Central ghrelin increases food foraging/hoarding that is blocked by GHSR antagonism and attenuates hypothalamic paraventricular nucleus neuronal activation

Michael A. Thomas, Vitaly Ryu, and Timothy J. Bartness†
Department of Biology, Center for Obesity Reversal, Georgia State University, Atlanta, Georgia

Submitted 19 May 2015; accepted in final form 7 November 2015


The stomach-derived “hunger hormone” ghrelin increases in the circulation in direct response to time since the last meal, increasing preprandially and falling immediately following food consumption. We found previously that peripheral injection of ghrelin potently stimulates food foraging (FF), food hoarding (FH), and food intake (FI) in Siberian hamsters. It remains, however, largely unknown if central ghrelin stimulation is necessary/sufficient to increase these behaviors regardless of peripheral stimulation of the ghrelin receptor [growth hormone secretagogue receptor (GHSR)]. We injected three doses (0.01, 0.1, and 1.0 μg) of ghrelin into the third ventricle (3V) of Siberian hamsters and measured changes in FF, FH, and FI. To test the effects of 3V ghrelin receptor blockade, we used the potent GHSR antagonist JMV2959 to block these behaviors in response to food deprivation or a peripheral ghrelin challenge. Finally, we examined neuronal activation in the arcuate nucleus and paraventricular hypothalamic nucleus in response to peripheral ghrelin administration and 3V GHSR antagonism. Third ventricular ghrelin injection significantly increased FI through 24 h and FH through day 4. Pretreatment with 3V JMV2959 successfully blocked peripheral ghrelin-induced increases in FF, FH, and FI at all time points and food deprivation-induced increases in FF, FH, and FI up to 4 h. c-Fos immunoreactivity was significantly reduced in the paraventricular hypothalamic nucleus, but not in the arcuate nucleus, following pretreatment with intraperitoneal JMV2959 and ghrelin. Collectively, these data suggest that central GHSR activation is both necessary and sufficient to increase appetitive and consummatory behaviors in Siberian hamsters.

Siberian hamsters; arcuate nucleus; food intake; food deprivation

Obesity is a primary health concern affecting developed and developing countries worldwide, with an incidence rate of 34.9% in the United States as of 2008 (58). Comorbidities associated with obesity include type 2 diabetes, stroke, heart disease, and some cancers (17, 38, 41, 60, 75, 85). As a result of the increasing rate of obesity and its comorbidities, the health costs associated with treatment have reached $147 billion (35), making obesity a major health and economic burden. Many factors, including genetics, contribute to excess weight gain, but the primary cause is a surplus of energy intake compared with energy expenditure. This is compounded by the problem of easy access to calorically dense, cheap food, as well as the ability to store more of these items for longer periods of time. Thus, an understanding of the underlying behaviors and neuroanatomic pathways involved in weight gain is critical to discovery of novel treatments.

Appetitive behaviors include driving to/shopping for food (foraging) and the storing (hoarding) of food in cupboards, refrigerators, freezers, and pantries, whereas consummatory behavior involves the consumption of food (21). The research in the field has largely focused on consummatory behavior [food intake (FI)] in mice and rats and, in the process, uncovered detailed central and peripheral pathways controlling this behavior. Although considerable attention has been paid to consummatory behavior, little attention has been paid to appetitive behaviors, such as the hoarding of food (FH) and foraging for food (FF) (for review see Ref. 9). Food restriction and deprivation in laboratory rats and mice trigger subsequent increases in FI upon reexposure to food (for review see Ref. 8); by contrast, humans and hamsters, contrary to popular belief and personal anecdotes, instead “overhoard,” storing considerably more food than normal, but do not overeat (33, 42, 53, 54). Furthermore, when faced with extended (~24–48 h) food deprivation (FD), humans and Siberian hamsters do not exhibit prolonged overeating. Rather than increasing FI for extended periods, humans and Siberian hamsters increase FH (7, 10, 12, 29, 33, 36, 54, 78). In humans and rodents, periods of negative energy balance directly correlate with increases in the circulating concentration of the endocrine hormone ghrelin (51, 72, 79). Furthermore, peripheral administration of ghrelin mimics substantial increases in FF, FH, and FI after 56 h of FD in Siberian hamsters (48, 73), indicating the importance of ghrelin in driving appetitive and consummatory behaviors.

Ghrelin acts on its receptor [growth hormone secretagogue receptor (GHSR)] in diverse central and peripheral areas, including the arcuate nucleus (Arc) (for review see Ref. 1), ventral tegmental area (for review see Ref. 31), suprachiasmatic nucleus (14, 84), paraventricular hypothalamic nucleus (PVH) (50), and vagus nerve (26). Although GHSRs are expressed on vagal afferents, vagal deafferentation does not block the marked increase in FI following a peripheral ghrelin challenge in rodents (3), indicating that central, but not peripheral, GHSR activation drives consummatory behavior. Within the Arc, ghrelin activates agouti-related protein (AgRP)/neuropeptide Y (NPY) neurons to increase FI (1, 14), and the coadministration of these peptides into the third ventricle (3V) increases FF, FH, and FI in Siberian hamsters (71). Ghrelin-sensitive neurons in the Arc project to diverse brain areas, including the PVH (18, 52) and parabrachial nucleus (82, 83), to stimulate FI, and peripheral administration of ghrelin triggers expression of c-Fos [a marker of neuronal activation (43,
The development of direct and indirect ghrelin antagonists has shed light on the physiological role of ghrelin in rodents. Peripheral administration of an anti-ghrelin Spiegelmer (69), as well as inhibition of ghrelin octanoylation and, thus, its conversion to its active form (70), blocks exogenous ghrelin-induced neural activation peripherally and centrally (given its route of administration peripherally) and short-term (<48 h) increases in FF, FI, and FH; however, long-term (>48 h) appetitive and consummatory behaviors remain unchanged. In addition to ghrelin-peptide antagonists, development of the specific GHSR antagonist JMV2959 (55, 56) allows for site-specific studies of ghrelin activity throughout the brain (62, 63, 67). These studies have partially elucidated the role of ghrelin in Siberian hamsters and other rodent models, but whether central GHSR activation is necessary and sufficient to drive appetitive ingestive behaviors independent of peripheral GHSR blockade remains unclear.

Here, we tested the sufficiency of 3V central ghrelin to increase FF, FI, and FH in Siberian hamsters. After confirming the sufficiency of 3V ghrelin to drive these behaviors, we tested the effects of 3V GHSR blockade by administering the GHSR antagonist JMV2959 followed by 1) a 48-h FD challenge and 2) a peripheral ghrelin challenge. Finally, we examined neuronal activation as seen by c-Fos immunoreactivity (43) in the Arc and PVH following peripheral ghrelin administration and 3V GHSR antagonism to further clarify neuronal activity from two structures implicated in driving appetitive behaviors in Siberian hamsters.

MATERIALS AND METHODS

Animals

Adult male Siberian hamsters (Phodopus sungorus, n = 39) from our breeding colony were housed in same-sex groups in polypropylene cages (48 x 27 x 15 cm), allowed ad libitum access to food (formula 5001, Purina, St. Louis, MO) and water, and raised in a long-day photoperiod (16:8 h light-dark cycle, light onset at 1800) from birth until they were used in the following experiments. Room temperature was maintained at 21.0 ± 2.0°C. At ~2.5–3.0 mo, the animals were housed singly in polypropylene cages (27.8 x 17.5 x 13.0 cm) with Alpha-dri (Specialty Papers, Kalamazoo, MI) bedding and one cotton Nestlet (Ancare, Belmore, NY). Animals were allowed ad libitum access to the experimental, pelleted test diet (purified 75-mg Dustless Precision Pellets, Bio-Serve, Frenchtown, NJ) and water and housed at 21.0 ± 2.0°C with 50 ± 10% humidity in a 16:8-h light-dark cycle (light offset at 1300) for 2 wk to acclimate to the new light offset. All procedures were approved by the Georgia State University Animal Care and Use Committee and were in accordance with Public Health Service and US Department of Agriculture guidelines.

Foraging-and-Hoarding Apparatus

Hamsters were acclimated for 1 wk before and after cannula implantation in a specially designed foraging-and-hoarding setup as previously described (28). Briefly, two cages were connected using convoluted polyvinylchloride tubing (38.1 mm inner diameter, ~1.5 m long) with three bends containing wire mesh to allow for vertical and horizontal climbing between cages. The top, foraging cage (456 x 234 x 200 mm [length x width x height]) was equipped with a pellet dispenser, running wheel (524 mm circumference), and water bottle. The bottom, hoarding cage [290 x 180 x 130 mm (length x width x height)] contained Alpha-dri bedding and one cotton Nestlet. To mimic the darkness of a burrow, the hoarding cage was opaque and covered with an aluminum pan throughout the experiment. Wheel revolutions were measured using a magnetic detection system connected to a computer with monitoring software (Med Associates, Georgia, VT). Food was available ad libitum for 2 days following introduction to the foraging-and-hoarding apparatus. Subsequent to this initial training period, food was removed and all food had to be earned (1 pellet/10 wheel revolutions) for 5 days, during which time wheel revolutions, FI, pellets earned (FF), FH, and body mass were measured daily. After 1 wk of acclimation, animals were returned to cages and allowed ad libitum access to food and further used for cannula surgery.

Measurement of FF, FH, and FI

FF was defined as the number of pellets earned following completion of the required 10 wheel revolutions. FH was defined as the number of pellets collected in the bottom, burrowing cage, as well as the number of pellets removed from cheek pouches. FI was defined as the total number of pellets earned minus the number of pellets left in the top cage and the number of pellets hoarded. An electronic balance set to “parts measurement” was used to count pellets (75 mg = 1 pellet). All whole food pellets and fractions of food pellets were recovered from each cage and hamster food pouches daily (0900) and then quantified as whole pellets using a precision balance set to “parts,” whereby one 75-mg food pellet = 1 and fractions of pellets were computed by the balance. FF, FH, and FI were measured daily at 0900 for the duration of the experiment.

Cannula Implantation, Injections, and Verification

Cannulas were stereotaxically implanted into the 3V of animals under isoflurane (Aerrane, Baxter Healthcare, Deerfield, IL) anesthe-

sis (23). Briefly, the head of each anesthetized animal was shaved to expose the skull. A guide cannula (26-gauge stainless steel; Plastics One, Roanoke, VA) was lowered and stereotaxically (coordinates: anterior-posterior from bregma 0 mm, medial-lateral from midsagittal sinus 0 mm, and dorsal-ventral from the top of the skull ~5.5 mm) targeted for the 3V. Cannulas were secured to the skull using cyanoacrylate ester gel, 3/16-mm jeweler’s screws, and dental acrylic. A removable dummy cannula was used to seal the opening of the guide cannula throughout the experiment. Animals received ketoprofen (5 mg/kg sc; Ketofen, Fort Dodge Animal Health, Fort Dodge, IA) to minimize postoperative discomfort and an apple slice to facilitate food and water intake for the first 2–3 days postsurgery. Animals were kept in shoebox-type caging for 2 wk postsurgery for recovery.

At 1 wk before each test day, each animal was lightly handled for 1 min, and the dummy cannula was removed and replaced to acclimate the animal to the injection protocol. On test days, an injection cannula (33-gauge stainless steel; Plastics One) extending 0.5 mm beyond the guide cannula was connected to a microsyringe via PE-20 tubing and inserted into the guide cannula. All hamsters were injected with 400 nl of ghrelin, JMV2959, or saline vehicle over the course of 30 s, and the injector was left in place for 30 s to minimize reflux up the sides of the cannula before it was removed, as previously described (23).

After the last behavioral test in experiment 3, bromophenol blue dye (400 nl) was injected into each animal to confirm placement of the cannula in the 3V. Animals were then given an overdose of pentobarbital sodium (100 mg/kg) transcardially perfused with 100 ml of heparinized saline followed by 125 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Brains were removed and postfixed in 4% paraformaldehyde for 2 days followed by a 30% sucrose solution until sectioning. Brains were sectioned on a freezing microtome at 80 μm, and cannula placement was verified using light microscopy. Cannulas were considered a “hit” if blue dye was visible...
in any part of the ventricle, and only these animals were included in the analysis.

**Experiment 1: Does 3V Injection of Ghrelin Increase FF, FI, and FH in Siberian Hamsters?**

After the initial acclimation period, all hamsters were assigned to four groups counterbalanced for body mass, FI, FF, and FH levels. On test days, animals were placed in a clean burrowing cage and blocked from accessing the top cage to prevent feeding 2 h before injections. At light offset, 0.01, 0.1, or 1.0 μg of ghrelin or vehicle was injected into the 3V, and access to food was restored. FI, FF (wheel revolutions), and FH were recorded at 2, 4, and 24 h postinjection and then daily for 8 days. After a 1-wk washout period, a length of time previously determined to yield a return to baseline, the same protocol was repeated until each animal had received all four treatments (n = 31).

**Measurement of circulating plasma ghrelin concentration.** To ensure that the 3V-administered ghrelin does not cross the blood-brain barrier, a separate cohort of 36 adult male hamsters were obtained from our breeding colony and singly housed in polypropylene cages with Alpha-dri bedding and one cotton Nestlet. Cannulas were implanted as described above, and animals were allowed to recover for 2 wk with ad libitum access to food. Daily FI and body mass were recorded. After recovery, the animals were counterbalanced for daily FI and body mass and, finally, assigned to four groups: 1) 0.01 μg of ghrelin, 2) 0.1 μg of ghrelin, 3) 1.0 μg of ghrelin, or 4) vehicle. Third ventricular injections were given at light offset, and retroorbital blood was taken from anesthetized animals according to our previously published method (70) at 2, 4, and 24 h postinjection. Circulating acylated ghrelin (acyl-ghrelin) concentrations were measured using mouse/rat acyl-ghrelin ELISA (Cayman Chemical) according to the manufacturer’s instructions and previously published methods (6). Briefly, 500 μl of blood were collected into heparinized Natelson tubes, transferred to prechilled EDTA-containing Microtainers (BD, Franklin Lakes, NJ), and inverted 10 times. Subsequently, 300 μl of blood were immediately transferred from EDTA-containing tubes to prechilled microcentrifuge tubes containing 300 μl of the transfer buffer (1.2% of 10 N NaOH, 2 mM p-hydroxymercuribenzoic acid, 500 mM NaCl, and 25 mM EDTA in deionized water), mixed thoroughly by gentle inversion, and spun at 5,000 rpm at 4°C for 10 min. Next, 300 μl of plasma were transferred to prechilled microcentrifuge tubes, acidified with 1 N HCl (1 μl/10 μl of plasma), spun at 5,000 rpm at 4°C for 5 min, and stored at −20°C until assayed.

**Experiment 2: Does 3V Injection of the GHSR Antagonist JMV2959 Prevent Peripheral Ghrelin-Induced Increases in FF, FI, and FH in Siberian Hamsters?**

After a 10-day washout period following the final test day of experiment 1, animals were assigned to four new treatment groups counterbalanced for body mass, FI, FF, and FH levels. In our pilot experiment, groups consisted of animals treated as follows: 1) intraperitoneal (ip) saline + 3V saline, 2) ip saline + 3V JMV2959 (10 μg), 3) ip ghrelin (30 μg/kg body mass) + 3V JMV2959 (10 μg), and 4) ip ghrelin (30 μg/kg body wt) + 3V saline. On test days, animals were placed in a clean burrowing cage and prevented from accessing the top cage as described for experiment 1. All injections were given concurrently at light offset, and access to food was restored. After these injections, FI, FF, and FH were recorded at 2, 4, and 24 h and then daily for 8 days. After a 1-wk washout period, the same injection protocol was repeated until each animal had received all treatments (n = 21).

**Experiment 3: Does 3V Injection of a GHSR Antagonist Block Food Deprivation-Induced Increases in FF, FI, and FH?**

After a 10-day washout period following the final test day of experiment 2, animals were divided into two groups counterbalanced for body mass, FI, FF, and FH levels: 1) 3V saline and 2) 3V JMV2959 (10 μg). Animals were injected with saline or JMV2959 at the onset of FD [time (T) = 0], T = 24 h, and T = 48 h. After the final injection, access to food was restored, and FI, FF, and FH were recorded at 2, 4, and 24 h and then daily until animals returned to behavioral baseline. After returning to baseline, the animals were transferred to shoebox cages, and brain tissue was processed as described above to verify cannula location (n = 20).

**Experiment 4: Does 3V Injection of a GHSR Antagonist Prevent Ghrelin-Induced Increases in c-Fos Expression?**

Twenty Siberian hamsters were selected from the breeding colony and housed individually in shoebox cages before and after 3V cannula implantation. A separate, age-matched cohort of animals was used for experiment 4, because the within-subject design of experiments 1, 2, and 3 did not allow for unconfounded test of neural activation. Animals were divided into four treatment groups: 1) ip saline + 3V saline, 2) ip saline + 3V JMV2959 (10 μg), 3) ip ghrelin (30 μg/kg body mass) + 3V saline, and 4) ip ghrelin (30 μg/kg body mass) + 3V JMV2959 (10 μg). Animals were injected at light offset and perfused 2 h later. This time point was chosen, because preliminary data showed the largest increases in FF, FI, and FH at 2–4 h (48, 69). Brains were sectioned at 40 μm on a freezing microtome. For immunohistochemistry, free-floating brain sections were rinsed in 0.1 M PBS (twice for 15 min) and then incubated for 30 min in a blocking and permeabilization solution consisting of 10.0% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS. Sections were incubated overnight with rabbit anti-c-Fos antiserum (1:800 dilution; catalog no. sc-52, Santa Cruz Biotechnology, Santa Cruz, CA) in 0.1 M PBS with 0.5% normal goat serum. For immunohistological controls, the primary antibody was either omitted or preadsorbed with the immunizing peptide overnight at 4°C, which abolished immunostaining. Subsequently, sections were washed in 0.1 M PBS (twice for 15 min) and incubated with biotinylated goat anti-rabbit antibody (1:200 dilution; Vector Laboratories, Burlingame, CA) for 2 h. Bound secondary antibody was then amplified with the Vector Elite ABC kit (1:800 dilution; Vector Laboratories), and antibody complexes were visualized by a 0.05% diaminobenzidine reaction. After the sections were mounted, they were dehydrated and cleared through a series of ethanol, isopropanol, and xylene solutions. Cleared slides were immediately mounted with Permount (Thermo Fisher Scientific, Waltham, MA), and coverslips were applied.

**Quantitative and Statistical Analysis**

Images were viewed and captured using ×100 and ×200 magnification with an imaging photomicroscope (model DP73, Olympus, Tokyo, Japan). Cellsens (Olympus) and Adobe Photoshop CS5 (Adobe Systems, San Jose, CA) software were used to evaluate the PVH and Arc c-Fos-immunoreactive images. The Arc and PVH were chosen for quantification, because previous studies showed increased neuronal activity in these areas in response to ghrelin (20, 69). The c-Fos-immunoreactive neurons were considered positively labeled on the basis of size and shape of the nuclei. Exhaustive counts of c-Fos-positive neurons at the same anatomic level in all animals were then averaged across all animals within each experimental group. A mouse brain atlas (58a) was used to identify brain areas, because no Siberian hamster brain atlas is available and because the size and...
shape of most of the brain structures of Siberian hamsters are similar to those of mice and much more similar to brain structures of mice than to those provided in the commercially available Syrian hamster stereotaxic atlas. For preparation of the photomicrographs, we used Adobe Photoshop CS5 only to adjust the brightness and contrast and to remove artifactual obstacles (i.e., obscuring bubbles) to make the composite plates.

In experiments 1 and 2, raw behavioral data for each individual animal were transformed to percent change from saline control before statistical analysis according to the following formula: \[(x - \text{vehicle})/\text{vehicle} \times 100\], where \(x\) is the measured value in response to experimental treatment and vehicle represents the value measured for the individual following control injection (3V saline in experiment 1 and ip saline + 3V saline in experiment 2). Thus each animal serves as its own control, and data are reported as percent change from baseline measurements. No statistical comparisons were made across the time intervals because of the unequal duration of the intervals. Behavioral data were analyzed using one-way repeated-measures ANOVA across all treatments (i.e., saline, 0.01 \(\mu\)g of ghrelin, 0.1 \(\mu\)g of ghrelin, and 1.0 \(\mu\)g of ghrelin for experiment 1 and ip saline + 3V saline, ip saline + 3V JMV2959, ip ghrelin + 3V saline, and ip ghrelin + 3V JMV2959 for experiment 2) within each time point followed by the post hoc Bonferroni’s test using NCSS (version 2009, Kaysville, UT). For all measures of FF, FI, and FH in experiments 1 and 2, data are reported as percent difference from vehicle control (100%); values are means \pm SE. Circulating acyl-ghrelin concentrations in experiment 1 were analyzed using one-way ANOVA at each time point. For experiment 3, data were not transformed to percent change, because animals were food-deprived only once and, therefore, cannot serve as their own control, because Siberian hamsters do not overeat after FD and, therefore, take weeks to return to their initial body mass. Raw data for FF, FI, and FH were analyzed using the Mann-Whitney U test. For experiment 4, c-Fos immunoreactivity for all groups was analyzed using one-way ANOVA. Differences were considered statistically significant if \(P < 0.05\).

RESULTS

Experiment 1: Does 3V Injection of Ghrelin Increase FF, FI, and FH in Siberian Hamsters?

**FF.** No significant increase in FF was seen across all three doses of 3V ghrelin at all time points examined compared with saline controls (Fig. 1A). A trend of increased FF was observed at 2–4 h postinjection for 0.1- and 1.0- \(\mu\)g ghrelin doses but only reached a maximum increase of \(\sim 160\%\) compared with saline \([F(3,90) = 2.11, P = 0.10; \text{Fig. 1A}]\).

**FI.** FI was significantly increased by \(\sim 200\%\) of control at 2–4 h postinjection for the 1.0- \(\mu\)g ghrelin dose \([F(3,90) = 3.23, P < 0.05; \text{3V saline; Fig. 1B}]\). No significant increase was seen for 3V injection of the 0.01- or 0.1- \(\mu\)g ghrelin dose at any time point (Fig. 1B).

**FH.** 3V ghrelin significantly increased FH at 0–2 h \([F(3,90) = 4.64, P < 0.05]\), 2–4 h \([F(3,90) = 4.93, P < 0.05]\), 4–24 h \([F(3,90) = 2.75, P < 0.05]\), 48 h \([F(3,90) = 4.24, P < 0.05]\), day 3 \([F(3,90) = 3.69, P < 0.05]\), and day 4 \([F(3,90) = 2.95, P < 0.05]\) for the 1.0- \(\mu\)g dose. FH reached a maximum of \(\sim 600\%\) following the 1- \(\mu\)g dose compared with control values at 48 h postinjection and gradually declined to baseline at day 5 (Fig. 1C). The 0.1- \(\mu\)g dose significantly increased FH at 2–4 h \([F(3,90) = 4.93, P < 0.05]\) and day 3 \([F(3,90) = 3.69, P < 0.05]\), reaching a maximum of \(\sim 400\%\) (Fig. 1C). No increase was observed at any time point for the 0.01- \(\mu\)g ghrelin dose (Fig. 1C) compared with the saline control.

Circulating acyl-ghrelin concentrations. To ensure that 3V-administered ghrelin was not crossing the blood-brain barrier, we repeated experiment 1 and measured peripheral circulating acyl-ghrelin concentrations at 2, 4, and 24 h postinjection. No dose of 3V-administered ghrelin significantly affected peripheral circulating acyl-ghrelin concentrations at any time point (Fig. 2).

Experiment 2: Does 3V Injection of the GHSR Antagonist JMV2959 Prevent Peripheral Ghrelin-Induced Increases in FF, FI, and FH in Siberian Hamsters?

**FF.** FF was significantly increased at 4–24 h postinjection for the ip ghrelin + 3V saline group \([F(3,60) = 3.10, P < 0.05]\) vs. ip saline + 3V saline; Fig. 3A) by \(\sim 350\%\) compared with

---

*Fig. 1. Food foraging (FF, A), food intake (FI, B), and food hoarding (FH, C) in response to injection of 0.01, 0.1, or 1.0 \(\mu\)g of ghrelin into the 3rd ventricle (3V). Values are means \pm SE; \(n = 31\) for each group. *\(P < 0.05\) vs. saline controls.*
the control group. FF was attenuated at 4–24 h postinjection in the ip ghrelin + 3V JMV2959 group, reaching a maximum of ~200% compared with saline (Fig. 3A). No change from the control treatment was seen across all time points in the ip saline + 3V JMV2959 group (Fig. 3A). FF returned to baseline at 48 h for all groups (Fig. 3A).

FI. FI was significantly increased by ~200% of control at 0–2 h for the ip ghrelin + 3V saline group \( [F(3,60) = 4.77, P < 0.05] \). FI was attenuated at 0–2 and 2–4 h in the ip ghrelin + 3V JMV2959 group and reached a maximum of ~160% at 4–24 h \( (Z = 2.05, P < 0.05) \). No change in pellets eaten was observed following ip saline + 3V JMV2959 treatment (Fig. 3B).

FH. The number of pellets hoarded were significantly increased at 0–2 h \( [F(3,60) = 3.11, P < 0.05] \), 2–4 h \( [F(3,60) = 3.43, P < 0.05] \), day 3 \( [F(3,60) = 3.60, P < 0.05] \), day 4 \( [F(3,60) = 3.23, P < 0.05] \), and day 5 \( [F(3,60) = 3.37, P < 0.05] \) in the ip ghrelin + 3V saline group (Fig. 3C) and reached a maximum of ~400% at day 5. FH was attenuated in the ip ghrelin + 3V JMV2959 group at all time points and reached a maximum of ~200% at day 5; however, the increase was not statistically significant (Fig. 3C). No change in pellets hoarded was observed for the ip saline + 3V JMV2959 group at all time points.

Experiment 3: Does 3V Injection of a GHSR Antagonist Block Food Deprivation-Induced Increases in FF, FI, and FH in Siberian Hamsters?

FF. FF was significantly decreased in the 3V JMV2959 group at 0–2 and 2–4 h postinjection compared with 3V saline control \( (P < 0.05) \) (Fig. 4A). No difference was observed between the two groups at 4–24 or 48 h postinjection (Fig. 4A).

FI. The number of pellets eaten was significantly decreased in the 3V JMV2959 group at 0–2 h postinjection compared with 3V saline control \( (P < 0.05) \) (Fig. 4B). No difference in pellets eaten was observed at 2–4, 4–24, and 48 h postinjection (Fig. 4B).

FH. FH was significantly decreased in the 3V JMV2959 group at 0–2 and 2–4 h postinjection compared with 3V saline control \( (P < 0.05) \) (Fig. 4C). No difference in pellets hoarded was observed at 4–24 h through day 7 for both groups (Fig. 4C). The 3V JMV2959 group displayed a trend of increased FH following the 48-h time point, although the increase did not reach statistical significance (Fig. 4C).
DISCUSSION

The current study was designed to test the sufficiency and necessity of central and peripheral GHSR activation to increase appetitive and consummatory behaviors in Siberian hamsters. Similar to previous reports in laboratory mice and rats (45, 73, 80, 81), 3V administration of ghrelin was sufficient to increase FI in ad libitum-fed Siberian hamsters. We showed here for the first time, however, that 3V ghrelin is sufficient to increase ingestive behaviors independent of peripheral GHSR stimulation/blockade. We next tested the hypothesis that central GHSR activation mediates the appetitive and consummatory response to peripheral ghrelin. The GHSR antagonist JMV2959 successfully blocked exogenous ghrelin-induced increases in FF and FI and attenuated FH from the first time point postinjection (2 h) through the end of the study (day 8). This finding further supports data showing that central GHSR activation, rather than vagal GHSR signaling, regulates peripheral ghrelin-induced increases in appetitive and consummatory behaviors (3). To further elucidate the neuronal mechanism underlying this effect, we measured c-Fos immunoreactivity in the Arc and PVH following administration of 3V JMV2959 and ip ghrelin. Pretreatment with JMV2959 significantly decreased the number of c-Fos-positive cells in the PVH, but not in the Arc, following a peripheral ghrelin challenge, suggesting that peripheral ghrelin-induced increases in FF, FI, and FH are mediated by neuronal activity in the PVH, rather than the Arc.

In line with previous experiments testing the effects of peripheral ghrelin, 3V ghrelin potently stimulated appetitive and consummatory behaviors in Siberian hamsters. By contrast to ip injections (48, 69), 3V administration circumvents possible peripherally mediated mechanisms driving FF, FI, and FH. For instance, activation of the stomach-vagal-hindbrain-midbrain axis (25–27) increases FI, whereas 3V administration prevents peripheral GHSR involvement. Third ventricular ghrelin at 0.1 and 1.0 μg potently stimulated FF, FI, and FH. Furthermore, 3V ghrelin administration closely mimicked the drastic increases in FH after ip ghrelin, reaching an apex of ~600%. Our data indicate for the first time that ghrelin acts directly on central, but not peripheral, vagal GHSRs to drive ingestive behaviors in Siberian hamsters. This is further supported by our findings that 3V ghrelin does not significantly affect peripheral circulating ghrelin concentrations and indicates that 3V ghrelin does not cross the blood-brain barrier (Fig. 2). We therefore hypothesize that centrally mediated ghrelin activity is sufficient to induce ingestive behaviors independent of peripheral GHSR stimulation/blockade. The marked increase in ghrelin plasma concentration following an exogenous ghrelin challenge or prolonged FD activates peripheral and central GHSRs and markedly increases FF, FI, and FH (48); however, by circumventing peripheral GHSR involvement, we have for the first time directly examined the discrete role of central GHSR activation in driving appetitive and consummatory behaviors in Siberian hamsters. The central and peripheral distribution of GHSRs suggests that the effects of ghrelin are mediated by a number of distinct areas (86). Furthermore, our findings that 3V ghrelin markedly increases FF, FI, and FH independent of peripheral GHSR activation further support the presence of multiple, redundant systems mediating these behaviors (40). Overall, we demonstrated that 3V ghrelin is sufficient to increase appetitive and consummatory behaviors independent of peripheral GHSR activation.

Our previous tests of the necessity of ghrelin focused on peripheral mechanisms to block ghrelin directly (69) or prevent its conversion to its physiologically active form (70) without directly blocking its receptor. Therefore, we used the potent GHSR antagonist JMV2959 to test the necessity of central GHSR activation to increase FF, FI, and FH in response to a peripheral ghrelin challenge. Third ventricular GHSR blockade allows for a robust test of central ghrelin in driving FF, FH, and FI independent of manipulation of peripheral ghrelin signaling. JMV2959 administration attenuates FI (39) and ghrelin-induced operant responding for food reward in rodents (67). Here, 3V injection of JMV2959 abolished the ghrelin-induced increases in appetitive and consummatory behaviors at all time points. Furthermore, 3V JMV2959 + ip saline did not affect FF compared with 3V saline + ip saline, indicating that blockade of FF, FI, and FH is not due to nonspecific effects on locomotor activity. The inhibition observed following central GHSR antagonism is in contrast to previous work attempting to...
block the appetitive and consummatory effects of exogenous ghrelin, both directly and indirectly, in Siberian hamsters (46, 47, 69, 70), in that appetitive and consummatory behaviors were completely blocked throughout the experiment. Furthermore, direct 3V administration of JMV2959 circumvents peripheral GHSR manipulation and, therefore, directly tests the discrete role of central GHSR activation. The blockade of appetitive behaviors in response to a peripheral ghrelin challenge suggests that central GHSR activation, and thus bypassing peripheral vagal GHSRs, is integral for these effects and could, in turn, be a more useful clinical treatment option if the mechanism for this complete blockade could be discerned. Our results therefore support previous findings that ghrelin-induced increases in feeding behavior are not dependent on intact vagal

Fig. 5. Number of c-Fos-immunoreactive cells and representative photographs in arcuate nucleus [Arc (A and a–d)] and paraventricular hypothalamic nucleus [PVH (B and e–h)] in response to ip saline + 3V saline (a and e), ip saline + 10 µg JMV2959 (b and f), 30 µg/kg ip ghrelin + 3V saline (c and g), or 30 µg/kg ip ghrelin + 10 µg 3V JMV2959 (d and h). Values are means ± SE; n = 4–5 for each group. *P < 0.05 vs. ip saline + 3V saline controls. Scale bars = 100 µm.
GHSR antagonism blocks fasting/ghrelin-induced hoarding increases

AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00216.2015 • www.ajpregu.org

affere (3). However, it remains unclear exactly which ghrelin-dependent pathway is being inhibited, as 3V administration of JMV2959 blocks GHSR activation throughout the brain, and future studies are needed to delineate the precise pathways driving appetitive and consummatory behaviors. We have collectively demonstrated for the first time that central GHSR activation is integral in driving ingestive behaviors in Siberian hamsters.

To further examine the ability of JMV2959 to block ghrelin-induced increases in appetitive and consummatory behaviors, we chronically injected JMV2959 throughout 48 h of FD, which has previously been shown to potently increase FF, FI, and FH and circulating ghrelin levels in Siberian hamsters (7, 11, 30, 74, 78). We chose to repeatedly inject JMV2959 in 12-h increments to block GHSR activation throughout FD, in line with previous experiments (69), because ghrelin is continuously released in response to the negative energy balance that would occur during the 48 h of food deprivation. Third ventricular treatment with JMV2959 blocked FF and FI through 2 h and FH through 4 h after refeeding (Fig. 4). By contrast to a single peripheral ghrelin challenge, food deprivation chronically increases circulating ghrelin concentrations. In turn, acute GHSR antagonism is able to block exogenous ghrelin-induced, but not food deprivation-induced, increases in long-term appetitive and consummatory behaviors. These data suggest that JMV2959 blocks the effects of ghrelin throughout food deprivation, but once its effectiveness is diminished, the food deprivation-induced circulating ghrelin begins to drive the appetitive ingestive behaviors. Taken together, experiments 2 and 3 demonstrate that central activation of GHSR is necessary for exogenous ghrelin-induced increases in FF, FI, and FH, but not for long-term increases induced by food deprivation. This result supports previous reports suggesting that ghrelin is integral in food deprivation increases in appetitive behavior but not necessary because of the abundance of other physiological mechanisms compensating in its absence, that is, the multiple redundant systems driving ingestive behaviors (13, 40).

To determine brain areas involved in ghrelin-induced changes in appetitive and consummatory behaviors in a preliminary examination, c-Fos immunoreactivity in the Arc and PVH was quantified following exogenous ghrelin treatment and 3V administration of JMV2959. We chose these areas because of previous work indicating significantly increased neuronal activity following peripheral ghrelin injection (20, 69). Here and in previous reports, exogenous ghrelin potently increased FI and FH at 0–4 h postinjection (46, 48, 69). We therefore examined c-Fos immunoreactivity in the Arc and PVH at 2 h following identical treatments as in experiment 2. Increased c-Fos immunoreactivity in response to exogenous ghrelin was observed in the Arc and PVH at 2 h postinjection, in line with previous reports (50, 61, 69). Pretreatment with JMV2959 blocked neuronal activation in the PVH following exogenous ghrelin, but, interestingly, the Arc was unaffected (Fig. 5). Approximately 94% of AgRP neurons express GHSRs (77), and exogenous ghrelin markedly increases AgRP/NPY expression (32). These neurons project to a number of downstream nuclei, including the PVH (5, 18), where they antagonize melanocortin receptors 3 and 4 to promote FI. Furthermore, AgRP neurons directly inhibit neighboring proopiomelanocortin neurons in the Arc (4, 19). As GHSR antagonism predominantly affects AgRP neurons, one potential explanation for our finding is that GHSR blockade concurrently inhibits AgRP → PVH projections and disinhibits proopiomelanocortin neurons, resulting in ghrelin-induced c-Fos immunoreactivity comparable to AgRP → Arc, regardless of GHSR antagonism. In addition to blockade of AgRP neurons, a number of hindbrain nuclei express GHSRs (86). These nuclei project to, and receive projections from, the PVH (22, 37, 65), indicating a distributed forebrain and hindbrain neuronal circuit regulating the effects of ghrelin. Moreover, 4V ghrelin injection markedly increases FI (34), and selective GHSR expression on hindbrain nuclei is sufficient to maintain blood glucose levels during caloric restriction (64), suggesting a physiologically relevant role for endogenous ghrelin signaling on these neurons. We therefore hypothesize that 3V GHSR antagonism blocks c-Fos expression in the PVH, but not the Arc, through inhibition of a distributed forebrain and hindbrain ghrelin signaling network. However, as the identity of these c-Fos-positive neurons remains a mystery, we can only speculate as to the reason for this finding.

Ghrelin acts primarily in the Arc to promote FI (80, 86); however, GHSRs are expressed in a number of brain nuclei, including the PVH (86). We hypothesize, however, that central GHSR blockade attenuates FF, FI, and FH in an Arc-dependent manner based on the following findings: 1) peripheral ghrelin administration markedly increases Arc AgRP expression, and these neurons project to the PVH (4, 20, 57); 2) complete Arc or NPY neuron ablation markedly decreases central and peripheral ghrelin-induced FI (16, 68), indicating that Arc activation mediates ghrelin-induced FI; and 3) photostimulation of AgRP neurons markedly increases FI (2, 82), whereas concurrent AgRP photostimulation and AgRP → PVH chemoinhibition attenuate this increase (82), indicating that AgRP → PVH activation primarily drives FI. Collectively, these data suggest that blockade of FI following 3V GHSR blockade and i.p. ghrelin is due to inhibition of AgRP → PVH activity. On the basis of behavioral results in experiment 2, we hypothesize that peripheral ghrelin may increase appetitive behaviors in the very least in a PVH-involved mechanism and that GHSR blockade attenuates ghrelin-induced neuronal activity of PVH inputs. It remains unclear, however, if PVH activity is a response to activity of the hindbrain (or vice versa) or other brain areas, or both, and future experiments will expand on this question. We are aware of the circumstantial evidence provided by c-Fos immunoreactivity, in that the absence of neuronal activity in the PVH does not necessarily indicate the absence of activity, as some neurons use other immediate-early genes (43). We therefore interpret these findings as being suggestive of a possible PVH-involved mechanism driving appetitive behaviors in Siberian hamsters.

These data collectively indicate that central activation of GHSR by exogenous ghrelin is integral in driving appetitive and consummatory behaviors in Siberian hamsters. In addition, central GHSR antagonism is able to block food deprivation-induced short-term, but not long-term, increases in appetitive behaviors because of the continued release of ghrelin (e.g., food deprivation). The blockade of appetitive behaviors in response to GHSR antagonism and a peripheral ghrelin challenge appears to involve the PVH, which receives projections from a number of discrete brain areas, including the Arc. Although GHSR antagonism successfully blocked ghrelin-
induced increases in ingestive behavior, future studies are needed to delineate the precise pathways involved.

**Perspectives and Significance**

An understanding of the complex relationship of central integration of peripheral satiety signals is an important step in the development of clinically useful obesity treatment options. We have developed a novel model to study human FF and FH, a relatively unstudied facet of obesity, in Siberian hamsters. Here, we show for the first time that central blockade of GHSRs prevents the marked increase in short- and long-term FF, FH, and FI following a peripheral ghrelin challenge. In addition, we show for the first time that this blockade prevents neuronal activation of the PVH, but not the Arc, suggesting that PVH activity is integral in driving appetitive and consummatory behaviors.

**ACKNOWLEDGMENTS**

The authors thank Babette Aicher (AEtna Zentaris, Inc.) for the GHSR antagonist JM2959 and Johnny Garretson and Fardowsa Robow for continued help with data collection.

**GRANTS**

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01 DK-078358 awarded to T. J. Bartness.

**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

M.A.T., V.R., and T.J.B. developed the concept and designed the research; M.A.T. performed the experiments; M.A.T. analyzed the data; M.A.T., V.R., and T.J.B. interpreted the results of the experiments; M.A.T. prepared the figures; M.A.T., V.R., and T.J.B. drafted the manuscript; M.A.T., V.R., and T.J.B. edited and revised the manuscript; M.A.T., V.R., and T.J.B. approved the final version of the manuscript.

**REFERENCES**


60. Leviatsky DA, DeRosino L. One day of food restriction does not result in an increase in subsequent daily food intake in humans. Physiol Behav 99: 495–499, 2010.


