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Full title: Chronic CNS Oxytocin Signaling Preferentially Induces Fat Loss in High Fat Diet-Fed Rats by Enhancing Satiety Responses and Increasing Lipid Utilization

Abbreviated title: Oxytocin Reduces Body Adiposity in High Fat Diet-Fed Rats

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Abstract:

Based largely on a number of short-term administration studies, growing evidence suggests that central oxytocin is important in the regulation of energy balance. The goal of the current work is to determine if long-term third ventricular (3V) infusion of oxytocin into the CNS is effective for obesity prevention and/or treatment in rat models. We found that chronic 3V oxytocin infusion between 21-26 days by osmotic minipumps both reduced weight gain associated with the progression of high fat diet (HFD)-induced obesity and elicited a sustained reduction of fat mass with no decrease of lean mass in rats with established diet-induced obesity. We further demonstrated that these chronic oxytocin effects result from *1)* maintenance of energy expenditure at pre-intervention levels despite ongoing weight loss, *2)* a reduction in respiratory quotient, consistent with increased fat oxidation, and *3)* an enhanced satiety response to CCK-8 and associated decrease of meal size. These weight-reducing effects persisted for approximately 10 days after termination of 3V oxytocin administration and occurred independently of whether sucrose was added to the HFD. We conclude that long-term 3V administration of oxytocin to rats can both prevent and treat diet-induced obesity.

Introduction

Published data suggest that in addition to its well-recognized peripheral effects on uterine contraction during parturition and milk ejection during lactation (33), the nonapeptide oxytocin plays an important role in the regulation of energy homeostasis (23, 53, 59, 103, 104). Transgenic mice with deficient oxytocin (18) or oxytocin receptor (OTR) signaling (93) exhibit adult-onset obesity, and copy number variations associated with the OTR gene (*OXTR*) are linked with an early-onset obesity phenotype in humans (96). Furthermore, impaired oxytocin release within the hypothalamic paraventricular nucleus (PVN) is evident in diet-induced obese (DIO) mice (103), which could lead to defects in peripheral release of oxytocin, and potentially explain the decreased circulating levels in DIO mice (103, 104), genetically obese rodents (32, 73), as well as obese humans and individuals with type 2 diabetes (74). Moreover, the pathogenesis of Prader-Willi syndrome, a rare human genetic disorder characterized by hyperphagia and severe obesity, is linked to a reduced size and number of PVN oxytocin neurons (91). Importantly, both acute and chronic administration of oxytocin is sufficient to bypass impaired leptin signaling to reduce weight gain and body weight in both DIO (23, 53, 59, 103, 104) and genetically obese rodent models (1, 42, 47, 54, 59, 73) as well as weight loss in DIO rhesus monkeys (10) and humans (105). While collectively these findings are indicative of an important physiological role for oxytocin in energy homeostasis, the mechanisms underlying this function have not been fully elucidated.

The effects of CNS administration of oxytocin to reduce body weight gain over 7-15 days in DIO mice and rats are known (23, 103, 104); however, its effectiveness to elicit sustained weight loss during chronic administration into the CNS beyond 15 days

has not been examined. This is a key unanswered question because the potential efficacy of oxytocin therapy as a treatment for obesity cannot be assessed without understanding the effects of chronic administration. It's been established that the anorectic effects of other peptides, such as exendin-4, peptide YY(3-36), and melanotan II (MTII), dissipate with chronic administration (52, 72, 75, 85). In the case of oxytocin, it's not known whether chronic CNS administration can prevent weight gain associated with the progression of diet-induced obesity or whether weight loss under oxytocin administration is specific for fat while sparing lean mass. Information about these critical questions is a prerequisite for understanding the mechanisms of oxytocin's effects on energy homeostasis and its potential for therapeutic use to treat obesity. The present article reports studies and data on rats that focus on these key issues.

Decreased food intake appears to contribute to the ability of oxytocin to reduce body weight in rodents (23, 53, 59, 103, 104). Oxytocin reduces consumption of not only low fat/high carbohydrate diets (1, 3, 4, 10, 23, 40, 53-55, 59, 66, 76, 103, 104), including sugars (10, 51, 61), but also high fat diets (HFDs) (23, 53, 59, 103, 104). Conversely, defective oxytocin signaling is linked to increased intake of fat (103, 104) and carbohydrates, including sugar (2, 38, 51, 61, 68) suggesting a physiological role for oxytocin to limit consumption of fat and sugar. However, existing data has failed to establish the extent to which exogenous oxytocin may preferentially reduce consumption of specific macronutrients. We therefore also sought to better understand whether oxytocin-mediated weight loss is dependent on the content of sucrose or fat.

Recent studies indicate that in addition to suppressing food intake, oxytocin may also reduce body weight in rodents and nonhuman primates by increasing energy

expenditure (10, 63, 90, 99, 102-104). Conversely, animals with either partial or complete loss in oxytocin signaling show decreased energy expenditure (18, 45, 93, 99, 104) and brown adipose tissue thermogenesis (18, 45, 93). Whether changes of energy expenditure contribute to the anti-obesity effect of oxytocin in rats with established diet-induced obesity, however, has not been fully established. Thus, we also investigated whether changes of energy expenditure contribute to oxytocin-mediate weight reduction in DIO rats.

Our findings demonstrate that chronic 3V oxytocin infusion (≈ 21-26 days) both reduces weight gain associated with the progression of diet-induced obesity and elicits sustained weight loss in rats with established diet-induced obesity, that this effect arises from reduced fat mass with no loss of lean mass, and that these chronic oxytocin effects are due, in part, to the fact that energy expenditure is maintained at pre-intervention levels despite ongoing weight loss. Chronic oxytocin treatment was also associated with evidence of increased fat oxidation as well as an enhanced satiety response to CCK and an associated reduction of meal size. In addition, we show that the effectiveness of oxytocin to reduce weight gain occurs independently of whether sucrose is added to the HFD. Following cessation of treatment, weight gain in oxytocin-treated animals remains below vehicle-treated controls for ≈ 10 days. Lastly, we demonstrate that, like chronic CNS administration, chronic subcutaneous administration of oxytocin is sufficient to reduce body weight gain in animals maintained on a HFD at a dose that does not appear to elicit aversive behavioral responses (e.g. nausea or malaise).

Methods

Animals

Adult male Sprague-Dawley (SD-SAS and CD® IGS) (≈ 2.5-6 months/323-887 g) were obtained from Charles River Laboratories International, (Wilmington, MA). CD® IGS rats were used for all central infusion studies as well as for the 3-day peripheral infusion study and 2-h saccharin preference ratio study whereas SD-SAS rats were used for all other peripheral infusion studies. By design, we chose the CD® IGS rat model because they grow more rapidly in comparison to SD-SAS rats and are thus are a more suitable model to determine if chronic increases in CNS oxytocin signaling (>15 days) are sufficient to prevent or delay the progression of diet-induced obesity over the course of the 26-day minipump infusion. SD-SAS rats were used to extend previous studies (59) to determine if chronic systemic infusions of oxytocin can recapitulate the effects of CNS administration to reduce food intake and weight gain at doses that do not elicit nausea or malaise. All animals were housed individually in Plexiglas cages in a temperature controlled room under a 12:12-h light-dark cycle (lights off at 1 p.m.). Animals had *ad libitum* access to water and either a low fat chow diet containing 13% kcal from fat (Harlan Teklad, Madison, WI) or a HFD containing 60% kcal from fat (Research Diets, D12492, New Brunswick, NJ), unless otherwise stated. A HFD containing 60% kcal from fat lacking sucrose was used in **Study 7** and **Study 8** (Research Diets, D08060104; corn starch replaced sucrose in D12492). Kaolin pellets were also purchased from Research Diets, Inc. The current research protocols were approved both by the Institutional Animal Care and Use Committee of the Veterans

Affairs Puget Sound Health Care System (VAPSHCS) and the University of Washington in accordance with NIH Guidelines for the Care and Use of Animals.

Drug Preparation

Fresh solutions of oxytocin acetate salt (Bachem Americas, Inc., Torrance, CA) were prepared the day of each experiment. Oxytocin was solubilized in sterile water and diluted with sterile saline. CCK-8 (Bachem) was dissolved in saline with 0.1% BSA (Bachem Americas).

3V cannulations

Animals were implanted with a cannula within the 3V with a side port that was connected to an osmotic minipump (model 2004, DURECT Corporation, Cupertino, CA). Briefly, animals under isoflurane anesthesia were placed in a stereotaxic apparatus with the incisor bar positioned 3.3 mm below the interaural line. A 26-gauge cannula (Plastics One Inc., Roanoke, VA) was stereotaxically positioned into the 3V [8.1 mm anterior to the interaural line; 0 mm lateral to the midline, and 8.6 mm ventral to the skull surface] and secured to the surface of the skull with dental cement and stainless steel screws. A 2.4” piece of plastic tubing (Tygon^{TM} Microbore Tubing, 0.020" x 0.060"OD, 100 ft./roll; Cole-Parmer, Vernon Hills, IL) was tunneled subcutaneously along the midline of the back and connected to the 21-gauge sidearm osmotic minipump-cannula assembly. A stainless steel 22-gauge pin plug (Instech Laboratories, Inc., Plymouth Meeting, PA) was temporarily inserted at the end of the tubing during a two week postoperative recovery period, after which it was replaced by an osmotic minipump

(DURECT Corporation) containing saline or oxytocin. Animals were treated with the analgesic ketoprofen (5 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and the antibiotic baytril (5 mg/kg; Patterson Veterinary, Devens, MA) at the completion of the 3V cannulations and were allowed to recover at least 10 days prior to implantation of osmotic minipumps.

Subcutaneous osmotic minipump implantations for systemic delivery

Osmotic minipumps (model 1003D or 2002, DURECT Corporation) were implanted subcutaneously along the midline of the back into animals that were placed under isoflurane anesthesia. The interscapular incision was closed with standard metal wound clips.

CCK-8 injections

Intraperitoneal (IP) injections were administered to rats via a 1.0 mL syringe with a 25-gauge needle (1 mL/kg injection volume) immediately prior to the start of the dark cycle, i.e. at a time when the animals normally begin eating and when CCK-8 has a potent effect on reducing food intake.

Body Composition

Determinations of lean body mass and fat mass were made on chow and HFD-fed animals, including HFD-fed DIO animals, by quantitative magnetic resonance using an EchoMRI 4-in-1^{TM}-700 instrument (Echo Medical Systems, Houston, TX) at the VAPSHCS Rodent Metabolic and Behavioral Phenotyping Core. Body composition

measurements were also made in DIO rats using an EchoMRI™-700 instrument (Echo Medical Systems, Houston, TX) at the University of Washington (UW) Nutrition Obesity Research Center Energy Balance and Glucose Metabolism Core.

Indirect calorimetry, locomotor activity and meal pattern measurements

Energy expenditure, locomotor activity, and meal pattern measures were obtained using a computer controlled indirect calorimetry system (Promethion; Sable Systems International, Las Vegas, NV) located in the Energy Balance and Glucose Metabolism Core of the Nutrition Obesity Research Center at the UW as previously described (59). Calorimetry cages (similar to home cages with bedding) were each equipped with water bottles and food hoppers connected to load cells for continuous food and water intake monitoring and housed in a temperature- and humidity-controlled Caron environmental chambers (Caron Products and Services, Marietta, OH). Respiratory quotient (RQ) was calculated as the ratio of CO_2 production over O_2 consumption. Energy expenditure was calculated using the Weir equation: kcal/h = 60 × (0.003941 × $\dot{V}o_2$ +0.001106 × $\dot{V}co_2$) (95) and expressed in units of kilocalories per hour. Ambulatory activity and meal patterns were determined simultaneously with the collection of the calorimetry data (59). Consecutive adjacent infrared beam breaks in the *y*-axes, i.e. the length of the cage, were scored as an activity count and a tally was recorded every 10 min. Meal patterns were defined using the following criteria: An individual meal was defined based on our previously published criteria (58) of at least 0.2 g of HFD that was separated from the end of the previous meal by at least 15 min. Daily food intake data were analyzed using a computer program (ExpeData version 1.7.22, Sable Systems

International). Meal duration was defined as the time from the beginning to the end of a single meal, and the inter-meal interval as the time from the end of one meal to the beginning of the next. Average meal size was calculated by dividing the number of feeding bouts (individual meals) by the total amount of food consumed (in grams) over the 72-h test period.

Study Protocols

Study 1: Effects of chronic 3V oxytocin infusions on food intake, body weight gain, and body composition in HFD-fed and chow-fed rats.

3V cannulated rats received implantations of 28-day minipumps to infuse vehicle (saline) or oxytocin (16 nmol/day) directly into the brain and were maintained on either chow or HFD for 26 days. Food intake and body weight were recorded daily in ad libitum fed rats over 26 days. Dosing was based on recently published data in a DIO rat model (23).

Study 2: Effects of chronic 3V oxytocin infusions on kaolin intake in chow-fed rats.

Ad libitum fed rats were maintained on chow prior to receiving implantations of 3V cannulas and 28-day minipumps to infuse vehicle (saline) or oxytocin (16 nmol/day). The amount of kaolin intake (g) was assessed across 15 days. The placement of kaolin and chow were reversed every other day within each treatment condition.

Study 3: Effects of chronic 3V oxytocin infusions on food intake, body weight gain, and body composition in HFD-fed and chow-fed rats.

Ad libitum fed rats were maintained on chow or HFD for 2 and 2.5 months prior to receiving implantations of 3V cannulas and 28-day minipumps to infuse vehicle (saline) or oxytocin (16 nmol/day), respectively (2-week interval between 3V cannula and minipump implantations). Food intake and body weight were recorded daily over 26 days.

Study 4: Effects of chronic 3V oxytocin infusions on food intake, body weight, and body composition in rats with established diet-induced obesity.

Ad libitum fed rats were maintained on chow or HFD for 4 and 4.5 months prior to receiving implantations of 3V cannulas and 28-day minipumps to infuse vehicle (saline) or oxytocin (16 nmol/day) over 21 days, respectively. Daily body weight was recorded on days 1-5, 9, 12, 14, 16, and 20-21. Daily food intake was recorded on days 1-5 and 21 while 72 h measurements were completed between days 9-12.

Study 5: Effects of chronic 3V oxytocin infusions on energy expenditure, locomotor activity, RQ, and meal patterns in DIO rats.

Ad libitum fed rats were maintained on a HFD for 4 and 4.5 months prior to receiving implantations of 3V cannulas and 28-day minipumps to infuse vehicle (saline) or oxytocin (16 nmol/day), respectively. DIO rats (from **Study 3A**) were acclimated to the Sable Systems indirect calorimetry cages for approximately 1 week prior to continuous

281 measurement of energy intake for meal pattern analysis (58, 59), energy expenditure
282 (59), locomotor activity (59), and RQ (59) between infusion days 12-14.
283
284 **Study 6: Effects of chronic 3V oxytocin infusions on CCK-8-induced satiety in**
285 **HFD-fed rats.**
286 Rats received implantations of 3V cannulas and 28-day minipumps prior to receiving
287 vehicle (saline) or oxytocin (16 nmol/day) and were maintained on a 6-h fast. Following
288 minipump implantations, rats were immediately placed on the HFD. Beginning on
289 infusion day 2, animals received an IP injection of either vehicle or CCK-8 (0.25, 0.5, 1,
290 2 nmol/kg, IP) immediately prior to the start of the dark cycle at 48-h intervals. Food
291 intake was measured at 0.5 h following access to food and the start of the dark cycle.
292
293 **Study 7: Effects of chronic 3V oxytocin infusions on food intake, body weight**
294 **gain, and body composition in DIO rats maintained on a HFD lacking sucrose.**
295 Ad libitum fed rats were maintained on HFD lacking sucrose for 4 and 4.5 months prior
296 to receiving implantations of 3V cannulas and 28-day minipumps to infuse vehicle
297 (saline) or oxytocin (16 nmol/day) over 28 days, respectively. Daily food intake and body
298 weight were recorded in 3-h fasted animals over 28 days.
299
300
301
302

Study 8: Effects of treatment cessation on food intake, body weight gain, and body composition in DIO rats maintained on a HFD lacking sucrose.

Following completion of **Study 7** on day 28, a subset of animals was euthanized and the remaining DIO animals had minipumps removed on day 38. Daily food intake and body weight were recorded in 3-h fasted animals for an additional 28 days.

Study 9: Effects of chronic subcutaneous oxytocin infusions on food intake, body weight gain, 2-h saccharin preference ratio and kaolin intake in chow-fed rats.

Ad libitum fed rats were implanted with 3- (0, 50, 100, 200 nmol oxytocin/day) or 14-day osmotic minipumps (0, 50 nmol oxytocin/day). Daily food intake and body weight were measured across the 3- and 12-day infusion period. Dosing was based on recently published data in a DIO rat model (23).

2-bottle saccharin preference test

Following implantation of the 3-day osmotic minipumps [0, 50, 100, 200 nmol oxytocin/day], both 0.1% saccharin solution and water were made available to the animals throughout the 3-day infusions. The next day (day 4) water was removed 2 h before onset of the dark cycle, and animals were presented with both water and saccharin solution at the onset of the dark cycle. Intake of water and saccharin were recorded over 2 h. The placement of water and saccharin was counterbalanced within each treatment condition. Saccharin was removed after the 2-h test. Preference ratios were calculated as the saccharin intake/water intake + saccharin intake (7, 30).

Kaolin intake test

The amount of kaolin intake (g) was assessed across 12 days following implantation of minipumps containing vehicle (saline) or oxytocin [50 nmol/day (subcutaneous)]. The placement of kaolin and chow were reversed every other day within each treatment condition.

Study 10: Effects of chronic subcutaneous oxytocin infusions on body weight gain in HFD-fed and chow-fed rats.

Ad libitum fed rats that had been maintained on chow were implanted subcutaneously with 14-day osmotic minipumps to infuse vehicle (saline) or oxytocin (50 nmol/day) systemically. Animals were then placed on either chow or HFD for the remainder of the 13-day infusion period. Daily food intake and body weight were measured across the 13-day infusion period.

Blood collection

Blood was collected in either 3 (**Study 4**) or 6-h (**Study 10**) fasted rats at the end of the light cycle within a 2 h window (11:00 a.m.-1:00 p.m.). Treatment groups were counterbalanced at time of euthanasia to avoid bias. Blood samples (3 mL) were collected immediately prior to transcardial perfusion by cardiac puncture in chilled serum separator tubes (SST-amber; Becton-Dickinson, Franklin Lakes, NJ). Whole blood was centrifuged at 6,000 rpm for 1.5-min at 4°C; serum was removed, aliquoted and stored at −80°C for subsequent analysis.

Serum hormone measurements

Serum adiponectin and leptin were measured using electrochemiluminescence detection [Meso Scale Discovery (MSD®), Rockville, MD] using established procedures (17). Intra-assay coefficient of variation (CV) for adiponectin and leptin were 0.8 and 3.6%, respectively. The limits of detectability for these assays are as follows: adiponectin (0.11-200 ng/mL) and leptin (0.055-100 ng/mL). Serum fibroblast growth factor-21 (FGF-21) (R&D Systems, Minneapolis, MN), irisin (AdipoGen, San Diego, CA) and serum oxytocin (ENZO Life Sciences, Farmingdale, NY) levels were determined by ELISA. The intra-assay CV for FGF-21, irisin and oxytocin were 3.3, 8.0 and 6.0%, respectively; the limits of detectability were 13.4-2000 pg/mL (FGF-21), 0.1-5 μg/mL (irisin) and 15-1000 pg/mL (oxytocin). ENZO Life Sciences recently switched to a different rabbit polyclonal detection antibody and, based on the information available from the company and comparisons with other assays across several species, results in at least 1.78- fold higher baseline levels.

Glucose and lipid measurements

Blood was collected for glucose measurements by tail vein nick and measured by the glucometer using the AlphaTRAK 2 blood glucose monitoring system (Abbott Laboratories, Abbott Park, IL) (11). Total cholesterol, triglycerides (TGs), and free fatty acids (FFAs) were measured using an enzymatic based kit (Wako Chemicals USA, Inc., Richmond, VA). Intra-assay CVs for total cholesterol, FFAs and TGs were 2.9, 2.2, and 3.5%, respectively. These assay procedures have been validated for rodents (22).

Statistical Analyses

All results are expressed as means ± SE. Comparisons between multiple groups involving between subjects designs were made using one- or two-way ANOVA as appropriate, followed by a post-hoc Fisher's least significant difference test. Comparisons involving within-subjects designs were made using a one-way repeated-measures ANOVA followed by a post-hoc Fisher's least significant difference test. Analyses were performed using the statistical program SYSTAT (Systat Software, Point Richmond, CA). Differences were considered significant at $P<0.05$, 2-tailed.

Results

Studies 1-2: Effects of chronic 3V oxytocin infusions on food intake, body weight gain, and body composition in HFD-fed and chow-fed rats

We first determined if the effects of long-term (≈ 26 days) infusion of oxytocin into the CNS was sufficient to prevent body weight gain associated with the progression of diet-induced obesity. At study onset, HFD-fed animals had lower body weights relative to chow-fed animals (HFD: 362±7 g; Chow: 408±6 g; $P<0.05$) but there was no significant difference in body adiposity between groups (HFD: 29.7±1.7 g; Chow: 28.2±1.6 g).

HFD: Relative to HFD-fed animals that received vehicle, there was a significant effect of 3V oxytocin to attenuate weight gain over a period of 26 days (**Figure 1A**; $P<0.05$) and 3V oxytocin also tended to reduce body weight throughout the duration of the study (Vehicle: 487±17 g; Oxytocin: 435±16 g) ($P=0.058$). There was a significant main effect of oxytocin to selectively reduce body fat (data not shown) as well as body

adiposity gain (**Figure 1B**; *P*<0.05) and serum leptin levels (data not shown; *P*<0.05) but it had no significant effect on lean mass gain. The effect of oxytocin to reduce body weight gain and body adiposity was mediated, at least in part, by a sustained reduction of energy intake (**Figure 1C-D**; *P*<0.05). To determine if this suppression of food intake was secondary to an aversive effect of central oxytocin, we measured its effect on kaolin intake over the 15-day testing period in a separate cohort of chow-fed rats that received the same dose of oxytocin. Chronic 3V administration of oxytocin failed to increase kaolin consumption (data not shown). These findings extend a previous report showing that acute 3V administration of oxytocin has no effect on kaolin consumption in chow-fed mice (103).

Chow: We then examined if these effects of oxytocin to reduce weight gain and food intake in HFD-fed rats were maintained in chow-fed control rats. By comparison, while there was only a transient effect of oxytocin to reduce body weight gain (**Figure 1D**; *P*<0.05) there was no significant main effect of oxytocin to reduce body weight in chow-fed animals (490 ± 12 g) relative to vehicle controls (475±15 g). Similarly, there was no significant main effect of chronic 3V oxytocin to reduce body adiposity gain (**Figure 1E**) or serum leptin (data not shown) in chow-fed animals and in fact, oxytocin caused an increase in lean mass gain relative to vehicle (*P*<0.05) in the absence of any effect on food intake (**Figure 1F-G**).

Two-way ANOVA revealed a significant diet*drug interaction of oxytocin to reduce weight gain across days 4-26 and to reduce energy intake in animals on HFD relative to

418 chow-fed controls on days 1-4, 6-10, 15, 18, 20-23 and 25-26 (P<0.05). This interactive
419 effect was maintained when weekly energy intake data were compiled across weeks 1-4
420 (P<0.05). There was a near significant diet*drug interactive effect of oxytocin to reduce
421 weight gain on days 1 (P=0.091) and 3 (P=0.054) as well as on energy intake on days 5
422 (P=0.075), 14 (P=0.051), 16 (P=0.052), and 17 (P=0.076). In addition, there was a
423 significant main effect of oxytocin to reduce total fat (F(1,24)=7.933; P<0.05) and fat
424 mass gain (F(1,24)=12.204; P<0.05) as well as an interactive effect of oxytocin to
425 reduce both total fat (F(1,24)=5.636; P<0.05) and fat mass gain in HFD-fed animals
426 relative to chow-fed controls (F(1,24)=10.101; P<0.05). Overall, the data indicate that
427 augmented CNS oxytocin signaling elicits sustained reductions in both body weight gain
428 and body adiposity while preserving lean mass in HFD-fed rats, effects which were
429 mediated, in part, through a reduction in energy intake. In contrast, increased 3V
430 oxytocin administration was largely ineffective on these outcomes in chow-fed rats.
431
432 **Study 3: Effects of chronic 3V oxytocin infusions on food intake, body weight**
433 **gain, and body composition in HFD-fed and chow-fed rats.**
434 Following the previous experiment, we then asked if a) the effects of oxytocin to reduce
435 food intake and body weight gain involved a potential macronutrient preference towards
436 fat, and b) the enhanced responsiveness to oxytocin among HFD-fed animals was
437 maintained throughout the progression of diet-induced obesity. At study onset, following
438 exposure to chow or HFD for 2.5 months, there was no significant difference in body
439 weight between HFD-fed animals and chow-fed animals (HFD: 531±16 g; Chow:

544±13 g), but HFD-fed animals had greater body adiposity (HFD: 131±12 g; Chow: 67±4 g; $P<0.05$).

HFD: Relative to HFD-fed animals that received vehicle, central administration of oxytocin reduced weight gain throughout the infusion period (**Figure 2A**; $P<0.05$), but there was no significant effect on body weight (Vehicle: 586±29 g; Oxytocin: 553±20 g). These effects of oxytocin were associated with a reduction in body adiposity gain (**Figure 2B**; $P<0.05$), serum leptin ($P<0.05$; data not shown), and a slight increase, and therefore protective effect, on lean mass ($P<0.05$). This effect was accompanied by a reduction in energy intake that was maintained until the last week of the study (**Figure 2C-D**). A lower dose of oxytocin (1.6 nmol/day) was also examined in HFD-fed rats, but it was ineffective in reducing weight gain, fat mass, or energy intake (data not shown).

Chow: We next asked if the effects of oxytocin to reduce weight gain and food intake were attenuated in weight-matched chow-fed control rats. In contrast to its effect in HFD-fed rats and consistent with our earlier observations, 3V oxytocin (16 nmol/day) reduced weight gain only on days 2 and 3 (**Figure 2D**; $P<0.05$) but had no significant effect on body weight in chow-fed rats relative to vehicle controls (Vehicle: 583±22 g; Oxytocin: 593±21 g). In addition, there was no significant effect of oxytocin to reduce fat ($P=0.053$) or lean mass gain (**Figure 2E**), serum leptin levels (data not shown), or energy intake (**Figure 2F-G**).

Two-way ANOVA revealed there was a significant diet*drug interactive effect for oxytocin to preferentially reduce weight gain in HFD-fed rats relative to chow-fed controls across day 5-6, 11, 13-16, and 22-26 (P<0.05). There was also a near significant diet*drug interaction of oxytocin to reduce weight gain on days 4 (P=0.055), 7 (P=0.078), 8 (P=0.073), 9 (P=0.051), 10 (P=0.096), 12 (P=0.084), 18 (P=0.091), 19 (P=0.055), 20 (P=0.050) and 21 (P=0.106). In addition, there was a near significant diet*drug interactive effect for oxytocin to reduce energy intake in HFD-fed animals relative to chow-fed controls on day 22 of the infusion period (P=0.060). Collectively, the data showed that there was enhanced sensitivity to oxytocin to elicit sustained reductions in weight gain and energy intake in weight-matched pre-obese rats through a specific reduction in body fat in HFD-fed rats at a dose that was largely ineffective in chow-fed rats.

Study 4: Effects of chronic 3V oxytocin infusions on food intake, body weight gain, body weight, and body composition in rats with established diet-induced obesity.

Following the previous experiment we next asked if long-term infusions of oxytocin into the 3V reduce food intake and body weight in an established DIO rat model. By design, DIO rats weighed more (706±20 g) and had increased adiposity (205±13 g) relative to chow-fed rats (583±15 g; 94±7 g) (P<0.05) at study onset (after exposure to chow or HFD for 4.5 months). We have found that 3-4 months of exposure to our HFD is required to elicit diet-induced obesity in male CD® IGS rats (both an increase in body weight and body adiposity relative to age-matched chow-fed control rats).

HFD: DIO rats that received 3V oxytocin treatment over 21 days experienced much greater weight loss than 3V-vehicle treated controls (**Figure 3A-B**; $P<0.05$). Consistent with our previous studies, 3V oxytocin also reduced body weight gain relative to vehicle treatment throughout the infusion period (**Figure 3B**; $P<0.05$). Oxytocin-elicited weight loss was attributed to a sustained reduction of body fat stores and was accompanied by a corresponding decrease of serum leptin concentrations ($P<0.05$; **Table 1**), and a slight increase, and therefore protective effect, on lean mass (**Figure 3C**; $P<0.05$). These effects were also accompanied by a transient reduction of energy intake (**Figure 3D**) during days 1-5 which persisted through days 9-12 (Vehicle: 222±6 kcal; Oxytocin: 195±10 kcal) ($P<0.05$). However, these effects were no longer significant by day 21.

Chow: In contrast, oxytocin failed to reduce body weight or body weight gain in age-matched chow-fed control rats (**Figure 3E-F**), although body adiposity gain following 3V oxytocin was reduced relative to vehicle treatment (**Figure 3G**; $P<0.05$). Oxytocin also failed to significantly reduce energy intake throughout the infusion period in chow-fed animals.

Two-way ANOVA revealed a significant diet*drug interactive effect of 3V oxytocin to produce a more pronounced reduction of body weight in addition to body weight gain in HFD-fed rats compared to chow-fed rats across days 2-5, 9, 12, 14, 16, and 20-21 as well as energy intake in HFD-fed rats relative to chow-fed rats across days 4 and 5 ($P<0.05$). There was a near significant diet*drug interactive effect of 3V oxytocin to suppress energy intake on days 2 ($P=0.132$) and 3 ($P=0.063$). Overall, these findings

implicate a preferential effect of oxytocin to reduce energy intake and produce sustained weight loss by decreasing fat mass while sparing lean mass in DIO rats at a dose that was largely ineffective in chow-fed rats.

Study 5: Effects of chronic 3V oxytocin infusions on energy expenditure, locomotor activity, RQ, and meal patterns in DIO rats.

Energy expenditure

To gain insight into the mechanisms whereby oxytocin induces weight loss in rats with diet-induced obesity induced by a HFD, we measured energy expenditure using respirometric indirect calorimetry across 72 h during oxytocin or vehicle infusions spanning days 12-14 of treatment. We found that, despite a marked effect of oxytocin to induce weight loss at this time point, total energy expenditure was similar to that of vehicle-treated controls (*P*=NS; **Figure 4A/Figure 4D**) and there was no significant main effect of oxytocin to increase energy expenditure relative to vehicle treatment during the light cycle ($F(1,13)=0.447$), dark cycle ($F(1,13)=0.224$) or total daily energy expenditure ($F(1,13)=0.337$) (**Figure 4D**). Energy expenditure data were examined for possible confounding by differences in measures of body size (43, 44). There were no significant correlations or suggestive trends when any measure of energy expenditure was regressed on any measure of body size either within groups or for both groups combined. Accordingly, we did not adjust energy expenditure for body mass, fat mass, or lean mass.

Locomotor activity

We next examined the effectiveness of long-term oxytocin infusions to alter locomotor activity in DIO rats. Although oxytocin treatment appeared to reduce activity during the early part of the dark cycle ($P<0.05$; **Figure 4B/Figure 4E**), consistent with earlier reports (23, 53), there was no significant main effect of oxytocin to increase activity during the light cycle ($F(1,13)=0.822$), dark cycle ($F(1,13)=0.638$) or when measured as total daily activity ($F(1,13)=0.779$) (**Figure 4E**).

RQ

Our next goal was to measure RQ in order to assess if long-term administration of oxytocin into the 3V may impact fat oxidation in DIO rats. In rats with diet-induced obesity, 3V oxytocin treatment lowered RQ during both the light and dark period ($P<0.05$; **Figure 4C/Figure 4F**), suggesting an effect of oxytocin to increase fatty acid utilization. Consistent with this, we found a significant main effect of oxytocin to reduce RQ during the light cycle ($F(1,13)=7.710$, $P<0.05$), dark cycle ($F(1,13)=5.610$, $P<0.05$) as well as total daily RQ ($F(1,13)=7.087$, $P<0.05$) (**Figure 4F**), consistent with other reports (23, 49, 53).

Serum hormones in chow-fed and HFD-fed DIO rats

There was an increase of serum leptin, FGF-21, blood glucose and total cholesterol in vehicle-treated DIO animals relative to chow-fed vehicle-treated animals ($P<0.05$; **Table 1**). Consistent with earlier reports and its selective action to reduce fat mass, oxytocin treatment was associated with a reduction in serum leptin in DIO animals (23), but not in

554 chow-fed control animals. Oxytocin treatment also reduced serum total cholesterol
555 levels (P<0.05) in DIO animals. In addition, oxytocin treatment was not associated with
556 a significant change in blood glucose, FFA, TG, adiponectin, irisin, or FGF-21.
557
558 **Meal Patterns**
559 Given previous evidence that endogenous oxytocin signaling enhance meal-related
560 satiety in chow-fed animals (6, 9, 57, 67), we sought to extend these findings by
561 determining the effect of exogenous oxytocin on meal patterning in HFD-fed DIO
562 animals that were housed in indirect calorimetry chambers equipped to continuously
563 measure energy intake. Chronic 3V infusion of oxytocin into HFD-fed DIO rats reduced
564 energy intake during both the light and dark cycle (**Figure 5A**). This effect was due to a
565 reduction in meal size (**Figure 5B**) with no change in meal frequency (**Figure 5C**) such
566 that the total number of meals consumed was not different between groups with diet-
567 induced obesity. Further analysis revealed a significant main effect of oxytocin to reduce
568 meal size during the light cycle ($F(1,12)=8.831$, P<0.05), dark cycle ($F(1,12)=5.173$,
569 P<0.05), and throughout the entire day ($F(1,12)=9.778$, P<0.05). This effect of oxytocin
570 treatment was associated with shorter meal duration (**Figure 5D**) and longer inter-meal
571 interval (**Figure 5E**). Overall, these findings indicate that the effect of 3V oxytocin to
572 reduce food intake in DIO rats occurs, in part, via reduced meal size without changing
573 meal frequency (P<0.05).
574
575

Study 6. Effects of chronic 3V oxytocin infusions on CCK-8-induced satiety in HFD-fed rats.

Based on these observations, in a separate cohort of rats, we next determined if increased oxytocin signaling enhanced the satiety response to CCK-8 in animals maintained on a HFD. As others have reported (21, 81) we found that low doses of CCK-8 (0.25, 0.5 nmol/kg) failed to reduce food intake in animals maintained on a HFD (*P*=NS). In the presence of increased oxytocin signaling, however, the ability of these lower doses of CCK-8 (0.25, 0.5 nmol/kg) to reduce food intake was markedly enhanced. At higher doses of CCK-8, however, the addition of oxytocin failed to enhance the effectiveness of CCK-8 to reduce food intake (**Figure 5F**). Our findings show that there was an overall significant main effect of CCK-8 to 1) reduce 0.5 h food intake in the absence of oxytocin treatment ($F(4,56)=3.483$, $P<0.05$), and 2) reduce 0.5 h food intake in the presence of oxytocin ($F(4,52)=3.141$, $P<0.05$).

Two-way ANOVA revealed a near significant main effect of CCK-8 (0.5 nmol/kg) (($F(1,54)=3.822$, $P=0.056$), a significant main effect of oxytocin ($F(1,54)=10.147$, $P<0.05$) and a near significant interactive effect of oxytocin and CCK-8 to suppress 0.5 h food intake $F(1,54)=2.433$, $P=0.125$). These findings support the hypothesis that increased oxytocin signaling enhances sensitivity to the meal-related satiety response to lower doses of CCK-8 in HFD-fed DIO rats.

Study 7: Effects of chronic 3V oxytocin infusions on food intake, body weight gain, and body composition in DIO rats maintained on a HFD lacking sucrose.

Our next goal was to examine if our earlier findings showing oxytocin to preferentially reduce weight gain in DIO rats relative to chow-fed controls (**Study 4**) was due, in part, to sucrose having been a component in the HFD (2, 68). Therefore, we examined the effectiveness of central oxytocin to reduce food intake and weight gain in DIO rats maintained on a HFD lacking sucrose. DIO rats maintained on the HFD lacking sucrose had similar body weights [HFD (-sucrose): 690±21 g vs HFD (+sucrose): 706±20 g] and, as expected, tended to have slightly reduced adiposity [HFD (-sucrose): 170±17 g vs HFD (+sucrose): 205±13 g] (P=0.123) relative to DIO rats maintained on a HFD containing sucrose (**Study 4**) after exposure to each respective HFD for 4.5 months. Central administration of oxytocin reduced weight gain throughout the infusion period (**Figure 6A**; P<0.05), an affect associated with a reduction in fat mass and a slight increase, and therefore protective effect, on lean mass (**Figure 6B**; P<0.05). This effect was also accompanied by a reduction in energy intake that was maintained until the third week of the study (**Figure 6C-D**).

Study 8: Effects of treatment cessation on food intake, body weight gain, and body composition in DIO rats maintained on a HFD lacking sucrose.

To determine the extent to which oxytocin-elicited reductions in weight gain, body adiposity, and food intake persist following the cessation of treatment we tracked daily food intake and body weight in animals following minipump removal in a subset of animals used in **Study 7**. Body composition was measured at the end of the washout

621 study. Oxytocin continued to reduce weight gain in this subset of animals between
622 infusion days 29-39 (**Figure 6E**). Following cessation of treatment (minipump removal
623 on day 38), weight gain in oxytocin-treated animals remained below that of vehicle-
624 treated controls for ≈ 10 days (**Figure 6E**; $P<0.05$). This effect was associated with an
625 increase in body adiposity (**Figure 6F**; $P<0.05$) and energy intake that was maintained
626 through the second week following minipump removal (**Figure 6G-H**).
627
628 **Study 9: Effects of chronic subcutaneous oxytocin infusions on food intake,**
629 **body weight gain, 2-h saccharin preference ratio and kaolin intake in chow-fed**
630 **rats.**
631 As a first step to assessing its translational potential to prevent or treat obesity we
632 determined whether chronic systemic administration of oxytocin was effective at
633 reducing weight gain without inducing nausea or food aversion. To address this, we
634 sought to identify the lowest dose of oxytocin that, following 3-day subcutaneous
635 infusion, reduced body weight gain without producing nausea. Oxytocin reduced weight
636 gain beginning on day 1 and this effect persisted throughout the 3-day measurement
637 period (**Figure 7A**). The effect of subcutaneous oxytocin on food intake was less clear
638 at these low doses. Oxytocin tended to reduce energy intake on day 2 at 50 ($P=0.055$),
639 100 ($P=0.1$) and 200 nmol/day ($P=0.092$; **Figure 7B**). Oxytocin (50, 100, 200 nmol/day)
640 also failed to significantly alter 2-h saccharin preference ratios relative to vehicle
641 (**Figure 7C**) and oxytocin (50 nmol/day) also failed to significantly increase kaolin
642 consumption throughout the 12-day analysis period (**Figure 7D**). Overall, the data
643 indicate that chronic subcutaneous administration of oxytocin, at doses that reduce

644 weight gain and food intake in chow-fed rats is unlikely to elicit nausea. These findings
645 extend other reports showing that acute bolus injections of oxytocin into the CNS or
646 periphery fail to increase kaolin intake (103) or elicit a conditioned taste aversion (42,
647 63, 103).
648
649 **Study 10: Effects of chronic subcutaneous oxytocin infusions on body weight**
650 **gain in HFD-fed and chow-fed rats.**
651 Following completion of the previous experiments, we next asked if chronic 13-day
652 subcutaneous administration of oxytocin (50 nmol/day), at a dose that failed to induce
653 nausea (**Study 9**), was sufficient to mimic the effects of 3V administration to prevent
654 diet-induced obesity.
655
656 ***HFD:*** In rats fed a HFD, subcutaneous administration of oxytocin significantly reduced
657 body weight gain throughout the 13 day infusion period (**Figure 8A**; $P<0.05$). These
658 effects were associated with a transient reduction in food intake on days 2 and 4
659 (**Figure 8B**).
660
661 ***Chow:*** By comparison, oxytocin produced only a transient reduction of weight gain of
662 chow-fed rats on days 1-3 ($P<0.05$; **Figure 8C**), an effect that was not associated with a
663 significant reduction in energy intake (**Figure 8D**).
664
665 There was a significant diet*drug interactive effect of oxytocin to preferentially reduce
666 body weight gain on day 4 ($P<0.05$) and a near significant interactive effect on days 3

and 7 (P=0.108). Similarly, there was also an interactive effect of oxytocin to preferentially reduce energy intake in HFD-fed rats relative to chow-fed controls across days 2 and 4 (P<0.05) and a near significant interactive effect on day 7 (P=0.091). We also compared the effects of chronic 14-day infusions of vehicle and oxytocin in a separate group of chow-fed rats (N=10/group). Oxytocin reduced body weight gain over days 9-10 and 12-14 (P<0.05). Consistent with our earlier findings oxytocin had a transient effect to reduce food intake over days 2-3 (P<0.05). When comparing chow-fed rats to the HFD-fed group there tended to be a significant diet*drug interactive effect of oxytocin to preferentially reduce body weight gain (P=0.070) as well as reduce energy intake on day 4 (P<0.05). Overall, the data showed that there was an enhanced effectiveness of oxytocin, following chronic subcutaneous administration, to reduce weight gain and energy consumption in HFD-fed rats at a dose that was largely ineffective in chow-fed rats.

Serum hormones in chow-fed and HFD-fed rats

There was an increase of serum leptin in vehicle-treated HFD-fed animals relative to chow-fed vehicle-treated animals (P<0.05; **Table 2**). In contrast to our central infusion data from DIO rats, peripheral infusions of OT failed to reduce serum leptin in pre-obese rats maintained on a HFD. As expected (23, 25, 73, 92), serum oxytocin levels were lower among rats receiving subcutaneous vehicle relative to oxytocin treatment on infusion day 13, irrespective of whether animals were maintained on HFD or chow (P<0.05 oxytocin vs. VEH) (**Table 2**). There were no differences in oxytocin levels between vehicle-treated animals maintained on chow or HFD (P=NS), consistent with

690 our previous data in rats (59). Peripheral oxytocin treatment was also not associated
691 with a significant change in blood glucose, FFA, total cholesterol, adiponectin, irisin, or
692 FGF-21.
693
694 **Discussion**
695 Our findings demonstrate that a chronic increase (≈ 21-26 days) of CNS oxytocin
696 signaling both reduced weight gain associated with the progression of diet-induced
697 obesity and evoked sustained weight loss in rats with established diet-induced obesity
698 by selectively reducing fat mass while preserving lean mass, even when administered at
699 a dose that did not appreciably affect energy balance in chow-fed controls. We also
700 report oxytocin-induced weight loss involves *1)* maintenance of energy expenditure at
701 pre-intervention levels despite ongoing weight loss, *2)* a robust reduction of respiratory
702 quotient, consistent with increased fat oxidation, and *3)* an enhanced satiety response
703 to CCK-8 and associated reduction of meal size. Moreover, oxytocin's weight-reducing
704 effects occur independently of whether sucrose is added to the HFD and persist for ~10
705 days following cessation of treatment. While the CNS mechanisms underlying this anti-
706 obesity effect remains unresolved, these data indicate that long-term CNS oxytocin
707 administration can both prevent and treat diet-induced obesity and that these effects are
708 selective for loss of body fat. Combined with our evidence that chronic subcutaneous
709 infusions of oxytocin are sufficient to reduce body weight gain in animals maintained on
710 a HFD at a dose that does not appear to elicit aversive behavioral responses (e.g.
711 nausea or malaise), these findings identify oxytocin as a viable agent for obesity
712 prevention and treatment.

These findings are key steps in extending our understanding of the CNS mechanisms by which oxytocin may influence and potentially regulate energy homeostasis and body adiposity in particular. Our findings that oxytocin limits food intake, body weight and body adiposity gain in animals on a HFD- but not on a chow diet, irrespective of whether obesity is present, suggests that the effect of oxytocin to inhibit food intake depends on the diet being consumed. Consistent with this view, acute or chronic central or systemic oxytocin administration reduces consumption of HFD (23, 53, 59, 103, 104), whereas impairment of oxytocin signaling is associated with increased consumption of fat (103, 104), implying a physiological role for oxytocin to limit fat consumption. These findings are also consistent with studies showing that ingestion of Intralipid™ stimulates Fos (marker of neuronal activation) in PVN oxytocin neurons (68). This observation raises the possibility that the mechanism of oxytocin's effects on body weight may involve a preferential macronutrient consumption of fat. While additional studies to determine if chronic increases in oxytocin signaling impact macronutrient preference will be helpful, together, these findings build on existing rodent data to suggest that oxytocin's effects to limit consumption of preferred (palatable) macronutrients helps to drive its effects on food intake and body weight. Collectively, these findings suggest that the effect of central oxytocin to reduce food intake, body weight, and body adiposity is specific to consumption of a HFD, irrespective of whether obesity is present.

Our findings are consistent with earlier reports in which daily acute 3V injections of oxytocin over 1 week into DIO mice were sufficient to reduce body weight gain through a specific reduction in fat mass (104). Deblon and colleagues also reported that

chronic infusions of oxytocin into the lateral ventricle reduce weight gain and adiposity gain in HFD-fed rats (23). However, oxytocin was infused into the lateral cerebral ventricle for a shorter period of time (14 days); it was also not clear to what extent obesity was actually induced in the rats because they were on the HFD for only 5 weeks prior to oxytocin treatment. Those reports also did not include body weight or total fat mass following diet exposure in either HFD-fed rats or age-matched chow-fed control rats (23) but instead focused on the effects of chronic subcutaneous infusions of oxytocin on changes of body weight gain and body composition relative to vehicle treatment. Consistent with other studies (23, 73), oxytocin treatment limited fat mass while eliciting either no change or a slight increase (and therefore a protective effect), on lean mass. Insight into the mechanism (s) underlying oxytocin's protective effect on lean mass awaits further investigation.

The findings across all studies consistently show that oxytocin reduces weight gain in pre-obese HFD-fed animals regardless of whether the animals were weight-matched at study onset. Potential limitations in this study, however, are that, despite both groups of animals in **Study 1** being age-matched, these groups were not weight-matched at study onset. Animals arrived at the VAPSHCS at different ages prior to study onset, which likely contributed to different growth rates. Nevertheless, these discrepancies are unlikely to have influenced the major overall conclusion because oxytocin also produced consistent and preferential reductions of weight gain in HFD-fed rats that were both age- and weight-matched to chow-fed controls in **Study 3**. In addition, the HFD used in this study and other studies (23, 53, 59, 103, 104) also contains more sucrose (6.7% kcal from sucrose) relative to chow (3.7% kcal from

sucrose), and although relatively small, it may have also contributed to the enhanced effectiveness of oxytocin in this model (2, 10, 38, 61, 68). However, we found that chronic 3V infusions of oxytocin reduce weight gain, body adiposity and energy consumption in DIO rats irrespective of whether sucrose is added to their HFD. Collectively, these findings suggest that oxytocin preferentially reduces consumption of highly palatable foods that are high in fat and/or high in fat and sugar.

In contrast to the reduction in circulating levels of oxytocin reported in DIO mice (103, 104), we found no difference in circulating levels between vehicle-treated chow-fed and HFD-fed rats. One potential explanation for the lack of observed reduction in serum oxytocin may be due, in part, to the length of time animals were placed on the HFD and severity of obesity. Serum oxytocin was measured from rats that were on the HFD for only 13 days (**Study 10**), whereas previous studies reported reductions in serum oxytocin in DIO mice that were maintained on the HFD for 12 (104) or 16 weeks (103). Furthermore, Zhang and colleagues demonstrated that circulating levels of oxytocin were lower in DIO mice particularly when blood was sampled during the mid to late light cycle (104). By design, blood sampling in our study occurred towards the end of the light cycle to match the time period where such differences occur between lean and DIO mice. While not the goal of our study, future studies aimed at collecting blood across multiple circadian time points in rats with already established DIO will be helpful in addressing the extent to which reductions in serum oxytocin may potentially contribute to the pathogenesis of diet-induced obesity in a rat model.

We and others have demonstrated that in HFD-fed animals oxytocin reduces body weight, in part, by reducing food intake (23, 53, 59, 103, 104), and that the

underlying mechanism, based on data from chow-fed animals, may involve increased sensitivity to meal-related satiety signals, such as CCK (6, 9, 57, 67). Leptin is similarly hypothesized to inhibit food intake by enhancing the hindbrain neuronal and satiety response to CCK (12, 34, 57, 58, 97), although this effect is impaired in diet-induced obesity (26, 59). Indeed the ability of low doses of CCK to reduce food intake and activate Fos expression (a marker of neuronal activation) in hindbrain neurons linked to the control of meal size are also impaired in animals maintained on a HFD (20, 21, 81). These observations raise the possibility that the effect of oxytocin, a downstream target of leptin action (12, 57, 70, 99), to inhibit food intake in DIO models is intact (10, 23, 53, 59, 103, 104) because oxytocin acts downstream of the site of leptin resistance. To our knowledge, there are no published studies investigating whether increased oxytocin signaling is sufficient to restore the impaired satiety response to low doses of CCK-8 in animals maintained on a HFD. Accordingly, we hypothesized that increased oxytocin signaling circumvents leptin resistance to reduce food intake in DIO animals by enhancing the satiety response to CCK. Our findings are consistent with this hypothesis, since oxytocin both reduced meal size (without affecting the number of meals consumed) and enhanced sensitivity to the effect of CCK to reduce food intake. One recent report, however, shows chronic administration of oxytocin into the lateral cerebral ventricle reduces meal frequency without impacting meal size in HFD-fed Wistar rats (23). However, it is not clear which criteria were used to define a meal in this study and further studies need to clarify whether strain, route of administration, extent of leptin resistance or obesity in HFD-fed animals at time of oxytocin treatment impacts meal patterning. Nevertheless, combined with previous evidence that reduced oxytocin

805 signaling reduces responsiveness to CCK (6, 9, 67) and is linked to increases in meal
806 size (13, 100), these observations support a physiological role for oxytocin to regulate
807 responsiveness to meal-related satiety signals and hence to participate in control of
808 meal size.

809 Our study also addressed the effects of oxytocin on energy expenditure.
810 Although we did not detect differences in total energy expenditure between 3V vehicle
811 and 3V oxytocin-treated animals, the latter group lost weight relative to the former and
812 previous evidence suggests that persistent, sustained reductions in food intake and
813 body weight are associated with a compensatory decrease of energy expenditure in
814 both rodent models (29) and humans (77-79, 84). We therefore interpret these findings
815 to suggest that oxytocin, like leptin, prevents the effect of weight loss to reduce energy
816 expenditure (77-79) and in this way enhances weight loss induced by reduced food
817 intake. Whether and how this observation is linked to the effect of oxytocin to selectively
818 reduce body fat stores while protecting lean body mass (39, 77) awaits further study.

819 The extent to which oxytocin may elicit weight loss through factors that elicit
820 “browning” of white adipose tissue has only recently been examined. One study
821 reported that chronic subcutaneous administration of oxytocin, at a dose that reduced
822 weight gain in obese *db/db* mice, also appeared to increase the number of uncoupling
823 protein-1 (+) cells in subcutaneous and visceral fat of obese *db/db* mice (73).
824 Furthermore, Lawson and colleagues demonstrated that oxytocin in amenorrheic
825 athletes is positively correlated with resting energy expenditure as well as FGF-21 and
826 irisin (48), two factors that induce the conversion of white adipose tissue to the “brite” or
827 “beige” adipose tissue (14, 28, 98). However, we did not measure a significant increase

828 in either FGF-21 or irisin in response to 3V or SC infusions of oxytocin that was effective
829 in limiting weight gain. As blood was sampled after the weight loss had already occurred
830 (day 21) in DIO rats, it is possible that oxytocin may have increased FGF-21 or irisin
831 had blood been collected prior to oxytocin elicited reductions in body weight. Future
832 studies will assess circulating levels of FGF-21 or irisin at multiple time points with
833 respect to the progression of diet-induced obesity.

834 We report that extended increases in CNS oxytocin signaling increased fatty acid
835 oxidation (as evidenced by a decreased RQ) in DIO rats. These findings extend those of
836 Deblon and colleagues who reported both a reduced RQ and increased expression of
837 lipolytic genes in adipose tissue following 14-day infusions of oxytocin into the lateral
838 cerebral ventricle in HFD-fed rats (23). Oxytocin also increases free fatty acids, glycerol,
839 and/or reduces triglycerides in cultured 3T3-L1 adipocytes (23, 101), rats (23) and DIO
840 nonhuman primates (10). Lawson and colleagues also reported a tendency for oxytocin
841 to reduce triglycerides in humans (49). In addition, chronic central or systemic oxytocin
842 increases epididymal white adipose tissue expression of hormone sensitive lipase (a
843 key mediator of adipocyte lipolysis) (1, 23) while reducing expression of fatty acid
844 synthase (an enzyme linked to lipogenesis) in rats (1). While it remains to be
845 determined whether chronic CNS infusions of oxytocin increase circulating levels (23) in
846 sufficient concentrations to trigger a peripheral effect on lipolysis, it is possible that
847 these effects in our model may be attributed, in part, to a direct effect on adipocytes (60,
848 83, 94) where OTRs are expressed (1, 31, 32, 60, 83, 94, 101) or an indirect
849 mechanism. The mechanism underlying these effects may involve outgoing
850 polysynaptic sympathetic nervous system projections from PVN oxytocin neurons to

851 both inguinal (86) and epididymal white adipose tissue (86, 89). With respect to the
852 question of which receptor populations contribute to the effects of oxytocin to impact
853 energy expenditure, acute microinjection of oxytocin into the median raphe increases
854 both heart rate and body temperature in mice (102), and oxytocin administration into the
855 VMH increases short-term energy expenditure in rats (63). Evidence that adeno-
856 associated viral expression of OTRs into the VMH/DMH of OTR null mice restores
857 impairments in cold-induced thermogenesis (45) and corrects defects in β3- and α2-
858 adrenceptor mRNA expression in interscapular brown adipose tissue, strengthen the
859 link between OTRs in these areas and control of energy expenditure. It is well
860 established that stimulation of the VMH increases activation of efferent nerves
861 innervating brown adipose tissue (62) and induces brown adipose tissue thermogenesis
862 (71), whereas VMH lesions are associated with deficits in sympathetic nervous system
863 activity (80). In addition, some cells in the DMH (65) and VMH (5, 65) have polysynaptic
864 projections to brown adipose tissue. Oxytocin may therefore stimulate brown adipose
865 tissue thermogenesis through either direct (82) or indirect (65) projections from PVN
866 oxytocin neurons to sympathetic premotor neurons (65).
867
868 **Perspectives**. Obesity and its associated metabolic complications (19, 24, 35) are
869 major health concerns (87). According to the World Health Organization, obesity rates
870 have increased at least 2-fold since 1980 with approximately 1.9 billion adults classified
871 as being overweight as of 2014 (Fact sheet No. 311, World Health Organization). In the
872 US alone, obesity impacts approximately 78 million adults and 12.5 million children and
873 adolescents (64). This increase is associated with increased consumption of diets high

874 in fat (16), and sugars, particularly fructose corn syrup and sucrose (15, 37, 56, 88), all
875 of which contribute to the metabolic irregularities observed in the metabolic syndrome
876 (e.g., visceral adiposity, insulin and leptin resistance, dyslipidemia, weight gain).
877 Unfortunately, existing pharmacotherapeutic strategies to treat obesity are relatively
878 ineffective and there is widespread need for improved treatments. We and others have
879 previously shown that chronic systemic administration of oxytocin elicits weight loss or
880 reductions of weight gain in DIO and genetically obese rodent models, DIO nonhuman
881 primates and obese humans through mechanisms that include reduction of food intake
882 (1, 10, 23, 49, 53, 59, 104, 105), increased energy expenditure (10), increases in
883 lipolysis (1, 8, 23), and/or reductions in RQ (23, 49, 53). (23, 49, 53). Our findings
884 demonstrate that chronic 3V oxytocin infusion both reduced weight gain associated with
885 the progression of HFD-induced obesity and elicited a sustained reduction of fat mass,
886 with no decrease of lean mass, in rats with established diet-induced obesity. We further
887 demonstrated that these chronic oxytocin effects result from *1)* maintenance of energy
888 expenditure at pre-intervention levels despite ongoing weight loss, *2)* a robust reduction
889 in respiratory quotient, consistent with increased fat oxidation, and *3)* an enhanced
890 satiety response to CCK-8 and associated reduction of meal size. In contrast to other
891 anti-obesogenic therapies, including melanocortin receptor ligands [MTII (72), BIM-
892 22493 (46)], topiramate (27), and the GLP-1 receptor agonist, liraglutide (50), which
893 reduce both fat mass and lean mass, oxytocin is one of the few hormones that we are
894 aware of (aside from leptin (36, 41, 69)) that protects against the loss in lean mass and
895 selectively reduces fat mass, thus highlighting its translational potential. Combined with
896 our evidence that chronic subcutaneous infusions of oxytocin are sufficient to reduce

body weight gain in animals maintained on a HFD at a dose that does not appear to elicit aversive behavioral responses (e.g. nausea or malaise), these findings identify oxytocin as a viable agent for obesity prevention and treatment. Future studies are warranted to determine the extent to which chronic intranasal administration of oxytocin evokes weight loss in obese men and women by reducing fat mass while preserving lean mass, which will be important if oxytocin is be considered as a potential anti-obesity treatment in humans.

Figure legend

Figure 1. Effects of chronic 3V oxytocin infusions on food intake, body weight gain, and body composition in HFD-fed and chow-fed rats.
Ad libitum fed rats were either placed on HFD (60% kcal from fat; N=5-6/group) or maintained on chow (N=8-9/group) at onset of continuous infusions of vehicle or oxytocin (16 nmol/day). *A/D*, Change in body weight gain in animals maintained on HFD or chow; *B/E*, Change in fat mass and lean mass in animals maintained on HFD or chow; *C/F*, Change in daily energy intake (kcal/day) in animals maintained on HFD or chow*; D/G,* Change in weekly energy intake (kcal/week) in animals maintained on HFD or chow. Note week 4 data represent data across only 5 days. Data are expressed as mean ± SEM. **P*<0.05 oxytocin vs. vehicle.

Figure 2. Effects of chronic 3V oxytocin infusions on food intake, body weight gain, and body composition in HFD-fed and chow-fed rats.
Ad libitum fed rats were maintained on HFD (60% kcal from fat; N=6-8/group) or chow (n=8-9/group) for 2.5 months prior to receiving continuous infusions of vehicle or oxytocin (16 nmol/day). *A/D*, Change in body weight gain in animals maintained on HFD or chow. *B/E*, Change in fat mass and lean mass in animals maintained on HFD or chow; *C/F*, Daily energy intake (kcal/day); *D/G,* Change in weekly energy intake (kcal/week) in animals maintained on HFD or chow. Note week 4 data represent data across only 5 days. Data are expressed as mean ± SEM. **P*<0.05 oxytocin vs. vehicle.

Figure 3. Effects of chronic 3V oxytocin infusions on food intake, body weight, and body composition in rats with established diet-induced obesity.
Ad libitum fed rats were either maintained on HFD (60% kcal from fat; N=7-8/group) or chow (N=7-8/group) for 4.5 months prior to receiving continuous infusions of vehicle or oxytocin (16 nmol/day). *A/E*, Change in body weight in HFD-fed DIO or chow-fed control animals; *B/F*, Change in body weight gain in HFD-fed DIO or chow-fed control animals; *C/G*, Change in fat mass and lean mass in HFD-fed DIO or chow-fed control animals; *D/H*, Daily energy intake (kcal/day) in HFD-fed DIO or chow-fed control animals. Data are expressed as mean ± SEM. **P*<0.05 oxytocin vs. vehicle.

Figure 4. Effects of chronic 3V oxytocin infusions on energy expenditure, locomotor activity, and RQ in DIO rats.
Ad libitum fed DIO rats received continuous infusions of vehicle or oxytocin (16 nmol/day) over 21 days and were maintained on HFD (60% kcal from fat; N=7-8/group). *A,* 24-h profile of energy expenditure during vehicle or oxytocin infusions; *B*, Energy expenditure measurements during the light, dark, and throughout the 24-h period; *C,* 24-h profile of locomotor activity; *D*, Locomotor activity measurement during the light, dark and throughout the 24-h period; *E*, 24-h profile of RQ; *F*, RQ measurement during the light, dark and throughout the 24-h period*.* Data are expressed as mean ± SEM. **P*<0.05 oxytocin vs. vehicle.

Table 1. Serum measurements following 3V Infusions of oxytocin or vehicle in chow-fed and HFD-fed DIO rats from **Study 4**.

Figure 5A-F. Effects of chronic 3V oxytocin infusions on meal patterns in DIO rats or on CCK-8-elicited satiety in HFD-fed rats. Ad libitum fed DIO rats received continuous infusions of vehicle or oxytocin (16 nmol/day) and were maintained on HFD (60% kcal from fat; N=7-8/group). *A*, Change in energy intake (kcal/day); *B*, Meal size; *C*, Meal frequency; *D*, Meal duration; *E*, Inter-meal interval; *F*, Rats (6-h fasted) received continuous infusions of oxytocin vehicle or oxytocin (16 nmol/day) in combination with intraperitoneal injections of CCK-8 (immediately prior to start of dark cycle). Data are expressed as mean ± SEM. A-E: **P*<0.05 oxytocin vs. vehicle, F: **P*<0.05 oxytocin vehicle + CCK-8 vs. oxytocin + CCK-8.

Figure 6A-D. Effects of chronic 3V oxytocin infusions on food intake, body weight gain and body composition in DIO rats maintained on a HFD lacking sucrose.
DIO rats were maintained on HFD (60% kcal from fat; N=8-10/group) for 4.5 months prior to receiving continuous infusions of vehicle or oxytocin (16 nmol/day). A subset of these rats was euthanized on day 28 while minipumps were left in place in the remaining rats until day 38 as indicated below.
Figure 6E-G. Effects of treatment cessation on food intake, body weight gain and body composition in DIO rats maintained on a HFD lacking sucrose.
DIO rats were maintained on HFD (60% kcal from fat; N=3-5/group) for the duration of the washout study. Minipumps were removed on day 38 (indicated by arrow) from DIO rats that had previously received continuous infusions of vehicle or oxytocin (16 nmol/day).
FIGURE 6A-G. Daily food intake and body weight were measured in 3-h fasted rats. *A/D*, Change in body weight gain. *B/E*, Change in fat mass and lean mass; *C/F*, Daily energy intake (kcal/day); *D/G,* Change in weekly energy intake (kcal/week).
Data are expressed as mean ± SEM. **P*<0.05 oxytocin vs. vehicle, **P*<0.05 post-oxytocin vs. post-vehicle.

Figure 7. Effects of chronic subcutaneous oxytocin infusions on food intake, body weight gain, 2-h saccharin preference ratio and kaolin intake in chow-fed rats. Ad libitum fed rats received continuous infusions of vehicle or oxytocin (0, 50, 100, 200 nmol/day) and were maintained on chow (N=7-9/group). *A*, Change in body weight gain in animals maintained on chow; *B*, Change in daily chow intake (g/day); *C*, Intake of saccharin using two-bottle conditioned taste aversion test. *D*, Daily kaolin consumption (N=6-10/group). Data are expressed as mean ± SEM. **P*<0.05 oxytocin vs. vehicle.

Figure 8. Effects of chronic subcutaneous oxytocin infusions on body weight gain in HFD-fed and chow-fed rats.
Ad libitum fed rats received continuous infusions of vehicle or oxytocin (50 nmol/day) across 3 or 13 days and were maintained on chow or HFD (60% kcal from fat; N=6-12/group). *A-B*, Change in body weight gain in animals maintained on chow or HFD; *C-D*, Daily energy intake (kcal/day). Data are expressed as mean ± SEM. **P*<0.05 oxytocin vs. vehicle.

Table 2. Serum measurements following subcutaneous infusions of oxytocin or vehicle in chow-fed and HFD-fed rats from **Study 10**.

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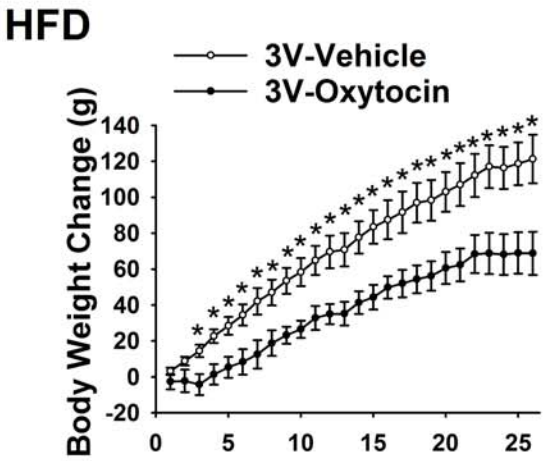

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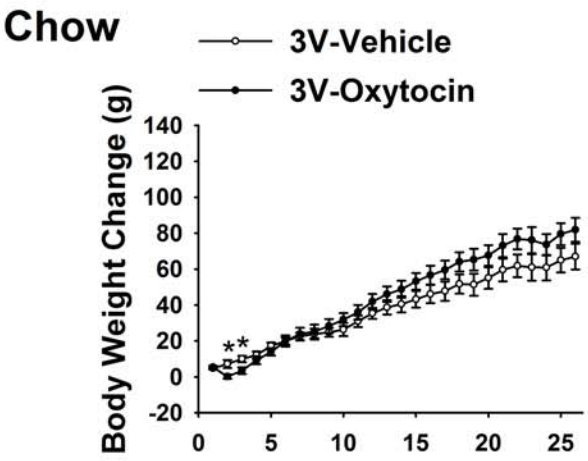
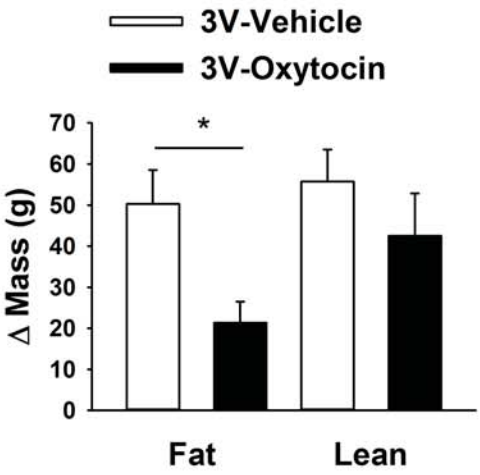


FIGURE 1D



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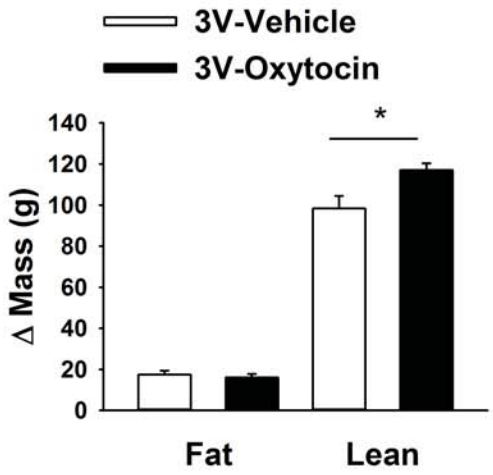

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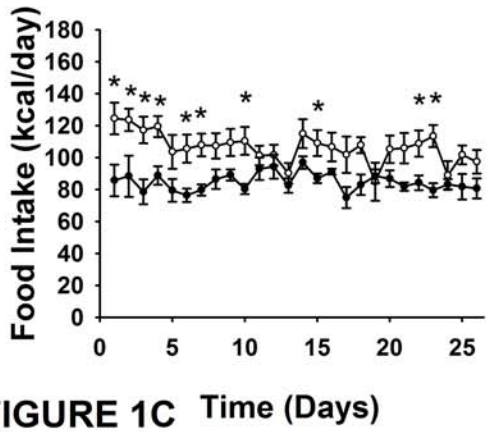

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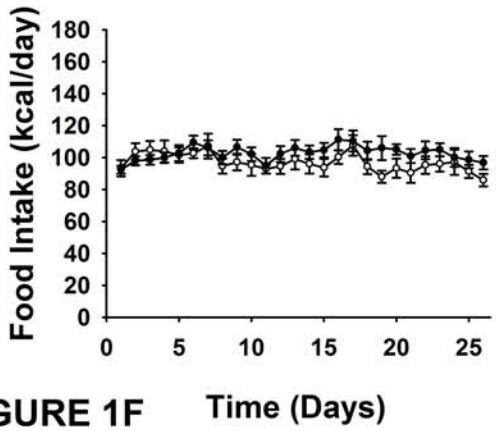

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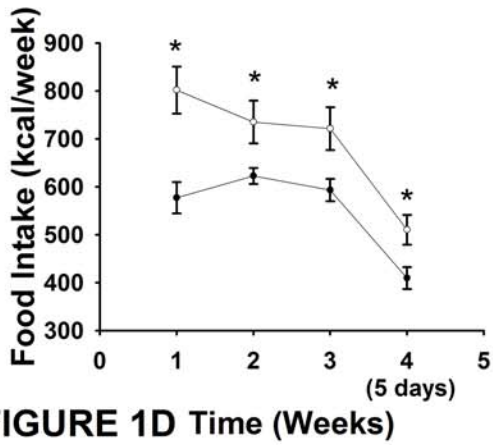

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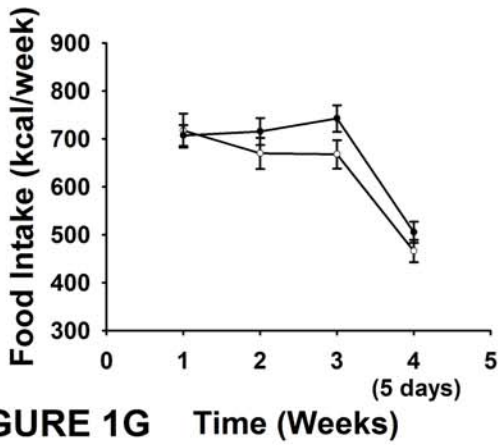

FIGURE 1G

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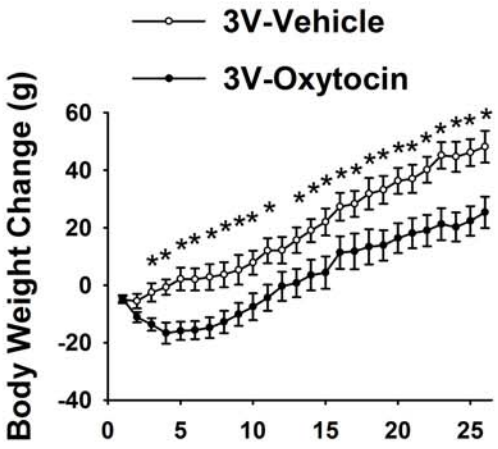

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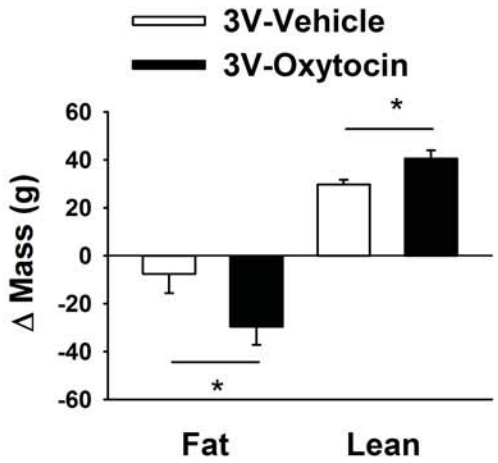

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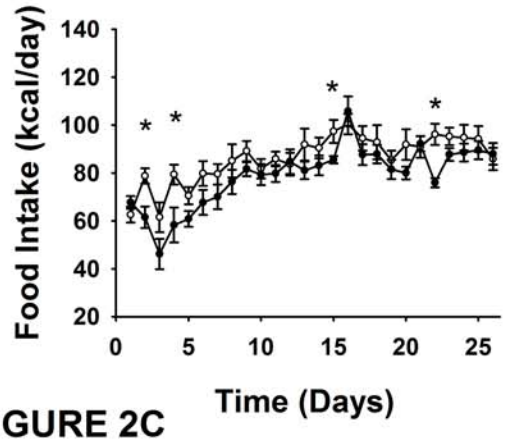

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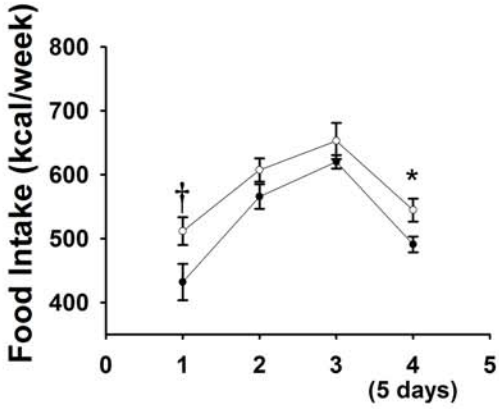

FIGURE 2D Time (Weeks)

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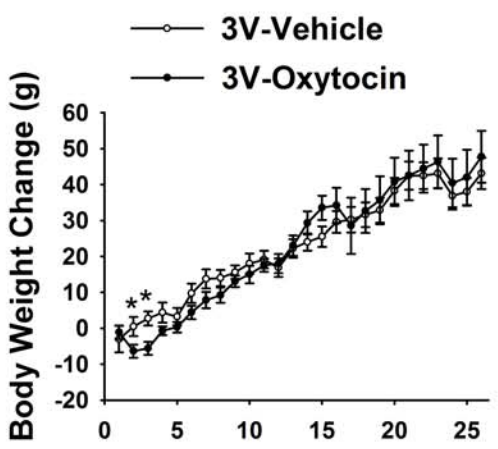

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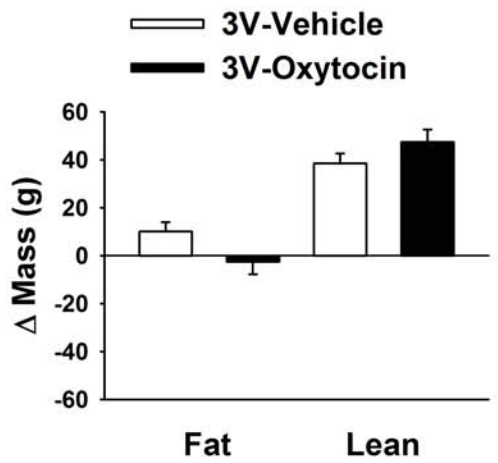

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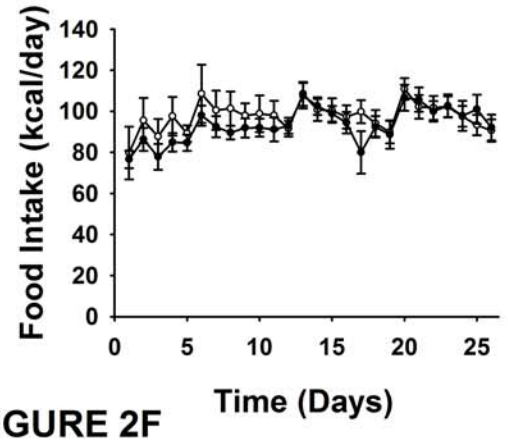


FIGURE 2F

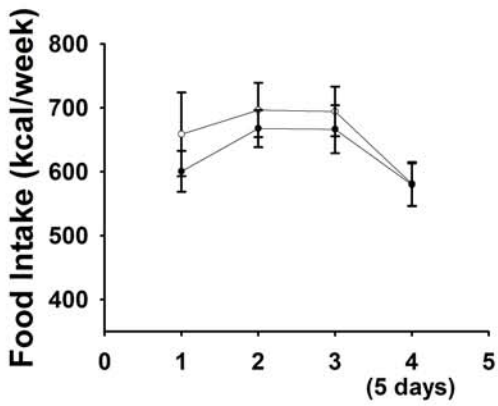


FIGURE 2G Time (Weeks)

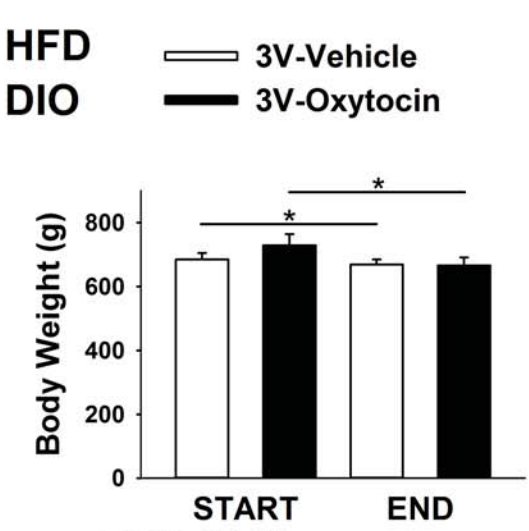
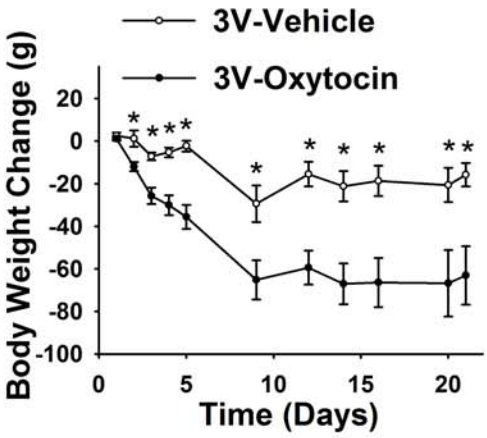


FIGURE 3A



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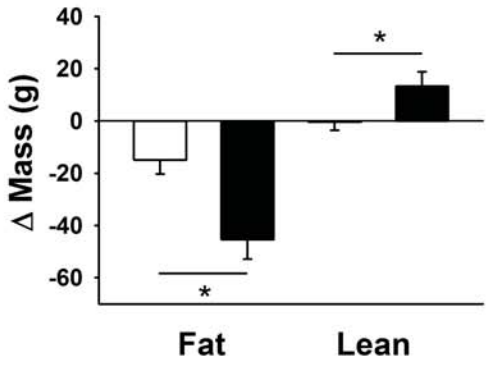

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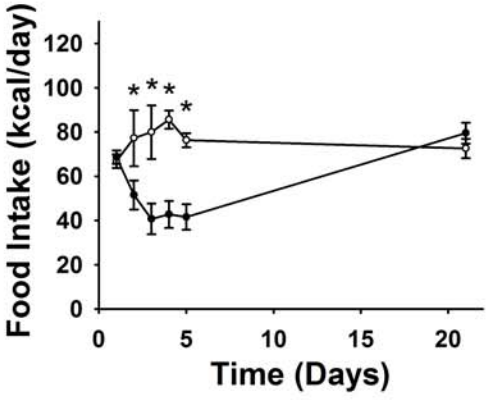

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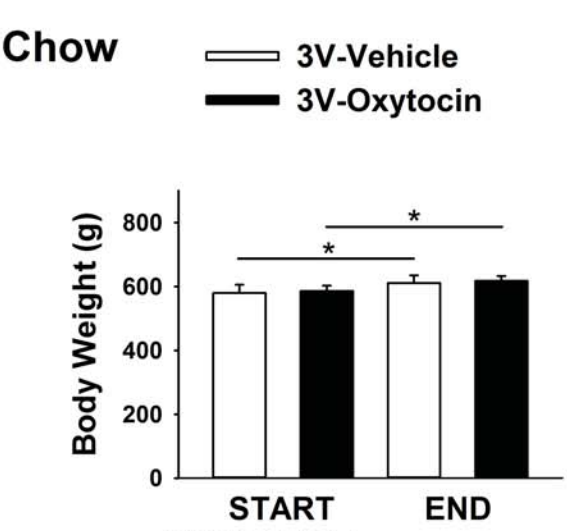

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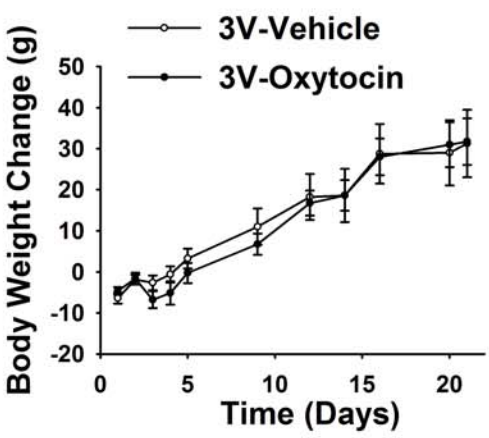

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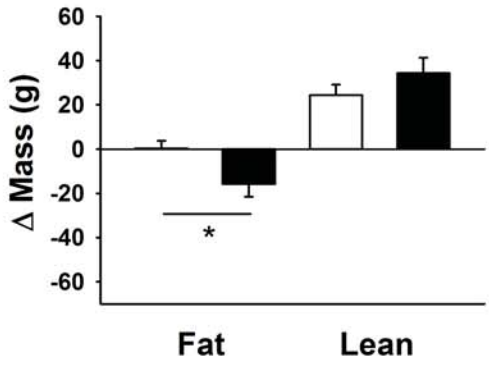

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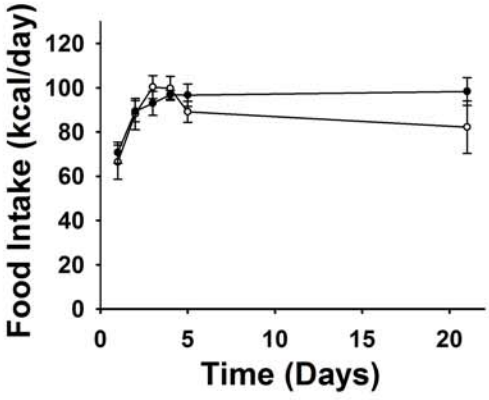


FIGURE 3H

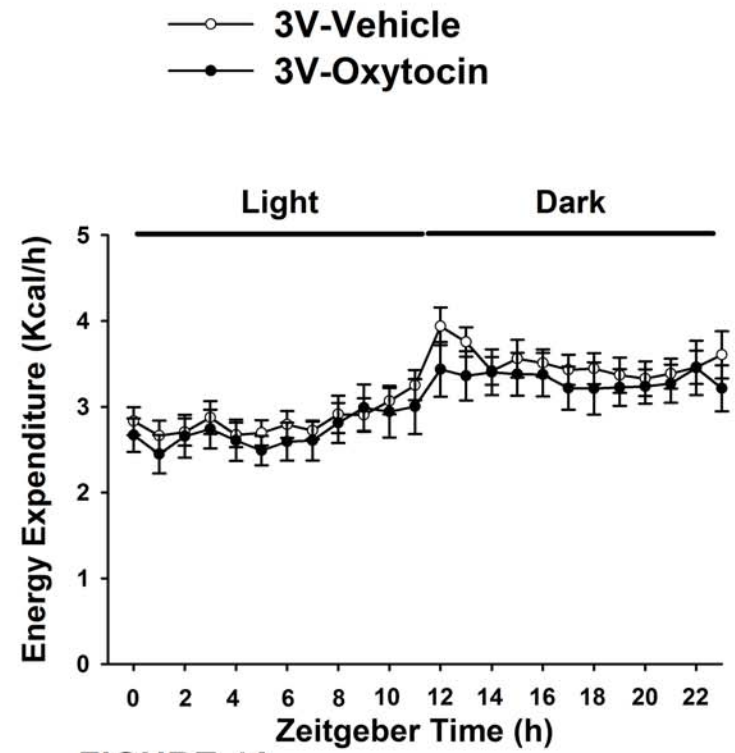
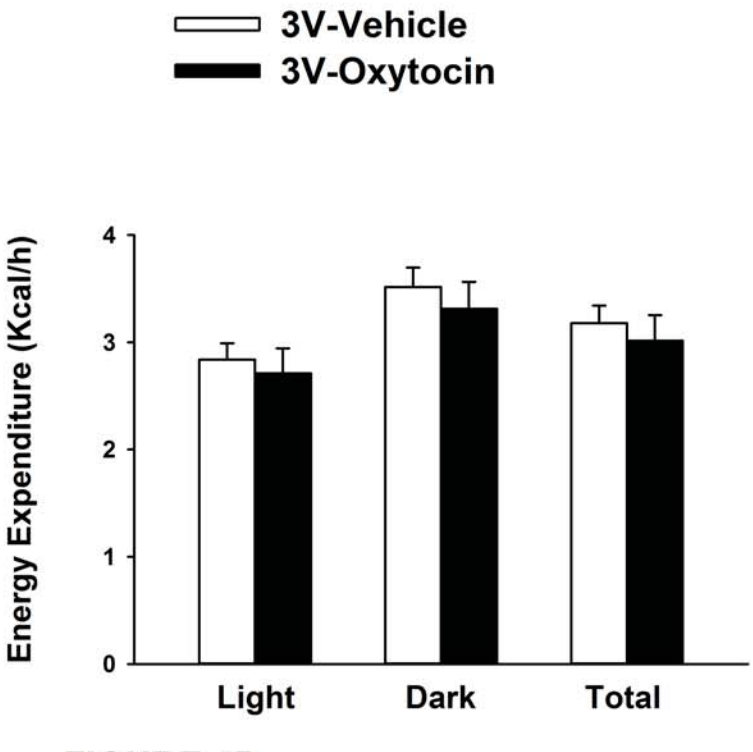


FIGURE 4A



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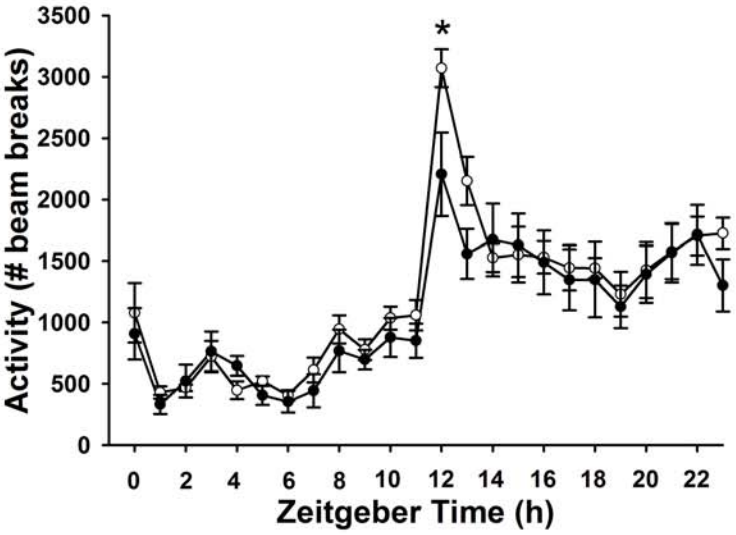

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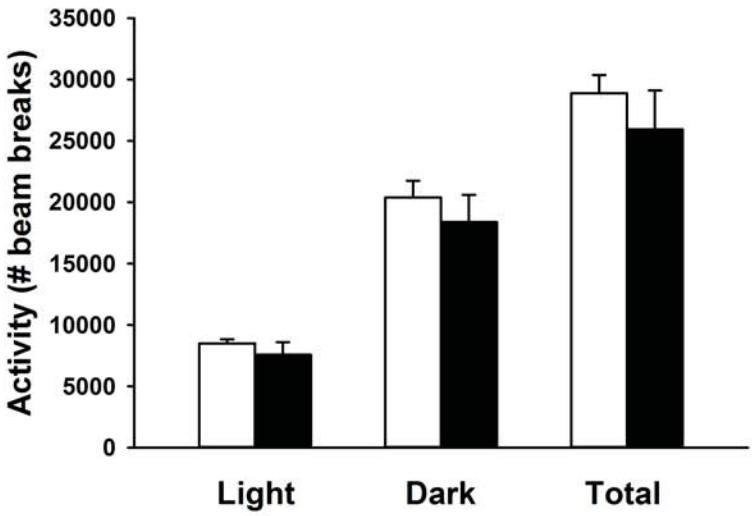

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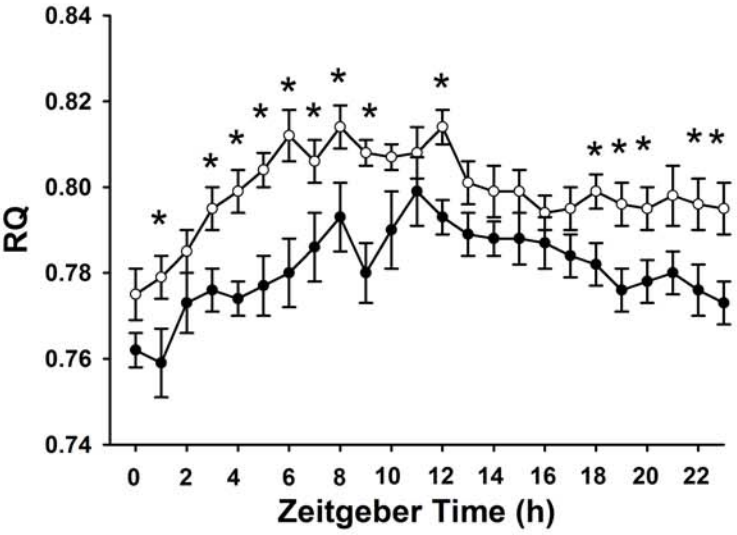

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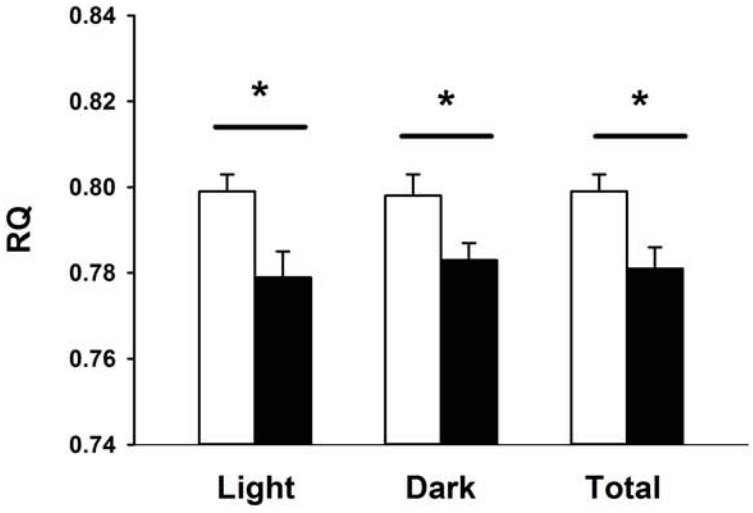

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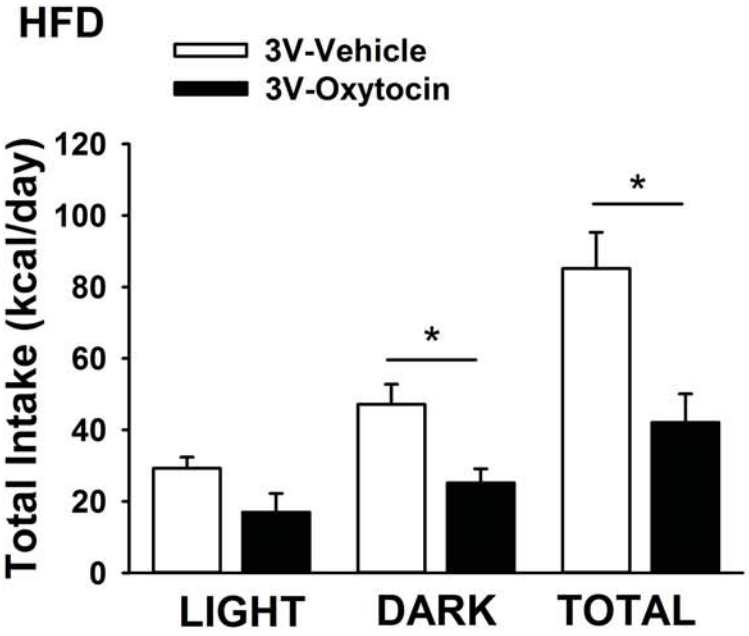
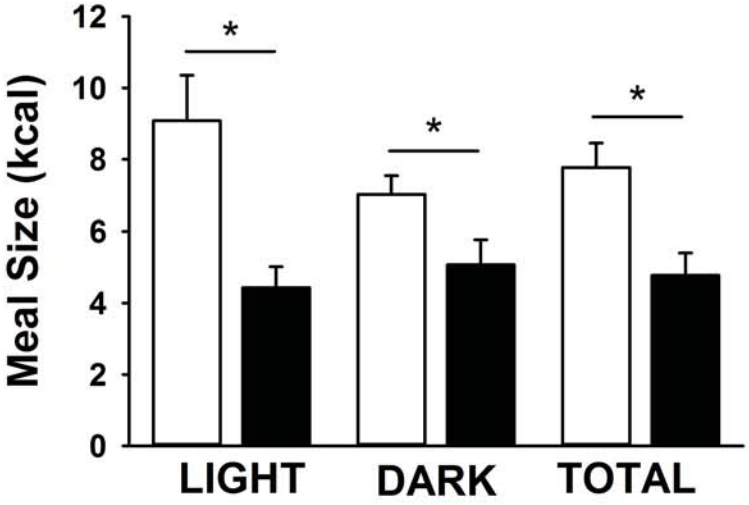


FIGURE 5A



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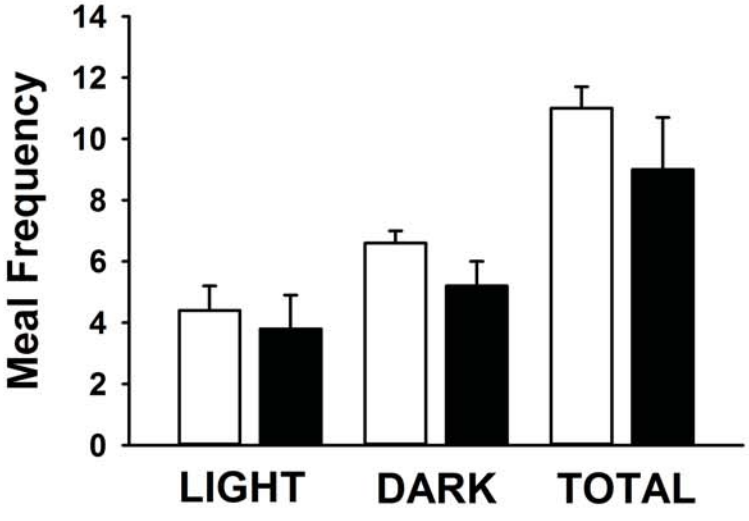

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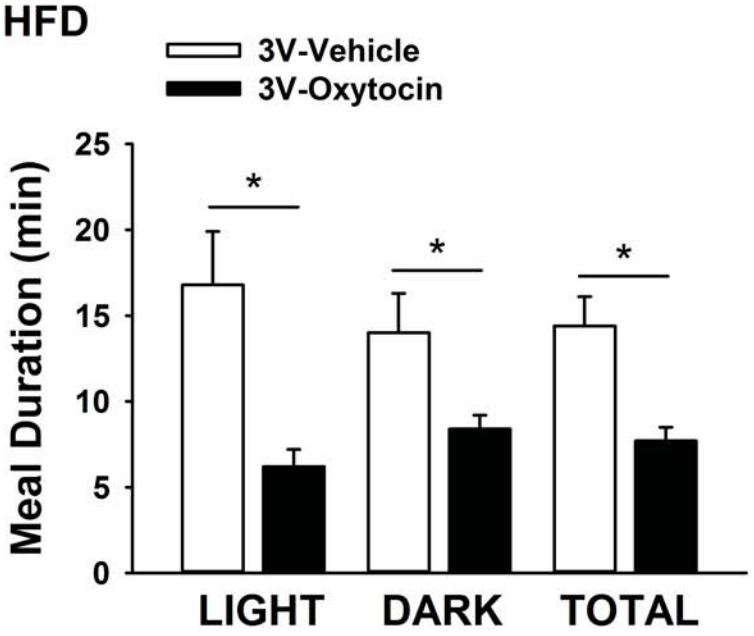

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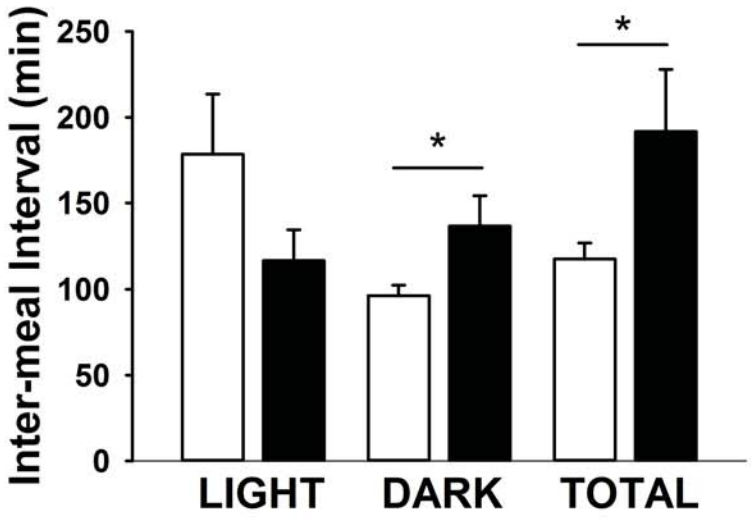

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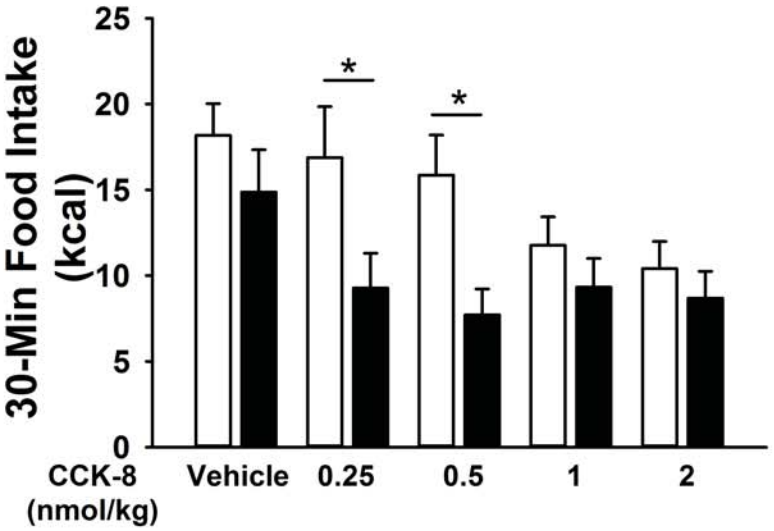

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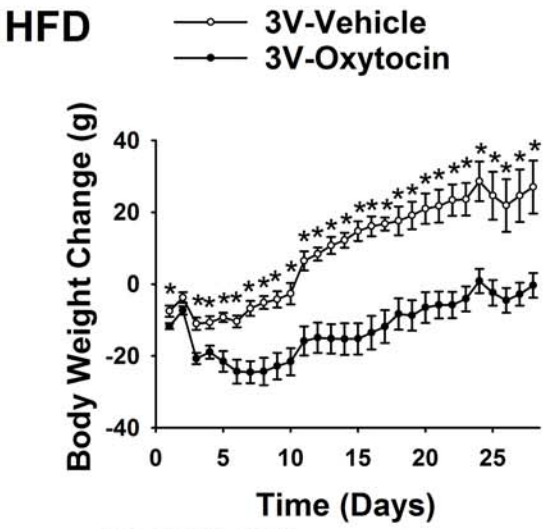

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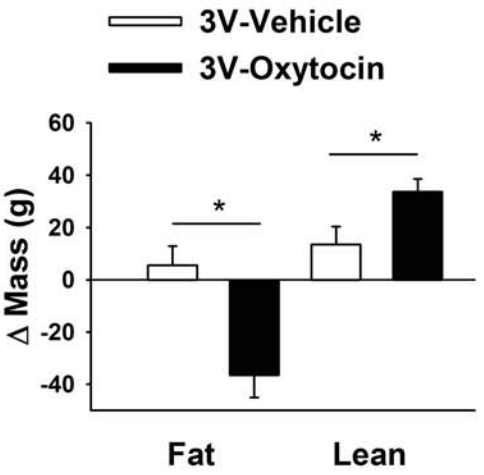

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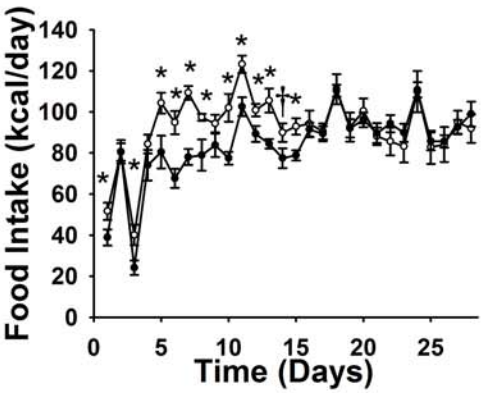

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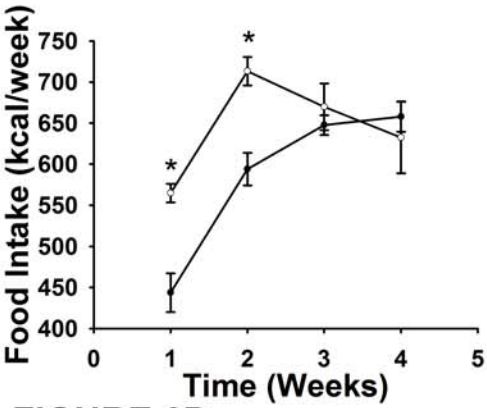

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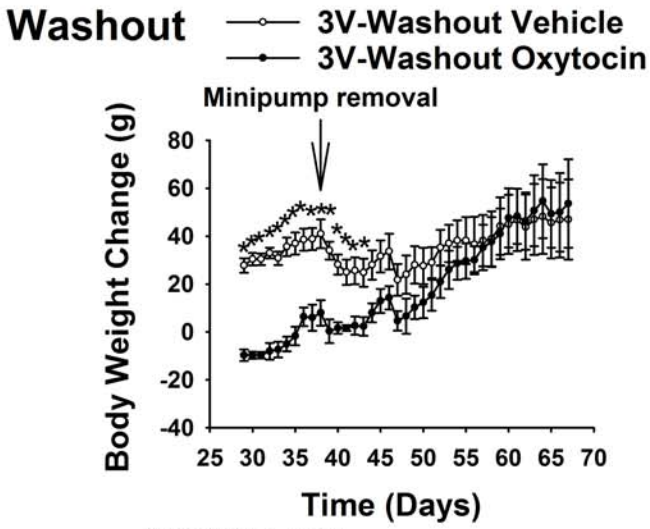

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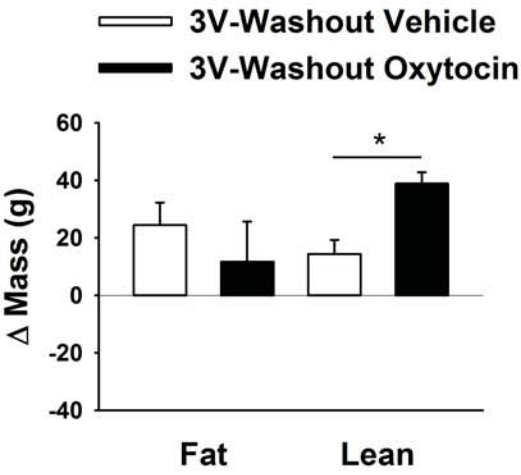

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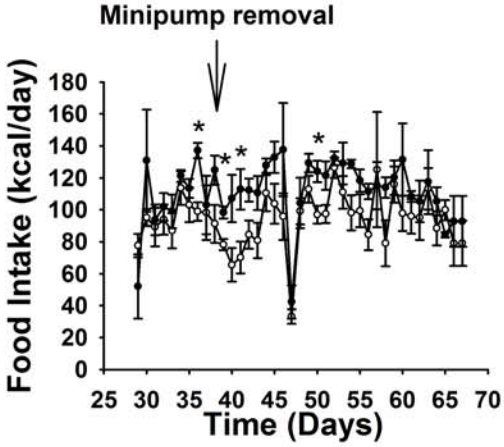

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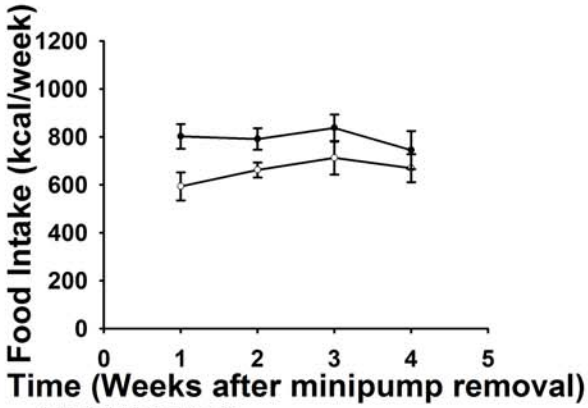

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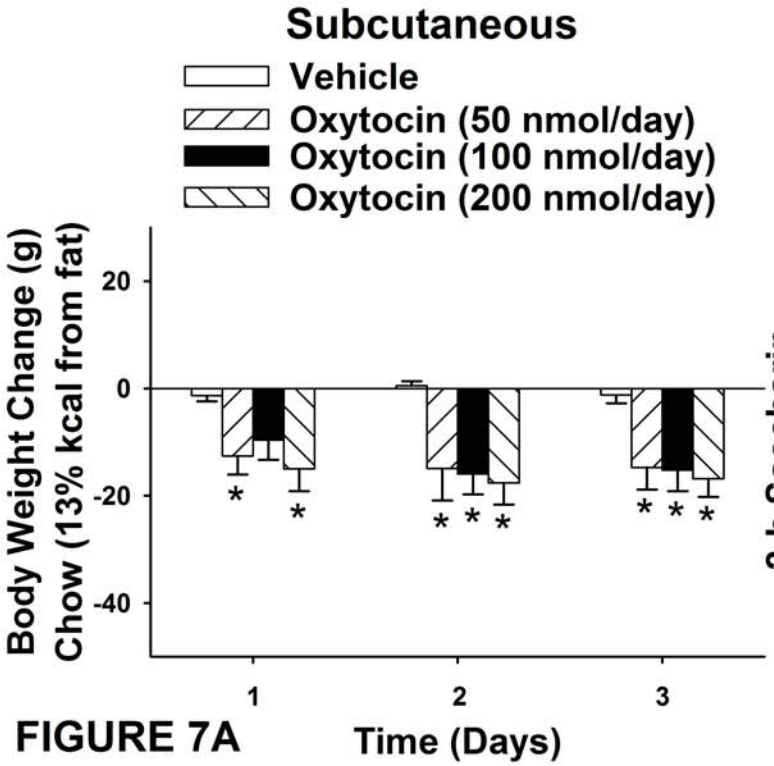
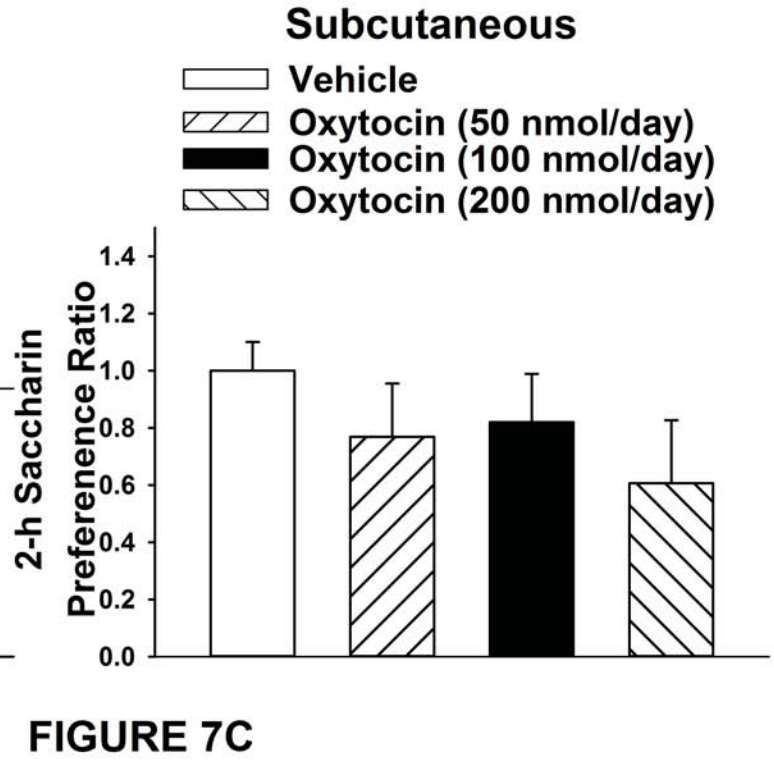


FIGURE 7A



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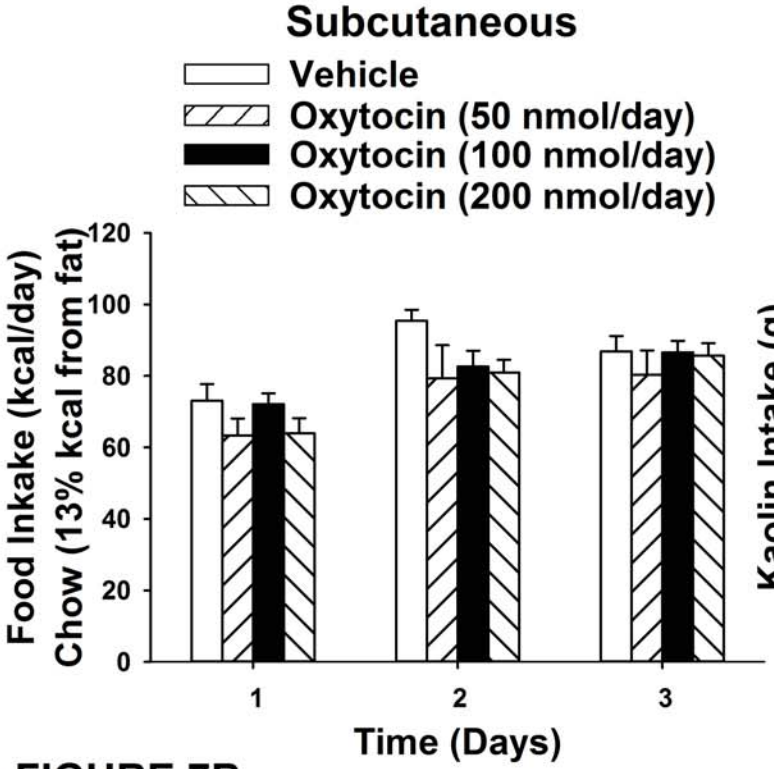

FIGURE 7B

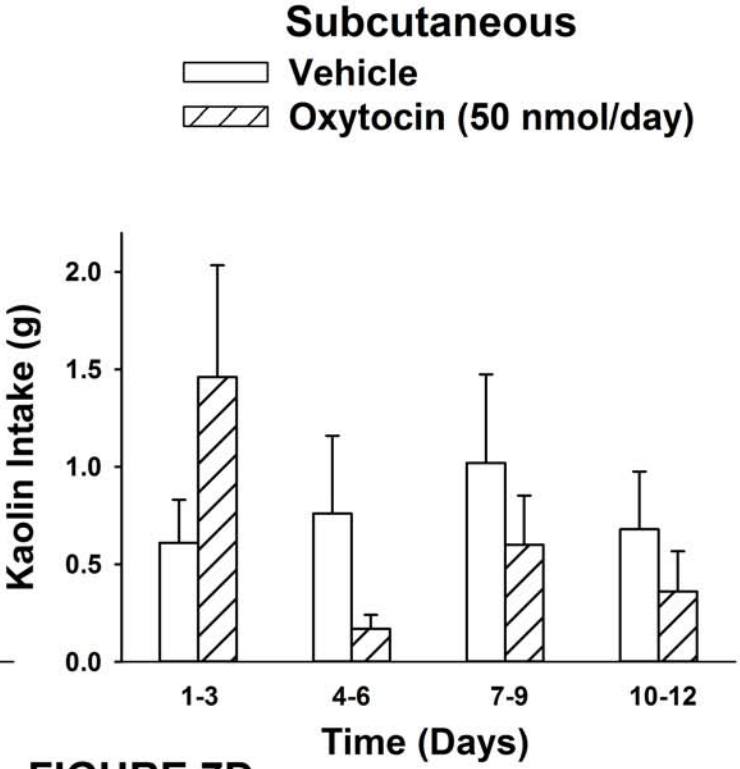

FIGURE 7D

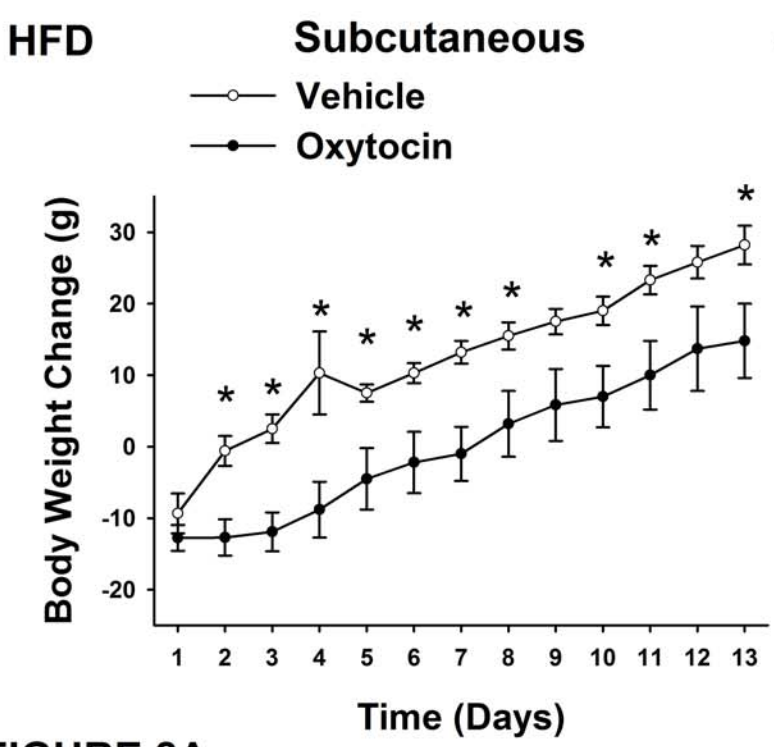

FIGURE 8A

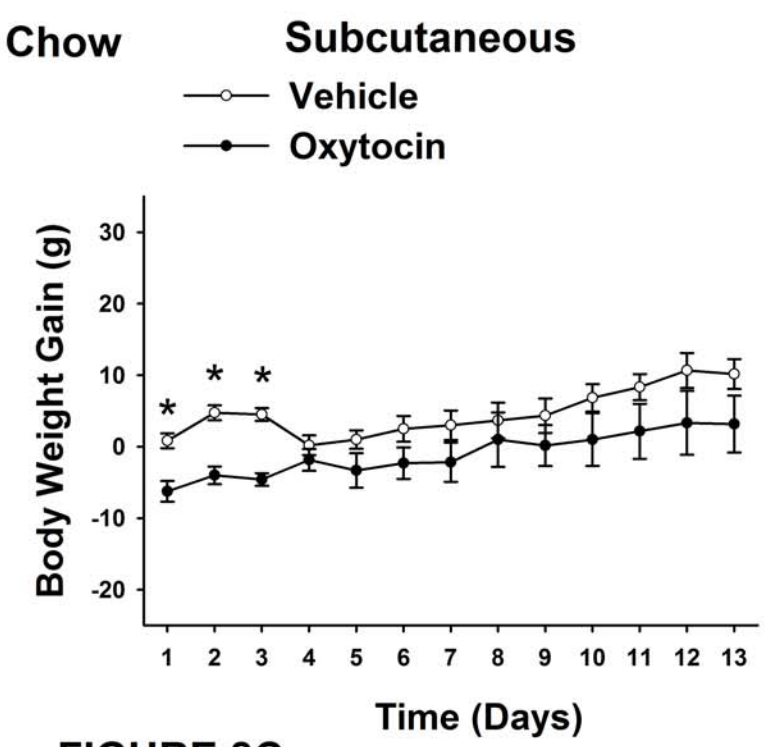

FIGURE 8C

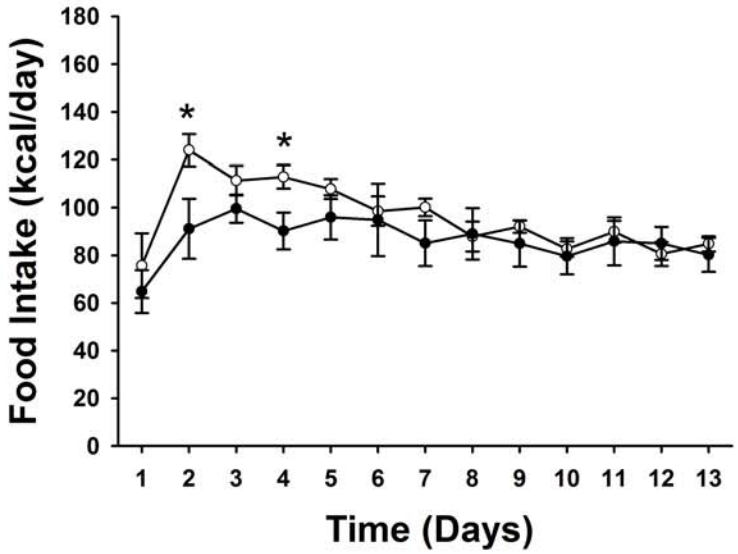

FIGURE 8B

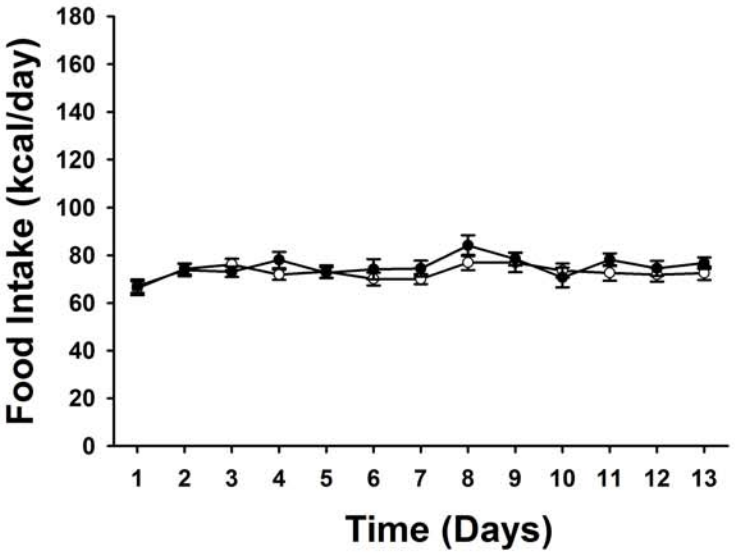

FIGURE 8D

Table 1. DIO Amelioration: Serum Measurements Following 3V Infusions of Oxytocin or Vehicle

3V Treatment	Vehicle	Oxytocin	Vehicle	Oxytocin
	CHOW	CHOW	HFD	HFD
Leptin (ng/mL)	8.5 ± 2.0^{ac}	4.7 ± 0.5^{a}	20.5 ± 2.6^{b}	11.6 ± 2.1^{c}
Adiponectin (µg/mL)	5.4 ± 0.5^{ab}	5.0 ± 0.3^{a}	6.4 ± 0.4^{b}	6.0 ± 0.4^{ab}
FGF-21 (pg/mL)	67.9 ± 12.8^{a}	80.5 ± 16^{ab}	119.8 ± 13.3^{bc}	128 ± 14.1^{c}
Irisin (µg/mL)	4.9 ± 0.2	4.9 ± 0.4	4.9 ± 0.5	5.3 ± 0.3
Blood Glucose (mg/dL)	97 ± 4.3^{a}	96 ± 3.0^{a}	108 ± 2.4^{b}	107.4 ± 3.1^{b}
FFA (mEq/L)	0.3 ± 0	0.2 ± 0	0.3 ± 0	0.4 ± 0.1
TG (mg/dL)	79.6 ± 10.9^{a}	50 ± 7.0^{b}	44.9 ± 5.5^{b}	34.5 ± 3.6^{b}
Total Cholesterol (mg/dL)	66.5 ± 3.3^{a}	64 ± 2.7^{a}	98.9 ± 4.7^{b}	84.3 ± 5.5^{c}

Different letters denote significant differences between treatments

Shared letters are not significantly different from one another

N=8-10/group

Table 2. Serum Measurements Following SC Infusions of Oxytocin or Vehicle

SC Treatment	Vehicle	Oxytocin	Vehicle	Oxytocin
	CHOW	CHOW	HFD	HFD
Leptin (ng/mL)	2.9 ± 0.6[a]	3.2 ± 0.1[ac]	10.3 ± 1.2[bc]	11.8 ± 4.7[b]
Adiponectin (µg/mL)	4.1 ± 0.3	5.4 ± 0.2	5.3 ± 0.6	5.6 ± 0.7
FGF-21 (pg/mL)	122.6 ± 26	76.2 ± 9.3	117.2 ± 16	119 ± 25.9
Irisin (µg/mL)	4.4 ± 0.4	4.3 ± 0.2	4.5 ± 0.4	4.6 ± 0.4
Blood Glucose (mg/dL)	99.3 ± 2.9[ac]	99.2 ± 4.4[ac]	106.8 ± 1.0[bc]	109.7 ± 2.4[b]
FFA (mEq/L)	0.18 ± 0.02	0.21 ± 0.01	0.21 ± 0.02	0.17 ± 0.03
Total Cholesterol (mg/dL)	67.8 ± 17	51.3 ± 6.2	77.2 ± 9.6	75.2 ± 12.0
Oxytocin (pg/mL)	1513.9 ± 253.3[a]	2882.9 ± 383.1[b]	1553.2 ± 233.3[a]	2894.2 ± 350.2[b]

Different letters denote significant differences between treatments

Shared letters are not significantly different from one another

N=5-6/group