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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

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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 (> 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 (Coulouchem 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 MgCl₂, 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^{ΔΔCT}. 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 α = 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.CO₂, 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.CO₂ than the HFD group \((p<0.01\) and \(p<0.05\), respectively), Figure 3A. For v.O₂, 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.O₂ 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
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 \( (p<0.05 \text{ for all}) \), 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 ≤ 34.9), or class II obese (BMI ≤ 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 libitum 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 α1A receptor in the IMF-HFD and LFD groups, whereas the expression of α1B 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 \textit{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, \textit{Agrp} relative mRNA levels in the ARC demonstrated a similar trend as \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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.

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References
12. Esghinia 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
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
44. Ritter S, Dinh TT, Li AJ 2006 Hindbrain catecholamine neurons control multiple glucoregulatory responses. Physiol Behav 89:490-500
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


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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.

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<th>LFD</th>
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<td>Pomc</td>
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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.

<table>
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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.

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<td>0.978 ± 0.069</td>
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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 IMF protocol with LFD (IMF-LFD; n = 8). Data are represented as means ± SEM. ** indicates difference from HFD (p<0.01).

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 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 IMF-HFD; $ indicates difference (p<0.05) from IMF-HFD; $$ indicates difference (p<0.01) from LFD; $$$ 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.
Figure 2.
Figure 3.
Figure 4.
Figure 5.