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Rats with a truncated ghrelin receptor (GHSR) do not respond to ghrelin, and show reduced intake of palatable, high-calorie food

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HIGHLIGHTS

- Rats lacking the ghrelin receptor (GHSR) eat less on a high-fat diet.
- GHSR knockout rats have normal glucose tolerance.
- GHSR knockout rats show reduced locomotion.
- The GHSR knockout rats eat less of a palatable cookie dough dessert after a meal.

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ABSTRACT

Ghrelin, a peptide hormone produced by the stomach, is the endogenous ligand for the Growth Hormone Secretagogue Receptor (GHSR). Ghrelin acts on the GHSR to increase food intake, appetitive behaviors, and adiposity. Recently, a rat model with a null mutation to the GHSR gene (FHH-GHSR$^{m1/Mcwi}$) was generated and used in behavioral studies, but the basic metabolic phenotype of this strain as well as that of the background strain (Fawn Hooded Hypertensive, FHH) has not been characterized in detail. Here we compared male FHH-GHSR$^{m1/Mcwi}$ rats with their wild-type littermates (FHH-WT) in a number of metabolic parameters. In the 24 h of recovery following an acute overnight fast, FHH-GHSR$^{m1/Mcwi}$ rats consumed less food than FHH-WT animals, and relative to their body weights, adult FHH-GHSR$^{m1/Mcwi}$ rats consumed fewer calories when placed on a high-fat diet. Despite this, FHH-GHSR$^{m1/Mcwi}$ rats did not show a difference in diet-induced obesity or weight gain. Fasted FHH-GHSR$^{m1/Mcwi}$ rats exhibited increased Agouti-Related Peptide (AgRP) and Neuropeptide Y (NPY) expression in the Arcuate Nucleus (ARC), indicative of altered central regulation of feeding and energy balance. FHH-GHSR$^{m1/Mcwi}$ rats exhibited lower levels of home cage locomotor behavior over the entire light/dark cycle, and reduced levels of food anticipatory activity when placed on a restricted feeding schedule. Finally, FHH-GHSR$^{m1/Mcwi}$ rats consumed less of a palatable dessert (cookie dough) given after the completion of the scheduled meal. Altogether, our data show that rats lacking a functional GHSR tend to eat less than their wild-type counterparts in the face of acute fasts, chronic high-fat diet exposure, and exposure to a palatable dessert, despite not showing differences in body weight and glucose homeostasis that are characteristic of GHSR null mice. These data indicate that many, but not all responses to GHSR ablation are conserved between rats and mice. The FHH-GHSR$^{m1/Mcwi}$ rat thus represents a novel and useful model for studying GHSR function in rats.

1. Introduction

Ghrelin is a 28 amino acid peptide hormone secreted primarily from the X/A-like cells of the gastric oxyntic mucosa in the stomach [1,2]. Ghrelin release is enhanced under conditions of negative energy balance and in anticipation of meals [3,4], and ghrelin administration has the effect of increasing food intake and fat storage [5,6]. In addition to its role in homeostatic feeding, ghrelin acts via the mesocorticolimbic dopamine system to mediate reward-seeking and motivated behaviors [7,8,9,10]. Ghrelin is also released in response to psychosocial stress, where it may mediate the metabolic and behavioral adaptations that accompany the stress response [11,12]. Ghrelin in circulation is found both in its native form and in the serine 3 acylated form commonly referred to as active ghrelin, since it is the only form capable of binding to the Growth Hormone Secretagogue Receptor 1a (GHSR1a), the only

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known receptor for ghrelin [13,14]. The acyl-modification is uniquely catalyzed by Ghrelin *O*-acyl Transferase (GOAT), a member of the membrane-bound *O*-acyltransferase (MBOAT) family [13,14], and mice lacking GOAT are entirely deficient in acylated forms of ghrelin [15].

The effects of ghrelin comprise not only consummatory behaviors, but also motivated and appetitive behaviors that often precede food intake. In humans there is an increase in circulating ghrelin in advance of meals [16], and a similar phenomenon occurs in rodents entrained to a scheduled mealtime [16,17,18,19]. This increase in circulating ghrelin is correlated with an increase in locomotor activity [17,20,21]. This pattern of behavior, termed food anticipatory activity, is thought to reflect the foraging behaviors that an animal in the wild would normally exhibit under conditions of restricted food access [22]. Food anticipatory activity is enhanced by ghrelin administration in sated animals [23]. Moreover, food anticipatory activity is attenuated in mice lacking the ghrelin receptor (GHSR) [24,23], as well as rats treated with ghrelin antagonists [21].

The Growth Hormone Secretagogue Receptor (GHSR), also known as the ghrelin receptor, is a G protein-coupled receptor (GPCR) expressed widely throughout the body and brain, with particular enrichment in brain regions concerned with homeostatic and motivational function such as the hypothalamus, hippocampus, as well as in a number of midbrain and brainstem nuclei [25,26]. In addition to its role as a receptor for active ghrelin, GHSR also participates in receptor dimers with numerous other GPCRs including those for dopamine (D1R, D2R), serotonin (5-HT2C) and the melanocortin-3 receptor (MC3R) [27,28,29,30]. In the arcuate nucleus of the hypothalamus (ARC), GHSR is expressed in neurons that co-express the orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), and both peripheral and central ghrelin administration activates these neurons, leading to upregulation of these neuropeptides [31,32]. Ghrelin inhibits the activity of pro-opiomelanocortin (POMC) neurons, though this is likely an indirect trans-synaptic effect since POMC neurons do not generally express GHSR [33,31]. Leptin and ghrelin have opposing effects on food intake and body weight, but in most hypothalamic nuclei, with the notable exception of the AgRP/NPY neurons of the ARC, the leptin receptor (ObRb) and GHSR are not co-localized [34].

Knowledge of ghrelin's physiological and behavioral effects has been advanced in large part by the use of knockout mouse models. The gene coding for ghrelin itself can be knocked out, leading to a complete absence of circulating ghrelin [35], as can GOAT, leading to a specific lack of acylated ghrelin [15]. Mice lacking GHSR ($GHSR^{-/-}$) have also been well-studied, showing mild hypophagia, resistance to diet-induced obesity, and resistance to diet-induced metabolic dysfunctions such as impaired glucose tolerance and insulin sensitivity [36,37]. Moreover, $GHSR^{-/-}$ mice show reduced food intake when placed on a restricted feeding schedule [7], reduced anticipatory behavior to scheduled meals [24], and preferential use of fat as an energy substrate under conditions of stress [11]. Importantly, these phenotypic effects are quite subtle and often reveal themselves only under the appropriate dietary, energetic, or behavioral challenges.

Though many advances in the ghrelin literature are owed to transgenic mouse models, there are some experimental situations, particularly those involving complex surgical procedures or behavioral tasks, to which rats are better suited. A GHSR knockout rat model based on the Fawn Hooded Hypertensive (FHH) inbred background strain was recently generated at the Medical College of Wisconsin using *N*-ethyl-*N*-nitrosourea (ENU)-induced mutagenesis (FHH-$GHSR^{m1/Mcwi}$) [38]. These rats resist the effects of exogenous ghrelin on gastrointestinal motility and food intake [39,40], as well as the locomotor sensitizing effect of chronic cocaine exposure [40]. Young (2–4 months) FHH-$GHSR^{m1/Mcwi}$ rats have reduced dendritic spine density and fewer doublecortin immunopositive neurons in the dentate gyrus relative to their wild-type counterparts, and in old age (5–8 months) knockouts go on to exhibit reduced performance in a food-motivated radial arm maze task [41].

Although this strain has been used to good effect in several studies so far, its body weight, food intake, and metabolic phenotype have not yet been evaluated in detail. The effect of this GHSR mutation on the consumption of normal and palatable food, for example, is an important consideration not only in studies of metabolism, but also in cognitive, motivational, and behavioral experiments in which food is used as a reinforcer. There are many such models that are already well-adapted to using rats as an experimental organism, and the usefulness and validity of the FHH-$GHSR^{m1/Mcwi}$ rat in these will be greatly enhanced if its commensurability with the $GHSR^{-/-}$ mouse is better understood, since this mouse is the basis for much of the pre-existing knowledge of ghrelin function. Therefore, in order to better understand the phenotype of this rat, we evaluated a number of metabolic, neurobiological, and behavioral traits that are well-established phenotypic markers of $GHSR^{-/-}$ mice.

2. Methods

2.1. Animals

Male FHH wild-type (FHH-WT) and GHSR KO (FHH-$GHSR^{m1/Mcwi}$) rats were developed at the Human and Molecular Genetics Center of the Medical College of Wisconsin as described in [39,40]. The $GHSR^{m1/Mcwi}$ mutant possesses a C > T transition at nucleotide 1027 (GenBank accession number NM032075.3), creating a stop codon (CAG > TAG) that yields a translated protein truncated by 21 amino acids at the c terminus. For more details see http://pga.mcw.edu.

All subjects in this study were bred at Carleton University from breeding pairs originally obtained from the Medical College of Wisconsin. Pups were genotyped using real time PCR with GHSR-specific primer and probe sets (Transetyx). Rats were housed in a temperature and humidity controlled vivarium at 21 °C and 50% humidity with a 12 h light-dark cycle (lights on at 8:00). Rats in our colony were weaned into multi-housed environments of 2–3 littermates per cage. Prior to entry into the study, rats were single-housed. Unless otherwise mentioned, standard laboratory chow (3.3 kCal/g) and water were provided ad-libitum throughout the study. All procedures were approved by the Carleton University Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care.

2.2. GHSR function

A total of 32 rats were randomly selected from the breeding colony at approximately 60 days of age. At zeitgeber time 2 (ZT2; 10:00) rats were given intraperitoneal injections of 15 μg freshly prepared rat ghrelin (Peptides International) or saline ($n = 8$ per genotype per injection). Rats were sacrificed 15 min post-injection by rapid decapitation. Trunk blood was collected for analysis of growth hormone levels.

2.3. Fast-refeed test

Adult rats ($n = 8$ FHH-WT and $n = 8$ FHH-$GHSR^{m1/Mcwi}$) were randomly selected from available animals in the breeding colony (age range 160–200 days at study start). Because of the variable age range in this cohort, we elected to express body weight and food intake in relative terms to correct for initial differences in body weight. After several days of baseline monitoring of food intake and body weight, we conducted a fast-refeed test. Animals were fasted overnight (food removed at ZT11). Food was returned at ZT1 the following morning, and food intake was monitored at 30 min, 1, 2, 4, 6, and 24 h post-return.

2.4. Diet-induced obesity

One week following the fast-refeed test, rats previously subject to the fast-refeed test were placed on an ad-libitum high fat diet (HFD) with 60% of caloric content from fat (5.24 kCal/g; Research Diets). Food intake and body weight were monitored weekly for 12 weeks.

2.5. Glucose tolerance test (GTT)

After 11 weeks of HFD exposure, rats were fasted overnight (19:00–9:00). The following morning, animals were given an intraperitoneal (IP) bolus injection of glucose (2 g/kg). Blood glucose was measured using a commercially available Bayer Contour glucometer (Bayer) at 0 (baseline), 15, 30, 60, and 120 min following injection.

2.6. Hormonal analysis

One week following the GTT, HFD-exposed rats were again fasted overnight and sacrificed the following morning by rapid decapitation. Trunk blood was collected at the time of sacrifice in EDTA coated vacutainers (BD) and stored on ice until plasma was separated by centrifugation at 3000 rpm for 15 min. Plasma was stored at −80 °C until assay. A total of 10 rats ($n = 5$ FHH-WT and $n = 5$ FHH-GHSR$^{m1/Mcwi}$) were used for hormonal assays. Active ghrelin was assayed using a Rat/Mouse Ghrelin (active) ELISA kit according to the manufacturer's directions (EMD Millipore). Insulin, leptin, and PYY3–36 were assayed by a MilliPlex Rat Gut Hormone kit according to the manufacturer's directions (EMD Millipore). Plasma was prepared in a similar manner for animals injected with ghrelin, and analyzed using a Rat Growth Hormone (GH) EIA kit (SPI Bio) according to the supplied protocol. Each assay was conducted using the same kit for all samples, and intra-assay coefficients of variation were <10%.

2.7. Carcass analysis

Carcasses from HFD-exposed rats were stored at −20 °C until analysis. The visceral fat pad (comprising the perigonadal and mesenteric depots), retroperitoneal, subcutaneous and intrascapular brown adipose tissue (BAT) depots were dissected and weighed by an observed blind to the animal's genotype.

2.8. qRT-PCR

Owing to sample loss during processing, a total of 9 HFD-exposed rats ($n = 5$ FHH-WT and $n = 4$ FHH-GHSR$^{m1/Mcwi}$) were used for gene expression analysis by qRT-PCR. Brains were rapidly removed at sacrifice and sliced into 1 mm coronal sections using an ice-chilled brain matrix (Ted Pella Inc.). The arcuate nucleus (ARC) was dissected and flash-frozen on dry ice. ARC samples were stored at −80 °C until analysis. Briefly, total RNA was extracted using TRIzol according to the manufacturer's protocol (Life Technologies). RNA integrity was verified by agarose gel electrophoresis; RNA purity and concentration were measured by Nanodrop spectrophotometry (Thermo Scientific). RNA was reverse transcribed using the SuperScript II kit with oligo(DT) according to the manufacturer's method (Life Technologies). Quantitative real-time PCR was performed in a MyiQ Single Colour Real-Time PCR Detection System (Bio-Rad). Gene expression was determined by the 2ddct method using β-actin as a housekeeping control gene. Primer sequences were as follows. NPY: Forward 5′-TCCGCTCTGCGACACTACAT-3′, Reverse 5′-GGAAGGGTCTTCAAGCCTTGT-3′. AgRP: Forward 5′-GCTCCACTGAAGGGCATCA-3′, Reverse 5′-TAGCACCTCCGCCAAAGCT-3′. POMC: Forward 5′-GCTCAAGGTCCTTCCTGGTG-3′, Reverse 5′-GCCCTGGATTGAATCACGCC-3′. ObRb: Forward 5′-GCTGGAAGCCTGTCGTACTCTTCAC-3′, Reverse 5′-TACACTGCGTCATAGGTAAACTTCCCTC-3′. β-actin: Forward 5′-GTGCCACCAGACAGCACTGTGTTG-3′, Reverse 5′-TGGAGAAGAGCTATGAGCTGCCTG-3′.

2.9. Food anticipation and dessert consumption

A total of 20 rats ($n = 10$ FHH-WT and $n = 10$ FHH-GHSR$^{m1/Mcwi}$) were selected at random from available animals in our colony and used in the food anticipation study. In preparation for the later dessert challenge, rats were given free access to 4 g of cookie dough overnight (Pillsbury) on a circular ceramic plate to reduce later neophobia. All rats finished this initial sample of cookie dough overnight. Food intake and home-cage locomotor activity was measured for a baseline period of four days. Locomotion was measured in one hour time bins using infrared activity monitor frames (Micromax, AccuScan Instruments). After the baseline period, rats were placed for 23 days on a restricted feeding schedule where regular chow was accessible ad libitum only from ZT2-6. Food consumed during the four hour period of food access was measured, as was locomotor activity in the hours leading up to food access. Food anticipation was defined as the number of infrared beam crossings observed during the 2 h preceding the scheduled meal. During the last hour of the food access period on the 24th day of food restriction, rats were given 30 g of cookie dough. Since by this time, rats had already consumed most of their daily caloric intake in chow, this additional access to a highly palatable food represents a model of dessert. Dessert consumption was defined as the amount of cookie dough eaten during this period on the 24th day.

2.10. Statistics

All data are expressed as mean ± SEM. Results from the ghrelin challenge were analyzed using two-way ANOVA with genotype and injection as factors. Significant interactions were further examined using Fisher's Protected LSD with a significance criterion set at $p < 0.05$. In cases where data from the two genotypes were compared directly without any additional factors, two-tailed independent *t*-tests were used, with a significance criterion set at $p < 0.05$. Timecourse data, including from the fast-refeed test, HFD exposure period, GTT, and locomotion monitoring, were analyzed by repeated measures ANOVA.

3. Results

3.1. Ghrelin-induced growth hormone release

Exogenous ghrelin stimulates the release of growth hormone within 15 min of administration in a GHSR-dependent manner [1]. To demonstrate the success of the ENU-induced mutagenesis method in disabling the GHSR, we examined ghrelin-induced growth hormone release 15 min post-injection in a cohort of rats. Two-way ANOVA revealed a significant interaction between genotype and injection ($F(1.24) = 12.78$, $p < 0.01$). Post-hoc analysis revealed the expected increase in circulating growth hormone in ghrelin-injected FHH-WT animals ($p < 0.001$; Fig. 1A), however this effect was absent in FHH-GHSR$^{m1/Mcwi}$ rats, confirming the loss of function mutation.

3.2. Fast-refeed test

Rebound feeding during the first 6 h of the fast-refeed test was equivalent between genotypes was assessed by repeated-measures ANOVA. This analysis did not reveal a significant interaction between genotype and time ($p > 0.05$; Fig. 1B), suggesting that there are no differences in acute rebound feeding in the 6 h following a fast. However, when cumulative food intake over the entire 24 h period following food return was considered, FHH-GHSR$^{m1/Mcwi}$ rats did eat less than their FHH-WT counterparts ($t(14) = 2.148$, $p < 0.05$; Fig. 1C).

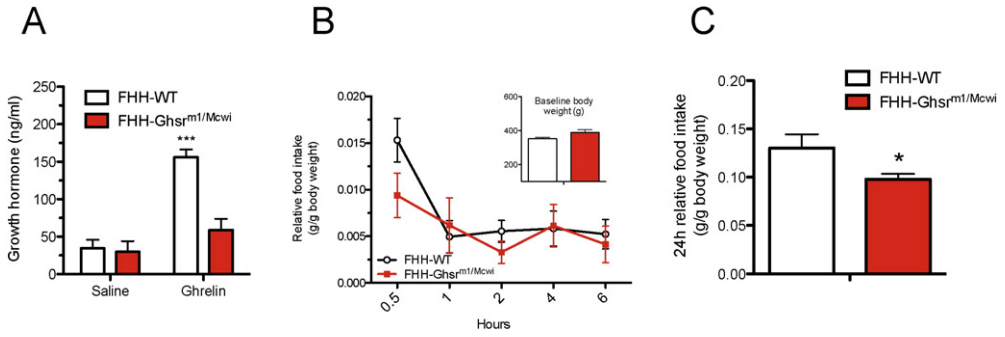

Fig. 1. Rats lacking GHSR do not respond to ghrelin, and show reduced food intake following an overnight fast. (A) Plasma growth hormone levels 15 min following 15 μg IP ghrelin injection. (B) Rebound feeding over 6 h following an overnight fast. Baseline, pre-HFD body weight inset. (C) Cumulative body weight adjusted food intake over 24 h following an overnight fast. *p < 0.05, ***p < 0.001 vs. FHH-WT. All values represent mean −/+ SEM.

3.3. Weight gain on high-fat diet

One week following recovery from the fast-refeed test, the same cohort of rats was placed on a HFD for 12 weeks. Both genotypes entered a dynamic phase of weight gain following placement on the diet, reaching a static plateau after 5–6 weeks on the diet. All animals gained approximately 20% over their initial baseline weights. Repeated measures ANOVA revealed no interaction between time and genotype ($p > 0.05$), indicating the two genotypes did not differ in their body weight response to the HFD (Fig. 2A). When caloric intake relative to body weight was averaged over the entire period of HFD exposure, FHH-GHSR$^{m1/Mcwi}$ rats were found to have consumed significantly fewer calories than FHH-WT rats ($p < 0.01$ by two-tailed independent samples *t*-test; Fig. 2B). When caloric intake was examined on a weekly basis by repeated measures ANOVA, we found a significant interaction between time and genotype ($F(1.4.405) = 2.96$, $p < 0.05$ by Greenhouse-Geisser correction; Fig. 2C). Post-hoc analysis showed that FHH-GHSR$^{m1/Mcwi}$ rats consumed significantly fewer calories per gram body weight at weeks 1 and 2 ($p < 0.01$) as well as 6 and 8 ($p < 0.05$).

3.4. Glucose tolerance test

After 11 weeks of HFD exposure, rats were fasted overnight and subjected to an intraperitoneal glucose tolerance test. There were no differences in fasting blood glucose on the morning of the glucose tolerance test ($p > 0.05$; Fig. 3A). Post-injection blood glucose was analyzed by repeated measures ANOVA, which revealed no significant interaction between time and genotype ($p > 0.05$; Fig. 3A). Similarly, there was no significant difference between genotypes when the entire test period was considered as a whole by area under the curve (AUC) ($p > 0.05$; Fig. 3B).

3.5. Body composition

Analysis of fat pad mass in the cohort of rats exposed to the HFD did not reveal any significant differences between the genotypes in any of the visceral, retroperitoneal, subcutaneous, or brown adipose tissue depots ($p > 0.05$; Fig. 4A).

3.6. Serum hormone levels

There were no differences in fasting insulin, PYY3-36, or active ghrelin between FHH-WT and FHH-GHSR$^{m1/Mcwi}$ rats from the HFD cohort ($p > 0.05$; Fig. 4B). FHH-GHSR$^{m1/Mcwi}$ animals showed a trend toward lower fasting leptin levels ($t(10) = 1.883$, $p = 0.089$; Fig. 4B). Blood glucose measurements collected at the time of sacrifice were also equivalent between the genotypes (FHH-WT: 4.49 ± 0.11 mMol/L, FHH-GHSR$^{m1/Mcwi}$: 4.61 ± 0.14 mMol/L; $p > 0.05$).

3.7. Hypothalamic gene expression

Gene expression in dissected arcuate nuclei from the HFD exposure cohort was analyzed by qRT-PCR. FHH-GHSR$^{m1/Mcwi}$ rats in showed a trend toward increased AgRP expression ($t(7) = 2.272$, $p = 0.058$; Fig. 4C) and a significant increase in NPY expression in the ARC ($t(7) = 4.230$, $p < 0.01$; Fig. 4C). There were no significant differences between the genotypes in ObRb or POMC mRNA expression ($p > 0.05$; Fig. 4C).

3.8. Food anticipation

A new cohort of rats was used for the studies of food anticipation and dessert consumption ($N = 20$). Locomotion data from a total of 4 rats had to be dropped due to technical problems with the activity

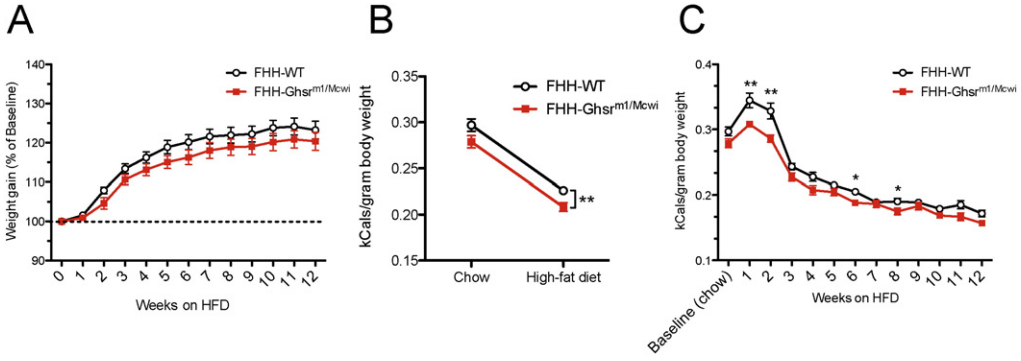

Fig. 2. Reduced food intake on a high-fat diet in rats lacking GHSR. (A) Weekly weight gain relative to baseline weight in rats placed on a high-fat diet. (B) Average baseline and post-highfat diet body weight-adjusted food intake. (C) Weekly body weight adjusted food intake following placement on a high-fat diet. *p < 0.05, **p < 0.01 vs. FHH-WT. All values represent mean −/+ SEM.

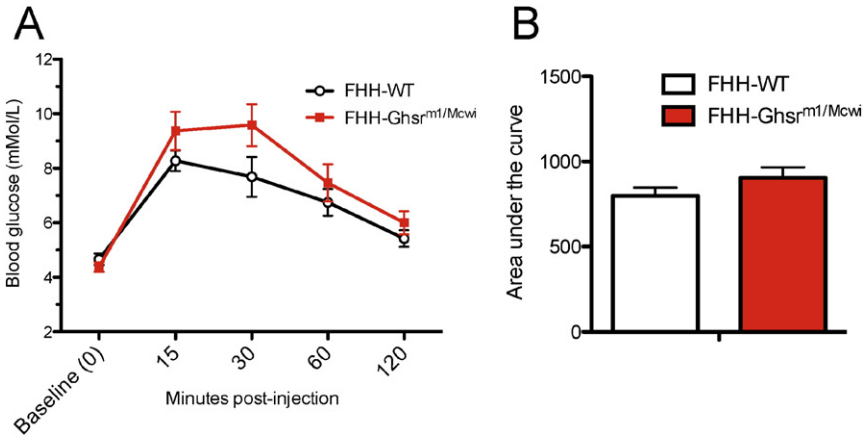

Fig. 3. Normal glucose tolerance in rats lacking GHSR. (A) Glucose tolerance test results at each measurement timepoint. (B) Glucose tolerance test area under the curve. All values represent mean −/+ SEM.

monitoring system. A repeated measures ANOVA was performed to examine average hourly locomotion in animals over the last three days of baseline (data from the first day were dropped to allow animals to adjust to the activity monitoring system). A significant main effect of strain indicated that FHH-GHSR$^{m1/Mcwi}$ rats are hypoactive compared to FHH-WT rats ($F(1.13) = 17.976$, $p = 0.001$; Fig. 5A). This analysis also revealed a significant main effect of hour ($F(23,299) = 45.724$, $p < 0.001$) and a significant interaction between hour and genotype ($F(19,299) = 8.096$, $p < 0.001$; Fig. 5A). This difference existed at each time bin, but it was the most pronounced during the dark phase of the light-dark cycle (Fig. 5B).

Owing to problems with the locomotor equipment, data were missing for three days of measurement during the restricted feeding phase. Initially we looked at the two hours immediately before meal presentation (ZT1,2) and analyzed total locomotion in this period over the food restriction phase. Using repeated measures ANOVA, we found a significant main effect of day ($F(19,247) = 3.435$, $p < 0.001$), revealing the gradual development of increased locomotion in anticipation of food presentation (Fig. 5C). We also found a main effect of genotype ($F(1.13) = 5.275$, $p < 0.05$), indicating reduced food anticipatory activity among FHH-GHSR$^{m1/Mcwi}$ rats (Figs. 5D,F). Next, we averaged locomotor data from the food restriction phase of this study, and analyzed these by repeated measures ANOVA. In this analysis, a significant main effect of genotype indicated that FHH-GHSR$^{m1/Mcwi}$ rats were still hypoactive relative to FHH-WT rats ($F(1.13) = 10.241$, $p < 0.01$; Fig. 5D). When data from the light and dark periods were averaged and analyzed by *t*-test, the difference was only significant during the dark phase ($p < 0.05$; Fig. 5E).

Food intake did not differ between the two genotypes at baseline, nor was there an effect of genotype during the food restriction paradigm ($p > 0.05$, Fig. 5G). Repeated measures ANOVA revealed a significant effect of day ($F(22,374) = 10.11$, $p < 0.001$) as rats increased their food intake after becoming accustomed to the restricted feeding schedule, but there was no effect of genotype at any time point (Fig. 5G, Days 1 and 23 shown).

3.9. Dessert intake

On the 24th day of food restriction, rats were challenged with cookie dough during the last hour of their 4 h chow access period. Since rats had already consumed the majority of their daily food by the last hour of access, this was not affected by cookie dough presentation (Fig. 5H). Interestingly, we found that FHH-GHSR$^{m1/Mcwi}$ rats consumed significantly less cookie dough relative to their body weights ($t(17) = -2.66$, $p < 0.05$; Fig. 5I).

4. Discussion

In this series of studies we hypothesized that FHH-GHSR$^{m1/Mcwi}$ rats would, relative to their WT counterparts, exhibit a metabolic and behavioral phenotype reminiscent of GHSR$^{-/-}$ mice. Through the use of the GHSR$^{-/-}$ mouse, a great deal about the role of ghrelin signaling in metabolism, behavior, and even cognition has been learned in recent years. One of the most important, and earliest findings regarding the GHSR$^{-/-}$ mouse was that it shows reduced weight gain when placed on a high-fat diet [37]. We did not observe this characteristic in our rats, as animals of both genotypes gained equivalent amounts of weight, and did so at equivalent rates. A similar pattern of diet-induced weight gain was observed in congenic GHSR$^{-/-}$ mice, suggesting that the effect of GHSR ablation on diet-induced obesity depends in part on an interaction with the genetic background of the subject [42]. When considering the lack of effects of our GHSR mutation on body weight, body composition, and glucose tolerance, it should be noted that in our hands, neither of the genotypes gained more than approximately 20% of its baseline body weight, nor did any rats develop fasting hyperglycemia or hyperinsulinemia, even after 12 weeks of HFD exposure. Given this, it may be that the FHH strain is inherently resistant to diet-induced obesity and type-II diabetes. If this is the case, then it would be difficult to ascertain a role for GHSR signaling in 'resisting' these effects, given that in the FHH strain, there is nothing to be resisted.

The choice of background strain in rodent studies inevitably presents challenges, as each strain possesses its own pre-existing traits against which the effects of genetic mutations must be compared. This is true of the FHH strain, which exhibits a number of important physiological and behavioral traits that set it apart from outbred strains that are more commonly used in behavioral and neurometabolic studies. Aside from their comparatively short lifespan (11–13 months) and tendency to develop spontaneous hypertension and renal disease in adulthood [43], FHH rats demonstrate increased preference for alcohol [44], more depressive-like behavior [44,45], and higher levels of social anxiety [46] than outbred strains. These rats also exhibit regionally-specific alterations in 5-HT turnover [47], blunted cocaine-induced striatal DA release [48], and a blunted behavioral response to serotonergic agents [49]. Relative to Wistar and Sprague–Dawley rats, FHH rats exhibit higher levels of spontaneous locomotion [50], eat less when faced with restricted food access [51], and gain less weight when stressed [49]. Many of these strain differences are in precisely the variables of most interest to the metabolic and neuropsychiatric models commonly employed in ghrelin studies, and thus FHH-specific strain effects must be accounted for when designing experiments. This highlights the broader implications of background strain control when using transgenic animals. A pertinent example of this from the mouse literature is the

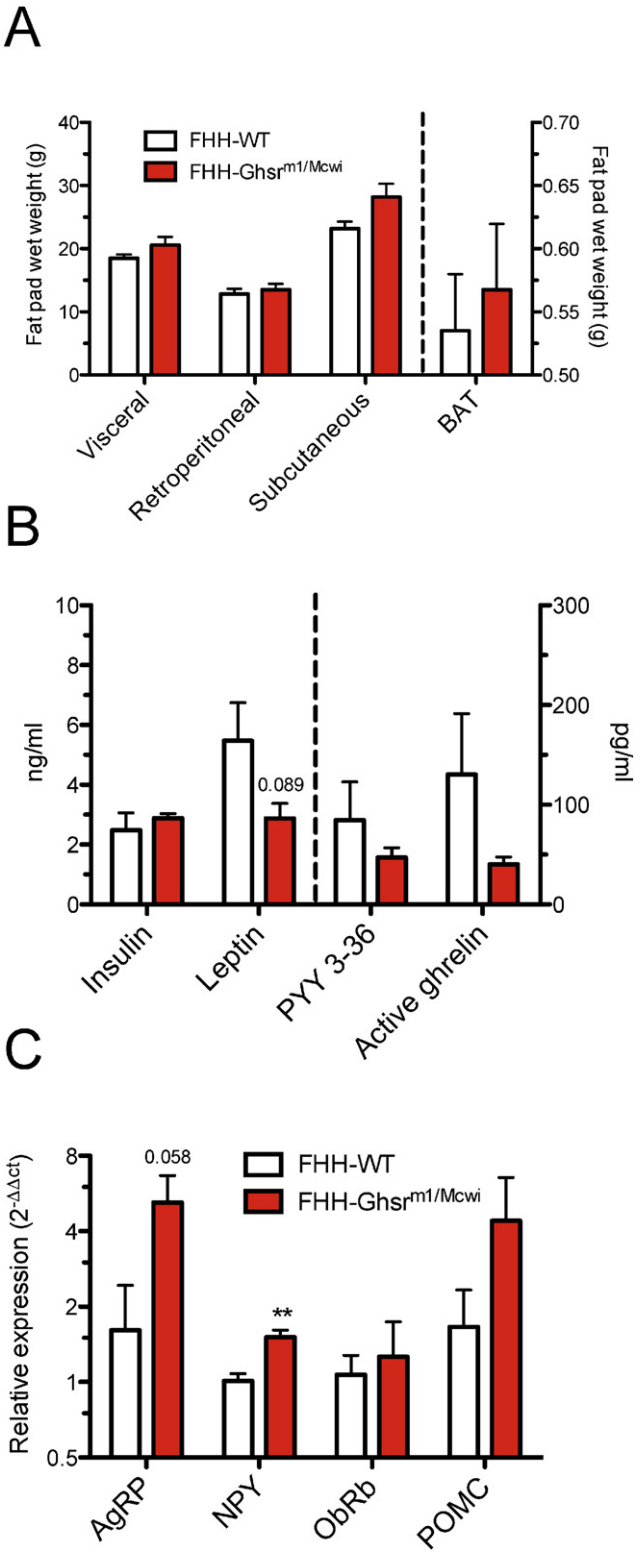

Fig. 4. Increased orexigenic hypothalamic gene expression in fasted GHSR-deficient rats. (A) Post-mortem fat pad weight in animals following 12 weeks of high-fat diet exposure. (B) Fasting circulating insulin, leptin, PYY3-36, and active ghrelin in overnight fasted high-fat diet exposed rats. (C) Arcuate nucleus (ARC) AgRP, NPY, ObRb, and POMC mRNA expression in overnight fasted high-fat diet exposed rats (B). $^{**}p < 0.01$ vs. FHH-WT. All values represent mean −/+ SEM.

issue of body weight in $GHSR^{-/-}$ mice, with some knockouts showing no difference in baseline body weight compared to wild-types [37], but knockouts thoroughly backcrossed onto a C57BL/6J line being reliably lighter than wild-types [42].

Both ghrelin and GHSR1a are expressed in pancreatic islet cells, and ghrelin acting on β-cells in either a paracrine or endocrine capacity tends to reduce glucose-stimulated insulin secretion [52,53,54], though this is not always observed in every model and dose [55]. Given this, and given the fact that $GHSR^{-/-}$ mice are reported to have improved insulin sensitivity relative to WT animals [36], we hypothesized that our FHH-$GHSR^{m1/Mcwi}$ rats would show improved glucose tolerance. Surprisingly this was not the case, as FHH-$GHSR^{m1/Mcwi}$ rats did not differ statistically from FHH-WT rats at any timepoint during the glucose tolerance test, in fact they showed a tendency toward impaired glucose tolerance, though this was not statistically significant. Fasting blood glucose collected prior to the GTT and at sacrifice did not differ between the genotypes, and unlike $GHSR^{-/-}$ mice that normally show reduced serum insulin in adulthood [37,36], fasting insulin collected at sacrifice was not different between our genotypes.

It was recently reported that aged $GHSR^{-/-}$ mice, in addition to showing alterations in meal pattern microstructure, exhibit NPY and AgRP mRNA upregulation in the ARC [56]. After an overnight fast, our animals exhibited a similar pattern of upregulated NPY and AgRP mRNA expression. Given that GHSR1a is expressed on NPY/AgRP neurons in the ARC, and that ghrelin administration tends to increase the expression of these neuropeptides [31,32], finding NPY and AgRP upregulation in animals lacking GHSR signaling is somewhat paradoxical, particularly in light of our finding of reduced refeeding in FHH-$GHSR^{m1/Mcwi}$ rats. There are two factors that may have contributed to this effect. Diet-induced obese animals are known to have attenuated central responses to ghrelin, with reductions in ghrelin-induced NPY and AgRP transcription, cFOS induction in NPY and AgRP neurons, and food intake relative to chow-fed animals [57]. Since our rats had been on a high-fat diet for 12 weeks, it is possible that FHH-WT rats had developed some degree of central ghrelin resistance, limiting its ability to upregulate NPY and AgRP expression. In addition, fasting leptin levels were reduced, albeit non-significantly, in our FHH-$GHSR^{m1/Mcwi}$ rats relative to FHH-WTs, and this may favor orexigenic gene expression [58]. In combination, these two factors may have contributed to increased fasting orexigenic neuropeptide gene expression in our FHH-$GHSR^{m1/Mcwi}$ rats relative to FHH-WT rats.

In contrast to the lack of effect of our GHSR mutation on body weight and glucose homeostasis, FHH-$GHSR^{m1/Mcwi}$ rats did show a number of differences in feeding behavior. Ghrelin levels rise under fasting conditions and play a role in driving food-seeking and consummatory behavior [3,4], and because of this we hypothesized that rats without functioning ghrelin signaling would be deficient in these capacities. To begin to test this, we decided to examine rebound feeding after an overnight fast. Mice given GHSR antagonists directly into the ventral tegmental area (VTA), a key structure in the mesocorticolimbic dopamine system, show blunted rebound feeding [7], however global $GHSR^{-/-}$ models show normal refeeding [59,42]. As with $GHSR^{-/-}$ mice, our FHH-$GHSR^{m1/Mcwi}$ rats did not show increased food consumption relative to wild-types during the first 6 h of food access. However, when food intake over the entire 24 h period following food presentation was considered, we found that FHH-$GHSR^{m1/Mcwi}$ rats ate less than wild-types. This may point to an altered response to satiety signals in FHH-$GHSR^{m1/Mcwi}$ rats, given that the difference in food intake presumably emerges some time after the initial bout of hyperphagia following food return.

Ghrelin signaling is important in mediating dietary preferences when food choice is available, biasing animals toward palatable, high-calorie diets [60]. We approached the question of palatable, high-calorie diets first by observing food intake and body weight while animals consumed a high-fat diet. Initially, we hypothesized that FHH-$GHSR^{m1/Mcwi}$ rats would consume less of a provided high-fat diet compared to wild-type rats. This did appear to be the case, because when calorie intake over the period of HFD exposure was viewed as a

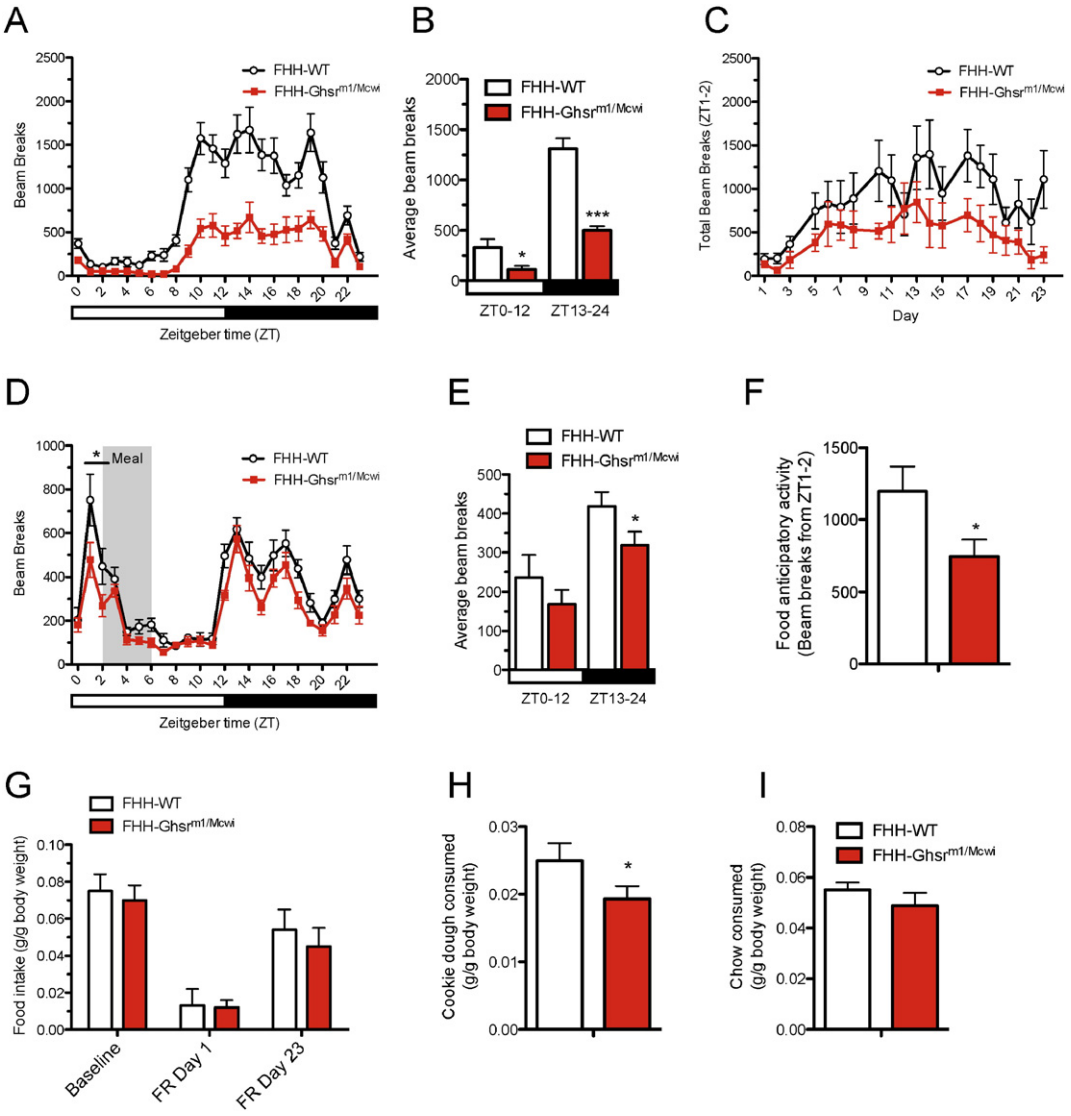

Fig. 5. Reduced locomotion, food anticipatory activity, and dessert consumption in GHSR-deficient rats. (A) Baseline 24 h home-cage locomotion averaged over three days of monitoring. (B) Baseline home-cage locomotion from three days of monitoring averaged across the light (ZT0-12) and dark (ZT13-24) periods. (C) Locomotor behaviour immediately preceding food presentation during the restricted feeding period (ZT1-2) over 23 days of food restriction. (D) 24 h home-cage locomotion averaged over 23 days of food restriction. (E) Home-cage locomotion from 23 days of food restriction averaged across the light and dark periods. (F) Food anticipatory locomotor activity demonstrated by cumulative beam breaks from ZT1 and 2. (G) Food intake adjusted to body weight at baseline, and during the first and last days of food restriction. (H) Chow intake adjusted to body weight on the last day of food restriction. (I) Body weight adjusted consumption of the cookie dough dessert following the regularly scheduled chow meal on the last day of food restriction. *$p < 0.05$, ***$p < 0.001$ vs. FHH-WT. All values represent mean −/+ SEM.

whole, FHH-GHSR$^{m1/Mcwi}$ rats consumed significantly less than FHH-WTs, though this effect was the strongest during the first two weeks of HFD exposure. The tendency to eat less when given an HFD is consistent with observations in GHSR$^{-/-}$ mice, which also tend to eat less than wild-types on a high-fat diet [37,42,36]. Since we did not offer our animals a choice of diet in this study, it is uncertain whether our observations reflect an alteration in the homeostatic regulation of caloric intake, or the absence of a hedonic response to a palatable, high energy diet. Interestingly, despite consuming fewer calories on average during the period of HFD exposure, FHH-GHSR$^{m1/Mcwi}$ rats gained weight at the same rate as FHH-WTs, suggesting that increased feed efficiency may be a unique feature in our model. While we were unable to measure energy expenditure directly, the argument for increased feed efficiency is supported by our finding that FHH-GHSR$^{m1/Mcwi}$ rats exhibit significantly reduced home-cage locomotor activity, and this suggests reduced energy expenditure, which could account for the difference in feed efficiency during the dynamic period of HFD-induced weight gain.

Knowing that our strongest phenotypic differences between the genotypes involved feeding behavior in various contexts, we decided to examine the case of restricted feeding and food anticipatory activity. Animals faced with a restricted schedule of food availability develop a series of anticipatory behaviors and metabolic adaptations in order to prepare for the ingestion, absorption and metabolism of the contained nutrients [61]. In the rat and mouse strains commonly used in laboratory environments, this anticipation manifests in part as an increase in locomotion and circulating active ghrelin in the period immediately prior to scheduled meal presentation [16,17,18,19]. These changes constitute a dramatic re-arrangement of the animal's normally nocturnal pattern of locomotion and ad libitum food intake. During the baseline period prior to food restriction, we found that FHH-GHSR$^{m1/Mcwi}$ rats moved significantly less over 24 h than FHH-WT animals, with the greatest magnitude of difference being found during the dark phase of the light–dark cycle. Placing these animals onto a restricted feeding schedule resulted in a large reduction in spontaneous locomotion in FHH-WT animals, and a much smaller reduction in FHH-GHSR$^{m1/Mcwi}$

Table 1
Comparison of FHH-GHSR$^{m1/Mcwi}$ rat and GHSR$^{-/-}$ mice in various behavioral and metabolic measures.

Measure	FHH-GHSRm1/Mcwi rat	GHSR −/− mouse	Reference
High fat diet-induced weight gain	ND	⇓/ND	[37,42]
High fat diet intake	⇓	⇓/ND	[37,42]
Glucose tolerance	ND	⇑	[36,71]
Circulating insulin	ND	⇓	[36]
Circulating leptin	ND	ND	[37]
Body fat	ND	⇓	[37]
AgRP expression	ND	⇑	[56]
NPY expression	⇑	⇑	[56]
POMC expression	ND	⇓	[56]
Home cage locomotion	⇓	⇓	[37]
Food anticipatory activity	⇓	⇓	[24,63]

rats. As animals became accustomed to a timing of food presentation, their locomotion during the hours immediately preceding it rose. Activity in this period was significantly greater in FHH-WT rats, and this is consistent with a number of reports showing that ghrelin enhances food anticipatory activity [23,62,21], and that food anticipatory activity is attenuated in mice lacking the gene for either ghrelin or GHSR [24,63, 23,21] (but see [20]). However, the observed reduction in food anticipatory activity did not result in any relative impairments in food intake during the restricted feeding schedule.

It may be that the reduced intake of the palatable, high-fat diet observed in FHH-GHSR$^{m1/Mcwi}$ rats is related to disrupted reward system functioning. Studies of palatable diet exposure and reward function in mice lacking functional ghrelin signaling support this conjecture [7, 64,65,66,67]. However, in a situation where animals are not offered a choice in diet, such a hypothesis is difficult to address. To more directly assess the role of GHSR signaling in hedonic feeding, we designed a 'dessert' challenge modelled after one recently carried out in mice. In this study, mice engineered to lack the enzyme ghrelin *O*-acyltransferase (GOAT), the enzyme that octanoylates and activates the ghrelin peptide, were presented with a high fat diet in the last hour of a scheduled meal and ate significantly less of it than their wild-type counterparts [68]. Since in this model, the animals had been trained to meet their daily homeostatic caloric requirements within the limited period of food availability, their consumption of the palatable treat immediately following is analogous to a high-calorie dessert offered following a meal. We used a similar approach in our rat model of disrupted ghrelin signaling, and indeed our finding of reduced cookie dough consumption in FHH-GHSR$^{m1/Mcwi}$ rats is consistent with observations in mice by Davis et al. [68]. Our findings support the perspective that in the presence of palatable foods, ghrelin signaling can make sated animals behave as hungry ones [10,69,9,70].

Together these data demonstrate that the FHH-GHSR$^{m1/Mcwi}$ rat does not respond to ghrelin, and in addition demonstrates a number of phenotypic effects in body weight, food intake, locomotor behavior, and gene expression that resemble, albeit in a generally milder form, the signature phenotypic markers of GHSR$^{-/-}$ mice (see Table 1). Of particular interest was our finding of reduced dessert consumption in FHH-GHSR$^{m1/Mcwi}$ rats, since this supports a role for ghrelin signaling in driving hedonic overconsumption of food, and points toward potentially useful future pharmacotherapies involving the ghrelin receptor. Studies such as this that demonstrate conserved functions of GHSR and ghrelin signaling are also of value from a comparative endocrinology standpoint. Because rats are often the preferred animal model for studying certain cognitive and behavioral tasks, reproductive behavior, and studies where repeated blood sampling or complex surgical procedures are needed, knowing that the FHH-GHSR$^{m1/Mcwi}$ rat approximates the GHSR$^{-/-}$ mouse in a number of ways constitutes an important piece of evidence justifying their validity in the study of ghrelin signaling in those fields.

Contributions

AA, HM, and VS designed the research. HM, VC, VS, EM, AW, and MW conducted the research. AA, HM, VC, and VS analyzed the data.

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