Anti-Ghrelin Spiegelmer Inhibits Exogenous Ghrelin-Induced Increases in Food Intake, Hoarding and Neural Activation, but Not Food Deprivation-Induced Increases

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\textbf{Running Head:} Ghrelin Ingestive Behavior Effects Inhibited by Spiegelmer

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ABSTRACT

Circulating concentrations of the stomach-derived ‘hunger-peptide’ ghrelin increase in direct proportion to the time since the last meal. Exogenous ghrelin also increases food intake in rodents and humans suggesting ghrelin may increase post-fast ingestive behaviors. Food intake after food deprivation is increased by laboratory rats and mice, but not by humans (despite dogma to the contrary) nor by Siberian hamsters; instead, humans and Siberian hamsters increase food hoarding, suggesting the latter as a model of fasting-induced changes in human ingestive behavior. Exogenous ghrelin markedly increases food hoarding by ad libitum-fed Siberian hamsters similarly to that after food deprivation indicating sufficiency. Here we tested the necessity of ghrelin to increase food foraging/hoarding/intake and neural activation [c-Fos immunoreactivity (c-Fos-ir)] using anti-ghrelin Spiegelmer NOX-B11-2 (SPM), an L-oligonucleotide that specifically binds active ghrelin inhibiting peptide-receptor interaction. SPM blocked exogenous ghrelin-induced increases in food hoarding the first two days after injection, and foraging and food intake at 1-2 h and 2-4 h, respectively, and inhibited hypothalamic c-Fos-ir. SPM given every 24 h across 48 h food deprivation inconsistently inhibited food hoarding after refeeding and c-Fos-ir, similarly to inabilities to do so in laboratory rats and mice. These results suggest that ghrelin may not be necessary for food deprivation-induced foraging/hoarding and neural activation. A possible compensatory response, however, may underlie these findings because SPM-treatment led to marked increases in circulating ghrelin concentrations. Collectively, these results show that SPM can block exogenous ghrelin-induced ingestive behaviors, but the necessity of ghrelin for food deprivation-induced ingestive behaviors remains unclear.

Key Words: foraging, Siberian hamster, c-Fos, immunohistochemistry
INTRODUCTION

Obesity is a critical health problem nearly worldwide due to its many secondary health consequences including stroke, Type II diabetes, heart disease, and some cancers (13; 26; 29; 55; 56; 65; 71). One result of the dire nature and pervasiveness of obesity is the increase in health care costs – for example, an estimated 147 billion USA dollars in 2008 (24). Therefore, preventing and reversing obesity will have overall health and financial benefits. Food intake that exceeds energy expenditure is the prima facie cause of obesity. We believe one contributing cause of the overconsumption of food is the increased availability of relatively inexpensive calorically dense foods/drinks, their longer shelf-lives, and the increasingly larger storage compartments (refrigerators, freezers, pantries) for these items [for review:(7)].

Ingestive behavior is a series of motor responses that begins with the search for food (foraging/food shopping) and eventual location of food, followed for its immediate consumption (feeding/eating) or delayed consumption with intervening steps whereby food is transferred to another location (burrow/‘home’) and at least temporarily hoarded/stored. In 1918, Wallace Craig (14) dichotomized regulatory behaviors, including food intake, into either the appetitive phase (i.e., the steps leading to the goal, in the present case foraging and hoarding of food) or the consummatory phase (i.e., as in consummation of the goal, here consumption of food). The vast majority of studies of ingestive behavior/obesity has been conducted using laboratory rats and mice and has focused on the consummatory phase (food intake) yielding considerable insights into a complex and often redundant set of physiological controls of feeding. By contrast, there has been significantly less research focused on the appetitive aspects of ingestive behavior that also are under physiological control [for review see: (7)]. We have approached this often ignored, yet critical feature of ingestive behavior (one cannot eat food not acquired), using
Siberian hamsters (*Phodopus sungorus*) because, unlike laboratory mice and rats (12; 32; 61), they hoard food in nature (25), a behavior that can be readily duplicated in the laboratory (48; 67) using our simulated foraging/burrow system (16). Moreover, one of the most frequent energetic challenges employed in ingestive behavior/obesity research is food deprivation-refeeding. Surprisingly few laboratory studies have tested food deprivation-refeeding responses in humans and field studies of religious prohibitions on eating followed by refeeding reveal a virtual absence of post-fast eating increases [for review see: (7)]. Some examples of the lack of overeating after fasting/food restriction is that there are no compensatory increases in food intake on subsequent days by non-restrained eaters fasted for 24 h or food restricted to 1200 kcal (46), or fasted for 19 h (31). We are aware of only one laboratory study reporting a modest (~20%) increase in food intake after a 36 h fast (36). There is no increase in food intake after the one-day fast at the beginning of each month for members of the Church of Jesus Christ of Latter-Day Saints (54). With the ~13 h daily fasting by Muslims during Ramadan daylight hours, eating after sunset shows significant, but relatively modest food intake increases [(37; 38; 45); ~20%, 5%, 6%, respectively], or no increases (2; 42). Thus, contrary to popular and perhaps personal intuitions about eating after a period of not doing so, there is essentially no or little post fast/food restriction-induced increases in eating by humans [for review see: (7)]. Rather both hungry humans and hamsters ‘overhoard’ after food access resumption, whereby hungry humans bring home more food even after mild periods of no food versus their fed counterparts (9; 22; 49), as do food deprived Siberian hamsters exhibiting markedly increased food hoarding, but not food intake (5; 6; 17; 67). Therefore, the consummatory and appetitive ingestive behaviors of humans and Siberian hamsters are more similar than different compared to laboratory rats or mice.
Therefore, we have exploited this commonality in our study of appetitive ingestive behaviors (food foraging/food hoarding).

One consequence of periods without food for humans, laboratory rats and mice, and Siberian hamsters is a significant increase in circulating concentrations of ghrelin [for review see: (50)]. Ghrelin is a 28-amino acid peptide released by the stomach in direct proportion to the time since food was last consumed and thereby may act as a “hunger signal” (44; 64). We also found monotonically increasing circulating concentrations of ‘active’ ghrelin (acylated ghrelin) with increasing food deprivations lengths in Siberian hamsters (39). Ghrelin is acylated from its desacylated form in the gastric L cells that synthesize the peptide via ghrelin O-acyl transferase [GOAT; (72)] converting ghrelin to is bioactive acylated form (27; 69). Acylated ghrelin increases food intake through its sole receptor, growth hormone secretagogue receptor (GHSR a.k.a. GHSR-1a), by stimulating GHSRs possessed by arcuate nucleus (ARC) neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons [for review: (3)], ventral tegmental area dopaminergic neurons, [for review: (21)], caudal brainstem neurons (23) and likely other neural sites.

The use of various knock-out mouse models to test the necessity of ghrelin in food intake and its role in obesity has been inconclusive. Several ghrelin loss of function mouse models, including those deleting ghrelin (60; 68), GOAT (72), or GHSRs (53; 74), generally do not produce the expected decreases in food intake or adiposity when the mice are maintained on a chow diet, suggesting that ghrelin is not necessary for normal day-to-day feeding. In some cases when given a high-fat diet, however, the body mass gain of the ghrelin knock-out mice is blunted, but variable across studies depending on genetic, environmental, and developmental factors [for review (3)]. One hypothesis that may explain the lack of an effect in ghrelin
signaling-deficient mice is that during development compensatory mechanisms are initiated that
are able to overcome the loss of ghrelin similar to what occurs in mice with fetal AgRP neuron
ablation (47).

Because laboratory rats and mice do not hoard food in nature [for review see: (7)] and
because of the inability to genetically modify Siberian hamsters, we only have tested the
sufficiency of ghrelin to stimulate food foraging. That is, exogenous ghrelin administration in ad
libitum-fed Siberian hamsters (that produces food deprivation-induced concentrations of
circulating acylated ghrelin) triggers impressive and persisting increases in food hoarding (39-
41) duplicating both the magnitude (>~300%) and duration (5-6 d) of food deprivation-induced
increases in food hoarding by this species (5; 6; 17; 20; 67). In addition, exogenous ghrelin
administration creates interoceptive cues that generalize to those produced by food deprivation in
ad libitum-fed laboratory rats (15). Together this suggests that exogenous ghrelin mimics
aspects of food deprivation making it a useful tool to test ad libitum-fed hamsters because it
eliminates many other changes associated with food deprivation per se.

To test the necessity of ghrelin for increases in food foraging, hoarding, and intake in
Siberian hamsters we took advantage of the development of non-natural nucleic acids designed
to bind to a specific target peptide to neutralize its effects (30). In addition, because they have an
L-ribose backbone, they are protected from degradation by nucleases creating a high affinity
binding to the peptide making them an attractive tool (30). These compounds are termed
Spiegelmers (from the German “Spiegel” or “mirror” because they are built from mirror-image
nucleotides) and anti-ghrelin Spiegelmer NOX-B11-2 (SPM) is designed to specifically
neutralize acylated ghrelin (30; 35). SPM prevents the orexigenic effects of exogenous ghrelin
in both laboratory rats and mice, but not of a synthetic GHSR agonist, Compound A, suggesting
no direct interaction with GHSRs (43; 59). Chronic, systemic infusion of SPM partially reverses 
diet-induced obesity and transiently inhibits food intake, but does not do so in ghrelin knock-out 
mice (59).

Here we tested the necessity of ghrelin in food foraging, hoarding, and intake in Siberian 
hamsters. This was accomplished by peripherally injecting SPM to neutralize exogenously 
administered ghrelin and any naturally-released ghrelin, and in a second experiment, to 
normalize food deprivation-induced increases in naturally-released circulating ghrelin. The 
subsequent changes in these ingestive behaviors and c-Fos immunoreactivity (c-Fos-ir), an 
indicator of neural activation (33), and acylated/desacylated ghrelin were assayed. Changes in c-
Fos-ir here and in other studies of ghrelin and SPM (8; 10; 43) indicate potential neural 
activation sites associated with ghrelin/lack of ghrelin alterations in ingestive behaviors.

MATERIALS AND METHODS

Animals

Adult male Siberian hamsters, ~2.5-3 months old and weighing 34-46 g were obtained 
from our breeding colony, previously described (11). Hamsters were raised from birth in group 
housing in a long-day photoperiod (16L:8D; light offset: 1800) until used in experiments. Tap 
water and food (Laboratory Rodent Diet 5001, Purina, St. Louis, MO) were available ad libitum 
unless otherwise indicated. Room temperature was maintained at 21 ± 2 ºC. All procedures 
were approved by the Georgia State University Institutional Animal Care and Use Committee 
and were in accordance with Public Health Service and United States Department of Agriculture 
guidelines.
Foraging and Hoarding Apparatus

Ingestive behavior was assessed using our foraging and hoarding apparatus, adapted from Perrigo and Bronson (52) and previously described (16). In brief, the foraging and hoarding apparatus consists of two cages (top: 456 x 234 x 200 mm and bottom: 290 x 180 x 130 mm) connected by polyvinylchloride tubing (38.1 mm inner diameter and ~1.52 m in length) with corners and straightaways for vertical climbs and horizontal runs, respectively. The top cage is exposed to vivarium light, contains a running wheel (524 mm circumference) connected to a software/hardware-based program that monitors wheel rotations (Med Associates, Georgia, VT), as indicated by a magnet and magnetic field-sensitive switch, and delivers food pellets (75 mg pellets: Dustless Precision Pellets, Purified 75 mg pellets; Bio-Serv, Frenchtown, NJ) from a pellet dispenser (Med Associates), and a water bottle. The opaque, bottom cage contains Alpha-Dri bedding (Specialty Papers, Kalamazoo, MI) and one cotton nestlet (Anacare, Belmore, NY) and its top is covered to simulate the darkness of a burrow. Quantification of food foraging, intake, and hoarding was done daily at 0900 (light offset: 1330). Food foraging was defined as the number of wheel rotations divided by 10 (as 10 wheel rotations were required for each pellet delivered). Food intake was defined as the number of pellets earned minus hoarded food and surplus food (food remaining in the top cage). Food hoarded was defined as the food found in the bottom cage and in the cheek pouches of the hamsters. An electronic scale used to weigh the food pellets was set to “parts” measurement, resulting in one 75 mg food pellet = 1 with fractions of pellets computed by the scale. After data collection the surplus and hoarded pellets were discarded.

Foraging and Hoarding Apparatus Acclimation and Baseline
Upon transfer to the foraging and hoarding room (16L:8D; light offset: 1330), animals were singly housed in polypropylene shoebox cages (290 x 180 x 130 mm) for 2 wk to acclimate to the new light cycle and the pellet test diet. After the 2 wk, each animal was placed into a foraging and hoarding apparatus and for the first 3 d animals were given 300 pellets as well as being able to earn a pellet for every 10 wheel rotations. Subsequent to the first 3 d, food only was available after the completion of 10 wheel rotations for the duration of the experiment unless otherwise noted. The animals were allowed to acclimate to the 10 wheel rotations/pellet for 10 d to ensure stabilization of baseline body mass, food intake and food hoarding. Pilot data indicated that SPM did not affect general locomotor activity (neither increases or decreases) suggesting no non-specific increases or decreases in locomotor activity that could be misinterpreted as increases or decreases in motivation to earn food or for the latter possible malaise ([e.g., (39)]).

Experiment 1: Does SPM inhibit exogenous ghrelin-induced increases in ingestive behavior?

The animals (N = 40) were placed into one of three groups balanced for percent change in body mass, absolute body mass, food intake, and food hoarding during the baseline period. The three groups were: 1) saline + 30 µg/kg ghrelin, 2) 18 mg/kg SPM + saline and 3) 18 mg/kg SPM + 30 µg/kg ghrelin (SPM was generously provided by NOXXON Pharma AG, Berlin, Germany). The ghrelin dose was selected based upon our previous study showing this dose produced circulating acylated ghrelin concentrations equivalent to that for 48 h food deprivation in Siberian hamsters (39), whereas the SPM dose was calculated by extrapolating from previous studies in laboratory rats and mice (8; 43) and our own pilot study in Siberian hamsters (data not shown). A saline + saline group was not included because pilot data showed no difference between saline + saline and SPM + saline treated animals for any measure (data not shown).
Animals were acclimated to the injection protocol the week before the first test day and for the duration of the study. In brief, hamsters were lightly restrained for 30 s similar to the process undertaken each test day. Each bottom cage was changed on test days (to eliminate any possibility of missed hoarded pellets or fractions of pellets) and hamsters were blocked from access to food for 1-2 h before injection as done previously (39). On test days, hamsters were injected intraperitoneally (i.p.) 30 min before light offset with either SPM or its vehicle (sterile physiological saline) and received ghrelin or its vehicle (sterile physiological saline) i.p. at light offset similar to a previous experiment (39). Food foraging, intake, and hoarding were measured at 1, 2, 4, and 24 h post-injection and each day until pre-test day baseline values for all behaviors were restored (~7-8 d). Each animal received all three treatments over the course of the study using a within-subject design that reduces the inherent variability in food hoarding, a strategy we successfully used previously (18; 19; 39-41), with the treatments counterbalanced. An 8 d washout period occurred after each test day. When pre-test (baseline) values were restored after each animal had received all three treatments, hamsters were given another week to establish a ‘new’ baseline for the food deprivation study (see Experiment 3).

**Experiment 2: Does SPM inhibit ghrelin-induced neural activation?**

A separate cohort of male Siberian hamsters (N=46) was obtained from our breeding colony and were singly housed in standard polypropylene shoebox cages. The photoperiod and light offset were the same as the breeding colony. Body mass and food intake were measured every other day for 2 wk. The animals were then separated into 4 groups balanced for percent body mass change, absolute body mass, and food intake over the 2 wk period. During the 2nd wk, animals were sham injected each day. In brief, each animal was lightly restrained for 30 s
twice, once at 30 min before light offset and once at light offset. The sham injection procedure
was followed to minimize neural activation (to be measured by c-Fos-ir) due to handling stress
as we have done previously (63). Each group received 2 injections separated by 30 min in the
same manner and dosage as Experiment 1. Brains were collected at 2 or 24 h post-treatment
resulting in the following groups: 1) saline + saline 2 h (n=6), 2) SPM + saline 2 h (n=6), 3)
saline + ghrelin (n=6), 4) SPM + ghrelin 2 h (n=6), 5) saline + saline 24 h (n=6), 6) SPM + saline
24 h (n=5), 7) saline + ghrelin 24 h (n=5), and 8) SPM + ghrelin 24 h (n=6). After tissue
collection, brains were stored, sectioned on a sliding freezing microtome at 30 µm, and stained
for c-Fos-ir as we previously described (63) using the c-Fos antibody sc-52 (Santa Cruz
Biotechnology, Santa Cruz, CA) according to manufacturer’s suggestions. The standard time
point for assaying c-Fos-ir is 60-120 min post treatment, but we and others have obtained
significant increases c-Fos-ir at 14-24 h post treatment (28; 63; 73). C-Fos-ir cells were counted
across each entire structure using light microscopy with the counter blind to the treatment
regime. Specific nuclei were selected a priori based upon the available literature where systemic
ghrelin resulted in c-Fos-ir and any other nuclei that appeared to have ghrelin-induced or SPM-
induced changes c-Fos-ir during quantification (see below). To reduce the probability of
counting the same neuron twice, Abercrombie’s correction factor was used (1).

Experiment 3: Is ghrelin necessary for food deprivation-induced increases in ingestive behavior?

Siberian hamsters (N=40) from Experiment 1 were re-divided into four groups balanced
for percent body mass change, absolute body mass, food intake, and food hoarding after the 1 wk
‘new’ baseline period that followed restoration of the original pre-Experiment 1 baseline: 1) Fed-
Saline, 2) Food-Deprived-Saline, 3) Food-Deprived-18 mg/kg SPM, or 4) Food-Deprived-36
mg/kg SPM. SPM dosages were based upon the known effective ratio of SPM to ghrelin and the circulating ghrelin concentration in Siberian hamsters after a 48 h food deprivation (39; 59). All animals, except Fed-Saline, were food deprived for 48 h (beginning at light-offset) by disconnecting the pellet dispenser from the computer thereby allowing continued assessment of wheel running with no pellet delivery. SPM was administered at 24 h intervals such that each animal received 3 i.p. injections at the initiation of food deprivation (time 0 h), 24, and 48 h later (30). After the final injections, the pellet dispensers were reconnected to the computer and food foraging, food intake, and food hoarding were measured at 1, 2, 4, 24 h and each day subsequent to refeeding until the pre-food deprivation baseline was recovered.

In two previous studies (8; 59), the inability of SPM to block food deprivation-induced increases in food intake and c-Fos-ir was speculated to be due to, among other possibilities, ghrelin not being necessary for these responses. This overlooked another possibility suggested by the increased ghrelin concentrations assayed (58; 59) -- that the ghrelin assay recognized acylated ghrelin bound to SPM and/or unbound SPM. Therefore, to more completely test the necessity of ghrelin in food-deprived animals with specific regard to the circulating concentrations of ghrelin, we obtained a separate cohort of male Siberian hamsters (N=24) from our breeding colony. Animals were individually housed in standard polypropylene shoebox cages for 2 wk before study initiation to acclimate them to the new housing conditions (16L:8D, light offset: 1800). Animals were weighed, food intake measured daily for 1 wk and then the animals were divided into four groups balanced for the percent change in body mass, absolute body mass, and average daily food intake: 1) ad libitum-fed/saline-injected, 2) ad libitum-fed/SPM-injected, 3) 48 h food-deprived/saline-injected, and 4) 48 h food-deprived/SPM injected. Food deprivation began at light-offset. Injections (SPM at 36 mg/kg body mass or
sterile physiological saline vehicle, i.p.) were given every 24 h beginning at the start of the food
deprivation period yielding 3 total injections. After the final injection, intraorbital blood was
taken according to our previously published method (62) and circulating acylated ghrelin and
desacylated ghrelin concentrations were assessed using ELISA (ALPCO Immunoassays, Salem,
NH) exactly according to manufacturer’s instructions and previous published methods (4). In
brief, ~500 µl blood was collected via the retro-orbital sinus using heparinized Natelson
collecting tubes. For the acylated ghrelin assay (A05117), 300 µl of the blood was put into a
pre-chilled BD microtainers containing EDTA (Franklin Lakes, NJ), inverted 10 times, and
placed on ice until all samples were obtained. The blood was then transferred to pre-chilled
microcentrifuge tubes containing 300 µl of blood transfer buffer (1.2 % 10N NaOH 2mM p-
hydroxymercuribenzoic acid, 500 mM NaCl, and 25 mM EDTA in deionized water) and then
spun at 5000 rpm at 4 ºC for 10 m. Plasma was then transferred to pre-chilled microcentrifuge
tubes, immediately acidified using 1 N HCl (1 µl HCl/10 µl plasma) and spun at 5000 rpm at 4
ºC for 5 min. The acidified plasma was transferred to a microcentrifuge tube and stored at -20 ºC
until assayed for acylated ghrelin. For desacylated ghrelin, 200 µl of the remaining blood was
put into a pre-chilled BD microtainers containing EDTA (Franklin Lakes, NJ), inverted 10 times,
placed on ice until all samples were obtained and then spun at 3500 rpm at 4 ºC for 10 min. The
samples were then transferred to microcentrifuge tubes and stored at -20 ºC until assayed for
desacylated ghrelin (A05118). Control samples were run for both assays to assure that our
values were not due to the assays recognizing unbound SPM, or SPM bound to ghrelin and to
ensure that the blood treatment procedure did not cause SPM to disassociate from the acylated
ghrelin. The three controls for both assays were prepared in 1.5 ml microcentrifuge tubes: 1)
SPM (90 ng/ml or 3.6 mg/ml), 2) acylated ghrelin (150 pg/ml) or des-acyl ghrelin (16 pg/ml),

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and 3) acylated ghrelin (150 pg/ml) with SPM (90 ng/ml) or des-acyl ghrelin (8 pg/ml) with SPM (3.6 mg/ml), respectively depending upon the assay. The acylated ghrelin with SPM control samples were acidified according to the procedure used above and stored in the same conditions and for the same length of time as the plasma samples. All control samples were diluted in the standard assay buffer that all other samples were diluted. Samples were diluted as necessary to be read in the midrange of the standard curve.

**Experiment 4: Is ghrelin necessary for food deprivation-induced neural activation?**

Additional male Siberian hamsters (N=24) were selected from our colony, housed, and treated as outlined in Experiment 2, unless otherwise noted. At the onset of treatment, half of the animals were food deprived for 48 h and the remaining animals were fed *ad libitum*. Each feeding treatment was further divided into two injection groups: 1) saline and 2) 36 mg/kg SPM. Groups were balanced for percent body mass change, absolute body mass, and average food intake during the 2 wk baseline period. Injections were administered with the same dosage and timing as in Experiment 3. After the final injection at the end of the 48 h food deprivation, animals were perfused and brains collected. c-Fos-ir was quantified as in Experiment 2.

**Statistical Analyses**

In Experiment 1, raw data was transformed into percent change from saline before statistical analyses according to the formula: [((X-Control)/Control)*100], where “Control” is the SPM + Saline group. For all measures of food foraging, intake, and hoarding in Experiment 1, the data are graphed as the mean percent difference from control treatment (100%) + SEM. No statistical comparisons are reported across time within a test day as the time intervals were of
unequal duration. Therefore, analyses were only run within time points using two-way repeated measures ANOVA (1st injection x 2nd injection). c-Fos-ir data from Experiment 2 and 4 were analyzed using two-way ANOVA, 1st injection x 2nd injection and injection x feeding state, respectively. For Experiment 3, behavioral data were analyzed using two-way ANOVA (feeding state x injection) within each time point. Post hoc tests for behavioral data were conducted with Duncan’s New Multiple Range test and for c-Fos-ir Bonferroni’s when appropriate. All analyses were done using NCSS (version 2007, Kaysville, UT) and exact probabilities and test values were omitted for simplicity and clarity of presentation. Statistical significance was considered if P<0.05.

RESULTS

Experiment 1: Does SPM inhibit exogenous ghrelin-induced increases in ingestive behavior?

Systemic ghrelin injection significantly increased food foraging at 1-2 h post-injection compared with SPM + saline-treated animals (Ps<0.05), an effect inhibited by SPM pretreatment (Ps<0.05; Fig 1A). Exogenous ghrelin significantly increased food intake above SPM + saline at 0-1, 1-2, and 2-4 h post-injection (Ps<0.05), an effect prevented by SPM pretreatment at the 0-1 and 2-4 h (Ps<0.05; Fig 1B), but not at 1-2 h (Fig 1B). Exogenous ghrelin increased food hoarding above SPM-saline treated animals at all time points (Ps<0.05, Fig 1C), with values returning to baseline after 7 d. SPM + ghrelin animals did not exhibit the typical ghrelin-induced increases in food hoarding the first two d post-treatment (Ps<0.05; Fig 1C); this inhibition waned, however, elevating food hoarding to that of ghrelin only-injected hamsters by Day 3 (Fig 1C). Both ghrelin injected groups had significantly increased food hoarding versus SPM + saline controls on Days 3-7 (Ps<0.05; Fig 1C; Means ± SE for SPM + saline treated animals, 0-1 h:
29.4 ± 4.7 (pellets earned), 4.6 ± 0.67 (pellets eaten), 1.3 ± 0.37 (pellets hoarded); 1-2 h: 20.1 ± 4.3 (pellets earned), 1.7 ± 0.34 (pellets eaten), 0.35 ± 0.13 (pellets hoarded); 2-4 h: 46.4 ± 8.5 (pellets earned), 7.0 ± 1.1 (pellets eaten), 4.2 ± 1.0 (pellets hoarded); 4-24 h: 43.3 ± 4.1 (pellets earned), 32.1 ± 1.3 (pellets eaten), 6.3 ± 1.7 (pellets hoarded); Day 2: 212.0 ± 23.2 (pellets earned), 60.1 ± 3.3 (pellets eaten), 18.7 ± 4.2 (pellets hoarded); Day 3: 229.5 ± 25.6 (pellets earned), 61.8 ± 4.2 (pellets eaten), 12.3 ± 3.1 (pellets hoarded); Day 4: 257.1 ± 23.9 (pellets earned), 63.5 ± 4.3 (pellets eaten), 15.2 ± 3.4 (pellets hoarded); Day 5: 252.7 ± 24.7 (pellets earned), 63.1 ± 4.1 (pellets eaten), 14.4 ± 3.9 (pellets hoarded); Day 6: 236.4 ± 22.4 (pellets earned), 60.9 ± 3.1 (pellets eaten), 17.9 ± 5.1 (pellets hoarded); Day 7: 259.4 ± 27.3 (pellets earned), 55.4 ± 3.1 (pellets eaten), 10.2 ± 2.0 (pellets hoarded); Day 8: (pellets earned), (pellets eaten), (pellets hoarded).

Experiment 2: Does SPM inhibit ghrelin-induced neural activation?

Saline (SPM vehicle) + ghrelin injection increased c-Fos-ir compared with saline + saline injected animals in the ARC (Fig 2), but not in the other nuclei examined [including but not limited to, the paraventricular nucleus (PVH), ventromedial hypothalamus (VMH), sub-zona incerta (sZI), perifornical area (pFA), area postrema (AP), and nucleus of the tractus solitarius (NTS; data not shown)] 2 and 24 h post-injection. SPM treatment alone did not increase c-Fos-ir compared to saline + saline at 2 or 24 h post-injection, but did inhibit ghrelin-induced c-Fos-ir in the ARC at 2 (Fig 2) and 24 h post injection (data not shown). Representative photographs of ARC c-Fos-ir are shown (Fig 2b) at approximately the same coronal level of the Arc as depicted for similarly SPM-treated mice (8).
Experiment 3: Is ghrelin necessary for food deprivation-induced increases in ingestive behavior?

Food deprivation significantly increased food foraging at 4-24 h and food hoarding at all time points until Day 8, with the exception of Day 5, compared with ad libitum fed saline-treated animals (Ps<0.05; Fig 3A and C). Systemic SPM injection (18 and 36 mg/kg) administered during food deprivation did not prevent increases in food foraging and hoarding at any time point compared to saline-treated animals, except food foraging at 4-24 h in the 18 mg/kg SPM treated animals (Fig 3A). On Days 6 and 7, the group receiving the low dose of SPM returned to baseline hoarding (Fig 3C) with the other two treatments (saline and 36 mg/kg SPM) doing so on Day 8 (Fig 3C). Food deprivation did not cause increased food intake (Fig 3B).

Because SPM did not affect these ingestive behaviors, similar to the lack of effect on food deprivation-induced increases in food intake by SPM-treated laboratory rats (58), we tested the in vivo ability of SPM to decrease circulating acylated ghrelin and desacylated ghrelin concentrations after 48 h food deprivation. Circulating desacylated ghrelin concentrations were significantly increased at the end of the 48 h food deprivation period in food-deprived saline-injected hamsters compared with saline-injected ad libitum-fed animals (P<0.05; Fig 4A). Desacyl-ghrelin concentrations of SPM-treated hamsters far exceeded (~100x) that of saline-treated animals regardless of whether they were food-deprived or ad libitum-fed (Ps<0.05; Fig. 4A). Acylated ghrelin concentrations of food-deprived animals treated with saline or SPM were significantly increased compared with their ad libitum-fed counterparts (Ps<0.05; Fig. 4C). Both SPM-treated groups had significantly increased circulating acylated ghrelin concentrations versus their respective feeding treatment groups (Ps<0.05; Fig. 4C). This effect was not due to the assay recognizing unbound SPM or SPM bound to ghrelin as ghrelin (Fig. 4B and D).
Experiment 4: Is ghrelin necessary for food deprivation-induced increases in neural activation?

Neural activation (c-Fos-ir) was significantly increased in hypothalamic nuclei (ARC, PVH, sZI, and pFA) and other regions examined [central nucleus of the amygdala (CeA), AP, and NTS] in food-deprived hamsters treated with saline or SPM compared with *ad libitum*-fed saline controls (Table 1 and Figure 5). SPM did not inhibit c-Fos-ir in 48 h food-deprived animals when compared to 48 h food-deprived, saline-treated animals in any of the brain nuclei examined. SPM-treated animals, regardless of feeding state, had increased c-Fos-ir when compared with *ad libitum*-fed, saline-treated animals (Table 1).

DISCUSSION

The present experiments were designed to test whether ghrelin is necessary for increases in food foraging, food hoarding, and food intake. To do so we used two separate conditions that increase circulating acylated (active) ghrelin -- peripheral acylated ghrelin injection and food deprivation -- and attempted to inhibit/block acylated ghrelin in each of the conditions. We used a SPM that is designed to specifically bind acylated ghrelin and prevent receptor/ligand interaction. Food deprivation triggered marked and prolonged increases in food hoarding by Siberian hamsters here, as we previously reported (5; 6; 17; 20; 67), and exogenous peripheral ghrelin administration mimicked these food deprivation-induced increases in hoarding in both magnitude and duration (39-41). Peripheral injection of SPM blocked exogenous ghrelin-triggered increases in food foraging, intake, and hoarding, as well as neural activation (c-Fos-ir). None of these responses were blocked by SPM in food-deprived hamsters. This inability of SPM to stem these food deprivation-induced increases in ingestive behavior and neural activation, however, is consistent with its inability to prevent food deprivation-induced increases in food...
intake in laboratory rats (58) and c-Fos-ir in laboratory mice (8). We assayed acylated (active) and desacyl (inactive) ghrelin in fed and food-deprived SPM-treated hamsters and found large, increases in acylated ghrelin and desacyl (inactive) ghrelin. The increase in acylated ghrelin is not unique to this study (58; 59) and may be a possible compensatory response that is strikingly engaged thereby increasing the secretion of both forms of ghrelin.

Our first two experiments were designed to test the ability of SPM to inhibit exogenous ghrelin-induced increases in ingestive behavior and neural activation. Exogenous ghrelin increases food intake in laboratory rats and mice [for review see: (50)], a finding extended to Siberian hamsters to include food foraging and food hoarding [present study; (39-41)]. Increases in food hoarding due to exogenous ghrelin last for up to 7 d post injection due to a currently elusive unknown mechanism underlying this persisting effect [e.g., (39)]. In Experiment 1, ghrelin caused short-term increases in food foraging (1-2 h) and intake (0-1, 1-2, and 2-4 h), similar to previous experiments (39-41), and SPM blocked the increase in food foraging and food intake at 0-1 and 2-4 h post-injection. Our results are similar to those in laboratory rats and mice where SPM inhibits exogenous ghrelin-induced increases in food intake (8; 43; 59). In Experiment 1, ghrelin caused the typical long-lasting increases in food hoarding persisting 6 d, an effect that was inhibited by SPM co-administration during the first two d post injection. The inhibition waned after the second day post treatment, perhaps due to possible compensatory increases of other stimulatory factors, including ghrelin (see below). SPM inhibited the increases in food intake by laboratory rats and mice during the first few hours post-injection, a time when the stimulatory effects of ghrelin on food intake are the greatest (43) and a result that is similar to the inhibition of the ingestive behaviors seen here in Siberian hamsters.
The SPM blockade of ghrelin-induced increases in ARC c-Fos-ir at 2 and 24 h post-injection compared with saline-injected hamsters is similar to the SPM-induced blockade of c-Fos-ir in laboratory rats and mice injected peripherally with ghrelin (8; 43). It is worth noting that we did not see significantly increased c-Fos-ir in the PVH that others previously reported after systemic ghrelin administration (57), perhaps due to species differences or the post injection time points for the collection of the neural tissue [2 h (present data) vs. 90 min (57)], although our finding is not unique (66). Our neural activation data are in agreement with the behavioral data. Thus, the first two experiments demonstrate the ability of SPM to inhibit exogenous ghrelin-induced increases in ingestive behavior and brain c-Fos-ir.

After confirming the ability of SPM to diminish ghrelin-induced increases in ingestive behaviors in Siberian hamsters, we tested the necessity of ghrelin in food deprivation-induced increases in ingestive behaviors. Forty-eight h food deprivation triggers increases in food foraging and hoarding in Siberian hamsters with increases in food hoarding lasting for up to 7 d (5; 6; 17; 20; 67), a response that is similar to that caused by ghrelin administration [e.g., (17; 39)]. Because circulating ghrelin concentrations significantly increase with food deprivation in this (17; 39) and other species, ghrelin appeared to be a logical candidate mechanism underlying these ingestive behaviors increases by Siberian hamsters. Therefore, we used SPM to eliminate endogenous bioactive (acylated) ghrelin from interacting with its receptors to test the necessity of ghrelin for food deprivation-induced increases in foraging, food intake, and food hoarding. Food deprivation caused the expected brief, but small, increases in food foraging and triggered long-term increases in food hoarding and increases in c-fos-ir compared with non-food-deprived animals, effects not blocked by SPM. The apparent inability of SPM to block the food deprivation-induced increases in food hoarding and c-Fos-ir appears might be interpreted as
ghrelin not being necessary for the increases in this appetitive ingestive behavior after food withdrawal-refeeding. Because, however, it was previously shown that SPM-treated laboratory mice and rats had circulating acylated ghrelin concentrations ~10 times that of controls (58; 59) and food deprivation-induced c-Fos-ir was not inhibited (8), we tested whether a possible compensatory increase in circulating acylated ghrelin may have overcome the SPM-induced neutralization thereby obfuscating this test of ghrelin necessity. Therefore, we assayed both acylated and desacylated circulating ghrelin in SPM- and vehicle-treated hamsters that were ad libitum-fed or 48 h food-deprived as in the behavioral tests. SPM treatment did not decrease circulating concentrations of acylated ghrelin, but instead increased it by several orders of magnitude and also increasing desacylated ghrelin concentrations. Others speculated that the rise of plasma acylated ghrelin with SPM treatment can be explained by a compensatory increase in ghrelin synthesis, a prolonged half-life and/or decreased clearance rate of the peptide when bound to SPM, or a combination of all of the above (43; 44). Becskei et al. and Sangiao-Alvarellos et al. argued that ghrelin might not be necessary for food deprivation-triggered increases in food intake and neuronal activation or possibly that the acylated ghrelin assays may measure both SPM bound to acylated ghrelin and unbound SPM (8; 58). We tested the latter notion by assaying SPM, acylated ghrelin with SPM, and acylated ghrelin with SPM in vitro where these solutions were treated exactly as the blood samples (EDTA and acidification) in an ELISA for acylated ghrelin. The ELISA accurately measured the dose of acylated ghrelin and critically, SPM alone and both acylated ghrelin with SPM samples (untreated and EDTA + acidification) returned non-detectable levels of acylated ghrelin. These results, therefore, eliminate the possibility that the assay recognizes unbound SPM or SPM bound to acylated ghrelin as acylated ghrelin.
Note that the amount of SPM we injected in the food deprived hamsters across the food deprivation would be sufficient to bind the apparent SPM-induced increased concentrations of circulating acylated ghrelin we measured. The increased desacylated plasma ghrelin concentrations in these animals could have resulted from increased production in vivo. Such SPM-induced increases in circulating ghrelin concentrations also could explain why we observed no attenuation of neural activation or behavior after SPM treatment during food deprivation, similar to that found in laboratory mice [c-Fos-ir, (8)] and laboratory rats [food intake, (58)]. SPM-triggered increases in circulating acylated ghrelin concentrations also have been previously shown in diet-induced obese mice receiving chronic infusions of SPM (33mg/kg d) that led to weight loss and reduced food intake (59) and in food-deprived rats injected intraperitoneally with SPM (30 mg/kg, every 24 hours) that thwarted full recuperation of body weight with refeeding (58). The increase in circulating acylated ghrelin concentrations of these latter two studies was, however, of a lesser magnitude than seen here and desacylated ghrelin concentrations were not assessed (58; 59).

It also is possible that there are non-physiological explanations for the large increases in acylated and desacylated plasma ghrelin such as the assays detecting hydrolyzation of the plasma acylated ghrelin during sample processing (34) or that the required processing of the blood samples before acylated ghrelin assay caused the SPM and acylated ghrelin to become disassociated. At face value, however, the impressively increased SPM-induced circulating acylated ghrelin concentrations appear to be due to the initial SPM neutralization of acylated ghrelin resulting in the vastly increased desacylated and acylated ghrelin production that eventually overwhelmed the SPM neutralization, thereby allowing the ingestive behaviors and neural activation by acylated ghrelin. Some support exists for this hypothesized compensatory
system. For example, desacylated ghrelin is increased in the mice lacking GOAT (70). In addition, circulating acylated ghrelin is suggestively, but not significantly, increased in female mice lacking GHSRs (70). Because these two genetically-engineered mouse models lack a significant portion of the mechanisms involved in ghrelin production or signaling, it is difficult to compare or contrast them with the use of SPM, but collectively these data suggest the possibility of an unknown compensatory mechanism on ghrelin production triggered when acylated ghrelin effects are blocked leading to an increase in desacylated ghrelin and subsequently acylated ghrelin.

Collectively, the present data does not impugn ghrelin as a prime factor in food deprivation-induced increases in ingestive behavior including food hoarding, nor the efficacy of SPM; rather it suggests support for sufficiency of ghrelin because of the possible induction, by the effective SPM binding of acylated ghrelin, of compensatory-induced increases in acylated ghrelin when initial acylated ghrelin is neutralized. Clearly, however, the exogenous ghrelin-induced increases in food hoarding and neural activation are effectively blocked by SPM neutralization.
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FIGURE CAPTIONS

Figure 1. Mean ± SEM percent difference from i.p. 18 kg/mg SPM + saline in response to i.p. injections of saline + 30 µg/kg ghrelin or 18 kg/mg SPM + 30 µg/kg ghrelin in (A) food foraging, (B) food intake, and (C) food hoarding. * P<0.05 vs SPM + saline controls.

Figure 2. (A) Mean ± SEM c-Fos immunoreactive cells per slice in response to intraperitoneal injection of saline + saline, 18mg/kg SPM + saline, saline + 30 µg/kg ghrelin, or 18mg/kg SPM + 30 µg/kg ghrelin (A) 2 h post-injection in the ARC nucleus. * P<0.05 vs all other groups. (B) Representative ARC photomicrographs [Bregma: -1.46 mm (51)] of the c-Fos-ir counts (a) saline + saline, (b) 18mg/kg SPM + saline, (c) saline + 30 µg/kg ghrelin, and (d) 18mg/kg SPM + 30 µg/kg ghrelin (x 20 magnification). Arc=arcuate nucleus, 3V=third ventricle.

Figure 3. Mean ± SEM of ingestive behaviors (A) food foraging, (B) food intake, and (C) food hoarding after treatment with saline injected ad libitum fed animals or saline, 18 mg/kg SPM, or 36 mg/kg SPM in 48 h food deprived animals. * P<0.05 vs saline injected ad libitum fed animals.

Figure 4. Mean ± SEM of (A) plasma des-acyl ghrelin concentrations and (C) plasma acylated ghrelin concentration in animals ad lib fed or food deprived for 48 hours and with i.p. injections of saline or 36 mg/kg SPM. Different letters indicate statistical differences P<0.05. (B) represents control wells run in tandem with (A) of 3.6 mg/ml SPM, 18 pg/ml des-acyl ghrelin, or 3.6 mg/ml SPM + 9 pg/ml des-acyl ghrelin (note the initial des-acyl ghrelin concentrations). (D)
represents control wells run of 90 ng/ml SPM, 150 pg/ml acyl ghrelin, or 90 ng/ml SPM + 150 pg/ml acylated ghrelin.

Figure 5. Representative photomicrographs [Bregma: -0.82 mm (51)], including the PVH and sub-zona incerta, of c-Fos immunoreactivity in A. ad libitum fed + saline, B. 48 hour food deprivation + Saline, C. ad libitum fed + 36 mg/kg SPM, and D. 48 hour food deprivation + 36 mg/kg SPM (x 10 magnification). sZI=subZona Incerta, PVH=hypothalamic paraventricular nucleus, 3V=third ventricle.

Table 1. The effect of three Spiegelmer injections (36 mg/kg) on mean c-Fos immunoreactive cells ± SEM per section. * indicates a significant increase when compared with the saline treated ad libitum fed group (P<0.05).

<table>
<thead>
<tr>
<th>Region</th>
<th>Ad Libitum Fed</th>
<th>48 h Food Deprivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Spiegelmer</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arcuate Nucleus</td>
<td>4.7 ± 1.1</td>
<td>24.7 ± 3.3*</td>
</tr>
<tr>
<td>Perifornical Area</td>
<td>5.4 ± 0.9</td>
<td>16.2 ± 2.8*</td>
</tr>
<tr>
<td>Paraventricular Nucleus</td>
<td>12.2 ± 3.3</td>
<td>218.7 ± 28.2*</td>
</tr>
<tr>
<td>subZona Incerta</td>
<td>4.2 ± 0.8</td>
<td>28.3 ± 5.1*</td>
</tr>
<tr>
<td>Central Amygdala</td>
<td>1.3 ± 0.6</td>
<td>13.6 ± 3.1*</td>
</tr>
<tr>
<td>Area Postrema</td>
<td>2.9 ± 1.2</td>
<td>9.2 ± 1.6*</td>
</tr>
<tr>
<td>Nucleus of the Tractus Solitarius</td>
<td>3.2 ± 0.7</td>
<td>14.5 ± 2.1*</td>
</tr>
</tbody>
</table>


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Figure 1

A. Percent Change in Food Foraging Compared to Saline

B. Percent Change in Food Intake Compared to Saline

C. Percent Change in Food Hoarding Compared to Saline
Figure 2

A. Arcuate Nucleus cFos-immunoreactive Cells 2-Hours Post-Injection

![Graph showing the number of cFos-ir cells per slice for different treatments.](attachment:image.png)

- Saline + Saline
- SPM + Saline
- Saline + Ghrelin
- SPM + Ghrelin

* Indicates a significant difference.
Figure 2 (cont.)

A

B

C

D
Figure 5

(A) sZI, PVH

(B) PVH, sZI

(C) sZI, PVH

(D) PVH, sZI