HHS Public Access

Author manuscript

Obesity (Silver Spring). Author manuscript; available in PMC 2015 January 01.

Published in final edited form as:

Obesity (Silver Spring). 2014 July ; 22(7): 1643–1652. doi:10.1002/oby.20725.

Spexin is a Novel Human Peptide that Reduces Adipocyte Uptake of Long Chain Fatty Acids and Causes Weight Loss in Rodents with Diet-induced Obesity*

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Abstract

Objective—Microarray studies identified Ch12:orf39 (Spexin) as the most dysregulated gene in obese human fat. Therefore we examined its role in obesity pathogenesis.

Design and Methods—Spexin effects on food intake, meal patterns, body weight, Respiratory Exchange Ratio (RER), and locomotor activity were monitored electronically in C57BL/6J mice or Wistar rats with dietary-induced obesity (DIO). Its effects on adipocyte [^{3}H]-oleate uptake were determined.

Results—In humans, Spexin gene expression was down-regulated 14.9-fold in obese omental and subcutaneous fat. Circulating Spexin changed in parallel, correlating (r = −0.797) with Leptin. In rats, Spexin (35 μg/kg/day s.c) reduced caloric intake ~32% with corresponding weight loss. Meal patterns were unaffected. In mice, Spexin (25 μg/kg/day i.p.) significantly reduced the RER at night, and increased locomotion. Spexin incubation *in vitro* significantly inhibited facilitated fatty acid (FA) uptake into DIO mouse adipocytes. Conditioned taste aversion testing (70μg/kg/day i.p.) demonstrated no aversive Spexin effects.

Conclusions—Spexin gene expression is markedly down-regulated in obese human fat. The peptide produces weight loss in DIO rodents. Its effects on appetite and energy regulation are

*A preliminary account of this work was presented at the 2012 Annual Meeting of The Obesity Society: Walewski, J.L., Ge, F., Lobdell IV, H., Schwartz G., Vasselli J, Berk, PD. A novel human peptide that reduces adipocyte uptake of long chain fatty acids and causes weight loss in mice. [Abstract]. Obesity 2012; 20(9) (Late-Breaking Abstract Supplement): p6. http://www.obesity.org/images/stories/obesity2012/2012_Late Breaking_abstracts.pdf

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Disclosures

All other authors report that they have no competing interests to declare.

presumably central; those on adipocyte FA uptake appear direct and peripheral. Spexin is a novel hormone involved in weight regulation, with potential for obesity therapy.

INTRODUCTION

The worldwide epidemic of obesity is expected to remain one of the greatest challenges to public health in the 21st century. The U. S. in particular is experiencing an obesity epidemic with profound consequences (1), e.g. ~300,000 deaths annually (2), a *decrease* in life expectancy (3), and enormous health care costs (4). Obesity is linked to well documented increases in the prevalence of many conditions that cause excess morbidity and mortality (5).

White adipose tissue (WAT) plays a major role in energy storage. In addition, distinct WAT depots also function as “endocrine organs” by secreting unique profiles of adipokines, a diverse collection of more than 50 cytokines, chemokines, and hormone-like factors which contribute to the maintenance of energy homeostasis. While not expressed exclusively by WATs, some locally secreted adipokines have been shown to affect appetite, satiety, and glucose and lipid metabolism (6). The actions of many of these adipokines are ultimately integrated to regulate glucose and energy metabolism, long chain fatty acid (LCFA) uptake and storage, and insulin activity via both paracrine and endocrine mechanisms.

Since 2008, microarray studies in our laboratory comparing gene expression in obese vs non-obese human omental and subcutaneous fat have identified both individual genes and biological pathways whose components are significantly dysregulated in obese fat. Among our earliest studies, using arrays with probes for ~55K genes and ESTs, we identified ~3,500 genes and ESTs that exhibited significant differences in expression (7). Of these, the most down-regulated gene was Ch12:orf39, whose mRNA was under-expressed 14.9-fold in obese fat. It appeared to encode a secreted peptide, which we subsequently recognized was identical to Spexin, a novel peptide identified by Mirabeau et al in 2007 using Markov modeling (8).

That Spexin was the single most down-regulated gene in obese human fat, coupled with observations by Mirabeau et al that Spexin induced muscarinic-like contractions in stomach smooth muscle *in vitro* (8), led us to postulate that it might normally function as an adipocyte-expressed satiety factor, and that the lack of Spexin expression by obese fat might lead to the loss of a key adipokine potentially involved in the regulation of gut motility, food intake, energy metabolism and long chain fatty acid (LCFA) uptake and storage in adipocytes. Therefore, we set out to define its biological role in rodent models of diet-induced obesity.

METHODS

Patients

Patients undergoing clinically indicated abdominal laparoscopic surgical procedures consented to removal of omental and subcutaneous fat samples for studies of LCFA transport, molecular studies, and a venous blood sample for the measurement of circulating

adipokines. Obese patients were undergoing bariatric surgical procedures, and the non-obese patients were undergoing other clinically indicated laparoscopic procedures at either the Weill Cornell or Columbia Presbyterian campuses of New York Presbyterian Hospital. The protocols, consent documents, and procedures for these studies were approved by the individual Institutional Review Boards of the Columbia University and Weill Cornell Medical Centers.

Materials

9,10-[^{3}H]-Oleic acid (OA) was purchased from NEN Life Science Products (Boston, MA, USA), type I collagenase from Sigma (St. Louis, MO, USA), and fatty acid free bovine serum albumin (BSA) from Boehringer Mannheim (Indianapolis, IN, USA).

Two preparations of Spexin were used: the first from Phoenix Pharmaceuticals (Burlingame, CA) and the second, a custom synthesis product from the Ferring Research Institute (San Diego, CA). Both were >99% pure by HPLC and ID'd by LC/MS.

Isolation of Adipocytes

Adipocytes from human omental and subcutaneous fat biopsies (9) and from epididymal fat pads of obese mice (18 weeks of age) (10) were isolated and sized by direct light microscopy as described (9, 10).

Studies of LCFA Uptake Kinetics

The initial rate of [^{3}H]-OA uptake by both human and mouse adipocytes was determined by rapid filtration (9, 11). The unbound oleate concentration ([OAu]) in each test solution was calculated from the OA/BSA molar ratio (11) (12) , using the LCFA/BSA binding constants of Spector et al (13). Data fitting used the SAAM II program of Berman and Weiss (14).

Statistical Considerations—Values for physiologic variables, unless otherwise noted, are reported as the mean ± standard error, and calculated according to standard methods of descriptive statistics (15). The significance of differences between groups was assessed with Student's two-tailed t tests, with $p \leq 0.05$ being considered significant.

Gene expression studies

Whole human genome microarray—Gene expression in omental and subcutaneous fat samples from obese versus non-obese subjects was compared by whole genome microarray analysis as previously described in detail (7). Median normalized gene expression data (arbitrary expression units) were analyzed using the GeneSifter Data package, with statistical treatment of the results as reported earlier (7).

qRT-PCR—Spexin gene expression was examined in the same adipose tissue samples and additional obese and non-obese human omental adipose tissues by qRT-PCR according to the methods reported earlier (7). Spexin primers for PCR were designed using Primer 3 software (v.0.4.0) at http://fokker.wi.mit.edu/primer3/input.htm. Similar methods were used to test for Spexin gene expression in mouse epididymal adipose tissues. Specific primer sequences for both human and mouse Spexin are presented in Supplemental Table 1. 18S

RNA was used as the control to normalize gene expression. The average fold change (AFC) was computed by using the mean difference in ΔCt between each test gene &18S for each sample, i.e. AFC = $2^{-(\text{average } \Delta\Delta Ct)}$.

Quantitative immunoassays

Quantitative immunoassays to determine circulating levels of Spexin by competitive EIA (Cat # EK-023-81) and human Leptin by antigen capture ELISA (Cat # EK-003-12) were performed with kits purchased from Phoenix Pharmaceuticals (Burlingame, CA). Unknown samples were measured in duplicate, and OD values were quantified by comparison to within-assay standard curves. Quantitative validation was also confirmed by "spike-in" experiments, in which known amounts of Spexin standard were added to individual serum samples (see Supplemental Data).

Metabolic assessments in DIO mice

Metabolic and behavioral measurements were performed as previously described (16). Individually housed C57BL/6J mice with diet-induced obesity (DIO) or age-matched controls (Jackson Labs, Bar Harbor, ME, USA) were maintained ad lib on a high fat diet (HFD, D12492, Research Diets, New Brunswick, NJ) which provided 60% of total calories as fat.

24 hr feeding behavior in DIO rats

Obese (DIO) adult female Wistar rats were maintained ad lib on 60% HFD (D12492) in individual chambers in which their feeding behavior was continuously recorded via a BioDaq Electronic Food Intake Monitoring System (Research Diets, Inc., New Brunswick, NJ).

Conditioned taste aversion studies in rats

Conditioned taste aversion studies were conducted using adult female Wistar rats according to established protocols (17-19). For training, rats were water, but not food, deprived for 20 hrs (18:00 -14:00 hrs the following day), then offered a single bottle of water ad libitum for 20 min. Training took place on 2 days of each week, spaced at 3-4 day intervals, to allow recovery of normal food intake and body weight. A total of 7 training sessions were conducted, by which time all animals had learned to drink promptly when fluid was offered.

RESULTS

Gene expression profiling of human fat

Gene expression in omental and subcutaneous fat samples from obese versus normal weight subjects was compared by whole genome microarray analysis (7). Median normalized gene expression ratios for each probe, representing 55K known genes and ESTs, were compared by log-log plot (**Figure 1A**). The mRNA for Ch12, ORF39 (Spexin) demonstrated a highly significant 14.9-fold drop in expression in obese compared to non-obese fat tissues (**Figure 1B**), the largest change in gene expression observed in obese human fat (p=0.00292).

Validation of gene expression results by qRT-PCR—Observed changes in Spexin gene expression were validated by qRT-PCR of all of the original fat samples assayed initially by microarray, another 12 omental samples from obese patients (final n=18), and 4 from additional non-obese tissue donors (final n=9). When assayed by qRT-PCR, Spexin mRNA demonstrated a 33.3-fold decrease in Spexin gene expression in human obese, compared to non-obese fat samples (**Figure 1C**; $p<0.001$).

Spexin gene expression was also analyzed in 20 week old DIO mice (n=10) and C57BL/6J controls (n = 8) by qRT-PCR. The normalized Spexin gene expression ratio in the DIO mice was 0.638 ± 0.107 compared to 1.00 ± 0.091 for the normal weight controls (p=0.047) (**Supplemental figure 1A**).

Spexin and Leptin concentrations in serum

Sera from non-obese and obese patients (BMIs = 23.5 ± 0.9 vs 49.3 ± 1.8 respectively) were assayed for circulating Spexin and Leptin concentrations by commercial immunoassays. The mean concentration of Spexin peptide in the serum of obese patients was approximately 10% of that in non-obese subjects (1.1 ± 0.7 vs 11.6 ± 1.3 ng/mL; obese vs non-obese; n=7/group; $p<0.0002$), in agreement with the 15-fold reduction in Spexin gene expression found in WATs from obese patients. Circulating Leptin averaged 8.5 ± 3.5 ng/mL in serum from non-obese patients, and 37.4 ± 4.7 ng/mL in serum from obese patients (p=0.014). There was a highly significant, non-linear, negative correlation $r = -0.797$, $p<0.01$) between Spexin and leptin concentrations in human sera (**Figure 2A**). In addition, Spexin concentrations were significantly negatively correlated (**Figure 2B**) and leptin concentrations positively correlated (**Figure 2C**) with the Vmax for LCFA uptake by omental adipocytes from the same patients. Circulating Spexin concentrations were also measured in 20 week old DIO mice (2.24 ± 0.07 ng/mL, n = 17) and age-matched C57BL5/6J background controls (4.28 ± 0.48 ng/mL, n= 5) (**Supplemental Figure 1B**). As in the human samples, circulating spexin concentrations were significantly higher in the non-obese Control mice than in the obese DIO animals ($p<0.001$). The less dramatic differences in Spexin expression and serum levels in the DIO mice may reflect either an intrinsic species-specific difference, or that the DIO mice weighed only approximately 1.5 X as much as the non-obese animals, while the obese patients in this study weighed 2.5 times as much as the non-obese study participants.

Effect of exogenous Spexin on body weight in C57BL/6J mice with DIO

To test our hypothesis that Spexin is a satiety factor that modulates feeding behavior, we administered various Spexin doses to DIO mice by daily IP injection for up to 50 days. Control animals received daily IP injections of equal volumes of 1XPBS. For these studies mice were housed 5 to a cage in standard plastic box cages. In addition to body weights, food and water consumption were measured daily in some of these studies. Spexin treatment consistently resulted in weight loss. In a representative study, animals treated with Spexin at 25 μg/kg (IP QD) lost weight over the course of the experiment while controls receiving 1X PBS continued to gain weight on the HFD (Figure 3). In parallel studies in which food consumption was also measured, food consumption declined progressively over time in Spexin-treated mice, but remained essentially unchanged in PBS-treated controls

(**Supplemental Figure 2**). By contrast to the obese DIO mice, normal weight C57BL/6J mice did not lose weight when treated with similar doses of Spexin.

Effects of exogenous Spexin on metabolic parameters in DIO Mice

In a subsequent study, after adaptation to metabolic cages, individually housed mice were treated daily either with Spexin (35 μg/kg i.p.) or an equivalent volume of PBS for 19 days. Body weights were recorded daily. As indicated by the slopes and correlation coefficients of the weight vs time regression lines, the vehicle treated animals continued to gain weight over the course of the experiment ($y = +0.1625x + 39.801$, $r = +0.9262$), while the Spexin-treated animals lost weight over the same period ($y = -0.0666x + 38.3$, $r = -0.7318$) (**Figure 4A**). All animals were monitored with measurements of O_2 consumption, CO_2 production, the respiratory exchange ratio (RER), and energy expenditure (EE) throughout the study (**Figure 4B through 4E**). Metabolic results such as these may be expressed either per mouse or per gram BW. They are displayed in Figure 4 on a per mouse basis. However, neither VO2 nor EE were significantly affected in Spexin treated mice, regardless of whether the data are expressed per mouse or per gram BW. Tracings of Ambulatory Events Counts and the Respiratory Exchange Ratio across representative 24 hr periods during this study are presented in **Figure 5**. Unfortunately, efforts to monitor the sizes of indvidual meals and cumulative food intake electronically during this study were unsuccessful.

Spexin inhibits LCFA uptake into isolated adipocytes

Multiple studies suggest that regulation of adipocyte LCFA uptake is an important control point for body adiposity, e.g. (9-11, 20). To study the potential role of Spexin in this process, epididymal fat pads were removed from Spexin-treated DIO mice, and [^{3}H]-oleic acid uptake kinetic constants in isolated adipocytes were determined (9-11, 21). The V_{max} for facilitated LCFA uptake in Spexin-treated mice, 61±9 pmol/sec/50,000 cells, was only 28% of that of the control mice (211±60 pmol/sec/50,000cells, p=0.047). Adipocyte suspensions were also prepared from untreated DIO mice. Paired aliquots were incubated with either PBS or PBS/Spexin (20ng/mL). Short incubations *in vitro* (2hrs) resulted in approximately a 40% down-regulation of adipocyte LCFA uptake (data not shown). Nearly identical results were obtained with both Spexin preparations tested (Phoenix Pharmaceuticals, Ferring Research Institute). Finally, adipocytes isolated from untreated DIO mice were incubated for 2 hours in either PBS alone or PBS + Spexin at one of 10 concentrations from 0.01 to 80 ng/ml. LCFA uptake kinetics were then determined. A total of 38 triplicate uptake inhibition studies were performed (**Figure 6**). They reveal a bimodal response of LCFA uptake inhibition to the concentration of Spexin employed, with maximal inhibition of 73 ± 6% of control LCFA uptake at a Spexin concentration of 1 ng/nl.

Effect of Spexin on 24 hour feeding behavior and body weight in DIO rats

Female DIO rats used in this study were approximately 20% overweight for age at the beginning of treatment. Individual body weights and total food consumed were measured daily. Reductions of daily food intake seen by day 3 (~ 32%) were statistically significant on days 4-6, the final treatment day, and first two washout days, (**Figure 7A**). The reduction in body weight noted by Day 4 persisted well beyond the treatment period (**Figure 7B**).

Cumulative 24-hr food intake curves from this study were obtained via continuous recording (**Figure 7 C-F**). At baseline, the two groups of animals demonstrated essentially identical feeding behavior. It is noteworthy that meal frequency and the temporal (light/dark) feeding pattern during the 24 hr feeding periods remained normal in the Spexin-treated group throughout the treatment and washout periods. However, both meal size (gms of food consumed/meal) and meal duration (time spent feeding/meal) were smaller in the Spexin-treated animals, who consumed approximately 32% fewer calories overall than controls during the last day of treatment and the first two days following treatment.

Conditioned taste aversion testing in Wistar Rats

The total amount of saccharin solution consumed by the LiCl-treated animals was significantly less than that consumed by either the saline or Spexin-treated animals (**Figure 8**, $p < 0.01$ or smaller). This was noted by day 2 of testing, and persisted throughout the challenge period. No significant differences in saccharin consumption from the vehicle-injected group were noted in the Spexin-treated animals, indicating that reductions in 24hr food consumption previously seen in Spexin-treated animals were not due to aversive effects of Spexin on ingestive behavior.

DISCUSSION

Spexin was first identified as a novel peptide hormone by Mirabeau et al (2007), using an approach based on hidden Markov model screening to identify novel peptide-encoding sequences in the human genome (8). Virtually nothing was known initially about its biological activity. Microarray studies on surgical fat biopsies in our laboratory starting in 2008 identified Ch12:orf39 as the gene with the greatest difference in expression between obese and non-obese human fat. We subsequently recognized that Ch12:orf39 encoded Spexin, the peptide identified by Mirabeau (8).

Our finding that Spexin was the most down-regulated gene in microarray studies in obese human fat (7), and the demonstration of its contractile activity in an *in vitro* rat stomach explant model system (8), led us to postulate that Spexin might function as an adipocyte-expressed satiety factor. We speculated that the lack of Spexin expression by obese fat might reflect the loss of a key adipokine, which would potentially impact the regulation of gut activity, food consumption, energy metabolism and long chain fatty acid (LCFA) uptake and storage in adipocytes. A commercial immunoassay allowed us to examine possible relationships between circulating levels of Spexin and those of known obesity-related adipokines in human sera.

Leptin is known to play a major role in the regulation of body weight and food consumption (10, 11, 22), and its expression is elevated in obesity (22). The strong negative correlation (see Figure 2A) between Leptin and Spexin in the serum of obese patients and normal weight controls supports the idea that these peptides might play antagonistic roles in the normal regulation of hunger, satiety, body adiposity and weight by serving as opposing components of a negative feedback loop. We therefore explored the biological role of Spexin in energy metabolism and storage in two rodent models of diet-induced obesity. Daily intraperitoneal injections of Spexin led to a reduction in food consumption and body

weight in DIO mice, while vehicle-injected DIO mice continued to gain weight. Analogous data were obtained in a separate study with DIO rats. The lack of detectable taste-aversive effects of Spexin in formal testing appreciably enhances the importance of these observations.

These findings were further pursued in separate cohorts of mice undergoing Spexin treatment for 19 days while in metabolic chambers. The calorimetry cage studies revealed that Spexin significantly reduced the RER, suggesting preferential fat oxidation, particularly during darkness, when the difference in RER between Spexin- and vehicle-treated mice was highly significant. The observed reduction in the RER at night appears to result from inhibition of the normal night-time elevation in carbohydrate metabolism, with an attendant shift to lipid oxidation. Spexin also significantly increased locomotor activity, but only in the Z-plain, by “rearing”. There was no evidence of randomly increased “anxiety” activity.

The basis for the reduction in RER and the increase in locomotor activity in Spexin-treated animals remains speculative. Nevertheless, the reduction of food intake without taste aversion, altered metabolism, and increased locomotor activity during Spexin administration suggest that these effects collectively may be centrally mediated. This question will be pursued going forward by studying the effects of Spexin administration in positron emission tomographic scanning of brain activity, and of the effects of low dose intracerebroventricular Spexin administration on food consumption and body weight.

The regulation of facilitated LCFA uptake into adipocytes has been proposed to be an important control point for body adiposity (9). This process has been studied extensively in our laboratory using dietary manipulations in intact rodents, and in adipocytes isolated from various genetic and diet-induced animal models of obesity and from obese patients (9-12, 20, 21) . To test the potential role of Spexin in the regulation of LCFA uptake into adipocytes, we first showed that Spexin treatment of DIO mice *in vivo* significantly inhibited facilitated LCFA uptake into adipocytes freshly isolated from the treated animals. We subsequently studied the pharmacodynamics of acute *in vitro* Spexin incubation on adipocytes freshly isolated from untreated DIO mice. The resulting concentration-response curve for Spexin-induced inhibition of LCFA uptake into adipocytes was biphasic, demonstrating a maximum 73% inhibition of LCFA uptake at a Spexin concentration of 1 ng/ml. These data clearly indicate that, in addition to effects that likely are centrally mediated, Spexin has direct effects on peripheral adipocytes. Such a curve is an example of ‘hormesis”, a phenomenon which is typically reflected in a bi-phasic peptide concentration-response curve (23-25). It is most often seen with peptide hormones whose effects are receptor mediated, when the peptide is tested at doses that span several orders of magnitude (23, 26). These results are consistent with the hypothesis that Spexin may play a role in the regulation of long chain fatty acid uptake into adipocytes via a receptor-mediated process.

Since the original report (8) seven more articles about Spexin have appeared (27-33), one of which identified the same gene sequences, designated NPQ, using an alternative bioinformatics approach (31). In rats, low level Spexin expression was detected by RT-PCR in all tissues studied, including esophagus, stomach , small intestine, liver, pancreas, lung, skeletal muscle, heart, uterus, thymus, spleen kidney, bladder, specific brain regions, and

multiple endocrine organs including anterior pituitary, adrenal, thyroid, testis and ovary. Spexin was also widely identified in rat tissues by immunohistochemistry (28). However, its expression in fat has not previously been described. It has been reported to have a number of endocrine functions (27, 29, 32) and to be a modulator of cardiovascular and renal function (32), although in the latter case the doses administered by rapid i.v. bolus injection to achieve the transient effects reported were very large and certainly supra-physiologic (up to 10 X what we administered daily intraperitoneally to produce weight loss). After this paper was initially submitted, two studies in goldfish (*Carassius auratus*) were published, demonstrating (A) that injection of goldfish Spexin into the CNS led to a decrease in feeding behaviors and total food consumption through selective alterations in the expression of orexigenic and anorexigenic signals in the telencephalon, optic tectum and hypothalamus (33), and (B) that Spexin specifically suppressed luteinizing hormone release (27). Thus, like leptin, Spexin appears to be an adipokine with not only major roles in the regulation of food intake and body weight, but also diverse endocrine effects.

To our knowledge, we are the first to demonstrate Spexin expression in human WAT, to identify the almost complete absence of Spexin expression in human *obese* WAT, to propose that Spexin has a role in the normal regulation of adipose tissue function including uptake of LCFA, that the absence of Spexin may be a major component of the hormonal dysreguation seen in obese fat, and that repletion of circulating Spexin may help restore normal feeding behaviors and energy balance in obese animals and man. The Spexin story is clearly just beginning and there are many unanswered questions, of which the effects of Spexin on body composition is an important one, but what is reported here clearly moves the field forward.

This study, one result of a productive collaboration between a laboratory focused on lipid metabolism and a team of bariatric surgeons, strongly supports the hypothesis that Spexin is a potent, natural satiety-inducing peptide that plays a key role in regulating feeding behavior, uptake of LCFAs into adipocytes, energy utilization and metabolism, and body weight in DIO mice and rats. Its therapeutic potential for the treatment of human obesity merits detailed exploration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Different aspects of this study have been supported by grants DK-52401, DK-72526 and DK072526-04eS1 from the National Institute of Diabetes and Digestive and Kidney Diseases, a Pilot and Feasibility Study from the New York Obesity and Nutrition Research Center (NYONRC) at St. Lukes Hospital (NYONRC grant DK-26687), a Sponsored Research Agreement between the Ferring Research Institute and Columbia University, a Sponsored Research Agreement between CovX Phamaceuticals and Columbia University, and the Columbia Liver Disease Research Fund. Drs. Walewski and Berk disclose that they are co-inventors on a patent application for the use of Spexin for the treatment of obesity and obesity-related disorders.

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What is already known about this subject?

- Spexin is a novel, recently described peptide hormone
- The Spexin gene (Ch12:orf39) maps to a locus associated with various phenotypic markers of obesity
- Spexin has recently been shown to play a role in satiety in goldfish

What does this study add?

- In gene expression microarray studies Spexin is, proportionally, the most down-regulated gene in obese human fat.
- Administration of exogenous Spexin reduces food intake and body weight in DIO rodents.
- Spexin inhibits long chain fatty acid uptake into adipocytes in DIO rodents, and may be a regulator of LCFA uptake into human adipose tissue.

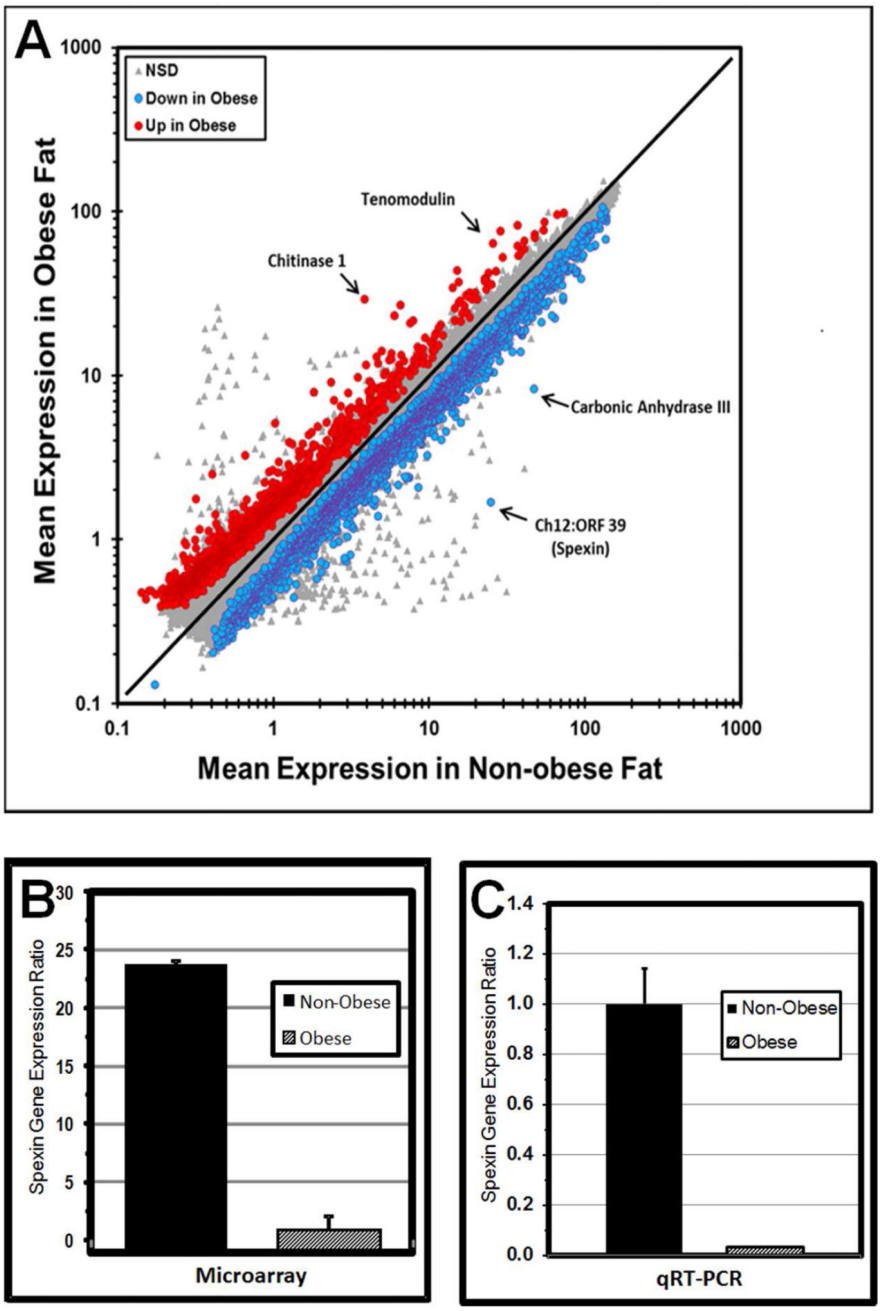

Figure 1. Differences in Gene Expression Between Normal and Obese Human Fat

A) Whole genome microarray analysis of non-obese fat vs obese (log-log plot): colored dots represent genes with significant differences ($p<0.05$) in expression between sample sets (red = genes over-expressed in obese fat, blue = genes over-expressed in non-obese fat; grey data points represent expression data not significantly different between the two fat groups, sometimes due to excessive variability in the data. Tenomodulin is reported to be over-expressed in obese fat (34, 35). Increased Chitinase 1 expression, a marker of activated macrophages in pulmonary disease (36), may reflect an inflammatory state common in

obesity. **B)** Spexin is 14.9 fold under-expressed in obese human fat by microarray, and **C)** Spexin is 33.3 fold under-expressed in obese human fat by qRT-PCR.

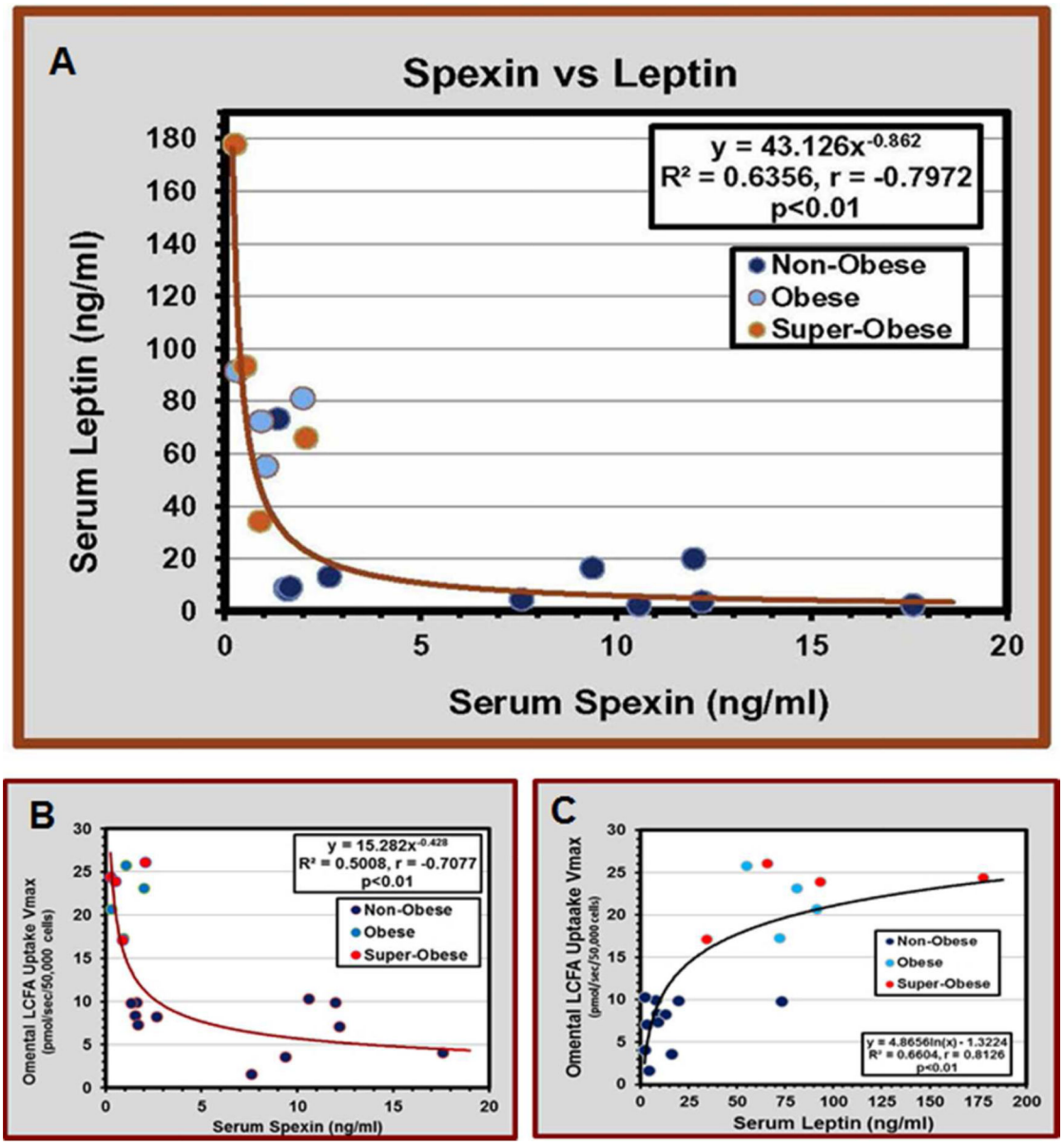

Figure 2. Serum Spexin and Leptin versus LCFA Uptake in Non-obese, Obese and Super-Obese Patients

A) There was a significant negative, non-linear correlation between human serum leptin and Spexin concentrations in the 18 available samples (r = −0.64, p<0.01). **B)** Spexin concentrations were significantly *negatively* correlated with the correponding Vmax for omental LCFA uptake in the same patient (r = −0.71, p<0.01). C) By contrast serum leptin was strongly *positively* correlated with this parameter: (r = +0.81, p<0.01.

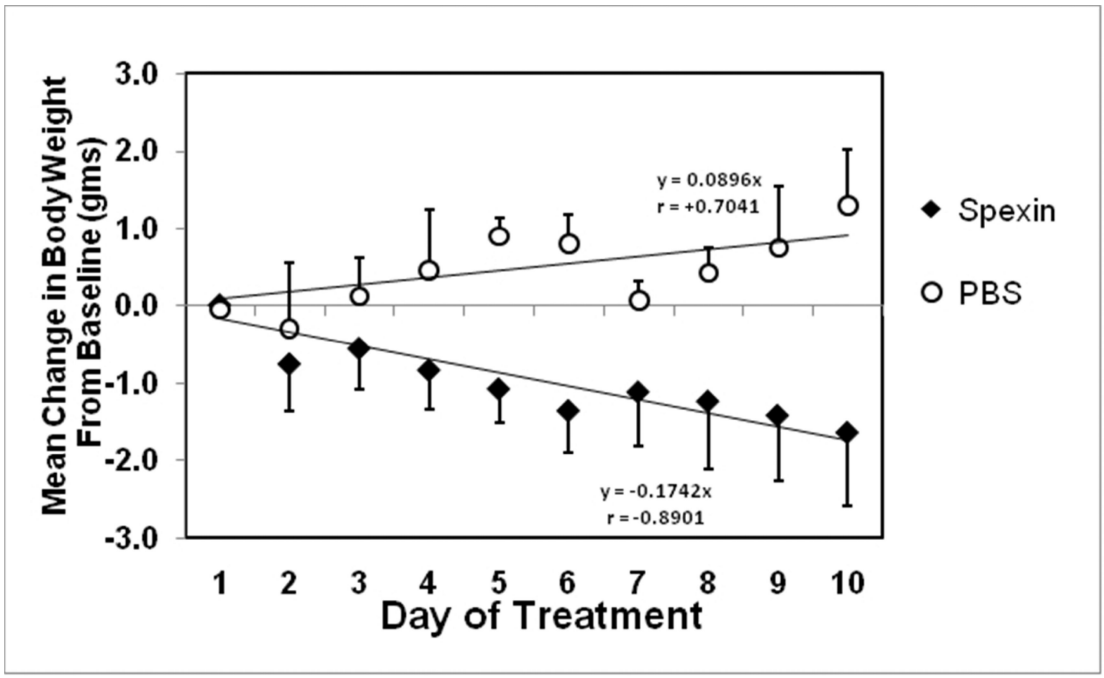

Figure 3. Daily Intraperitoneal Injections of Spexin Result in Weight Loss in DIO Mice

Adult male C57BL/6J DIO mice (Jackson Labs) received daily injections of Spexin (25 μg/kg IP in 0.1 mL of 1X PBS) for 10 days. Controls received daily IP injections of vehicle. All mice were maintained on ad lib high fat diet (D12492, Research Diets, New Brunswick, NJ). Body weights were measured daily. Data are mean change from baseline weight ± SE, (n = 5/group). Day 1 is the first day of injection. All Spexin-treated animals lost weight progressively (Slope = − 0.1742 g/day, r = − 0.8901). Controls continued to gain weight on the high-fat diet (Slope = + 0.0896, r = 0.7041).

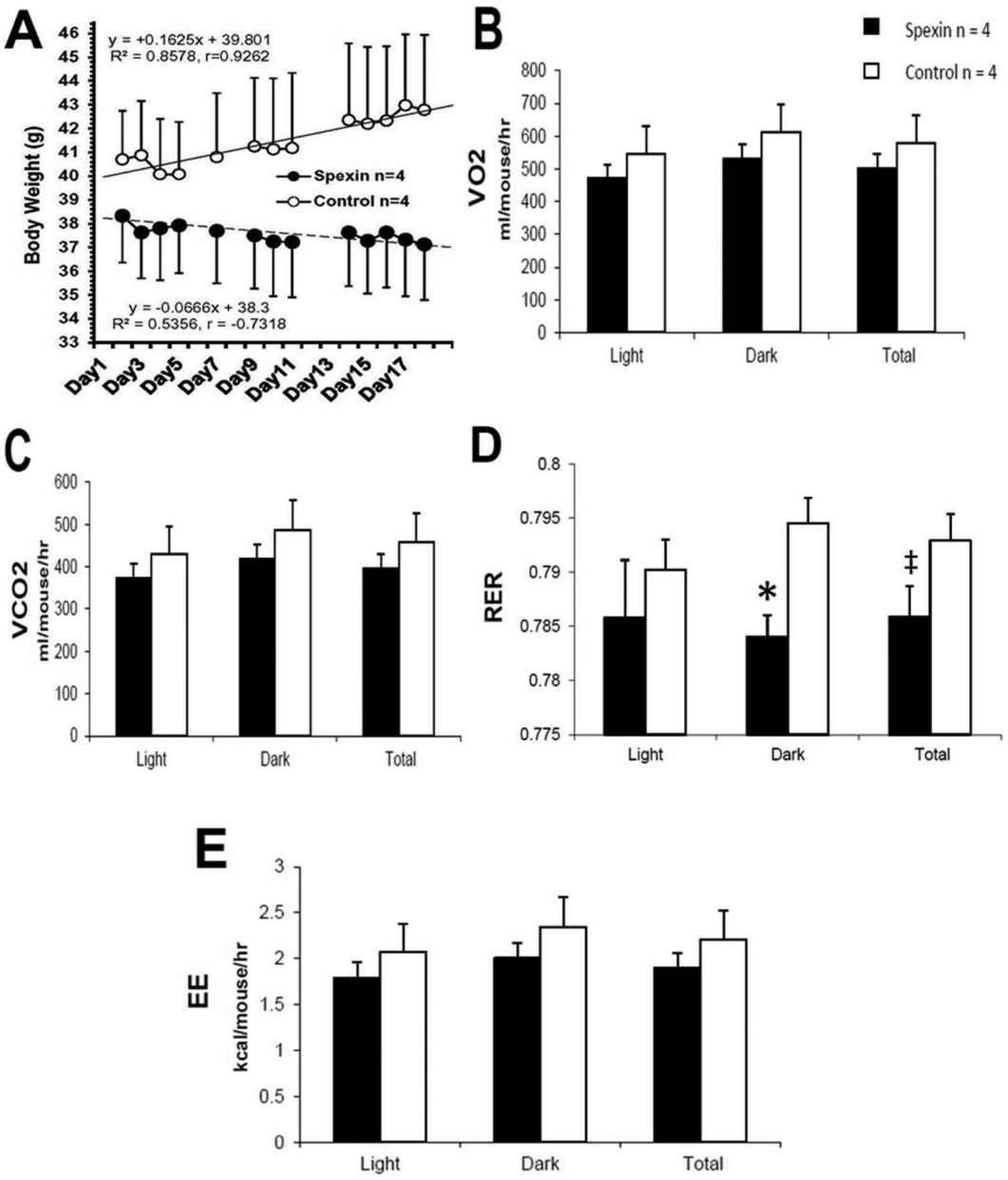

Figure 4. Effects of Spexin on Metabolic Parameters in DIO Mice

After adaptation, 18 – 20 week old DIO mice were dosed with Spexin (35 μg/kg ip in 1X PBS) daily for 19 days while individually housed in metabolic chambers. Individual body weights, 24 hour locomotor activity (ambulation in the X, Y and Z planes), oxygen consumption, CO_2 output, and the respiratory exchange ratio (RER) were monitored continuously using a CLAMS (Columbus Instruments, Columbus, OH) open-circuit indirect calorimetry system (16, 37). As in earlier studies (e.g. Figure 3), Spexin-treated animals lost weight but control mice continued to gain weight on the **HFD (4A).** Metabolic parameters such as VO2, VCO2, and EE may be expressed on either a per mouse or per gram BW basis. We have displayed them in Figure 4 on a per mouse basis. However, neither VO2 nor EE were significantly affected in Spexin treated mice regardless of whether the data are

expressed per mouse or per gram BW. Oxygen consumption (VO_2) **(4B)** was modestly increased during the dark cycle (lights off), when the mice are more active, *in both* control and Spexin-treated animals, compared to the light cycle. Overall, the Spexin-treated mice demonstrated a very modest, non-significantly lower VO_2 through the light, dark, and total periods compared to that in control animals. Carbon dioxide production (VCO_2) during the light and dark cycles **(4C)** exhibited a similar pattern to that of VO2, being somewhat lower in Spexin-treated vs control mice. The cumulative RER results over 24 hours **(4D)** , which represent the ratio of VCO_2/VO_2, show different patterns between the light (12 hours) and dark (12 hours) periods, and between the Spexin treated and control mice. The control mice exhibited an expected, modest elevation in RER during the dark compared to the light cycle, whereas that in the Spexin treated animals changes very little during the dark cycle. Consequently, the Spexin-treated animals have significantly *lower* RERs compared to the control mice during the dark cycle (0.784 ± 0.002 versus 0.795 ± 0.002, p = 0.006, respectively). The 24 hr total RERs were also lower in the Spexin-treated animals, although this difference did not quite achieve statistical significance (p =0.056) (Figure: * = $p < 0.05$, ‡ = 0.10 - p - 0.05). Total energy expenditure (EE) showed little difference in any comparison **(4E)**. The total kcal/mouse/hr expended increased slightly during the dark versus the light periods in both groups, however the total amounts of energy consumed during each period were almost identical between the Spexin-treated animals and the controls.

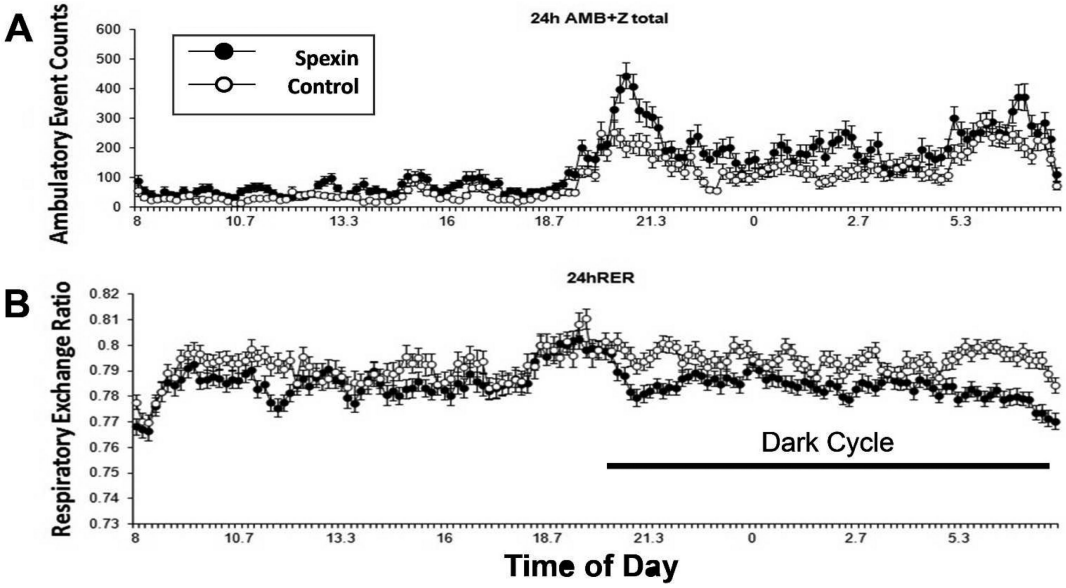

Figure 5. Effects of Spexin over 24hr on Selected Metabolic Parameters in DIO Mice

Twenty-four hour locomotor activity (ambulation in the X, Y and Z planes) **(Panel A)** and the respiratory exchange ratio (RER) **(Panel B)** were monitored continuously using a CLAMS (Columbus Instruments) open-circuit indirect calorimetry system. During dark periods increases in ambulation (especially in the Z plane, i.e. increased rearing), and reductions in RER reached statistical significance. The results indicate: 1] that Spexin increases both rearing (Z plane) and TOTAL (= X, Y and Z planes summed) locomotor activity relative to vehicle-treated DIO controls **(5A)**, and 2] that Spexin significantly reduced the RER, relecting an increase in lipid oxidation **(5B)**, particularly at night. It should be noted that the main diurnal variations normally seen in these parameters are preserved in both groups of animals, as can be seen by the generally parallel tracks of the recordings over the 24 hour observation period. One example is the clear presence of the pre-anticipatory spike in ambulation just before “lights out”, as the animals exhibit the expected increase in activity in advance of normal nightime feeding behaviors **(5A)**.

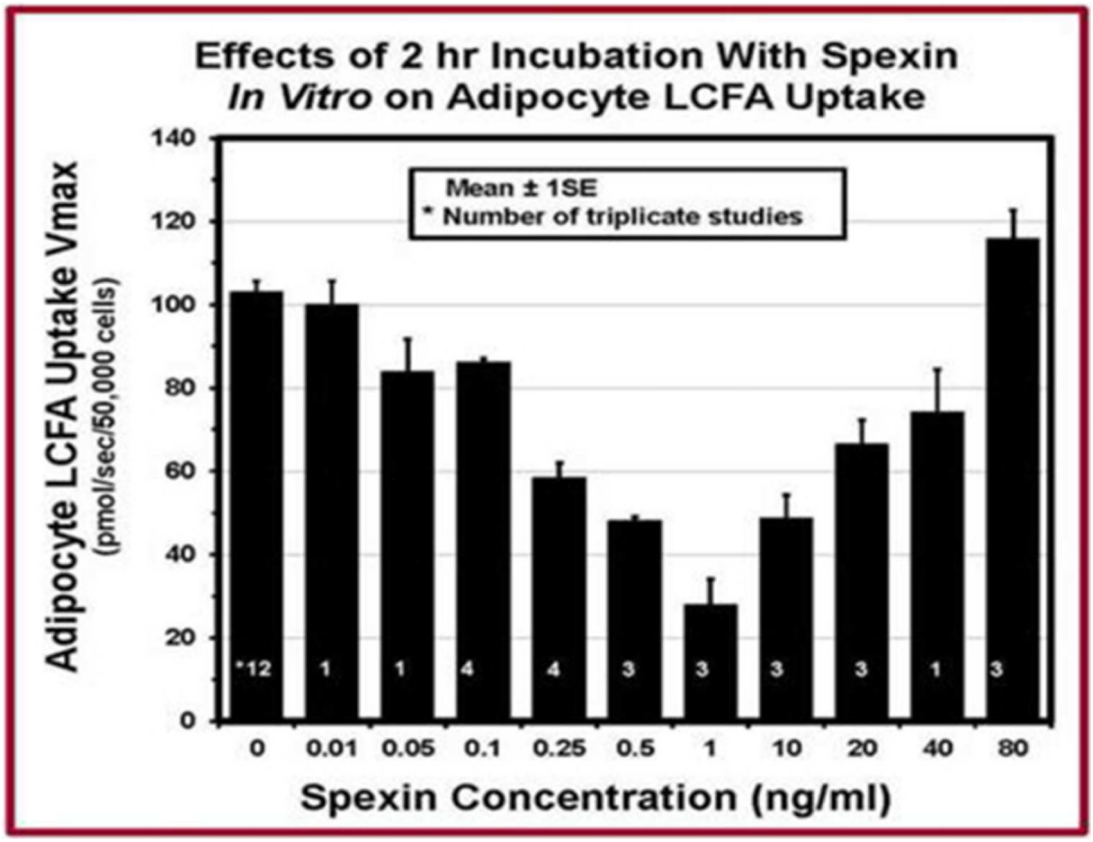

Figure 6. Concentration-response Studies of Spexin Inhibition of LCFA Uptake into Isolated Murine Adipocytes

Adipocytes isolated from untreated DIO mice were suspended in iced PBS until ready for study. After being warmed to 37°C, the cells were then incubated for two hours in either PBS alone (controls) or PBS + Spexin at 10 different concentrations, ranging from 0.01 to 80 ng/ml. The cells were then washed, and their LCFA uptake kinetics determined. The biphasic dose response curve is consistent with hormesis (see Discussion).

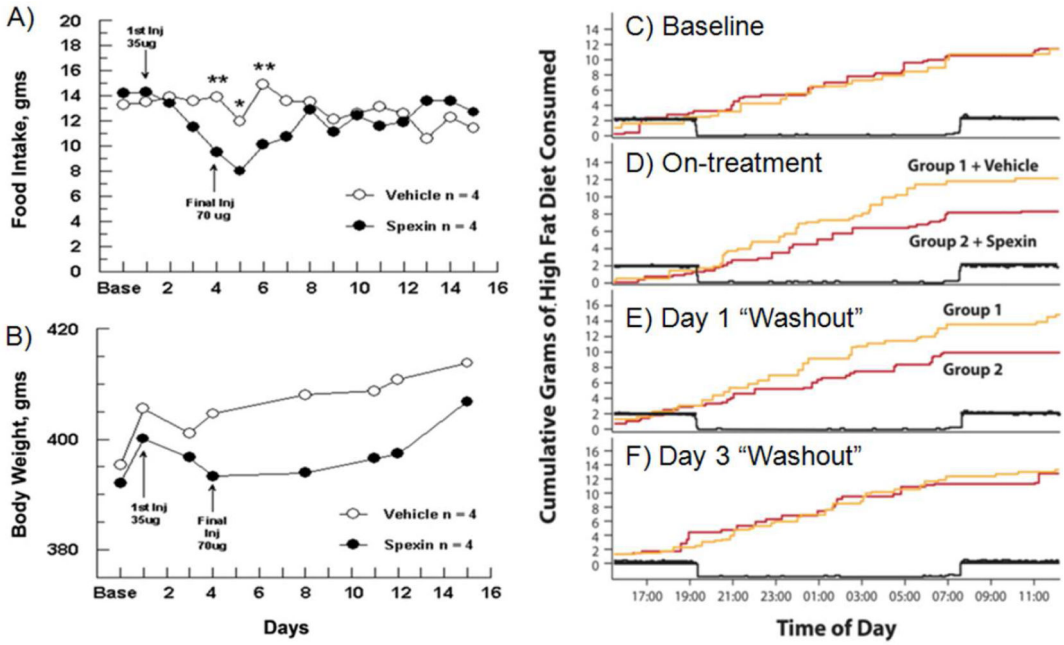

Figure 7. Daily Injections of Spexin into DIO Rats Lead to Reductions in Food Intake (A), Body Weight (B), and Size and Duration of Individual Meals (C-F)

Female DIO rats received single daily injections (sc) of vehicle (phosphate-buffered saline, PBS) or Spexin (n = 4/group) over a 4-day period, with Spexin doses set at 35 μg/kg BW for the first 3 days and 70μ/kg BW on the fourth day. Body weight and 24-hr food intake of the rats were recorded daily during the treatment period, and for 11 consecutive post-treatment days ("washout" days). **(A)** Significant reductions (ca 32%) of daily food intake were seen on days 4-6 of the experiment (the final treatment day, and first two washout days) that persisted at least to day 7 (*$p < 0.05$ ** $p < 0.01$ vs PBS controls). **(B)** A reduction in body weight was apparent by day 4 and persisted well beyond the treatment period.

An analysis of the number of feeding events or "meals" during each 24 hour light/dark cycle, and the size and duration of individual meals was conducted using computer-recorded feeding data. Individual body weights and total food consumed were measured daily. Thicker horizontal blue lines at the base of the figures indicate the "lights on" period, with the "lights off" period running from 19.30 to 7:30 hours. As expected, the vast majority of feeding occurs during "lights off". **C) Baseline:** When the lights are turned off (dark period), both groups of DIO rats begin to consume food at a relatively steady rate. Meal pattern analysis revealed that meal frequency, meal duration and meal size were very comaprable between the two groups at this point. **D) On-treatment:** By the fourth treatment day Spexin-treated animals (70Lg/kg) demonstrated reduced meal size and duration, without altering their overall feeding pattern. Food consumption was lower during the normal feeding period (lights out; 19:00 to 07:00 hours), and the animals maintained the normal cessation of feeding behaviors during the "lights on" period. No signs of overt toxicity or nausea were observed in the spexin-treated rats. **E) Day 1 Wash-out;** during the intial day of post-Spexin treatment, the satiety effect of the peptide begins to diminish. **F) Day 3 Wash-out;** several days after the last injection of Spexin, both groups of animals again demonstrate equivalent total 24hr food consumption and meal patterns. It is noteworthy that

meal frequency and the temporal (light/dark) feeding pattern during the 24 hr feeding periods remained normal in the peptide-treated group throughout the treatment and washout periods. However, both meal size (gms of food consumed/meal) and meal duration (time spent feeding/meal) were smaller in the Spexin-treated animals, who consumed approximately 32% fewer calories overall during the last day of treatment and the first two days following treatment.

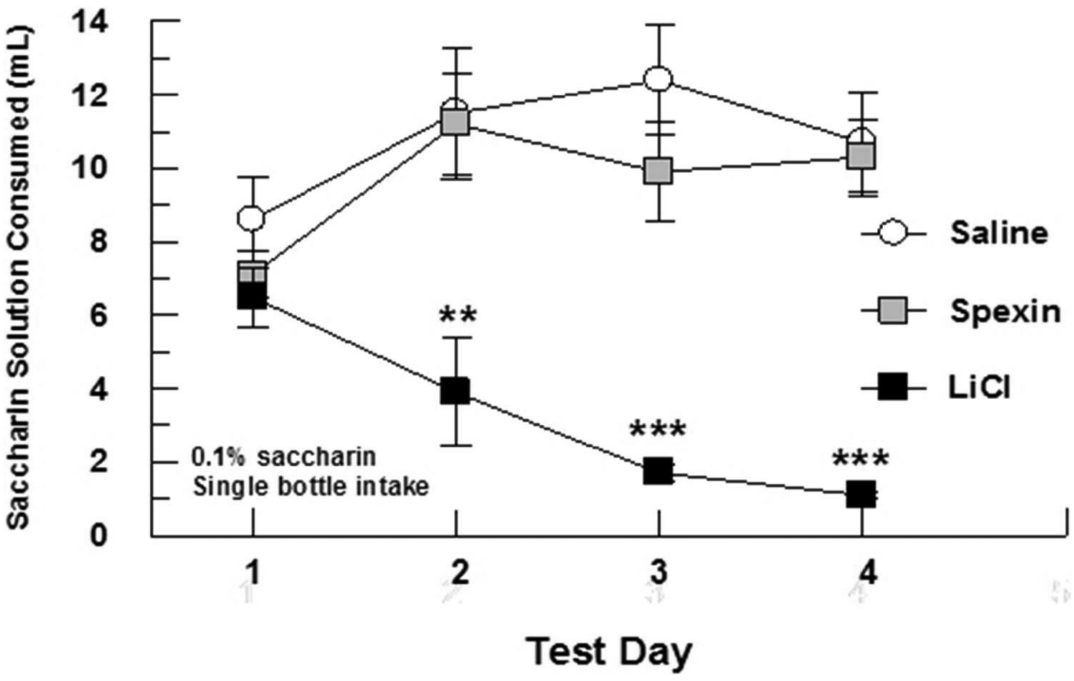

Figure 8. Conditioned taste aversion testing in adult female Wistar rats

After 20 hrs of water deprivation, rats were offered a single bottle containing 0.1% sodium saccharin for 20 min, followed 20 min later by an ip injection of either physiological saline (n=6), 70μg spexin/kg body weight (n=6), or 63.5 mg LiCl/kg body weight (0.15M LiCl) (n=5). Spexin and LiCl were mixed in 1X PBS in concentrations of 7μg/ml and 0.15M, respectively, and a solution dose of ml injected = 1% of body weight (g) was used for all injections. All groups experienced saccharin exposure (20 min), followed by their first injection on test day 1. Injections were administered on test days 1-3, with no injection on the final test day. The average amount of saccharin solution consumed during each 20 min test period was recorded for each group. A mild dose of LiCl was used over repeated test sessions to generate a slowly developing conditioned taste aversion. LiCl results served as a positive control arm (for taste aversion) with which to assess the effects of repeated injections of Spexin. The total amount of saccharin solution consumed during the 20 min test by the LiCl-treated animals on the 2nd injection day and following was significantly less than consumed by either the saline or spexin-treated animals, ** $p < 0.01$, *** $p < 0.001$. Data points represent mean ± SEM. No significant difference in the volumes of saccharin solution consumed were observed in the Spexin-treated animals.