Evidence Supporting a Role for Constitutive Ghrelin Receptor Signaling in Fasting-Induced Hyperphagia in Male Mice

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Ghrelin is a potent orexigenic peptide hormone that acts through the growth hormone secretagogue receptor (GHSR), a G protein–coupled receptor highly expressed in the hypothalamus. *In vitro* studies have shown that GHSR displays a high constitutive activity, whose physiological relevance is uncertain. As GHSR gene expression in the hypothalamus is known to increase in fasting conditions, we tested the hypothesis that constitutive GHSR activity at the hypothalamic level drives the fasting-induced hyperphagia. We found that refed wild-type (WT) mice displayed a robust hyperphagia that continued for 5 days after refeeding and changed their food intake daily pattern. Fasted WT mice showed an increase in plasma ghrelin levels, as well as in GHSR expression levels and ghrelin binding sites in the hypothalamic arcuate nucleus. When fasting-refeeding responses were evaluated in ghrelin- or GHSR-deficient mice, only the latter displayed an ~15% smaller hyperphagia, compared with WT mice. Finally, fasting-induced hyperphagia of WT mice was significantly smaller in mice centrally treated with the GHSR inverse agonist K-(D-1-Nal)-FwLL-NH_2, compared with mice treated with vehicle, whereas it was unaffected in mice centrally treated with the GHSR antagonists D-Lys3-growth hormone–releasing peptide 6 or JMV2959. Taken together, genetic models and pharmacological results support the notion that constitutive GHSR activity modulates the magnitude of the compensatory hyperphagia triggered by fasting. Thus, the hypothalamic GHSR signaling system could affect the set point of daily food intake, independently of plasma ghrelin levels, in situations of negative energy balance. (***Endocrinology*** **159: 1021–1034, 2018)**

Ghrelin is a 28-residue octanoylated peptide predominantly secreted from endocrine cells of the stomach (1). Ghrelin is recognized as a highly potent orexigenic peptide hormone (2). In addition, ghrelin plays a variety of other physiological roles that include, but are not limited to, modulation of growth hormone secretion, blood glucose homeostasis, and stress response, among others (2). In humans and rodents, plasma ghrelin levels rise before meals and then decrease postprandially (3, 4). Ghrelin acts via its unique receptor, the

ISSN Online 1945-7170

Received 1 November 2017. Accepted 21 December 2017.
First Published Online 28 December 2017

Abbreviations: 2-d, after 2 days; 4-d, after 4 days; 6-d, after 6 days; AgRP, agouti-related protein; ANOVA, analysis of variance; ARC, arcuate nucleus; DAB, diaminobenzidine; F-ghrelin, fluorescein-ghrelin(1–18); GHRP-6, growth hormone–releasing peptide 6; GHSR, growth hormone secretagogue receptor; GPCR, G protein–coupled receptor; ICV, intracerebroventricular; IR, immunoreactive; KO, knockout; NPY, neuropeptide Y; POMC, proopiomelanocortin; SEM, standard error of the mean; WT, wild-type.

growth hormone secretagogue receptor (GHSR), which is a G protein–coupled receptor (GPCR) highly expressed in the hypothalamus (5). GHSR is particularly enriched in neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons of the hypothalamic arcuate nucleus (ARC), which are a key target of circulating ghrelin to increase food intake (6–9).

GHSR is recognized as a GPCR that displays an unusually high constitutive activity. Specifically, *in vitro* studies showed GHSR signals with ~50% of the maximum activity in the absence of ghrelin (10, 11). Notably, most studies concerning constitutive GHSR activity have focused on molecular aspects of this phenomenon, whereas the magnitude of its *in vivo* effects and physiological relevance are uncertain (12). To study the *in vivo* impact of constitutive GHSR activity, two studies in rodents have tested the effect of GHSR inverse agonists, which reduce constitutive activity. In the first one, *ad libitum*–fed rats chronically treated with central infusions of the GHSR inverse agonist [*d*-Arg1, *d*-Phe5,*d*-Trp7,9,Leu11]-substance P reduced their food intake and body weight (13). However, this analog of substance P also acts on other GPCRs, raising some concerns about its specificity for GHSR when used *in vivo* (14, 15). The other study used central administration of the GHSR inverse agonist K-(D-1-Nal)-FwLL-NH_2 and also found a decrease in food intake in *ad libitum*–fed rats (16). Genetic manipulations of the ghrelin system in rodents also suggest that constitutive GHSR activity may have *in vivo* implications. In particular, most studies using ghrelin knockout (KO) mouse models, fed with either regular chow or a high-fat diet, do not show substantial differences in terms of food intake or body weight, compared with wild-type (WT) mice (17). In contrast, some studies using GHSR-deficient mouse models fed with regular chow showed a subtle but substantial decrease in body weight, compared with WT mice, and such differences were enhanced when mice were either aged or fed with a high-fat diet from weaning (17). The fact that mice lacking GHSR exhibit more robust alterations in eating behaviors, compared with ghrelin KO mice, may be an indication that the latter retains high constitutive GHSR activity (17). In humans, a role for constitutive GHSR activity is suggested by a naturally occurring mutation (Ala204Glu) that selectively abolishes constitutive activity without altering ghrelin-evoked activity and leads to familial short stature (18). In addition, another study reported some mutations of GHSR that impact on its constitutive GHSR activity and also lead to short stature in humans (19). Altogether, these findings suggest that constitutive GHSR activity may play a role *in vivo*, independently of ghrelin action.

The ghrelin/GHSR system helps to cope against energy deficit conditions. Thus, the relevance of the GHSR signaling becomes more evident in situations such as fasting or caloric restriction, when the ghrelin/GHSR system is up-regulated and activates a number of responses that contribute to maintain glycemia and drive food intake (20). Plasma ghrelin levels increase under fasting in both humans and rodents (21, 22). In addition, GHSR mRNA levels increase in the hypothalamus of fasted rodents (23–25), and the hypothalamic responsiveness to a GHSR agonist, measured by the induction of the marker of neuronal activation c-Fos, increases in fasted rats (26). Notably, central GHSR signaling seems to be more relevant during prolonged fasting, compared with short fasting periods, despite that similar plasma ghrelin levels are found in 24 or 48 hour fasted mice (24, 27). Under fasting conditions, ARC NPY/AgRP neurons are activated, and the ARC neurons that produce anorexigenic peptides derived of the proopiomelanocortin (POMC) precursor are inhibited (28–30). The upregulation of NPY signaling is one of the key players known to drive the compensatory hyperphagia that fasted animals display when they have access to food (9, 31). Thus, it can be hypothesized that constitutive GHSR activity at the hypothalamic level modulates the compensatory hyperphagia that follows a 48-hour fasting event. To test this hypothesis, we studied the response of mice with genetic and pharmacological manipulations of the ghrelin/GHSR system to a fasting-refeeding protocol.

Materials and Methods

Animals

This study was performed using 3- to 5-month-old male mice generated in the animal facility of either the Multidisciplinary Institute of Cell Biology (IMBICE; La Plata, Buenos Aires, Argentina) or the Centre de Psychiatrie et Neurosciences (Paris, France). Experimental mice included the following: (1) WT mice, on a pure C57BL/6 background, (2) GHSR-null mice, which do not express the GHSR (32), and (3) ghrelin-KO mice, which lack the preproghrelin gene (33). GHSR-null and ghrelin-KO mice were obtained from crosses between heterozygous animals backcrossed for >10 generations onto a C57BL/6 genetic background. Animals were maintained under controlled temperature (21°C) and photoperiod (12-hour light/dark cycle from 0600 hours to 1800 hours) with regular chow and water available *ad libitum*. For fasting-refeeding studies, mice were housed individually under the same controlled conditions and maintained on a chow diet for 1 week before the experiments. Studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council (United States) (34) and the European Communities Council Directive (86/609/European Economic Community). All experimentations received approval from the Institutional Animal Care and Use Committee of each institution.

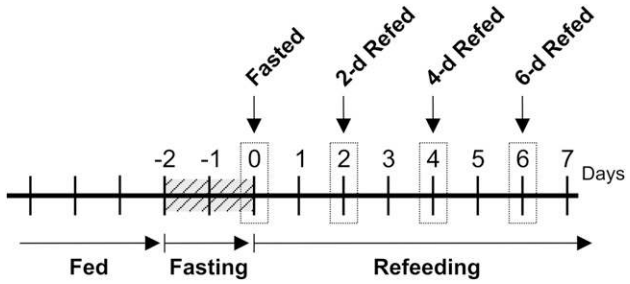

Figure 1. Overall experimental design. The figure summarizes the experimental design used in the current study. Mice were fasted by removing the chow diet from the home cages at 1000 hours and were refed 48 hours later. WT mice were exposed to the fasting-refeeding protocol and studied at the different times of refeeding indicated over the time line. Mice, with genetic manipulation of the GHSR signaling, included GHSR-null mice and ghrelin-KO mice. Mice with pharmacological manipulations of the GHSR signaling included WT mice treated with [D-Lys3]-growth hormone–releasing peptide 6 ([D-Lys3]-GHRP-6), JMV2959, or K-(D-1-Nal)-FwLL-NH_2 during the fasting period. In particular, mice were intracerebroventricular (ICV) injected every 8 hours, starting at 1600 hours of the first day of fasting and finishing at 0800 hours of the second day of fasting.

Fasting-refeeding protocol

The overall experimental design for the fasting-refeeding protocol is diagrammed in Fig. 1. Initially, individually housed WT mice were either fed *ad libitum* (n = 9) or exposed to a fasting-refeeding protocol (n = 17), in which mice were fasted by removing the chow diet from the home cages at 1000 hours and refed 48 hours later. Body weight and food intake were monitored daily and manually at 1000 hours for 7 days after refeeding. Food intake was calculated by subtracting the weight of the remaining food at 1000 hours by the weight of the initial food. In an independent study, acclimated and singly housed WT mice were either fed *ad libitum* (n = 4) or exposed to a fasting-refeeding protocol (n = 4) in cages placed in the Labmaster device (TSE Systems GmbH), which automatically monitors food intake and locomotor activity using feeding sensors and an infrared light beam-based system. In both cases, mice were monitored, at least, from the third day before fasting to the seventh day after fasting (experimental day 7).

In independent studies, WT mice were euthanized at 1000 hours, at different experimental days along the fasting-refeeding protocol. In one experiment, mice were euthanized in *ad libitum*–fed (n = 6) or 2 days fasted (n = 6) conditions (hereafter, named fed and fasted groups, respectively), as well as after 2 (n = 6) or 4 (n = 6) days of refeeding (hereafter referred to as 2-d refed and 4-d refed groups, respectively). Here, blood samples were used to quantify plasma ghrelin and desacyl-ghrelin levels, whereas their brains were used to obtain ARC punches that were, in turn, used to quantify the mRNA levels of POMC, NPY, and GHSR genes. In another experiment, fed (n = 4), fasted (n = 7), 2-d refed (n = 4), and 4-d refed (n = 4) mice were used to estimate the presence of GHSR protein using the ghrelin binding assay described later. Finally, another set of fed (n = 7), fasted (n = 8), 2-d refed (n = 6), 4-d refed (n = 6), and 6-day (6-d) refed (n = 6) mice were anesthetized and perfused with formalin to obtain their brains, which were used to perform immunostainings against NPY and POMC and estimate the levels of these food intake-regulating signals in the ARC.

Fasting-refeeding response in mice with genetic manipulations of the ghrelin system

GHSR-null mice (n = 11) and their WT littermates (n = 15) were exposed to the fasting-refeeding protocol, and their body weight and food intake were manually monitored, as described previously. In other experiment, GHSR-null mice and their WT littermates were fed *ad libitum* (n = 6 and n = 7, respectively) or 2 days fasted (n = 7 and n = 8, respectively). On the morning of the experimental day, fed and fasted mice were anesthetized and perfused with formalin to obtain their brains for immunostaining. In another study, ghrelin-KO mice (n = 5) and their WT littermates (n = 7) were exposed to the fasting-refeeding protocol, and their body weight and food intake were automatically monitored, as described previously.

Fasting-refeeding response in mice with pharmacological manipulations of the GHSR signaling

For central infusion of drugs, mice were first stereotaxically implanted with a single indwelling guide cannula into the lateral ventricle [intracerebroventricular (ICV); placement coordinates: anteroposterior: −0.34, lateral: +1.0, and ventral: −2.3 mm]. After surgery, mice were individually housed and allowed to recover for at least 5 days. To block pharmacologically ghrelin-induced GHSR activation, WT mice were ICV treated during the fasting period with [D-Lys3]-growth hormone–releasing peptide 6 ([D-Lys3]-GHRP-6; catalog no. SLBN1014V; Sigma-Aldrich) or JMV2959 [synthesized as previously described (35)]. In particular, mice were treated with vehicle (artificial cerebrospinal fluid) alone (n = 10) or containing [D-Lys3]-GHRP-6 (2 nmol/mouse, n = 5) or JMV2959 (3 nmol/mouse, n = 8) every 8 hours, starting at 1600 hours of the first day of fasting and finishing at 0800 hours of the second day of fasting. Thus, each mouse received six ICV injections. Mice were refed at 1000 hours, and body weight and food intake were manually monitored during refeeding, as described previously. The dose of [D-Lys3]-GHRP-6 was chosen based on a previous study (36) and on our own work, which showed that it reduced by 42.4% ± 4.0% the 2-hour food intake induced by 0.02 nmol/mouse of ICV-injected ghrelin. The dose of JMV2959 was chosen based on a previous study (37) and on our own work, which showed that it reduced a 2-hour food intake induced by ICV-injected ghrelin (0.02 nmol/mouse) by 72.0% ± 12.8%. To block pharmacologically constitutive GHSR signaling, WT mice were ICV treated with K-(D-1-Nal)-FwLL-NH_2 during the fasting period. The K-(D-1-Nal)-FwLL-NH_2 is an inverse agonist that was synthesized by automated solid-phase peptide synthesis, as described elsewhere (16). Mice were treated with artificial cerebrospinal fluid alone (n = 12) or containing K-(D-1-Nal)-FwLL-NH_2 (1 nmol/mouse, n = 13) every 8 hours during the fasting period, as described previously. Mice were refed at 1000 hours, and body weight and food intake were manually monitored during the refeeding period. The dose of K-(D-1-Nal)-FwLL-NH_2 was chosen based on a previous study (16) and on our own work, which showed the following: that (1) it significantly reduced *ad libitum* food intake in the early dark-phase period (from 1800 hours to 2300 hours) when injected at 1800 hours (160.0 ± 41.3 mg vs 669.0 ± 123.7 mg, respectively) and (2) it reduced 2 hour food intake induced by ICV-injected ghrelin (0.02 nmol/mouse) by 81.0% ± 5.6%. Importantly,

the same dose of K-(D-1-Nal)-FwLL-NH_2 did not reduce *ad libitum* food intake in GHSR-null mice during the early dark-phase period when injected at 1800 hours (n = 5 each). Importantly, mice exposed to pharmacological manipulations in GHSR signaling 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.

Determination of plasma ghrelin levels

Ghrelin and desacyl-ghrelin plasma concentrations were assayed by specific enzyme immunoassay (A05118 and A05117, respectively; Bertin Pharma). Blood samples were collected on EDTA (1 mg/mL final) and *p*-hydroxy-mercuribenzoic acid (0.4 mM final). Then, plasmas were immediately acidified with HCl (0.1 N final) to preserve acylation and stored frozen at −80°C.

Quantification of mRNA levels in hypothalamic punches

Brains were extracted, placed in cold diethylpyrocarbonate phosphate-buffered saline, and sectioned into 1 mm coronal slices by use of a mouse brain matrix. Punches of tissue corresponding to the location of the ARC, identified by comparing the coronal slices with a mouse brain atlas (38), were excised using a 15-gauge needle. Punches were collected in TRIzol reagent (Invitrogen), and total RNA was isolated, according to the manufacturer's protocol. The concentration and purity of RNA were estimated in a NanoDrop Lite Spectrophotometer (Thermo Scientific). One microgram of total RNA from each region was reverse transcribed into cDNA using random hexamer primers and Moloney murine leukemia virus reverse transcription (Promega). Quantitative polymerase chain reaction for NPY, POMC, and GHSR was performed in triplicate with HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne), using a real-time polymerase chain reaction system StepOne Cycler (Applied Biosystems). Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Fold change from fed values was determined using the relative standard curve method, normalizing the expression to the ribosomal protein L19 (reference gene). Primer sequences for NPY were sense: 5′-GCCAGATACTACTCCGCTCTG-3′, antisense: 5′-GATCTCTTGCCATATCTCTGTCTG-3′ (GenBank accession no. NM_023456.3), product size 68 bp. Primer sequences for POMC were sense: 5′-CCTCCTGCTTCAGACCTCCATA-3′, antisense: 5′-TGTTCATCTCCGTTGCCTGG-3′ (GenBank accession no. NM_008895.3), product size 159 bp. Primer sequences for GHSR were sense: 5′-GCTCTGCAAACTCTTCCA-3′, antisense: 5′-AAGCAGATGGCGAAGTAG-3′ (GenBank accession no. NM_177330.4), product size 99 bp. Primer sequences for ribosomal protein L19 were sense: 5′-AGCCTGTGACTGT-CCATTCC-3′, antisense: 5′-TGGCAGTACCCTTCCTCTTC-3′ (GenBank accession no. NM_009078.2), product size 99 bp.

Immunohistochemistry

As previously described (39), brains of perfused mice were removed, postfixed, immersed in 20% sucrose, and cut coronally at 40 μm into three equal series on a sliding cryostat. To perform immunohistochemistry, sections were pretreated with 0.5% H_2O_2, treated with blocking solution (3% normal donkey serum and 0.25% Triton-X), and incubated with a rabbit anti-NPY (Table 1), a rabbit anti-c-Fos (Table 1), or a rabbit anti-POMC antibody (Table 1) for 48 hours at 4°C. Next, all sections were incubated with a biotinylated goat anti-rabbit antibody (catalog no. BA-1000; 1:3000; Vector Laboratories) and then with the Vectastain Elite ABC (catalog no. PK-6200; Vector Laboratories), according to the manufacturer's protocols. Finally, a visible signal was developed with diaminobenzidine (DAB)/nickel solution for NPY and c-Fos immunostainings, giving a black/purple precipitate and only with DAB for POMC immunostaining. Negative controls were also performed using the same procedure for each immunostaining but omitting either the primary antibody or secondary antibody. Sections were sequentially mounted on glass slides and coverslipped with mounting media.

Assessment of ghrelin-binding sites

A fluorescein-ghrelin(1–18) (hereafter referred to as F-ghrelin) tracer, provided by Dr. Luyt (University of Western Ontario, Canada) was used. F-Ghrelin is an 18-residues analog of the hormone with a fluorescein moiety attached at its C terminus. F-Ghrelin behaves similarly to endogenous ghrelin in terms of GHSR affinity and specificity (40, 41). Here, anesthetized mice were stereotaxically implanted with a single indwelling sterile guide cannula into the lateral ventricle and injected with F-ghrelin (60 pmol/mouse). Mice were perfused with formalin, 30 minutes after treatment, as described previously. Brains were processed, as described previously to generate coronal brain sections, which were then used for immunostaining against fluorescein using a goat anti-fluorescein antibody (Table 1) for 48 hours at 4°C. Then, sections were treated with a biotinylated donkey anti-goat antibody (catalog no. BA-5000; 1:1500; Vector Laboratories) and then with the Vectastain Elite ABC kit, according to the manufacturer's protocols. Finally, a visible signal was developed with DAB/nickel solution, giving a black/purple precipitate. Negative controls were also performed using the same procedure but omitting the primary or secondary antibodies. Sections were sequentially mounted on glass slides and coverslipped with mounting media.

Table 1. Antibodies Used

Peptide/Protein Target	Name of Antibody	Manufacturer, Catalog No.	Species Raised in (Polyclonal)	Dilution Used	RRID
NPY	Anti-NPY	Abcam, ab30914	Rabbit	1/30,000	AB_1566510
c-Fos	Anti-c-Fos (H-125)	Santa Cruz Biotechnology, sc7202	Rabbit	1/2000	AB_2106765
Fluorescein/Oregon Green	Anti-fluorescein	Molecular Probes, A-11096	Goat	1/1500	AB_221558
POMC 27–52	Anti-POMC	Phoenix Pharmaceuticals, H-029-30	Rabbit	1/6000	AB_2307442

Abbreviation: RRID, Research Resource Identifier.

Quantitative analysis

Low- and high-magnification bright-field images were acquired with a Nikon Eclipse 50i and a DS-Ri1 Nikon digital camera. Quantifications were bilaterally performed in digital high-magnification images of one complete series of coronal ARC sections between bregma −1.58 and −2.06 mm, using the anatomical limits, according to the mouse brain atlas (38). Total NPY-immunoreactive (IR) cells, POMC-IR cells, and c-Fos-IR cell nuclei were quantified, and data were expressed as IR cells per side. Fluorescein-IR signal was quantified as either positive cells or punctuates; these data were expressed as IR cells per coronal section per side or IR-punctas/100 μm^2. All data were corrected for double counting, according to the method of Abercrombie (42). The mean diameter of the positive signal was determined using Fiji. Blind quantitative analysis was performed independently by at least two observers.

Statistical analyses

Data are expressed as the means ± standard error of the mean (SEM). Equality of variance was analyzed using Bartlett or Levene tests. When variances were equal, one-way analysis of variance (ANOVA), followed by the Tukey test, was used. When variances significantly differed, one-way ANOVA, followed by the Games–Howell test was used. Unpaired *t* test with Welch correction was performed to compare cumulative food intake data of WT mice, ICV treated with each pharmacological manipulation and WT vs either GHSR-null or ghrelin-KO mice. Two-way ANOVA, followed by the Bonferroni test, was used to compare data from fasting-refeeding responses of WT, GHSR-null, ghrelin-KO, and WT ICV-treated mice. Differences were considered significant when $P < 0.05$.

Results

Daily food intake is affected in a long-term fashion after an event of fasting

First, we quantified food intake and body weight responses of WT mice under the fasting-refeeding protocol. Daily food intake was 3.43 ± 0.07 g/day in *ad libitum*–fed WT mice. As compared with fed mice, daily food intake of refed mice significantly increased and remained elevated until day 5 of refeeding [Fig. 2(a)]. Cumulative food intake from day 1 to day 5 of refeeding was 24.07 ± 0.58 g, which represented an average of 3.48 ± 0.90 g/day for the overall period of fasting plus the 5 days of refeeding. The body weight of fasted mice significantly decreased at the end of the fasting period, compared with fed mice, and then fully recovered by day 3 of refeeding [Fig. 2(b)]. An independent set of WT mice tested in an automatized system showed an increase in the daily food intake that was also significantly higher until day 5 of refeeding, similarly as seen using the manual method. The automatized system, however, showed that most of the daily food intake of *ad libitum*–fed mice occurred in the dark cycle (86.2% ± 0.6% of the total daily food intake) and that such pattern was unchanged through the study [Fig. 2(c)]. In refed mice, daily food intake not only increased but also changed its daily pattern [Fig. 2(c)]. As compared with food intake of fed mice, light-phase food intake of refed mice significantly increased the first 6 days of refeeding [Fig. 2(d)], whereas dark-phase food intake of refed mice significantly decreased in the same period of time [Fig. 2(e)].

ARC GHSR levels increased after an event of fasting

We then studied the impact of the fasting-refeeding protocol on plasma ghrelin levels and hypothalamic GHSR expression. As expected, plasma ghrelin and desacyl ghrelin levels were 1.9 ± 0.5- and 1.8 ± 0.3-fold higher in fasted mice compared with fed mice [Fig. 3(a) and (b)]. Plasma ghrelin levels decreased at 2 days of refeeding compared with levels detected in fasted mice. GHSR mRNA levels in the ARC were 3.1 ± 0.6-fold higher in fasted mice, compared with fed mice, and then decreased in refed mice [Fig. 3(c)]. To estimate the amount of GHSR protein, we used a F-ghrelin binding assay that provides distinct cell body-like and punctate labelings. The number of cell bodies binding F-ghrelin in the ARC was similar in all experimental groups (not shown). However, the density of F-ghrelin binding punctate in the ARC of fasted mice was 3.8 ± 0.6-fold higher compared with *ad libitum*–fed mice. The density of F-ghrelin binding punctate in the ARC decreased after 2 days of refeeding, compared with the amount found in fasted mice, but remained 2.3 ± 0.2-fold higher than the density found in the ARC of fed mice. The density of F-ghrelin binding punctate in the ARC at 4 days of refeeding was similar to the values found in fed mice [Fig. 3(d) and (e)].

As NPY is a key target of GHSR signaling in the ARC, we studied the effect of the fasting-refeeding protocol on the biosynthesis of this NPY. ARC NPY mRNA levels were 11.1 ± 2.9-fold higher in fasted mice compared with fed mice [Fig. 4(a)]. After 2 days of refeeding, ARC NPY mRNA levels remained 6.4 ± 1.1-fold higher than in fed mice. After 4 days of refeeding, ARC NPY mRNA levels were similar to fed mice. In terms of NPY peptide, most of the NPY-IR signal was observed with a dendritic localization, and few NPY-IR cell bodies were identified in the ARC of fed mice. The number of NPY-IR cells significantly increased in the ARC of fasted mice (46.4 ± 4.3-fold higher compared with fed mice) and then decreased after 2 and 4 days of refeeding, compared with numbers detected in fasted mice, but remained 23.5 ± 3.7- and 7.7 ± 1.4-fold higher, respectively, compared with the values detected in fed mice [Fig. 4(b) and (c)]. After 6 days of refeeding, the number of NPY-IR cells in the ARC was similar to the number found in fed mice. POMC mRNA levels and the number of POMC-IR cells in the ARC were 0.5 ± 0.1- and 0.5 ± 0.1-fold smaller, respectively, in fasted mice compared with fed mice [Fig. 4(d)–(f)]. However, POMC mRNA levels and the number POMC-IR cells in the ARC were not statistically different after 2 or more days of refeeding compared with fed mice.

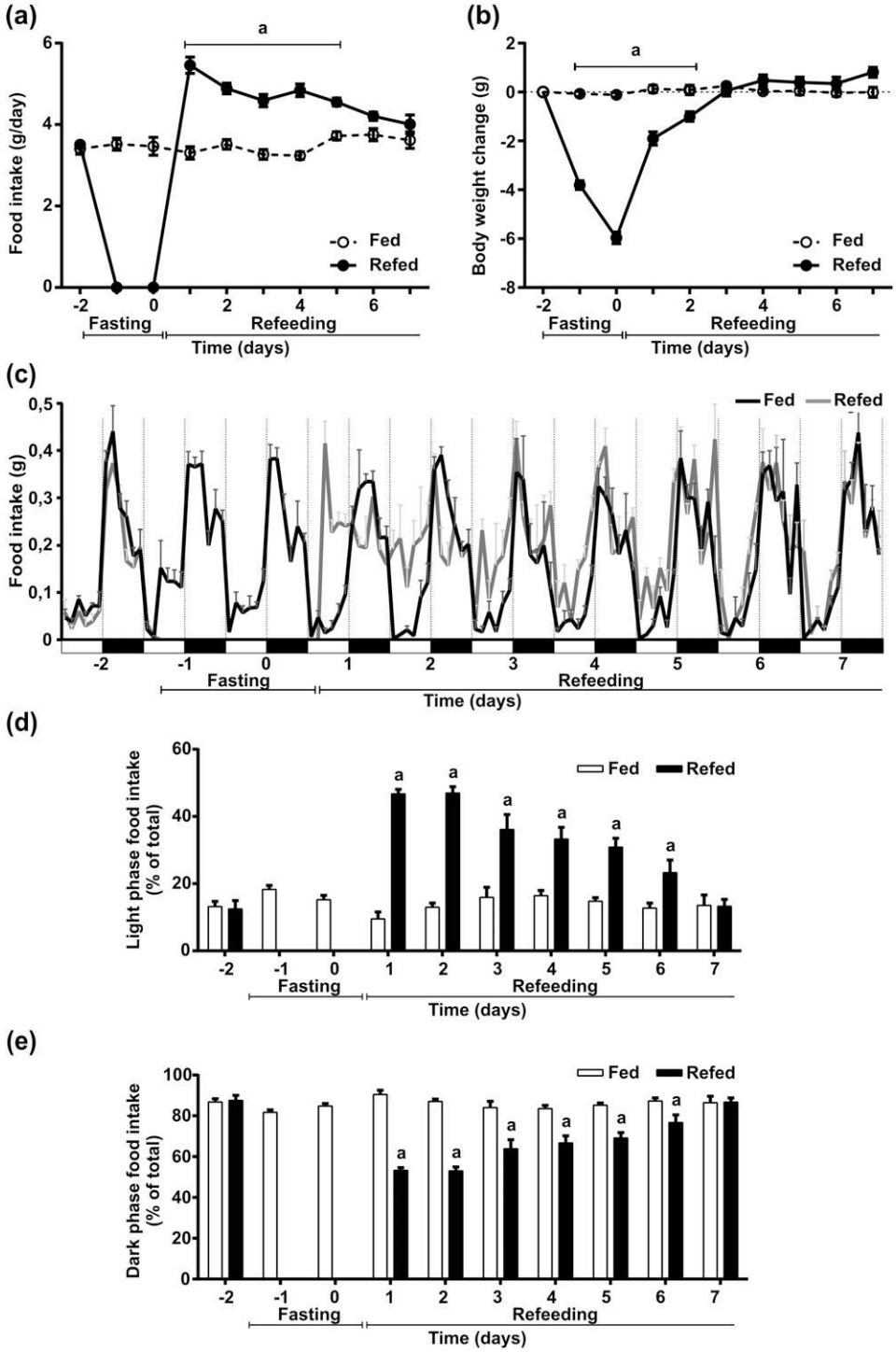

Figure 2. Daily food intake is affected in a long-term fashion after an event of fasting. (a and b) Changes in food intake and body weight, respectively, of WT mice that were maintained with *ad libitum* access to regular chow (n = 9) or fasted for 2 days and then allowed free access to food at 1000 hours (n = 17). (c) The daily feeding pattern monitored using an automated feeding/activity station (TSE system, GmbH). Light and dark phases are denoted by white and black rectangles on the *x*-axis. (d and e) Percentage of light- and dark-phase feeding, respectively (n = 4 for *ad libitum*–fed mice and n = 4 for refed mice). Data represent the means ± SEM and were compared by two-way ANOVA. a, $P < 0.05$ vs *ad libitum*–fed mice on the same day.

GHSR signaling is required for a full compensatory hyperphagia

Then, we studied the food-intake response of GHSR-null mice to the fasting-refeeding protocol. Daily food intake of refed GHSR-null mice significantly increased and remained elevated until day 5 of refeeding, as seen in WT mice [Fig. 5(a)]. However, cumulative food intake in GHSR-null mice between days 1 and 5 of refeeding was decreased by 14.4% ± 3.5% compared with WT mice [Fig. 5(b)]. Body weights of WT and GHSR-null mice did

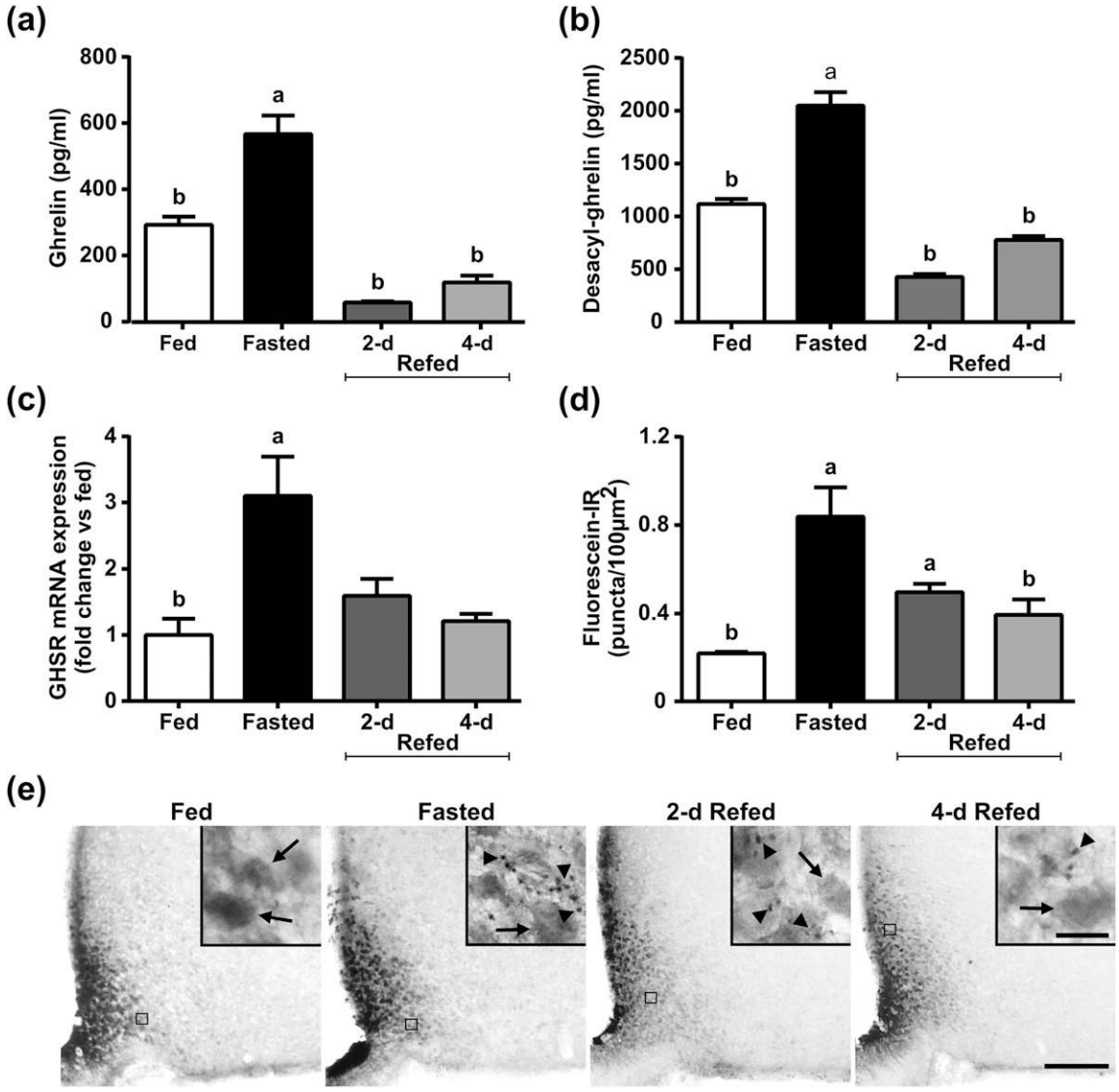

Figure 3. ARC GHSR binding sites but not circulating ghrelin remain increased several days after an event of fasting. (a and b) Plasma ghrelin and desacyl-ghrelin levels evaluated by specific enzyme immunoassay, respectively (n = 6 for each experimental group). (c) Comparative values of GHSR mRNA, relative to the ribosomal protein L19 gene, in ARC punches obtained from each experimental group (n = 6 per group). (d) Bar graph displaying the quantitative analysis of the number of fluorescein-IR puncta per area unit in the ARC of each experimental group. (e) Representative photomicrographs of ARC coronal sections of ICV F-ghrelin-treated mice (n = 4–7 per group) subjected to chromogenic immunostaining against fluorescein. Insets in each image show high magnification of areas marked in low-magnification images. Arrows and arrowheads point to fluorescein-IR somas and puncta, respectively. Original scale bars, 100 and 10 μm for the low- and high-magnification images, respectively. Data represent the means ± SEM and were compared by one-way ANOVA. a, $P < 0.05$ vs *ad libitum*–fed mice; b, $P < 0.05$ vs fasted mice.

not differ throughout the experiment [Fig. 5(c)]. An independent set of WT and GHSR-null mice exposed to fasting showed that the number of both NPY-IR cells and c-Fos-IR cells was significantly reduced in the ARC of fasted GHSR-null mice compared with the numbers found in the ARC of fasted WT mice [Fig. 5(d)–(g)].

Constitutive, but not ghrelin-evoked, GHSR signaling is required for a full compensatory hyperphagia

Then, we studied the response to the fasting-refeeding protocol of mice with other manipulations of the ghrelin system. We found that daily food intake, as well as the compensatory hyperphagia, after the fasting period of ghrelin-KO mice [Fig. 6(a) and (b)] and of WT mice treated with the GHSR antagonists [D-Lys3]-GHRP-6 or JMV2959 [Fig. 6(c) and (d)] was similar compared with the control group. The daily food intake of refed WT mice treated with the GHSR inverse agonist K-(D-1-Nal)-FwLL-NH_2 significantly increased and remained elevated until day 5 of refeeding, but the cumulative food intake between days 1 and 5 was significantly decreased by 14.8% ± 3.8% in K-(D-1-Nal)-FwLL-NH_2-treated mice compared with vehicle-treated mice [Fig. 6(e) and (f)]. In an independent set of mice treated with either

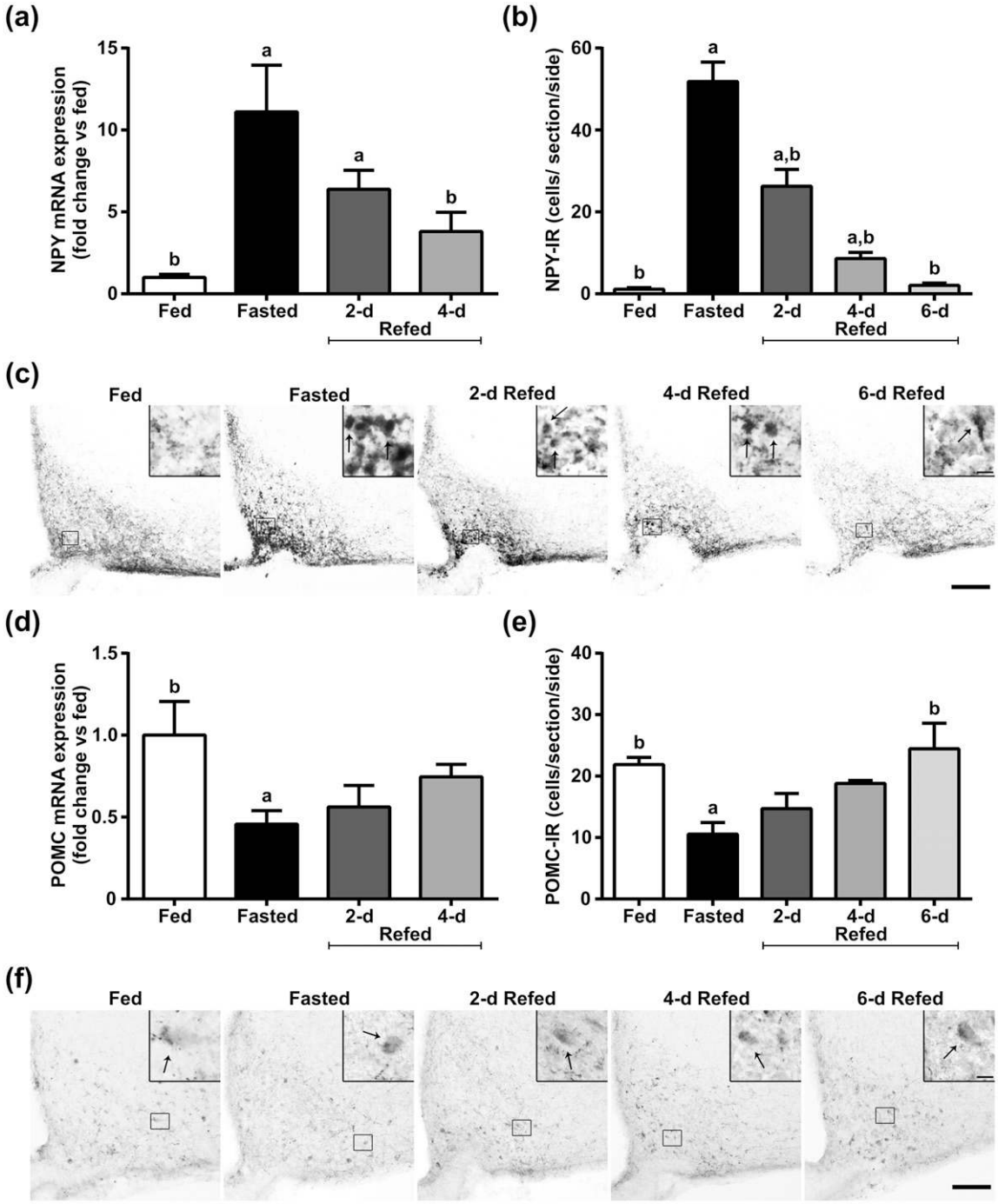

Figure 4. ARC NPY levels increased and POMC levels decreased after an event of fasting. (a and d) Comparative values of NPY and POMC mRNA, respectively, relative to the ribosomal protein L19 gene, in ARC punches obtained from each experimental group (n = 6 per group). (c and f) Representative photomicrographs of ARC coronal sections of mice in each experimental group (n = 6–8 per group) subjected to chromogenic immunostaining against NPY and POMC, respectively. Insets in each image show high magnification of areas marked in low-magnification images. Arrows point to NPY- or POMC-IR cells. Original scale bars, 100 and 10 μm for the low- and high-magnification images, respectively. (b and e) Bar graphs displaying the quantitative analysis of the number of NPY and POMC cells, respectively, in the ARC of each experimental group. Data represent the means ± SEM and were compared by one-way ANOVA test. a, $P < 0.05$ vs *ad libitum*–fed mice; b, $P < 0.05$ vs fasted mice.

vehicle or K-(D-1-Nal)-FwLL-NH_2 during the fasting period, the number of both NPY-IR and c-Fos-IR cells in the ARC was significantly reduced in fasted mice treated with K-(D-1-Nal)-FwLL-NH_2 compared with the numbers found in fasted mice treated with vehicle [Fig. 6(g)–(j)].

Discussion

In vitro studies, using heterologous expression systems or lipid discs, have shown that GHSR displays an unusually high constitutive activity that signals with ~50% of its maximal capacity in the absence of ghrelin (11, 43–45). In

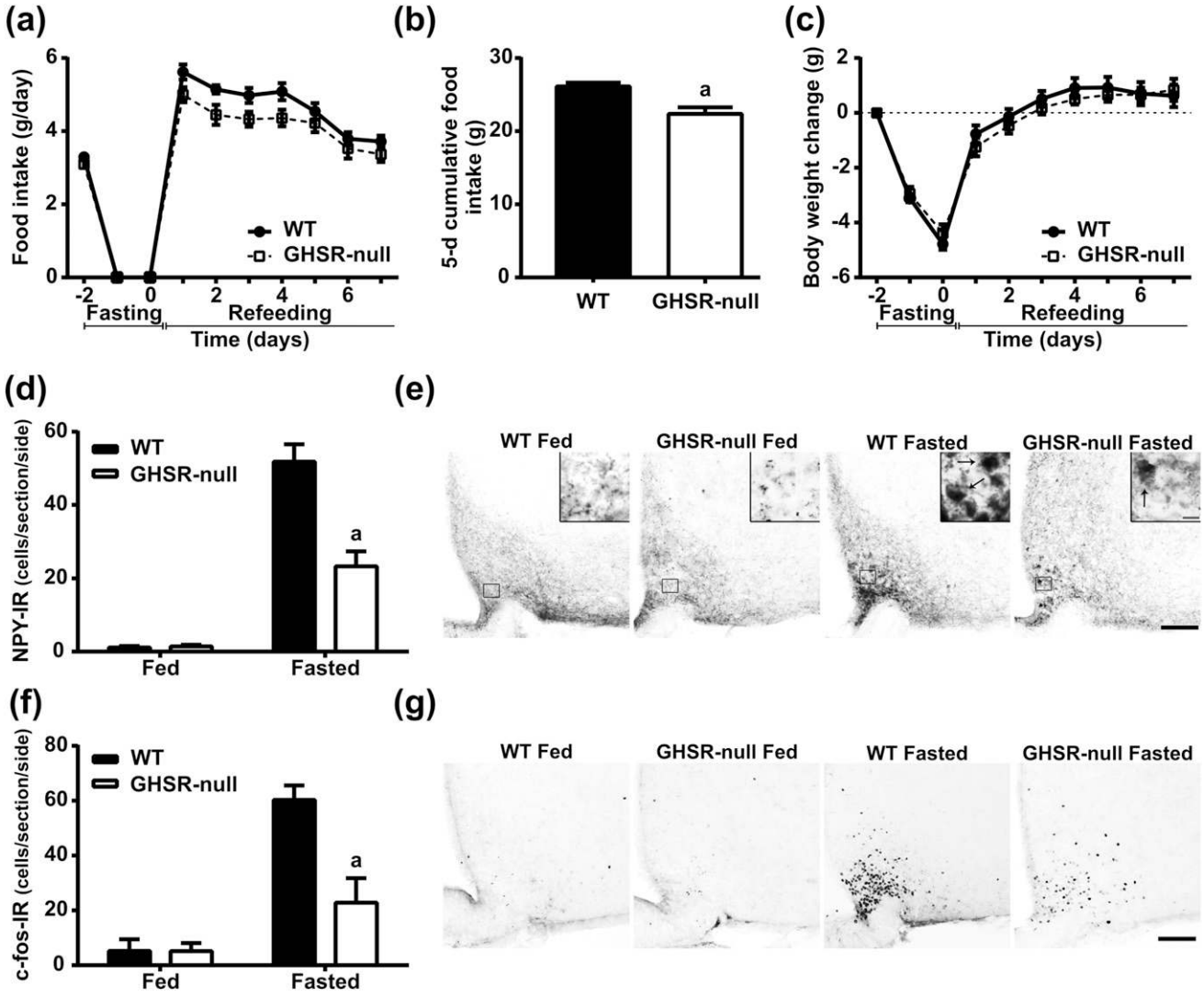

Figure 5. GHSR signaling is required to display a full fasting-induced hyperphagia. (a and c) Changes in food intake and body weight, respectively, of WT (n = 15) and GHSR-null (n = 11) mice during 2 days of fasting and refeeding, as described in Fig. 1. Data represent the means ± SEM and were compared by two-way ANOVA. (b) The 5-day cumulative food intake during refeeding of WT and GHSR-null mice, respectively. Data represent the means ± SEM and were compared by unpaired *t* test with Welch correction. a, $P < 0.05$ vs WT mice. (e and g) Representative photomicrographs of ARC coronal sections of WT and GHSR-null mice, respectively, belong to *ad libitum*–fed and fasted groups, subjected to chromogenic immunostaining against NPY and c-Fos, respectively. Arrows point to NPY-IR cells. Original scale bars, 100 and 10 μm for the low- and high-magnification images, respectively. (d and f) Bar graphs displaying the quantitative analysis of the number of NPY-IR and c-Fos-IR cells, respectively, in the ARC of each experimental group. Data represent the means ± SEM and were compared by two-way ANOVA. a, $P < 0.05$ vs WT mice under fasting conditions.

addition, the constitutive GHSR activity has been shown to affect growth hormone secretion in human somatotroph adenomas *in vitro* (12). Whether GHSR displays *in vivo* ghrelin-independent signaling is unknown; however, some naturally occurring GHSR mutations that impair its constitutive activity have been linked to short stature in human populations, suggesting that constitutive GHSR activity may be physiologically relevant (18, 19). As indicated in the Introduction, some pharmacological or genetic manipulations of the ghrelin/GHSR system in mouse models also provided circumstantial evidence that the constitutive GHSR activity plays a role in regulating body weight and/or food intake. However, observations have been subtle and inconsistent in *ad libitum*–fed conditions. Thus, the relevance of ghrelin-independent GHSR signaling on food intake regulation has remained uncertain. It is well established that the ghrelin/GHSR system becomes more relevant under conditions of energy deficit, such as 48-hour fasting, a situation where plasma ghrelin levels, as well as the hypothalamic expression of GHSR, are increased (23, 24). Notably, hypothalamic GHSR mRNA levels, as well as the sensitivity to ghrelin, seem to be higher in mice fasted for longer periods of time (24, 27). Thus, we reasoned the following: that (1) the central GHSR signaling is likely to be increased when hypothalamic GHSR expression levels are high and (2)

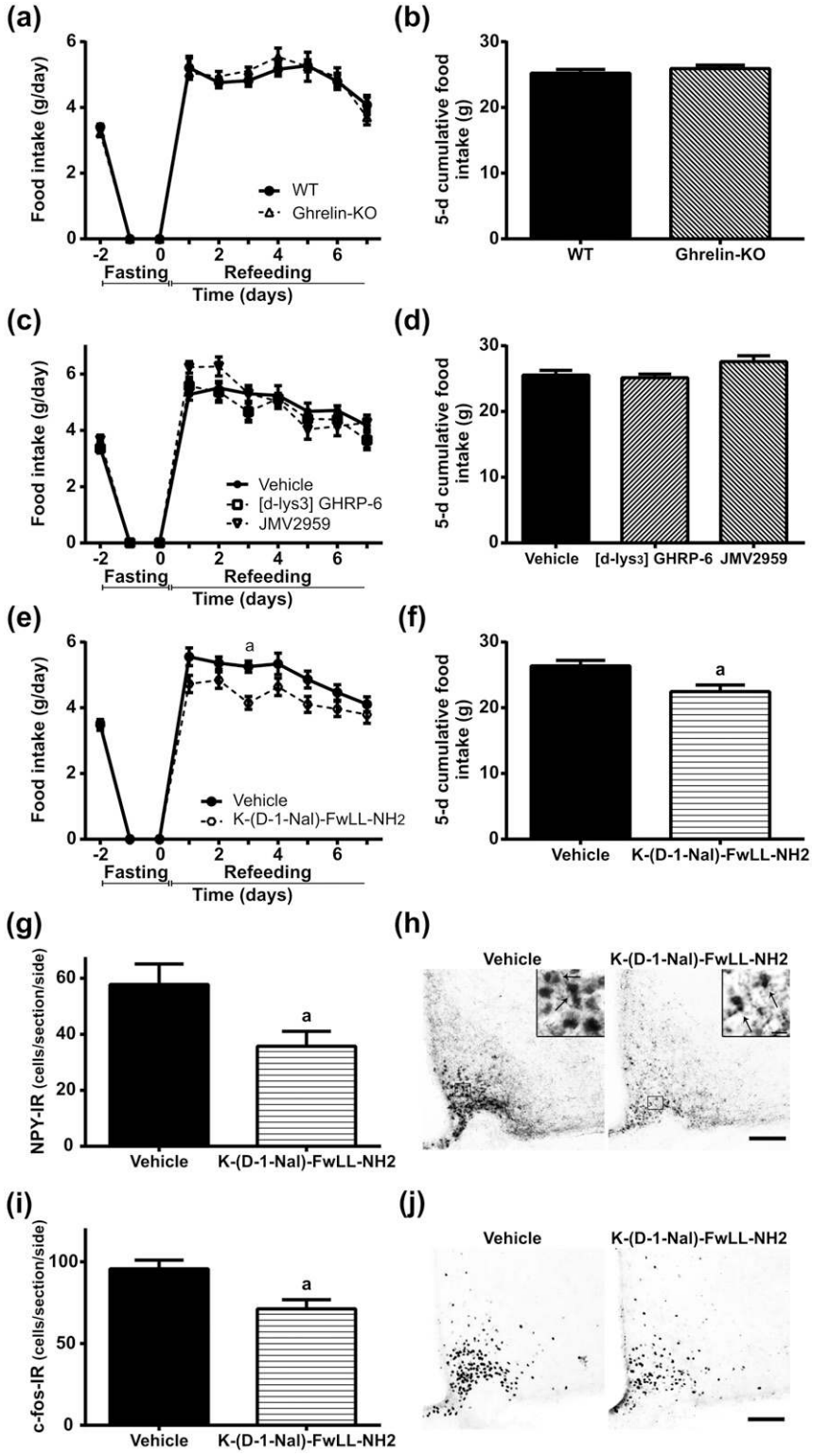

Figure 6. Central administration of the GHSR inverse agonist K-(D-1-Nal)-FwLL-NH_2, but not genetic deficiency of ghrelin or GHSR antagonists, impairs the fasting-induced hyperphagia. (a, c, and e) Changes in food intake of WT (n = 7) or ghrelin-KO (n = 5) mice; WT mice ICV treated with vehicle (n = 7) or with the GHSR antagonists [D-Lys]-GHRP-6 (n = 5) or JMV2959 (n = 8); and WT mice ICV treated with vehicle (n = 12) or with the GHSR inverse agonist K-(D-1-Nal)-FwLL-NH_2 (n = 13), respectively. Mice were fasted and refed as described in Fig. 1. Data represent the means ± SEM and were compared by two-way ANOVA. (b, d, and f) The 5-day cumulative food intake during refeeding of WT or ghrelin-KO mice; WT mice ICV treated with vehicle, [D-Lys]-GHRP-6, or JMV2959; and WT mice ICV treated with vehicle or with K-(D-1-Nal)-FwLL-NH_2, respectively. Data represent the means ± SEM and were

the refeeding period that follows a 48-hour fasting event would be an interesting condition to unmask a potential effect of constitutive GHSR signaling on food intake regulation.

Here, we confirmed that mice display several days of hyperphagia after an event of fasting and then return to basal food-intake levels, as previously reported (46, 47). Interestingly, the extra amount of calories that refed mice ingested during the fasting-induced hyperphagia period exactly matched the amount of calories that mice did not consume during the fasting period. Thus, the compensatory hyperphagia seems to depend on homeostatic aspects of feeding, which drive food intake according to energy balance. Notably, ghrelin levels increased in fasted mice and quickly returned to basal levels when the fasting state was over, as expected based on the short half-life of circulating ghrelin (48). Indeed, ghrelin levels in fasted rats return to basal levels a few hours after refeeding (22, 49, 50). GHSR mRNA levels have been shown to be increased in the ARC of fasted mice and rats (44, 51). A more recent study showed that GHSR mRNA levels are ~3.4-fold increased, specifically within ARC NPY/AgRP neurons (25, 52). In line with these observations, the ghrelin-induced increase in the number of c-Fos-IR cells in the ARC has been shown to be higher in fasted animals (26). Here, we not only confirmed that ARC GHSR mRNA levels increased in fasted mice but also showed that ghrelin binding increased in the ARC of these animals, suggesting that gene transcription results in GHSR protein biosynthesis. We did not detect an increase in the number of ghrelin binding cells in the ARC of fasted mice, but rather, we detected an increase in the presynaptic bouton-like shape-labeling in this hypothalamic region. Ghrelin binding has been already shown in the NPY and γ-aminobutyric acid synaptic terminals within the ARC and its targets (*e.g.*, the

hypothalamic paraventricular nucleus), respectively, where it regulates neurotransmitter release via the regulation of presynaptic calcium channels (28, 44, 53, 54). Here, we found that the NPY-IR signal displayed a dendritic localization in the ARC of fed mice but had both a dendritic and a cell body localization in the ARC of fasted mice. These observations, together with the fact that the NPY mRNA levels were increased in the ARC of fasted mice, suggest that the NPY biosynthesis is increased during fasting. In parallel, POMC mRNA levels and the number of POMC-IR cells were decreased in the ARC of fasted mice. Thus, it can be hypothesized that the fasting-induced increase in GHSR signaling promotes ARC NPY/AgRP neuron activation, which in turn, inhibits the ARC POMC neurons (28). Interestingly, ARC GHSR mRNA levels returned to basal levels at day 2 of refeeding, whereas the density of ghrelin binding sites in the ARC remained elevated at this time point and returned to basal levels at day 4 of refeeding. In addition, we found that the NPY levels remained elevated at days 2 and 4 of refeeding, whereas POMC levels returned to fed levels after refeeding. To our knowledge, the *in vivo* half-lives of the GHSR mRNA and protein have not been estimated. As the half-life for other GPCRs, such as the κ-opiod receptor, has been estimated to several days (55), it seems reasonable to hypothesize that GHSR mRNA gene expression in the ARC returns to basal levels when the fasting state is over, whereas the GHSR protein remains present in NPY/AgRP neurons for a longer time period. Such increments of GHSR levels in ARC NPY/AgRP neurons at the first days of refeeding would impact NPY biosynthesis and as a consequence, feeding behavior.

To test if the behavioral and neuronal changes detected at the first days of refeeding require GHSR signaling, we studied GHSR-deficient mice. Mice lacking GHSR displayed a smaller hyperphagia during the refeeding period compared with WT mice. In parallel, the increments of the NPY levels and the neuronal activation, as indicated by c-Fos signal, in the ARC of fasted GHSR-null mice were also smaller compared with fasted WT mice. These observations indicate that GHSR signaling is required to display the full compensatory hyperphagia that follows a fasting event. Remarkably, GHSR-null mice displayed fasting-induced hyperphagia and showed a subtle decrease in their total cumulative food intake during the refeeding period (~15%) without substantial differences in body weight compared with refed WT mice. Such results are not unexpected, as a result of inherent redundancies in the mechanisms responsible for body weight homeostasis-related food intake in which many hormonal [*e.g.*, leptin (46)] and metabolic [e.g. glucose (56)] systems are involved (57). It is interesting to note that stress strongly influences eating behaviors and that ghrelin signaling also modulates the response to stress (39, 58, 59). Thus, the smaller fasting-induced hyperphagia observed in GHSR-null mice may be impacted by a different susceptibility to fasting-induced stress of these animals. Further studies are required to investigate this aspect of the study in detail. Notably, a previous study did not detect differences in food intake of WT and GHSR-KO mice exposed to a fasting-refeeding paradigm similar to the one used in the current study (60). However, the study to which we refer was performed with mice previously fed a high-fat diet that weighed ~40 to 45 g, whereas we used young mice ranging from 22 to 25 g of body weight, fed with regular chow diet. As obese and/or aged mice are less prone to adapt to metabolic changes (61), it is likely that such differences may explain the diverse outcomes of the studies.

Mice lacking the ghrelin gene and mice centrally treated with two unrelated GHSR antagonists displayed a full compensatory hyperphagia after a fasting event. Some, but not all, previous studies using different pharmacological strategies (*e.g.*, anti-ghrelin RNA spiegelmers, anti-ghrelin antibodies, GHSR antagonists) have shown that the action of endogenous ghrelin is required for the fasting-induced hyperphagia (8, 62–66). The reason for these discrepancies is unknown, although differences among experimental designs (*e.g.*, dose, administration protocols) are likely among the critical factors that impact the results. To our knowledge, no adverse effects have been reported for the tested GHSR ligand; however, toxicity is always a concern when drugs are centrally administered. For the current study, pharmacological tests were carefully set up, and mice were treated with the maximum dose of GHSR antagonists that did not induce any sign of sickness-like behavior to avoid nonspecific effects of the compounds on food intake. Under these experimental conditions, GHSR antagonists successfully, but partially, blocked the orexigenic effect of exogenously administered ghrelin. The fact that not only two completely unrelated GHSR antagonists but

Figure 6. (Continued). compared by unpaired *t* test with Welch correction. a, $P < 0.05$ vs WT mice ICV treated with vehicle. (h and j) Representative photomicrographs of ARC coronal sections of WT mice ICV treated with vehicle or K-(D-1-Nal)-FwLL-NH_2 during the fasting period, subjected to chromogenic immunostaining against NPY and c-Fos, respectively. Arrows point to NPY-IR cells. Original scale bars, 100 and 10 μm for the low- and high-magnification images, respectively. (g and i) Bar graphs displaying the quantitative analysis of the number of NPY-IR cells and c-Fos-IR cells, respectively, in the ARC of each experimental group. Data represent the means ± SEM and were compared by unpaired *t* test with Welch correction. a, $P < 0.05$ vs WT mice ICV treated with vehicle.

also the genetic deficiency of endogenous ghrelin failed to affect the compensatory hyperphagia after a fasting event suggests that the ghrelin-evoked GHSR activation was not required for such behavior. Interestingly, we found that mice centrally treated with K-(D-1-Nal)-FwLL-NH_2 displayed a smaller compensatory hyperphagia after fasting compared with mice treated with vehicle. Importantly, the extent to which this pharmacological treatment impacted hypothalamic, constitutive GHSR activity cannot be directly measured; however, the observation that WT mice treated with K-(D-1-Nal)-FwLL-NH_2 and GHSR-null mice displayed a similar reduction of the fasting-induced hyperphagia suggests that this GHSR inverse agonist fully abrogated the receptor activity. It is interesting to stress that fasted mice were centrally treated with K-(D-1-Nal)-FwLL-NH_2 exclusively during the fasting period and that they displayed smaller increments of both NPY levels and neuronal activation in the ARC at the end of the fasting period. Thus, GHSR activity seems to play a role during both fasting and refeeding periods. The fact that K-(D-1-Nal)-FwLL-NH_2 did not affect the food intake of GHSR-null mice strongly indicated that the effects were specific. Notably, K-(D-1-Nal)-FwLL-NH_2 partially blocked ghrelin-induced food intake (see Materials and Methods), suggesting that this GHSR ligand may also affect the ghrelin-evoked GHSR activation; however, the observations that the compensatory hyperphagia after fasting was not affected in ghrelin-KO mice and in mice treated with GHSR antagonists suggest that this eating behavior is independent of the action of ghrelin.

Given the high constitutive activity of GHSR, both antagonism and inverse agonism properties should be taken in consideration when testing GHSR ligands as potential drugs for clinical use. Here, we found evidence that the decrease in GHSR signaling in mice mitigates the hyperphagia that follows an event of food deprivation. Further studies are necessary to test if changes in GHSR signaling also contribute to the mechanisms controlling long-term body weight after chronic caloric restriction. Notably, a recent study showed that the suppression of ghrelin signaling in obese mice prevents postdieting body weight rebound, a problem commonly observed in dieters (67). Thus, treatments capable of blocking constitutive GHSR signaling may help to maintain a reduced calorie intake after dieting and contribute to the long-term management of obese patients (68). Further studies are required to test if the suppression of ghrelin signaling is also useful to treat other eating disorders that have been linked to ghrelin signaling, such as binge eating (69, 70). Importantly, an oral GHSR inverse agonist, named PF-05190457, has been recently developed and already tested in patients (71, 72). Thus, our observations in a mouse model may have clinical applications in the near future.

Acknowledgments

We thank MinCyT-ECOS-Sud (A13B01) and Bec.Ar for support covering exchanges, Dr. Jeff Zigman (University of Texas Southwestern Medical Center, Dallas, TX) for providing the GHSR-null mice, and Dr. Catherine Tomasetto (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France) for providing the ghrelin-KO mice. The technical assistance of Dr. María Jose Tolosa, Dr. Guadalupe García-Romero, and Lic. Mirta Reynaldo is also acknowledged.

Financial Support: This work was supported by the PICTO2013-0065 grant, which is cofinanced by the National Agency of Scientific and Technological Promotion of Argentina and GlaxoSmithKline (to M.P.) and by grants of the INSERM and Agence Nationale pour la Recherche (ANR-12-JSV1-0013-01; to V.T.). G.F. was supported by CONICET.

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Disclosure Summary: The authors have nothing to disclose.

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