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Rodent models of obesity induced by consuming high-fat diet (HFD) are characterized by inflammation both in peripheral tissues and in hypothalamic areas critical for energy homeostasis. Here we report that unlike inflammation in peripheral tissues, which develops as a consequence of obesity, hypothalamic inflammatory signaling was evident in both rats and mice within 1 to 3 days of HFD onset, prior to substantial weight gain. Furthermore, both reactive gliosis and markers suggestive of neuron injury were evident in the hypothalamic arcuate nucleus of rats and mice within the first week of HFD feeding. Although these responses temporarily subsided, suggesting that neuroprotective mechanisms may initially limit the damage, with continued HFD feeding, inflammation and gliosis returned permanently to the mediobasal hypothalamus. Consistent with these data in rodents, we found evidence of increased gliosis in the mediobasal hypothalamus of obese humans, as assessed by MRI. These findings collectively suggest that, in both humans and rodent models, obesity is associated with neuronal injury in a brain area crucial for body weight control.

Introduction

Obesity has emerged as a major health problem in industrialized nations. Despite substantial progress in understanding the neurobiology of energy homeostasis (the biological process through which energy intake and expenditure are matched to one another so as to promote stability in the amount of food stored as fat) (1), little is known regarding how brain systems designed to promote weight stability are altered in common forms of obesity (2, 3).

Growing evidence implicates immune cell–mediated tissue inflammation as an important mechanism linking obesity to insulin resistance in metabolically active organs, such as liver, skeletal muscle, and adipose tissue (4–6). In rodent models of diet-induced obesity (DIO), increased inflammatory signaling in the mediobasal hypothalamus (MBH) similarly contributes to leptin resistance and weight gain (7–12), but the cellular interactions underlying this inflammatory response remain uncharacterized. The goal of the current study was to identify the neuroanatomical correlates of obesity-associated hypothalamic inflammation and to determine whether similar responses occur in humans.

We report that unlike inflammation in peripheral tissues, a process that develops over weeks to months of high-fat diet (HFD) feeding in rodent models (13–15), markers of hypothalamic inflammation are elevated within 24 hours of HFD exposure. Within the first week of HFD, markers of neuron injury also become evident in the hypothalamic arcuate nucleus (ARC) and adjacent median eminence (ARC-ME) in association with reactive gliosis involving recruitment of both microglia and astrocytes. Although initially transient, suggesting an effective neuroprotective response, inflammation and gliosis return and become established with continued HFD exposure. Using an established MRI method (16–19), we also report evidence of increased gliosis in the MBH of obese humans. These findings collectively suggest that, in both humans and rodent models, obesity is associated with injury to a key brain area for energy homeostasis.

Results

Time course of HFD-induced hypothalamic inflammatory gene expression. Consistent with a large volume of literature (7–11, 13, 14, 20), we found that expression of several proinflammatory genes increased by approximately 50% in both hypothalamus (Figure 1A) and liver (Figure 1B) of adult male rats subjected to long-term (20 weeks) consumption of a HFD (60% of calories from fat) relative to that in controls fed standard chow. By comparison, whereas inflammation was not detected in either liver or adipose tissue after only 4 weeks of HFD, hypothalamic inflammation was clearly evident at this earlier time point (Figure 1, C–E). Thus, the effect of HFD feeding to induce hypothalamic inflammatory gene expression seems unlikely to arise from a systemic inflammatory process.

Since rats fed the HFD for 4 weeks gained more than 50 g more body weight and more than 10% more fat mass than chow-fed controls over the same time frame (data not shown), it remains possible that hypothalamic inflammation at this time point is a consequence of obesity. To address this question, we analyzed hypothalamic proinflammatory gene expression in rats during the
initial phase of HFD feeding. Relative to that of chow-fed controls, rats placed on the HFD exhibited a transient but robust (50%–100%) increase of food intake (Figure 1, G and H) that gave rise to a small but significant increase of body weight and fat mass by day 7 (Figure 1F). In this cohort, gene expression analysis revealed a complex “on-off-on” pattern, with elevated hypothalamic levels of Il6, Tnfa, Socs3, Ikkb, and Ikbke mRNA observed within the first 3 days of HFD exposure, followed by a decline to baseline values from days 7 to 14 and a subsequent return to elevated levels by day 28 (Figure 1I). Thus, increases of hypothalamic proinflammatory gene expression closely mirrored changes of energy intake during the first days of HFD feeding, and both occurred prior to significant expansion of body fat mass (P = NS for fat mass gain of HFD-fed rats at days 1 or 3 compared with chow-fed controls; Figure 1F). C57BL/6 mice displayed a similar, transient increase of proinflammatory gene expression during the first 7 days of HFD feeding.
HFD-induced neuronal injury. The rapid onset of inflammation and reactive gliosis observed in the hypothalamus of rats and mice consuming a HFD is a hallmark of the response to neuron injury (e.g., induced by ischemia or excitotoxicity) (21–24). To more directly test the hypothesis that HFD exposure causes ARC neuron injury, we performed immunohistochemical staining to detect induction of the chaperone Hsp72, a component of the neuroprotective response to neuron injury (26), in the ARC-ME of rats fed the HFD for 7 days relative to that of chow-fed controls (Figure 6, A and B). As predicted, Hsp72 immunostaining was increased in HFD-fed rats relative to that in chow-fed controls, and hypothalamic Hsp72 mRNA expression was also detectably elevated within 3 days of HFD exposure (data not shown). Among ARC neurons in which Hsp72 induction was detected are those containing proopiomelanocortin (POMC) that are components of a critical network for energy balance regulation (ref. 1 and Figure 6, C and D).

We next used electron microscopy to investigate whether autophagy, an independent marker of neuronal stress/injury (27, 28), was induced in POMC neurons from C57BL/6 mice fed the HFD (Figure 6, E–I). After 20 weeks of HFD feeding, the percentage of POMC neurons with detectable autophagosomes increased more than 10 fold (black arrows in Figure 6F; higher-magnification view in Figure 6G), such that the majority of POMC cells examined now exhibited this organelle (6.4% ± 4.1% in chow fed vs. 81.2% ± 8.7% in HFD fed; 

(Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI59660DS1), indicating that hypothalamic inflammatory signaling occurs prior to substantial weight gain in mice, as it does in rats.

Markers of gliosis during early HFD feeding. Activation, recruitment, and proliferation of microglia (macrophage-like immune cells of the brain) and astrocytes, collectively termed “reactive gliosis,” are hallmarks of the brain response to neuronal injury (21–24). We observed that, in rats fed a HFD for up to 28 days, hypothalamic expression of mRNA encoding cytokine–specific markers Cd68 and Emr1 (which encodes F4/80) increased by 50%–100% by day 3 (Figure 2A), suggesting an effect of HFD feeding to promote microglial accumulation in this brain area. Interestingly, the level of hypothalamic Emr1 gene expression was correlated with fat mass gain (r = 0.70; P < 0.001; Figure 2B). Combined with our finding that hypothalamic expression of mRNA encoding the astrocyte marker gene Gfap is comparably increased at the same early time point (Figure 2A), these results suggest that the rapid onset of hypothalamic inflammation induced by HFD feeding is associated with robust glial responses.

Using immunohistochemistry to detect the microglia-specific cytoplasmic marker Iba1 (ref. 25 and Figure 3A), we found that microglial number increased in rat ARC by day 3 of HFD exposure compared with that in chow-fed controls (Figure 3, B–D) and remained elevated throughout the initial 2-week period (Figure 3, E and F; quantified in Figure 3G). Concomitantly, microglial cells enlarged (Figure 3H) and adopted a more activated morphology (compare Figure 3I with Figure 3J). In separate cohorts of rats fed a HFD for up to 8 weeks, microglial accumulation in the ARC correlated with the degree of fat mass gain (r = 0.54, P = 0.019; Figure 3K), and animals with greater increases of fat mass also had larger microglia in the ARC (r = 0.52, P = 0.028; Figure 3L). Moreover, changes of both microglial accumulation and cell size appear to be limited to the ARC-ME region, as they were not observed in other hypothalamic (lateral hypothalamic area, ventromedial nucleus) or extrahypothalamic (hippocampus, cerebral cortex) brain areas (Supplemental Figure 2). Since an analogous accumulation of microglia occurred in the ARC-ME of wild-type C57BL/6 mice (Supplemental Figure 3), this effect of HFD feeding is not unique to rats.

The effect of HFD on astroglial responses was assessed in C57BL/6 mice by GFAP immunostaining. As expected, GFAP-positive astrocytes were scattered throughout the ARC-ME of mice fed standard chow, and their processes resembled those in other brain areas (Figure 4A). Within just 1 week of HFD exposure, however, the intensity of GFAP staining in the ARC-ME increased by approximately 50% (Figure 4, B and G). Like hypothalamic inflammatory markers (and unlike the microglial response), this astrogliosis was transient, returning to baseline in mice fed the HFD for 2 to 3 weeks (Figure 4, C and D, respectively; quantified in Figure 4G) but recurring with long-term HFD feeding relative to that in age-matched controls fed chow for the same duration (Figure 4, E and F; quantified in Figure 4H). A similar pattern of hypothalamic gliosis was observed in rats over the first 8 weeks of HFD feeding (data not shown), confirming that comparable microglial, astroglial, and inflammatory responses to HFD occur in both species.
and shape of the mitochondria varied greatly in the HFD-fed animals but not in the chow-fed animals (Figure 6F, white arrows). Furthermore, the size and shape of the mitochondria varied greatly in the HFD-fed animals but not in the chow-fed animals (Figure 6F, white arrows). To determine whether cellular injury (Figure 6, A–D) and autophagy (Figure 6, E–I) ultimately give way to permanent alteration of POMC neurons, we quantified POMC-immunopositive cells in the ARC of mice fed a HFD (white arrows in Figure 6, E and F; higher-magnification view in Figure 6H). Whereas mitochondria in POMC neurons from chow-fed rats uniformly demonstrated homogenous, compact electron-dense lumens with well-organized, parallel-oriented cristae (Figure 6H), mitochondria from HFD-fed animals manifested irregular swellings and less electron-dense lumen with nonparallel cristae that were frequently discontinuous (Figure 6H). Furthermore, the size and shape of the mitochondria varied greatly in the HFD-fed animals but not in the chow-fed animals (Figure 6F, white arrows).

To determine whether mitochondrial morphology was observed in POMC neurons from mice fed a HFD (white arrows in Figure 6, E and F; higher-magnification view in 6H), we investigated whether MBH gliosis is also associated with obesity in humans. We used magnetic resonance imaging obtained from a retrospective cohort of 34 subjects who had clinical MRI examinations without identified abnormalities. BMI ranged from lean to obese (17.7–44.1 kg/m²). To detect gliotic changes below the visual detection threshold, ratios were created to compare mean signal intensity within regions of interest (ROIs) placed in the MBH with ROIs in adjacent amygdalar tissue (ROIs indicated by green circles and arrows in Figure 7, A and B, on representative images from a lean and an obese subject). The putamen was used as a control ROI. Mean signal intensities were highly correlated between ROIs in the right and left hemispheres for all 3 brain regions (MBH: \( r = 0.99, P < 0.0001 \); amygdala: \( r = 0.99, P < 0.0001 \); putamen: \( r = 0.97, P < 0.0001 \)), such that MBH/amygdala mean signal ratios were similar on both sides of the brain (left: 1.13 ± 0.12; right: 1.08 ± 0.14; \( r = 0.54, P = 0.001 \)).

Across all subjects, left MBH/amygdala signal intensity was positively correlated with BMI, both by simple linear regression of mean intensity ratios (\( r = 0.38, P = 0.027 \); Figure 7C) and multivariate analysis using MBH signal as the independent variable and amygdala signal as covariate (\( P < 0.05 \); data not shown). By comparison, the left MBH/amygdala mean signal ratio was not associated
response suggests that neuroprotective responses are mounted that the initiation of a HFD. The transient nature of this hypothalamic limit or reverse the injury during its initial phases, but, with sustained exposure to the HFD, ARC-ME gliosis and injury responses are reestablished. Combined with MRI-based evidence for gliosis in the MBH of obese humans, our findings suggest that, in both humans and rodent DIO models, obesity is associated with neuron injury in a brain area crucial for body weight control.

Several mechanisms have been forwarded to explain obesity-induced inflammation in both peripheral tissues and hypothalamus, including activation of TLR4, induction of endoplasmic reticulum stress, and activation of serine/threonine kinases, such as IKKβ (reviewed in ref. 8). While the contribution made by these mechanisms remains uncertain, the much earlier onset of inflammation in hypothalamus relative to that in peripheral tissues raises the possibility that different processes are involved. Moreover, the nature of the hypothalamic inflammation occurring during the first days of HFD feeding may differ fundamentally from that involved with chronic HFD exposure. The conclusion that the rapid onset of MBH inflammation is a manifestation of neuron injury and associated neuroprotective responses is consistent with previous evidence of apoptosis and glial ensheathment of ARC neurons in animals rendered obese by chronic HFD feeding (29, 30). Moreover, these responses were detected specifically in ARC POMC cells (29, 30), which is consistent with our finding of an approximately 25% reduction in the number of hypothalamic POMC neurons in mice chronically fed a HFD. In this context, it is noteworthy that POMC cells play an essential role to protect against obesity and that loss of these cells is sufficient in and of itself to cause excess weight gain in mice (31).

In experimental models of brain injury, astrocytes play a key neuroprotective role, limiting the extent of both inflammation and neuron loss (21, 22). The reactive gliosis we observed in the MBH of both rats and mice may therefore be neuroprotective in nature, limiting local injury induced by HFD feeding. Consistent with this hypothesis is our finding that the return of proinflammatory markers in the ARC-ME area to basal, preintervention values (on day 7 of HFD feeding) coincides with the appearance of a reactive gliosis in the same brain area. Like markers of inflammation, however, this gliosis is initially transient, but both responses are reestablished as obesity develops (within 4 weeks of HFD feeding). These observations suggest that with sustained exposure the capacity of supportive glial cells to control the damage associated with HFD feeding is exceeded and that neuron injury and loss can no longer be prevented.

This hypothesis is consistent with our finding that during HFD feeding, Hsp72 was rapidly induced in neurons in the ARC-ME. Heat shock proteins are induced in response to many forms of brain injury, including stroke, neurodegenerative disease, epilep-
Autophagy is a lysosomal degradative pathway that maintains cellular homeostasis by turning over cellular components. Interestingly, this process was recently suggested to participate in the physiological response of MBH neurons to fasting, based on evidence that fasting-induced autophagy liberates nutrient-related signals that regulate neuron firing (33). Beyond the response to starvation, however, autophagy is also a prominent feature of numerous pathological processes (e.g., neurodegeneration) and can lead to apoptosis of neurons and other cell types (27, 28, 34). Although the precise interpretation of increased autophagy in the setting of HFD feeding awaits additional study, recent evidence suggests it may serve to minimize neuronal inflammation and injury (35). Consistent with this interpretation, the apoptosis of ARC POMC cells in rats with DIO (30) and the 25% reduction in POMC cell number we observed in HFD-fed mice support the hypothesis that increased numbers of autophagosomes in this cell population reflect ongoing cell injury and that this protective response along with others, such as upregulated chaperone expression and endoplasmic reticulum stress (11, 36), are ultimately insufficient to prevent cell loss with continued exposure to HFD. This model warrants further study with interventions to determine whether susceptibility to DIO is altered by manipulation of these protective responses.

Although microglia can be activated in a proinflammatory manner and cause tissue damage (37, 38), their diverse roles to regulate support neuronal function are a topic of intense recent interest (37–39). For example, microglia participate in synaptic pruning both during development (39) and in adult brain (37, 38), and they play an important neuroprotective role under conditions in which the initial insult is directed at neurons (e.g., demyelinating or neurodegenerative disease) (23, 24), rather than at microglia themselves. Available evidence suggests that in the first few days of HFD feeding, the inflammation and neuron injury we have observed in the ARC-ME is not due to actions of microglia. Rather, we hypothesize a neuroprotective effect of these cells based on our findings that (a) accumulation and enlargement of microglia becomes detectable in the ARC only after inflammation is established in this brain area, and (b) this microglial response persists even as local inflammation resolves (albeit transiently). With chronic HFD feeding, however, a pathogenic role for proinflammatory microglia has not been excluded, and future studies are warranted to address this issue. An additional unanswered question is why astrocytosis in the ARC resolves temporarily (along with a decrease of proinflammatory markers) after the first week of HFD feeding, whereas microglial accumulation continues to increase during this time.

It is important to note that the current studies were undertaken in rodent strains known to be genetically predisposed to DIO, and the important question of whether the hypothalamic response to HFD feeding differs in obesity-resistant strains remains unanswered. This point is particularly germane in light of evidence that the balance between excitatory and inhibitory synaptic contacts on ARC neurons is altered by HFD feeding (29, 40) in a manner that differs between animals that are genetically predisposed to DIO and those that are obesity resistant (29). Given the capacity of both microglia and astrocytes to participate in synaptic remodeling and modulate synaptic levels of various neurotransmitters (21–24, 37, 41), it will be of interest in future studies to determine whether hypothalamic responses to HFD feeding are influenced by genetic factors that impact predisposition to DIO.

Based on literature establishing structural MRI as a reliable method for visualizing and quantifying gliosis in human brain (16–19), we undertook a retrospective analysis of T2-weighted magnetic resonance images obtained previously in a cohort of young human subjects undergoing clinical examination. We found hyperintensity of the T2-weighted signal in human MBH that was significantly increased ($P < 0.05$) in obese individuals compared with that in
Ikbke - I16sis detection by MRI), these data lend translational relevance to these findings is MRI evidence for gliosis in the hypothalamus of obese humans. Collectively, this work identifies a potential link between obesity and hypothalamic injury in humans as well as animal models.

Methods

Animals. Weight-matched male Long-Evans rats (300–350 g; Harlan) or male C57BL/6 mice (20–25 g) were housed individually in a specific pathogen-free environment, maintained in a temperature-controlled room with a 12-hour-light/12-hour-dark cycle, and provided with ad libitum access to water and either standard laboratory chow (3.34 kcal/g; PMI Nutrition International) or a diet containing 60% kcal fat (HFD, 5.24 kcal/g; D12492; Research Diets) for periods ranging from 1 day to 8 months. Body weight and food intake were monitored daily.

Real-time PCR. For expression analyses, RNA from liver, epididymal white adipose tissue, and MBH (rectangular block excised as previously described; ref. 43) was extracted using TRIzol B according to the manufacturers’ instructions (MRC). RNA was quantified by spectrophotometry at 260 nm (Nanodrop 1000; Thermo Scientific) and reverse transcribed with avian myeloblastosis virus reverse transcriptase (1 μg; Promega). Levels of mRNA for Nfkbia, Ikbkb, Ikbbe, Il6, Il1b, Tnfα, Gfap, Emr1, Cdb8, Gapdh (internal control), and 18S RNA (internal control) were measured by semiquantitative real-time PCR on an ABI Prism 7900 HT (Applied Biosystems). The primer sequences were designed using Primer Express (version 2.0.0; Applied Biosystems) as follows: Nfkbia, forward, TGCCCTGGCCAGTGTACAGCTTT; reverse, CAAAGTCGACGTTGCCACGAT; rat Ikbkb, forward, AGGTTGAC-TAAGTCCAGAC; reverse, ACAGCCGAGATGTGACG; mouse Ikbkb, forward, GCCACCTGGATGACTCTA; reverse, CACTATCCGTGGCT-GTACCT; Ikbbe, forward, ACCACCTAATCATTGGC; reverse, ACTGCGAATAGCTTCACGATG; rat Il6, forward, CAGAGGATAC-GACGACAGCAG; reverse, CAGTGGTATCTGCTGTTCTTACA; mouse Il6, forward, GTGGCTAGGACGACGAA; reverse, GGTCTGCG-GATGAGACCTCA; Il1b, forward, TAAAGGAGGACAGCAACGACA; reverse, GATCCACACTCTCCGCTGAC; rat Tnfα, forward, GCTCCCTCT-CATCAGTTGAC; reverse, CTCCCTCAGTGCTGTTTG; mouse Tnfα, forward,
to identify cell nuclei. GFAP and Iba1 antibodies have been widely validated in the literature as markers for astrocytes and microglia, respectively. In addition, we saw no overlap between these markers and NeuN, a neuronal marker (data not shown). The absence of detectable immunoreactivity in chow-fed controls and presence of positive staining in HFD brains indicates that the Hsp72 antibody staining is not nonspecific, we cannot absolutely rule out the possibility that the antibody may recognize other antigens in hypothalamic neurons of HFD-fed rats.

Images were captured on an Eclipse E600 upright microscope equipped with a color digital camera (Nikon). Quantification was performed in a blinded fashion on anatomically matched brain regions identified in x20 images. Both sides of bilateral structures (e.g., ARC, VMH, etc.) were counted on 2 to 4 slides per animal, and replicate values from each animal were individually averaged before determining group means (n = 4–8/group). For Iba1 and POMC immunostaining in which discrete cells could be identified, cell number was counted manually within pre-specified ROIs using Photoshop (Adobe) or ImageJ (http://rsbweb.nih.gov/ij/). Signal intensity was scored in a semiquantitative fashion and was found to covary with diet group. For microglial cell size (using Iba1) and GFAP immunoreactivity, thresholding was performed in ImageJ, followed by densitometric quantification.

**Figure 7**
Radiologic evidence of gliosis in the MBH of obese humans. Representative coronal T2-weighted images through the hypothalamus from (A) a normal weight and (B) an obese subject. Insets show the placement of right and left ROIs (green circles) in the MBH and amygdala (AMy). In the MBH (thick arrows) of the obese subject, signal ratios demonstrated subtle hyperintensity (brightness) relative to the amygdala (thin arrows). Scale bar: 20 mm; 10 mm (insets). (C) Correlation of BMI with MBH hyperintensity as measured by left (L) MBH/amygdala signal ratio (n = 34 subjects; r = 0.38; P = 0.027).
tin levels (21%), and seizure (15%). On targeted visual inspection of the MBH, no studies were rated as abnormal, 7 were rated as equivocal (4 left, 1 right, 2 bilaterally), and 27 were rated as normal. There was no difference in the proportion of normal weight versus obese subjects who had an equivocal finding (Fisher’s exact = 1.000; P = NS).

Single coronal slices through the hypothalamus, using T2-weighted fast spin echo (with or without fat saturation) or fluid-attenuated inversion recovery sequences, were identified for each subject. The MBH was visually inspected for abnormalities and rated as normal, equivocal, or abnormal. ROIs in the right and left MBH, the right and left amygdala, and the right and left putamen were defined by a neuroradiologist, who was blinded to all clinical information. Mean ROI signal intensity, standard deviation, and area of ROI were measured by using proprietary software on the PACS workstation (Centricity, GE Healthcare). Ratios were calculated by comparing the mean signal intensity in the MBH on each side with that in the ipsilateral amygdala ROI. A control ratio compared mean signal intensity in the putamen with that in the ipsilateral amygdala. Subjects were classified into 2 groups: obese (BMI > 30 kg/m²) and normal weight (BMI 19–24.9 kg/m², without any previously recorded BMI > 30). Subjects with BMIs outside these ranges were excluded from group analyses, but data from all subjects were used in correlation analyses.

**Statistics.** All results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad PRISM (version 4.0b; GraphPad Software). One-way ANOVA with Dunnett’s least significant difference post-hoc tests was used to compare mean values against those for chow controls, while 2-tailed Student’s t-tests were used for 2-group comparisons. For the human MRI study, group differences were assessed by unpaired 2-tailed Student’s t-test for continuous variables and Fisher’s exact test for categorical variables. Normal distributions were confirmed for continuous variables. Pearson’s correlation coefficients were calculated, and univariate linear regression was used to test for significance. A secondary analysis was performed using a multiple linear regression to test for significance. A secondary analysis was performed using a multiple linear regression to test for significance. A secondary analysis was performed using a multiple linear regression to test for significance.

**Study approval.** All study protocols involving rats and mice were approved by the Animal Care and Use Committees at the University of Washington, University of Cincinnati, or Yale University and conducted in accordance with the NIH guidelines for care and use of animals. The study involving analysis of human MRI scans was approved by the University of Washington Institutional Review Board (IRB). The IRB granted a waiver of informed consent; because this was a retrospective study that involved minimal risk to the involved subjects, it was not possible to contact all of the subjects, and appropriate safeguards were in place to protect subject confidentiality and privacy.

**Acknowledgments**

We thank A. Cubelo, L. Nguyen, C. Davis, J. David, and K. Ogimoto for technical assistance with the studies described in this manuscript. This work was supported by a NIH Career Development Award (DK088872) and Diabetes Endocrinology Research Center (DERC) Pilot and Feasibility Award (DK017047) to J. Thaler; NIH grants to M. Schwartz (DK068384, DK083042 and DK052989), M. Tschöp (DK077975), and T. Horvath (DK080000); The Netherlands Organization for Scientific Research – ALW Rubicon to C. Yi; and the Merit Review Research and Research Enhancement Award Programs of the Office of Research and Development, Department of Veterans Affairs to D. Baskin. D. Baskin is the recipient of a Department of Veterans Affairs Senior Research Career Scientist Award. Additional assistance and support was provided by the Nutrition Obesity Research Center (DK035816), Mouse Metabolic Phenotyping Center (U24 DK076126), and Cellular and Molecular Imaging Core of the DERC (DK017047) at the University of Washington.

Received for publication September 30, 2011, and accepted in revised form November 2, 2011.

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