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Ghrelin signaling is not essential for sugar or fat conditioned flavor preferences in mice

Anthony Sclafani, Khalid Touzani, and Karen Ackroff

Brooklyn College of CUNY, Brooklyn, NY, USA

Abstract

The oral and post-oral actions of sugar and fat stimulate intake and condition flavor preferences in rodents through a process referred to as appetite. Ghrelin is implicated in food reward processing, and this study investigated its involvement in nutrient conditioning in mice. In Exp. 1 ghrelin receptor-null (GHSR-null) and C57BL/6 wildtype (WT) mice learned to prefer a flavor (CS+) mixed into 8% glucose over another flavor (CS–) mixed into a "sweeter" but non-nutritive 0.1% sucralose + saccharin (S+S) solution. In Exp. 2 treating WT mice with a ghrelin receptor antagonist [(D-Lys3)-GHRP-6] during flavor training did not prevent them from learning to prefer the CS+glucose over the CS–S+S flavor. GHSR-null and WT mice were trained in Exp. 3 to drink a CS+ paired with intragastric (IG) infusion of 16% glucose and a CS– paired with IG water. Both groups drank more CS+ than CS– in training and preferred the CS+ to CS– in a choice test. The same (Exp. 4) and new (Exp. 5) GHSR-null and WT mice learned to prefer a CS+ flavor paired with IG fat (Intralipid) over a CS– flavor paired with IG water. GHSR-null and WT mice also learned to prefer a CS+ flavor added to 8% fructose over a CS– added to water. Together, these results indicate that ghrelin receptor signaling is not required for flavor preferences conditioned by the oral or post-oral actions of sugar and fat. This contrasts with other findings implicating ghrelin signaling in food reward processing and food-conditioned place preferences.

1. Introduction

Obesity and its associated disease states remain major public health concerns. While obesity has multiple causes, there is widespread agreement that the availability of sugar- and fat-rich foods contributes to overeating and weight gain [8]. The sweet and fatty flavors of these foods stimulate brain reward systems and can override homeostatic satiety systems that limit intake [29,39,54]. In addition, sugar and fat can have post-oral appetite stimulating actions that increase food consumption and condition flavor preferences [40,42]. We refer to this process as appetite, to distinguish it from the post-oral nutrient satiation process that suppresses feeding [40]. Post-oral sugar and fat appetite is demonstrated by the findings that intragastric (IG) self-infusions of sugar or fat stimulate the intake of and preference for

Correspondence: Dr. Anthony Sclafani, Department of Psychology, Brooklyn College of CUNY, 2900 Bedford Avenue, Brooklyn, NY 11210 USA, Voice: 718-951-5606, Fax: 718-951-4824, ASclafani@gc.cuny.edu.

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a flavored non-nutritive solution (the CS+) paired with the IG infusions [1,59,61]. This post-oral conditioning effect is mediated in part through the activation of brain dopamine reward circuits [12,47]. Although incompletely understood, post-oral appetite is mediated at least in part by the stimulation of intestinal nutrient sensors [48,60,61]. Vagal and splanchnic afferent fibers are activated by intestinal nutrients and carry satiation signals to the brain [7]. However, lesioning these afferent fibers by abdominal vagotomy, selective afferent abdominal vagotomy, celiac-superior mesenteric ganglionectomy, or systemic capsaicin treatment does not block flavor preference conditioning by IG sugar or fat infusions [28,44,46,59]. Hormonal signaling is another way gut nutrient sensors can activate brain feeding and reward centers. However, most hormones (e.g., CCK, GLP-1, PYY) that are released by sugar and fat in the gut suppress rather than stimulate feeding and in some cases produce conditioned flavor aversions [10,38,57].

Ghrelin is the only known gut hormone that stimulates feeding. However, sugar and fat ingestion suppress ghrelin release [20,56], which seems incompatible with a role for peripheral ghrelin in nutrient-stimulated intake and flavor conditioning. Nevertheless, there is extensive evidence implicating ghrelin in the reward evaluation of foods as well as drugs of abuse [31,33,36,50]. In particular, exogenous ghrelin injections increase the intake of sweet and/or high-fat foods and fluids and operant responding for such foods [9,15,16,35,52], while ghrelin receptor antagonists or receptor deletion suppress eating and food motivated behavior [16,18,27]. With respect to food conditioned preferences, place preferences conditioned by palatable foods or ethanol are attenuated by ghrelin receptor antagonism or genetic deletion [4,11,14,23,35]. In addition, stimulation of feeding by a learned food-related cue (cue-potentiated eating) is attenuated by ghrelin receptor antagonisms while GHSR-null mice showed a non-selective increase in responding to both positive and negative conditioned stimuli [55]. Other evidence also suggests a connection between ghrelin signaling and nutrient-conditioned preferences. That is, ghrelin stimulation of operant responding for sucrose was blocked by D1 but not D2 dopamine receptor antagonism [32], which parallels the suppression of IG sugar conditioned flavor preferences by D1 but not D2 receptor antagonists [3]. In view of these findings, it is possible that ghrelin signaling at central brain sites is critical for nutrient conditioned flavor preferences and appetite stimulation [22,40].

In the present study we investigated the role of ghrelin signaling in sugar and fat conditioned flavor preferences using genetic and pharmacological approaches that deleted the ghrelin receptor (growth hormone secretagogue receptor, GHSR) or blocked the receptor. Three different flavor conditioning designs were used that involved orally consumed non-nutritive and nutritive sweeteners, IG infusions of glucose or fat, and orally consumed fructose.

2. Experiment 1. Flavor conditioning by oral glucose in GHSR-null mice

Food-restricted C57BL/6 (B6) mice trained 1 h/day to drink a flavored non-caloric 0.8% sucralose solution (the CS-) and a differently flavored 8% glucose solution (CS+/Glu) consumed more glucose than sucralose and displayed a significant preference for the glucose-paired CS+ flavor [59]. This was attributed to a conditioned preference based on the post-oral actions of glucose rather than the sugar's sweet taste. This is indicated by finding

that B6 mice actually prefer 0.8% sucralose to 8% glucose in 1-min choice tests that minimize post-oral nutritive effects. Experiment 1 compared glucose-induced stimulation of intake and flavor conditioning in GHSR-null mice and normal wildtype (WT) mice using a modified version of the 1-h oral paradigm. The mice were first given a 1-min, two-bottle choice test to determine if the GHSR-null and WT mice differed in their taste preferences for the non-caloric and caloric sweeteners. As noted above, ghrelin signaling alters the intake of sugar and saccharin solutions and Experiment 1 determined if it is essential for animals to learn preferences based on post-oral sugar actions (see [31]).

2.1 Materials and methods

2.1.1. Subject—Adult GHSR-null mice (5 male, 4 female) and WT mice (5 male, 5 female) were singly housed in tub cages in a room with a 12 h light-dark cycle (lights on 9 AM). GHSR-null mice and WT littermates expressed on a C57BL/6 background (Charles River, Wilmington, MA) were generated as previously described [58]. The genotype status of the GHSR-null and WT mice was confirmed by real-time PCR of ear biopsies (Transnetyx, Cordova, TN). The mice were fed lab chow (5001; PMI Nutrition International, Brentwood, MO) or, when food restricted, fixed-size chow pellets (0.5 or 1 g, F0171, F0173; Bio-Serv, Frenchtown, NJ) that allowed for precise adjustment of daily food rations. All tests were conducted 3 to 5 hours after lights on. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Brooklyn College and were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.1.2. Apparatus—Drinking tests were conducted in plastic test cages with one or two stainless-steel sipper spouts as previously described [59]. Motorized bottle holders positioned the sipper spouts in front of the cages at the start of a session and retracted them at the end of the session. Licking behavior was monitored with electronic lickometers (model ENV-250B; Med Associates) interfaced to a computer.

2.1.3. Test solutions—The non-nutritive sweet solution contained 0.1% sucralose (Tate & Lyle; Dayton, OH) and 0.1% sodium saccharin (Sigma Aldrich, St. Louis, MO) and the nutritive solution contained 8% glucose (food grade, Honeyville Food Products, Rancho Cucamonga, CA). The solutions were prepared daily using deionized water on a w/w basis because intakes were measured by weight. The 0.1% sucralose+saccharin mixture (S+S) replaced the 0.8% sucralose solution used in our prior work [59] because it is preferred to 8% glucose but, unlike 0.8% sucralose, does not have post-oral inhibitory effects on ingestion [45]. During flavor conditioning sessions, the S+S and glucose solutions were flavored with 0.01% ethyl acetate or 0.01% propyl acetate (Sigma). Half of the mice had ethyl acetate added to the S+S solution (the CS−/S+S) and propyl acetate added to the glucose solution (the CS+/GLU); the flavors were reversed for the remaining mice in each group. During flavor choice tests, both flavors were added to S+S solutions and are referred to as the CS+/S+S and CS−/S+S or simply CS+ and CS−.

2.1.4. Procedure—The mice were water deprived overnight and given 5-min access to water from two sipper spouts in the test cages; water was then provided in the home cages

for 1 h. The following day the mice were given 1-min choice tests with the S+S vs. glucose in the test cages. They were then given water ad libitum but restricted access to food to maintain them at 90% of their ad libitum weight. While food restricted, the mice were given 1-min S+S vs. glucose tests on two successive days. In these tests, one solution was presented for 5 sec and then the other for 5 sec to allow the mice to sample the contents of each sipper tube before being presented with both tubes for the 1-min choice test. The timing of each session for each mouse began with its 10th lick and the sipper tubes were automatically retracted at the end of the session. The mice were then returned to their home cages, and 1 h later given a second 1-min choice test in the test cages with the left-right position of the solutions reversed. Because solution intakes were minimal during the 1-min tests, solution preferences were expressed as licks recorded for each solution.

The mice were then given flavor preference training, with a series of one-bottle training sessions (1 h/day) with the CS-/S+S (days 1–3) and CS+/GLU (days 4–6) followed by four additional 1-h sessions with the CS-/S+S (days 7 and 9) and CS+/GLU (days 8 and 10). The mice were then given two-bottle tests (1 h/day) on days 11 and 12 with the CS+ vs. CS– presented in the S+S mixture. The left-right positions of the CS+ and CS– solutions were counterbalanced throughout training and testing and 1-h solution intakes were recorded by weight. This training and test series was based on our prior studies [60,61].

2.1.5. Data analysis—S+S and glucose licks during the 1-min choice conducted with the food-restricted mice were averaged and analyzed using a mixed model ANOVA with a group factor (GHSR-null vs. WT) and repeated-measure factor (S+S vs. glucose). Intakes of CS+/GLU and CS-/S+S during the five 1-h training sessions were averaged, as were the 1-h intakes of CS+ and CS– during the two bottle tests. These data were evaluated in separate ANOVAs. Preliminary analyses revealed no main effects of sex or genotype $\times$ sex interactions and therefore the data for male and female mice were combined.

2.2. Results and Discussion

In the 1-min tests the GHSR-null mice licked significantly more for S+S than glucose (171.3 vs. 48.3 licks/min) as did the WT mice (171.2 vs. 74.9 licks/min; $F(1,17) = 21.3$, $P < 0.01$). The GHSR-null and WT groups did not differ in their 1-min licks or S+S preferences (79% vs. 69%, respectively).

In the one-bottle training sessions (Fig. 1) the mice consumed more CS+/GLU than CS-/S+S ($F(1,17) = 16.4$, $P < 0.001$). The GHSR-null mice consumed somewhat less of the training solutions than the WT mice but this difference was not significant. In the two-bottle choice tests, both groups consumed more CS+ than CS– ($F(1,17) = 61.6$, $P < 0.001$) and their intakes were quite similar. The percent CS+ intakes of the GHSR-null and WT groups were also similar at 76% and 77%, respectively.

The GHSR-null and WT groups strongly preferred the S+S solution to the 8% glucose solution, which indicates that S+S had the more preferred, presumably sweeter, taste. Yet both groups consumed more CS+/GLU than CS-/S+S during 1-h training and more CS+ than CS– in the two-bottle test. Together, these findings indicate that the preference for the glucose-paired CS+ flavor was due to the post-oral rather than the taste effects of the

glucose solution, which is consistent with prior studies [41,59,61]. The new finding here is that GHSR-null mice were like WT mice in preferring the S+S solution in 1-min tests but developing a preference for the CS+ flavor paired with glucose in 1-h tests.

3. Experiment 2. Flavor conditioning by oral glucose in mice treated with an GHSR antagonist

The first experiment indicated that ghrelin receptor signaling is not critical for mice to acquire a preference for a flavor paired with glucose over a flavor paired with a non-nutritive S+S solution. Because of the possibility of functional recovery following genetic deletion of the receptor, Experiment 2 investigated the role of ghrelin in glucose conditioning by using the ghrelin receptor antagonist (D-Lys3)-GHRP-6. This antagonist is reported to decrease food intake, high-fat diet preference, alcohol intake, body weight, and lever pressing for food in rodents [2,17,18,19,21,24,25]. Here we determined if treating WT mice with (D-Lys3)-GHRP-6 during training sessions with CS+/GLU and CS-/S+S solutions altered their acquisition of a CS+ flavor preference.

3.1. Method

3.1.1. Animals—Adult C57BL/6J (B6) mice (20 male, Jackson Laboratory, Bar Harbor, MA) were housed as in Experiment 1.

3.1.2. Test solutions and Drugs—The CS+/GLU and CS-/S+S solutions were prepared as in the first experiment. The GHSR antagonist (D-Lys3)-GHRP-6 (Tocris Bioscience, Ellisville, MO) was dissolved in sterile 0.9% saline and administered intraperitoneally (IP) at a dose of 66.6 nmol/10 g of body weight [17,18,19]. The mice were injected with 0.1 ml/10 g body weight of the drug or saline (0.9%) 30 min prior to the daily training sessions.

3.1.3. Procedure—The mice were water restricted and trained (1 h/day) to drink unflavored 0.1% S+S and water in the test cages for two days. They were then switched to the food restriction schedule as in Experiment 1 and given three additional 1 h/day sessions with S+S vs. water following IP saline injections. Six flavor training sessions (1 h/day) were then conducted using a simplified version of the Experiment 1 flavor conditioning protocol: the CS-/S+S was presented on days 1, 3 and 5 and the CS+/GLU on days 2, 4 and 6. A two-bottle choice test with the CS+ and CS- presented in the S+S mixture was conducted on days 7 and 8. The Drug and Saline groups (n=10 each), equated for unflavored S+S intakes and body weights, were injected with (D-Lys3)-GHRP-6 and saline, respectively, prior to each training session; they were not injected prior to the two-bottle test sessions.

3.2. Results and Discussion

During one-bottle training (Fig. 2) both groups consumed more CS+/GLU than CS-/S+S ($F(1,18) = 52.6$, $P < 0.001$). Overall, the Drug group consumed less CS+/GLU and CS-/S+S than did the Saline group ($F(1,18) = 5.2$, $P < 0.05$). In the two-bottle test, both groups consumed significantly more CS+ than CS- ($F(1,18) = 103.6$, $P < 0.001$) and their overall intakes did not differ. The percent CS+ intakes of the Drug and Saline groups were also comparable at 74% and 78%, respectively.

Pharmacological antagonism of the GHSR receptor did not prevent the mice from drinking more CS+/GLU than CS-/S+S during training or acquiring a significant preference for the glucose-paired CS+ flavor. The results were quite similar to those obtained with the GHSR-null mice in Experiment 1 except that (D-Lys3)-GHRP-6 significantly reduced the CS+/Glu and CS-/S+S training intakes by 25% and 20%, respectively. This drug effect may have resulted from ghrelin receptor antagonism although (D-Lys3)-GHRP-6 also acts on other receptors (e.g., 5-HT(2B) [13]).

Only one dose of (D-Lys3)-GHRP-6 was used and it is conceivable that a higher drug dose might inhibit glucose conditioning. We evaluated the effect of a higher drug dose on glucose intake in the Saline animals. Following the two-bottle test the mice were given 1 h/day sessions with unflavored 8% glucose following injections of saline or (D-Lys3)-GHRP-6 at a 133.3 nmol/10 g body weight dose [17,19]. Relative to the saline treatment, the drug suppressed glucose intake by 40% and most of the animals appeared distressed just prior to the glucose sessions, i.e., lying on their bellies in their home cage. In addition, the 133.3 nmol dose increased the latency to drink glucose compared to the saline treatment (median latency = 132.5 vs. 5.0 sec, Wilcoxon T = 2.80, $p < 0.01$). In contrast, the Drug animals treated with the 66.6 nmol dose during training showed no obvious signs of distress and their latency to consume CS+/GLU was not much different from that of the Saline mice (median latency = 10.2 vs. 7.1 sec). Given the “side effects” of the 133.3 nmol dose (see [17]), we did not evaluate its effect on glucose conditioning in mice.

4. Experiment 3. Flavor conditioning by intragastric glucose in GHSR-null mice

The glucose-conditioned flavor preferences observed in Experiments 1 and 2 suggest that GHSR signaling is not essential for post-oral sugar conditioning. The present experiment sought direct evidence for this interpretation by using an intragastric (IG) conditioning paradigm. In prior studies, B6 mice trained to drink a flavored saccharin solution (CS+) paired with IG self-infusions of 16% glucose increased their CS+ intake and acquired a significant preference for the CS+ flavor over an alternate flavor (CS-) paired with IG water infusions [59,61]. In Experiment 3 we compared the flavor conditioning response of GHSR-null and WT mice to IG self-infusions of 16% glucose. Note that the IG glucose was diluted by the orally consumed CS+ saccharin solution to a net 8% solution, the sugar concentration used in the first two experiments.

4.1. Method

4.1.1. Animals—Adult GHSR-null mice (6 male, 6 female) and WT mice (7 male, 3 female) were fitted with chronic gastric catheters while anesthetized with isoflurane (2%) inhalation as previously described [45]. Analgesia was provided by a subcutaneous injection of Buprenorphine SRTM (1 mg/kg, ZooPharm, Windsor, CO) at the time of surgery.

4.1.2. Apparatus—IG infusion tests were conducted in plastic test cages [45]. The sipper spouts were interfaced via electronic lickometers to a computer that operated a syringe pump which infused liquid into the gastric catheters as the animals licked; the oral-to-infusion

intake ratio was maintained at ~1:1. The pump rate was nominally 0.5 ml/min, but the overall infusion rate and volume was controlled by the animal's licking behavior. Daily oral fluid intakes were measured to the nearest 0.1 g, and IG infusions were recorded to the nearest 0.5 ml.

4.1.3. Test solutions—The mice were initially trained to drink in the infusion cages using unflavored 0.025%–0.1% saccharin solutions. They were then trained with flavored solutions (CS) containing 0.025% or 0.05% saccharin (see below) flavored with 0.02% ethyl acetate or propyl acetate (Sigma). Dilute saccharin solutions were used to keep initial CS intakes relatively low to permit stimulation of intake by IG sugar self-infusions [61]. During training, the CS– solution was paired with IG infusion of water while the CS+ solution was paired with IG infusion of 16% glucose. For half the animals the CS– solution contained ethyl acetate and the CS+ solution contained propyl acetate; the flavors were reversed for the remaining animals.

4.1.4. Procedure—Two weeks after surgery the mice were water restricted (1 h/day) and trained in the test cages to drink unflavored 0.025% saccharin paired with matched IG infusions of water for two 1 h/day sessions. They were then switched to a food restriction schedule that maintained them at ~90% body weight as in Experiment 1. Initially, 0.025% saccharin intakes were low in the food restricted GHSR-null and WT mice (0.8–1.0 g/1 h) and the sweetener concentration was increased to 0.05% and then 0.1% to stimulate drinking. This increased saccharin intakes but the GHSR-null mice consumed less fluid (oral + IG) than the WT mice at each concentration (1.4 vs. 2.6, 2.6 vs. 3.2 g/h, $P < 0.05$). The saccharin concentrations were then adjusted to 0.05% and 0.025%, respectively, for the GHSR-null and WT mice which equated their intakes (1.8 vs. 1.9 g/h). The GHSR-null and WT groups were therefore tested with flavored CS solutions containing 0.05% and 0.025% saccharin, respectively, for the remainder of the experiment.

The mice were given a series of one-bottle training sessions (1 h/day) with the CS– paired with IG water (days 1–3) and CS+ paired with IG glucose (days 4–6). This was followed by four additional 1-h sessions with the CS– (days 7 and 9) and CS+ (sessions 8 and 10) paired with their respective IG infusions. A two-bottle choice test was conducted on days 11 and 12 (1 h/day) with the CS+ vs. CS– solutions without IG infusions. The left-right positions of the CS+ and CS– solutions were counterbalanced throughout training and testing. This training series was based on our prior studies [60,61] and parallels that of Exp. 1.

4.1.5. Data analysis—Intakes (oral + IG) during the first three CS– training sessions (referred to as CS– Test 0) were averaged and compared to the intakes during the subsequent three CS+ sessions (referred to as CS+ Tests 1 to 3). These data were evaluated with an ANOVA with a Group factor (GHSR-null vs. WT) and repeated measure factor (Tests 0–4). In addition, CS– and CS+ intakes were averaged over the 5 sessions which each CS and evaluated with a separate ANOVA (Group $\times$ CS). Mean CS+ and CS– intakes during the two-bottle choice test were also evaluated with an ANOVA.

4.2. Results and Discussion

During the first three CS[−] training sessions (Test 0) the 1-h intakes of the GHSR-null and WT mice were similar (Fig. 3). When switched to the CS⁺ flavor paired with IG glucose infusions, both groups increased their 1-h intakes and consumed more fluid in each of the first three CS⁺ tests than in the CS[−] Test 0 ($F(3,60) = 18.7$, $P < 0.001$). They also consumed more CS⁺ than CS[−] in the alternating training sessions 7–10. Over all five training sessions with each CS, the GHSR-null and WT mice drank 24% and 26% more, respectively, of the CS⁺/IG glucose than the CS[−]/IG water (2.3 vs. 1.9, 2.5 vs. 2.1 g/session, $F(1,20) = 45.3$, $P < 0.001$). In the two-bottle choice test, both groups consumed significantly more CS⁺ than CS[−] ($F(1,20) = 34.5$, $P < 0.001$). The GHSR-null and WT mice did not significantly differ in their percent CS⁺ preferences although the preference was numerically higher in the GHSR-null group (76% vs. 67%).

In agreement with prior results [59,60,61], IG glucose infusions in mice increased the intake of the CS⁺ solution from the very first training session and conditioned a significant preference for the CS⁺ over the CS[−]. The new finding here is that GHSR-null mice displayed similar intake and conditioning responses to the IG glucose infusions as did the WT mice. This extends the results of the first two experiments and indicates that normal ghrelin receptor signaling is not required for the post-oral appetite effects of glucose.

5. Experiment 4. Flavor conditioning by intragastric fat in GHSR-null mice

In Experiments 1 and 3 GHSR-null and WT mice did not differ in their flavor conditioning response to oral or IG glucose. These findings contrast with previous reports of impaired food-conditioned place preferences in GHSR-null mice [11,14,35]. In these studies place conditioning was produced using high-fat foods, and it may be that ghrelin signaling is more critical for fat than sugar conditioning. We tested this possibility in Experiment 4 by comparing the flavor conditioning response of GHSR-null and WT mice to IG fat infusions.

5.1. Methods

5.1.1. Subjects—The GHSR-null and WT mice from Experiment 3 were used and maintained on the same food restriction schedule.

5.1.2. Test solutions—The CSs consisted of 0.05% saccharin (GHSR-null mice) or 0.025% saccharin (WT mice) solutions flavored with 0.05% grape or cherry Kool Aid mix (Kraft Foods, White Plains, NY). The CS⁺ was paired with IG self-infusions of 6.4% Intralipid (isocaloric to 16% glucose) and the CS[−] was paired with IG water infusions. The Intralipid was prepared by diluting 20% Intralipid (Baxter, Deerfield, IL), a stable soybean oil emulsion, with water. For half of the animals the cherry was the CS⁺ paired with IG Intralipid, and grape was the CS[−] flavor paired with IG water; the flavors were reversed for the remaining animals.

5.1.3. Procedure—At the end of Experiment 3 the mice were given a single 1 h/day session with unflavored saccharin paired with IG water. They were then given six 1-h training sessions with the CS[−] paired with IG water (days 1, 3, and 5) and the CS⁺ paired

with IG Intralipid (days 2, 4, and 6). A two-bottle test (1 h/day) with the CS+ vs. CS– was then conducted on days 7 and 8 without IG infusions.

5.1.4. Data analysis—Intakes during the three CS– and CS+ training sessions were averaged and evaluated with an ANOVA with a Group factor (GHSR-null vs. WT) and repeated measure factor (CS– vs. CS+). Mean intakes during the CS+ vs. CS– two-bottle test were evaluated in a separate ANOVA.

5.2. Results and Discussion

Overall, intake of the CS+ exceeded that of the CS– during one-bottle training ($F(1,20) = 11.1$, $P < 0.001$) and there were no group differences in training intakes (Fig. 4). In the two-bottle choice test, the GHSR-null and WT groups both consumed significantly more CS+ than CS– ($F(1,20) = 44.4$, $P < 0.001$). The GHSR-null and WT mice were also similar in their percent CS+ preferences at 77% and 73%, respectively.

The flavor conditioning results were quite similar to those obtained with IG glucose infusions in Experiment 3. The findings therefore do not support the idea that ghrelin receptor signaling is more critical for fat than sugar flavor conditioning.

6. Experiment 5. Flavor conditioning by intragastric fat in GHSR-null mice: effects of deprivation level

In one conditioned place preference study, food-restricted GHSR-null mice showed impaired place conditioning to a high-fat diet when maintained at 95% of their ad libitum body weight but not when maintained at an 85% body weight level [35]. The GHSR-null and WT rats in Experiment 3 were given restricted food rations designed to keep them at a 90% body weight level but body weight records indicated that their mean weights during the experiment were 87% of ad libitum levels. It is possible, therefore, that GHSR-null mice maintained at a 95% weight level might be deficient in learning to prefer a fat-paired flavor just as they are deficient in learning a fat-paired place preference. We investigated this possibility in the fifth experiment.

6.1. Methods

6.1.1. Subjects—Adult GHSR-null mice (6 male, 6 female) and WT mice (5 male, 5 female) were fitted with chronic gastric catheters and housed as in Experiments 3. The mice were given larger daily food rations that maintained their body weights throughout training and testing at 95% of ad libitum levels (mean weight levels: GHSR-null mice = 95.2%, WT mice = 94.6%).

6.1.2. Procedure—Two weeks after surgery the mice were trained 1 h/day in the test cages to drink unflavored 0.1% saccharin paired with IG water infusions for two days while water restricted and then for three days while food restricted. The higher saccharin concentration was used in this experiment to stimulate adequate CS intakes in mice maintained at the 95% body weight level. The GHSR-null and WT mice consumed

comparable amounts of the unflavored 0.1% saccharin (2.6 vs. 2.5 g/h). Therefore both groups were trained with cherry and grape Kool-Aid flavored 0.1% saccharin solutions.

As in Experiment 4, the mice were given six 1-h training sessions with the CS– paired with IG water (days 1, 3, and 5) and CS+ paired with IG 6.4% Intralipid (days 2, 4, and 6). A two-bottle test (1 h/day) with the CS+ vs. CS– was then conducted on days 7 and 8 without IG infusions.

The data were analyzed as in Experiment 4.

6.2. Results and Discussion

During one-bottle training the mice consumed similar amounts of CS+ and CS– paired with IG Intralipid and water infusions, respectively, and the intakes of the GHSR-null and WT mice were comparable (Fig. 5). In the two-bottle choice test (Fig. 5), the GHSR-null and WT groups consumed substantially more CS+ than CS– ($F(1,20) = 128.3$, $P < 0.001$). The two-bottle CS intakes of the GHSR-null and WT mice were similar as were their percent CS+ preferences at 84% and 86%, respectively.

Training and testing the mice at a 95% body weight level did not reveal a deficit in fat-conditioned flavor preferences in the GHSR-null mice. Instead, the GHSR-null and WT mice were quite similar in their absolute and percent preferences for the fat-paired CS+ flavor. The present findings differed from Experiment 4 in that the mice did not consume more CS+ than CS– during training. Overall, the mice consumed more CS+ and CS– than the animals in the fourth experiment, perhaps because of the higher saccharin concentration in the CS solutions.

7. Experiment 6. Flavor conditioning by oral fructose in GHSR-null mice

While glucose and Intralipid have potent post-oral appetite effects in B6 mice, this is not case with all nutrients. In particular, IG infusions of fructose (8–16%) failed to condition flavor preferences in B6 mice in 1 or 24 h/day training sessions [41,60]. Yet, B6 mice learn to prefer a flavor (CS+) added to an 8% fructose solution over a different flavor (CS–) added to water during 24-h one-bottle training sessions [43]. This represents a form of flavor-flavor learning in which the sweet taste of fructose reinforces the CS+ preference. In Experiment 6 we determined if GHSR-null mice are impaired in this type of flavor-sugar conditioning.

7.1. Methods

7.1.2. Subjects—Adult GHSR-null mice (6 male, 6 female) and WT mice (6 male, 6 female) were singly housed in tub cages in a room with a 12-h light-dark cycle. Lab chow was available ad libitum in a food hopper on the cage top. Fluid was available through sipper spouts attached to 50 ml plastic tubes placed on the top of the cage adjacent to the food hopper. The sipper spouts were inserted through holes positioned 3.7 cm apart in a stainless-steel plate, and the drinking tubes were fixed in place with clips. Fluid intakes were measured to the nearest 0.1 g. Fluid intakes were corrected for spillage which was estimated by recording the change in weight of two drinking tubes placed on an empty cage.

7.1.3. Test solutions—The training solutions consisted of 8% fructose (food grade, Honeyville Foods) and water flavored with 0.05% orange or lemon-lime Kool Aid mix. For half of the animals orange was added to the CS+/fructose solution and lemon-lime was added to the CS−/water solution; the flavors were reversed for the remaining animals. For the two-bottle choice test the CS+ and CS− flavors were both presented in water.

7.1.4. Procedure—The mice were adapted to their home cages with two water bottles for one week. They were then given one-bottle training sessions (24 h/day) with the CS−/water (days 1, 3) and CS+/fructose (days 2, 4). Water only was available for one day and the mice were then given a two-bottle preference test with the CS+ vs. CS− flavors presented in water for 2 days. The left-right positions of the two CS+ and CS− solutions were counterbalanced throughout training and testing. The data were analyzed as in prior experiments.

7.2. Results

During training both groups consumed significantly more CS+/fructose than CS−/H₂O [$F(1,22) = 201.8$, $P < 0.001$] (Fig. 6). The GHSR-null mice, however, consumed less CS+/fructose than did the WT mice; CS−/H₂O intakes were similar in the two groups (Group $\times$ Fluid interaction, $F(1,22) = 7.6$, $P < 0.05$). In the two-bottle test (Fig. 6), both the GHSR-null and WT groups consumed more CS+ than CS− [$F(1,22) = 83.7$, $P < 0.001$] and they did not differ in CS intakes or percent CS+ intakes (74 vs. 76%). Overall, female mice consumed more fluid than male mice during training (9.5 vs. 7.9 g/day) and testing (7.3 vs. 5.5 g/day) ($F(1,20) = 8.6$, $P < 0.01$) but female and male mice did not significantly differ in their CS+ preference.

8. General Discussion

The present results confirm our prior findings that the post-oral actions of glucose and fat stimulate intake and condition flavor preferences in B6 mice [1,59,60]. The new finding here is that these post-oral appetite effects do not require an intact ghrelin signaling system. The findings also indicate that flavor preference conditioning by the sweet taste of fructose is not impaired in GHSR-null mice. Yet, impaired ghrelin receptor signaling was associated with reduced intakes of sweet solutions in some experiments, which is consistent with prior reports [16,18,27].

The first two experiments investigated the ability of mice to learn a preference for a flavor added to a less “tasty” glucose solution over a flavor added to a more “tasty” non-caloric sucralose+saccharin solution. An initial 1-min choice test in Experiment 1 established that GHSR-null mice, like WT mice preferred the S+S solution to the glucose solution by ~75%. Yet, in the 1-h tests, which allowed post-oral actions to influence intake, the GHSR-null and WT mice consumed more glucose than S+S. Then in the subsequent 1-h flavor choice test the GHSR-null and WT mice preferred the glucose-paired flavor over the S+S paired flavor by ~75%. Comparable results were obtained in the Experiment 2 with B6 mice treated with the GHSR antagonist (D-Lys3)-GHRP-6 or saline during 1-h training trials: the drug and control mice displayed ~75% preferences for the glucose-paired flavor over the S+S paired flavor. The two experiments differed only in that the mice treated with the GHSR antagonist, but not the GHSR-null mice, consumed significantly less of the glucose and S+S

solutions during training than did control mice. These findings are consistent with prior reports of reduced saccharin intake in mice treated with a GHSR antagonist but not in GHSR-null mice [4,15,27]. The finding that disrupting ghrelin receptor signaling using genetic deletion of the receptor or pharmacological blockade provides converging evidence that the glucose-conditioned flavor preferences are not dependent upon intact ghrelin receptor activity.

The glucose-stimulated flavor preference observed in the first two experiments and elsewhere [49] is attributed to the post-oral rather than the taste properties of the sugar, given that naïve mice prefer S+S to the sugar in brief access tests. Direct evidence for post-oral glucose conditioning is provided by the IG infusion procedure used in Experiment 3. With this paradigm the GHSR-null mice, like WT mice, consumed significantly more of the CS+Glu flavor paired with IG glucose infusions than of the CS– flavor paired with IG water during 1-bottle training. Both groups then significantly preferred the CS+ to the CS– in two-bottle choice tests conducted without infusions. The groups differed only in that the GHSR-null mice initially consumed less of the unflavored saccharin solutions compared to the WT mice. To equate the CS intakes and glucose infusions of the two groups, the GHSR-null and WT mice were trained with CS solutions containing 0.05% and 0.025% saccharin, respectively. The GHSR-null mice in Experiment 1 tended to consume less than WT mice of 0.1% S+S (2.2 vs. 2.5 g, $P < 0.072$) solution, so the differential intakes of the GHSR-null and WT mice of the dilute saccharin solutions are not surprising. As noted above, prior studies reported comparable saccharin intakes in GHSR-null and WT mice, but these animals were tested non-deprived in longer sessions (4–24 h/day).

The first three experiments provided no evidence that post-oral glucose conditioning is impaired in mice with reduced ghrelin receptor signaling. The next two experiments investigated post-oral flavor conditioning by fat. This was of interest because published studies reported impaired place preference conditioning in GHSR-null mice trained with a high-fat food [14,35]. Experiment 4 revealed similar fat-conditioned flavor preferences in GHSR-null and WT mice with prior IG glucose conditioning experience. Experiment 5 obtained equally robust (~85%) fat-conditioned preferences in naïve GHSR-null and WT mice. An important feature of the fifth experiment is that the mice were trained at a body weight level (95% of ad lib weight) that was found to impair a high-fat conditioned place preference in GHSR-null mice [35].

The last experiment determined if impaired ghrelin signaling attenuated a flavor preference conditioned by the sweet taste of fructose. The GHSR-null mice consumed less flavored fructose solution during training than did the WT mice, yet they displayed a CS+ preference that was nearly identical to that of the WT mice (74% vs. 76%). The reduced fructose intake of the GHSR-null mice is consistent with other findings indicating that ghrelin modulates the reward value of sweet fluids and foods [15,31,36,51]. It is somewhat surprising that fructose preference conditioning was not impaired in the GHSR-null mice. There are other treatments, however, that differentially affect fructose intake and flavor conditioning. That is, fructose-conditioned flavor preferences in rats were not attenuated by treatment with the opiate receptor antagonist naltrexone even though the drug reduced fructose intakes during training [6]. Similar naltrexone effects were observed in BALB/c mice, although naltrexone

did somewhat attenuate fructose conditioned preferences in SWR mice; the drug's effect on fructose conditioning in B6 mice has not been investigated [26]. In contrast to naltrexone, the dopamine D1 receptor antagonist SCH23396 was found to block fructose preference conditioning in mice (BALB/c, SWR) and rats [5,26]. The D1 antagonist also blocked flavor conditioning by IG sucrose or glucose infusions in rats [3,53]. Yet, despite the important role of brain dopamine circuits to flavor conditioning and the modulation of dopamine function by ghrelin [36], flavor preference conditioning was not disrupted by GHSR deletion or its pharmacological antagonism in the present study.

As previously mentioned, the flavor conditioning findings reported here contrast with prior reports of impaired place preference conditioning in animals with compromised ghrelin signaling. The different conditioning results may reflect fundamental differences in flavor vs. place learning processes. Note, for example, that amphetamine simultaneously conditioned a place preference but a flavor avoidance in rats [37]; see also [30,34]. It is possible that ghrelin receptor signaling is more closely involved in appetitive actions, which may be more closely associated with place conditioning, than in consummatory actions, which may be more closely associated with flavor conditioning. On the other hand, there are procedural differences in the flavor conditioning experiments of the present study and prior place preference experiments which may contribute to the different outcomes. In particular, place preferences were conditioned with high-fat or high-fat/sugar diets available during 20–30 min training sessions, whereas in the present study flavor preferences were conditioned by sugar solutions or fat emulsions presented orally or by IG infusion during 1 or 24 h training sessions. It would be of interest in a future experiment to simultaneously train mice to associate a flavor and a place with the intake of a sugar solution and determine if impaired ghrelin signaling selectively impaired the conditioning of the place and flavor preferences.

In summary, despite extensive evidence implicating ghrelin in homeostatic and reward aspects of feeding, the present experiments provided no evidence that ghrelin has an essential role in flavor preference learning. This parallels findings obtained with manipulations of brain opioid systems, which influence food reward but have minimal effects of flavor conditioning, but differs from those involving brain dopamine systems which are implicated in food reward and flavor conditioning.

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Highlights

Flavor preferences are conditioned by sugar and fat in mice

Ghrelin signaling is implicated in food reward processing

Ghrelin receptor-deficient mice displayed normal flavor conditioning

Ghrelin receptor antagonism did not block sugar-conditioned flavor preferences

Ghrelin signaling is not essential for sugar or fat conditioned flavor preferences

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Figure 1.

Experiment 1. Mean (+sem) intakes of GHRSR-null and WT mice given flavored 8% glucose (CS+/Glucose) and flavored 0.1% sucralose + 0.1% saccharin mixture (CS-/S+S) during one-bottle training and CS+ flavored S+S (CS+/S+S) and CS- flavored S+S (CS-/S+S) during two-bottle tests. Numbers atop bars represent mean percent preference for the CS+/S+S solution. Significant ($P < 0.05$) differences between the flavored solutions within each group are indicated by an asterisk (*).

Figure 2.

Experiment 2. Mean (+sem) intakes of Drug and Saline groups given flavored 8% glucose (CS+/Glucose) and flavored 0.1% sucralose + 0.1% saccharin (CS-/S+S) during one-bottle training and CS+ flavored S+S (CS+/S+S) and CS- flavored S+S (CS-/S+S) during two-bottle tests. The Drug group was injected with the GHSR antagonist (D-Lys3)-GHRP-6 and the Saline group with saline prior to the daily training sessions. No injections were administered prior to the two-bottle tests. Numbers atop bars represent mean percent preference for the CS+/S+S solution. Significant ($P < 0.05$) differences between the flavored solutions within each group are indicated by an asterisk (*).

Fig. 3.

Experiment 3. A. Mean (+sem) 1-h total intakes (oral + IG) of the GHSR-null and WT groups are plotted for one-bottle Tests 0–3. The mice were maintained at ~90% of their body weight during the experiment. The mice drank (1 h/day) a CS– flavored saccharin solution paired with IG water infusions in Test 0 before being switched to a CS+ flavored saccharin solution paired with IG infusions of 16% glucose in CS+ Tests 1–3. B. Mean (+sem) 1-h oral intakes are plotted for CS+ and CS– flavored saccharin solutions during the two-bottle tests for the GHSR-null and WT groups. CS+ and CS– intakes were not paired

with IG infusions during the tests. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between Test 0 vs. Tests 1–3 intakes and between CS+ vs. CS– intakes are indicated by an asterisk.

Fig. 4.

Experiment 4. One-Bottle Training. Mean (+sem) 1-h total (oral + IG) training intakes of GHSR-null and WT groups given CS+ paired with IG infusion of 6.4% Intralipid (CS+/IG Intralipid) and CS- paired with IG water infusion (CS-/IG Water). The mice were maintained at ~90% of their body weight during the experiment. Two-Bottle Tests. Mean (+sem) 1-h oral intakes of CS+ and CS- flavored saccharin solutions (CS+, CS-) during the two-bottle tests for the GHSR-null and WT groups. CS+ and CS- intakes were not paired with IG infusions during the tests. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between CS+/IG Intralipid vs. CS-/IG water and between CS+ and CS- intakes during the Training and Test sessions are indicated by an asterisk.

Fig. 5.

Experiment 5. One-Bottle Training. Mean (+sem) 1-h total (oral + IG) training intakes of GHSR-null and WT groups given CS+ paired with IG infusion of 6.4% Intralipid (CS+/IG Intralipid) and CS- paired with IG water infusion (CS-/IG Water). The mice were maintained at ~95% of their body weight during the experiment. Two-Bottle Tests. Mean (+sem) 1-h oral intakes of CS+ and CS- flavored saccharin solutions (CS+, CS-) during the two-bottle tests for the GHSR-null and WT groups. CS+ and CS- intakes were not paired with IG infusions during the tests. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between CS+/IG Intralipid vs. CS-/IG water and between CS+ and CS- intakes during the Training and Test sessions are indicated by an asterisk.

Fig. 6.

Experiment 6. Mean (+sem) 24-h training intakes of GHSR-null and WT groups given flavored 8% fructose (CS+/Fructose) and flavored water (CS-/water) during one-bottle training and CS+ flavored water (CS+/Water) and CS- flavored water during two-bottle tests. Numbers atop bars represent mean percent preference for the CS+ solution. Significant ($P < 0.05$) differences between the flavored solutions within each group are indicated by an asterisk (*).