Intermittent Fasting Promotes Fat Loss with Lean Mass Retention, Increased Hypothalamic Norepinephrine Content, and Increased Neuropeptide Y Gene Expression in Diet-Induced Obese Male Mice

Intermittent Fasting Promotes Fat Loss with Lean Mass Retention, Increased Hypothalamic Norepinephrine Content, and Increased Neuropeptide Y Gene Expression in Diet-Induced Obese Male Mice

Juliet D. Gotthardt[1,2], Jessica L. Verpeut[1,3], Bryn L. Yeomans[1,2], Jennifer A. Yang[1,3], Ali Yasrebi[1], *Troy A. Roepke[1-4], Nicholas T. Bello[1-4]

[1]Department of Animal Sciences, School of Environmental & Biological Sciences, Rutgers, The State University of New Jersey, New Brunswick, NJ. USA
[2]Nutritional Sciences Graduate Program, Rutgers, The State University of New Jersey, New Brunswick, NJ. USA
[3]Graduate Program in Endocrinology and Animal Biosciences, Rutgers, The State University of New Jersey, New Brunswick, NJ. USA
[4]New Jersey Institute for Food, Nutrition, and Health, Rutgers, The State University of New Jersey, New Brunswick, NJ. USA

Abbreviated title: IMF Increases Hypothalamic Norepinephrine Content
Word Count: 5550
Key Words: Dieting, appetite, adrenergic receptors, feeding, meal-feeding, short-term weight loss, therapeutic strategies

*Corresponding author:
Troy A. Roepke
Department of Animal Sciences
Rutgers, The State University of New Jersey
School of Environmental & Biological Sciences
Bartlett Hall, 84 Lipman Drive
New Brunswick, NJ 08901
Phone: 848-932-9454
Fax: 732-932-6996

Disclosure statement: The authors do not have any financial disclosures or conflicts of interest.

Abstract

Clinical studies indicate alternate day, intermittent fasting (IMF) protocols result in meaningful weight loss in obese individuals. To further understand the mechanisms sustaining weight loss by IMF, we investigated the metabolic and neural alterations of IMF in obese mice. Male C57/BL6 mice were fed a high-fat diet (HFD; 45% fat) *ad libitum* for 8 weeks to promote an obese phenotype. Mice were divided into 4 groups and either maintained on *ad libitum* HFD (HFD), received alternate day access to HFD (IMF- HFD), switched to *ad libitum* low fat diet (LFD; 10% fat), or received IMF of LFD (IMF- LFD). After 4 weeks, IMF-HFD (~13%) and IMF-LFD (~18%) had significantly lower body weights than HFD. Body fat was also lower (~40-52%) in all diet interventions. Lean mass was increased in the IMF-LFD (~12-13%) compared with HFD and IMF-HFD groups. Oral glucose tolerance AUC was lower in the IMF-HFD (~50%), whereas insulin tolerance AUC was reduced in all diet interventions (~22-42%). HPLC measurements of hypothalamic tissue homogenates indicated higher (~55-60%) norepinephrine (NE) content in the anterior regions of the medial hypothalamus of IMF compared with *ad libitum* fed groups, whereas NE content was higher (~19-32%) in posterior regions in the IMF-LFD group only. Relative gene expression of *Npy* in the arcuate nucleus was increased (~65-75%) in IMF groups. Our novel findings indicate that intermittent fasting produces alterations in hypothalamic NE and NPY, suggesting an involvement in the counter regulatory processes of short-term weight loss are associated with an IMF dietary strategy.

Introduction

Calorie restriction is the most widely prescribed and self-imposed strategy for treating excessive weight gain and obesity (1-3). In the US, most common commercial programs for calorie reduction include reducing daily caloric intake by portion control, low calorie meals, and/or meal-replacement options (1). Rather than reducing daily total caloric intake, intermittent fasting (IMF) has received attention as a possible approach for long-term weight loss (4). Although varying in period of fasting (e.g., alternate day fasting or once/twice a week fasting days), IMF protocols have a similar advantage in that bouts of unrestricted eating occur following fasting periods (5, 6). Several human studies have indicated that a short-term (i.e., 8-24 weeks) IMF protocol results in weight loss (i.e., 3-8%) in overweight or obese subjects (5-13). Weight loss occurs over several weeks because, despite overeating on refeeding days, individuals do not fully compensate for the calorie-deficit realized on the fasting days (6). One appealing feature of IMF protocols is that dieters do not have to count calories during the bouts of unrestricted eating (4). However, one common obstacle to the long-term adherence to IMF is intense feelings of hunger during the fasting periods (14). These subjective feelings of hunger can be mitigated by reducing the period of fasting or providing a small meal (10).

The influence of IMF on the hypothalamic control of energy homeostasis in obesity provides an investigative avenue from which research-based strategies to reduce hunger during fasting periods may be elucidated. In normal weight individuals, energy homeostasis, and subsequently perceived hunger, is tightly controlled through various peripheral and central signaling factors. The hypothalamus is considered one of the central regulatory regions in this regard and responds directly to peripheral signals as well as to inputs from hindbrain noradrenergic nuclei (A1 and A2) (15). Within the hypothalamus, the arcuate nucleus (ARC), the paraventricular nucleus (PVN), and the ventromedial nucleus (VMH) are involved in energy homeostasis (16, 17). The ARC contains orexigenic neuron populations, including neuropeptide Y (NPY)/agouti-related protein (AgRP) expressing neurons, and anorexigenic neuron populations, including proopiomelanocortin (POMC) expressing neurons (16, 17). The PVN contains both corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) expressing

neurons. Elevations in CRH and TRH levels have been shown to decrease food intake and reduce body weight (18, 19). Among the circulating peripheral modulators of energy homeostasis are a number of peptide hormones whose receptors can be found in these hypothalamic nuclei. One example is ghrelin, which is a gastrointestinal peptide that stimulates feeding and promotes positive energy balance (16). Ghrelin exerts its orexigenic response by stimulating NPY/AgRP neurons and simultaneously inhibiting POMC neurons (17). Alternatively, the adipokine leptin functions to decrease food intake by inhibiting NPY/AgRP while stimulating POMC neurons (17). While the actions of these peripheral and central signals are well-defined in non-obese and lean animals, the role of these signals are diminished or attenuated in states of excess weight gain and obesity.

Obesity results in distinct neural and metabolic alterations that support overconsumption and weight gain (20-22). For instance, leptin and insulin resistance, dysregulation of hypothalamic neuropeptides, and reduced satiety signals are some of the broad physiological impairments that accompany diet-induced obesity (20, 23-26). As such, physiological changes that result in lower body weight in non-obese or lean phenotypes do not accurately represent the mechanisms of weight loss in obesity. Despite the wealth of animal studies examining how IMF improves markers for aging (27, 28), cognitive performance (29-31), and immune responses (32, 33), there are no studies in obese animals to determine how IMF promotes weight loss. An understanding of the neural and metabolic alterations that promote weight loss by IMF in obese animals can provide greater insight into developing research-based modifications to IMF protocols to reduce hunger, increase long-term compliance, and enhance maintenance of weight loss.

The goal of this study was to examine the central and peripheral changes in response to IMF in a DIO model. C57 male mice at post-natal date (PND) 49 were fed a high-fat diet (HFD; 45% fat) *ad libitum* for 8 weeks. Following this 8-week period, mice were either maintained on *ad libitum* HFD, received IMF of HFD (IMF-HFD), switched to *ad libitum* low-fat diet (LFD; 10% fat), or received IMF of LFD (IMF-LFD). While other DIO protocols have used an extended period of high-fat feeding ($\geq$ 12 weeks) (34), the rationale for the 8 week initial high fat diet feeding study was to model the target

population of overweight and obese individuals that have reported the most beneficial weight loss with intermittent fasting protocols. We hypothesized that, despite being on a high fat diet, mice fed IMF-HFD would display improved glucose metabolism, enhanced metabolic profiles, and distinct monoamine signaling in the hypothalamus comparably to LFD and IMF-LFD groups.

Materials and Methods

Animals

Male C57BL/6 mice (n=64) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). At PND 49, all were fed an *ad libitum*, high fat diet (HFD; 4.73 kcal/g, 45% fat, 20% protein, 35% carbohydrate; D12451) for 8 weeks. Mice were then equally divided by bodyweight and transitioned to one of four experimental groups as follows: *ad libitum* HFD, IMF of HFD (IMF-HFD), *ad libitum* low fat diet (LFD; 3.85 kcal/g, 10% fat, 20% protein, 70% carbohydrate; D12450B), or IMF of LFD (IMF-LFD). All diets were obtained from Research Diets (New Brunswick, NJ). IMF mice were food deprived every other 24-hour period beginning at 9:00 AM (fasting day), 2 hours into the light cycle. On fasting days, all animals were weighed, food intake was recorded, and cages were changed. Mice were pair housed and maintained on a 12-hour light/dark cycle; lights on from 0700HR to 1900HR. All procedures were approved by the Institutional Animal Care and Use Committee of Rutgers University.

Body Composition and RER

Body composition was assessed using the EchoMRI 3-in-1 Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA) in all mice. The Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH, USA), an indirect calorimeter, was used to measure v.O2, v.CO2, and respiratory exchange ratio (RER; v.CO2/v.O2). Mice were maintained on their respective feeding protocols and housed in the system for 48 hours, beginning on a fast day for IMF-HFD and IMF-LFD mice. The second 24-hour epoch (feeding day for IMF mice) was used for analysis.

Oral Glucose and Insulin Tolerance Tests

An oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT) were performed on all groups. For IMF-HFD and IMF-LFD animals, these were performed on fasting days and food was not replaced after testing. Six hours prior to the OGTT, all mice were placed in clean cages, weighed, and food deprived. At the start of the test, mice were placed in Plexiglas restrainers and a tail nick was performed to obtain a baseline glucose reading using a glucometer (AlphaTRAK 2). Immediately thereafter, mice were gavaged with a bolus of glucose (2.0 g/kg body weight) and placed in an individual clean cage without food and water. Blood samples were collected from the tail in their individual cages at 15, 30, 60, 90, 120, and 180 min post-gavage. After 180 min, all mice were returned to their home cages, water was replaced, and food was returned to HFD and LFD animals. After sufficient recovery (2-3 days), an ITT was performed after a fast in a similar manner as the OGTT with an intraperitoneal (i.p.) injection of insulin (0.75 units/kg). Blood samples were collected from the tail in their individual cages at 15, 30, 60, 90, and 120 min post-injection.

Plasma Hormones

After a 5-hour fast (fast day for IMF-HFD and IMF-LFD), animals were euthanized by decapitation. Blood was collected; a protease inhibitor, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), at 1mg/mL was added to each sample, and samples were maintained on ice until centrifugation at 3,000 rpm for 10 min at 4°C. Plasma was stored at -80°C until analysis. Insulin, ghrelin (active), and leptin were determined by multiplex assay (EMD Millipore). A radioimmunoassay was performed to determine plasma corticosterone (sensitivity: 25 ng/ml; MP Biomedicals, Santa Ana, CA, USA) levels.

Biogenic Amines

Brain samples were dissected from the anterior (containing the anterior hypothalamus and the paraventricular hypothalamus) and posterior portions of the medial hypothalamus (containing the arcuate

nucleus and the ventromedial hypothalamus). Biogenic amines for each brain section were extracted and analyzed as previously described by reverse-phase HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific, Sunnyvale, CA, USA) with electrochemical detection (Coulochem III, Thermo Fisher Scientific) (35). An acetonitrile-based phosphate buffer mobile phase (MD-TM; Thermo Fisher Scientific) was used for all experiments. The internal standard, 3,4-dihydroxybenzylamine (DHBA), was added to all samples prior to extraction. Quantification of norepinephrine (NE), epinephrine (EPI), dopamine (DA), and serotonin (5-HT), plus metabolites homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), was determined by Chromeleon 7.1 software (Thermofisher). Values were expressed as picograms (pg) divided by wet tissue weight (milligrams) of each sample.

Tissue dissections for qPCR

Hypothalamic nuclei were micro-dissected for RNA extraction and gene expression analysis. The PVN, ARC, and VMH were cut into 1 mm coronal slices using a brain matrix (Ted Pella, Inc., Redding, CA, USA), anterior (Bregma: -0.70 to -1.34mm) and posterior (Bregma: -1.35 to -1.94mm) (36). The brain blocks were transferred to RNAlater (Life Technologies, Inc., Grand Island, NE, USA) and stored overnight at 4°C. Samples were dissected from slices using a dissecting microscope. Dissected tissue was stored at -80°C. Total RNA was extracted from the PVN, VMH, and ARC using Ambion RNAqueous-Micro Kits (Life Technologies, Inc). Total RNA was also DNase I-treated, using the extraction kits, at 37°C for 30 min to minimize any genomic DNA contamination. RNA quantity and quality were determined using a NanoDrop ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA).

Quantitative real-time PCR

cDNA was synthesized from 200 ng of total RNA using Superscript III reverse transcriptase (Life Technologies, Inc.), 4 µL 5X Buffer, 25 mM $MgCl_2$, 10 mM dNTP (Clontech Laboratories, Inc., Mountain View, CA, USA), 100 ng random hexamer primers (Promega Corporation, Madison, WI, USA), 40 U/µL Rnasin (Promega), and 100 mM DTT in DEPC-treated water (Gene Mate, Bioexpress,

Inc., Kaysville, UT, USA) in total volume of 20 µL. Reverse transcription was conducted using the following protocol: 5 min at 25°C, 60 min at 50°C, 15 min at 70°C. The cDNA was diluted to 1:20 with Nuclease-free water (Gene Mate, Bioexpress) for a final cDNA concentration of 0.5 ng/µL and stored at -20°C. Basal hypothalamus (BH) test tissue RNA was used for positive and negative controls (no reverse transcriptase) and processed simultaneously with the experimental samples.

All primers were designed to span exon–exon junctions and synthesized by Life Technologies using Clone Manager 5 software (Sci Ed Software, Cary, NC, USA). See Table 1 for a listing of all the primer sets used for quantitative real-time PCR (qPCR). For qPCR, 4 µL of cDNA template (an equivalent of 2 ng total RNA) was amplified using Sso Advanced SYBR Green (BioRad, Inc., Hercules, CA, USA) on CFX-Connect Real-time PCR instrument (BioRad). Standard curves for each primer pair were prepared using serial dilutions of BH cDNA in duplicate to determine the efficiency [$E = 10^{(-1/m)} - 1$, m = slope] of each primer pair. All efficiencies expressed as percent efficiency were approximately equal (one doubling per cycle, 90–100%). The relative mRNA expression data was analyzed using the ΔΔCT method (37, 38). The amplification protocol for all the genes was as follows: initial denaturing at 95°C for 3 min followed by 40 cycles of amplification at 94°C for 10s (denaturing), 60°C for 45s (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95°C for 10s, 65°C to 95°C (in increments of 0.5°C) for 5s and 95°C for 5s. The reference genes used were *Actb* and *Gapdh*. Positive and negative controls were added to each amplification run, which included a water blank. Quantification values were generated only from samples showing a single product at the expected melting point.

Final relative quantitation was done using the comparative CT method (37, 38). The data were reported as relative mRNA expression. To determine the CT for each transcript, the threshold was consistently set at the lowest point of the exponential curve where the slope of the curve was the steepest for all plates. The relative linear quantity of target molecules was calculated using the formula $2^{-\Delta\Delta CT}$. All gene expression data were expressed as an n-fold difference relative to the HFD group.

Statistical Analyses

The data are presented as mean ± SEM. A two-way ANOVA or two-way ANOVA with repeated measures was performed to determine schedule, diet, and diet X schedule effects. An ANCOVA with body weight as a covariate was also performed on the RER measurements (39). A Newman-Keuls post-hoc was performed unless otherwise specified. All statistical analyses were performed using Statistica 7.1 software (StatSoft, Tulsa, OK, USA) and significance was set at $\alpha = 0.05$.

Results

IMF Feeding Reduces Body Weight, Fat Mass, and Caloric Intake Comparably to a Low Fat Diet

For all groups (HFD, LFD, IMF-HFD, and IMF-LFD), we measured bodyweight, food intake, and body composition. For bodyweight, there were significant effects of diet [$F(1, 28)=13.2$, $p<0.01$], schedule [$F(1, 28)=11.2$, $p<0.01$], and time X diet X schedule [$F(13, 364)=2.4$, $p<0.01$]. As demonstrated in Figure 1A, at PND133 (4 weeks following diet interventions) the IMF-HFD and IMF-LFD groups had lower ($p<0.05$) body weights than did the HFD group. Total caloric intake was measured over the course of the experimental period. For cumulative caloric intake, there were significant effects of diet [$F(1, 12)=24.5$, $p<0.001$], schedule [$F(1, 12)=13.0$, $p<0.01$], time [$F(13, 156)=1528.4$, $p<0.001$], and time X schedule [$F(13, 156)=3.5$, $p<0.001$]. At the end of the 4 weeks, all groups had lower cumulative intakes than the HFD group ($p<0.001$), Figure 1B.

For fat mass (g), there were significant effects of diet [$F(1, 28)=18.9$, $p<0.001$], schedule [$F(1, 28)=6.8$, $p<0.05$], and diet X schedule [$F(1, 28)=13.1$, $p<0.01$]. All groups had lower fat mass than HFD group ($p<0.001$), Figure 2A. For lean mass, there was only an effect of diet [$F(1, 28)=7.8$, $p<0.01$], whereas the IMF-LFD had higher lean mass than the IMF-HFD and HFD groups ($p<0.05$ for both), Figure 2B.

IMF Increases RER

For v.CO_2, there were significant effects of diet [$F(1, 28)=11.9$, $p<0.01$] and diet X schedule [$F(1, 28)=25.6$, $p<0.001$]. Both LFD and IMF-LFD groups had higher v.CO_2 than the HFD group ($p<0.01$ and $p<0.05$, respectively), Figure 3A. For v.O_2, there were significant effects of diet [$F(1, 28)=5.5$, $p<0.05$], schedule [$F(1, 28)=9.3$, $p<0.01$], and diet X schedule [$F(1, 28)=26.6$, $p<0.001$]. Additionally, v.O_2 was lower in the IMF-LFD group compared with all other groups ($p<0.001$), Figure 3B. Respiratory exchange ratio (RER) analysis indicated a diet effect [$F(1, 28)=243.4$, $p<0.001$] and a schedule effect [$F(1, 28)=45.1$, $p<0.001$]. RER was elevated in the IMF-HFD group relative to HFD ($p<0.001$) and lower relative to LFD ($p<0.05$), whereas the IMF-LFD group was elevated in respect to all other groups ($p<0.001$), Figure 3C. Because body weight can influence energy metabolism, RER was analyzed by ANCOVA with body weight as a covariate. Accounting for body weight, there was a diet effect [$F(1, 27) = 209$, $p< 0.0001$] and a schedule effect [$F(1, 27) = 39.7$, $p< 0.0001$]. All groups were different from HFD ($p < 0.05$). RER was plotted as a function of body weight to illustrate the effect of diet and schedule, Figure 3D.

Glucose and Insulin Tolerances are Altered by an IMF Schedule of Feeding

Glucose tolerance was determined over 180 minutes following an oral bolus of glucose. For glucose tolerance, there were significant effects of diet [$F(1, 28)=40.1$, $p<0.001$], schedule [$F(1, 28)=47.2$, $p<0.001$], and time X diet X schedule [$F(6, 168)=5.3$, $p<0.001$]. At 15 min, all groups had lower blood glucose levels compared with the HFD ($p<0.001$). The IMF-LFD was also lower than IMF-HFD and LFD ($p<0.001$). At 30 and 60 min, the IMF-LFD group maintained lower blood glucose than all other groups ($p<0.001$ and $p<0.05$ for all, respectively). At 90 min, IMF-LFD group had lower blood glucose levels than the HFD group only ($p<0.05$), Figure 4A. AUC analysis showed an overall reduction in oral glucose tolerance in the IMF-LFD mice compared to all other groups ($p<0.001$), Figure 4B.

Insulin tolerance was measured after an i.p. injection of insulin over 120 minutes. For insulin tolerance, there were significant effects of diet [$F(1, 28)=27.3$, $p<0.001$], diet X schedule [$F(1, 28)=9.9$, $p<0.01$], and time X diet X schedule [$F(5, 140)=10.0$, $p<0.001$]. IMF-LFD group had lower baseline

glucose than all other groups ($p<0.01$), but for 60, 90, and 120 min the IMF-LFD was elevated compared with LFD group ($p<0.05$). For all time points, except for baseline and 15 min, the LFD was lower than the HFD group ($p<0.05$). In addition, the LFD group also had lower blood glucose than the IMF-HFD group at 60, 90, and 120 min ($p<0.05$ for all), Figure 4C. AUC analysis showed a reduction as a consequence of the intermittent schedule; IMF-HFD and IMF-LFD were lower than HFD and LFD groups, respectively ($p< 0.05$ for both). Also, the LFD group had lower AUC than the HFD group ($p<0.05$), Figure 4D.

IMF and HFD influence terminal plasma levels of Insulin and Leptin but Not Ghrelin or Corticosterone.

Plasma levels of hormones were assessed by multiplex assay. For plasma insulin levels there was an effect of diet [$F(1, 28)=5.8$, $p<0.05$]. Insulin was significantly lower in all groups compared with the HFD group ($p<0.05$), Figure 5A. Likewise, for leptin concentrations, there were effects of diet [$F(1, 28)=25.9$, $p<0.001$], schedule [$F(1, 28)=7.6$, $p<0.05$], and diet X schedule [$F(1, 28)=11.531$, $p<0.01$]. Plasma leptin were lower in all groups compared with the HFD group ($p<0.001$), Figure 5B. There were no effects of diet, schedule, or diet X schedule on terminal plasma ghrelin, Figure 5C. Similarly, we did not observe any effects on terminal corticosterone (data not shown), suggesting that the IMF protocols did not induce a stress response.

Medial Hypothalamic Norepinephrine and Dopamine Increase in Response to IMF

Biogenic amines were measured in the anterior and posterior medial hypothalamus by HPLC. These regions are inclusive of the PVN and ARC/VMH, respectively. For NE content in the anterior medial hypothalamus, there were effects of diet [$F(1, 27)=5.4$, $p<0.05$] and schedule [$F(1, 27)=26.0$, $p<0.001$]. There was an elevation as a consequence of the intermittent schedule. The IMF-HFD and IMF-LFD groups were higher than HFD and LFD groups, respectively ($p<0.05$ for both), Figure 6A (left). In the posterior medial hypothalamus, there were effects of schedule [$F(1, 27)=15.1$, $p<0.001$] and diet X schedule [$F(1, 27)=4.9$, $p<0.05$]. NE was increased in the IMF-LFD compared with all other groups ($p < 0.05$), Figure 6A (right). For DA content in the anterior medial hypothalamus, there were effects of diet

[F(1, 26)=18.6, p<0.001], schedule [F(1, 26)=22.1, p<0.001], and diet X schedule [F(1, 26)=4.7, p<0.05]. DA concentrations were significantly higher in the anterior medial hypothalamus of IMF-LFD animals than all other groups (p<0.001), Figure 6B (left). For 5-HT, 5-HIAA, and HVA there were no effects of diet or schedule in either hypothalamic region, Figure 6C-E.

NPY and POMC mRNA expression in the ARC and Adrenergic Receptor in the PVN of the Hypothalamus Respond to an Intermittent Schedule of Feeding.

Gene expression in the ARC, PVN, and VMH was measured by qPCR. For ARC *Npy* expression, there was an effect of schedule [F(1, 26)=21.7, p<0.001]. There was an elevation as a consequence of the intermittent schedule. The IMF-HFD and IMF-LFD groups had significantly greater *Npy* expression than both the HFD and LFD groups (p<0.05 for all), Table 2. For ARC *Pomc* expression, there was a diet effect [F(1, 27)=14.8, p<0.001] and schedule effect [F(1, 27)=8.3, p<0.01]. *Pomc* expression was lower in all groups compared with the HFD group (p<0.05), Table 2. For ARC growth hormone secretagogue receptor (GHSR) gene expression, there was an effect of schedule [F(1, 27)=9.4, p<0.01]. The IMF-HFD and IMF-LFD groups demonstrated higher levels of *Ghsr* gene expression than the HFD group (p<0.05), Table 2. Conversely, there were no significant differences in ARC expression of *Agrp*, glucagon-like peptide 1 receptor (*Glp1r*), or the adrenergic receptors, *Adra1a*, *Adra1b*, or *Adra2c*, Table 2. In the PVN, there was an effect of schedule on *Adra1a* [F(1, 26)=8.3, p<0.01] and *Adra1b* [F(1, 26)=5.1, p<0.05], Table 3. For expression of *Adra1a*, the IMF-HFD and LFD were significantly lower than the HFD group (p<0.05). For expression of *Adra1b*, the LFD had lower levels than the HFD group (p<0.05), Table 3. Gene expressions of PVN *Adra2c*, *Crh*, *Trh,* and *Glp1r* were not significantly different between groups, Table 3. In the VMH, expressions of *Glp1r*, *Adra1a*, *Adra1b*, *Adra2b*, and *Adra2c* showed no significant effects of either diet or schedule of the diet, Table 4.

Discussion

Several clinical studies have indicated that intermittent fasting is an effective weight loss treatment for some obese and overweight populations (7, 9, 11, 40). However, there have not been any preclinical studies examining the effects of this diet strategy in animal models of obesity. The intermittent fasting protocol used in our study was an alternate day fasting regimen with repeated 24-hour intervals of food deprivation followed by 24-hour *ad libitum* food access. Our study sought to further understand the neural and metabolic consequences of an intermittent fasting protocol in adult male DIO mice. In particular, our study promoted an obese phenotype by exposing mice to *ad libitum* high-fat feeding for 8 weeks before beginning the intermittent fasting protocol or low fat/low calorie diet switch (LFD). One group of mice was maintained on the high-fat diet throughout the study (HFD; 12 weeks total), which was the control group in these experiments. Indeed, most intermittent fasting protocols in humans have been validated in overweight, class I obese (BMI $\leq$ 34.9), or class II obese (BMI $\leq$ 39.9) individuals (7-10, 40). However, most of the subjects in these studies were either overweight or class I obese (40).

In order to uncover the neural and metabolic changes that promote weight loss by intermittent fasting, our measurements were taken after significant body weight loss was achieved. This was achieved at the 4 week time point in the present set of experiments. At 4 weeks, body weights were significantly lower in IMF-HFD (~13 % reduction) and IMF-LFD (~18% reduction) groups compared with the HFD group. It is important to note that all three groups (IMF-HFD, LFD, and IMF-LFD) consumed statistically similar cumulative caloric intakes over the 4-week period (~15-20% reduction compared with the HFD group). While the study did not have a pair-fed control groups, there was complete overlap in cumulative caloric intakes between the IMF-HFD and LFD diet groups (i.e., calorie-matched). As a result, there was a reduction in fat mass in all groups compared with the HFD group. This was also reflected in reduced terminal plasma leptin levels by approximately 65% in all groups compared with the HFD group. Thus, it appears that IMF of a HFD is similarly effective at reducing caloric intake and, therefore, fat accumulation, as a low fat/low calorie diet.

One interesting finding in our study was that the IMF-LFD had higher lean mass than the HFD and IMF-HFD groups. While the cause for this increase in lean mass is unknown, retention of lean mass

has been reported in humans undergoing a modified intermittent fasting protocol for 7 weeks (6). In a study by Klempel and colleagues (6), overweight or obese subjects (BMI 30-39.9; n= 32 completers) were randomly assigned to receive a high-fat diet (45% fat) or lower fat diet (25% fat). To reduce feelings of hunger, subjects were able to consume 25% of their energy needs on fast days. At the completion of the study, subjects in either the high-fat or low-fat intermittent fasting regimen lost weight from baseline (~ 4.5%), but there was no difference in amount of weight loss or body composition between diets (6). The retention (or elevation in lean mass) is in contrast to the findings observed by Chausse and colleagues (2014) in non-obese male Sprague Dawley rats exposed to an alternate fasting protocol for 3 weeks (41). In that study, intermittent fasting produced a reduction in epididymal fat mass, it also resulted in a reduction of soleus and plantaris muscle mass compared with *ad libitum* feeding of the same AIN-93 diet (13.8% protein, 76% carbohydrates, 10.2 % fat).

Chausse and colleagues (2014) also found similar relative RER levels between *ad libitium* and intermittent fasting (on fed days) rats (41), whereas our findings indicate an increase in RER on fed days with intermittent fasting with respect to diet (i.e., IMF-HFD was increased relative to HFD and IMF-LFD was increased relative LFD). Besides the difference in rodent species and diets, one prominent difference in the design of our study and that of Chausse et al., (2014) was that our mice were placed on high-fat diet for 8 weeks prior to the intermittent fasting regimen. Future studies will be designed to determine if the metabolic changes associated with DIO promote the retention of lean mass during an intermittent fasting regimen.

Another major finding of the present study was that oral glucose tolerance was slightly improved with an intermittent fasting of high-fat diet. Although there was not a difference in AUC, 15 min following the oral glucose load, blood glucose levels were lower in the IMF-HFD and LFD groups. Possibly related to the retention in lean mass, the IMF-LFD had lower glucose levels, over time and when expressed as AUC, following the oral glucose load. Improvements with intermittent fasting and a LFD were also observed in the insulin tolerance tests. Notably, terminal plasma insulin levels were reduced by approximately 45% in all groups compared with the obese HFD group. Improvements in insulin levels

have been noted with long-term intermittent fasting protocols in overweight and obese women (5). In a 24-week study, overweight or obese women (30 ± 5 kg/m2 BMI) were randomly divided into either a continuous calorie restriction of 25% or an IMF protocol of 2 consecutive fasting days (75% calorie restriction) per week. At 6 months, weight reduction was 7% in both groups, but the IMF subjects had significantly improved fasting insulin (5.2 vs. 6.3 μU/ml) and lower HOMA scores (1.1 vs. 1.3 μU/mmd/L) than continuously calorie restricted subjects (5). Reductions in plasma levels of insulin levels also have been noted with intermittent fasting protocols in non-obese rodents (27-29, 33, 42). Therefore, combination of intermittent fasting regimen with a lower fat diet could be beneficial for the glucose homeostatic impairments associated with obesity. Because we did not measure free fatty acids, lipoproteins, or markers for fat oxidation, it is unclear how an intermittent fasting regimen with a lower fat diet improves obesity-related impairments associated with obesity.

The increase in hypothalamic norepinephrine (NE) content as a consequence of the intermittent fasting schedule is another major finding of our study. Hypothalamic NE content was measured in the anterior section of the medial hypothalamus, inclusive of the paraventricular nucleus (PVN). In the PVN, there was a decrease in the gene expression of $\alpha 1_A$ receptor in the IMF-HFD and LFD groups, whereas the expression of $\alpha 1_B$ receptor was decreased in the LFD group compared with the HFD group. In the posterior section of the hypothalamus, inclusive of the ARC and VMH, NE content was elevated in the IMF-LFD group compared with all other groups. A predominant source of NE for hypothalamic nuclei are from a group of neurons located in caudal hindbrain (mostly the A2 cell group) (43). Hypothalamic NE levels have been associated with the neuroendocrine response to stress and reproductive behaviors (15). With respect to feeding behavior, elevated hypothalamic NE is critically involved in the hyperphagia that accompanies conditions of acute glucoprivation (44). In addition, orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons in the arcuate nucleus (ARC) of the hypothalamus, which project to the PVN, are activated and NPY/ AGRP gene expression levels are increased following glucoprivation with the non-metabolized glucose analog, 2-deoxyglucose (2-DG) (45, 46). Using immunotoxic lesions of the NE and epinephrine (E) neurons (by using saporin-conjugated to antibody for

dopamine β-hydroxylase) that project to ARC, Fraley and Ritter (47) demonstrated the critical role of NE/E neurons to glucoprivic response. In particular, Fraley and Ritter found that immunotoxin lesions of NE/E projecting neurons in the medial hypothalamus abolished the hyperphagic response to 2DG. In addition, they demonstrated NPY and AgRP basal levels were elevated, but did not alter expression levels, in response to 2DG following an immunotoxin lesion of NE/E (47). Long-term bouts (14 days) of glucoprivation in intact animals have demonstrated the feeding response is attenuated over time, but NPY levels in the ARC remain elevated at day 14 (48). For the reason that our measurement were taken at the 4 week time point during the initial phases of weight loss, one issue that needs examination is how *maintenance* of the weight loss achieved by intermittent fasting alters hypothalamic NE target regions. In addition, we need to determine whether elevated hypothalamic NE is directly involved with the glucose homeostasis improvements.

Findings from our study indicate that *Npy* relative mRNA levels in the ARC, similar to NE in the PVN, were elevated in response to an intermittent fasting protocol (regardless of diet) compared with HFD and LFD conditions. Although not reaching statistical significance, *Agrp* relative mRNA levels in the ARC demonstrated a similar trend as *Npy*. Interestingly, relative mRNA expression levels of the hypothalamic, anorexigenic precursor polypeptide POMC were elevated in all groups compared with HFD group. This is in agreement with previous findings that arcuate *Pomc* gene expression is reduced in animals fed a high fat diet (20) and those prone to diet-induced obesity (49). Our findings suggest that hypothalamic NE content and *Npy* mRNA are elevated after a 5 hour fast as a consequence of the 4-week exposure to an intermittent fasting regimen. Acute bouts of food deprivation have been shown to increase hypothalamic *Npy* mRNA expression (50, 51), an effect that may potentially be augmented by the repetitive nature of an intermittent fasting protocol. The role of NE in PVN and interactions with orexigenic peptide, NPY, are certainly mechanisms that require further attention. In addition, the elevation of *Npy* and NE as a consequence of the entrainment, not weight loss, of the intermittent feeding paradigm is another possible that needs further investigation. Because our measurements of NE content and *Npy* gene expression were performed on regions rather than distinct nuclei, future experiments will

use targeted more mechanistic approaches to uncover the role of the NE and NPY in the weight loss and glucose regulatory alterations that accompany intermittent fasting.

Another finding of our study was that dopamine (DA) content in the posterior region of the medial hypothalamus was increased in the IMF-LFD compared with the IMF-HFD and LFD groups. In a study by Martin and colleagues, regional monoamine content was measured after 6 month on different dietary feeding protocols, including intermittent fasting, in male and female non-obese rats (29). Although hypothalamic regions were not examined, there were differences in the catecholamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the cerebellum of male and female rats exposed to the intermittent fasting schedule (29). Female rats exposed to a 40% caloric restriction for 6 months had increased performance in behavioral cognitive task which was accompanied by a decrease in DA and increase in serotonin content in the hippocampus compared with *ad libitum* fed rats (29). Thus, the significance of the increase in DA in the posterior medial hypothalamus in DIO mice exposed to the IMF-LFD schedule is unclear. In addition, another limitation of our methods is that measurements of biogenic amine tissue content do not provide an accurate index of biogenic amine turnover or steady-state concentrations (52).

An obstacle for intermittent fasting regimens is the feelings of hunger that accompany prolonged bouts of food deprivation (14). Ghrelin is a gastrointestinal hormone that is elevated during period of fasting and associated with subjective feeding of hunger in non-obese adults (53). Fasting increases GHSR in the hypothalamus (54) while diet-induced obesity via a high-fat diet has been shown to induce ghrelin resistance in arcuate NPY/AgRP neurons (55). In addition, though the actions of peripheral ghrelin are thought to be primarily through the afferent vagal nerve to the hindbrain (56), there exist ghrelin-containing cells within the hypothalamus and specifically the ARC (57, 58). Interestingly, hypothalamic ghrelin release decreases in glucoprivic states, such as with fasting or 2DG administration, whereas the opposite effect is observed with peripheral ghrelin release (59). In our study, terminal ghrelin levels were not elevated, but the relative gene expression of the ghrelin receptor, *Ghsr*, was increased in the ARC of animals placed on the intermittent feeding schedules (i.e., IMF-HFD and IMF-LFD). Future

work is needed to understand the feed-forward increase in *Ghsr* gene expression, subjective feelings of hunger, and the weight loss associated with intermittent fasting protocols.

Because our study design used pair-housed mice, one limitation of our findings is that diet-specific calorie restricted groups were not included in our study. Information gathered from a restricted access to diet, but not exposed to prolonged periods of food deprivation, would provide insight into whether fasting has benefits over simple calorie restriction. As it stands, our studies do not resolve the ongoing debate as to whether intermittent fasting provides an added benefit beyond daily caloric restriction (4). Keeping in mind the limitation of extrapolating animal studies and fasting periods to clinical practice, these studies do provide a starting point for research–based human studies to examine the efficacy of long-term intermittent fasting regimens for weight loss in certain populations of overweight or obese individuals.

Acknowledgment.

Support by NJ06156 (USDA-NIFA) to NTB and R00DK083457, R00DK083457-S1, P30ED005022, and NJ06107 (USDA-NIFA) to TAR. Technical assistance with the feeding protocols was provided by Brandon Smith, Ami Patel, Thissa Thambugala, Lauren Palena, and Brittany Wilhite.

References

1. **Gudzune KA, Doshi RS, Mehta AK, Chaudhry ZW, Jacobs DK, Vakil RM, Lee CJ, Bleich SN, Clark JM** 2015 Efficacy of commercial weight-loss programs: an updated systematic review. Ann Intern Med 162:501-512
2. **Julia C, Peneau S, Andreeva VA, Mejean C, Fezeu L, Galan P, Hercberg S** 2014 Weight-loss strategies used by the general population: how are they perceived? PloS one 9:e97834
3. **McAllister EJ, Dhurandhar NV, Keith SW, Aronne LJ, Barger J, Baskin M, Benca RM, Biggio J, Boggiano MM, Eisenmann JC, Elobeid M, Fontaine KR, Gluckman P, Hanlon EC, Katzmarzyk P, Pietrobelli A, Redden DT, Ruden DM, Wang C, Waterland RA, Wright SM, Allison DB** 2009 Ten putative contributors to the obesity epidemic. Critical reviews in food science and nutrition 49:868-913
4. **Johnstone A** 2015 Fasting for weight loss: an effective strategy or latest dieting trend? Int J Obes (Lond) 39:727-733
5. **Harvie MN, Pegington M, Mattson MP, Frystyk J, Dillon B, Evans G, Cuzick J, Jebb SA, Martin B, Cutler RG, Son TG, Maudsley S, Carlson OD, Egan JM, Flyvbjerg A, Howell A** 2011 The effects of intermittent or continuous energy restriction on weight loss and metabolic disease risk markers: a randomized trial in young overweight women. Int J Obes (Lond) 35:714-727
6. **Klempel MC, Kroeger CM, Varady KA** 2013 Alternate day fasting (ADF) with a high-fat diet produces similar weight loss and cardio-protection as ADF with a low-fat diet. Metabolism: clinical and experimental 62:137-143
7. **Bhutani S, Klempel MC, Kroeger CM, Trepanowski JF, Varady KA** 2013 Alternate day fasting and endurance exercise combine to reduce body weight and favorably alter plasma lipids in obese humans. Obesity 21:1370-1379
8. **Hoddy KK, Kroeger CM, Trepanowski JF, Barnosky AR, Bhutani S, Varady KA** 2015 Safety of alternate day fasting and effect on disordered eating behaviors. Nutrition journal 14:44
9. **Klempel MC, Kroeger CM, Bhutani S, Trepanowski JF, Varady KA** 2012 Intermittent fasting combined with calorie restriction is effective for weight loss and cardio-protection in obese women. Nutrition journal 11:98
10. **Varady KA, Bhutani S, Church EC, Klempel MC** 2009 Short-term modified alternate-day fasting: a novel dietary strategy for weight loss and cardioprotection in obese adults. The American journal of clinical nutrition 90:1138-1143
11. **Varady KA, Bhutani S, Klempel MC, Kroeger CM, Trepanowski JF, Haus JM, Hoddy KK, Calvo Y** 2013 Alternate day fasting for weight loss in normal weight and overweight subjects: a randomized controlled trial. Nutrition journal 12:146
12. **Eshghinia S, Mohammadzadeh F** 2013 The effects of modified alternate-day fasting diet on weight loss and CAD risk factors in overweight and obese women. J Diabetes Metab Disord 12:4
13. **Johnson JB, Summer W, Cutler RG, Martin B, Hyun DH, Dixit VD, Pearson M, Nassar M, Telljohann R, Maudsley S, Carlson O, John S, Laub DR, Mattson MP** 2007 Alternate day calorie restriction improves clinical findings and reduces markers of oxidative stress and inflammation in overweight adults with moderate asthma. Free Radic Biol Med 42:665-674
14. **Heilbronn LK, Smith SR, Martin CK, Anton SD, Ravussin E** 2005 Alternate-day fasting in nonobese subjects: effects on body weight, body composition, and energy metabolism. The American journal of clinical nutrition 81:69-73
15. **Itoi K, Sugimoto N** 2010 The brainstem noradrenergic systems in stress, anxiety and depression. J Neuroendocrinol 22:355-361
16. **Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW** 2006 Central nervous system control of food intake and body weight. Nature 443:289-295
17. **Sobrino Crespo C, Perianes Cachero A, Puebla Jimenez L, Barrios V, Arilla Ferreiro E** 2014 Peptides and food intake. Frontiers in endocrinology 5:58

18. **Choi YH, Hartzell D, Azain MJ, Baile CA** 2002 TRH decreases food intake and increases water intake and body temperature in rats. Physiol Behav 77:1-4
19. **Krahn DD, Gosnell BA, Levine AS, Morley JE** 1988 Behavioral effects of corticotropin-releasing factor: localization and characterization of central effects. Brain Res 443:63-69
20. **Lin S, Storlien LH, Huang XF** 2000 Leptin receptor, NPY, POMC mRNA expression in the diet-induced obese mouse brain. Brain Res 875:89-95
21. **Ma Y, Bertone ER, Stanek EJ, 3rd, Reed GW, Hebert JR, Cohen NL, Merriam PA, Ockene IS** 2003 Association between eating patterns and obesity in a free-living US adult population. Am J Epidemiol 158:85-92
22. **Molnar D, Jeges S, Erhardt E, Schutz Y** 1995 Measured and predicted resting metabolic rate in obese and nonobese adolescents. J Pediatr 127:571-577
23. **Inui A, Asakawa A, Bowers CY, Mantovani G, Laviano A, Meguid MM, Fujimiya M** 2004 Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 18:439-456
24. **Moran TH** 2006 Gut peptide signaling in the controls of food intake. Obesity 14 Suppl 5:250S-253S
25. **Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ, Baskin DG** 2000 Central nervous system control of food intake. Nature 404:661-671
26. **Williams KW, Elmquist JK** 2012 From neuroanatomy to behavior: central integration of peripheral signals regulating feeding behavior. Nat Neurosci 15:1350-1355
27. **Anson RM, Guo Z, de Cabo R, Iyun T, Rios M, Hagepanos A, Ingram DK, Lane MA, Mattson MP** 2003 Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. Proceedings of the National Academy of Sciences of the United States of America 100:6216-6220
28. **Wan R, Camandola S, Mattson MP** 2003 Intermittent food deprivation improves cardiovascular and neuroendocrine responses to stress in rats. The Journal of nutrition 133:1921-1929
29. **Martin B, Pearson M, Kebejian L, Golden E, Keselman A, Bender M, Carlson O, Egan J, Ladenheim B, Cadet JL, Becker KG, Wood W, Duffy K, Vinayakumar P, Maudsley S, Mattson MP** 2007 Sex-dependent metabolic, neuroendocrine, and cognitive responses to dietary energy restriction and excess. Endocrinology 148:4318-4333
30. **Vasconcelos AR, Yshii LM, Viel TA, Buck HS, Mattson MP, Scavone C, Kawamoto EM** 2014 Intermittent fasting attenuates lipopolysaccharide-induced neuroinflammation and memory impairment. Journal of neuroinflammation 11:85
31. **Li L, Wang Z, Zuo Z** 2013 Chronic intermittent fasting improves cognitive functions and brain structures in mice. PloS one 8:e66069
32. **Varady KA, Roohk DJ, Bruss M, Hellerstein MK** 2009 Alternate-day fasting reduces global cell proliferation rates independently of dietary fat content in mice. Nutrition 25:486-491
33. **Wan R, Ahmet I, Brown M, Cheng A, Kamimura N, Talan M, Mattson MP** 2010 Cardioprotective effect of intermittent fasting is associated with an elevation of adiponectin levels in rats. The Journal of nutritional biochemistry 21:413-417
34. **Buettner R, Scholmerich J, Bollheimer LC** 2007 High-fat diets: modeling the metabolic disorders of human obesity in rodents. Obesity 15:798-808
35. **Verpeut JL, Walters AL, Bello NT** 2013 Citrus aurantium and Rhodiola rosea in combination reduce visceral white adipose tissue and increase hypothalamic norepinephrine in a rat model of diet-induced obesity. Nutrition research 33:503-512
36. **Paxinos G FS** 2008 The mouse brain in stereotaxic coordinates, compact, (3rd ed.): the coronal plates and diagrams. Amsterdam: Elsevier Academic Press
37. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408

38. **Pfaffl MW** 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research 29:e45
39. **Tschop MH, Speakman JR, Arch JR, Auwerx J, Bruning JC, Chan L, Eckel RH, Farese RV, Jr., Galgani JE, Hambly C, Herman MA, Horvath TL, Kahn BB, Kozma SC, Maratos-Flier E, Muller TD, Munzberg H, Pfluger PT, Plum L, Reitman ML, Rahmouni K, Shulman GI, Thomas G, Kahn CR, Ravussin E** 2012 A guide to analysis of mouse energy metabolism. Nat Methods 9:57-63
40. **Barnosky AR, Hoddy KK, Unterman TG, Varady KA** 2014 Intermittent fasting vs daily calorie restriction for type 2 diabetes prevention: a review of human findings. Translational research : the journal of laboratory and clinical medicine 164:302-311
41. **Chausse B, Solon C, Caldeira da Silva CC, Masselli Dos Reis IG, Manchado-Gobatto FB, Gobatto CA, Velloso LA, Kowaltowski AJ** 2014 Intermittent fasting induces hypothalamic modifications resulting in low feeding efficiency, low body mass and overeating. Endocrinology 155:2456-2466
42. **Martin B, Pearson M, Brenneman R, Golden E, Keselman A, Iyun T, Carlson OD, Egan JM, Becker KG, Wood W, 3rd, Prabhu V, de Cabo R, Maudsley S, Mattson MP** 2008 Conserved and differential effects of dietary energy intake on the hippocampal transcriptomes of females and males. PloS one 3:e2398
43. **Rinaman L** 2011 Hindbrain noradrenergic A2 neurons: diverse roles in autonomic, endocrine, cognitive, and behavioral functions. Am J Physiol Regul Integr Comp Physiol 300:R222-235
44. **Ritter S, Dinh TT, Li AJ** 2006 Hindbrain catecholamine neurons control multiple glucoregulatory responses. Physiol Behav 89:490-500
45. **Sergeyev V, Broberger C, Gorbatyuk O, Hokfelt T** 2000 Effect of 2-mercaptoacetate and 2-deoxy-D-glucose administration on the expression of NPY, AGRP, POMC, MCH and hypocretin/orexin in the rat hypothalamus. Neuroreport 11:117-121
46. **Fraley GS, Dinh TT, Ritter S** 2002 Immunotoxic catecholamine lesions attenuate 2DG-induced increase of AGRP mRNA. Peptides 23:1093-1099
47. **Fraley GS, Ritter S** 2003 Immunolesion of norepinephrine and epinephrine afferents to medial hypothalamus alters basal and 2-deoxy-D-glucose-induced neuropeptide Y and agouti gene-related protein messenger ribonucleic acid expression in the arcuate nucleus. Endocrinology 144:75-83
48. **Ozawa Y, Arima H, Watanabe M, Shimizu H, Ito Y, Banno R, Sugimura Y, Ozaki N, Nagasaki H, Oiso Y** 2011 Repeated glucoprivation delayed hyperphagic responses while activating neuropeptide Y neurons in rats. Peptides 32:763-769
49. **Betley JN, Xu S, Cao ZF, Gong R, Magnus CJ, Yu Y, Sternson SM** 2015 Neurons for hunger and thirst transmit a negative-valence teaching signal. Nature 521:180-185
50. **Hahn TM, Breininger JF, Baskin DG, Schwartz MW** 1998 Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. Nat Neurosci 1:271-272
51. **Palou M, Sanchez J, Rodriguez AM, Priego T, Pico C, Palou A** 2009 Induction of NPY/AgRP orexigenic peptide expression in rat hypothalamus is an early event in fasting: relationship with circulating leptin, insulin and glucose. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 23:115-124
52. **Estes KS, Simpkins JW** 1984 Age-related alteration in catecholamine activity within microdissected brain regions of ovariectomized Fischer 344 rats. J Neurosci Res 11:405-417
53. **Rahmouni K, Correia ML, Haynes WG, Mark AL** 2005 Obesity-associated hypertension: new insights into mechanisms. Hypertension 45:9-14
54. **Kim MS, Yoon CY, Park KH, Shin CS, Park KS, Kim SY, Cho BY, Lee HK** 2003 Changes in ghrelin and ghrelin receptor expression according to feeding status. Neuroreport 14:1317-1320
55. **Briggs DI, Enriori PJ, Lemus MB, Cowley MA, Andrews ZB** 2010 Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. Endocrinology 151:4745-4755

56. **Date Y, Shimbara T, Koda S, Toshinai K, Ida T, Murakami N, Miyazato M, Kokame K, Ishizuka Y, Ishida Y, Kageyama H, Shioda S, Kangawa K, Nakazato M** 2006 Peripheral ghrelin transmits orexigenic signals through the noradrenergic pathway from the hindbrain to the hypothalamus. Cell metabolism 4:323-331
57. **Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL** 2003 The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37:649-661
58. **Lu S, Guan JL, Wang QP, Uehara K, Yamada S, Goto N, Date Y, Nakazato M, Kojima M, Kangawa K, Shioda S** 2002 Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. Neuroscience letters 321:157-160
59. **Sato T, Fukue Y, Teranishi H, Yoshida Y, Kojima M** 2005 Molecular forms of hypothalamic ghrelin and its regulation by fasting and 2-deoxy-d-glucose administration. Endocrinology 146:2510-2516

Table 1. Primer sequences used for qPCR.

Gene Name	Product Length	Primer Sequence	Base Pair #	Accession #
Adra1a	187	F: TCTGCTGGCTGCCATTCTTC	1638-1657	NM_013461
		R: CACTGGATTCGCAGCACATTC	1805-1824	
Adra1b	84	F: CTTCATCGCTCTCCCACTTG	1174-1193	NM_007416
		R: TAGCCCAGCCAGAACACT	1240-1257	
Adra2b	130	F: GCAGAGGTCTCGGAGCTAA	905-923	NM_009633.3
		R: GCCTCTCCGACAGAAGATA	1016-1034	
Adra2c	154	F: CTCATGGCCTACTGGTACTTC	1657-1677	NM_007418.3
		R: TGCGCTTCAGGTTGTACTC	1792-1810	
Agrp	146	F: CTCCACTGAAGGGCATCAGAA	287–307	NM_007427.2
		R: ATCTAGCACCTCCGCCAAA	414–432	
Actb	63	F: GCCCTGAGGCTCTTTTCCA	849–867	NM_007393.3
		R: TAGTTTCATGGATGCCACAGGA	890-911	
Crh	86	F: AGGAGGCATCCTGAGAGAAGT	152-173	NM_205769.2
		R: CATGTTAGGGGCGCTCTC	906-923	
Gapdh	98	F: TGACGTGCCGCCTGGAGAAA	778–797	NM_008084.2
		R: AGTGTAGCCCAAGATGCCCTTCAG	852–875	
Ghsr	122	F: CAGGGACCAGAACCACAAAC	1003-1022	NM_177330
		R: AGCCAGGCTCGAAAGACT	1107-1124	
Glp1r	190	F: TTCAAGCTGTATCTGAGCATAG	806-827	NM_021332
		R: AGATGACACGGATGAAGATAAG	974-995	
Npy	182	F: ACTGACCCTCGCTCTATCTC	106–125	NM_023456
		R: TCTCAGGGCTGGATCTCTTG	268–287	
Pomc	200	F: GGAAGATGCCGAGATTCTGC	145–164	NM_008895
		R: TCCGTTGCCAGGAAACAC	327–344	
Trh	238	F: TTCGGCTTAACGTCTTC	150-170	NM_009426.3
		R: CTTCGTCGTAACTGGTATCC	369-387	

Table 2 Relative gene expression in the arcuate nucleus (ARC) of DIO mice after 4 weeks on a HFD, IMF-HFD, LFD, or IMF-LFD feeding schedule (n=8/group). All gene expression data were expressed as an *n*-fold difference relative to the mean of the HFD group. (*: $p<0.05$ from HFD; #: $p<0.05$ from LFD). Data are represented as means ± SEM.

Gene	HFD	IMF-HFD	LFD	IMF-LFD
Npy	0.936 ± 0.103	1.929 ± 0.269*,#	1.153 ± 0.112	2.035 ± 0.224 *,#
Pomc	1.016 ± 0.069	0.807 ± 0.063*	0.743 ± 0.055*	0.569 ± 0.073*
Agrp	1.058 ± 0.134	1.329 ± 0.189	1.004 ± 0.131	1.277 ± 0.163
Glp1r	1.060 ± 0.135	1.311 ± 0.191	1.014 ± 0.128	1.279 ± 0.167
Ghsr	1.022 ± 0.083	1.733 ± 0.171*	1.405 ± 0.204	1.744 ± 0.171*
Adra1a	1.055 ± 0.130	1.344 ± 0.199	1.010 ± 0.132	1.302 ± 0.171
Adra1b	1.058 ± 0.134	1.311 ± 0.197	1.004 ± 0.131	1.280 ± 0.168
Adra2c	1.032 ± 0.100	1.128 ± 0.237	0.945 ± 0.169	0.787 ± 0.099

NPY = neuropeptide Y; POMC = pro-opiomelanocortin; AgRP = agouti related peptide; GLP-1R = glucagon-like peptide 1 receptor; GHSR = growth hormone secretagogue receptor; Adra1A = alpha adrenergic receptor 1A; Adra1B = alpha adrenergic receptor 1B; Adra2C = alpha adrenergic receptor 2C

Table 3 Relative gene expression in the paraventricular nucleus (PVN) of DIO mice after 4 weeks on a HFD, IMF-HFD, LFD, or IMF-LFD feeding schedule. All gene expression data were expressed as an *n*-fold difference relative to the mean of the HFD group. (*: $p<0.05$ from HFD). Data are represented as means ± SEM.

Gene	HFD	IMF-HFD	LFD	IMF-LFD
Glp1r	0.933 ± 0.138	1.060 ± 0.080	0.806 ± 0.123	0.948 ± 0.090
Adra1a	1.013 ± 0.062	0.829 ± 0.035*	0.787 ± 0.039*	0.894 ± 0.054
Adra1b	1.066 ± 0.132	0.783 ± 0.044	0.726 ± 0.066*	0.836 ± 0.065
Adra2c	1.053 ± 0.110	0.803 ± 0.082	0.892 ± 0.082	0.827 ± 0.077
Crh	1.151 ± 0.163	0.842 ± 0.143	0.994 ± 0.127	1.101 ± 0.169
Trh	1.040 ± 0.111	1.121 ± 0.146	1.091 ± 0.144	1.400 ± 0.116

GLP-1R = glucagon-like peptide 1 receptor; Adra1A = alpha adrenergic receptor 1A; Adra1B = alpha adrenergic receptor 1B; Adra2C = alpha adrenergic receptor 2C; CRH = corticotropin releasing hormone; TRH = thyrotropin-releasing hormone

Table 4. Relative gene expression in the ventromedial medial hypothalamus nucleus (VMH) of DIO mice after 4 weeks on a HFD, IMF-HFD, LFD, or IMF-LFD feeding schedule. All gene expression data were expressed as an *n*-fold difference relative to the mean of the HFD group. Data are represented as means ± SEM.

Gene	HFD	IMF-HFD	LFD	IMF-LFD
Glp1r	1.043 ± 0.114	1.184 ± 0.100	0.930 ± 0.054	1.068 ± 0.101
Adra1a	1.003 ± 0.030	1.034 ± 0.067	0.949 ± 0.065	0.965 ± 0.052
Adra1b	1.036 ± 0.114	1.006 ± 0.066	1.031 ± 0.109	0.842 ± 0.065
Adra2c	1.018 ± 0.068	0.978 ± 0.069	1.174 ± 0.170	1.172 ± 0.165

GLP-1R = glucagon-like peptide 1 receptor; Adra1A = alpha adrenergic receptor 1A; Adra1B = alpha adrenergic receptor 1B; Adra2C = alpha adrenergic receptor 2C

Figure Legends

Figure 1. Male mice were placed on a high-fat diet (HFD; 45% Fat) to promote diet-induced obesity (DIO). After 8 weeks mice continued on the HFD (control group; n =8) were placed on an alternate day calorie deprivation intermittent fasting (IMF) protocol with HFD (IMF-HFD; n=8), switched to a control low-fat diet (LFD; 10% Fat; n=8) diet, or placed on placed on IMF protocol with LFD (IMF-LFD; n=8). Data are represented as means ± SEM. **A:** Body weights of mice at the end of 4 weeks of the diet intervention. **B:** Average cumulative food intake (Kcal) over 4 weeks. * indicates difference ($p<0.05$) from HFD, *** indicates difference from HFD ($p<0.001$).

Figure 2. Body composition as assessed by EchoMRI in all groups at the end of 4 weeks of the diet intervention. Data are represented as means ± SEM. **A**: Fat mass (g). **B:** Lean body mass (g). *** indicates difference from HFD ($p<0.001$); * indicates difference ($p<0.05$) from HFD; $ indicates difference ($p<0.05$) from IMF-HFD.

Figure 3. Respiratory exchange ratio (RER) measured by indirect calorimetry (24 h; fed day) in all groups at the end of 4 weeks of the diet intervention. Data are represented as means ± SEM. **A:** v.CO2. **B:** v.O2 **C:** RER. **D:** RER data as a function of body weight. * indicates difference ($p<0.05$) from HFD; **indicates difference ($p<0.01$) from HFD; # indicates difference ($p<0.05$) from LFD; ### indicates difference ($p<0.001$) from LFD; $$$ indicates difference ($p<0.001$) from IMF-HFD.

Figure 4. Oral glucose and insulin tolerance tests in all groups at the end of 4 weeks of the diet intervention. Data are represented as means ± SEM. **A:** Blood glucose (mg/dl) response to an oral bolus of glucose (2 g/kg) over 180 minutes. Values for IMF-HFD and LFD overlap. **B:** Area under the curve (AUC) of glucose tolerance test. **C:** Blood glucose response to intraperitoneal injection of insulin (0.75 U/kg) over 120 minutes. **D:** AUC of insulin tolerance test. * indicates difference ($p<0.05$) from HFD; **indicates difference ($p<0.01$) from HFD; ***indicates difference ($p<0.001$) from HFD; # indicates difference ($p<0.05$) from LFD; ### indicates difference ($p<0.001$) from LFD; ## indicates difference ($p<0.01$) from LFD; $ indicates difference ($p<0.05$) from IMF-HFD; $$ indicates difference ($p<0.01$) from IMF-HFD; $$$ indicates difference ($p<0.001$) from IMF-HFD.

Figure 5. Terminal levels of plasma hormones in all groups at the end of 4 weeks of the diet intervention. Data are represented as means ± SEM. **A**: Insulin **B:** Leptin **C:** Ghrelin. * indicates difference ($p<0.05$) from HFD; ***indicates difference ($p<0.001$) from HFD.

Figure 6. Biogenic amines were measured by HPLC in the anterior and posterior medial hypothalamus in all groups at the end of 4 weeks of the diet intervention (n=8 per group). **A:** Norepinephrine (NE). **B:** Dopamine **(**DA). **C:** Serotonin (5-hydroxytryptamine; 5-HT) **D:** 5-Hydroxyindoleacetic acid (5HIAA) **E:** Homovanillic acid (HVA) Data are represented as means ± SEM. * indicates difference ($p<0.05$) from HFD; # indicates difference ($p<0.05$) from LFD; $ indicates difference ($p<0.05$) from IMF-HFD.

Figure 1.

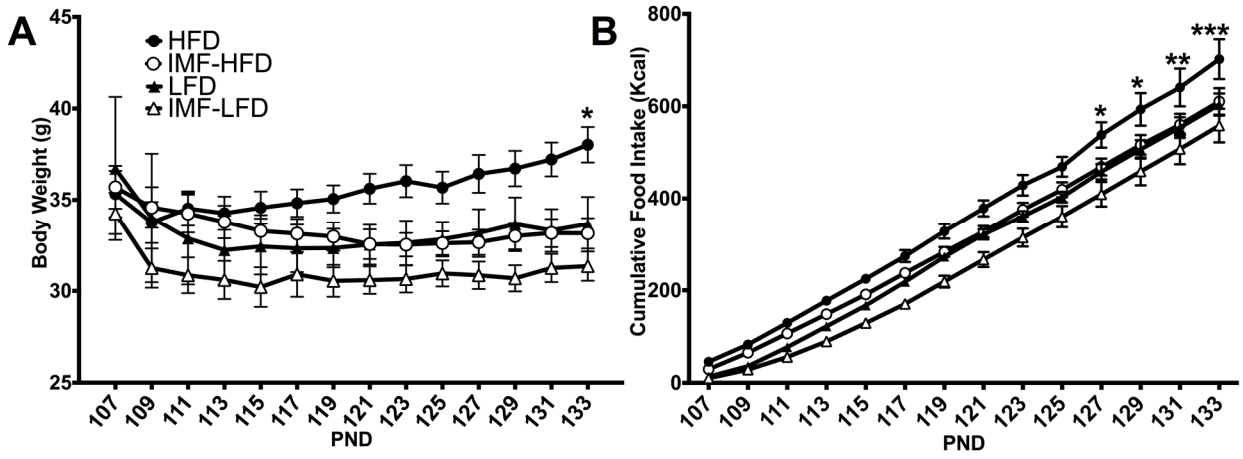

A
HFD
IMF-HFD
LFD
IMF-LFD
Body Weight (g)
45
40
35
30
25
107
109
111
113
115
117
119
121
123
125
127
129
131
133
PND
*
B
Cumulative Food Intake (Kcal)
800
600
400
200
0
*
*
**
***
PND

Figure 2.

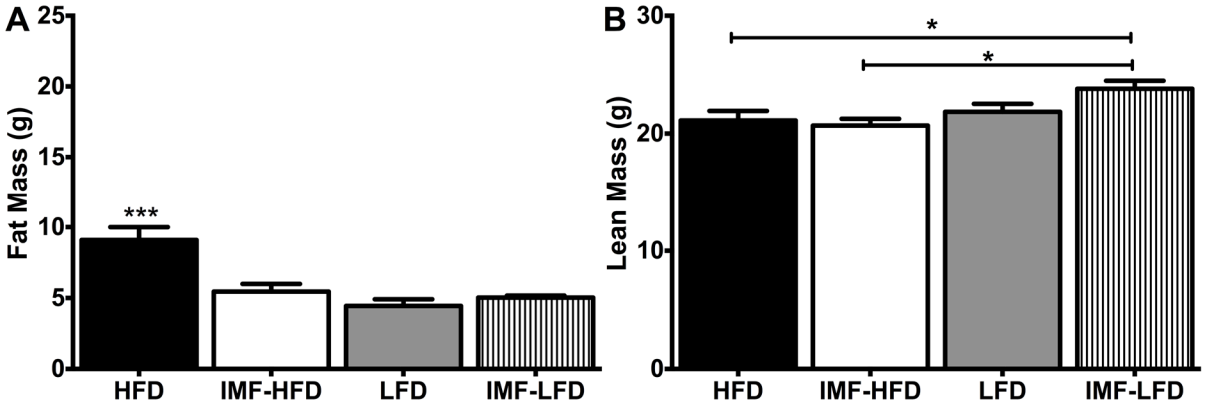

A
Fat Mass (g)
25
20
15
10
5
0
***
HFD
IMF-HFD
LFD
IMF-LFD
B
Lean Mass (g)
30
20
10
0
*
*
HFD
IMF-HFD
LFD
IMF-LFD

Figure 3.

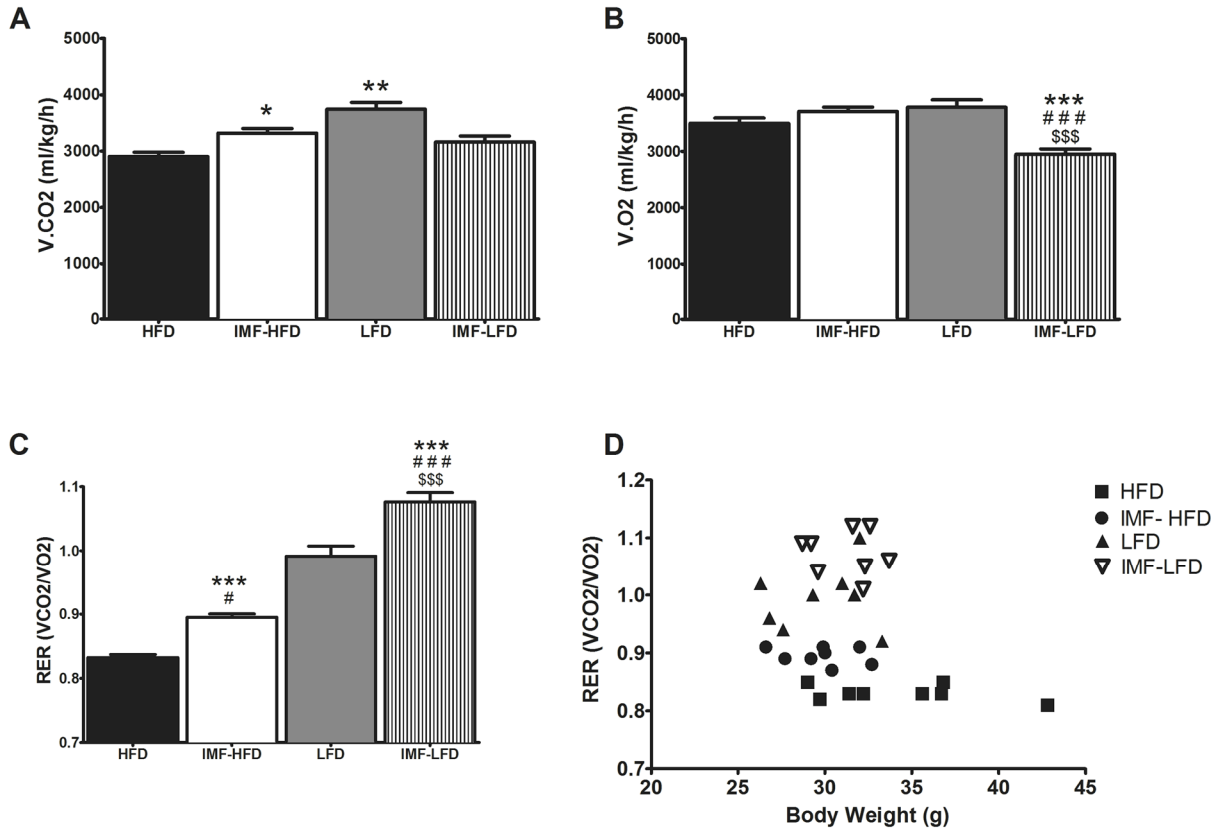

A
V.CO2 (ml/kg/h)
5000
4000
3000
2000
1000
0
*
**
HFD
IMF-HFD
LFD
IMF-LFD
B
V.O2 (ml/kg/h)
***
###
$$$
C
RER (VCO2/VO2)
1.1
1.0
0.9
0.8
0.7
***
#
D
RER (VCO2/VO2)
1.2
1.1
1.0
0.9
0.8
0.7
20
25
30
35
40
45
Body Weight (g)
HFD
IMF- HFD
LFD
IMF-LFD

Figure 4.

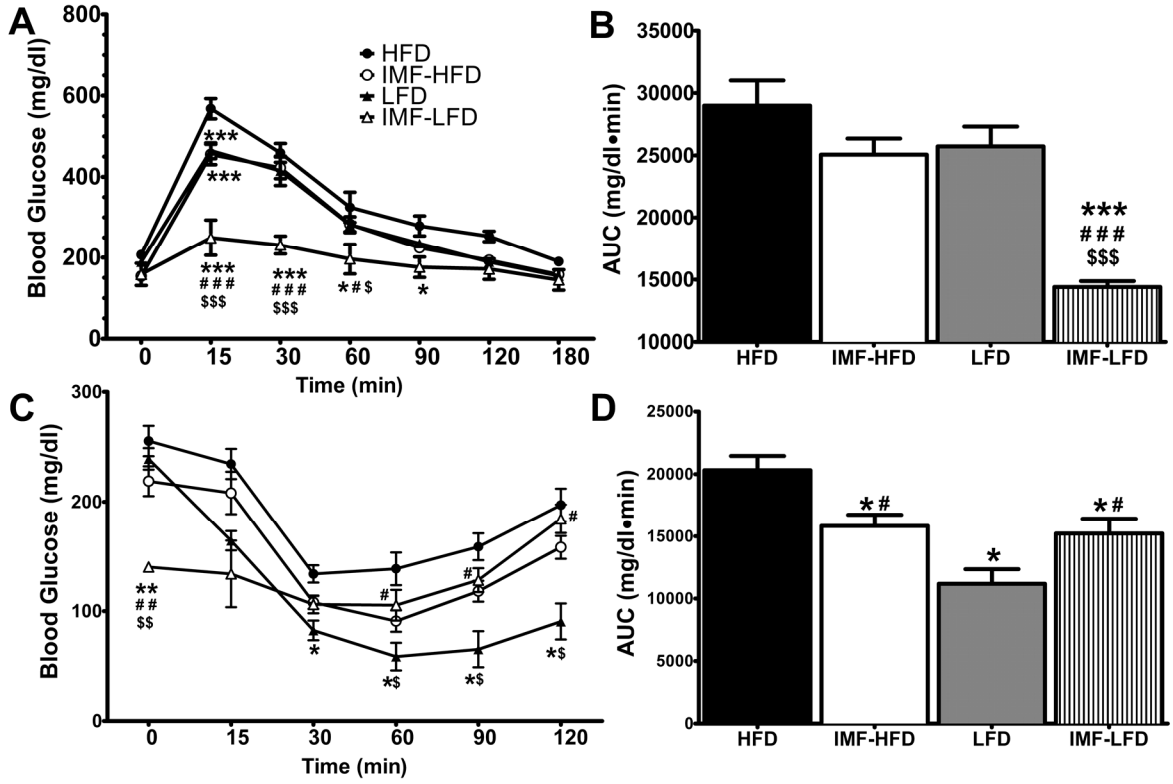

A
Blood Glucose (mg/dl)
Time (min)
HFD
IMF-HFD
LFD
IMF-LFD
B
AUC (mg/dl•min)
HFD
IMF-HFD
LFD
IMF-LFD
C
Blood Glucose (mg/dl)
Time (min)
D
AUC (mg/dl•min)
HFD
IMF-HFD
LFD
IMF-LFD

Figure 5.

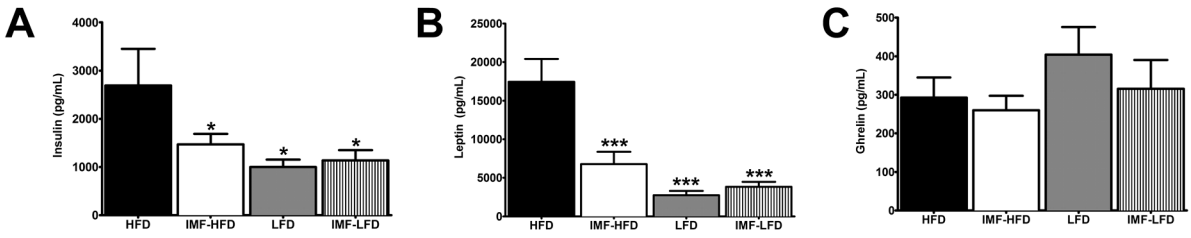
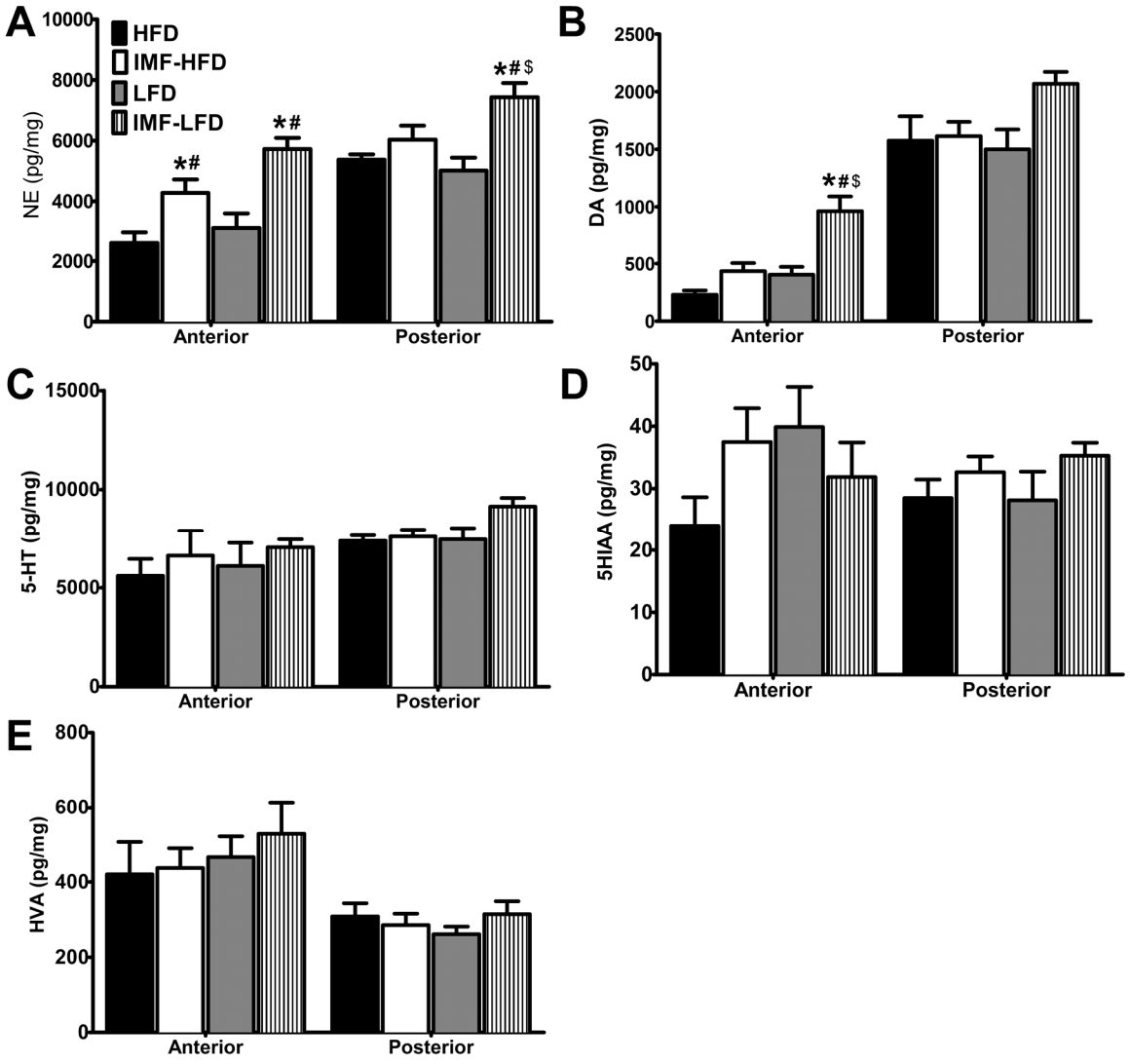

A
Insulin (pg/mL)
4000
3000
2000
1000
0
*
*
*
HFD
IMF-HFD
LFD
IMF-LFD
B
Leptin (pg/mL)
25000
20000
15000
10000
5000
0
***
***
***
HFD
IMF-HFD
LFD
IMF-LFD
C
Ghrelin (pg/mL)
500
400
300
200
100
0
HFD
IMF-HFD
LFD
IMF-LFD

Figure 6.


A
HFD
IMF-HFD
LFD
IMF-LFD
NE (pg/mg)
10000
8000
6000
4000
2000
0
*#
*#
*#$
Anterior
Posterior
B
DA (pg/mg)
2500
2000
1500
1000
500
0
*#$
Anterior
Posterior
C
5-HT (pg/mg)
15000
10000
5000
0
Anterior
Posterior
D
5HIAA (pg/mg)
50
40
30
20
10
0
Anterior
Posterior
E
HVA (pg/mg)
800
600
400
200
0
Anterior
Posterior