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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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Teubner BJ, Bartness TJ. Anti-ghrelin Spiegelmer inhibits exogenous ghrelin-induced increases in food intake, hoarding, and neural activation, but not food deprivation-induced increases. Am J Physiol Regul Integr Comp Physiol 305: R323–R333, 2013. First published June 26, 2013; doi:10.1152/ajpregu.00097.2013.—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) or 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, food hoarding, and food 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 2 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 and 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.

Obesity is a critical health problem nearly worldwide because of its many secondary health consequences, including stroke, Type 2 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 U.S. $147 billion 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, see Ref. 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 by 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 and 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 Ref. 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 fasting followed by refeeding reveal a virtual absence of post-fast eating increases (for review, see Ref. 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 nonrestrained eaters fasted for 24 h or food-restricted to 1,200 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 1-day fast at the

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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 (~20%, 5%, and 6% in Refs. 37, 38, and 45, respectively), or no increases (2, 42). Thus, contrary to popular and perhaps personal intimations 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 Ref. 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 vs. 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 with 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 Ref. 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 deprivation lengths in Siberian hamsters (39). Ghrelin is acylated from its des-acyl form in the gastric L cells that synthesize the peptide via ghrelin O-acyl transferase [GOAT; (72)] converting ghrelin to its bioactive acylated form (27, 69). Acylated ghrelin increases food intake through its sole receptor, growth hormone secretagogue receptor (GHSR, also known as GHSR-1a), by stimulating GHSRs possessed by arcuate nucleus (ARC) neuropeptide Y/agouti-related protein (AgRP) neurons (for review, see Ref. 3), ventral tegmental area dopaminergic neurons (for review, see Ref. 21), caudal brainstem neurons (23), and likely other neural sites.

The use of various knockout 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 knockout mice is blunted but variable across studies, depending on genetic, environmental, and developmental factors (for review, see Ref. 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 Ref. 7) and because of the inability to genetically modify Siberian hamsters, we only have tested the necessity 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 (more than ~300%) and duration (5–6 days) 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 nonnatural nucleic acids designed to bind to a specific target peptide to neutralize its effects (30). Rather, 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 knockout 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 neutralize 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 acyl/des-acyl ghrelin were assayed. Changes in c-Fos-ir in this and other studies of ghrelin and SPM (8, 10, 43) indicate potential neural activation sites associated with alterations in ingestive behaviors due to ghrelin or lack of ghrelin.

**MATERIALS AND METHODS**

**Animals.** Adult male Siberian hamsters, ~2.5–3 mo old and weighing 34–46 g, were obtained from our breeding colony, as previously described (11). Hamsters were raised from birth in group housing in a long-day photoperiod (16:8-h light-dark cycle; 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°C. All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee and were in accordance with Public Health Service and U.S. 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: 456 234 × 200 mm and bottom: 290 × 180 × 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
GHRELIN INGESTIVE BEHAVIOR EFFECTS INHIBITED BY SPIEGELMER

software/hardware-based program that monitors wheel rotations (Med Associates, St. Albans, VT), as indicated by a 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, Belmont, 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 chamber (16:8-h light-dark cycle; light offset: 1330), animals were singly housed in polypropylene shoebox cages (290 × 180 × 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 days, animals were given 300 pellets, as well as one pellet for every 10 wheel rotations. Subsequent to the first 3 days, 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 days to ensure stabilization of baseline body mass, food intake, and food hoarding. Pilot data indicated that SPM did not affect general locomotor activity (neither increased nor decreased it), suggesting no nonspecific increases or decreases in locomotor activity that could be misinterpreted as increases or decreases in motivation to find food or for the latter possible malaise (e.g., Ref. 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 on the basis of 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) intraperitoneally at light offset, similar to a previous experiment (39). Food foraging, intake, and hoarding were measured at 1, 2, 4, and 24 h postinjection and each day until pretest day baseline values for all behaviors were restored (~7–8 days). 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 that we successfully used previously (18, 19, 39–41), with the treatments counterbalanced. An 8-day washout period occurred after each test day. When pretest (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 four 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 two 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 have 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 after treatment, but we and others have obtained significant increases c-Fos-ir at 14–24 h after 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 on the basis of 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 in 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 redivided 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 those in the fed–saline group, 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 three intraperitoneal injections at the initiation of food deprivation (time 0 h), 24, and 48 h later (30). After the final injections, the pellet dispensers were reconnect to the computer, and food foraging, food intake, and food hoarding were measured at 1, 2, 4, 24, 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 was 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 wt ip or sterile physiological saline vehicle) were given at the first 2 days posttreatment (Experiment 1), the 2nd injection and injection of the food-deprived/saline-injected, and 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 ± SE. 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 × 2nd injection). c-Fos-ir data from Experiment 2 and 4 were analyzed using two-way ANOVA, 1st injection × 2nd injection and injection × feeding state, respectively. For Experiment 3, behavioral data were analyzed using two-way ANOVA (feeding state × 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 post hoc tests 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 when 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 postinjection compared with SPM + saline-treated animals (P < 0.05), an effect inhibited by SPM pretreatment (P < 0.05; Fig. 1A). Exogenous ghrelin significantly increased food intake above SPM + saline at 0–1, 1–2, and 2–4 h postinjection (P < 0.05), an effect prevented by SPM pretreatment at the 0–1 and 2–4 h (P < 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 (P < 0.05, Fig. 1C), with values returning to baseline after 7 days. SPM + ghrelin animals did not exhibit the typical ghrelin-induced increases in food hoarding the first 2 days posttreatment (P < 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 vs. SPM + saline controls on days 3–7 (P < 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); and day 8: 267.5 ± 25.0 (pellets earned), 62.9 ± 4.0 (pellets eaten), 10.5 ± 2.3 (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 of the hypothalamus (PVH), ventromedial hypothalamus, subzona incerta (S2Z), perifornical area (pFA), area postrema (AP), and nucleus of the tractus solitarius (NTS; data not shown)] 2 and 24 h

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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 (P < 0.05; Fig. 3, A 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 with saline-treated animals, except for 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 acyl ghrelin and des-acyl ghrelin concentrations after 48-h food deprivation. Circulating des-acyl 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).

Des-acyl-ghrelin concentrations of SPM-treated hamsters far exceeded (~100×) that of saline-treated animals, regardless of whether they were food-deprived or ad libitum-fed (P < 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 (P < 0.05; Fig. 4C). Both SPM-treated groups had significantly increased circulating acylated ghrelin concentrations vs. their respective feeding treatment groups (P < 0.05; Fig. 4C). This effect was not due to the assay recognizing unbound SPM or SPM bound to ghrelin as ghrelin (Fig. 4, B 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, sZ1, and pFA) and other regions examined [central nucleus of the amygdala, AP, and NTS] in food-deprived hamsters treated with saline or SPM compared with ad libitum-fed saline controls (Table 1 and Fig. 5). SPM did not inhibit c-Fos-ir in 48-h food-deprived animals compared with 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 [and as previously reported (5, 6, 17, 20, 67)], and exogenous peripheral ghrelin administration mimicked these food deprivation-induced increases in food intake in laboratory rats (58) and c-Fos-ir in laboratory mice (8). We assayed acylated (active) and des-acyl (inactive) ghrelin in fed and food-deprived SPM-treated hamsters and found large, increases in acyl ghrelin and des-acyl (inactive) ghrelin. The increase in acyl ghrelin is not unique to this study (58, 59) and may be a possible compensatory response that is strikingly

![Graph](image)

**Fig. 2.** Means ± SE of c-Fos-immunoreactive cells per slice in response to intraperitoneal injection of saline + saline, 18 mg/kg SPM + saline, saline + 30 μg/kg ghrelin, or 18 mg/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 saline + saline (a), 18 mg/kg SPM + saline (b), saline + 30 μg/kg ghrelin (c), and 18 mg/kg SPM + 30 μg/kg ghrelin (×20 magnification) (d). (Brown, cresyl violet counterstaining is blueish.) Arc, arcuate nucleus; 3V, third ventricle.
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 Ref. 50), a finding extended to Siberian hamsters to include food foraging and food hoarding (present study and Refs. 39–41). Increases in food hoarding due to exogenous ghrelin last for up to 7 days postinjection due to a currently elusive unknown mechanism underlying this persisting effect (e.g., Ref. 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 postinjection. Our results are similar to those in laboratory rats and mice in which 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 days, an effect that was inhibited by SPM coadministration during the first 2 days post injection. The inhibition waned after the second day posttreatment, 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 postinjection, 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 postinjection 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 have previously reported after systemic ghrelin administration (57), perhaps because of species differences or the postinjection 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 hour-food deprivation triggers increases in food foraging and hoarding in Siberian hamsters, with increases in food hoarding lasting for up to 7 days (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 possible mechanism underlying these increases in ingestive behaviors in 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

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**Fig. 3.** Means ± SE of ingestive behaviors food foraging (A), food intake (B), and food hoarding (C) 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.
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 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 \[10 \text{ times that of controls (58, 59)}\] 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 des-acyl 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 des-acyl 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. (8) and Sangiao-Alvarellos et al. (58) argued that ghrelin might not be necessary for food deprivation-triggered increases in c-Fos-ir, which was consistent with the results of our study.

### Table 1. The effect of three Spiegelmer injections (36 mg/kg) on mean c-Fos immunoreactive cells ± SE per section

<table>
<thead>
<tr>
<th>Region</th>
<th>Ad Libitum Fed</th>
<th>Spiegelmer</th>
<th>48-h Food Deprivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arcuate nucleus</td>
<td>4.7 ± 1.1</td>
<td>24.7 ± 3.3*</td>
<td>27.6 ± 3.9*</td>
</tr>
<tr>
<td>Perifornical area</td>
<td>5.4 ± 0.9</td>
<td>16.2 ± 2.8*</td>
<td>12.2 ± 2.4*</td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>12.2 ± 3.3</td>
<td>28.3 ± 28.2*</td>
<td>182.5 ± 17.1*</td>
</tr>
<tr>
<td>Subzona incerta</td>
<td>4.2 ± 0.8</td>
<td>28.3 ± 5.1*</td>
<td>23.9 ± 4.1*</td>
</tr>
<tr>
<td>Central amygdala</td>
<td>1.3 ± 0.6</td>
<td>13.6 ± 3.1*</td>
<td>16.2 ± 2.1*</td>
</tr>
<tr>
<td>Area postrema</td>
<td>2.9 ± 1.2</td>
<td>9.2 ± 1.6*</td>
<td>8.2 ± 1.3*</td>
</tr>
<tr>
<td>Nucleus of the tractus solitarius</td>
<td>3.2 ± 0.7</td>
<td>14.5 ± 2.1*</td>
<td>12.6 ± 4.1*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. *Significant increase when compared with the saline-treated ad libitum-fed group \((P < 0.05)\).
in food intake and neuronal activation or possibly that the acylated ghrelin assays may measure both SPM bound to acylated ghrelin and unbound SPM. 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 nondetectable 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 that we injected in the food-deprived hamsters across the food deprivation time period would be sufficient to bind the apparent SPM-induced increased concentrations of circulating acyl ghrelin that we measured. The increased des-acyl 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 with food deprivation, similar to that found in laboratory mice (c-Fos-ir; Ref. 8) and laboratory rats (food intake; Ref. 58). SPM-triggered increases in circulating acylated ghrelin concentrations also have been previously shown in diet-induced obese mice receiving chronic infusions of SPM (33 mg·kg\(^{-1}\)·day\(^{-1}\)) that led to weight loss and reduced food intake (59) and in food-deprived rats injected intraperitoneally with SPM (30 mg/kg, every 24 h) 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 des-acyl ghrelin concentrations were not assessed (58, 59).

It also is possible that there are nonphysiological explanations for the large increases in acyl and des-acyl 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 des-acyl 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, des-acyl 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’s effects are blocked, leading to an increase in des-acyl ghrelin and subsequently acyl ghrelin.

Collectively, the present data do not impugn ghrelin as a prime factor in food deprivation-induced increases in ingestive behavior, including food hoarding, nor the efficacy of SPM; rather the data lend support for sufficiency of ghrelin through the possible induction, by the effective SPM binding of acylated ghrelin, of compensatory increases in acylated ghrelin when initial acylated ghrelin is neutralized. Clearly, however, the exogenous ghrelin-induced increases in food hoarding and

![Image](https://example.com/image.jpg)
neural activation are effectively blocked by SPM neutralization.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: B.J.T. and T.J.B. conception and design of research; B.J.T. performed experiments; B.J.T. analyzed data; B.J.T. and T.J.B. interpreted results of experiments; B.J.T. prepared figures; B.J.T. drafted manuscript; B.J.T. and T.J.B. edited and revised manuscript; B.J.T. and T.J.B. approved final version of manuscript.

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