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**GROWTH HORMONE SECRETAGOGUE RECEPTOR SIGNALING AFFECTS
HIGH FAT INTAKE INDEPENDENTLY OF PLASMA LEVELS OF GHRELIN AND
LEAP2, IN A 4-DAY BINGE EATING MODEL**

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Short title: Ghrelin-independent GHSR activity affects binge-like high fat intake.

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The authors have nothing to disclose.

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ABSTRACT

The growth hormone secretagogue receptor (GHSR) is a G protein-coupled receptor highly expressed in the central nervous system. GHSR acts as a receptor for ghrelin and for liver antimicrobial peptide 2 (LEAP2), which blocks ghrelin-evoked activity. GHSR also displays ligand-independent activity, including a high constitutive activity that signals in the absence of ghrelin and is reduced by LEAP2. GHSR activity modulates a variety of food intake-related behaviors, including binge eating. Previously, we reported that GHSR-deficient mice daily and time-limited exposed to high fat (HF) diet display an attenuated binge-like HF intake, as compared to wild-type mice. Here, we aimed to determine if ligand-independent GHSR activity affects binge-like HF intake in a 4-day binge-like eating protocol. We found that plasma levels of ghrelin and LEAP2 were not modified in mice exposed to this binge-like eating protocol. Moreover, systemic administration of ghrelin or LEAP2 did not alter HF intake in our experimental conditions. Interestingly, we found that central administration of LEAP2 or K-(D-1-Nal)-FwLL-NH₂, both blockers of constitutive GHSR activity, reduced binge-like HF intake, while central administration of ghrelin or the ghrelin-evoked GHSR activity blockers [D-Lys3]-GHRP-6 and JMV2959 did not modify binge-like HF intake. Together, current data indicate that GHSR activity in the brain affects binge-like HF intake in mice independently of plasma levels of ghrelin and LEAP2.

Keywords: constitutive GHSR activity, palatable food, food reward.

INTRODUCTION

The growth hormone secretagogue receptor (GHSR) is a G protein-coupled receptor (GPCR) highly expressed in the brain^{1,2}. GHSR signaling controls a variety of functions including food intake, growth hormone secretion, glucose homeostasis, among others³. Endogenous ligands of GHSR include: ghrelin, an acylated peptide mainly produced in the gastrointestinal tract that activates GHSR, and the recently described liver-expressed antimicrobial peptide 2 (LEAP2), mainly expressed in the liver and small intestine that antagonizes ghrelin-evoked GHSR activation⁴. GHSR also displays some ligand-independent actions. *In vitro*, GHSR shows an unusually high constitutive activity that signals with ~50% of its maximum activity, in the absence of ghrelin^{5,6}. Constitutive GHSR activity is reduced by LEAP2, indicating that this ligand also acts as an inverse agonist of the receptor^{7,8}. A role for constitutive GHSR activity in humans is supported by a naturally occurring mutation of GHSR that abolishes constitutive activity without altering ghrelin-evoked activity and leads to familial short stature⁹. Furthermore, other mutations of GHSR that impact on its constitutive activity are linked to short stature in humans¹⁰. Besides its intrinsic constitutive activity, GHSR can allosterically interact with other GPCRs and induce mutual changes in their signaling activities¹¹. Thus, different molecular mechanisms can modulate GHSR activity and, as a consequence, its physiological impact.

GHSR activity potently regulates food intake. Ghrelin administration rapidly stimulates feeding in satiated humans and rodents¹², and LEAP2 administration abolishes ghrelin-induced food intake in mice^{4,7}. The fact that plasma ghrelin in humans and rodents rises before meals and decreases post-prandially suggests that endogenous ghrelin is linked to food intake regulation^{13,14}. Plasma levels of ghrelin and LEAP2 display an inverse relationship in response to changes in energy balance: ghrelin levels increase and LEAP2 levels decrease under fasting, whereas ghrelin levels decrease and LEAP2 levels increase upon refeeding⁴. Some studies using pharmacological blockage of ghrelin action (e.g., anti-ghrelin RNA spiegelmers, anti-ghrelin antibodies, GHSR antagonists) show that ghrelin-evoked GHSR activity is required for a full compensatory hyperphagia after a fast¹⁵⁻²⁰. Thus, GHSR ligands appear to synchronously adjust GHSR activity in order to modulate homeostatic aspects of food intake. Notably, studies using genetically-modified mice indicate that GHSR activity can impact on food intake in a ghrelin-independent manner. In particular, most studies show that mice lacking GHSR exhibit more robust alterations in eating behaviors, as compared to ghrelin knock-out mice²¹. In wild-type (WT) mice, the relevance of ghrelin-independent GHSR activity on food intake regulation can be uncovered after a prolonged fast. In particular, plasma ghrelin levels and GHSR gene expression in the

hypothalamic arcuate nucleus (ARC) increase in 48-h fasted mice, and they decline to basal levels soon after refeeding²². However, GHSR protein remains elevated in ARC neurons during the first days of refeeding and its action stimulates the biosynthesis of orexigenic peptides that, in turn, enhance the compensatory hyperphagia several days after the fast²². Thus, GHSR appears to regulate homeostatic aspects of food intake through ligand-dependent and ligand-independent mechanisms.

GHSR also regulates non-homeostatic aspects of food intake^{23,24}. Central administration of ghrelin to satiated rodents enhances behaviors associated to rewarding aspects of food intake such as food seeking behavior and the motivation to obtain palatable foods^{25–27}. Some studies show that peripherally injected ghrelin also modulates food reward-related behaviors^{18,26,28,29}; however, no direct evidence currently exists showing that circulating ghrelin can reach reward-related brain targets, such as the ventral tegmental area (VTA)¹². The effect of LEAP2 on rewarding aspects of food intake has not been investigated, yet. Since GHSR activity enhances the rewarding value of palatable foods^{18,25,30}, it has been proposed to modulate binge eating behaviors. Binge eating refers to the consumption of excessive amounts of usually palatable foods in a short time frame and is often associated with a sense of loss of control over the ability to cease eating once nutritional needs have been met³¹. Binge eating is observed in patients suffering some eating disorders (e.g. bulimia nervosa, binge eating disorder), in some people with obesity, and even in non-clinical individuals under specific situations (e.g. stress). Individuals with binge eating disorder have lower fasting and post-prandial ghrelin levels, as compared to obese subjects without binge eating disorder^{32–34}. Also, a single nucleotide polymorphism of the ghrelin gene has been associated with binge eating disorder³⁵. Another study in humans, however, failed to detect a correlation between fasting ghrelin levels and the frequency and severity of bingeing³⁶. Studies in rodents provided additional evidence linking GHSR activity and binge eating³⁷. In particular, satiated GHSR-deficient mice daily and time-limited exposed to high fat (HF) diet display an attenuated binge-like HF intake, as compared to WT mice^{38,39}. Interestingly, the lack of GHSR reduces binge-like intake over the initial accesses to palatable food, when daily HF intake gradually escalates and is recognized as the transition period from a controlled to an uncontrolled behavior.⁴⁰ Studies using mice lacking GHSR, however, do not allow to discriminate if binge-like HF intake depends, or not, on plasma levels of GHSR ligands. Since the accessibility of circulating ghrelin to mesolimbic brain areas appears to be strikingly low¹², we hypothesized that GHSR activity in the mouse brain affects binge-like HF intake independently of plasma levels of ghrelin and LEAP2. To test our hypothesis, we exposed WT mice to a 4-day binge-like eating protocol and explored the relationship between binge eating and plasma levels of ghrelin and

LEAP2. Additionally, we investigated if systemic or central treatment with the natural GHSR ligands ghrelin or LEAP2 affected binge-like HF intake. Finally, we tested if central administration of synthetic GHSR ligands affected binge-like HF intake.

MATERIAL AND METHODS

Animals and diets. All experiments were carried out with adult (9-12 weeks old) male C57BL/6J WT mice that were generated at the animal care facility of the IMBICE. Mice were housed under a 12-h light/dark cycle with food and water available *ad libitum*. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council, USA. Procedures were approved by the Institutional Animal Care and Use Committee of the IMBICE (approval ID 10-0122). Both regular chow (RC) and HF diet were provided by Gepsa (Grupo Pilar, www.gepsa.com). RC pellets provided 2.5 kcal/g energy and its percent weight composition was: carbohydrates 28.8, proteins 25.5, fat 3.6, fibers 27.4, minerals 8.1 and water content 6.7. HF diet pellets provided 3.9 kcal/g energy and their percent weight composition was: carbohydrates 22.5, proteins 22.8, fat 21.1, fibers 23.0, minerals 5.6 and water content 5.0. For details see⁴¹.

Drugs. Ghrelin was purchased from Global Peptides (cat# PI-G-03), dissolved in phosphate-buffered saline (PBS, pH 7.4) and prepared fresh on experimental days. For subcutaneous (SC) injections, mice were injected with 60 pmol/g BW of ghrelin, a dose that transiently increases plasma ghrelin levels resembling concentrations observed in mice under energy deficit conditions and elicits a robust increase in food intake^{42,43}. For intracerebroventricular (ICV) injections, mice were injected with 300 pmol/mouse of ghrelin, a dose that induces maximal food intake response in our experimental conditions⁴³. Since we recently demonstrated that the N-terminal sequence of LEAP2 confers full receptor binding and activity⁷, a peptide containing the initial 14 residues of N-terminal LEAP2 sequence (hereafter named LEAP2₁₋₁₄) was employed as an analog of full-length LEAP2. LEAP2₁₋₁₄ was assembled on solid support using Fmoc chemistry and starting from Agilent Amphisphere 40 RAM resin, purified by RP-HPLC and characterized by LC-MS and MALDI-MS/MS with a purity > 95%, as described previously⁷. LEAP2 was dissolved in PBS. For SC injections, mice were injected with 600 pmol/g BW of LEAP2₁₋₁₄ since this dose of SC-injected LEAP2 fully blocks the orexigenic effects of 60 pmol/g BW of ghrelin⁷. For ICV injections, mice were injected with 600 pmol/mouse of LEAP2₁₋₁₄, this dose of LEAP2₁₋₁₄ reduces the orexigenic effects of ICV-injected ghrelin (60 pmol/mouse) in the same extent than full-length LEAP2 (35.5±6.2 and 49.6±14.2 %, respectively). [D-Lys3]-growth hormone-releasing peptide 6 ([D-Lys3]-GHRP-6) was purchased from Sigma-Aldrich (cat# SLBN1014V). Mice were ICV-treated with 2 nmol/mouse of [D-Lys3]-GHRP-6, this dose was chosen based on ours and other's previous work showing that it blocks the orexigenic effects of ICV-injected ghrelin^{22,44}. JMV2959 was synthesized as previously described⁴⁵. Mice were ICV-

treated with 3 nmol/mouse of JMV2959, this dose was chosen based on our previous experience showing that it blocks the orexigenic effects of ICV-injected ghrelin²². K-(D-1-Nal)-FwLL-NH₂ was synthesized by automated solid-phase peptide synthesis, as described previously⁴⁶. Mice were ICV-treated with 1 nmol/mouse of K-(D-1-Nal)-FwLL-NH₂, this dose that was chosen based on our previous experience showing that it blocks the orexigenic effects of ICV-injected ghrelin and reduces the fasting-induced compensatory hyperphagia²². Importantly, mice ICV-injected with GHSR ligands did not show any sickness-like behavior such as a spiky coat, hunched posture, altered breathing rate, labored movements, reduced activity and/or subdued behavior.

HF diet binge-like eating protocol. *Ad libitum* fed mice were single-housed three days before the experiment. At 9.00 am of experimental day 1, RC pellets were removed from home cages and mice were exposed to a pre-weighed pellet of HF diet. At 11.00 am, HF pellets were removed from home cages and RC was re-added to the food hoppers. The same procedure was repeated during 4 consecutive days. Additionally, an independent group of mice, hereafter named RC group, was daily subjected to the same experimental protocol but exposed to a RC pellet, instead of the HF pellet, from 9.00 am to 11.00 am for 4 successive days. Daily food intake in the 2-h food access was calculated by subtracting the weight of the remaining food pellet at 11.00 am to the initial weight of the food pellet. In some cases, body weight and 22-h food intake was also assessed all over the study.

Assessment of plasma concentrations of ghrelin and LEAP2. Blood was collected from the facial vein of WT mice randomly assigned into the following experimental groups: 1) RC group (n=7), which was bled at 11.00 am, right after 2-h food intake, the experimental day 4, 2) pre-HF intake group (n=8), which was daily exposed to a HF pellet for 3 successive days and bled at 9.00 am, right before HF pellet exposure, the experimental day 4, and 3) post-HF intake group (n=11), which was daily exposed to a HF pellet for 4 successive days and bled at 11.00 am, right after HF intake, the experimental day 4. Blood was collected in tubes containing EDTA (1 mg/mL final) and the protease inhibitor *p*-hydroxy-mercuribenzoic acid (0.4 mM final). Then, blood samples were centrifuged at 5000 rpm for 10 min at 4°C. Plasmas were collected and acidified with 1 M HCl (0.1 M final) to preserve ghrelin acylation and stored frozen at -80°C until processing. Plasma ghrelin levels were assessed using an enzymatic immunoassay from Bertin Pharma (cat# A05118), as we did in the past²². This commercial assay specifically detects the bioactive acylated form of the peptide and does not cross react with des-acylghrelin. Plasma LEAP2 levels were assessed using an enzymatic immunoassay from Phoenix Pharmaceuticals (cat# EK-075-40). EIA LEAP2 kit demonstrated linearity of dilution and parallelism, and detected lower levels of

LEAP2 in plasma samples from 60% calorie restricted mice, as compared to *ad libitum* fed mice, as previously reported⁴.

Systemic treatments in mice exposed to the HF diet binge-like eating protocol. Single-housed mice were daily and SC-injected with sterile PBS three days before experimental day 1 in order to acclimate them to handling. From experimental day 1 to 4 of the binge-like eating protocol, mice received daily SC injections of vehicle alone (n=16) or containing ghrelin (n=9) or LEAP2₁₋₁₄ (n=10) and were then exposed to the HF diet pellet for 2-h. SC injections took no more than 20 seconds and caused no discomfort in mice, which were exposed to pre-weighed HF pellets immediately after treatment.

Central treatments in mice exposed to the HF diet binge-like eating protocol. One week before the experiment, mice were stereotaxically implanted with a single indwelling guide cannula into the lateral ventricle, as previously described in detail⁴⁷, using the following placement coordinates: antero-posterior: -0.34 mm, medio-lateral: +1.0 mm and dorso-ventral: -2.3 mm. Mice implanted with ICV cannulas and single-housed were daily ICV-injected with artificial cerebrospinal fluid (aCSF) for three days in order to acclimate them to handling. In one set of experiments, mice were daily ICV-injected with aCSF alone (n=18) or containing ghrelin (n=16) or LEAP2₁₋₁₄ (n=12), from experimental day 1 to 4 of the binge-like eating protocol, and then exposed to the HF diet pellet for 2-h. In another set of experiments, mice were daily ICV-injected with aCSF alone (n=16) or containing [D-Lys3]-GHRP-6 (n=13), JMV2959 (n=7) or K-(D-1-Nal)-FwLL-NH₂ (n=11), from experimental day 1 to 4 of the binge-like eating protocol, and then exposed to the HF diet pellet for 2-h. For ICV injections, mice were manually restrained and dummy cannulas were removed from guide cannulas and replaced by injector cannulas. Injections were slowly performed over the course of a minute in order to avoid rapid pressure changes inside the cranial cavity. Then, the injector cannulas were left in place for 2 min in order to prevent back flow of the injected solution. Finally, the injector cannulas were removed and replaced by the dummy cannulas. ICV injections were performed 20 min before mice were exposed to the pre-weighed HF pellets.

Statistical analyses. Data are expressed as the mean±standard error of the mean (SEM). Normal distribution of the data was tested using D'Agostino & Pearson's test. When data distribution was normal, data was analyzed using either: 1) one-way ANOVA followed by Tukey's, Holm-Sidak's or Dunnett's post-test, 2) two-way repeated measures (RM) ANOVA followed by Tukey's or Dunnett's post-test or 3) two-way ANOVA followed by Dunnett's post-test. When data

distribution differed from a normal distribution, Kruskal-Wallis test followed by Dunn's post-test was used. Figure legends describe the number of mice and the specific statistical analysis used for each experiment. Differences were considered significant when $P < 0.05$.

RESULTS

1. Plasma levels of ghrelin and LEAP2 are unaltered in mice exposed to a HF diet binge-like eating protocol. As previously reported^{39,48}, RC group showed a small 2-h food intake when exposed to the fresh pellet into the home cage, and such 2-h food intake did not change over the experimental days. In contrast, HF intake groups displayed a higher 2-h food intake all over the experiment, as compared to the RC group, as well as an escalating profile of 2-h HF intake over the successive days (Figure 1A). As also previously reported^{39,48}, 22-h RC intake during the rest of the day and body weight across the experiment did not differ in HF intake groups, as compared to RC group (not shown). Plasma ghrelin levels did not differ among RC, pre-HF intake, or post-HF intake groups, although ghrelin levels in post-HF intake group tended to be lower than in RC group (Figure 1B). Plasma LEAP2 levels did not differ among experimental groups (Figure 1C).

2. Systemic injection of ghrelin or LEAP2₁₋₁₄ did not affect HF intake in a binge-like eating protocol. In order to test if increments of circulating levels of ghrelin or LEAP2₁₋₁₄ affect HF intake in mice exposed to the HF diet binge-like eating protocol, *ad libitum* fed mice were daily SC-injected with PBS alone or containing each of these peptides and subsequently exposed to a HF pellet for 4 consecutive days (Figure 2A-B). Ghrelin- and LEAP2₁₋₁₄-treated groups showed daily 2-h HF intake, escalation profile of 2-h HF intake and cumulative 4-day HF intake similar as vehicle-treated group. Ghrelin- and LEAP2₁₋₁₄-treated groups also showed similar daily 22-h RC intake (Figure 2C) and body weight (not shown), as compared to vehicle-treated group all over the study.

3. Centrally-injected LEAP2₁₋₁₄ reduced cumulative HF intake in a binge-like eating protocol. In order to test if central administrations of ghrelin or LEAP2₁₋₁₄ affect HF intake in mice exposed to the binge-like eating protocol, *ad libitum* fed mice were daily ICV-injected with aCSF alone or containing each of these peptides and subsequently exposed to a HF pellet for 4 consecutive days (Figure 3A-B). Notably, mice ICV-injected with vehicle displayed a smaller 2-h HF intake at all experimental days, as compared to mice SC-injected with vehicle likely due to differences inherent to the procedure required for each type of injection. As compared to vehicle-treated group, ghrelin-treated group displayed: 1) a tendency to display a higher 2-h HF intake at experimental day 1 ($P=0.1001$), 2) a lack of 2-h HF intake escalation over the successive days ($P<0.0001$ and $P=0.0605$ for day 1 vs. day 4 HF intake in vehicle- and ghrelin-treated groups, respectively), and 3) a similar cumulative 4-day HF intake ($P>0.9999$). As compared to vehicle-treated group, LEAP2₁₋₁₄-treated group displayed: 1) a tendency to display a smaller 2-h HF

intake, although it only reached statistical significance at experimental day 4, 2) a lack of 2-h HF intake escalation over the successive days ($P < 0.0001$ and $P = 0.4435$ for day 1 vs. day 4 HF intake in vehicle- and LEAP2₁₋₁₄-treated groups, respectively), and 3) a reduced cumulative 4-day HF intake. Ghrelin- and LEAP2₁₋₁₄-treated groups showed similar daily 22-h RC intake (Figure 3C) and body weight (not shown), as compared to vehicle-treated group all over the study.

4. Pharmacological blockade of GHSR activity using K-(D-1-Nal)-FwLL-NH₂ reduces HF intake in a binge-like eating protocol. In order to test if pharmacological manipulations of GHSR activity affect HF intake in mice exposed to the HF diet binge-like eating protocol, *ad libitum* fed mice were daily ICV-injected with aCSF alone or containing [D-Lys3]-GHRP-6, JMV2959 or K-(D-1-Nal)-FwLL-NH₂ and then exposed to a HF pellet for 4 consecutive days (Figure 4A-B). [D-Lys3]-GHRP-6- and JMV2959-treated groups showed daily 2-h HF intake, escalation profile of 2-h HF intake and cumulative 4-day HF intake similar as vehicle-treated group. As compared to vehicle-treated group, K-(D-1-Nal)-FwLL-NH₂-treated group displayed: 1) a smaller 2-h HF intake at experimental days 3 and 4, 2) a lack of 2-h HF intake escalation over the successive days ($P < 0.0001$ and $P = 0.2351$ for day 1 vs. day 4 HF intake in vehicle- and K-(D-1-Nal)-FwLL-NH₂-treated groups, respectively), and 3) a reduced cumulative 4-day HF intake. [D-Lys3]-GHRP-6-, JMV2959- and K-(D-1-Nal)-FwLL-NH₂-treated groups showed similar daily 22-h RC intake (Figure 4C) and body weight (not shown), as compared to vehicle-treated group all over the study.

DISCUSSION

Here, we show that plasma levels of ghrelin and LEAP2 were not modified in mice exposed to a 4-day HF diet binge-like eating protocol, and that systemic administration of ghrelin or LEAP2 did not alter HF intake in our experimental conditions. Also, we show that central administration of GHSR ligands, which are known to potently block constitutive GHSR activity, reduced binge-like HF intake, while central administration of synthetic GHSR ligands that mainly block ghrelin action did not modify binge-like HF intake. Thus, current data suggest that GHSR activity in the brain affects binge-like HF intake in mice independently of plasma levels of ghrelin and LEAP2.

We found that plasma levels of ghrelin and LEAP2 in mice daily and time-limited fed with a HF diet for 4 days are similar after and before HF intake. Despite plasma ghrelin levels tended to be lower after the last event of 2-h HF intake, a phenomenon that could have contributed to HF intake cessation, the magnitude of such decrease did not reach statistical significance. Thus, binge-like HF intake seems independent of endogenous fluctuations of these hormones. We also found that systemic administration of ghrelin, in a dose that transiently increases plasma ghrelin levels by ~2-fold at 30 minutes after injection⁴⁹, did not affect binge-like HF intake. It seems likely that the spontaneous tendency of vehicle-treated mice to eat HF diet unmasked the orexigenic effect of ghrelin as HF intake had already reached a maximum magnitude. Moreover, we found that systemic administration of LEAP2₁₋₁₄ did not affect binge-like HF intake. The same dose of systemically-injected LEAP2₁₋₁₂, which is even shorter than LEAP2₁₋₁₄, blocks the orexigenic effects of systemically-injected ghrelin in the same extent that full-length LEAP2 indicating that experimental conditions were accurate to unmask effects of circulating LEAP2₁₋₁₄⁷. Thus, acute increments of plasma LEAP2, and the resulting blockade of endogenous ghrelin action, do not affect HF intake in our experimental conditions. ICV-injected ghrelin tended to increase HF intake on experimental day 1, and such increment masked HF intake escalation over the successive days. ICV-injected LEAP2₁₋₁₄ reduced binge-like HF intake in a similar fashion as previously seen in GHSR-deficient mice³⁹. These observations indicate that GHSR activity in the brain affects binge-like HF intake in mice. Current results also suggest that centrally-injected hormones act on brain areas involved in HF intake that were not targeted by systemically-injected hormones. Of note, LEAP2₁₋₁₄ displays full bioactivity⁷; however, it may not fully resemble all physiological features of LEAP2, such as plasma clearance or brain accessibility. Thus, the ability of circulating LEAP2 to act on brain areas regulating HF intake should be tested using an intact version of the hormone. Despite these considerations, current results strongly indicate that rapid fluctuations in circulating levels of ghrelin or LEAP2 do not affect binge-like HF intake in mice.

Studies in which rodents have daily or intermittent access to a palatable diet for either short- or long-term periods of time have made evident a link between GHSR activity and different features of binge eating. Here, we studied the role of GHSR activity on the early stage of our binge-like eating protocol, when total daily food intake and body weight are unaffected³⁹. At this early stage, 2-h HF intake increases across the binge-like eating events and then stabilizes⁵⁰⁻⁵⁴. Current observations together previous results, showing that GHSR-deficient mice do not increase 2-h HF intake in the same binge-like eating protocol³⁸, indicate that GHSR activity affects HF intake escalation. Intake escalation is a complex behavior observed in the early stages of binge eating models, as well as in rodents successively exposed to drugs of abuse. In the addiction field, escalation of drugs of abuse self-administration is considered an operant conditioning model of early stages of drug intake and has been used to investigate mechanisms of tolerance, behavioral sensitization and habit formation⁵⁵⁻⁵⁷. Since intake escalation would be associated to the feeling of loss of control over eating, a typical characteristic of binge eating episodes in humans, and to the genesis of addictive behaviors^{37,40,55}, future studies aimed to elucidate the specific role of GHSR activity on the neurobiology of intake escalation will be crucial. Importantly, GHSR signaling may play different roles in animals exposed to binge eating protocols for longer periods of time, when changes in body weight become evident. For instance, a recent study found that ICV-injected ghrelin shifts diet preference, increasing RC intake and reducing HF intake, in rats exposed to a 2-week binge eating protocol with simultaneous access to both diets⁵⁸. Also, it was shown that rats intermittently and time-limited fed with different palatable diets for several weeks displayed higher plasma ghrelin levels before each binge eating event⁵⁹⁻⁶³. In particular, one study found that increments of plasma ghrelin levels positively correlated with food anticipatory activity and that a GHSR antagonist decreased such behavior⁶⁰, while another study showed that blockade of plasma ghrelin increments attenuated the magnitude of binge-like HF intake⁶³. Moreover, two studies found that mice or rats intermittently and time-limited fed with a palatable diet over several weeks displayed a reduction of plasma ghrelin levels immediately after the last binge eating event^{64,65}. Other studies, however, could not confirm these observations and found that rats time-limited fed with palatable diets, either daily or intermittently, over several weeks did not change plasma ghrelin levels in anticipation to the scheduled meals as compared to control rats^{50,66}. Also, another study found that female rats with daily and limited access to a palatable diet over several weeks displayed lower plasma ghrelin levels under fasting, as compared to control rats⁶⁷. The reasons for these discrepancies are uncertain. The neurobiology of binge eating behaviors is complex, and plasma ghrelin levels depend not only on the short-term feeding patterns and long term energy balance⁶⁸ but also on the dietary lipid composition⁶⁹.

Thus, different experimental designs (e.g. type of palatable diet, frequency of exposure, or duration of the protocol) may help to gain insights about diverse roles of the GHSR activity on binge eating behaviors.

Here, we tested several well-characterized synthetic GHSR ligands. The peptide [D-Lys3]-GHRP-6 was initially generated by replacing alanine by D-lysine in position 3 of the GHSR agonist peptide GHRP-6⁷⁰, and shown to inhibit spontaneous and ghrelin-induced food intake in mice⁴⁴. [D-Lys3]-GHRP-6 is a potent GHSR antagonist, but also binds to chemokine and melanocortin receptors^{71,72}. JMV2959 is based on a trisubstituted 1,2,4-triazole structure⁴⁵ and was first reported as a selective GHSR antagonist⁷³. Further *in vitro* studies showed that JMV2959 displays a partial agonist activity on GHSR towards Gq signaling⁷⁵; however, such agonist activity is not evident in *in vivo* settings, where JMV2959 blocks the effects ghrelin in a GHSR-dependent manner^{74,76} presumably because it blocks the binding of the hormone to GHSR. The hexapeptide K-(D-1-Nal)-FwLL-NH₂ was developed based on modifications of the substance P analogue, which was the first reported GHSR inverse agonist⁵. K-(D-1-Nal)-FwLL-NH₂ is a potent inverse agonist *in vitro* and reduces spontaneous and ghrelin-induced food intake in rodents in a GHSR-dependent manner^{22,46}. Given the complexity of the biology of GHSR, a selective pharmacological manipulation of specific features of the receptor has been proved to be challenging⁷⁵. Indeed, it is likely that the interaction of these synthetic ligands with GHSR involves a variety of partially biased effects^{75,77}. Moreover, we cannot rule out that handling-induced stress affected, in some extent, our observations since central infusions *per se* slightly decreased 2-h HF intake in our experiments. Thus, current observations should be cautiously interpreted with these considerations in mind. In summary, we found here that central administration of the GHSR antagonist [D-Lys3]-GHRP-6 or JMV2959 did not affect HF intake escalation over successive days or cumulative 4-day HF intake supporting the notion that circulating ghrelin does not affect brain areas regulating binge-like HF intake. In contrast, ICV-injected K-(D-1-Nal)-FwLL-NH₂ reduced daily HF intake, HF intake escalation over the successive days and cumulative 4-day HF intake, in a similar fashion as seen for ICV-injected LEAP2₁₋₁₄. Notably, central administration of these GHSR synthetic ligands did not affect 22-h food intake and body weight. Thus, the overall observation that ghrelin-independent GHSR activity in the brain plays a modulatory role on binge-like HF intake in mice indicates that inverse agonism properties of synthetic GHSR ligands is a key feature to consider in potential drugs developed to treat binge eating. In this regard, the N-terminal end of LEAP2, rather than full LEAP2 sequence, emerges as a novel reference for the structure-based design of more potent GHSR inverse agonist.

Binge-like HF intake in the current experimental conditions is presumably driven by the palatable nature of the stimulus and involves rewarding aspects of appetite. Reward-related behaviors are regulated by the mesolimbic system, which connects the VTA with regions of the limbic system, including the nucleus accumbens (Acb)⁷⁸. There is much evidence showing that ghrelin affects the mesolimbic pathway and, as a consequence, rewarding aspects of appetite^{23,24}. For instance, GHSR is expressed in VTA dopamine neurons and ghrelin increases both action potential frequency of dopamine VTA neurons and release of dopamine in the Acb⁷⁹⁻⁸¹. GHSR activity in the VTA appears to regulate food intake independently of homeostatic aspects of eating since ghrelin treatment in mice lacking orexigenic ARC neurons increases palatable food intake in a VTA-dependent manner⁸². Importantly, genetic deficiency of GHSR in mice impacts on the activity of the mesolimbic pathway and, as a consequence, on rewarding aspects of eating²¹. GHSR knock-out mice show impaired dopamine release in the Acb in response to rewarding foods³⁰. In contrast to WT mice, GHSR-deficient mice do not display conditioned place preference for HF diet upon caloric restriction or chronic stress^{18,42}; while mice with expression of GHSR selectively in catecholaminergic cells, including the dopamine VTA neurons, show a similar conditioned place preference for HF diet after chronic stress as seen in WT mice⁴². Previous studies showing that GHSR-deficient mice exhibit a smaller binge-like HF intake also reported that these mice display a concomitant reduced activation of the mesolimbic pathway^{38,39}. Thus, it seems reasonable to hypothesize that centrally-injected GHSR inverse agonists reduce binge-like HF intake due its action on the mesolimbic pathway. Notably, the magnitude of the constitutive GHSR activity in neurons of the mesolimbic pathway has not been directly assessed *in vivo*. Thus, we cannot rule out that the central administration of these GHSR ligands may reduce binge-like HF intake by affecting other ligand-independent features of the receptor, such as its ability to heterodimerize with other GPCRs. Finally, it is interesting to point out that we previously provided evidence indicating that rapid increments of plasma ghrelin levels do not impact on the mesolimbic pathway. In particular, we reported that SC-injected fluorescent-ghrelin is found in brain areas located in apposition to circumventricular organs but not in the VTA⁴⁹. Also, we reported that SC-injected ghrelin does not increase c-Fos levels in the VTA or in the Acb nor affect locomotor activity in mice⁸³. Altogether, previous and current observations suggest that the effects of GHSR activity on the mesolimbic pathway and its further impact on binge-like HF intake occurs independently of circulating ligands. It is interesting to stress that a recent study reported that ghrelin-independent GHSR activity in the brain modulates cocaine-induced reward in mice⁸⁴. Similarly, GHSR activity can affect alcohol-induced reward and compulsive alcohol intake in rodents, independently of circulating ghrelin levels⁸⁵⁻⁸⁷. Notably, a recent preliminary study reported that administration of the GHSR inverse agonist PF-5190457 reduces alcohol and food

craving in humans⁸⁸. Therefore, pharmacological manipulation of ligand-independent GHSR activity appears as a promising strategy to treat not only binge eating but also a variety of other pathological consummatory behaviors with important clinical applications.

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FIGURE LEGENDS

Figure 1: *Plasma levels of ghrelin and LEAP2 are not modified in mice exposed to a HF diet binge-like eating protocol. A* 2-h food intake of RC (n=7), pre-HF intake (n=8) and post-HF intake (n=11) groups of mice in the successive days of the HF diet binge eating protocol. Note that pre-HF intake group lacks HF intake on experimental day 4 because these mice were bled before HF exposure and no exposed to HF diet. a, $P < 0.05$ vs day 1 same group and b, $P < 0.05$ vs RC group same day. Two-way RM ANOVA ($F(6, 69)_{\text{interaction}}=13.22$, $P < 0.0001$; $F(3, 69)_{\text{time}}=12.14$, $P < 0.0001$; $F(2, 23)_{\text{group}}=7.253$, $P=0.0036$; $F(23, 69)_{\text{matching}}=10.48$, $P < 0.0001$) followed by Tukey's post-test (pre-HF intake group: $P=0.0545$ and $P=0.0001$ for day 2 and day 3 vs day 1, respectively; post-HF intake group: $P=0.0010$, $P=0.0051$ and $P=0.0119$ for day 2, day 3 and day 4 vs day 1, respectively; day 2: $P=0.0111$ and $P=0.0002$ for pre- and post-HF intake group vs RC group, respectively; day 3: $P=0.0004$ and $P=0.0005$ vs for pre- and post-HF intake group RC group, respectively; day 4: $P=0.0031$ vs for post-HF intake group RC group). **B** plasma levels of ghrelin in RC, pre-HF intake and post-HF intake groups. One-way ANOVA ($F(2, 23)=2.824$, $P=0.0800$) followed by Dunnett's post-test ($P=0.0526$ for post-HF intake group vs RC group). **C** plasma levels of LEAP2 in RC, pre-HF intake and post-HF intake groups. One-way ANOVA ($F(2, 23)=2.379$, $P=0.1150$) followed by Dunnett's post-test. Data represent the mean \pm SEM.

Figure 2: *Systemically-injected ghrelin or LEAP2₁₋₁₄ do not affect HF intake in a binge-like eating protocol. A* 2-h HF intake of mice SC-treated with vehicle (n=16), ghrelin (n=9) or LEAP2₁₋₁₄ (n=10) and then exposed to the HF diet binge-like eating protocol. a, $P < 0.05$ vs day 1 same group; two-way RM ANOVA ($F(6, 96)_{\text{interaction}}=0.4374$, $P=0.8522$; $F(3, 96)_{\text{time}}=46.67$, $P < 0.0001$; $F(2, 32)_{\text{treatment}}=0.7530$, $P=0.4791$; $F(32, 96)_{\text{matching}}=7.313$, $P < 0.0001$) followed by Tukey's post-test (vehicle: $P=0.0032$, $P < 0.0001$ and $P < 0.0001$ for day 2, day 3 and day 4 vs day 1, respectively; ghrelin: $P=0.0050$, $P < 0.0001$ and $P < 0.0001$ for day 2, day 3 and day 4 vs day 1, respectively; LEAP2₁₋₁₄: $P=0.0011$, $P < 0.0001$ and $P < 0.0001$ for day 2, day 3 and day 4 vs day 1, respectively). **B** cumulative 4-day HF intake of mice that were SC-injected with vehicle, ghrelin or LEAP2₁₋₁₄ and exposed to the HF diet binge-like eating protocol. **C** 22-h RC intake of mice that were SC-injected with saline (n=5), ghrelin (n=4) or LEAP2₁₋₁₄ (n=5) and exposed to the HF diet binge-like eating protocol. Two-way ANOVA ($F(6, 44)_{\text{interaction}}=0.1756$, $P=0.9821$; $F(3, 44)_{\text{time}}=2.164$, $P=0.1057$; $F(2, 44)_{\text{treatment}}=0.3841$, $P=0.6833$) followed by Tukey's post-test. Data represent the mean \pm SEM.

Figure 3: Centrally-injected LEAP2₁₋₁₄ reduces HF intake in a binge-like eating protocol. **A** 2-h HF intake of mice ICV-treated with vehicle (n=18), ghrelin (n=16) or LEAP2₁₋₁₄ (n=12) and then exposed to the HF diet binge-like eating protocol. a, P<0.05 vs day 1 same group and b, P<0.05 vs ICV-injected vehicle same day; two-way RM ANOVA (F(6, 129)_{interaction}=2.031, P=0.0661; F(3, 129)_{time}=13.37, P<0.0001; F(2, 43)_{treatment}=4.608, P=0.0154; F(43, 129)_{matching}=6.006, P<0.0001) followed by Tukey's post-test (vehicle: P<0.0001 and P<0.0001 for day 3 and day 4 vs day 1, respectively; day 4: P=0.0037 for LEAP2₁₋₁₄ vs vehicle). **B** cumulative 4-day HF intake of mice that were ICV-treated with vehicle, ghrelin or LEAP2₁₋₁₄ and then exposed to the HF diet binge-like eating protocol. *, P<0.05 vs vehicle; Kruskal-Wallis test (KW statistic=8.459, P=0.0146) followed by Dunn's post-test (P=0.0363 for LEAP2₁₋₁₄ vs vehicle). **C** 22-h RC intake of mice that were ICV-treated with vehicle (n=10), ghrelin (n=10) or LEAP2₁₋₁₄ (n=7) and then exposed to the HF diet binge-like eating protocol. Two-way ANOVA (F(6, 96)_{interaction}=0.5666, P=0.0.7559; F(3, 96)_{time}=1.855, P=0.1424; F(2, 96)_{treatment}=2.319, P=0.1039) followed by Tukey's post-test. Data represent the mean±SEM.

Figure 4: ICV-administered K-(D-1-Nal)-FwLL-NH₂ reduces HF intake in a binge-like eating protocol. **A** 2-h HF intake of mice ICV-treated with vehicle (n=16), [D-Lys3]-GHRP-6 (n=13), JMV2959 (n=7) or K-(D-1-Nal)-FwLL-NH₂ (n=11) and then exposed to the HF diet binge-like eating protocol. a, P<0.05 vs day 1 same group and b, P<0.05 vs vehicle same day; two-way RM ANOVA (F(9, 129)_{interaction}=1.219, P=0.2891; F(3, 129)_{time}=19.47, P<0.0001; F(3, 43)_{treatment}=2.321, P=0.0886; F(43, 129)_{matching}=2.894, P<0.0001) followed by a Tukey's post-test (vehicle: P=0.0003 and P<0.0001 for day 3 and day 4 vs day 1, respectively; [D-Lys3]-GHRP-6: P=0.0002 and P<0.0001 for day 3 and day 4 vs day 1, respectively; JMV2959: P=0.0443 and P=0.0364 for day 3 and day 4 vs day 1; day 3: P=0.0270 for K-(D-1-Nal)-FwLL-NH₂ vs vehicle; day 4: P=0.0092 for K-(D-1-Nal)-FwLL-NH₂ vs vehicle). **B** cumulative 4-day HF intake of mice ICV-treated with vehicle, [D-Lys3]-GHRP-6, JMV2959 or K-(D-1-Nal)-FwLL-NH₂ and then exposed to the HF diet binge-like eating protocol. *, P<0.05 vs ICV-injected vehicle; One-way ANOVA test (F(3, 43)=2.323, P=0.0884), Holm-Sidak's post-test (P=0.0346 for K-(D-1-Nal)-FwLL-NH₂ vs vehicle). **C** 22-h RC intake of mice that were ICV-treated with vehicle (n=7), [D-Lys3]-GHRP-6 (n=4), JMV2959 (n=7) or K-(D-1-Nal)-FwLL-NH₂ (n=9) and then exposed to the HF diet binge-like eating protocol. Two-way ANOVA (F(9, 92)_{interaction}=0.7218, P=0.6877; F(3, 92)_{time}=0.4021, P=0.7518; F(3, 92)_{treatment}=0.3068, P=0.8204) followed by Tukey's post-test. Data represent the mean±SEM.

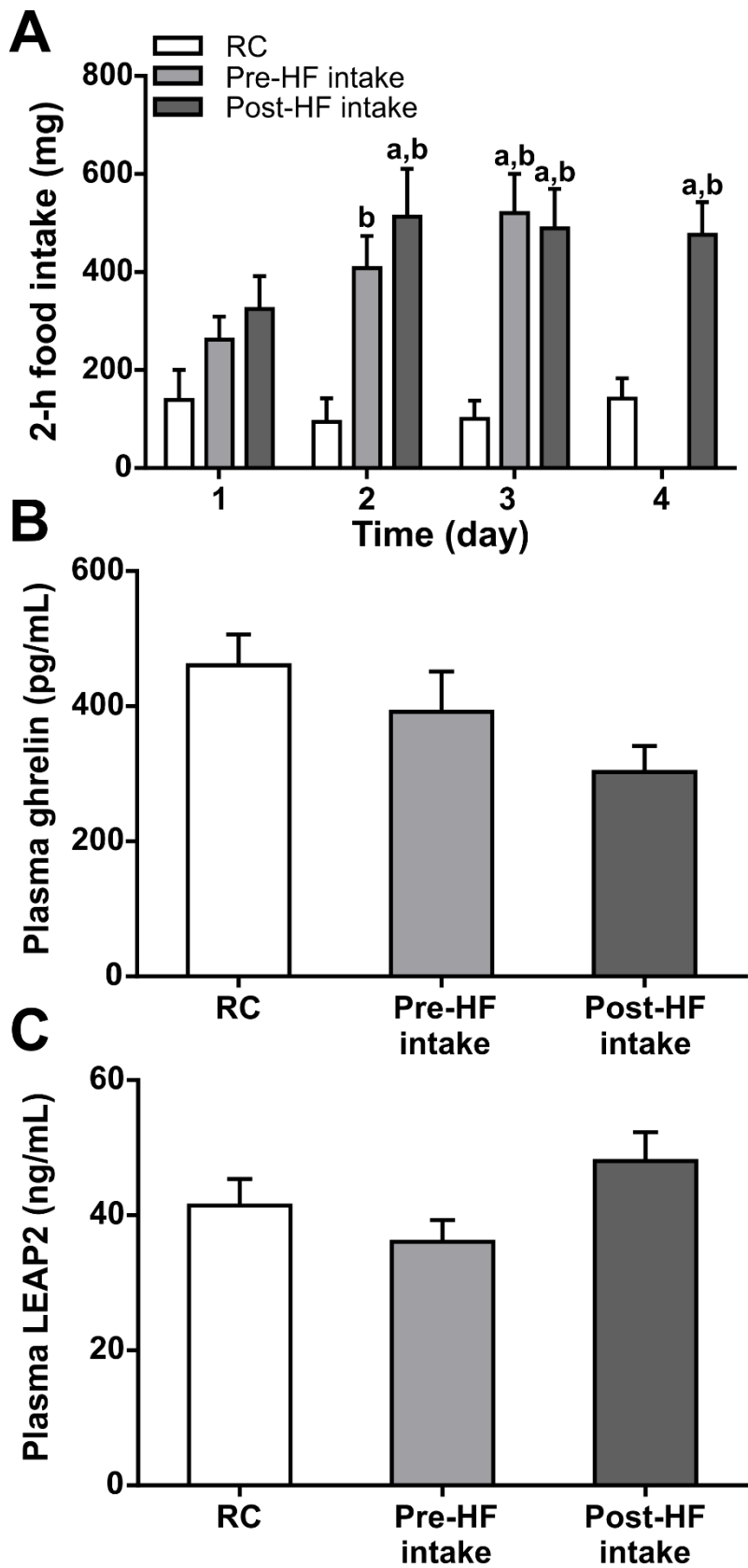


FIGURE 1

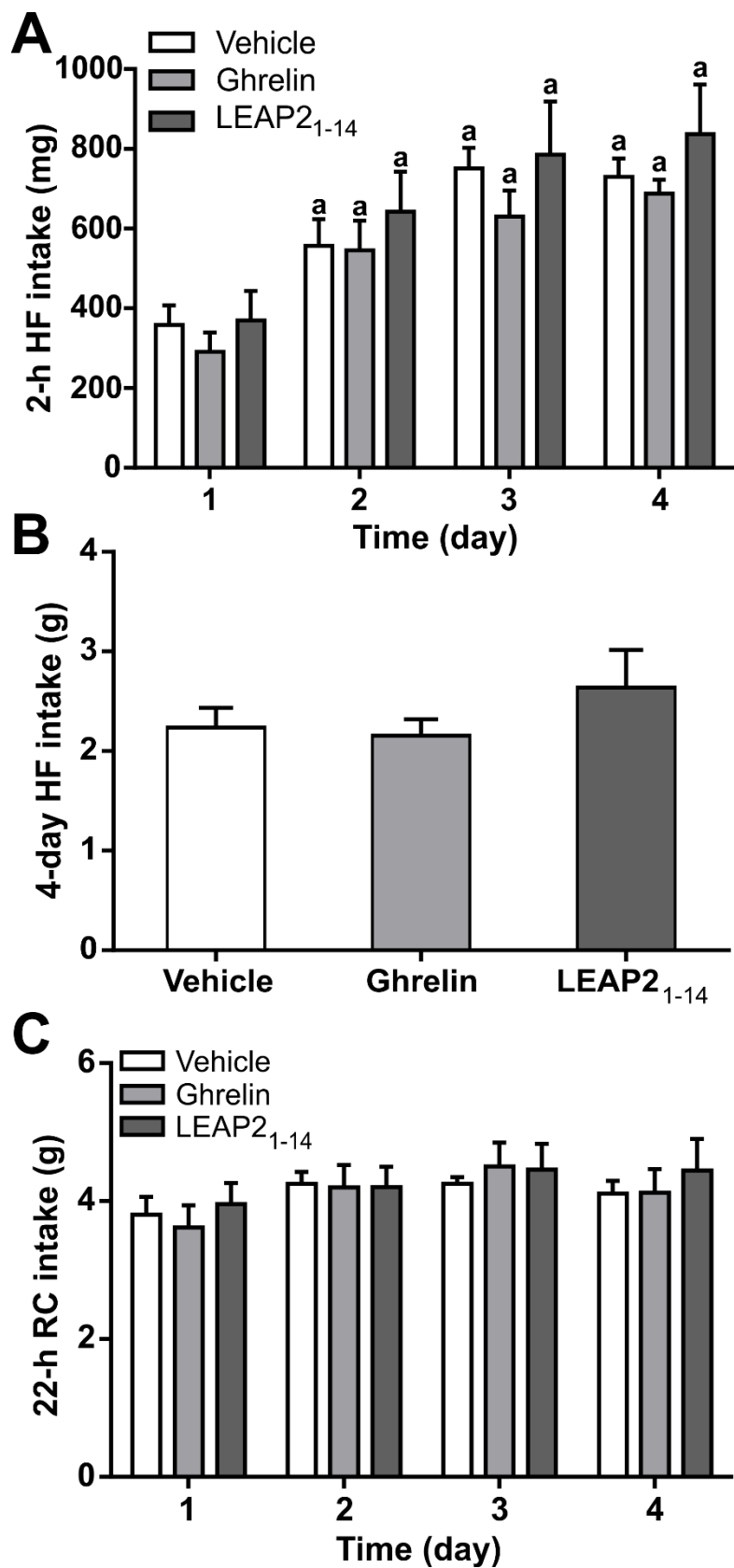


FIGURE 2

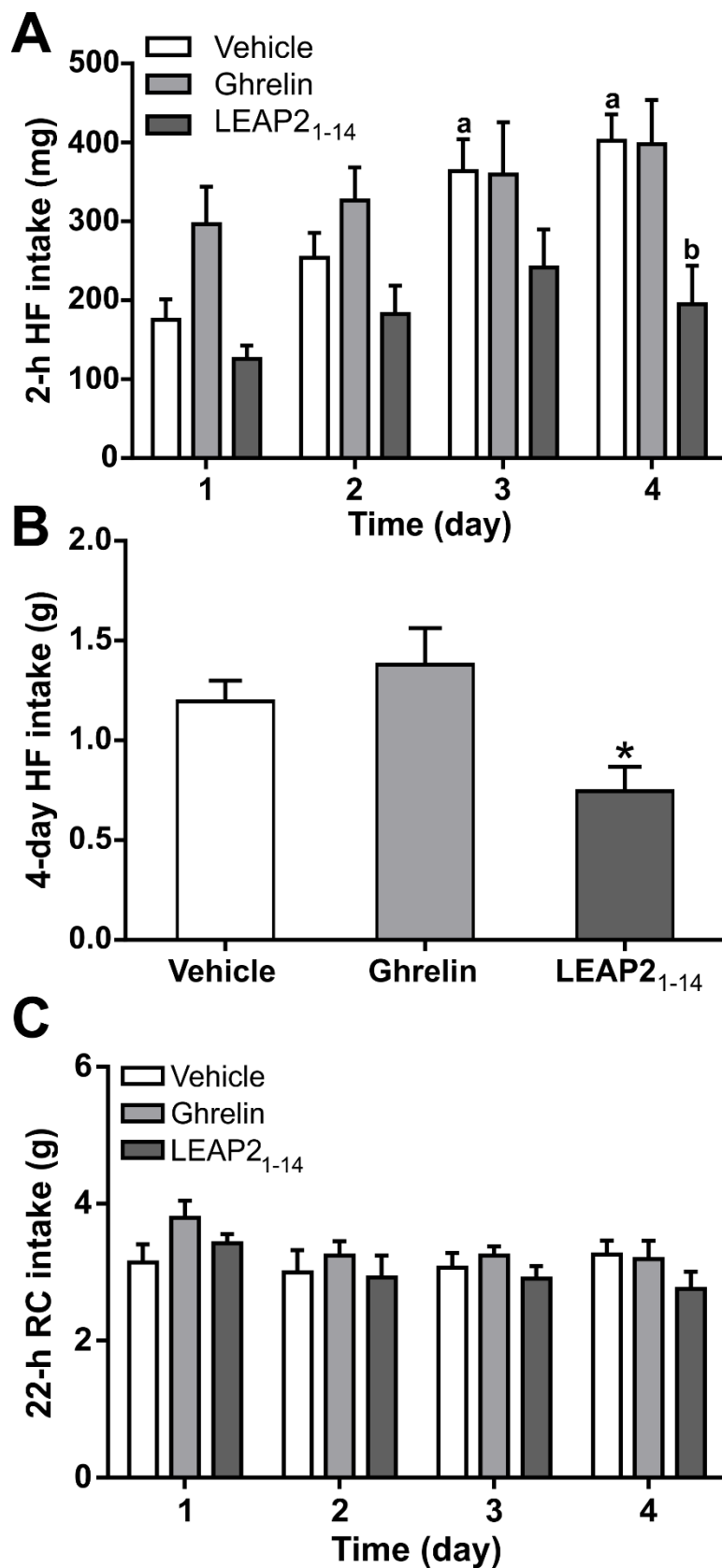


FIGURE 3

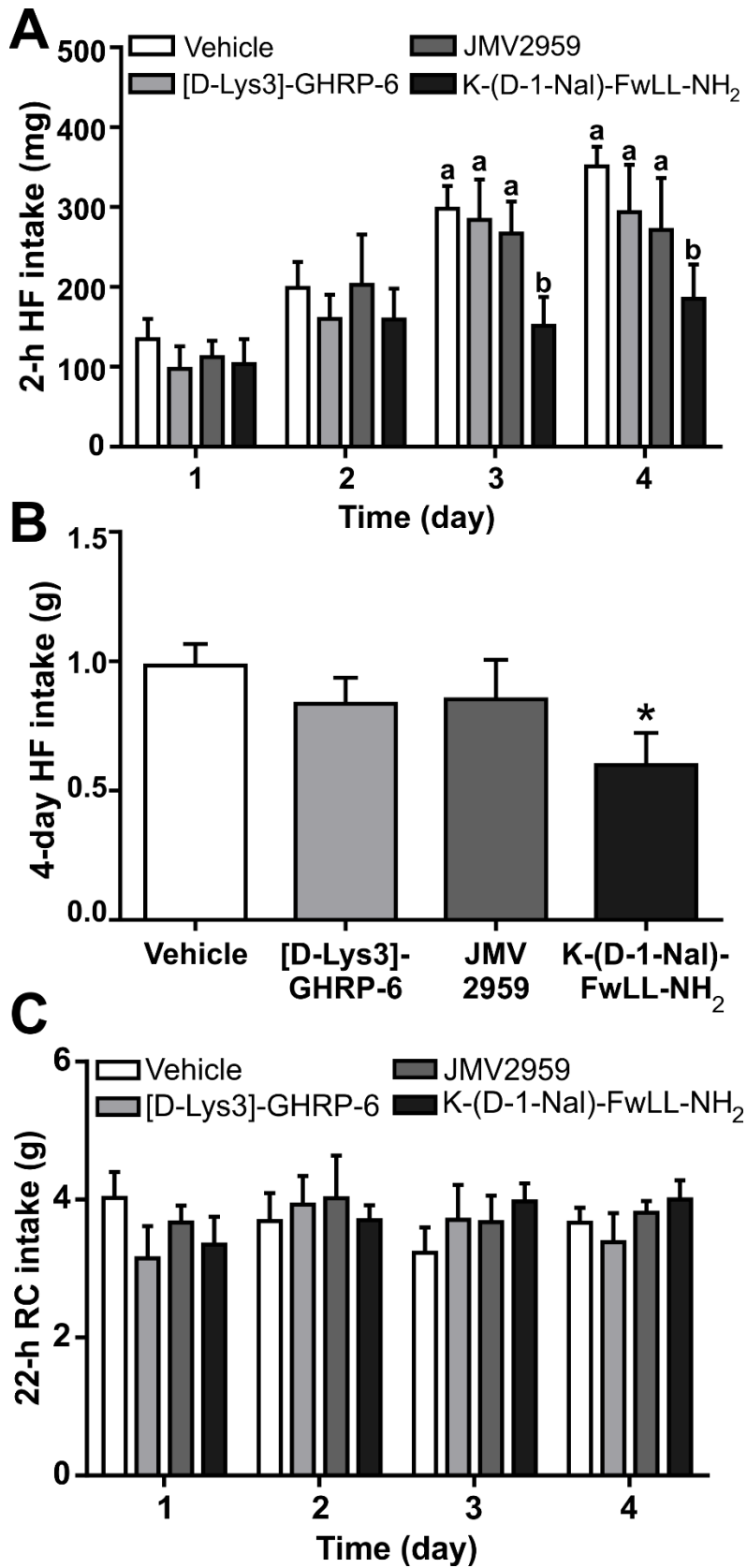


FIGURE 4