted in weight loss that was more rapid and more pronounced in the higher dose group, declining to roughly 50% of pre-leptin weights. When switched to HFD, mice implanted with saline pumps significantly gained weight above their already obese levels and above those of LFD controls within 1–2 days; this was replicated in both leptin dose studies. In animals infused with 6 μg/day leptin, HFD halted the weight loss compared to LFD counterparts (Fig. 5a), while in animals infused with 12 μg/day leptin, HFD resulted in resumption of weight gain (Fig. 5b).

Despite the increase in body weight induced by HFD in saline-treated *ob*/*ob* mice, there was no significant effect of diet on energy consumption in these mice as determined by periodic measurements of caloric intake (data not shown).

Table 2 Changes in hypothalamic and hepatic gene expression at 2 days, 3 weeks, and 15 weeks of HFD versus LFD feeding

	2 days ($n = 8$)	3 weeks ($n = 8$)	15 weeks ($n = 6$)
Hypothalamus			
KPNA2	0.95 (0.73–1.21)	1.32* (1.01–1.71)	3.8**·*** (3.0–4.88)
KPNB1	1.23 (1.04–1.43)	1.19 (1.01–1.41)	2.2** (1.71–2.89)
OBRa	1.07 (0.72–1.61)	1.64* (1.04–2.54)	Undetectable in HFD
OBRb	1.05 (0.79–1.39)	1.97* (1.03–2.53)	0.4** (0.28–0.67)
PIAS3 isoform 2	1.31 (1.13–1.54)	2.14 (1.86–2.47)	1.7** (1.30–2.24)
StIP1	1.04 (0.90–1.20)	1.37* (1.18–1.59)	1.9**·*** (1.45–2.57)
Liver			
OBRa	0.53 (0.39–0.72)	0.72 (0.51–1.02)	2.9** (2.4–3.4)
OBRb	1.09 (0.68–1.74)	1.21 (0.99–1.47)	1.0 (0.81–1.11)

Note: Values are the mean fold changes (HFD/LFD) for each time point (statistical ranges shown in parentheses). Statistical treatment was performed on individual ΔΔCt values (not shown). See Table 1 for abbreviations

* $P < 0.05$ for 3-week HFD vs 2-day HFD; ** $P < 0.05$ for 15-week HFD vs 3-week and 2-day HFD; *** $P < 0.05$ vs LFD for that timepoint only

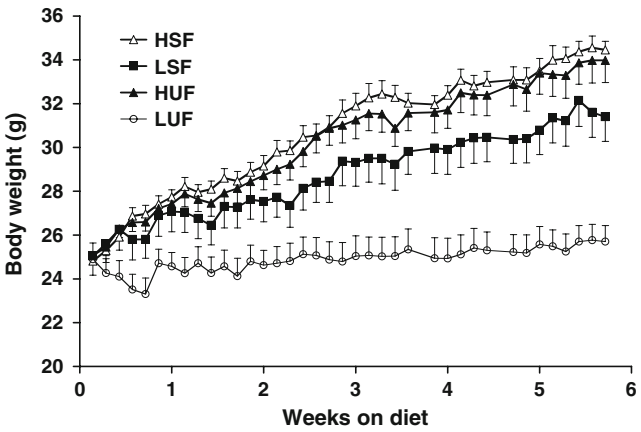

Fig. 4 Effects of saturated and unsaturated fats on body weights of C57BL/6 mice. Male mice were fed either a diet high (60%; H) or low (10%; L) in total fat. Each type of diet was further divided into those comprised of primarily saturated (S) or unsaturated (U) fat. Thus, HSF indicates a diet that was high in total fat content, and which contained mostly saturated fats. Values are the mean and SEM of eight animals per group. ANOVA indicated a significant difference between LUF and all other groups, and between LSF and all other groups. HUF and HSF were not different

Thus, the saline-treated animals gained weight on HFD without increasing their energy consumption. Both doses of leptin resulted in a significant decrease in energy consumption compared to saline-treated animals, as expected, but the effect was transient and did not persist throughout the experiment (data not shown).

Plasma hormone levels at the conclusion of each study in Fig. 5 are given in Table 4. Plasma leptin levels were undetectable in saline-treated *ob*/*ob* mice, as expected, and were increased to within and beyond the usually reported physiological range for this strain of mouse by the 6 and 12 μg/day leptin pumps, respectively. Plasma insulin levels of *ob*/*ob* mice were significantly lower in the 12 μg/day leptin-treated animals compared to saline controls, confirming the expected improved insulin sensitivity. As in wild-type mice, plasma levels of adiponectin were not significantly altered in *ob*/*ob* mice by diet, nor were they altered by leptin. In addition, adiponectin mRNA was determined in extracts of subcutaneous and omental adipose tissue and was not significantly altered by diet or leptin (data not shown). mRNA levels of OBRa and OBRb were determined in extracts of hypothalami and livers (Table 5). In hypothalami, the short isoform OBRa was significantly and markedly decreased in leptin-treated mice, but was unaffected by diet alone. The effect of leptin on hypothalamic OBRa mRNA was specific as it was not observed in livers of leptin-treated mice. mRNA of OBRb also was significantly decreased in the hypothalamus following chronic leptin treatment. As was true for ObRa, hypothalamic mRNA levels of OBRb were not significantly affected by diet. The decrease in hypothalamic OBRb mRNA was also tissue-specific, as it was increased in the livers of 12 μg/day leptin-treated mice.

Discussion

We report here that an HFD results in obesity in C57BL/6 mice without an increase in energy intake. This occurred in both female and male animals, unlike in rats for which sex differences in HFD-induced hyperphagia and obesity have been reported [29]. Additionally, weight gain in the present study was not related to a detectable increase in energy absorption, had a latency of at least 12 days, and resulted from either the presence of saturated fats or the total fat content of the diets. Weight gain was paralleled by significantly greater plasma levels of leptin, insulin, and

Table 3 Effects of saturated and unsaturated fats on body composition, plasma leptin, and energy consumption and absorption in male wild-type mice

	Diet			
	Low/unsaturated	Low/saturated	High/unsaturated	High/saturated
BMI	0.29 ± 0.26^{a}	$0.33 \pm 0.05^{a,b}$	0.36 ± 0.15^{b}	0.37 ± 0.14^{b}
Fat Index	2.5 ± 0.2^{a}	5.3 ± 0.4^{b}	8.8 ± 0.5^{c}	8.5 ± 0.2^{c}
Plasma leptin (ng/ml)				
Day 0	4.6 ± 0.8^{a}	4.0 ± 0.5^{a}	9.3 ± 1.8^{b}	$5.6 \pm 1.3^{a,b}$
Day 10	6.4 ± 0.5^{a}	$24.9 \pm 6.0^{a,b}$	$45.8 \pm 6.1^{b,c}$	$43.6 \pm 3.4^{b,c}$
Terminal	5.9 ± 0.5^{a}	39.4 ± 5.8^{b}	82.0 ± 12.8^{c}	$64.6 \pm 3.2^{b,c}$
Energy consumption (kcal/24 h/mouse)	16.7 ± 0.3	16.6 ± 0.9	18.0 ± 0.7	16.9 ± 0.2
Energy absorbed (kcal/24 h/mouse)	14.6 ± 0.6	15.0 ± 0.9	17.1 ± 0.9	14.7 ± 0.8

Note: Values are means and SE of 7–8 mice except those for day 0 leptin ($n = 4$–8) and those for energy absorbed ($n = 6$–8). Values for energy consumption are from 21 to 28 determinations (3–4 cages of two mice per cage, determined on seven different days during the course of the experiment). BMI: g/cm^2 (nose-anus distance); Fat index: combined weights of both perirenal and both inguinal fat pads, divided by residual body mass ($\times 100$). Plasma leptin was determined prior (day 0) to initiating diets, 10 days after, and at the conclusion of the study (terminal; 6 weeks). Energy consumption was calculated from seven separate 24 h food intake determinations. Energy absorbed was estimated as the difference in total energy intake and total energy excreted (in feces) from two of the 24 h collection periods. Values with identical lettered superscripts are not significantly different from each other within a row. No superscripts within a row indicates that none of the values were statistically different from each other

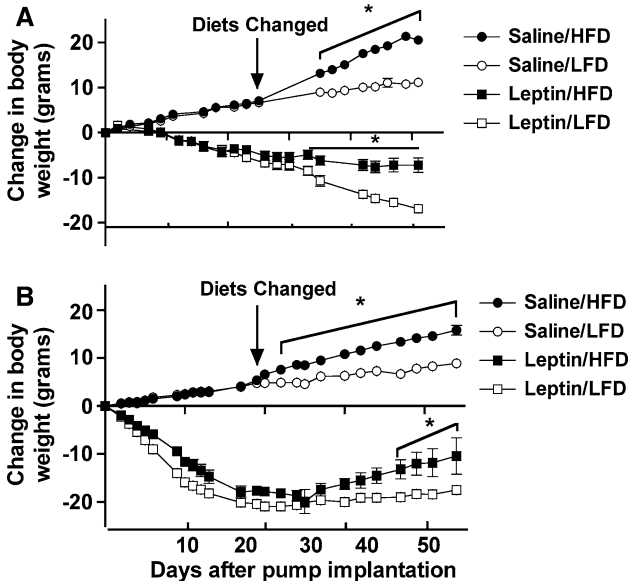

Fig. 5 The effects of high-fat diets on body weights of *ob*/*ob* mice with or without leptin-replacement. Male *ob*/*ob* mice were implanted subcutaneously with Alzet mini-osmotic pumps on day 1. The pumps released saline or (**a**) 6 μg/day or (**b**) 12 μg/day recombinant murine leptin and were present throughout the duration of the experiment (fresh pumps were implanted midway through the experiment to replace the original pumps before they became depleted of leptin). Values represent the mean and SEM of the changes in body weight from day zero (just prior to pump implantation) of 4–5 animals per group. Many error bars are obscured by the data symbols. Areas indicated by the asterisks indicate significant differences between LFD and HFD in a given group (saline or leptin). The time when half the animals were switched from LFD to HFD is indicated by the arrow. Prior to that time, all animals were on LFD as in Fig. 1. Starting body weights on day zero ranged from 44.4 ± 1.1 g to 48.1 ± 1.6 g

glucose, but no change in plasma adiponectin. We also report for the first time that hypothalamic mRNA levels for several genes whose products are associated with signaling steps that occur before and after the activation of STAT3 by leptin were altered by HFD. Finally, we report here that HFD induces weight gain in leptin-deficient mice with or without leptin replacement at physiological or supraphysiological levels.

Consumption of an HFD is well known to trigger pathophysiological changes in rodents and other mammals, including humans. Among these are the development of obesity with increased adiposity [4], and leptin and insulin resistance [1, 21] characterized by reduced target cell sensitivity to leptin and insulin and increased circulating levels of the hormones. The increased body weights, adiposity and circulating hormones and glucose levels observed in the present study confirmed the expected phenotype of wild-type mice chronically consuming an HFD.

Whether HFD induces obesity in part via increased energy consumption is equivocal. Several groups have reported increased energy consumption in mice or rats fed an HFD [1, 4, 6, 30]. Others, however, have reported no change [2, 3] or only a transient increase [31] in energy consumed on an HFD, including in humans [32]. These discrepancies may be related to strain and genetic background of the rodent models, the composition of fat in the diet, and the duration of the study [5, 33–35]. Notwithstanding, our results clearly demonstrate that an increase in energy consumption in C57BL/6 mice is not a prerequisite for HFD-induced weight gain; thus, the diet-induced

Table 4 Hormone values for *ob*/*ob* mice on low-fat diets (LFD) or high-fat diets (HFD), and implanted with either saline- or leptin-filled pumps

Treatment	Diet	Leptin (ng/ml)	Insulin (ng/ml)	Adiponectin (ng/ml)
Saline	LFD	Undetectable	6.1 ± 2.1*	11.4 ± 3.1
Saline	HFD	Undetectable	8.3 ± 1.2*	9.9 ± 1.4
Leptin (6 μg/day)	LFD	3.6 ± 0.3	n/a	n/a
	HFD		n/a	n/a
Leptin (12 μg/day)	LFD	15.0 ± 7.5	0.3 ± 0.2	11.4 ± 1.5
	HFD		2.5 ± 1.1	13.6 ± 0.8

Note: Values are means and SEM ($n = 4$–10 animals/group). The plasma levels of leptin achieved by the pumps are reported for all animals of a given dose regardless of diet

n/a, not available

* At least $P < 0.05$ vs 12 μg/day leptin/LFD

Table 5 Real-time PCR quantification of two isoforms of the leptin receptor in hypothalamus and liver of *ob*/*ob* mice treated with saline or leptin, and fed LFD or HFD

Treatment	Diet	Hypothalamus		Liver	
		OBRa	OBRb	OBRa	OBRb
Saline	LFD	1.00 (0.67–1.49)	1.00 (0.75–1.33)	1.00 (0.89–1.13)	1.00 (0.71–1.41)
Leptin (6 μg/day)	LFD	0.06* (0.05–0.08)	0.13* (0.11–0.15)	1.54 (1.10–2.15)	0.77 (0.59–0.99)
Leptin (12 μg/day)	LFD	0.14* (0.08–0.25)	0.19* (0.16–0.23)	1.31* (0.74–2.34)	8.50* (6.44–11.24)
Saline	HFD	0.60 (0.28–1.23)	0.47 (0.26–0.85)	0.86 (0.62–1.35)	1.73 (1.24–2.41)
Leptin (6 μg/day)	HFD	0.04* (0.04–0.05)	0.13* (0.09–0.15)	0.85 (0.51–1.8)	2.01 (1.06–4.17)
Leptin (12 μg/day)	HFD	0.91 (0.49–1.50)	0.17* (0.14–0.21)	0.64* (0.49–0.83)	11.40* (6.84–18.95)

Note: Tissues were collected from the animals in Table 1. Data are expressed as the mean fold-change relative to saline-treated animals on LFD for that tissue and for that isoform. The range of fold changes (in parentheses) was calculated from the standard error of the ΔΔCt values according to Livak and Schmittgen [62]. Statistical analyses were performed on individual ΔΔCt values and are only valid for comparisons within a given column

* $P < 0.05$ vs saline-LFD within a column

increase in body weight is due to factors other than increased energy consumption.

An alternative hypothesis is that HFD results in a reduction in metabolic rate, shifting the energy equation toward energy storage. However, energy expenditure in C57BL/6 mice fed a 45% fat diet for 12 or 16 weeks does not differ from LFD controls [34, 35]. Likewise, rats fed a 59% fat diet have a transient decrease in energy expenditure at 30 days, but no difference at 70 days compared to LFD controls [31], and humans fed a 40% fat diet can gain weight without decreased energy expenditure [32]. Thus, the literature does not support the hypothesis that a long-term decrease in metabolic rate accounts for HFD-induced obesity. Since we did not attempt to confirm these observations, however, it remains possible that metabolic rate did decrease in the mice used in our study under our particular housing and experimental conditions.

The divergence in body weights between wild-type LFD and HFD mice did not occur immediately. Our results and those of others [21, 31] demonstrate that at least 12 days or longer are required before the body weights of LFD and HFD mice and rats diverge. One possible explanation for the latency is that consumption of an HFD gradually induces changes in intestinal morphology and surface area, enterocyte function, and fatty acid transport such that the efficiency of nutrient absorption from the gut is increased [36, 37]. These changes could result in a greater percentage of consumed nutrients entering the blood. Consistent with this hypothesis are reports that HFDs are associated in rodents with numerous changes in the small intestine, including enterocyte expression of fatty acid binding proteins and transporters [38], ratios of different cell populations [39], and intestinal morphology and re-growth in an experimental model of short-bowel syndrome [40]. However, we did not observe a difference in the estimated energy absorption in wild-type mice on diets of different fat content and saturated/unsaturated fat ratios. This result suggests that energy deposition in white adipose tissue is upregulated in response to factors directly or indirectly attributable to the amount of fat in the diet and not the total amount of energy consumed or absorbed.

Even on LFDs (10%), increasing the ratio of saturated/unsaturated fats resulted in significant weight gain. Interestingly, however, the type of fat had no impact on weight gain when the total fat content of the diet was high (45%). At least some component of the weight gain that resulted from the high-fat/high-unsaturated fat diet, however, may have been the result of saturated fats. Despite the lower percentage of saturated fat in the diet, increasing the total fat content of the diet from 10% to 45% would have resulted in an absolute intake of saturated fat that was not much different from that consumed by mice in the low-fat/high-saturated fat group. The mechanisms by which saturated fats—particularly those containing medium and long-chain fatty acids—alter weight gain may include changes in fluidity of enterocyte plasma membranes and transport processes [37], or could be related to transport of fatty acids into white adipose tissue and synthesis into triglycerides. For example, it is possible that the expression and/or activity of lipoprotein lipase and acyl CoA:diacylglycerol transferase (DGAT) are chronically increased in white adipose tissue in mice after exposure to HFDs. Indeed, mice deficient in DGAT are resistant to diet-induced obesity [41].

The increase in body mass in wild-type HFD mice coincided with significantly elevated plasma leptin levels, consistent with the advent of leptin resistance. Of the various signaling pathways induced by the binding of leptin to its receptor (OBR) on target cells, the JAK2/STAT3 pathway is believed to be the major mediator of the anorexigenic actions of leptin on the hypothalamus [14]. Mice fed an HFD for 15 weeks have reduced leptin-activated STAT3 signaling in the hypothalamus [42], although the mechanism of this is uncertain. One way in which leptin resistance might arise is a decrease in hypothalamic leptin receptor content, as occurs, for example, during the period of weight gain in pregnant rats [43]. We hypothesized that HFD may induce changes in expression of genes associated with the activation and/or inhibition of the JAK2/STAT3 pathway in hypothalami. We report here quantitative assessment by qPCR of HFD-associated changes in mRNA levels of hypothalamic genes with known or putative functions related to the OBR/STAT3 signaling pathway. mRNA for hypothalamic OBRb, the only receptor isoform with full signaling capability through the STAT3 pathway [7, 44], was decreased by over 50% in wild-type HFD animals, confirming previous reports in rodents using non-quantitative methods or in which one but not both receptor subtypes were determined [7, 16, 45].

We also report for the first time that the change in hypothalamic OBRb mRNA was tissue-specific, as no change was observed in the liver. The change in OBRb may account in part for reduced hypothalamic signaling by leptin at 15 weeks as reported elsewhere [46], but it is important to note in our study that expression of OBRb mRNA actually increased in HFD mice at 3 weeks, when body mass and plasma leptin levels were already increasing. Interestingly, the other major signaling isoform of the receptor, OBRa, also first increased at 3 weeks and then decreased at 15 weeks in hypothalami of HFD mice. Again, this effect was tissue-specific, as an entirely different pattern of expression was observed in the liver. Thus, if receptor mRNA is correlated with receptor protein in hypothalami, as is likely [47], it is possible that the combined reduced hypothalamic expression of the two major isoforms of the leptin receptor contribute to leptin resistance and obesity in long term, but not short-term wild-type HFD animals.

In contrast to wild-type mice, there was no significant effect of diet on hypothalamic OBRa or OBRb mRNA in leptin-deficient *ob*/*ob* mice. Leptin treatment in those mice, however, resulted in a significant and marked decrease in hypothalamic expression of both receptor isoforms on either diet. The effect of leptin on leptin receptor isoform expression was specific as it did not occur in the liver. Thus, the reduced leptin signaling observed in wild-type rodents fed an HFD may be at least partly secondary to decreased hypothalamic OBRb levels, but other mechanisms may be equally or more important, particularly in leptin-deficient mice. In addition, the diet-independent decrease in expression of hypothalamic leptin receptor mRNA that occurred following leptin treatment in *ob*/*ob* mice suggests a mechanism by which elevations in circulating leptin may contribute to a worsening of diet-induced obesity, without being an initial cause of the obesity. Notwithstanding, in *ob*/*ob* mice, the quantity of receptors remaining after long-term leptin treatment is clearly sufficient for the animals to retain significant leptin responsiveness, and the weight gain induced by HFD in those animals cannot be attributable to an additional decrease in leptin receptor mRNA beyond that induced by leptin treatment.

Once leptin binds to its receptor, the occupied receptors form dimers which activate an associated janus kinase (JAK2) to cross-phosphorylate four tyrosine residues on the receptor, including tyr1138 which binds STAT3. STAT3 is then phosphorylated at tyr705 and ser727 and forms a dimer. The phosphorylated STAT3 dimers act as transcription factors that mediate the anorexigenic and metabolic actions of leptin in the brain [24, 48]. We did not detect a decrease in constitutive levels of the active, phosphorylated form of STAT3 protein in whole hypothalami of HFD mice at 15 weeks, which could suggest that signaling through this step is intact in wild-type HFD mice, despite the reduced receptor expression. However, we did not determine whether leptin-induced phosphorylation of STAT3 was affected, nor did we assess individual hypothalamic nuclei; Martin et al. [49], however, noted an attenuated leptin-induced phosphorylation of STAT3 in hypothalami of male FVB mice fed a 55% fat diet.

Once STAT3 is activated by phosphorylation, it forms dimers that translocate to the nucleus and bind to specific regions of DNA. The steps that mediate this translocation have not been elucidated, but are believed to require the presence of several other proteins, including the importins. Importins are a family of heteromeric proteins involved in nuclear transport of proteins that contain a nuclear localization sequence, such as that present in STAT3. The importins bind their cargo, and then bind each other, forming part of the nuclear pore complex [50]; phosphorylated dimers of STAT3 bind to members of the α importin family [51]. Thus, it is reasonable to suggest that increased expression of importins may facilitate nuclear translocation of active phosphorylated STAT3, the constitutive levels of which were unchanged in this study. We observed that HFD was associated with increased hypothalamic expression of mRNAs for importins $\alpha 2$ and $\beta 1$. These results appear to contradict the hypothesis that HFD results in reduced leptin signaling in the hypothalamus. It is possible, therefore, that upregulation of importins may be part of a complex compensatory mechanism that partially offsets other factors that reduce leptin signaling in diet-induced obesity, and could one day provide an additional target for experimental manipulation of leptin sensitivity in the hypothalamus.

Several intracellular factors have been identified that inhibit the action of leptin on its target tissues, including PIAS3 and StIP1. PIAS3 isoform 2 is an inhibitor of STAT3 binding to DNA (reviewed in Ref. [24]). In the present study, mRNA levels of this gene were increased in the hypothalami of HFD mice. StIP1 encodes a protein whose function is not well defined, but is believed to have contrasting functions related to STAT3 activity at different concentrations. At low concentrations, StIP1 may act as a scaffold that facilitates phosphorylation of STAT3 by activated JAK2, but at high concentrations it may sequester STAT3 in the cytosol and thereby prevent its nuclear translocation [18]. HFD induced a nearly two-fold increase in StIP1 mRNA in hypothalamus, which is consistent with its latter putative activity. These results, along with those described above for the leptin receptor, suggest that HFD induces a variety of changes in gene expression that are consistent with and may explain in part the reduced leptin signaling via pSTAT3 DNA-binding in the hypothalamus observed by others [42, 46].

The changes in mRNA observed in this study were restricted to whole hypothalami. Also, our studies do not distinguish between a change in gene expression and a change in mRNA half-life. It must be cautioned that different nuclei within the hypothalamus may respond differently to HFD, or not at all, or at different times after initiation of an HFD. Thus, we cannot conclude at this time that the changes in mRNA we observed were specific to leptin-sensitive cells. The anorexigenic and metabolic actions of leptin appear to be primarily confined to the arcuate and paraventricular nuclei of the rodent hypothalamus [52–55]. It is possible, therefore, that the magnitude of the changes in mRNA we observed were obscured by the use of whole hypothalami. Finally, in the absence of protein measurements, we cannot conclude that changes in mRNA were absolutely correlated with changes in protein content, although this is not unlikely.

Recent evidence suggests that exposure of the rodent brain to chronically elevated concentrations of leptin may itself contribute to leptin resistance, thereby exacerbating obesity [22, 23, 52]. If true, we would predict that when mice are fed an HFD superimposed on a background of leptin clamped at physiological levels, there should be less weight gain than in corresponding animals treated with chronically supraphysiological levels of leptin. To test this hypothesis, we clamped circulating leptin levels in leptin-deficient *ob*/*ob* mice at physiological or supraphysiological concentrations found in wild-type C57BL/6 of the same genetic background and age (see Table 1, and Refs. [13, 56, 57]). When control (saline-treated) mice were fed an HFD, their weight increased to very obese levels in the absence of leptin. Leptin replacement resulted in the expected dose-dependent weight loss while the animals were on an LFD. Seven weeks of leptin replacement at either dose did not appear to result in leptin resistance as indicated by continued weight loss in LFD animals. However, when switched to the HFD, the weight loss induced by leptin was halted and even reversed. These data indicate that HFD results in weight gain in *ob*/*ob* mice independent of the presence or absence of leptin at physiological or supraphysiological levels. Interestingly, however, body weights of HFD mice exposed to the higher dose of leptin for several weeks began to increase on HFD, whereas those on the lower dose stopped losing weight but did not regain any. This suggests that leptin and HFD interact in previously unrecognized ways to control leptin sensitivity in this one animal model. It should be noted, however, that the hypothalamic circuitry of *ob*/*ob* mice differs significantly from that of wild-type C57BL/6 mice, possibly due to the lack of leptin during critical windows of development [27, 28, 58], making it difficult to fully extrapolate the present data to the wild-type condition.

Adiponectin is an adipokine that improves insulin sensitivity and, like leptin, is associated with inhibition of appetite and a decrease in body weight in rodents [59]. Adiponectin levels have been reported to decrease in plasma of obese rodents [60], although a recent study reported no change in circulating adiponectin in wild-type C57BL/6 mice that were fed a high-fat/high-sucrose diet for 4 weeks [61]. In that study, circulating adiponectin levels actually increased after 16 weeks of the altered diet [61]. Changes in plasma adiponectin do not appear from our studies to be involved in the early stages of obesity onset. We did not observe a

diet-induced change in circulating adiponectin levels in either wild-type or leptin-deficient mice, although we did observe a significant age-dependent decrease in wild-type mice, consistent with a recent report [62]. Therefore, a decrease in circulating adiponectin does not appear to be a ubiquitous marker of the progression of obesity. However, it is noted that the radioimmunoassay (RIA) used to quantify adiponectin in the current study does not distinguish among all forms of circulating adiponectin; recent studies suggest that the different circulating forms are not equally well correlated with, for example, metabolic syndrome in humans [63]. We did, however, observe that omental, but not subcutaneous, fat released less adiponectin into the medium of incubated fat samples obtained from HFD mice than from LFD mice. Thus, it is important to distinguish between the two fat depots when determining how or if adiponectin regulation is altered during diet-induced obesity.

We conclude that in C57BL/6 mice a diet enriched in saturated fats induces weight gain regardless of the total fat content of the diet, independent of changes in energy consumption, energy absorption, and circulating adiponectin. Changes in hypothalamic OBR isoform expression, hypothalamic STAT3-interacting proteins, and circulating leptin levels may all contribute to various degrees to weight gain in such mice.

Materials and methods

Effects of LFD and HFD on body weight, energy consumption, and hormone levels in wild-type mice

All procedures were approved by the Boston University Institute Animal Care and Use Committee. In the first experiment, female C57BL/6 mice were obtained from Taconic Laboratories at 7 weeks of age, and housed 4/cage with lights on from 0700 to 1900 h. Mice were randomly assigned to one of two dietary groups. LFD animals were fed a diet composed of 10% kcal from fat consisting of soybean oil and lard (Research Diets, Inc.). HFD animals were fed a diet consisting of 60% kcal from fat, prepared by increasing the percentage of lard from 4% (LFD) of total kilocalories to 54% (HFD) without changing soybean oil content, and reducing the carbohydrate content from 70% to 20% of total calories. The amount of protein was identical between the diets (20% of total calories). The complete compositions of the diets are available through the supplier's website, http://www.researchdiets.com (Catalog #s D12450B and D12492).

Body weights were determined periodically for 15 weeks. Food intake was measured every week by filling cylindrical, plexiglass food cups with crushed powdered versions of the above diets. The powdered diets were wetted and formed into a mash which prevented the mice from extracting food from the cup. The cups were attached to a plexiglass base that prevented the cups from tipping. The opening to the cups was sufficiently large to allow the mice to feed, but not to enter the cup. Dry weights of food were obtained after filling the cups with the mash and again after removing the cups from the cage 24 h later and drying them in an oven to a constant weight. Bedding was carefully examined for the possible presence of food that may have been removed from the cups, although this was never observed. The total amount of energy consumed per cage of mice was determined using the following conversions provided by the supplier: LFD = 3.85 kcal/g, HFD = 5.2 kcal/g.

In a separate study designed to more precisely document the early changes in body mass and gene expression resulting from a switch to an HFD, female C57BL/6 mice were placed on the respective diets for 2 days or 3 weeks, with caloric intake and body weight measurements as above.

Two days, three weeks, or fifteen weeks after beginning their respective diets, mice were euthanized by CO_2 gas followed by decapitation between 1000 and 1100 h. Food was removed from the cages at the time of lights-on, 3–4 h prior to sacrifice. Hypothalami and livers were dissected, frozen in a dry ice/acetone bath, and stored at −85°C until use. To confirm the expected endocrine and metabolic profiles, trunk blood was collected from a subset of animals into heparin-containing tubes, and the plasma was frozen at −20°C for future hormone and other assays.

Effects of saturated and unsaturated fats on body weight, adiposity, energy consumption, and energy absorption in wild-type mice

Saturated and unsaturated fats were altered in four additional diets to determine the contribution of each type of fat to weight gain. These four new diets were specifically designed to differ in the percentage of saturated and unsaturated fat. Sixteen adult male C57BL/6 mice were fed LFDs (10%) or HFDs (45%; it was necessary to reduce the total fat content from 60% as above in order to maintain physical consistency of the unsaturated diets). Half of the animals in each group were further assigned to a diet in which the fat content consisted of either 9.5 or 76.7% saturated, with the remaining fat being a mix of mono- and polyunsaturated fats. Sucrose and protein contents were the same in all four diets. Body weights and food intakes were determined periodically as above; in the case of food intakes, seven different measurements were determined over the course of the experiment, and the mean caloric intake per dietary group over seven measurements was calculated. Blood samples were taken via submandibular venipuncture prior to and 10 days after initiating the diets. Trunk blood was collected at the time

of sacrifice as described above; plasma samples were subjected to RIA of leptin. Body mass index was calculated as g/cm^2 (nose-anus distance). In addition, inguinal and perirenal fat pads were removed and weighed to obtain an estimated fat index, according to Ref. [56]. Finally, the feces accumulated during two separate 24 h food intake periods were collected from the bedding, dried to a constant mass, homogenized using a mortar and pestle, and then an approximately 1 mg sample was subjected to bomb calorimetry with a Phillipson Oxygen Microbomb Calorimeter (Gentry Instruments, Inc., Aiken, SC). Total energy content of a fecal sample was corrected for total fecal mass over the 24 h period for each mouse. Energy absorption on each diet was estimated as the difference in the total energy consumption for that collection period, and total energy excreted (feces) in that same period. The collections were made on two separate occasions.

Interaction of HFD and leptin replacement on weight gain in *ob*/*ob* mice

Two separate experiments were performed with different doses of leptin. In each experiment, 20 *ob*/*ob* male mice (Taconic) purchased at 7 weeks of age were singly housed with lights on from 0700 to 1900 h and at an ambient temperature of 22.6–23.8°. Mice of this genetic background are prone to develop idiopathic dermatitis and thus were housed singly to minimize this possibility after surgery. *ob*/*ob* mice are less able to thermoregulate at cold ambient temperatures than are their wild-type counterparts, and this could potentially be exacerbated by being singly housed. However, the mice were provided with ample bedding of a type that facilitated adequate nesting and consequently provided insulation when the mice were in the nest. Moreover, the cages were plastic-bottomed to minimize heat conduction, and filter-topped, which tends to raise intra-cage temperatures by approximately 2°C above ambient. The mice were fed standard rodent lab chow for 1 week while they acclimated to their surroundings. All of the animals were then switched to LFD containing 10% total kilocalories as fat as in the experiments reported in Fig. 1. At a later time (see below), half the animals were changed to HFD containing 60% fat as in Fig. 1.

To clamp circulating leptin levels, animals were anesthetized with isoflurane, and a single 200 µl Alzet miniosmotic pump was surgically implanted subcutaneously in the midscapular region using aseptic technique. The incision was sealed with veterinary grade superglue, and the animals were returned to their cages. In one experiment, the pumps contained either 0 (saline) or 1 µg/µl recombinant mouse leptin; in a second experiment, pumps contained either 0 (saline) or 2 µg/µl leptin. Separate saline groups were included with each leptin dose study; this not only controlled for the effect of pump implantation but permitted an additional measure of replicability across experiments. The pumps are specified by the manufacturer to deliver product at a constant rate (0.25 µl/h) for up to 28 days; thus the daily doses of leptin were 6 and 12 µg/day, respectively.

Three or four weeks after the minipumps were implanted, when the leptin-treated animals had lost weight and approached the typical wild-type body mass for C57BL/6 male mice, the diets of half of the animals were switched from LFD to HFD. The other half of the animals remained on LFD. Also at that time, the spent minipumps were removed under isoflurane anesthesia and replaced with freshly filled minipumps, again containing either saline, 6 or 12 µg/day leptin. Each mouse received the same treatment (saline or 6 or 12 µg/day leptin) with both pumps.

The study was continued for an additional 3–4 weeks. At the conclusion of each study, mice were short-term fasted by removing food from the cages at lights-on, 3–4 h prior to sacrifice. Mice were euthanized by CO_2 gas followed by decapitation between 1000 and 1100 h. Hypothalami and livers were dissected, frozen in a dry ice/acetone bath, and stored at −85°C until use. Trunk blood was collected into heparinized tubes for determination of plasma leptin, insulin, and adiponectin by RIA. Sufficient blood was not always available from each mouse to complete each of these measurements.

Characterization of hypothalamic gene expression

Hypothalami and livers were processed for RNA using the Qiagen RNeasy mini kit with Qiazol. RNA concentration was measured by Nanodrop spectroscopy. RNA was reverse-transcribed to cDNA using the Applied Biosystems reverse transcriptase system. The cDNA was used for real-time PCR using SyBR green PCR master mix and ABI Prism 7900HT Sequence Detection System from Applied Biosystems. Primer sequences for each gene are given in Table 6. Primers were designed using Primer Express software to amplify ideal target sequences for qPCR; primers were optimized before use to confirm equivalent rates of amplification with housekeeping genes. All samples were run in quadruplicate. Real-time PCR data were analyzed using the ΔΔCt method [64] with 18S RNA as a control. Data are presented as mean fold-increases (HFD/LFD).

Adiponectin release from subcutaneous and omental fat

Approximately 100 mg samples of subcutaneous and perirenal omental fat were collected from mice at 15 weeks in

Table 6 Primer sequences for real-time PCR analysis of genes expressed in mouse tissue

Gene	Forward primer	Reverse primer
KPNA2	AGACGTTTCCACAGCCGC	TCAGATTTCCACGAAAGGCG
KPNB1	CAGAGGTGGCTCGCTATTGAT	CACTGTGAGGCCGAACTAGG
OBRa	GAAGAAGCTGTTTTGGGATGATGT	GTCCGTACTCTTTTGAAAAT TAAGTCC
OBRb	CAAGAATTGTTCCTGGGCACA	TCAGCTCCAAAAGAAGAGGA
PIAS3 isoform 2	GAAGGAGGCATCAGAGGTTTG	TAGACAGGAAATCACTGCCCA
StIP1	AGGAGCACGCAGCTATCATT	CCACAAGACCAGGTTCGAT
18S RNA	CGCCGCTAGAGGTGAAATTC	CCAGTCGGCATCGTTTATGG

Abbreviations: KPNA2 (importin α2); KPNB1 (importin β1), OBRa/b (leptin receptor isoforms a and b); PIAS3 (protein inhibitor of activated STAT3); StIP1 (STAT-interacting protein 1)

both the LFD and HFD groups from the animals in Fig. 1. The fat was minced and washed twice in 50 ml ice-cold Krebs buffer (pH 7.4) containing 1 mg/ml each of glucose and BSA, plus protease inhibitors. Each sample was then incubated for 1 h in 1 ml fresh buffer as above at 37°C in a humidified 95% oxygen/5% CO_2 atmosphere. At the end of the incubation period, the tubes were decanted over several layers of cheesecloth. The medium was frozen at −20°C for future RIA of adiponectin. The fat was then removed from the cheesecloth and dried to a constant weight in a drying oven. Data were normalized to mg dry fat mass.

Hormone and metabolic assays

Leptin and insulin were determined by RIA (Linco) in 10 µl unextracted plasma. Adiponectin was determined by RIA (Linco) in 1 µl of plasma and 50 µl media, respectively. The adiponectin RIA measures total adiponectin (all circulating forms). Glucose was determined using the glucose oxidase method (Pointe Scientific) in 10 µl plasma.

Data analysis

Statistical significance was determined by ANOVA to compare dietary groups with respect to each other and to time. In some cases, pairwise comparisons were then made using the Bonferonni multiple comparison method. In vitro adiponectin data were analyzed by ANOVA with Tukey post-hoc test. Differences in hormone and glucose levels between dietary groups were determined by student's unpaired *t*-test for leptin, insulin, and glucose at any timepoint. For adiponectin, data were analyzed by ANOVA followed by Tukey post-hoc test to evaluate age-dependent changes in plasma adiponectin as well as possible diet-dependent differences. Real-time PCR data were analyzed using individual ΔΔCt values to determine variance and statistical significance by ANOVA with Tukey post-hoc assessment; data are reported as the mean and range of the fold-change in mRNA in HFD mice with respect to LFD controls, as described elsewhere [64].

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