The Chimeric Peptide (GEP44) Reduces Body Weight, Energy Intake, and Energy Expenditure in Diet-Induced Obese Rats

Abbreviated title: Effects of GEP44 on Energy Expenditure in HFD-Fed Rats

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Abstract

We recently reported that a chimeric peptide (GEP44) targeting the glucagon-like peptide-1 receptor (GLP-1R) and neuropeptide Y1- and Y2- receptors decreased body weight (BW), energy intake and core temperature in diet-induced obese (DIO) male and female mice. Given that GEP44 was found to reduce core temperature (surrogate measure of energy expenditure (EE)) in DIO mice, we hypothesized that GEP44 would reduce EE in male and female high fat diet (HFD)-fed rats. To test this, rats were maintained on a HFD for at least 4 months to elicit DIO prior to undergoing a sequential 2-day vehicle period, 2-day GEP44 (50 nmol/kg) period and a minimum 2-day washout period and detailed measures of energy homeostasis. GEP44 (50 nmol/kg) reduced EE (indirect calorimetry), respiratory exchange ratio (RER), core temperature, activity, energy intake and BW in male and female rats. As in our previous study in mice, GEP44 reduced BW in male and female HFD-fed rats by 3.8 ± 0.2% and 2.3 ± 0.4%, respectively. These effects appear to be mediated by increased lipid oxidation and reductions of energy intake as GEP44 reduced RER and cumulative energy intake in male and female HFD-fed rats. The strong reduction of body weight in response to GEP44 is related to a robust reduction of energy intake, but not to stimulation of EE. The paradoxical finding that GEP44 reduced EE might be secondary to a reduction of diet-induced thermogenesis or might indicate an important mechanism to limit the overall efficacy of GEP44 to prevent further weight loss.

INTRODUCTION

The control of body weight involves a complex interaction of factors that control food intake and energy expenditure (EE). While many risk factors (i.e. dietary, environmental, genetic and psychological) are thought to contribute to obesity, increased energy intake without corresponding increases in energy output (EE, activity) contribute, in part, to weight gain and subsequent obesity (1). The obesity epidemic and its associated complications, including cardiovascular disease, type 2 diabetes, osteoarthritis, depression, certain types of cancer and COVID-19 related hospitalizations, are global health concerns. More than one billion people in the world are obese (2). Up to 50% of adults in the US alone are predicted to become obese by 2030 (3). Furthermore, the costs to treat obesity in the US by 2030 are estimated to be an alarming 3 trillion/year (4). Once-weekly treatment with semaglutide has resulted in prolonged loss in body weight that has ranged from ≈6.7% over 40 weeks (5), ≈14.9% over 68 weeks (6), and ≈10.2% over 208 weeks (7), further highlighting the need to optimize anti-obesity treatment options that result in more marked weight loss over more sustained treatment intervals. In addition, there is rapid weight regain when such treatment options are discontinued (8). The limitation of pharmacotherapies to promote long-term weight loss (>10%) in humans who are overweight or obese is thought to occur, in part, by activation of counter-regulatory orexigenic mechanisms in the CNS that drive hunger and energy conservation mechanisms that reduce EE, thus preventing further weight loss and promoting weight regain (9).

Recent findings indicate that combination therapy (co-administration of different compounds) or treatment with monomeric dual- and triple-agonists at low-dose or

subthreshold dose combinations may be more effective than monotherapy for producing sustained weight loss (10). The increased efficacy of these compounds is likely due, in part, to their ability to both suppress energy intake and/or increase EE as well as to prevent the count-regulatory orexigenic effects mentioned above. One example of a dual-agonist is Tirzepatide (Zepbound^{TM}), which targets both glucose-dependent insulinotropic polypeptide receptor (GIPR) and GLP-1R, was approved by the FDA in November, 2023, for weight management. It has been found to elicit more profound weight loss in humans when compared to semaglutide monotherapy. Prolonged treatment over a 72- (11) and 88-week (12) period has resulted in a profound weight loss of 20.9% and 25.3%, respectively More recently, retatrutide, a triple-agonist, targets GLP-1R, GIPR and glucagon receptors (GCGR), was able to elicit a sustained reduction of body weight by 24.2% over a 48-week period (13). While these compounds evoke profound weight loss in the absence of bariatric surgery, they are still associated with a number of mild to moderate adverse gastrointestinal side effects, namely diarrhea, nausea, vomiting and/or abdominal pain (11). This stresses the need to develop new, effective, and safe strategies to treat the growing obesity epidemic.

We recently developed the triple agonist, GEP44, which is a partial agonist at GLP-1R, Y1R and Y2R (14). GEP44 was originally designed to treat type 2 diabetes (T2DM) and obesity. We recently found that GEP44 reduced energy intake, body weight, core temperature, and glucose levels in male and female DIO mice primarily through a GLP-1R dependent mechanism. Given that GEP44 was found to reduce core temperature (surrogate measure of EE), we hypothesized that chimeric peptide GEP44 would also reduce EE (15). We further hypothesized that GEP44 would elicit weight loss primarily

through reductions of energy intake. However, the remaining open question was if the reduction in food intake is accompanied by a reduction in EE (using indirect calorimetry) which can limit the overall success of sustained weight loss. We also measured gross motor activity to test if spontaneous physical activity (16) or shivering and non-shivering thermogenesis in skeletal muscle (17) might contribute to GEP44-elicited reductions of EE. The selective GLP-1R agonist, exendin-4, was included as a control to assess GLP-1R mediated effects on EE (18).

METHODS

Animals

Adult male (~ 600–907 g at study onset) and female (~ 275–479 g at study onset) Long-Evans rats were initially obtained from Charles River Laboratories (Wilmington, MA) and maintained for at least 4 months on a high fat diet (HFD) prior to study onset. All animals were housed individually in Plexiglas cages in a temperature-controlled room (22 ± 2°C) under a 12:12-h light-dark cycle. All rats were maintained on a 1 a.m. (lights on)/1 p.m. (lights off) light cycle. Rats had *ad libitum* access to water and a HFD providing 60% kcal from fat (Research Diets, D12492, New Brunswick, NJ). Animals were maintained on a 1-h fast during the week prior and throughout the vehicle and drug treatment phases of **Study 1.1, Study 1.2 and Study 2.1** as listed below. The research protocols were approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Puget Sound Health Care System (VAPSHCS) in accordance with NIH's *Guide for the Care and Use of Laboratory Animals* (NAS, 2011) (19).

Drug Preparation

GEP44 was synthesized in the Doyle lab as previously described (14). Fresh solutions of GEP44 and exendin-4 (ENZO; Farmingdale, NY) were prepared, frozen and thawed prior to the onset of each experiment (**Studies 1-3**).

Implantation of implantable telemetry devices (PDT-4000 E-Mitter) into the abdominal cavity

Animals were anesthetized with isoflurane and subsequently underwent a sham surgery (no implantation) or received implantations of a sterile PDT-4000 E-Mitter (23 mm long × 8 mm wide; Starr Life Sciences Company) into the intraperitoneal cavity. The abdominal opening was closed with 4-0 Vicryl absorbable suture and the skin was closed with 4-0 monofilament nonabsorbable suture. Vetbond glue was used to seal the wound and bind any tissue together between the sutures. Sutures were removed within two weeks after the PDT-4000 E-Mitter implantation. All PDT-4000 E-Mitters were confirmed to have remained within the abdominal cavity at the conclusion of the study.

Acute SC injections of GEP44 and Exendin-4

GEP44 (or saline vehicle; 1 mL/kg injection volume) or exendin-4 were administered immediately prior to the start of the dark cycle following 1 h of food deprivation. Rats underwent all treatments. The study design consisted of one round of a 2-day baseline phase (vehicle treated), a 2-day treatment phase (single dose repeated over 2 days), and a washout phase (a minimum of 4 days for **Study 1** and 3 days for **Study 2**). During the drug treatment phase one-half the animals received exendin-4 (10 nmol/kg)

while the other half received GEP44 (50 nmol/kg). Treatments were counterbalanced so that each animal received both treatments [GEP44 (50 nmol/kg/day) or exendin-4 (10 nmol/kg/day)]. Body weight and energy intake were assessed daily at approximately 1-h prior to the start of the dark cycle.

Body Composition

Determinations of lean body mass and fat mass were made on conscious rats by quantitative magnetic resonance using an EchoMRI 4-in-1-700™ instrument (Echo Medical Systems, Houston, TX) at the VAPSHCS Rodent Metabolic Phenotyping Core.

Study Protocols

Study 1.1: Determine the effects of GEP44 and exendin-4 on signs of pain or discomfort, body weight and energy intake in male and female HFD-fed rats.

Signs of pain or discomfort

Animals were observed for facial signs of pain or discomfort (narrow eyes and flattened or elongated nose) during the period immediately following GEP44 and exendin-4 treatment in male and female HFD-fed rats.

Changes of Body Weight and Energy Intake. Prior to the onset of vehicle and drug treatment within each sex, both treatment groups were matched for body weight and adiposity. Study design was as described earlier under “Acute SC injections of GEP44 and Exendin-4”. By design, male rats were DIO as determined by both body weight (730.2 ±19.1 g) and adiposity (219±14 g fat mass; 29.6±1.2% adiposity) after

maintenance on the HFD for approximately 4 months. As expected, female HFD-fed rats were overweight/obese as confirmed by both body weight (340.8 ± 9.9 g) and adiposity (79.6 ± 8.2 g fat mass; 22.5 ±1.6% adiposity). The selective GLP-1R agonist, exendin-4, was included as a control to assess GLP-1R mediated effects on energy intake (18). Body weight and energy intake were measured daily. Body weight change reflects the total change over each sequential 2-day vehicle and 2-day drug treatment period. Energy intake data reflects the cumulative intake throughout the 2-day vehicle and 2-day drug treatment period.

Study 1.2A-B: Determine the effects of GEP44 and exendin-4 on A) core temperature and B) gross motor activity in male and female HFD-fed rats using an E-Mitter telemetry system.

Study 1.2A-B: Changes of A) Core Temperature and B) Gross Motor Activity. The protocol for measuring core temperature and gross motor activity has been described previously (20). Briefly, core temperature (surrogate marker of EE) and gross motor activity were recorded from each rat in the home cage every 5 min throughout the study. The last hour of the light cycle (during which time drug administration and assessments of energy intake and body weight occurred) was excluded from the telemetry analysis. Core temperature and activity were averaged throughout each sequential 2-day vehicle and 2-day drug treatment period.

Study 2.1A-C: Determine the effects of GEP44 and exendin-4 on A) ambulatory activity, B) EE and C) RER and in male and female HFD-fed rats using the CLAMS-HC indirect calorimetry system.

Study 2.1: Changes of A) Ambulatory Activity, B) EE and C) RER.

Animals from **Study 1.1** were used for these studies. The protocol for measuring EE have been previously described (21). Similar to **Study 1.1**, male and female HFD-fed rats underwent a 2-day vehicle period, 2-day drug treatment (GEP44 at 50 nmol/kg vs the selective GLP-1R agonist, exendin-4 at 10 nmol/kg). Following a 3-day washout period, treatments were given in a counterbalanced design so that each animal received both treatments over the 2-week experimental period. Rats were acclimated to indirect calorimetry cages prior to the study and data collection. EE measures were obtained using a computer-controlled indirect calorimetry system (CLAMS-HC, Columbus Instruments) located in the Rodent Metabolic Phenotyping Core at the VAPSHCS. Animals were individually housed throughout the testing period, in a home-cage-like setting (Tecniplast cage 1250; floor area 580 cm^2), in a light-and temperature-controlled enclosure (Powers Scientific, Pipersville, PA), with temperature set to 27± 1°C, and 12h light/ dark schedule. Irradiated corncob bedding and environmental enrichment (e.g., nylabone, gnawing sticks) were supplied on the floor of the cage. Feeders and water bottles were suspended from load cells, which enabled continuous measurement of food and water intake. Physical activity, cage temperature and humidity were all continuously measured, as was respiratory rate. Respiratory gases were measured by an integrated zirconia oxygen sensor and single-beam non-dispersed infrared CO_2 sensor. Gas sensors were calibrated at the beginning of each experiment with a span

gas containing known concentrations of O_2 and CO_2 with a balance of N2 (PraxAir, Tacoma WA). O_2 consumption (VO_2) and CO_2 (VCO_2) production were measured for each rat for 20 seconds with 9 measurements per animal per hour. Incurrent air reference values were determined after measuring every sixteen cages. RQ was calculated as the ratio of CO_2 production over O_2 consumption. EE was calculated by Columbus Instrument's software using the equation: VO_2 X (3.815 X 1.232 RQ) and was expressed in units of kilocalories per hour. [Note: The Columbus system does not use the Weir equation, namely, kcal/h= 60 X (0.003941 X VO_2 + 0.001106 X VCO_2). Total and ambulatory activity was determined simultaneously with the collection of the calorimetry data. Consecutive adjacent infrared beam breaks in the X and Y-axes, i.e., the length and width of the cage, were scored as activity counts, and the tally was recorded every 6.7 min. Data acquisition, instrument control, and data processing were coordinated by Oxymax software (v5.68, Columbus Instruments).

Study 3.1 Determine the effects of GEP44 and exendin-4 on brown adipose tissue (BAT) thermogenesis in male and female HFD-fed rats.

Study 3.1: Tissue Collection for Quantitative Real-Time PCR (qPCR). Following a washout period of at least 16 days at the end of **Study 1.1**, animals were fasted for 4-h prior to being treated 1x daily for 3 consecutive days with either vehicle, GEP44 or exendin-4. Tissue [interscapular brown adipose tissue (IBAT) was collected from 6-h fasted rats at 2-h post-injection. Rats were euthanized with an overdose of ketamine cocktail at 2-h post-injection. Tissue was rapidly removed, wrapped in foil and frozen in liquid N2. Samples were stored frozen at -80°C until analysis. IBAT was collected within

a 5-h window towards the end of the light cycle (9:00 a.m.-2:00 p.m.) as previously described in DIO CD® IGS/Long-Evans rats and C57BL/6J mice (22,23,25).

Study 3.1: qPCR

RNA extracted from samples of IBAT was analyzed using the RNeasy Lipid Mini Kit (Qiagen Sciences Inc, Germantown, MD) followed by reverse transcription into cDNA using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Quantitative analysis for relative levels of mRNA in the RNA extracts was measured in duplicate by qPCR on an Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA) using the following TaqMan® probes (Thermo Fisher Scientific Gene Expression Assay probes; **Table 1**): rat *Nono,* glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), uncoupling protein-1 (*Ucp1*), β1-adrenergic receptor (*Adrb1*), β3-adrenergic receptor (*Adrb3*), type 2 deiodinase (*Dio2*), PR domain containing 16 (*Prdm16*), G-protein coupled receptor 120 (*Gpr120*), cell death-inducing DNA fragmentation factor alpha-like effector A (*Cidea*), and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*Ppargc1a)*. Relative amounts of target mRNA were determined using the Comparative C_T or $2^{-\Delta\Delta C_T}$ method (26) following adjustment for the housekeeping gene, *Nono* and *Gapdh*. Specific mRNA levels of all genes of interest were normalized to the cycle threshold value of Nono and *Gapdh* mRNA in each sample and expressed as changes normalized to controls (vehicle treatment).

Study 3.2A-B: Determine the effects of GEP44 and exendin-4 on blood glucose and plasma hormones.

Study 3.2: Blood collection

Following a 6-h fast, blood samples [up to 2 mL] from animals in **Study 3.1** were collected by cardiac stick in chilled K2 EDTA Microtainer Tubes (Becton-Dickinson, Franklin Lakes, NJ) at 2-h post-injection. Whole blood was centrifuged at 6,000 rpm for 1.5-min at 4°C; plasma was removed, aliquoted and stored at −80°C for subsequent analysis.

Study 3.2A: Blood Glucose Measurements

Following at least a 16-day washout period, animals were divided into three groups (VEH, GEP44 and exendin-4) matched for body weight and adiposity. Animals were fasted for 4 h prior to receiving vehicle, GEP44 or exendin-4 1x daily for 3 consecutive days. On day 3, both tail vein glucose and tissues were collected at 2-h post-injection. Blood was collected from 6-h fasted animals at 2-h post-injection in a subset of rats for glucose measurements by tail vein nick and measured using a glucometer (AlphaTRAK 2, Abbott Laboratories, Abbott Park, IL) (22,23).

Study 3.2B: Plasma hormone measurements

Plasma leptin and insulin were measured using electrochemiluminescence detection [Meso Scale Discovery (MSD®), Rockville, MD] using established procedures (23,24). Intra-assay coefficient of variation (CV) for leptin was 2.8%. Intra-assay CV for insulin was 2.7%. Plasma glucagon (Mercodia, Winston Salem, NC), fibroblast growth factor-

21 (FGF-21) (R&D Systems, Minneapolis, MN) and irisin (AdipoGen, San Diego, CA) levels were determined by ELISA. The intra-assay CV for glucagon was 1.6%. The intra-assay CV for FGF-21 was 2.7%. The intra-assay CV for irisin was 6.9% for mouse (not obtained for rat). Plasma adiponectin was also measured using ELISA (Alpco, Salem, NH) using established procedures (23,24). Intra-assay CV for adiponectin was 1.7%. The range of detectability for each hormone is listed in **Supplemental Table 1**. The data were normalized to historical values using a pooled plasma quality control sample that was assayed in each plate.

Statistical Analyses

All results are expressed as mean ± SE. Planned comparisons within respective sex between vehicle and drug (plasma measures, tail vein glucose, and gene expression data) involving between-subjects design were made using one-way ANOVA. Planned comparisons within respective sex to examine treatment means of vehicle and drug (body weight change, energy intake, core temperature, activity, and EE) involving within-subjects designs were made using a one-way repeated-measures ANOVA. In addition, planned comparisons were used to examine drug or sex differences on body weight change and energy intake using a one-way ANOVA. Analyses were performed using the statistical program SYSTAT (Systat Software, Point Richmond, CA). Differences were considered significant at $P<0.05$, 2-tailed.

RESULTS

Study 1.1: Determine the effects of GEP44 and exendin-4 on signs of pain or discomfort, body weight and energy intake in male and female HFD-fed rats.

The overall goal of these studies was to determine the extent to which the chimeric peptide, GEP44, produces signs of pain or discomfort and impacts core temperature (as surrogate for EE) and activity, and EE at doses that reduce body weight and energy intake in both male DIO and female HFD-fed rats. In addition, we incorporated use of the selective GLP-1R agonist, exendin-4, to assess GLP-1R mediated effects on body weight, energy intake, core temperature, activity, and EE in male and female HFD-fed rats.

Observational Signs of Pain or Discomfort

The number of animals that displayed signs of pain or discomfort (narrow eyes, elongated or flattened nose) as described in (27) is shown in **Table 2**. Given that the incidents of pain or discomfort occurred in both the same and different animals over the 2-day treatment periods, we did not run statistical comparisons. However, as previously shown (14), there appeared to be increased signs of pain or discomfort following exendin-4 treatment in male and female rats.

Body Weight

Both male and female Wistar rats were weight-matched within respective groups prior to treatment onset. GEP44 treatment reduced cumulative 2-day body weight in both male DIO (**Fig. 1A**; $P<0.05$) and female HFD-fed rats (**Fig. 1A**; $P<0.05$). Specifically, GEP44

reduced body weight after 2-day treatment in male DIO and female HFD-fed rats by -29.3 ± 1.7 and -8.3 ± 1.5 grams, respectively (*P*<0.05). This amounted to approximately 3.8 ± 0.2% and 2.3 ± 0.4% weight loss in male and female rats, respectively.

Similarly, the selective GLP-1R agonist, exendin-4, reduced body weight in both male (**Fig. 1B**; *P*<0.05) and female HFD-fed rats (**Fig. 1B**; *P*<.05). Specifically, exendin-4 reduced body weight after 2-day treatment in male and female HFD-fed rats by -26.5 ± 1.7 and -7.6 ± 1.4 grams, respectively (*P*<0.05). This amounted to approximately 3.4 ± 0.2% and 2.2 ± 0.4% weight loss in male and female rats, respectively. Note that exendin-4 was administered at a slightly lower dose (5 nmol/kg instead of 10 nmol/kg) on treatment day 2. However, there was no difference in 24-h energy intake in response to exendin-4 treatment at 5 (41.9 ± 5.0 kcal) and 10 nmol/kg from either the same animals (43.8 ± 4.1 kcal; *P*=NS) or a different subset of animals (44.2 ± 3.5 kcal; *P*=NS). Therefore, we combined the data together as part of the analysis with the animals that received exendin-4 at 10 nmol/kg.

Effect of GEP44 vs Exendin-4 on Body Weight in Male and Female Rats

There was no significant difference in the effectiveness of GEP44 and exendin-4 to reduce total body weight at 48-h post-injection in male or female rats (*P*=NS).

Energy intake

GEP44 treatment significantly reduced cumulative energy intake following treatment days 1 and 2 in both male (**Fig. 1C**; *P*<0.05) and female rats (**Fig. 1C**; *P*<0.05). In

males, GEP44 reduced cumulative 24- and 48-h energy intake by approximately 76.6 ± 3.1% and 74.6 ± 2.3%, respectively. In females, GEP44 reduced cumulative 24- and 48-h energy intake by approximately 57.4 ± 3.0% and 63 ± 2.4% suppression, respectively. Similarly, exendin-4 treatment also significantly reduced cumulative energy intake following treatment days 1 and 2 in both male **(Fig. 1D**; *P*<0.05) and female rats (**Fig. 1D**; *P*<0.05). In males, exendin-4 reduced cumulative 24- and 48-h energy intake by approximately 70.8 ± 2.8% and 70.0 ± 2.8%, respectively. In females, exendin-4 reduced cumulative 24- and 48-h energy intake by approximately 68.5 ± 3.6% and 69 ± 2.4% suppression, respectively.

Effect of GEP44 vs Exendin-4 on Energy Intake in Male and Female Rats

GEP44 was more effective than exendin-4 at reducing cumulative energy intake over 24- [F(1,18)= 4.848, *P*=0.041] and 48-h post-injection [F(1,18)= 14.005, *P*=0.001] in male rats. On the other hand, exendin-4 was more effective than GEP44 at reducing cumulative energy intake over 24- [F(1,21)= 13.586, *P*=0.001] and 48-h post-injection [F(1,21)= 12.375, *P*=0.002].

Study 1.2A-B: Determine the effects of GEP44 and exendin-4 on A) core temperature and B) gross motor activity in male and female HFD-fed rats.

Study 1.2A-B: Changes of A) Core Temperature and B) Gross Motor Activity

The analysis focused on the effects of GEP44 and exendin-4 on core temperature and activity at 6-h post-injection (dark cycle) and 23-h post-injection period. We also analyzed the extent to which these effects carried into the 12-h dark cycle and 11-h light

cycle. Note that the last hour of the light cycle (hour 12) was excluded from the core temperature and activity analysis as this was during the time that the animals were being handled and injected. The core temperature and gross motor activity data represent data that were averaged over the 6-h, 12-h dark cycle, 11-h light cycle, and 23-h post-injection period. The gross motor activity from the female rats was excluded from this analysis due, in part, to defective gross motor activity data that was evident in the females. This strengthened the rationale for measuring ambulatory activity using the CLAMS in both male and female rats (**Study 2**).

Study 1.2A: Core temperature (males):

GEP44 and exendin-4 both reduced core temperature throughout the post-treatment period. GEP44 reduced core temperature over the 6-h post-injection (**Fig. 2A**; $P<0.05$) and 23-h post-injection period in both male rats (**Fig. 2B**; $P<0.05$). Notably, similar results were observed during the 12-h dark cycle in response to GEP44 ($P<0.05$; **Supplemental Figure 1A**). GEP44 also elicited a slight elevation of core temp during the 11-h light period ($P<0.05$; **Supplemental Figure 1A**).

Exendin-4 also reduced core temperature over the 6-h post-injection (**Fig. 2C**; $P<0.05$) and 23-h post-injection period in male rats (**Fig. 2D**; $P<0.05$). Notably, similar results were observed during the 12-h dark cycle in response to exendin-4 ($P<0.05$; **Supplemental Figure 1B**). Like GEP44, exendin-4 also elicited a slight elevation of core temp during the 11-h light period ($P<0.05$; **Supplemental Figure 1B**).

Core temperature (females):

GEP44 and exendin-4 both reduced core temperature throughout the post-treatment period. GEP44 reduced core temperature over the 6-h post-injection (**Fig. 2A**; $P<0.05$) and 23-h post-injection period in female rats (**Fig. 2B**; $P<0.05$). Notably, similar results were observed during the 12-h dark cycle in response to GEP44 ($P<0.05$; data not shown). GEP44 also elicited a slight elevation of core temp during the 11-h light period in female rats ($P<0.05$; data not shown).

Exendin-4 also reduced core temperature over the 6-h post-injection (**Fig. 2C**; $P<0.05$) and 23-h post-injection period in female rats (**Fig. 2D**; $P<0.05$). Notably, similar results were observed during the 12-h dark cycle in response to exendin-4 in female rats ($P<0.05$; data not shown). Like GEP44, exendin-4 also elicited a slight elevation of core temp during the 11-h light period ($P<0.05$; data not shown).

Effect of GEP44 vs Exendin-4 on Core Temperature in Male and Female Rats

GEP44 was more effective than exendin-4 at reducing average core temperature over 6-h [$F(1,14)= 7.685$, $P=0.015$], dark cycle [$F(1,14)= 25.173$, $P<0.01$], and 23-h [$F(1,14)= 9.809$, $P=0.007$]. GEP44 also tended to be more effective at reducing core temperature during the light cycle [$F(1,14)= 3.631$, $P=0.077$] in male rats.

GEP44 was also more effective than exendin-4 at reducing average core temperature over 6-h [$F(1,6)= 15.570$, $P=0.008$], dark cycle [$F(1,6)= 10.696$, $P=0.017$], and 23-h [$F(1,6)= 6.828$, $P=0.04$] in female rats.

Study 1.2B: Gross Motor Activity (males):

GEP44 and exendin-4 both reduced gross motor activity throughout the post-treatment period. GEP44 reduced core temperature over the 6-h post-injection (**Supplemental Figure 2A**; $P<0.05$) and 23-h post-injection period in male rats (**Supplemental Fig. 2B**; $P<0.05$). Notably, similar results were observed during the 12-h dark cycle in response to GEP44 in male rats ($P<0.05$; **Supplemental Figure 3A**). GEP44 failed to significantly impact activity during the 11-h light cycle (P=NS).

Exendin-4 also reduced gross motor activity over the 6-h post-injection (**Supplemental Figure 2A**; $P<0.05$) and 23-h post-injection period in male rats (**Supplemental Figure 2B**; $P<0.05$). Notably, similar results were observed during the 12-h dark cycle in response to GEP44 in male rats ($P<0.05$; **Supplemental Figure 3B**). Exendin also tended to reduce activity during the 11-h light cycle, but this was not significant (P=0.114).

Effect of GEP44 vs Exendin-4 on Gross Motor Activity in Male and Female Rats

There were no differences in the effectiveness of GEP44 and exendin-4 on gross motor activity over 6-h, light cycle or 23-h post-injection in male rats. GEP44 tended to produce a more profound reduction of activity during the dark cycle [$F(1,14)= 2.511$, P=0.135] when compared to exendin-4 in male rats. There were also no differences in the effectiveness of GEP44 and exendin-4 on gross motor activity over 6-h, dark cycle, light cycle, or 23-h post-injection in female rats.

Sex Differences Linked to Effects of GEP44 and Exendin-4 on Body Weight, Energy Intake and Core Temperature

There was a significant difference in the effectiveness of GEP44 (50 nmol/kg) to reduce cumulative body weight over the 48-h treatment period with males showing a more pronounced effect of GEP44 to reduce body weight change [$F(1,39)= 87.147$, $P<0.01$] and weight loss [$F(1,39)= 6.793$, $P=0.013$]. In addition, there was a more pronounced effect of GEP44 to reduce cumulative 24- [$F(1,39)= 19.721$, $P<0.01$] and 48-h [$F(1,39)= 12.118$, $P=0.001$] cumulative food intake in males relative to females.

We also found a reduction of core temperature following vehicle treatment in male rats relative to female rats. This was evident during the 6-h post-injection period, 12-h dark cycle, 11-h light cycle, and 23-h post-injection period. Similarly, we found a reduction of core temperature in male rats during baseline measurements (in the absence of vehicle treatment) during 6-h, 12-h dark, 11-h light and 23-h periods following the start of the dark cycle on the day prior to vehicle treatment.

There was also a significant difference in the effectiveness of GEP44 to reduce core temperature during the dark cycle [$F(1,20)= 8.251$, $P=0.009$], light cycle [$F(1,20)= 6.241$, $P=0.021$] and over the 23-h [$F(1,20)= 8.012$, $P=0.010$] with males showing a more pronounced effect.

There was also a significant difference in the effectiveness of exendin-4 (10 nmol/kg) to reduce body weight change [$F(1,39) = 76.915$, $P<0.01$] and weight loss [$F(1,39) = 10.873$, $P=0.002$] in male and female rats with males showing the more heightened

effect. There was, however, no significant difference on the ability of exendin-4 to suppress 24- and 48-h cumulative energy intake between males and females.

There was also a near significant difference in the effectiveness of exendin-4 to reduce core temperature at 6-h post-injection [F(1,20)= 4.144, *P*=0.055]. This reached significance during the dark cycle [F(1,20)= 8.893, *P*=0.007], light cycle [F(1,20)=10.652, *P*=0.004] and over the 23-h [F(1,20)= 12.536, *P*=0.002] with males showing a more pronounced effect.

Study 2.1A-C: Determine the effects of GEP44 and exendin-4 on ambulatory activity, EE, and RER in male and female HFD-fed rats.

Study 2.1A: Ambulatory Activity (males):

We also measured ambulatory activity as a complementary strategy to our earlier measurements on gross motor activity that were obtained from the same animals in **Study 1.2B**. In addition, we assessed both EE and RER to further delineate the mechanisms underlying the anti-obesity effects of both GEP44 and exendin-4 and to complement our earlier measurements on core temperature in **Study 3**.

GEP44 and exendin-4 both reduced activity throughout the post-treatment period.

Consistent with our observations on gross motor activity during the dark cycle, GEP44 also reduced activity over the dark cycle [F(1,15)= 43.364, *P*<0.01]. In addition, GEP44 also reduced activity during the light cycle [F(1,15)= 15.293, *P*<0.01] in male rats (**Fig. 3A**; *P*<0.05).

Similarly, exendin-4 also reduced activity over the dark cycle [F(1,15)= 39.856, *P*<0.01]. In addition, exendin-4 also reduced activity during the light cycle [F(1,15)= 7.265, *P*<0.05] in male rats (**Fig. 3C**).

Ambulatory Activity (females):

GEP44 and exendin-4 both reduced activity throughout the post-treatment period. GEP44 reduced activity over the dark cycle [F(1,15)= 21.168, *P*<0.01] but failed to impact ambulatory activity during the light cycle in female rats (*P*=0.167; **Fig. 3B**). Exendin-4 also reduced activity over the dark cycle [F(1,15) = 18.893, *P*<0.01] but failed to significantly impact activity during the light cycle in female rats (*P*=0.341; **Fig. 3D**).

Effect of GEP44 vs Exendin-4 on Ambulatory Activity in Male and Female Rats

GEP44 was more effective than exendin-4 at reducing ambulatory activity over the light cycle [F(1,15)= 6.446, *P*=0.023] in male rats but there were no differences between GEP44 and exendin-4 on ambulatory activity during the dark cycle. In contrast to male rats, there were no differences between GEP44 and exendin-4 on ambulatory activity over light or dark cycles in female rats.

Study 2.1B: EE (males):

Consistent with our finding that GEP44 and exendin-4 both reduced core temperature in male rats (surrogate marker of EE), GEP44 and exendin-4 also reduced EE throughout the post-treatment period. GEP44 reduced EE over the dark cycle [F(1,15)= 64.902,

P<0.01] in male rats (**Fig. 4A**; *P*<0.05). In contrast, GEP44 treatment was associated with an increase in EE during the light cycle [F(1,15)= 40.588, *P*<0.01].

Exendin-4 also reduced EE over the dark cycles in male rats [F(1,15)= 24.032, *P*<0.01] (**Fig. 4C**). Similar to what was observed following GEP44 treatment, exendin-4 treatment was also associated with an increase in EE during the light cycle [F(1,15)= 40.588, *P*<0.01].

EE (females):

Consistent with our finding that GEP44 and exendin-4 both reduced core temperature in female rats, GEP44 and exendin-4 both reduced EE throughout the post-treatment period. GEP44 reduced EE over the dark cycle in female rats [F(1,15)= 26.106, *P*<0.01] (**Fig. 4B**). In contrast, GEP44 treatment was associated with an increase in EE during the light cycle [F(1,15)= 5.150, *P*<0.05].

Exendin-4 also reduced EE over the light and dark cycles in female rats [F(1,15)= 30.036, *P*<0.01] (**Fig. 4D**). Similar to what was observed following GEP44 treatment, exendin-4 treatment was also associated with an increase in EE during the light cycle [F(1,15)= 23.463, *P*<0.01].

Effect of GEP44 vs Exendin-4 on EE in Male and Female Rats

There were no differences between GEP44 and exendin-4 on EE during the light or dark cycles in male or female rats.

Study 2.1C: RER (males):

We measured RER to assess the extent to which GEP44 and exendin-4 impacted lipid oxidation in male rats. GEP44 and exendin-4 both reduced RER throughout the post-treatment period. GEP44 reduced RER over both the dark cycle [$F(1,15)= 99.686$, $P<0.01$] and light cycle [$F(1,15)= 38.185$, $P<0.01$] in male rats (**Fig. 5A**).

Exendin-4 also reduced RER over both the dark cycle [$F(1,15)= 144.771$, $P<0.01$] and light cycle [$F(1,15)= 31.887$, $P<0.01$] in male rats (**Fig. 5C**).

RER (females):

We measured RER to assess the extent to which GEP44 and exendin-4 impacted lipid oxidation in female rats. GEP44 and exendin-4 both reduced RER throughout the post-treatment period. GEP44 reduced EE over both the dark cycle [$F(1,15)= 155.091$, $P<0.01$] (**Fig. 5B**) and light cycle [$F(1,15)= 31.915$, $P<0.01$] **(Fig. 5D)** in female rats. Exendin-4 also reduced RER over both the dark [$F(1,15)= 327.949$, $P<0.01$] and light cycles [$F(1,15)= 111.788$, $P<0.01$] in female rats (**Fig. 5C**).

Effect of GEP44 vs Exendin-4 on RER in Male and Female Rats

There were no differences in response to GEP44 and exendin-4 on RER during the light and dark cycles in male and female rats.

Sex Differences Linked to Effects of GEP44 and Exendin-4 on Ambulatory Activity, EE, and RER

Ambulatory Activity:

There were no significant differences in ambulatory activity (post-vehicle treatment) between male and female rats. There was a tendency for GEP44 to reduce ambulatory activity more effectively in males during the dark cycle [F(1,30)= 3.051, *P*=0.091]. However, there was no significant difference of GEP44 to impact ambulatory activity in males and females during the light cycle. There was also no significant difference of exendin-4 to impact ambulatory activity in males and females during the dark or light cycles.

EE:

There was a significant reduction of EE (post-GEP44 vehicle treatment) during both the dark [F(1,30)= 80.799, *P*<0.01] and light cycles [F(1,30)= 74.041, *P*<0.01] in male rats compared to female rats. In addition, GEP44 reduced EE more effectively in males during both the dark [F(1,30)= 96.293, *P*<0.01] and light cycles [F(1,30)= 61.955, *P*<0.01].

Similar to what we observed following GEP44 vehicle treatment, we also found a reduction of EE during both the dark [F(1,30)= 77.411, *P*<0.01] and light cycles [F(1,30)= 59.002, *P*<0.01] following exendin-4 vehicle treatment in male rats compared to female rats. Exendin-4 also reduced EE more effectively in males during both the dark [F(1,30)= 72.077, *P*<0.01] and light cycles [F(1,30)= 77.073, *P*<0.01].

RER:

There tended to be a reduction of RER (post-GEP44 vehicle treatment) during the dark cycle [F(1,30)= 2.552, *P*=0.121] in male rats compared to female rats but there were no sex differences in baseline RER during the light cycle. Similar to what we observed following GEP44 vehicle treatment, there tended to be a reduction of RER (post-exendin-4 vehicle treatment) during the dark [F(1,30)= 3.450, *P*=0.073] in male rats compared to female rats but there were no sex differences in baseline RER during the light cycle. There was also no sex difference in the response to GEP44 or exendin-4 on RER during the dark or light cycles.

Study 3.1: Determine the effects of GEP44 and exendin-4 on BAT thermogenesis in male and female HFD-fed rats.

Study 3.1: qPCR

IBAT: As an additional readout of GEP44 and exendin-4-elicited thermogenic effects in IBAT, relative levels of *Ucp1*, *Adrb1*, *Adrb3*, *Gpr120*, *Ppargc1a*, *Cidea*, *Prdm16*, and *Dio2* mRNA expression were compared by PCR in response to GEP44 (50 nmol/kg) and exendin-4 (10 nmol/kg) or vehicle treatment at 2-h post-injection in male (**Table 3A**) and female rats (**Table 3B**). We recently extended these findings and found that exendin-4 resulted in a reduction of thermogenic gene expression in IBAT (consistent with the reduction of core temperature). We found that exendin-4 reduced *Adrb1* mRNA expression and increased both *Cidea* and *Ppargc1a* mRNA expression in IBAT from male rats (*P*<0.05). In addition, we found that exendin-4 reduced *Adrb1*, *Dio2* and *Gpr120* mRNA expression from IBAT in female rats. Similarly, we found that GEP44

reduced *Adrb1* and *Gpr120* mRNA expression from IBAT in male rats ($P<0.05$) and it also tended to reduce *Ucp1 and Dio2* from IBAT in male rats ($0.05<P<0.1$). In addition, it also reduced *Adrb1*, *Ucp1, Dio2* and *Gpr120* mRNA expression in female rats ($P<0.05$) and tended to increase *Prdm16* mRNA expression from IBAT of female rats ($0.05<P<0.1$).

IWAT: In addition, we found that both exendin-4 and GEP44 stimulated *Gpr120* mRNA expression in IWAT from male rats ($P<0.05$). Furthermore, GEP44 also stimulated *Ppargc1a* mRNA expression in IWAT of male rats ($P<0.05$), while exendin-4 tended to stimulate *Ppargc1a* mRNA expression from IWAT in male rats ($0.05<P<0.1$). In female rats, exendin-4 tended to both reduce *Dio2* and increase *Gpr120* mRNA expression in IWAT. In addition, we found that GEP44 reduced *Dio2* mRNA expression from IWAT.

While the qPCR data were normalized to the housekeeping gene, *Nono*, similar results were obtained when normalizing the data to *Gapdh*.

Together, these findings suggest that GLP-1 receptor agonists result in a reduction of BAT thermogenesis in both male and female rats. Given that we also found this to be the case with EE, these findings suggest that both exendin-4 and GEP44-elicited weight loss is primarily due to reductions of energy intake. Furthermore, the reductions of BAT thermogenesis and EE associated with GEP44 and exendin-4 might be secondary to a reduction of diet-induced thermogenesis or might indicate an important mechanism to limit the overall efficacy of GEP44 and exendin-4 to prevent further weight loss.

Study 3.2A-B: A) Blood Glucose and B) Plasma Hormones

Consistent with previous findings in rats, we found that that GEP44 tended to reduce tail vein glucose (collected at 2-h post-injection) in male rats (**Table 4A**; *P*=0.099). In female rats, GEP44 produced a significant reduction of tail vein glucose [F(1,13) = 8.450, *P*=.012] (**Table 4B**)**.** In addition, exendin-4 reduced fasting tail vein glucose in male rats [F(1,10) = 5.365, *P*=.043] (**Table 4A**) but exendin-4 was not effective at reducing fasting tail vein glucose in female rats (**Table 4B**; P=NS). The reduction of blood glucose in response to GEP44 in male and female rats did not appear to be mediated by glucagon as there was no significant change in glucagon in response to GEP44. However, glucagon was reduced in response to exendin-4 in male rats [F(1,10) = 5.284, *P*=.044] but not in female rats, thus raising the possibility that the reduction of blood glucose in response to exendin-4 in male rats may be mediated, in part, by the reduction of glucagon in male rats. Neither GEP44 or exendin-4 reduced plasma leptin in male or female rats (**Table 4A/4B**; *P*=NS). GEP44 increased plasma insulin in both male [F(1,11) = 5.270, *P*=.042] and female rats [F(1,13) = 9.232, *P*=.010], consistent with what we had observed in male DIO mice (15). Exendin-4 also increased plasma insulin in female [F(1,12) = 11.704, *P*=.005], but not male rats. In addition, GEP44 also reduced plasma adiponectin in male [F(1,11) = 32.059, *P*<0.01] and female rats [F(1,13) = 33.492, *P*<0.01]. Similarly, exendin-4 also reduced plasma adiponectin in both male [F(1,10) = 14.979, *P*=0.003] and female rats [F(1,12) = 13.017, *P*=0.004]. There was a tendency for GEP44 to increase irisin in male rats (*P*=0.064) and female rats (*P*=0.065). Likewise, exendin-4 also tended to increase irisin in male rats (*P*=0.088) and female rats (*P*=0.057). GEP44 also increased free fatty acids in female [F(1,13) = 11.534,

P=0.005] but not in male rats. Similarly, exendin-4 tended to increase FFA in female (*P*=0.057) but not in male rats. GEP44 also elevated total cholesterol in male rats [F(1,11) = 5.483, *P*=0.039] and tended to elevate total cholesterol in female rats (P=0.080). In contrast, exendin-4 failed to significantly alter total cholesterol levels in either male or female rats. Lastly, both GEP44 [F(1,13) = 18.195, *P*=0.001] and exendin-4 [F(1,12) = 30.669, *P*<0.01] produced a robust increase in FGF-21 in female rats, similar to what has been reported in other studies in male mice (28).

DISCUSSION

The goal of the current study was to determine whether the novel chimeric triple agonist, GEP44, which targets GLP-1R, Y1R and Y2R, reduced EE and RER at doses that elicit reductions of core temperature, energy intake and weight loss in adult male and female HFD-fed rats. As observed in the mouse model (15), GEP44 also reduced core temperature, energy intake and body weight in both male and female HFD-fed rats. Furthermore, confirming data we have seen in our mouse experiments, and against our initial hypothesis, we found that GEP44 reduced EE and RER at doses that reduced core temperature, body weight and energy intake. In addition, we found that GEP44 and exendin-4 elicited sexually dimorphic effects on core temperature and change in body weight with GEP44 being able to produce more profound effects in male rats. Similar to what we have previously reported in male and female rats (14,29,30) and mice (15), GEP44-elicited reductions of body weight appear to be driven largely by reductions in energy intake but here we also found that it increased lipid oxidation (denoted by reduced RER). Furthermore, our finding that GEP44 also reduced EE might be

secondary to a reduction of diet-induced thermogenesis or might indicate an important mechanism to limit the overall efficacy of GEP44 to prevent further weight loss.

Most studies that have administered GLP-1R agonists systemically have either found a decrease or no change in EE or have failed to measure both core temperature and EE in the same group of animals. We extend previous findings from our laboratory (14,29) to demonstrate that GEP44 reduces core temperature (surrogate measure of EE) in both male and female rats, similar to what we described earlier in response to exendin-4 treatment. Our findings in male and female rats are also largely consistent with our recently published study in male and female DIO mice (15). Previous studies have also reported that peripheral administration of the GLP-1R agonist, exendin-4, reduced core temperature for up to 4 hours in rats (31). In addition, others have reported that peripheral administration of exendin-4 decreased either resting EE over 2 and 21 hours in adult male wild-type mice (18) or EE at 4-h post-injection in rats (32). These findings have translated to humans as others have also found that liraglutide decreased resting EE after 4 weeks of treatment in humans with type 2 diabetes (33). On the other hand, others found that semaglutide decreased dark phase EE through six days of treatment in DIO mice, but these findings were not significant after adjusting for lean mass (34). Similarly, others also found that semaglutide reduced resting EE after 12 weeks of treatment in humans but these effects were not significant after adjusting for lean mass (35).

Similar to what others have reported on core temperature being lower in response to thermoneutral temperatures or a cold challenge in male rats, we found that core temperature was lower in male rats following vehicle administration or during

baseline (untreated) measurements. McDonald and colleagues (36) determined that female rats had elevated BAT mass and more consistent guanosine 5'-diphosphate (GDP) binding to mitochondria derived from BAT during the aging process, while male rats had both reduced BAT mass and GDP binding. These sex differences with respect to core temperature might explain, in part, why we found what appeared to be an enhanced effectiveness of GEP44 and exendin-4 to reduce core temperature in male rats relative to female rats.

Consistent with our findings that GEP44 and exendin-4 reduced core temperature and EE, we found that both GEP44 and exendin-4 reduced thermogenic markers (biochemical readout of BAT thermogenesis) within IBAT and T_{IBAT} (functional readout of BAT thermogenesis). These findings are consistent with the notion that BAT thermogenesis is important in maintaining core temperature in other contexts such as fever or stress (37,38). Our findings, however, that exendin-4 and GEP44 reduced thermogenic markers within IBAT are consistent with what others have reported following intraperitoneal administration of exendin-4 in a rat model (32). Krieger found that exendin-4 reduced BAT *Adrb3* mRNA expression in rats. While we did not find a change in *Adrb3* mRNA expression in our rat model, we did find that exendin-4 reduced IBAT *Adrb1* mRNA expression in IBAT in both male and female DIO rats. In addition, exendin-4 reduced IBAT *Adrb1*, Ucp1, *Dio2* and *Gpr120* mRNA expression in female rats. In addition, GEP44 reduced also reduced IBAT Adrb1 mRNA expression in female rats, but, similar to what we found following exendin-4 in female rats, GEP44 also reduced IBAT *Adrb1*, *Ucp1*, *Dio2*, and *Gpr120* mRNA expression. In addition, we found that GEP44 also stimulated IWAT *Gpr120* and *Ppargc1a* mRNA expression in male rats

while it, like exendin-4, reduced *Dio2* mRNA expression in female rats. Overall, our findings suggest that systemic administration of both GEP44 and exendin-4 may reduce EE and BAT and WAT thermogenesis through both overlapping and distinct mechanisms in male and female rats. It remains to be determined whether browning or beiging of WAT is playing a major role in how GEP44 reduces body weight in male DIO rats.

Our findings showing that GEP44 and exendin-4 reduced both core temperature and ambulatory activity in both male and female rats are consistent with what we recently reported in both male and female DIO mice (15). Similarly, others have also found that systemic administration of exendin-4 at reduces locomotor activity in lean male Sprague-Dawley rats (39,40). While others did not find any change in activity in response to systemic exendin-4 treatment in lean male Sprague-Dawley rats (31), it might be possible that differences in timing of injections, body weight, animal model, and/or dosing may explain, in part, the different results between studies. Given that we found reductions in both core temperature and gross motor activity in male rats, our data raise the possibility that reductions in shivering and non-shivering thermogenesis in skeletal muscle (17) and/or spontaneous physical activity-induced thermogenesis (16) may have contributed to the hypothermic effects of both GEP44 and exendin-4 in male rats. Collectively, our findings indicate that GEP44 and exendin-4 may reduce 1) activity in male rats but not in female rats and 2) core temperature in both male and female rats.

Similar to what we reported in diet-induced obese mice (15), we found that both GEP44 and exendin-4 treatment improved glucose homeostasis in the rat model. GEP44 increased fasting plasma insulin in both male and female HFD-rats. This effect

was also observed following exendin-4 treatment in female HFD-rats and is consistent with the reduction of blood glucose (tail vein) in response to GEP44 in male and female rats as well as the reduction of blood glucose in response to exendin-4 in male rats. It is possible that, similar to what we found in the mouse model (15), reductions in glucagon may contribute, in part, to the glucose lowering effects. Increased FGF-21 levels (not measured in male rats) in response to both GEP44 and exendin-4 in female rats may also contribute, in part, to the reduction of blood glucose (41). These findings are similar to what has been reported in other studies in male mice (28). We also found evidence that both GEP44 and exendin-4 may also increase lipolysis, as indicated by the increase in plasma FFA in female HFD-fed rats. Similar to our findings, Nanchani reported that exendin-4 treatment appeared to reduce adiponectin levels in male rats although the results were not significant (42). How adiponectin might contribute to the metabolic or glucoregulatory effects of both GEP44 and exendin-4 remains to be determined. In contrast to what we observed in male and female mice (15), GEP44 and exendin-4 appeared to reduce total cholesterol levels. Elfers and colleagues recently reported that cholesterol levels in response to GEP44 were elevated relative to male DIO rats that were pair-fed to the GEP44 treatment group (30) yet there did not appear to be any differences between vehicle and the GEP44 treatment group. Note that cholesterol levels appeared to increase in response to GEP44 treatment relative to vehicle but it was not significant. It remains to be determined whether the differences in our studies and the earlier studies represent differences in species or paradigm differences.

One limitation to our studies is the lack of weight restricted or pair-fed controls for studies that assessed the effects of GEP44 and exendin-4 on IBAT and IWAT gene expression and EE in adult DIO male and female rats. As a result, it is possible that the effects of GEP44 and exendin-4 on the reduction of thermogenic gene expression or EE in HFD-fed rats may also be due, in part, to the reduction in diet-induced thermogenesis. Future studies incorporating the use of similar measurements in fasted animals will be helpful in more fully establishing the role of GEP44 and exendin-4 on thermogenesis and EE in male and female rats.

CONCLUSION

In summary, the results presented in this manuscript highlight the effects of the novel chimeric peptide, GEP44, on EE, RER, core temperature, ambulatory and gross motor activity, body weight, and energy intake in adult male DIO and female HFD-fed rats at doses that have not been found to elicit visceral illness in rats (as determined by facial observations of nausea or pain (14) and kaolin intake (29)) or emesis in shrews (14,29). The anti-obesity effects of GEP44 and exendin-4 appear to be mediated, in part, by increased lipid oxidation and reductions of energy intake. Our findings further demonstrate that there may be overlapping and distinct mechanisms that mediate the effects of GEP44 and exendin-4 on BAT thermogenesis and EE in male and female rats. Furthermore, our finding that GEP44 reduced EE might be secondary to a reduction of diet-induced thermogenesis or might be an important safety override mechanism to prevent clinically extreme weight loss. The findings to date indicate that

GEP44 is a promising drug that overcomes the adverse side effects associated with existing GLP-1R agonist medications (5,43) to treat obesity and/or T2DM.

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Figure legend

Figure 1A-D. Effects of the chimeric peptide, GEP44, or selective GLP-1R agonist, exendin-4, on body weight and energy intake in male and female HFD-fed rats. Male (N=19/group) and female (N=22/group) rats were maintained on HFD (60% kcal from fat for at least 4 months prior to being implanted with PDT-4000 telemetry devices into the abdominal cavity. Animals were fasted for 1-h prior to receiving SC injections of vehicle (sterile saline/water) followed by GEP44 (50 nmol/kg; 1 mL/kg injection volume) or exendin-4 in a counterbalanced design. *A*, Effect of GEP44 on change in body weight in male and female HFD-fed rats; *B*, Effect of exendin-4 on change in body weight in male and female HFD-fed rats; *C*, Effect of GEP44 on energy intake in male and female HFD-fed rats; *D*, Effect of exendin-4 on energy intake in male and female HFD-fed rats. The change in body weight and cumulative energy intake reflects the total change in both measurements over the sequential 2-day vehicle and 2-day drug treatment period. Data are expressed as mean ± SEM. *P<0.05 GEP44 or exendin-4 vs. vehicle.

Figure 2A-D: Effects of the chimeric peptide, GEP44, or selective GLP-1R agonist, exendin-4, on core temperature in male and female HFD-fed rats. Male (N=15/group) and female (N=7/group) rats were maintained on HFD (60% kcal from fat for at least 4 months prior to being implanted with PDT-4000 telemetry devices into the abdominal cavity. Animals were fasted for 1-h prior to receiving SC injections of vehicle (sterile saline/water) followed by GEP44 (50 nmol/kg; 1 mL/kg injection volume) or exendin-4 in a counterbalanced design. *A*, Effect of GEP44 on 6-h core temperature in male and female HFD-fed rats; *B*, Effect of GEP44 on daily (23-h) core temperature in male and female HFD-fed rats; *C*, Effect of exendin-4 on 6-h core temperature in male and female HFD-fed rats; *D*, Effect of exendin-4 on daily (23-h) core temperature in male and female HFD-fed rats. Core temperature was averaged at 6-h and 23-h post-treatment periods over the 2-day vehicle and 2-day drug treatment period. Data are expressed as mean ± SEM. *P<0.05 GEP44 or exendin-4 vs. vehicle.

Figure 3A-D: Effects of the chimeric peptide, GEP44, or selective GLP-1R agonist, exendin-4, on ambulatory activity in male and female HFD-fed rats. Male (N=16/group) and female (N=16/group) rats were maintained on HFD (60% kcal from fat for at least 4 months prior to being implanted with PDT-4000 telemetry devices into the abdominal cavity. Animals were fasted for 1-h prior to receiving SC injections of vehicle (sterile saline/water) followed by GEP44 (50 nmol/kg; 1 mL/kg injection volume) or exendin-4 in a counterbalanced design. *A/B*, Effect of GEP44 on ambulatory activity during the light and dark cycle periods in *A)* male and *B)* female HFD-fed rats; *C/D*, Effect of exendin-4 on ambulatory activity during the light and dark cycle periods in *C)* male and *D)* female HFD-fed rats. Ambulatory activity was averaged over the light (12-h) and dark (12-h) cycles across the 2-day vehicle and 2-day drug treatment periods. Data are expressed as mean ± SEM. **P*<0.05 GEP44 or exendin-4 vs. vehicle.

Figure 4A-D: Effects of the chimeric peptide, GEP44, or selective GLP-1R agonist, exendin-4, on EE (indirect calorimetry) in male and female HFD-fed rats. Male (N=16/group) and female (N=16/group) rats were maintained on HFD (60% kcal from fat for at least 4 months prior to being implanted with PDT-4000 telemetry devices into the abdominal cavity. Animals were fasted for 1-h prior to receiving SC injections of vehicle (sterile saline/water) followed by GEP44 (50 nmol/kg; 1 mL/kg injection volume) or exendin-4 in a counterbalanced design. *A/B*, Effect of GEP44 on EE during the light and dark cycle periods in *A)* male and *B)* female HFD-fed rats; *C/D*, Effect of exendin-4 on EE during the light and dark cycle periods in *C)* male and *D)* female HFD-fed rats. EE was averaged over the light (12-h) and dark (12-h) cycles across the 2-day vehicle and 2-day drug treatment periods. Data are expressed as mean ± SEM. **P*<0.05 GEP44 or exendin-4 vs. vehicle.

Figure 5A-D: Effects of the chimeric peptide, GEP44, or selective GLP-1R agonist, exendin-4, on RER (indirect calorimetry) in male and female HFD-fed rats. Male (N=16/group) and female (N=16/group) rats were maintained on HFD (60% kcal from fat for at least 4 months prior to being implanted with PDT-4000 telemetry devices into the abdominal cavity. Animals were fasted for 1-h prior to receiving SC injections of

vehicle (sterile saline/water) followed by GEP44 (50 nmol/kg; 1 mL/kg injection volume) or exendin-4 in a counterbalanced design. *A/B*, Effect of GEP44 on RER during the light and dark cycle periods in *A)* male and *B)* female HFD-fed rats; *C/D*, Effect of exendin-4 on RER during the light and dark cycle periods in *C)* male and *D)* female HFD-fed rats. RER was averaged over the light (12-h) and dark (12-h) cycles across the 2-day vehicle and 2-day drug treatment periods. Data are expressed as mean ± SEM. *$P<0.05$ GEP44 or exendin-4 vs. vehicle.

Table 1. TaqMan probes used in qPCR analysis of IBAT and IWAT.

Table 2. Observational signs of pain or discomfort following GEP44 and Exendin-4 treatment in male and female Rats. Animals were observed for both narrow eyes and a flattened or elongated nose during the immediate period following treatment on days 1-2. The + sign denotes an animal that displayed these signs of pain or discomfort.

Table 3A-D. Gene expression in IBAT and IWAT following SC vehicle, GEP44 (50 nmol/kg) or exendin-4 (10 nmol/kg) treatment: *A)* IBAT gene expression following GEP44 and exendin-4 treatment in male HFD-fed rats (N=6-7/group); *B)* IWAT gene expression following GEP44 and exendin-4 treatment in male HFD-fed rats (N=7-8/group); *C)* IBAT gene expression following GEP44 and exendin-4 treatment in female HFD-fed rats; and *D)* IWAT gene expression following GEP44 and exendin-4 treatment in female HFD-fed rats. Animals were fasted for 4 h prior to receiving vehicle, GEP44 or exendin-4 1x daily for 3 consecutive days. IBAT and IWAT tissue from male and female HFD-fed rats were collected on treatment day 3 at 2-h post-injection. Data are expressed as mean ± SEM. Different letters denote significant differences between treatments (Vehicle vs GEP44 or Exendin-4; $P<0.05$).

Table 4A-B: Effects of the chimeric peptide, GEP44 (50 nmol/kg) and the selective GLP-1R agonist, exendin-4 (10 nmol/kg), on plasma hormones in A) male (N=6-7/group) and B) female HFD-fed rats (N=7-8/group). Animals were fasted for 4 h prior to receiving vehicle, GEP44 or exendin-4 1x daily for 3 consecutive days. Blood was

collected on treatment day 3 by tail vein nick (glucose) or cardiac stick (leptin, insulin, FGF-21, glucagon, irisin, adiponectin, total cholesterol, free fatty acids) at 2-h post-injection of VEH, exendin-4 or GEP44. Data are expressed as mean ± SEM. Different letters denote significant differences between treatments (Vehicle vs GEP44 or Exendin-4; $P<0.05$).

Supplemental Table 1. Range of detectability for plasma hormones.

Supplemental Figure 1A-B: Effects of the chimeric peptide, GEP44, or selective GLP-1R agonist, exendin-4, on core temperature in male and female HFD-fed rats. Male (N=15/group) and female (N=7/group) rats were maintained on HFD (60% kcal from fat for at least 4 months prior to being implanted with PDT-4000 telemetry devices into the abdominal cavity and subsequently receiving SC injections of vehicle (sterile saline/water) followed by GEP44 (50 nmol/kg; 1 mL/kg injection volume) or exendin-4 in a counterbalanced design. *A*, Effect of GEP44 on core temperature during the light and dark cycle periods in A) male and female HFD-fed rats; *B*, Effect of exendin-4 on core temperature during the light and dark cycle periods in *B)* male and female HFD-fed rats. Core temperature was averaged at 11-h light and 12-h dark cycle periods over the 2-day vehicle and 2-day drug treatment period. Data are expressed as mean ± SEM. *$P<0.05$ GEP44 or exendin-4 vs. vehicle.

Supplemental Figure 2A-D: Effects of the chimeric peptide, GEP44, or selective GLP-1R agonist, exendin-4, on gross motor activity in male HFD-fed rats. Male (N=15/group) rats were maintained on HFD (60% kcal from fat for at least 4 months prior to being implanted with PDT-4000 telemetry devices into the abdominal cavity and subsequently receiving SC injections of vehicle (sterile saline/water) followed by GEP44 (50 nmol/kg; 1 mL/kg injection volume) or exendin-4 in a counterbalanced design. *A*, Effect of GEP44 on 6-h activity in male HFD-fed rats; *B*, Effect of GEP44-4 on daily (23-h) activity in male HFD-fed rats; *C*, Effect of exendin-4 on 6-h activity in male HFD-fed rats; *D*, Effect of exendin-4 on daily (23-h) activity in male female HFD-fed rats. Activity was averaged at 6-h and 23-h post-treatment periods over the 2-day vehicle and 2-day

drug treatment period. Data are expressed as mean ± SEM. *P<0.05 GEP44 or exendin-4 vs. vehicle

Supplemental Figure 3A-B: Effects of the chimeric peptide, GEP44, or selective GLP-1R agonist, exendin-4, on gross motor activity in male HFD-fed rats. Male (N=15/group) rats were maintained on HFD (60% kcal from fat for at least 4 months prior to being implanted with PDT-4000 telemetry devices into the abdominal cavity and subsequently receiving SC injections of vehicle (sterile saline/water) followed by GEP44 (50 nmol/kg; 1 mL/kg injection volume) or exendin-4 in a counterbalanced design. *A*, Effect of GEP44 on gross motor activity during the light and dark cycle periods in A) male and female HFD-fed rats; *B*, Effect of exendin-4 on gross motor activity during the light and dark cycle periods in *B)* male and female HFD-fed rats. Gross motor activity was averaged at 11-h light and 12-h dark cycle periods over the 2-day vehicle and 2-day drug treatment period. Data are expressed as mean ± SEM. *P<0.05 GEP44 or exendin-4 vs. vehicle

Effects of GEP44 and Exendin-4 on Body Weight and Energy Intake in Male and Female HFD-Fed Rats

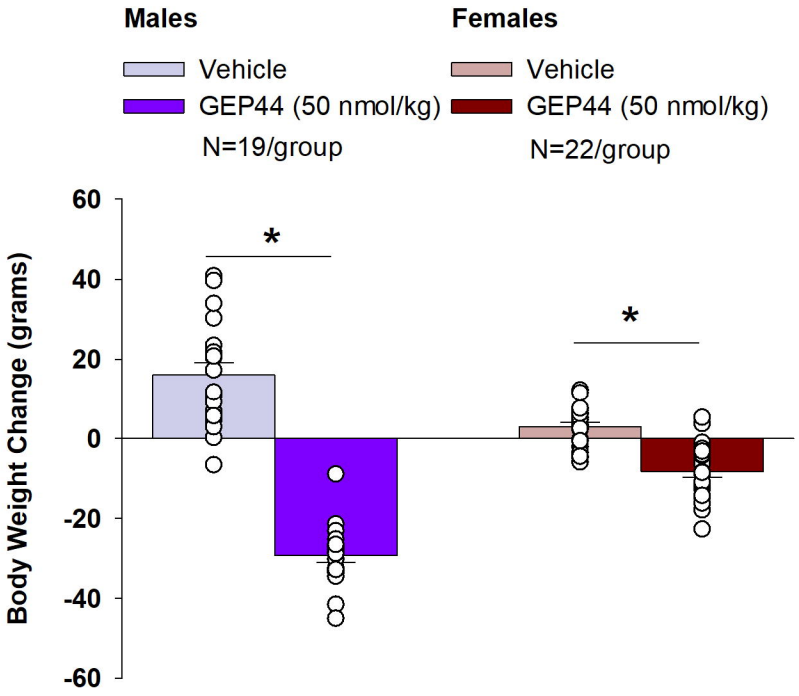


Figure 1A

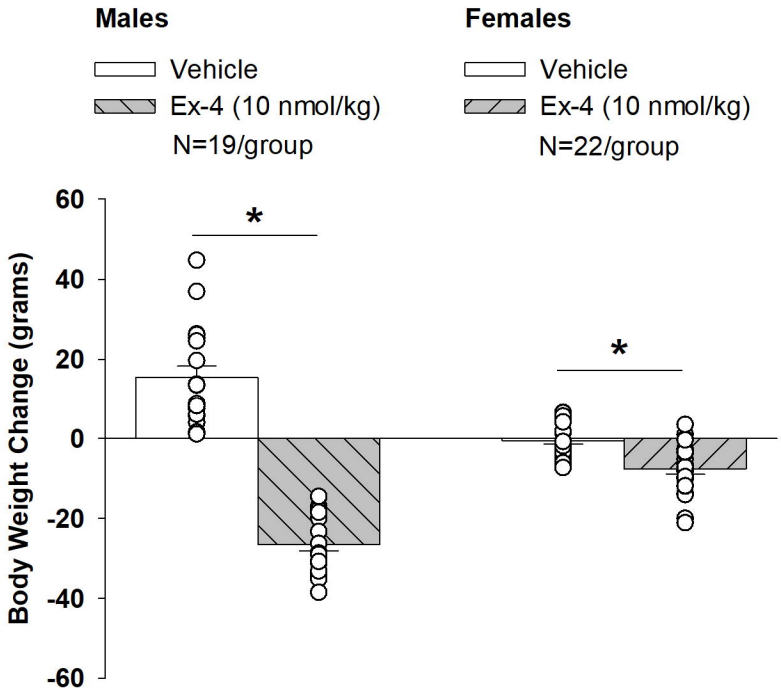


Figure 1B

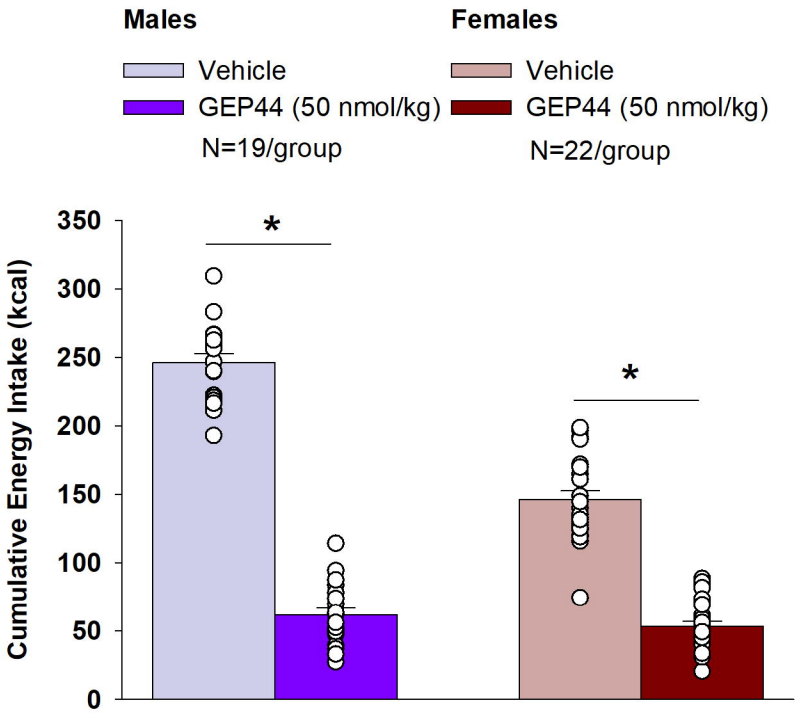


Figure 1C

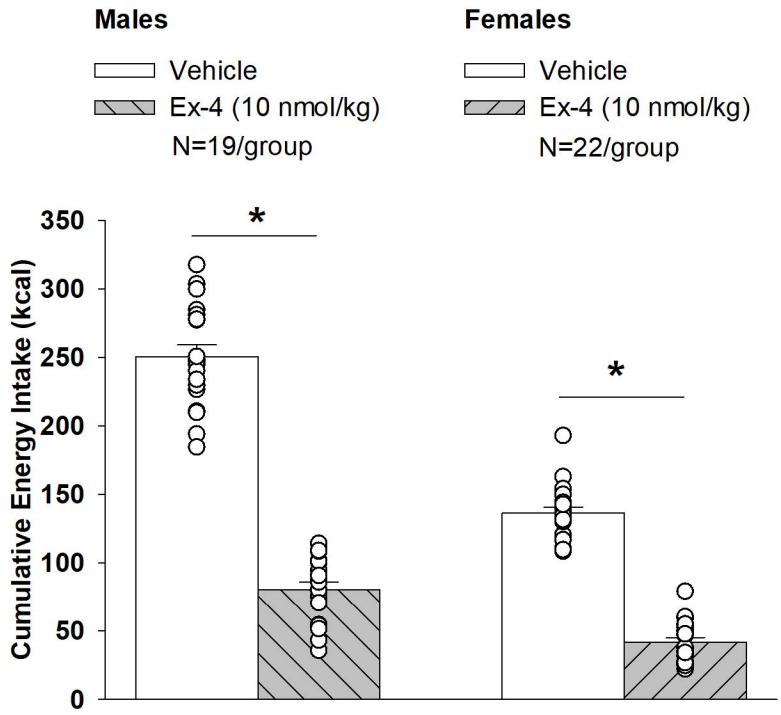
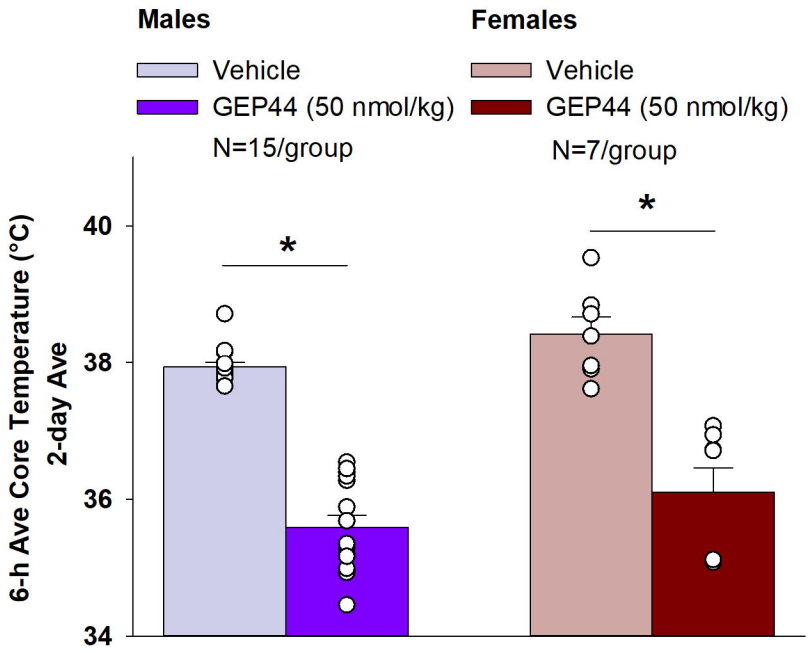


Figure 1D

Effects of GEP44 and Exendin-4 on Core Temperature in Male and Female HFD-Fed Rats



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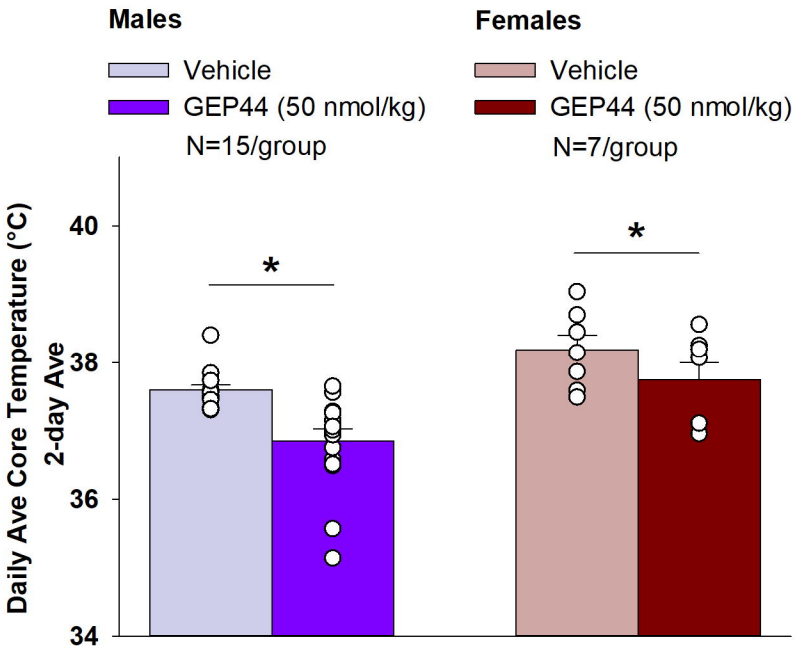

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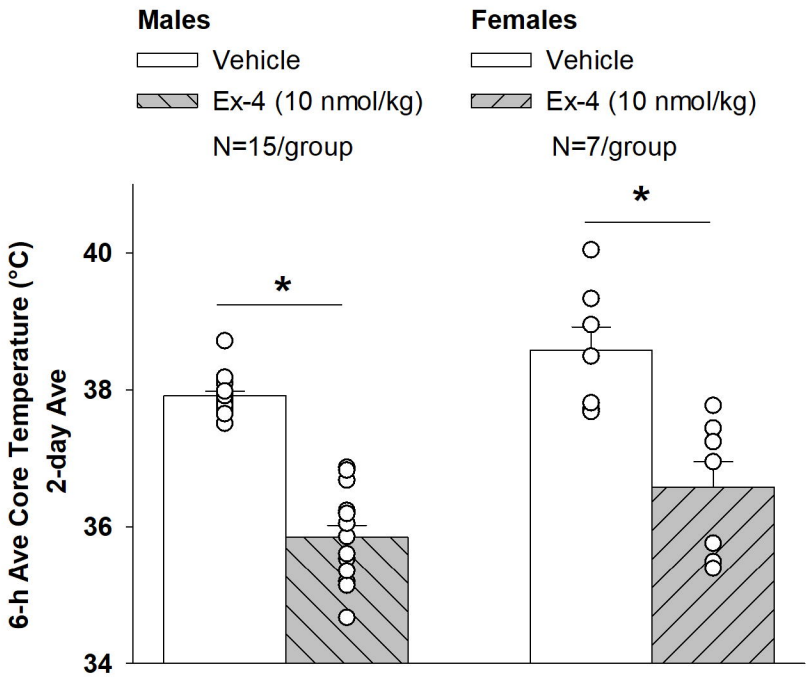

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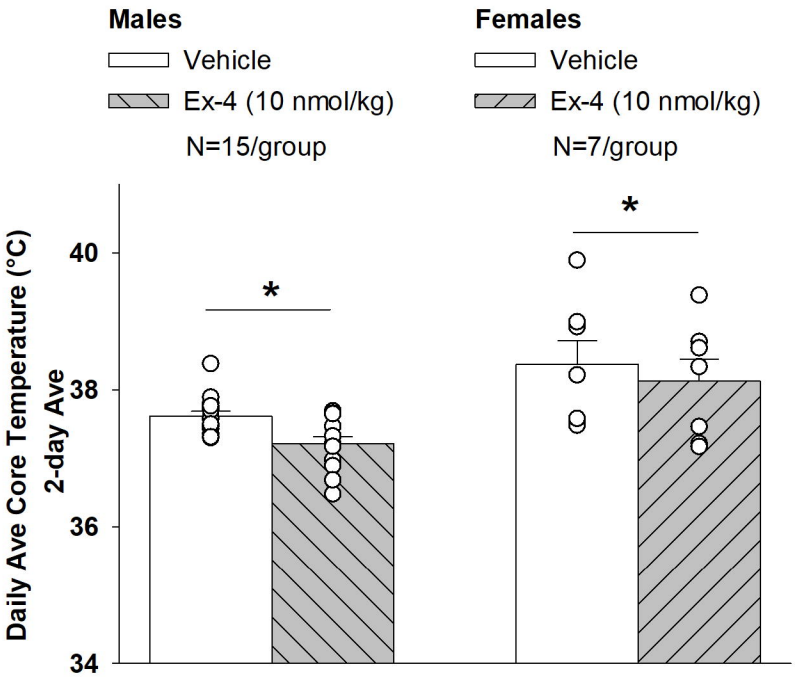
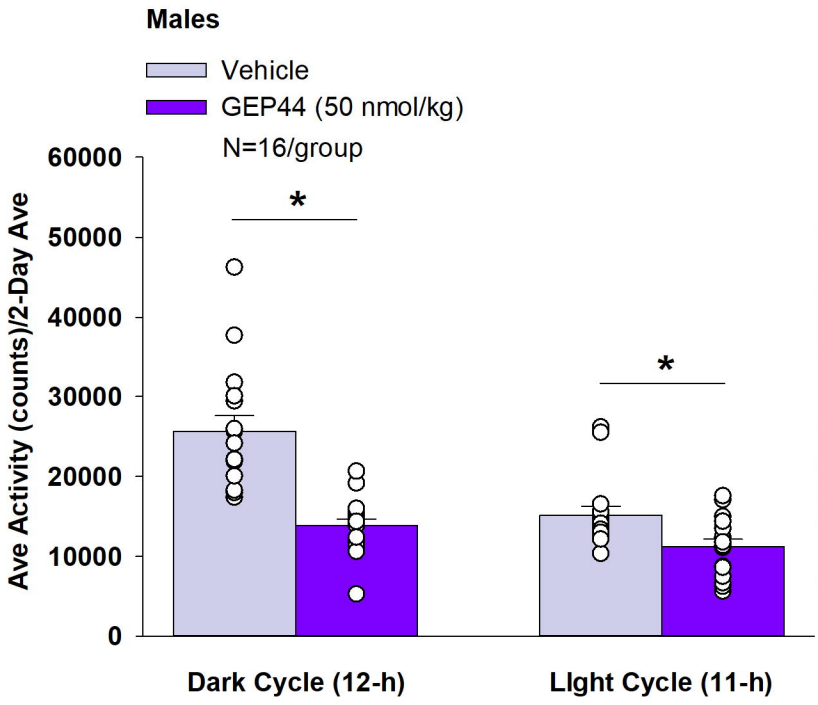


Figure 2D

Effects of GEP44 and Exendin-4 on Ambulatory Activity in Male and Female HFD-Fed Rats



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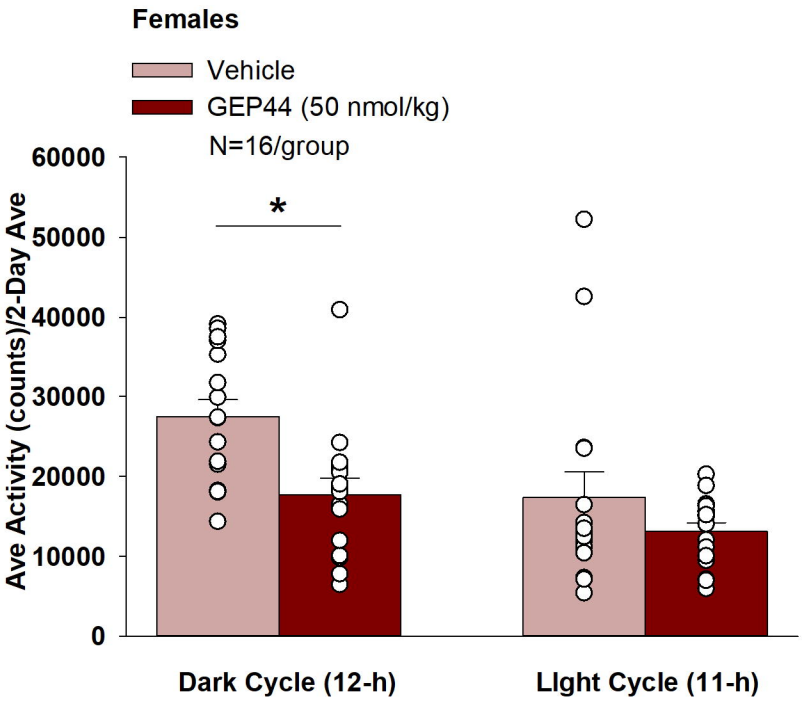
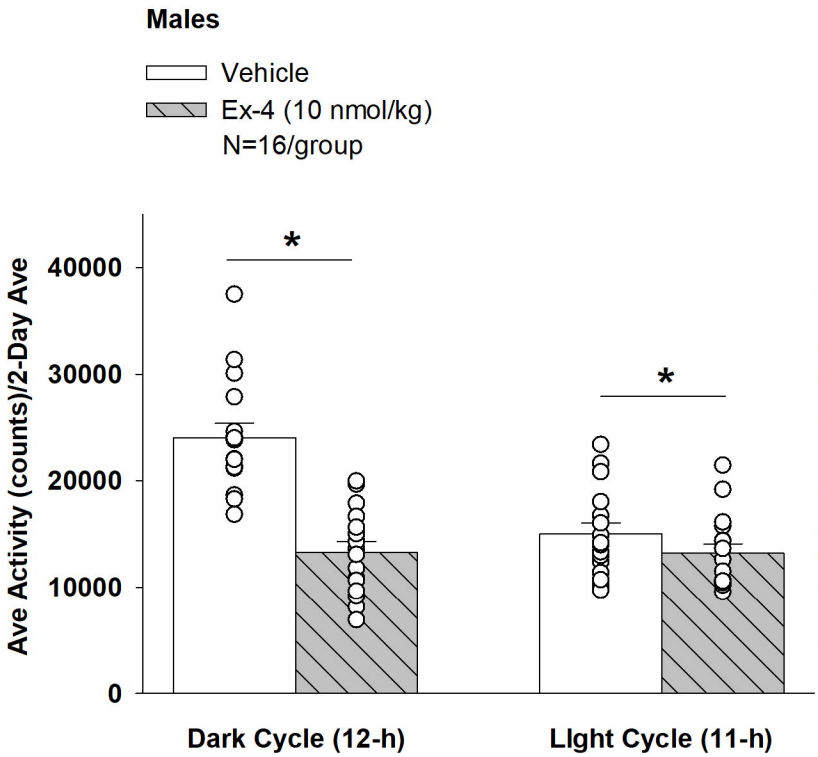


Figure 3B



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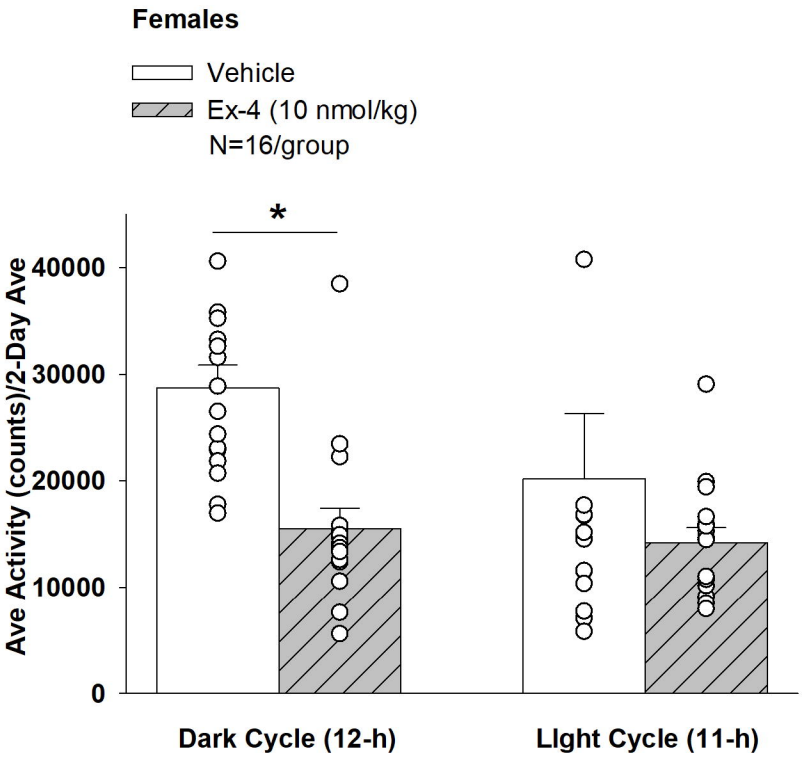
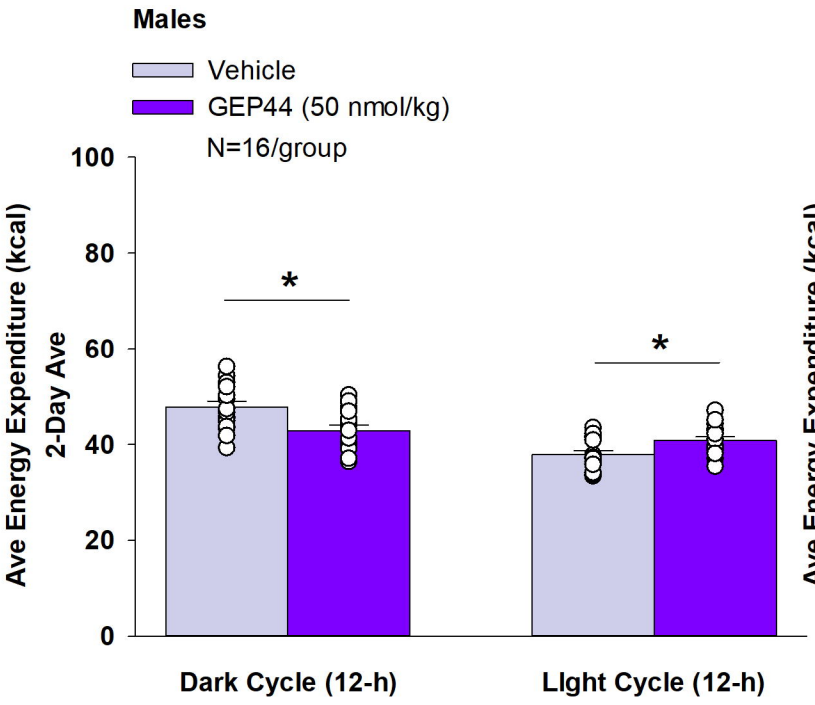


Figure 3D

Effects of GEP44 and Exendin-4 on Energy Expenditure in Male and Female HFD-Fed Rats



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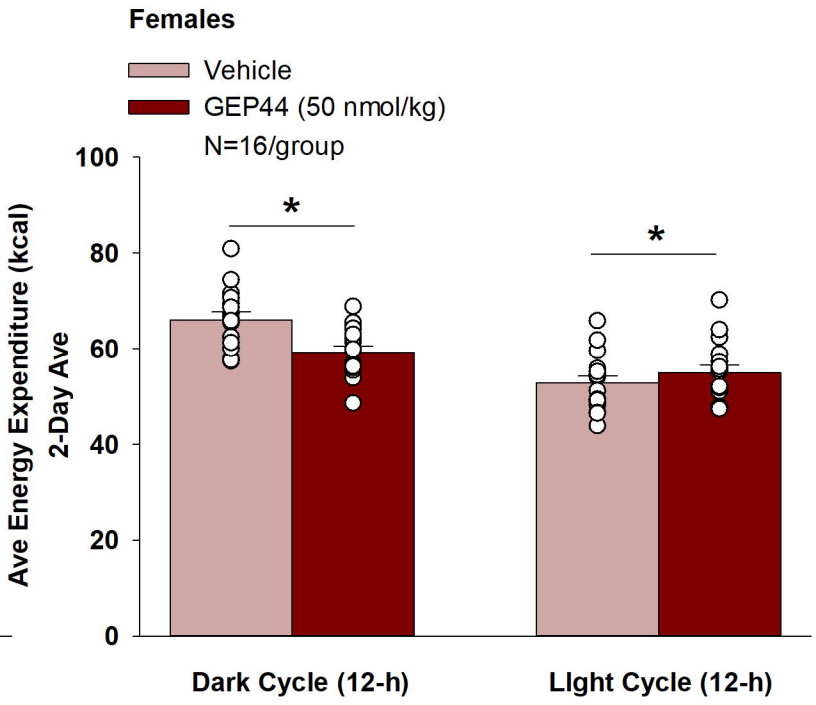

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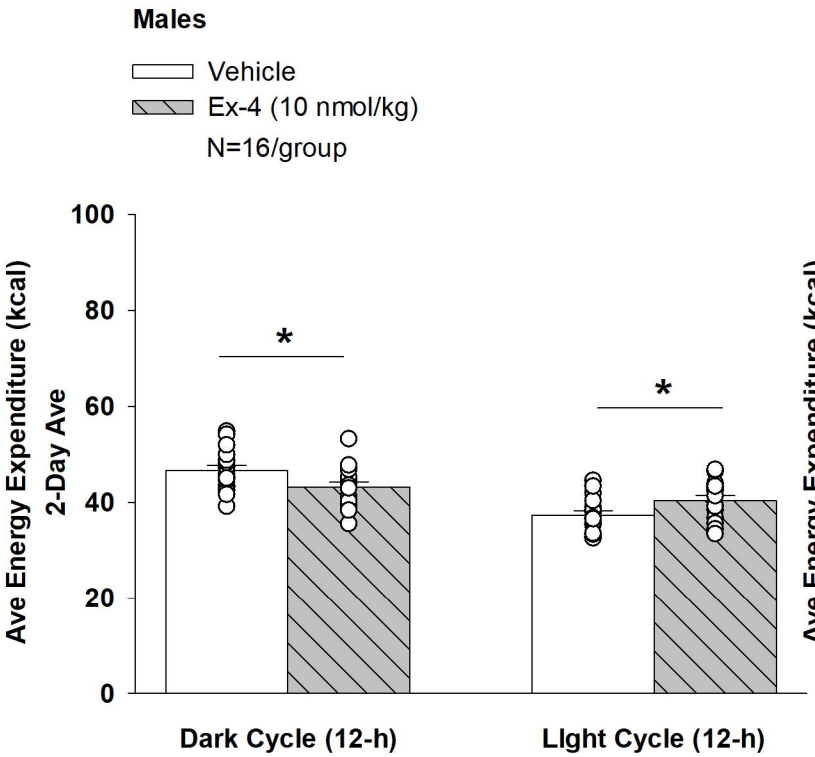

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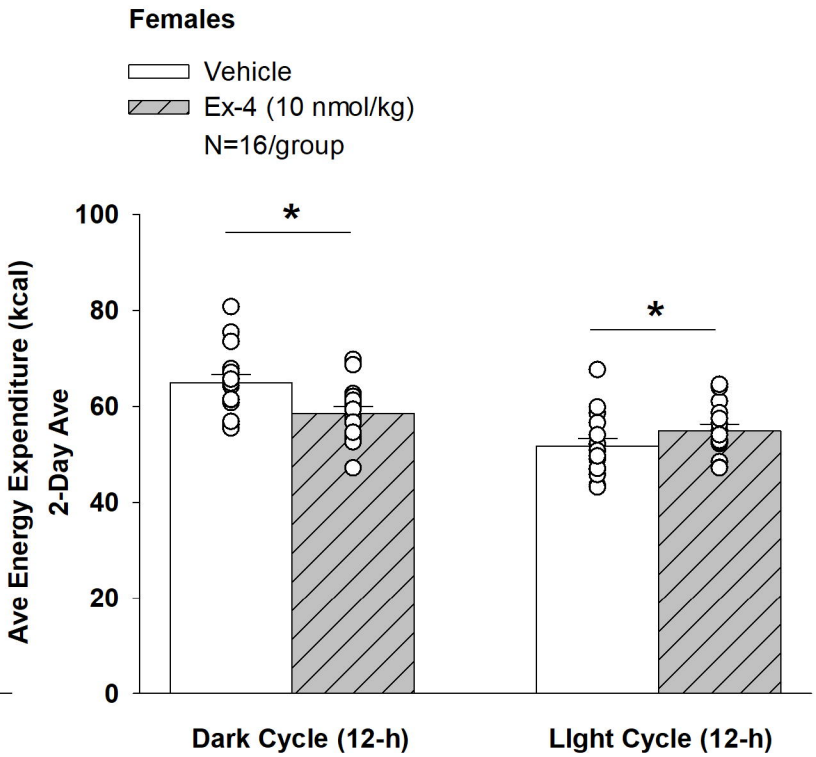


Figure 4D

Effects of GEP44 and Exendin-4 on Respiratory Exchange Ratio (RER) in Male and Female HFD-Fed Rats

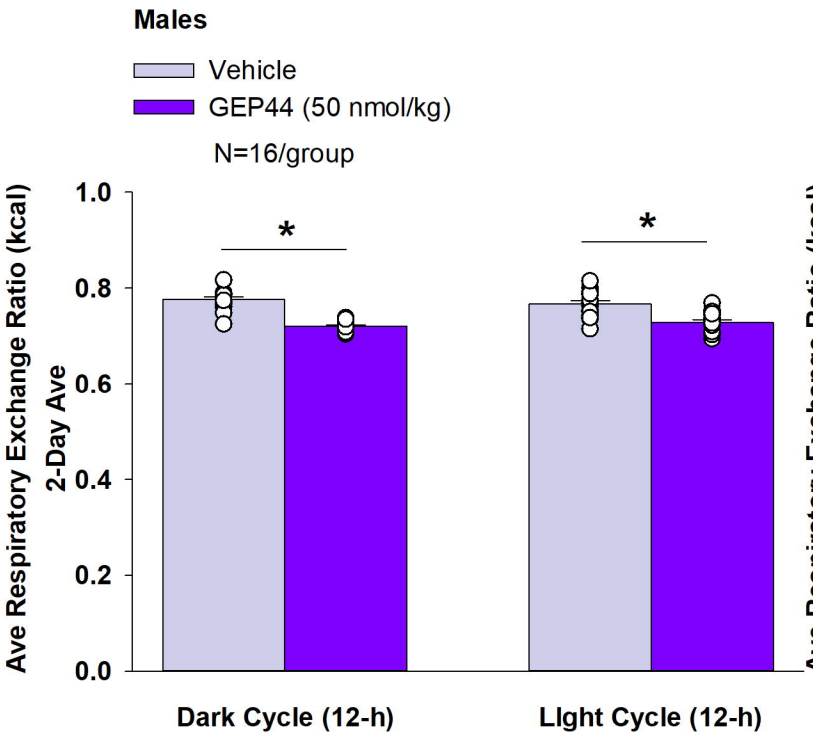


Figure 5A

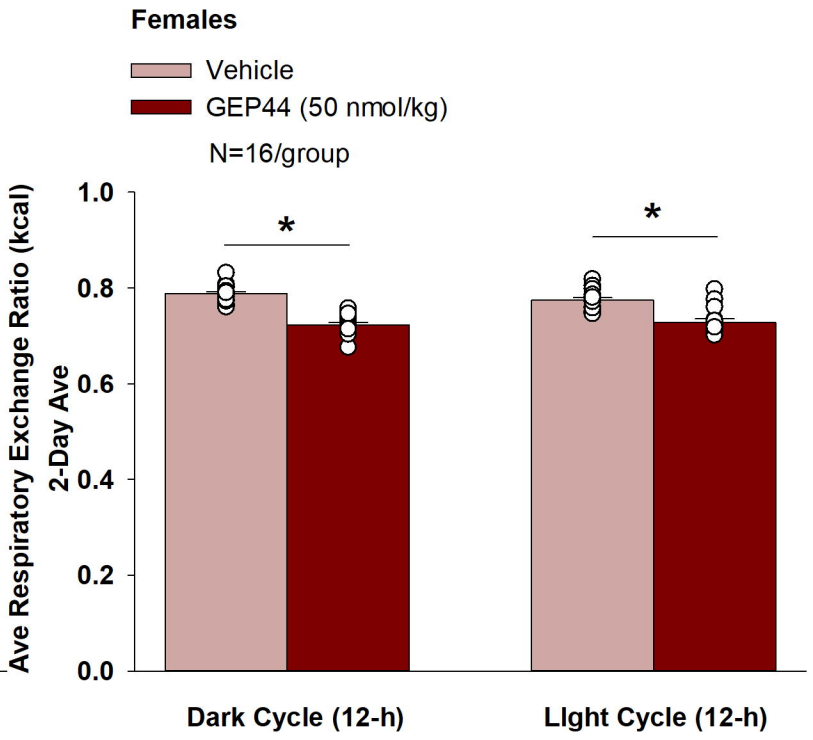


Figure 5B

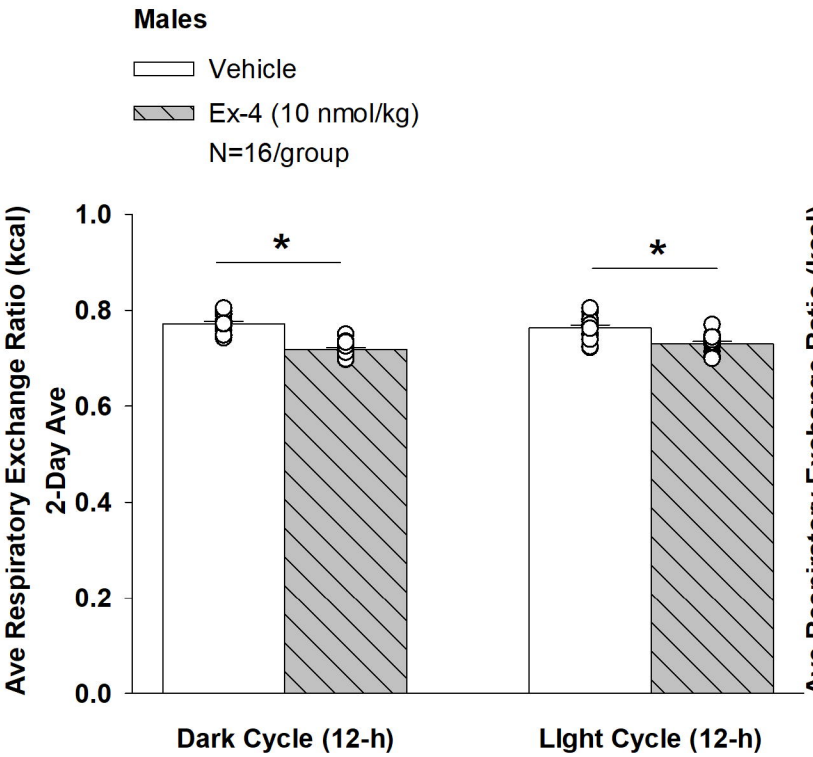


Figure 5C

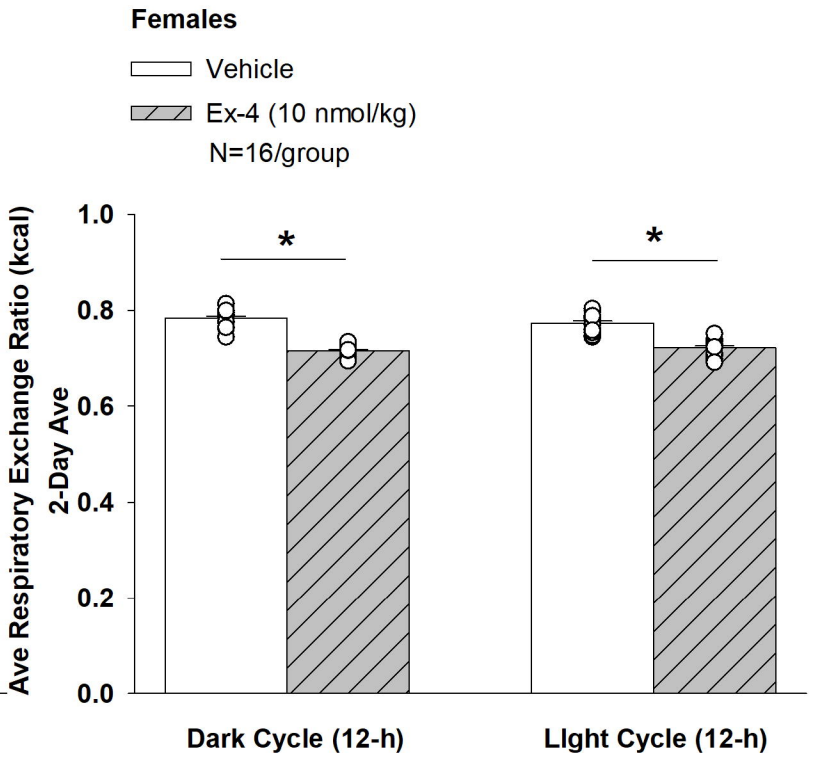


Figure 5D

Table 1. TaqMan probes used in qPCR analysis of IBAT and IWAT

Gene	Applied Biosystems Assay No.
Adrb1	Rn00824536_s1
Adrb3	Rn01478698_g1
Ucp1	Rn00562126_m1
Cidea	Rn04181355_m1
Dio2	Rn00581867_m1
Gpr120	Rn01759772_m1
Prdm16	Rn01516224_m1
Ppargc1a	Rn00580241_m1
Nono	Rn01418995_g1
Gapdh	Rn01775763_g1

TABLE 2. Observational Signs of Pain or Discomfort Following GEP44 and Exendin-4 Treatment

	VEH	GEP44	VEH	Exendin-4
Treatment Day 1				
Males				
Narrow eyes		+		++++++++++++
Flattened/Elongated nose		+		+++++++++++++++++
Females				
Narrow eyes		++++		+++++++++
Flattened/Elongated nose		+++		++++++++
Treatment Day 2				
Males				
Narrow eyes		+++		++++++++++++++++
Flattened/Elongated nose		++++		+++++++++++++++
Females				
Narrow eyes		++++		+++++++++
Flattened/Elongated nose		++++		++++++++++++

N=19/group (males)

N=23/group (females)

Table 3A. Changes in IBAT mRNA Expression Following GEP44 and Exendin-4 Treatment in Male DIO Rats

SC Treatment	Vehicle	Exendin-4	GEP44
IBAT			
Adrb1	1.0 ± 0.1	0.4 ± 0.03*	0.5 ± 0.1*
Adrb3	1.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.1
Ucp1	1.0 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
Cidea	1.0 ± 0.3	2.2 ± 0.3*	1.2 ± 0.2
Dio2	1.0 ± 0.3	0.8 ± 0.1	0.4 ± 0.1†
Gpr120	1.0 ± 0.3	0.5 ± 0.1	0.3 ± 0.1*
Prdm16	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.1
Ppargc1a	1.0 ± 0.1	1.4 ± 0.1*	1.0 ± 0.2

IBAT was collected in 6-h fasted rats at 2-h post-injection of vehicle, exendin-4 (10 nmol/kg) or GEP44 (50 nmol/kg).

***P*<0.05 vs vehicle**

†0.05<*P*<0.1 vs vehicle

N=6-7/group

Table 3B. Changes in IWAT mRNA Expression Following GEP44 and Exendin-4 Treatment in Male DIO Rats

Treatment	Vehicle	Exendin-4	GEP44
IWAT			
Adrb1	1.0 ± 0.1	1.1 ± 0.2	1.3 ± 0.2
Adrb3	1.0 ± 0.2	1.0 ± 0.3	1.9 ± 0.5
Ucp1	1.0 ± 0.2	2.5 ± 0.9	4.8 ± 1.9
Cidea	1.0 ± 0.1	1.1 ± 0.2	0.9 ± 0.1
Dio2	1.0 ± 0.2	0.8 ± 0.3	0.7 ± 0.2
Gpr120	1.0 ± 0.1	1.5 ± 0.2*	1.7 ± 0.2*
Prdm16	1.0 ± 0.1	1.2 ± 0.2	1.1 ± 0.1
Ppargc1a	1.0 ± 0.1	1.4 ± 0.1†	1.6 ± 0.2*

IWAT was collected in 6-h fasted rats at 2-h post-injection of vehicle, exendin-4 (10 nmol/kg) or GEP44 (50 nmol/kg).

***P*<0.05 vs vehicle**

†0.05<*P*<0.1 vs vehicle

N=6-7/group

Table 3C. Changes in IBAT mRNA Expression Following GEP44 and Exendin-4 Treatment in Female HFD-Fed Rats

SC Treatment	Vehicle	Exendin-4	GEP44
IBAT			
Adrb1	1.0 ± 0.2	0.4 ± 0.04*	0.4 ± 0.04*
Adrb3	1.0 ± 0.2	0.9 ± 0.2	1.2 ± 0.2
Ucp1	1.0 ± 0.2	0.6 ± 0.1†	0.5 ± 0.1*
Cidea	1.0 ± 0.2	1.5 ± 0.2	1.3 ± 0.3
Dio2	1.0 ± 0.1	0.4 ± 0.1*	0.3 ± 0.1*
Gpr120	1.0 ± 0.2	0.3 ± 0.1*	0.4 ± 0.1*
Prdm16	1.0 ± 0.1	1.4 ± 0.2	1.5 ± 0.2†
Ppargc1a	1.0 ± 0.2	1.2 ± 0.1	1.1 ± 0.1

IBAT was collected in 6-h fasted rats at 2-h post-injection of vehicle, exendin-4 (10 nmol/kg) or GEP44 (50 nmol/kg).

***P*<0.05 vs vehicle**

†0.05<*P*<0.1 vs vehicle

N=7-8/group

Table 3D. Changes in IWAT mRNA Expression Following GEP44 and Exendin-4 Treatment in Female HFD-Fed Rats

Treatment	Vehicle	Exendin-4	GEP44
IWAT			
Adrb1	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
Adrb3	1.0 ± 0.3	0.5 ± 0.2	0.6 ± 0.3
Ucp1	1.0 ± 0.4	2.4 ± 0.9	1.3 ± 0.5
Cidea	1.0 ± 0.4	1.0 ± 0.2	0.9 ± 0.3
Dio2	1.0 ± 0.3	0.4 ± 0.1†	0.4 ± 0.05*
Gpr120	1.0 ± 0.3	1.8 ± 0.3†	0.9 ± 0.3
Prdm16	1.0 ± 0.2	1.0 ± 0.1	0.8 ± 0.1
Ppargc1a	1.0 ± 0.4	2.5 ± 2.0	1.6 ± 0.6

IWAT was collected in 6-h fasted rats at 2-h post-injection of vehicle, exendin-4 (10 nmol/kg) or GEP44 (50 nmol/kg).

***P*<0.05 vs vehicle**

†0.05<*P*<0.1 vs vehicle

N=7-8/group

Table 4A. Plasma Measurements Following SC Administration of GEP44 or Exendin-4 in Male DIO Rats

SC Treatment	Vehicle	GEP44	Exendin-4
Leptin (ng/mL)	70.6 ± 11.2	85.4 ± 10.4	80.1 ± 8.8
Insulin (ng/mL)	2.2 ± 1.1	5.3 ± 0.9*	3.4 ± 0.6
Glucagon (pmol/L)	6.3 ± 1.0	5.2 ± 0.8	3.4 ± 0.6*
Adiponectin (µg/mL)	10.9 ± 0.7	5.8 ± 0.6*	7.3 ± 0.5*
Irisin (µg/mL)	5.4 ± 0.4	7.1 ± 0.6†	7.4 ± 0.9†
FFA (mEq/L)	0.38 ± 0.2	0.66 ± 0.04	0.75 ± 0.11
Total Cholesterol (mg/dL)	89.0 ± 8.9	120 ± 9.6*	111.8 ± 11.9
Tail Vein Glucose (mg/dL)	139.8 ± 7.5	121 ± 6.6*	115.3 ± 7.4*

Blood was collected in 6-h fasted rats by tail vein nick (glucose) or cardiac stick (leptin, insulin, glucagon, adiponectin, irisin, FFA, and total cholesterol) at 2-h post-injection of vehicle, exendin-4 (10 nmol/kg) or GEP44 (50 nmol/kg).

*P<0.05 vs vehicle

†0.05<P<0.1 vs vehicle

N=6-7/group

Table 4B. Plasma Measurements Following SC Administration of GEP44 or Exendin-4 in Female High Fat Diet-Fed Rats

SC Treatment	Vehicle	GEP44	Exendin-4
Leptin (ng/mL)	16.3 ± 2.8	20.2 ± 5.5	30.3 ± 8.5
Insulin (ng/mL)	0.6 ± 0.04	4.2 ± 1.1*	2.1 ± 0.5*
Glucagon (pmol/L)	46.6 ± 6.5	37.0 ± 8.0	40.9 ± 4.1
Adiponectin (µg/mL)	11.0 ± 0.9	5.8 ± 0.3*	6.5 ± 0.9*
Irisin (µg/mL)	3.1 ± 0.3	4.6 ± 0.7†	4.9 ± 0.8†
FGF-21 (pg/mL)	80.9 ± 18.6	440.5 ± 81.9*	458.2 ± 65.5*
FFA (mEq/L)	0.36 ± 0.06	0.74 ± 0.09*	0.56 ± 0.07†
Total Cholesterol (mg/dL)	67.1 ± 4.0	85.5 ± 8.3†	78.6 ± 7.0
Tail Vein Glucose (mg/dL)	119.1 ± 6.0	92.5 ± 6.8*	118.3 ± 8.9

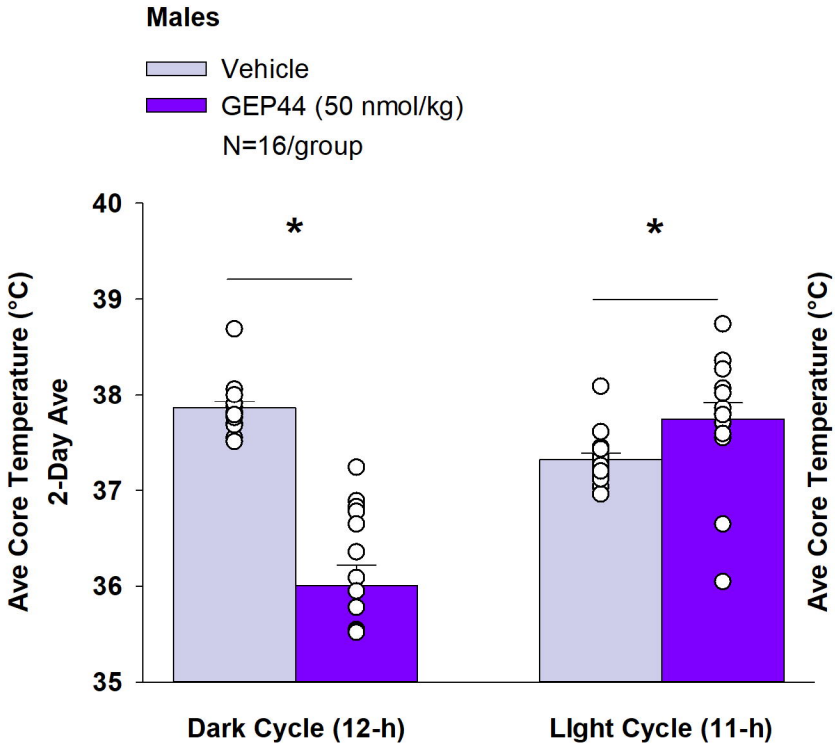
Blood was collected in 6-h fasted rats by tail vein nick (glucose) or cardiac stick (leptin, insulin, glucagon, adiponectin, irisin, FFA, and total cholesterol) at 2-h post-injection of vehicle, exendin-4 (10 nmol/kg) or GEP44 (50 nmol/kg).

*P<0.05 vs vehicle

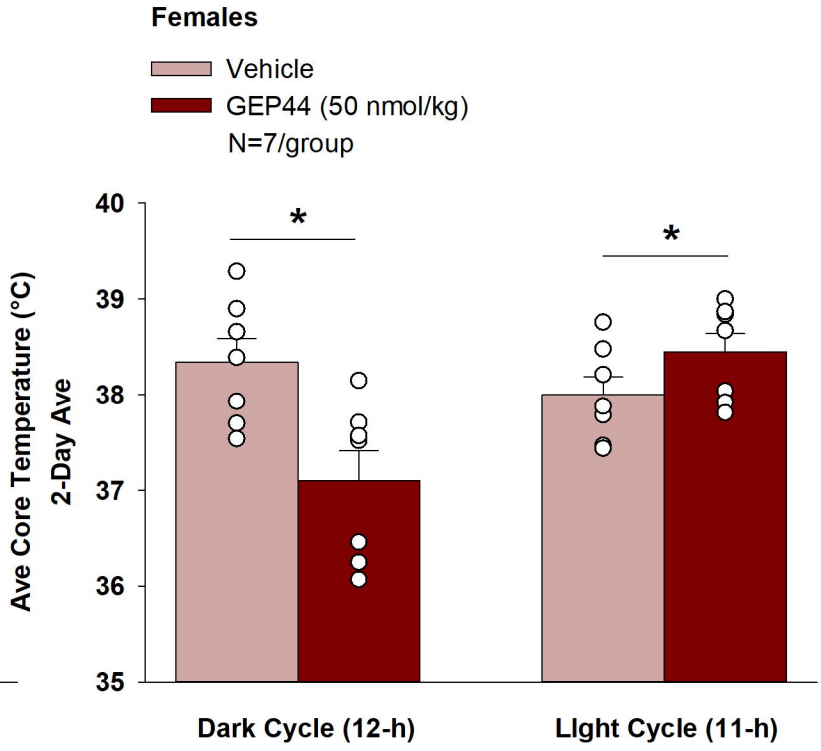
†0.05<P<0.1 vs vehicle

N=7-8/group

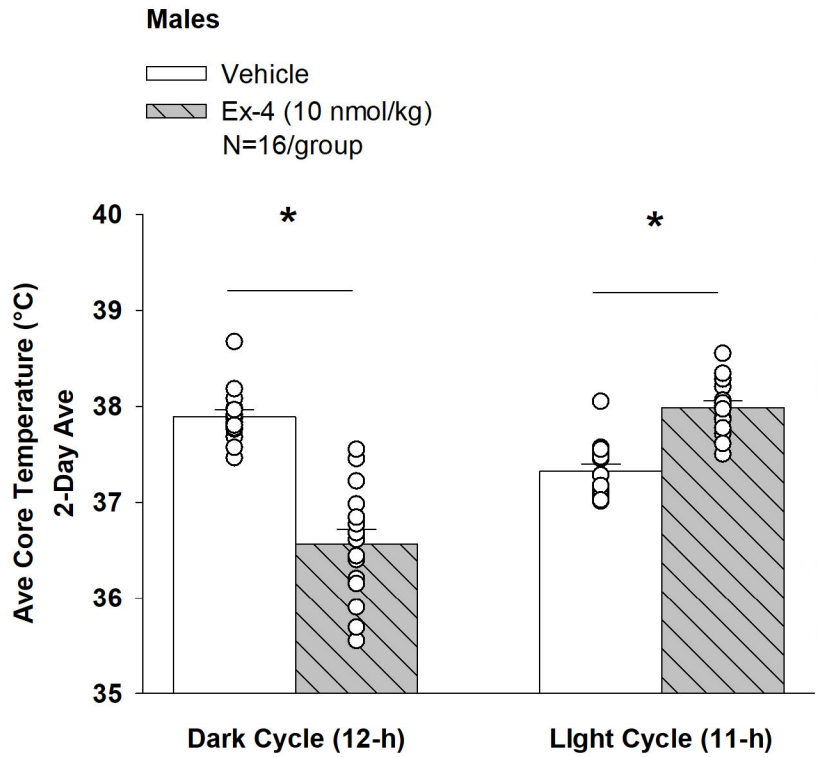
Effects of GEP44 and Exendin-4 on Core Temperature in Male and Female HFD-Fed Rats



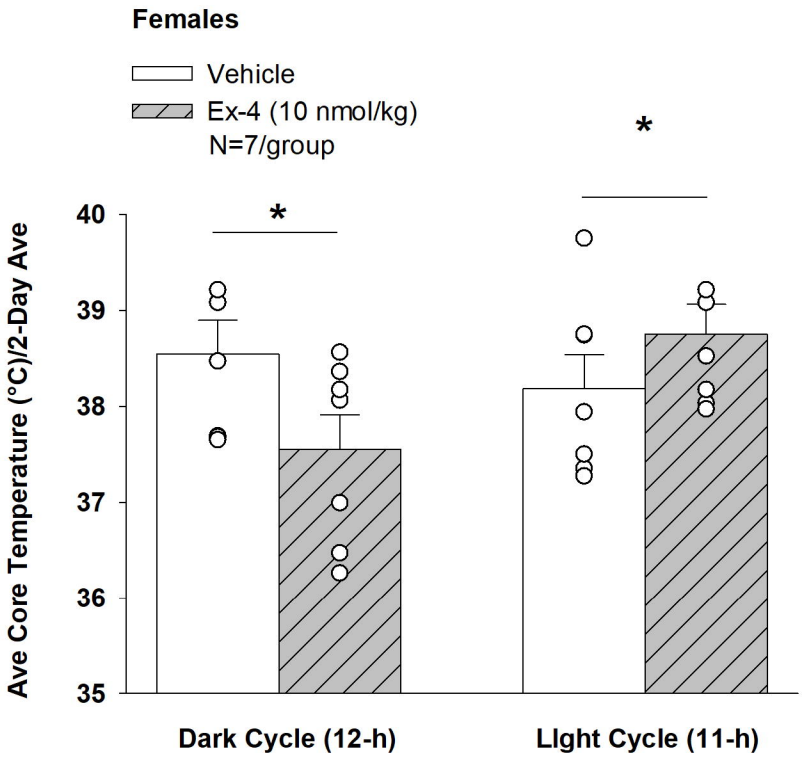
Supplemental Figure 1A



Supplemental Figure 1B

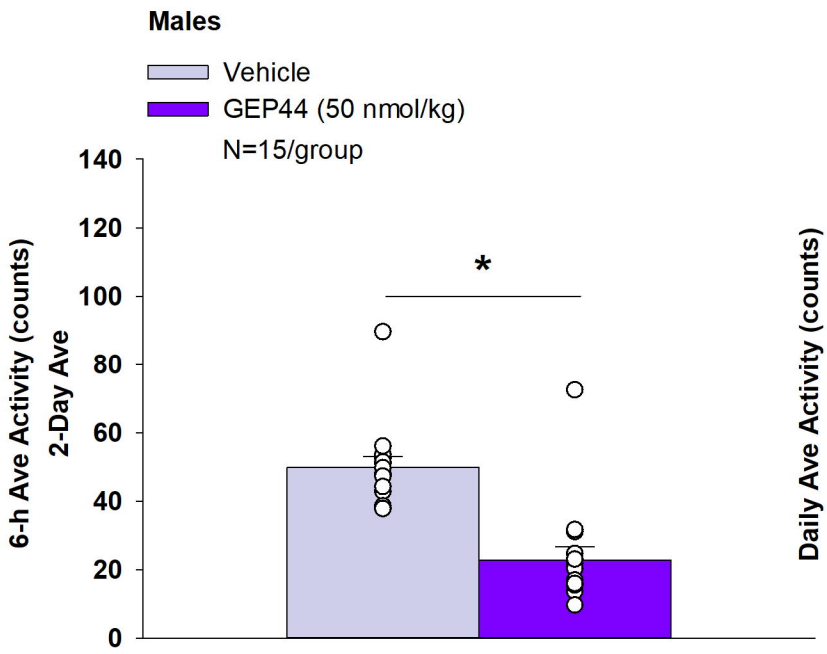


Supplemental Figure 1C

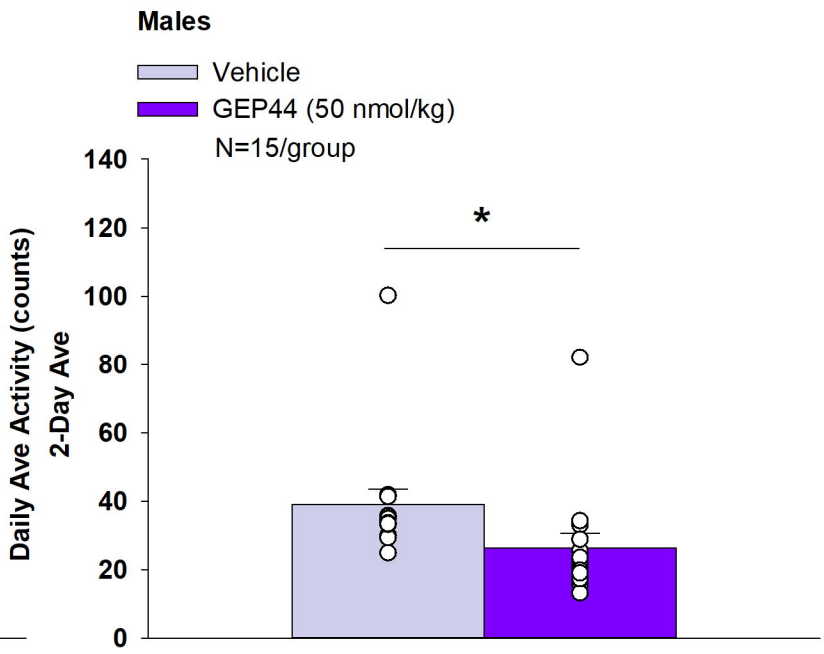


Supplemental Figure 1D

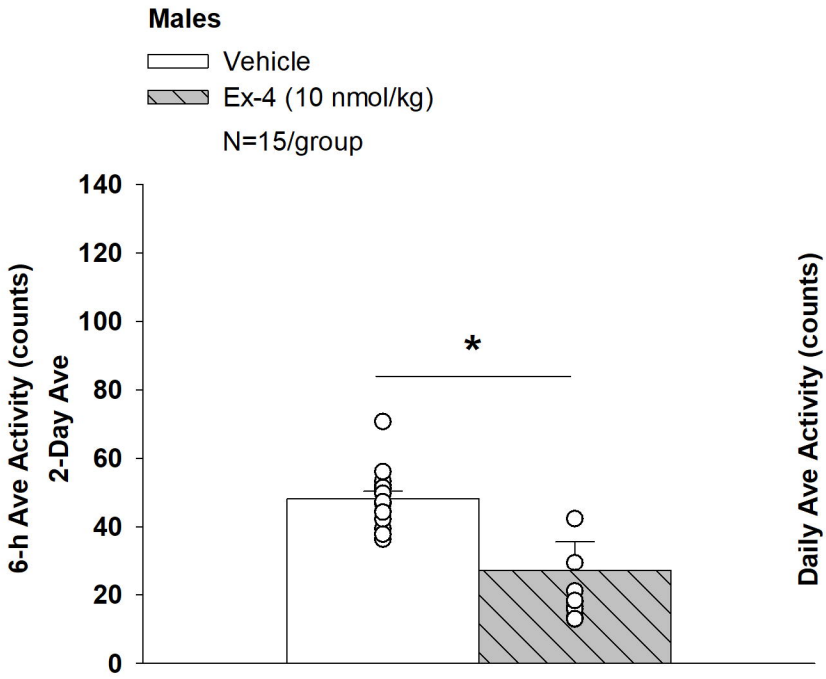
Effects of GEP44 and Exendin-4 on Activity in Male HFD-Fed Rats



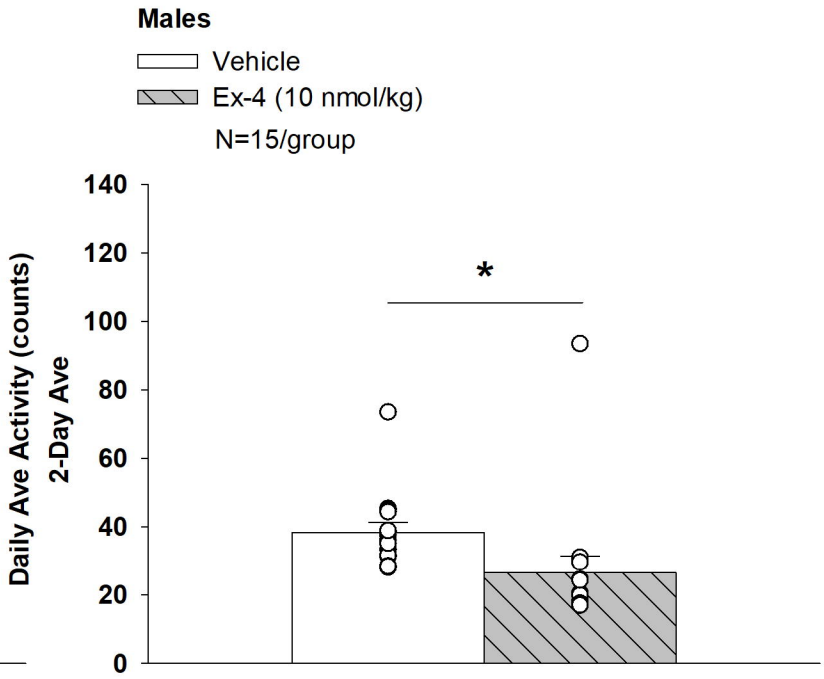
Supplemental Figure 2A



Supplemental Figure 2B

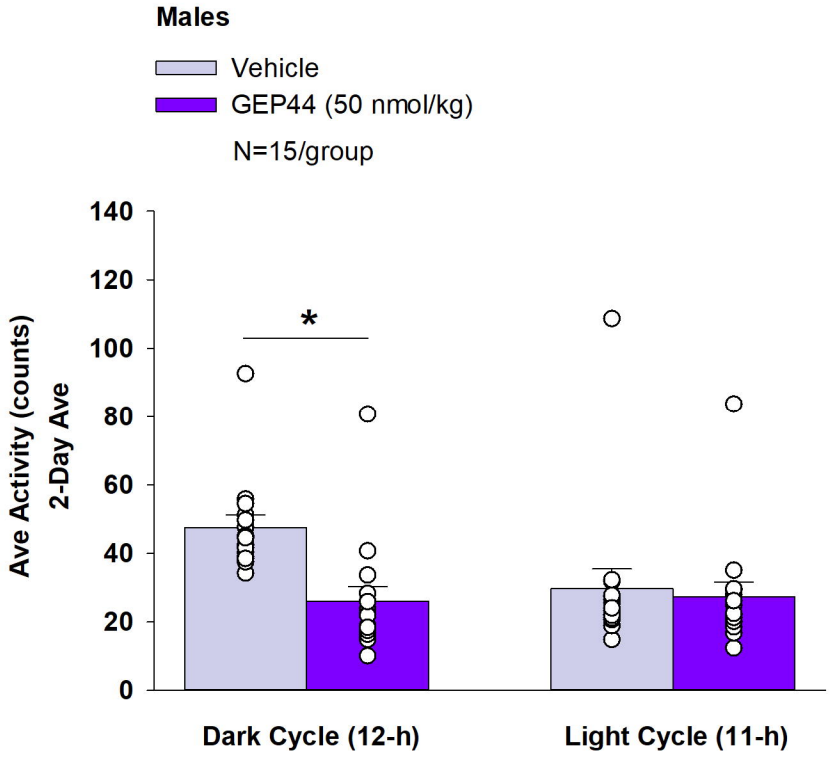


Supplemental Figure 2C

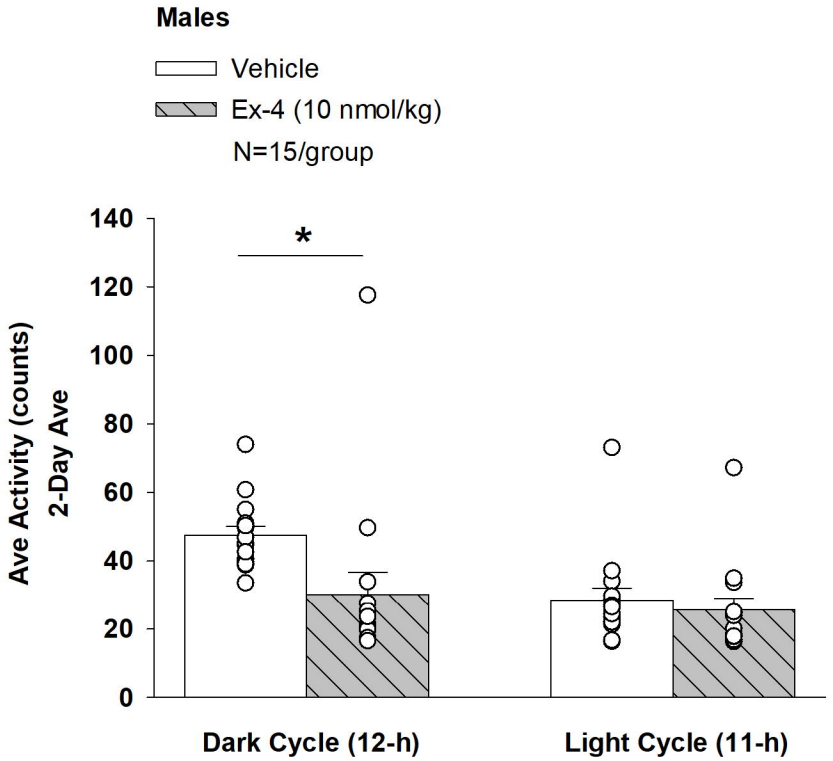


Supplemental Figure 2D

Effects of GEP44 and Exendin-4 on Activity in Male HFD-Fed Rats



Supplemental Figure 3A



Supplemental Figure 3B

Supplemental Table 1. Range of detectability for plasma hormones

Plasma hormone	Range of Detectability
Adiponectin	0.25-10 ng/mL
FGF-21	0.0313-2 ng/mL
Glucagon	0.00000696-0.000633 ng/mL
Insulin	0.069-50 ng/mL
Irisin	78-5,000 ng/mL
Leptin	0.07-51.9 ng/mL