Ghrelin treatment induces rapid and delayed increments of food intake: a heuristic model to explain ghrelin’s orexigenic effects

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INTRODUCTION

Ghrelin is an octanoylated peptide hormone synthesized in the stomach fundus that acts via the growth hormone secretagogue receptor (GHSR), which is mainly expressed in the brain. Ghrelin plays key roles regulating energy balance and metabolism [1]. The role of ghrelin becomes more evident in energy deficit conditions, such as calorie restriction or fasting, when plasma ghrelin levels increase and contribute to up-regulate food seeking, food intake and hyperglycemic mechanisms [2, 3]. In this regard, ghrelin is recognized as the most powerful known orexigenic hormone. In particular, systemically injected ghrelin potently (~ seven-fold) and rapidly (~ 15-min after injection) increases food intake in satiated fed mice [4–6]. Ghrelin also increases food intake in many other species, including humans [7]. The orexigenic effect of circulating ghrelin appears to mainly involve neurons of the hypothalamic arcuate nucleus (ARH) that synthesize the orexigenic neuropeptides agouti-related protein (AgRP) and neuropeptide Y (NPY), hereafter named ARHAgRP/NPY neurons, which are critical regulators of feeding [8]. Indeed, optogenetic or chemogenetic activation of ARHAgRP/NPY neurons potently increases food intake [9, 10], while its inhibition or ablation reduces food intake or even leads to aphagia [10–12]. ARHAgRP/NPY neurons express high levels of GHSR and rapidly sense elevations of plasma ghrelin [13]. Systemically injected ghrelin fails to induce food intake in mice with ablation of either the entire ARH or exclusively ARHAgRP/NPY neurons [4, 12]. Also, ghrelin increases food intake in mice with selective expression of GHSR in ARHAgRP/NPY neurons [14], and fails to increase food intake in mice lacking GHSR in ARHAgRP/NPY neurons [15]. Besides food intake, some studies found that ghrelin also affects energy balance through the regulation of the respiratory exchange ratio (RER), locomotor activity or energy expenditure (EE). The RER represents the CO₂ volume produced per O₂ volume consumed and is an indirect

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measurement of the type of nutrients that are oxidized to obtain energy, with RER = 1 for carbohydrate and RER = 0.7 for lipid-based substrate utilization [16]. Early evidence showed that a single systemic injection of ghrelin in ad libitum fed mice increases the RER ~ 60 min after treatment, suggesting that ghrelin favors carbohydrate oxidation over fat [17]. The putative effects of ghrelin on locomotor activity or EE are more controversial. Some studies found that systemically injected ghrelin induces a two-fold increase of locomotor activity in mice within the 0–60-min post-treatment period [18–20], whereas other studies could not confirm such observation [17, 21]. Similarly, a study reported that systemically injected ghrelin transiently decreases EE in mice in the ~30–60-min post-treatment period [22], whereas other studies reported that systemic or central administration of ghrelin does not affect EE in rodents [17, 23]. Thus, the orexigenic effect of ghrelin and its neurobiological basis are well established; in contrast, the degree to which ghrelin affects energy balance via the regulation of locomotor activity, EE or the RER remains more uncertain.

The potent orexigenic effect of ghrelin treatment supports the notion that this hormone (or ghrelin-mimetic compounds) could be used to treat patients suffering loss of appetite or weight loss. However, it seems clear that many intricacies regarding the coordinated action of ghrelin in the modulation of energy balance are still unsolved. Thus, the current study was performed to comprehensively characterize the interplay among ghrelin’s effects on energy homeostasis in mice. For this purpose, we first performed a systematic, dose–response and time-response analysis of the effects of ghrelin treatment on food intake, locomotor activity, EE and the RER in ad libitum fed or mice deprived of food during the measurements. Since our results indicated that ghrelin plays a major role as an orexigenic signal, we performed additional studies aimed to better understand the effect of ghrelin on food intake over time as well as the endogenous factors that sculpt the shape of such effect. Based on our observations, we propose a heuristic model to explain ghrelin’s effects on energy balance.

Materials and methods

Animals

Mice were generated in the animal facility of either the IMBICE, at La Plata, or the animal core facility “Buffon” of the Université de Paris/Institut Jacques Monod. Mouse models included were: (1) C57BL/6J wild-type (WT) mice (n = 371), (2) heterozygous NPY-GFP mice (n = 11), which express green fluorescent protein (GFP) under the control of the NPY promoter (Jackson Laboratory, Stock #006417) [24], and (3) homozygous AgRP-DTR mice (n = 26), which express the human diphtheria toxin receptor (DTR) under the control of Agrp gene regulatory elements [12]. All genetically modified mice were derived from crosses between mice back-crossed for >10 generations onto a C57BL/6J genetic background and were previously shown to display a high degree of specificity [12, 24]. ARH neurons-ablated mice were generated as previously described [4]. Briefly, 4-day-old WT mice were subcutaneously treated with 10–15 μl of saline alone or containing monosodium glutamate (Sigma-Aldrich, cat. G1626, 2.5 mg/g body weight (BW)) giving ARH-intact or ARH-ablated mice, respectively. The ablation of the ARH was histologically confirmed, as described in the past [4]. ARHAgRP/NPY neurons-ablated mice were generated as previously described [12]. Briefly, homozygous AgRP-DTR mice were subcutaneously injected with 20 μl of saline alone or containing diphtheria toxin (75 ng/mouse) during the first week after birth giving control or ARHAgRP/NPY neurons-ablated mice, respectively. Diphereria toxin (List Biological Laboratories) was dissolved in saline and administered at 50 mg/kg, based on a previous report [12]. The extent of the ablation of ARHAgRP/NPY neurons was confirmed using neuroanatomical and behavioral assessments, as also described in the past [12, 25]. Male mice were maintained under controlled temperature (21 °C) and photoperiod (12-h light/dark cycle from 6:00 to 18:00) with regular chow and water available ad libitum, and used for experiments at adulthood (10–14 weeks old). At La Plata, chow was provided by Gepsa (Grupo Pilar) and provided 2.5 kcal/g energy (weight composition: carbohydrates 28.8%, proteins 25.5%, fat 3.6%, fibers 27.4%, minerals 8.1%, and water 6.7%). At Paris, chow was provided by SAFE and provided 3.438 kcal/g energy (weight composition: carbohydrates 55.0%, proteins 19.3%, fat 5.1%, fibers 4.0%, minerals 4.6%, and water 12.0%). All 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, USA [26] and the European Communities Council Directive (86/609/EEC). All experiments received approval from the Institutional Animal Care and Use Committee of the IMBICE (ID 15-0132) and the Animal Care Committee of the Université de Paris (CEB 02-2017).

Experimental procedures and drugs

Plasma ghrelin assessment

To characterize plasma ghrelin levels in our experimental conditions, WT mice were intraperitoneally (IP) injected with vehicle alone or containing ghrelin (from Global Peptides, cat. PI-G-03 or from Tocris, cat. 1465/1). Ghrelin was dissolved in sterile phosphate-buffered saline (PBS, pH 7.4), frozen and prepared fresh on experimental days.
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and IP injected at 15, 60 or 150 pmol/g BW (which corresponds to 0.05, 0.2 or 0.5 mg/Kg BW, respectively). Blood was collected from the facial vein at 15, 30, 45 and 60 min post-injection in tubes pre-treated with ethylenediaminetetraacetic acid (1 mg/mL final) and the protease inhibitor post-injection in tubes pre-treated with ethylenediamine-tetraacetic acid (1 mg/mL final) and the protease inhibitor p-hydroxy-mercuribenzoic acid (0.4 mM final). Blood samples were centrifuged at 2655 g for 10 min at 4 °C. Plasma was separated, acidified with 1 M HCl (0.1 M final) to preserve ghrelin acylation and stored frozen at −80 °C until processing. Plasma ghrelin levels were assessed using an enzymatic immunoassay from Bertin Pharma (cat. A05118), as reported previously [2, 27].

Metabolic assessments

Some mice were used to investigate the effect of ghrelin on food intake, locomotor activity (beam breaks), whole EE (Kcal/h), oxygen consumption and carbon dioxide production (VO2, VCO2 where V is volume), and RER using calorimetric cages (Labmaster, TSE Systems GmbH, Bad Homburg, Germany). Each cage combined a set of sensitive feeding and drinking sensors for automated online measurement and was embedded in a frame with an infrared light beams-based activity monitoring system, which allowed measurement of total locomotion. Ratio of gases was determined through an indirect open circuit calorimeter [28], which monitors O2 and CO2 concentration at the inlet ports of a tide cage through which a flow of air (0.4 L/min) is ventilated and compared regularly to a reference empty cage. Whole EE was calculated according to the Weir equation, which indicates that EE (kcal/min) = 3.941 × VO2 + 1.106 × VCO2 [29]. The flow was calibrated with a O2 and CO2 mixture of known concentration (Air Liquide, S.A. France). Mice were individually housed in metabolic cages 2 days before experimental measurements, at 22 ± 1 °C room temperature with food and water available ad libitum, and habituated to handling with daily IP injections during the 3 days before experiments. On the experimental day, all food pellets were removed from the home cage hoppers and the bedding was confirmed to be free of chow remains. Then, mice were subjected to the different procedures/treatments and exposed to a single pre-weighed chow pellet (~1500 mg, Gepsa) on the floor of the home cages. Mice were injected with 60 pmol/g BW of ghrelin in all these experiments. Chow pellets together to any additional chow spillage were collected and weighed at 30, 60, 120, 180, 240 and 300 min after food exposure using a calibrated scale that had a precision of 1 mg. Food intake is calculated subtracting the remaining weight of the pellet to the weight of the pellets at the previous time point and expressed in mg. This procedure was used on:

i. WT mice that had a delayed access to chow after treatment in order to estimate the duration of the orexigenic effect of ghrelin. In particular, mice were injected with saline or ghrelin and exposed to a food pellet immediately (saline n = 10; ghrelin n = 11) or 60 (saline n = 16; ghrelin n = 19), 120 (saline n = 9; ghrelin n = 9), 180 (saline n = 19; ghrelin n = 22), 240 (saline n = 8; ghrelin n = 9) or 300 (saline n = 9; ghrelin n = 9) min after treatment.  

ii. ARH-intact (n = 13) and ARH-ablated (n = 7) mice that were injected with ghrelin and immediately exposed to a food pellet to test if ghrelin effect requires the presence of the ARH. 

iii. WT mice in which melanocortin receptor 4 (MC4R) was pharmacologically blocked using melanotan-II (MT-II), after the first event of ghrelin-induced food intake in order to test if the delayed orexigenic effect of ghrelin depends on the melanocortin signaling. Here, WT mice were first implanted with an intra-cerebroventricular (ICV) guide cannula, as we described in the past [30]. Then, ICV-cannulated mice were IP injected with ghrelin and immediately exposed to a food pellet. After 60 min, mice were ICV injected with either artificial cerebrospinal fluid alone (aCSF,
n = 7) or containing MT-II (0.5 µg/mouse, n = 6). MT-II (Phoenix Pharmaceuticals, cat. 043-23) dose was based on previous reports [31]. The correct placement of the cannula was confirmed by histological observation at the end of the experiment.

iv. WT mice in which the availability of plasma glucose was manipulated after the first event of ghrelin-induced food intake. Here, WT mice were initially injected with ghrelin and immediately exposed to a food pellet. Then, a set of mice was injected with vehicle alone (n = 10) or containing the non-metabolizable glucose analogue 2-deoxy-d-glucose (2-DG, Sigma-Aldrich, cat. D8375, 250 mg/Kg BW, n = 12), which decreases glucose availability, at 30 min after ghrelin injection, whereas another set of mice was injected with vehicle alone (n = 9) or containing glucose (Biopack, cat. 2000963808, 2.25 mg/Kg BW, n = 9) at 90 min after ghrelin injection. The doses selected were based on a previous report [32]. For glucose experiments, an independent set of WT mice was used to assess plasma glucose levels in mice that display ghrelin-induced food intake. Briefly, ad libitum fed mice were IP injected with saline (n = 4) or ghrelin (n = 2) and returned to their home cages in the presence of food. Blood was sampled from the facial vein right at 30 and 60 min after treatment, and glycemia was assessed using a glucometer (Accu-Chek® Performa). In addition, an independent set of ad libitum fed WT mice was used to assess plasma levels of glucose at 30, 60 and 120 min after glucose treatment (2.25 mg/Kg BW, n = 4), as described above.

v. WT mice in which gastric emptying was blocked using the anticholinergic agent hyoscine butyl-bromide, after the first event of ghrelin-induced food intake. Here, mice were IP injected with ghrelin and immediately exposed to a food pellet. After 60 min, mice were IP injected with saline alone (n = 14) or containing hyoscine butyl-bromide (6 mg/Kg BW, n = 13). Hyoscine butyl-bromide (Buscapina®, Boehringer Ingelheim) dose was based on a previous report [33]. In this case, an independent set of WT mice was first used to test the capability of such dose of hyoscine butyl-bromide to affect ghrelin-induced gastric emptying using the phenol red method [34, 35]. Briefly, ad libitum fed mice were administered by oral gavage with 0.1 mL of test solution (0.5 mg/mL phenol red and 5% glucose in water). After 1 min, mice were IP injected with ghrelin (60 pmol/g BW) and immediately after with vehicle (n = 8) or hyoscine butyl-bromide (n = 4). After 20 min, mice were euthanized by decapitation and their stomachs were ligated at both ends and removed. Stomachs were homogenized in 0.1 N NaOH; proteins were precipitated using 20% trichloroacetic acid and further centrifuged. Finally, supernatants were mixed with 4 M NaOH and 540 nm absorbance was measured. Gastric emptying was expressed as a percentage and calculated according to the following equation: \[ \left( 1 - \frac{\text{absorbance in test stomach/average of initial absorbance}}{100} \right) \times 100. \] The initial absorbance was obtained with an independent group of mice (n = 3), which was euthanized 1 min after the administration of the test solution and processed as described above.

vi. WT mice that were treated with leptin after the first event of ghrelin-induced food intake. In particular, WT mice were injected with ghrelin and immediately exposed to a food pellet. After 60 min, mice were IP injected with vehicle alone (n = 8) or containing recombinant murine leptin (0.1 mg/g BW, n = 15). Recombinant murine leptin (The National Hormone and Pituitary Program, USA) was dissolved in alkaline PBS (pH 8.25) and used at a dose based on previous reports [36]. Here, an independent set of WT mice was used to assess plasma leptin levels achieved under such experimental conditions. Briefly, ad libitum fed mice were treated with mouse leptin (0.1 mg/g BW) and blood samples were obtained from the tail vein in heparin tubes right before treatment as well as 30, 60 and 120 min after treatment. Plasma was separated by centrifugation and leptin levels were assessed using an enzymatic immunoassay from Linco Research (cat. EZML-82 K), as we have done in the past [36].

vii. WT mice that were treated with cholecystokinin sulfated octapeptide (CCK-8S; Asp-Tyr(SO₃H)-Met-Gly-Trp-Met-Asp-Phe-amide; American Peptide Company) after the first event of ghrelin-induced food intake. WT mice were IP injected with ghrelin and immediately exposed to a food pellet. After 60 min, mice were IP injected with vehicle alone (n = 11) or containing CCK-8S (5 µg/Kg BW, n = 10). The dose of CCK-8S is in the range previously shown to decrease food intake after a fasting period [37].

**Immunohistochemistry**

Individually housed WT mice were acclimated to handling 3 days before the experiment with daily IP injections of saline. On the experimental day, food was removed from the hoppers and mice were IP injected with saline alone or containing ghrelin (60 pmol/g BW). Mice remained without access to food and were anesthetized and transcardially perfused at 60 (saline n = 2; ghrelin n = 4), 120 (saline n = 7; ghrelin n = 7), 180 (saline n = 1; ghrelin n = 4), 240 (saline n = 2; ghrelin n = 5) or 300 (saline n = 1; ghrelin n = 4) min after treatment. Brains were removed, frozen and coronally cut at 45 µm. A series of brain sections was immuno-stained
GFP-expressing cells compared with the total number of was expressed as a percentage, which represents c-Fos + /GFP-expressing cells. Sections were mounted on glass slides and coverslipped with mounting media. Fluorescent images were acquired with a Zeiss AxioObserver D1 equipped with an AxioCam 506 monochrome camera. The number of GFP-expressing, c-Fos + and double c-Fos + /GFP-expressing cells were estimated in the ARH, as described above. The relationship between bregma – 1.58 and – 2.06 mm, using the anatomical limits described in the mouse brain atlas. Low-magnification bright-field representative images were acquired with a Nikon Eclipse 50i and a DS-Ri1 Nikon digital camera.

A similar study was performed using NPY-GFP mice. Briefly, individually housed NPY-GFP mice were IP injected with saline alone (n = 3) or containing ghrelin (60 pmol/g BW). One group of ghrelin-injected NPY-GFP mice remained with access to food (Gepsa, n = 3), while a second group (n = 5) were kept in their home cages in the absence of food for 180 min. Then, mice were perfused and their brains were obtained and coronally cut. A series of sections was processed as described above and incubated with the anti-c-Fos antibody (1:1000) for 2-h at 24 °C. Sections were mounted on glass slides and coverslipped with mounting media. Fluorescent images were acquired with a Zeiss AxioObserver D1 equipped with an Apotome.2 structured illumination module and an AxioCam 506 monochrome camera. The number of GFP-expressing, c-Fos + and double c-Fos + /GFP-expressing cells were estimated in the ARH, as described above. The relationship was expressed as a percentage, which represents c-Fos + /GFP-expressing cells compared with the total number of GFP-expressing cells.

Quantification of mRNA levels in the hypothalamus

Individually housed WT mice were IP injected with vehicle alone or containing ghrelin (150 pmol/g BW). Mice remained without access to food and were euthanized by decapitation at 60 (vehicle n = 4, ghrelin n = 6), 180 (vehicle n = 4, ghrelin n = 6) or 300 (vehicle n = 4, ghrelin n = 5) min after treatment. Brains were extracted and placed in a mouse brain matrix with the ventral surface on top and cut using the optic chiasm and rostral edge of mammillary bodies as rostral and caudal limits, respectively. Hypothalamic sulci were used as lateral limits and a 1.5-mm-thick slice was taken parallel to the base of the hypothalamus. Hypothalamus was collected in TRIzol Reagent (Invitrogen Inc), and total RNA was isolated according to the manufacturer’s protocol. To remove residual DNA contamination, RNA samples were treated with RNAse-free DNAs (Qiagen). The concentration of RNA samples was ascertained by measuring optical density at 260 nm. RNA integrity was confirmed by the detection of 18S and 28S bands after agarose-formaldehyde gel electrophoresis, and RNA quality was verified by optical density absorption ratio 260 nm/280 nm. Total RNA from each sample was reverse-transcribed into cDNA using random hexamer primers and M-MLV reverse transcriptase (Invitrogen, Life Technologies). Real-time quantitative PCR for NPY and AgRP was carried out using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, cat. 172-5271). All reactions were carried out in duplicate with no template control. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Fold change from vehicle-treated mice values was determined using the relative standard curve method, normalizing the expression to the ribosomal protein L19 (reference gene). Primers sequences for NPY were sense: 5′-GCCAGATACTACTCCGCTCTG-3′, antisense: 5′-GATCTCTTGCCATATCTCTGTCTG-3′ [GenBank Accession NM_023456.3], product size 68 bp. Primers sequences for AgRP were sense: 5′-TTGGCAGAGGTGCTAGATCCA-3′, antisense: 5′-AGGACTCGTGACGCTTACAC-3′ [GenBank Accession NM_033650.1], product size 108 bp. Primers sequences for L19 were sense: 5′-AGCCTGACGTGCCATCTCC-3′, antisense: 5′-TGCGATACCCCTCTCTCTC-3′ [GenBank Accession NM_009078.2], product size 99 bp.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 6.0 and differences were considered significant when P < 0.05. Datasets were analyzed with the ROUT method (Q = 1.0%) to detect and remove outliers. In most cases, two-way ANOVA was used to analyze data, considering repeated measures when appropriate, and three different post hoc tests were utilized: (1) Tukey’s multiple comparisons test, when the mean of each group was compared to every other mean; (2) Dunnett’s multiple comparisons test, when the mean of each group was compared to the mean of a “control” group; and (3) Bonferroni’s multiple comparisons test, when the mean of selected pairs of means was compared. For comparison of two datasets, we used either unpaired t-test, if distributions were normal and variances were homoscedastic, unpaired t-test with Welch’s correction, if distributions were normal but variances were not homoscedastic, or Mann–Whitney test, if distributions were not normal. The rapid and delayed time periods in each
experiment were determined as the timepoints in which a significant difference was obtained from control groups plus the timepoints immediately before and after. No significant differences ($P>0.09$) were observed between the number of c-Fos + cells in the ARH of vehicle-treated mice that were perfused 60 ($n=2$), 120 ($n=7$), 180 ($n=1$), 240 ($n=2$) or 300 ($n=1$) min after treatment, and thus their data were pooled and termed vehicle-treated group. All statistical tests performed are indicated in the figure captions.

Results

Ghrelin treatment induces rapid and delayed effects on food intake, EE and the RER in ad libitum fed mice

We tested three doses of ghrelin: 15, 60 and 150 pmol/g BW, among which 15 pmol/g BW was the minimum dose of ghrelin that significantly increased food intake, and 150 pmol/g BW was the minimum dose of ghrelin that induced the maximal food intake response in our experimental conditions. The 15 pmol/g BW dose induced a ~50-fold increase of plasma ghrelin levels at 15 min post-treatment that returned to basal levels at 45 min post-treatment (Fig. 1a). The 150 pmol/g BW dose induced a ~250-fold increase of plasma ghrelin levels at 15 min post-treatment, similar to those detected in mice that underwent a 60% calorie restriction for five days (Fig. 1b), that returned to basal levels at 45 min post-treatment (Fig. 1a). We first investigated the effects of the lower and the higher doses of ghrelin on food intake, locomotor activity, EE and the RER using automated measure of $O_2$, $CO_2$, feeding and activity. Both doses of ghrelin rapidly increased food intake. The low dose of ghrelin increased food intake at 30 min post-treatment, while the high dose of ghrelin increased food intake at 30 and 45 min post-treatment. Interestingly, both doses of ghrelin induced a delayed increase of food intake at 150 min post-treatment (Fig. 2a). Analysis of cumulative food intake indicated that both doses of ghrelin significantly increased food intake in the 30- to 60-min and the 135- to 165-min post-treatment periods (hereafter named rapid and delayed effects, Fig. 2b). Considering the 240-min cumulative food intake, the rapid effect of ghrelin represented 52 ± 7 and 52 ± 6% of food intake for the low and high dose, respectively, whereas the delayed effect represented 28 ± 7 and 24 ± 7% of food intake for the low and the high dose of ghrelin, respectively.

Ghrelin did not affect locomotor activity, at any time point (Fig. 2c), but the low dose of ghrelin induced a rapid increase of cumulative locomotor activity in the 15- to 45-min post-treatment period (Fig. 2d). Regarding EE, the low dose of ghrelin induced a transient stimulatory effect at 150 min post-treatment while the high dose of ghrelin affected EE at 30, 75 and 90 min after treatment (Fig. 2e). In terms of average EE, both doses of ghrelin induced a delayed decrease in EE (Fig. 2f). Regarding the RER, the low dose of ghrelin significantly increased the RER at 45, 60 and 75 min post-treatment, while the high dose of ghrelin significantly increased the RER from 45 min post-treatment until 240 min post-treatment, when the measurements were stopped (Fig. 2g). Since the effect of ghrelin on the RER seemed to occur right after the rapid ghrelin-induced increase of food intake, we calculated the rapid and delayed effects of ghrelin on the RER in the 60- to 105-min and in the 180- to 210-min periods of time, respectively, after the rapid and delayed effects of ghrelin on food intake. Ghrelin treatment induced a significant dose–response effect on the rapid changes in the RER, while only the high dose of ghrelin displayed a positive effect on the delayed changes in the RER (Fig. 2h).
**Ghrelin treatment does not affect EE and the RER in mice that are not allowed to eat**

In order to test if the effects of ghrelin on EE and the RER were secondary to ghrelin-induced food intake, we investigated the effects of ghrelin on locomotor activity, EE and the RER in mice that were housed in calorimetric cages but were not allowed to eat after ghrelin treatment. In this case, ghrelin did not affect locomotor activity (Fig. 3a, b), EE (Fig. 3c, d) or the RER (Fig. 3e, f), calculated on either a point-to-point or cumulative basis.

**Ghrelin treatment involves long-term orexigenic effects that involve the ARH**

First, we estimated how long the orexigenic effect of ghrelin lasts after treatment. For this purpose, a set of mice was injected with ghrelin and exposed to food at 0, 60, 120, 180, 240 or 300 min after injection. Here, mice were injected with 60 pmol/g BW of ghrelin, a dose that consistently increased food intake in our experimental conditions and induced a rapid increase of plasma ghrelin levels that returned to basal levels at 45 min after treatment, similar as seen for the low dose of ghrelin, as described above (Fig. 1a). Ghrelin treatment rapidly increased food intake in mice that had immediate access to food as well as in mice that were exposed to food at 60, 120, 180 and 240 min after injection (Fig. 4a). Thus, ghrelin seemed to induce a persistent orexigenic effect that lasted for ~4 h. In order to test if a single injection of ghrelin induces a long-lasting activation of ARH neurons, we estimated the number of c-Fos+ cells in the ARH of mice that were perfused 60, 120, 180, 240 or 300 min after ghrelin treatment and had no access to food (Fig. 4b, c). Interestingly, ghrelin increased the number of c-Fos+ cells in the ARH at 60, 120 and 180 min after treatment (Fig. 4b). Then, we tested if the ARH is required for the rapid and delayed effects of ghrelin on food intake, and for this purpose we tested the effect of systemically injected ghrelin in ARH-ablated mice, which show a selective ablation of this hypothalamic region (Fig. 4d) but conserved the morphology of other brain regions [4]. Manual assessment of food intake indicated that ghrelin treatment induced rapid and delayed increments of food intake in ARH-intact mice, similar as revealed by the automatized method in WT mice. In contrast, ghrelin treatment did not induce rapid nor delayed increments of food intake in ARH-ablated mice (Fig. 4e), suggesting that the orexigenic effects of ghrelin at both periods require the presence of the ARH.

**The rapid and delayed orexigenic effects of ghrelin treatment involve ARHAgRP/NPY neurons**

In order to investigate the role of ARHAgRP/NPY neurons on the rapid and delayed orexigenic effects of ghrelin, we tested the orexigenic effect of systemically injected ghrelin in mice with selective ablation of ARHAgRP/NPY neurons. Ghrelin treatment did not induce rapid nor delayed increments of food intake in ARHAgRP/NPY neurons-ablated mice (Fig. 5a), suggesting that both orexigenic effects of ghrelin require the presence of the ARHAgRP/NPY neurons. In order to test if the long-term effect of ghrelin on c-Fos in the ARH involves ARHAgRP/NPY neurons, we injected ghrelin to NPY-GFP mice that had no access to food after treatment and perfused them at 180 min after treatment. We found that ghrelin increased the fraction of GFP-expressing cells positive for c-Fos, (Fig. 5b, c). To test if ghrelin-induced c-Fos in ARHAgRP/NPY neurons is affected by food intake, we assessed the fraction of GFP-expressing cells positive for c-Fos in the ARH of NPY-GFP mice that were allowed to eat after ghrelin treatment and found a similar increase in the ARH of mice that were not allowed to eat after ghrelin treatment. We found that ghrelin increased NPY mRNA levels at 60 min after treatment (Fig. 5d) and AgRP mRNA levels at 60 and 180 min after treatment, as compared to vehicle treatment (Fig. 5e). Since the delayed increase of food intake in response to ghrelin correlates with an increase of AgRP mRNA levels, we assessed delayed ghrelin-induced food intake in WT mice in which AgRP action was pharmacologically blocked using ICV administration of the melanocortin 3/4 receptor ligand MT-II, which competes with AgRP action. We found that mice ICV injected with MT-II showed a decreased delayed ghrelin-induced food intake, as compared to mice ICV injected with vehicle (Fig. 5f).

**The orexigenic effect of ghrelin is transiently counteracted by satiation signals**

Our results indicate that ghrelin treatment induces a long-lasting activation of ARH neurons that correlates with a long-term orexigenic effect, which can be unmasked even 240 min after treatment in mice that were not allowed to eat. Since ghrelin-induced food intake involves a rapid and a delayed intake event in mice that are allowed to eat, we hypothesized that the orexigenic effect of ghrelin is transiently neutralized by the activation of post-prandial pathways that take place after the rapid event of food intake.
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Food intake increases plasma glucose levels [39, 40], and hyperglycemia has been shown to reduce ghrelin-induced food intake [41]. Under our experimental conditions, plasma glucose levels transiently increased 30 min after ghrelin treatment in mice allowed to eat (247 ± 22 vs. 163 ± 1 mg/dL in vehicle-treated mice, two-way ANOVA: $P_{interaction} = 0.0167, P_{time} = 0.0020, P_{treatment} = 0.0153$; Bonferroni’s post-test $P = 0.0026$) and then returned to basal levels at 60 min post-treatment (166 ± 2 vs. 172 ± 10 mg/dL in vehicle-treated mice). Thus, we theorized that the post-prandial increment of glycemia could transiently counteract the orexigenic effects of ghrelin treatment, and hypothesized that blocking or increasing glucose availability after the first event of food intake could affect the delayed event of ghrelin-induced food intake. In order to increase or decrease the availability of glucose after the rapid increase of food intake, mice were injected with glucose or 2-DG, respectively.

Glucose treatment transiently increased plasma glucose levels, which were 242 ± 29, 180 ± 12, and 171 ± 30 mg/dL at 30, 60, and 120 min after treatment (Two-way ANOVA: $P_{interaction} = 0.2851, P_{time} = 0.0958, P_{treatment} = 0.0280$; Bonferroni’s post-test $P = 0.0427$), and did not affect food intake in mice that were not treated with ghrelin (not shown). In ghrelin-treated mice, however, glucose treatment abrogated the delayed effect of ghrelin on food intake (Fig. 6a). In mice that were not treated with ghrelin, 2-DG treatment rapidly and transiently increased food intake exclusively in the 30- to 60-min post-treatment period (92.3 ± 37.0 vs. 27.3 ± 10.2 mg, $P = 0.03$). In ghrelin-treated mice, 2-DG treatment also increased food intake in the 30- to 60-min post-treatment period, which occurred after the rapid event of ghrelin-induced food intake, and then increased the magnitude of the delayed effect of ghrelin on food intake (Fig. 6b).

Food intake induces a transient increase of plasma CCK levels [42], and the simultaneous administration of ghrelin and CCK was shown to abrogate ghrelin-induced food intake in rats [43]. Thus, we theorized that a sustained increment of CCK signaling could affect the delayed orexigenic effect of ghrelin. Since previous studies showed that CCK in plasma has a short half-life and that its levels return to baseline ~30 min after fasting-induced food intake [44, 45], mice were first injected with ghrelin, allowed to eat, and then injected with CCK-8S at 60 min after ghrelin treatment. We found that CCK-8S treatment abrogated the capability of ghrelin to induce a delayed increment of food intake (Fig. 6c). Food intake also causes gastric filling, and the post-prandial increase of gastric size is a well-established factor that reduces eating [46, 47]. Thus, we theorized that slowing gastric emptying could affect the delayed event of ghrelin-induced food intake. In order to test our hypothesis, mice were first treated with ghrelin and then injected with hyoscyamine butyl-bromide [48, 49], in a dose that was confirmed to reduce ghrelin-stimulated gastric emptying as compared to vehicle-treated mice (7.0 ± 3.7 vs. 46.3 ± 4.1% of gastric emptying, respectively; two-way ANOVA: $P_{interaction} = 0.0322, P_{treatment1} = 0.0002, P_{treatment2} = 0.9790$; Bonferroni’s post-test: $P = 0.0003$). We found that hyoscyamine butyl-bromide impaired the capability of ghrelin to induce a delayed increment of food intake (Fig. 6d). Since leptin pretreatment also impairs the rapid orexigenic effect of ghrelin in rats [50], we tested if leptin treatment after the rapid event of ghrelin-induced food intake could modulate the delayed orexigenic effects of ghrelin. In leptin-treated mice, plasma leptin levels were 3.1 ± 0.6, 62.3 ± 8.5, 34.5 ± 5.6, and 25.1 ± 2.7 ng/mL at 0, 30, 60, and 120 min after treatment. Leptin treatment after the rapid event of ghrelin-induced food intake abrogated the capability of ghrelin to induce a delayed increment of food intake (Fig. 6e).

Discussion

Here, we provide a compelling characterization of food intake, locomotor activity, EE and RER in mice systemically treated with ghrelin. We found that ghrelin treatment induces a rapid and a delayed increment of food intake. Ghrelin treatment also induced some marginal changes on locomotor activity, EE and the RER, which seemed to mainly depend on food intake, as ghrelin-treated mice...
that were food deprived during the measurements showed no changes in these parameters. Furthermore, we found that the orexigenic effect of ghrelin was long lasting, required the presence of the ARHAgRP/NPY neurons, and mainly involved AgRP signaling. Finally, we provided evidence that some post-prandial satiation signals transiently counteract the stimulatory effect of ghrelin on food intake.

Some technical aspects of our experiments should be considered for a more accurate interpretation of the results. We chose to assess mice behavior in the morning in order

Fig. 3 Ghrelin treatment does not affect EE and the RER in mice not allowed to eat after treatment. a, c, and e show locomotor activity (expressed as cumulative beam breaks in 15 min), EE (c), and the RER (e) of 150 pmol/g BW and 15 pmol/g BW of ghrelin- and saline-treated mice. Pink rectangles indicate the rapid and delayed time periods used to calculate the cumulative values showed in b, d and f. RM two-way ANOVA [In a $F_{(32,240)} = 0.8060, P = 0.7637$; $F_{(16,240)} = 10.51, P < 0.0001$; $F_{(2,15)} = 2.267, P = 0.1379$. In c: $F_{(32,240)} = 1.407, P = 0.0800$; $F_{(16,240)} = 5.167, P < 0.0001$; $F_{(2,15)} = 2.267, P = 0.1379$. In e: $F_{(32,240)} = 0.4661, P = 0.9943$; $F_{(16,240)} = 17.55, P < 0.0001$; $F_{(2,15)} = 1.362, P = 0.2860$] followed by Dunnett’s multiple comparisons test was performed ($P < 0.05$ vs 150 pmol/g BW of ghrelin). b, d, and f quantitative analysis of the cumulative locomotor activity (b), average EE (d) and the changes in the RER (f) between ghrelin- and saline-treated mice in the rapid and delayed time periods. White-filled circles represent individual values.
to increase our capability to detect the effects of ghrelin, as food intake, locomotor activity, EE and the RER are low in the morning [51]. All tested doses of ghrelin induced transient supraphysiological increments of ghrelin levels, which are known to be required to consistently detect food intake responses to ghrelin [6]. Thus, current findings address the effects of ghrelin treatment, but their physiological implications are uncertain. The lowest tested dose of ghrelin transiently mimicked the hormone levels detected in severely calorie-restricted mice, in which a ~12–18-fold increase of ghrelin levels is detected, as compared to ad libitum fed mice [3]. However, plasma ghrelin levels remain elevated during long periods of time (e.g., days) under energy deficit states [3]. In addition, such sustained elevations of ghrelin levels are concomitant to a countless number of neuroendocrine adaptations, including an increment of GHSR gene expression in the ARH and a GHSR-dependent remodeling of hypothalamic neuronal circuits, which may affect the sensitivity to ghrelin [2, 52]. Thus, our experimental conditions may mimic the impact of lower increments of plasma ghrelin levels in some specific metabolic conditions. Finally, it is important to stress that food intake and locomotor activity were assessed as accumulated values every 15 min; in contrast, RER and EE were assessed every 15 min and reported as the average values at each time point. Such different sampling rates, which was inherent to the metabolic chambers design, have limited our capability to perform some correlation analysis.

Both the lower and the higher doses of ghrelin induced a similar ~ four-fold increase of food intake at 30 min post-treatment, but the high dose of ghrelin induced a longer orexigenic effect, which remained significant at 45 min post-treatment. Despite the 10 times difference in the dose of ghrelin, the rapid ghrelin-induced food intake event showed a poor dose–response profile, as it has been previously reported by us and by others [4, 6, 7, 53] and may be attributed to a ceiling effect. The delayed effect of ghrelin on food intake was ~50% smaller, as compared to the rapid event, usually took place at ~150 min post-treatment (although sometimes was detected ~180 min post-treatment), and also lacked a dose–response profile. Strikingly, ghrelin-induced food intake showed a marked level of variability independently of the strategy used to measure food intake (automated vs. manual) or of the diet source (SAFE vs. Gepsa). Thus, it seems likely that such variability results from the inherent inter-individual phenotypic variability that C57BL/6 mice are known to display for some traits, including food intake [54, 55]. Importantly, however, the biphasic pattern of ghrelin-induced food intake was observed in all experimental designs, highlighting the robustness of the reported effect.

In mice with access to food, ghrelin treatment did not significantly affect locomotor activity when data were analyzed at 15-min timeframes, but slightly increased the 15- to 45-min cumulative locomotor activity (compare 2c vs 2d), suggesting that circulating ghrelin plays a rather minor role regulating such behavior, even at supraphysiological levels. The subtle effect of ghrelin on locomotor activity together to the fact that locomotion can be assessed using different experimental strategies (e.g., beam breaks, video tracking, etc.) may explain previous inconsistencies in terms of the reported ghrelin’s effects on locomotor activity [17–21]. Notably, we found that ghrelin treatment did not induce locomotor activity in mice that were not allowed to eat, confirming that circulating ghrelin does not stimulate ambulatory activity. Rather, the locomotor behaviors detected in ghrelin-treated mice exposed to food may be related to behavioral rituals associated to food consumption such as handling or hoarding of food pellets.

We found that ghrelin treatment failed to affect EE in mice that were not allowed to eat. Current observations agree with previous reports showing that systemic or central administration of ghrelin in rodents does not affect total EE [17, 23], and with the finding that systemically injected ghrelin does not acutely affect the expression of the uncoupling protein 1 (UCP1), a mitochondrial protein of the brown adipose tissue responsible for non-shivering thermogenesis [56]. In mice allowed to eat after treatment, however, we found that ghrelin affected EE in a complex manner. Since total EE includes resting EE, activity-induced EE and diet-induced EE, which refers to the post-prandial increase of thermogenesis due to the stimulation of energy-requiring processes [57], it is likely that the effect of ghrelin on EE at 150 min post-treatment depends on diet-induced EE since it occurs in parallel with the delayed effect of ghrelin on food intake. The reasons why ghrelin did not affect, or even sporadically reduced, EE when ghrelin-induced food intake was highest are uncertain. It could be hypothesized that ghrelin initially decreases EE, until 90 min post-treatment, in a magnitude that transiently neutralizes diet-induced EE. In this regard, a study found that systemically injected ghrelin decreases EE, in the 30–60-min post-treatment period, in mice without access to food [22]. Also, some studies have inferred that ghrelin decreases EE based on the observations that systemically injected ghrelin over 7 days decreased UCP1 gene expression in the brown adipose tissue [58] or that centrally injected ghrelin reduced sympathetic nerve activity and temperature in the brown adipose tissue [59]. Overall, the current study indicates that acute ghrelin treatment does not affect total EE in the absence of food intake, and that the mechanisms by which ghrelin jointly affects food intake and total EE are complex and need to be specifically investigated.

Ghrelin treatment induced a robust and dose–response stimulatory effect on the RER, which was absent in ghrelin-treated mice without access to food, suggesting that this
a

IP administration

Exposure to food

Rapid food intake

Minutes

Cumulative 30 min food intake (mg)

Time between ghrelin injection and exposure to food (min)

Vehicle

Ghrelin

b

IP administration

Perfusion

Minutes

c-Fos+ cells (cells/side)

Time between ghrelin injection and perfusion (min)

Vehicle

Ghrelin

60

120

180

240

Veh

60

120

180

240

300

c

Vehicle

Ghrelin 60 min

Ghrelin 120 min

Ghrelin 180 min

Ghrelin 240 min

Ghrelin 300 min

d

ARH-intact

ARH-ablated

3V

3V

e

ARH-intact + Ghrelin

ARH-ablated + Ghrelin

Cumulative food intake (mg)

Rapid

Delayed


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Fig. 4 Rapid and delayed effects of ghrelin treatment on food intake involve the ARH. a schematic representation of the experimental design (left timeline) and the quantitative analysis of the 30-min cumulative food intake (right bar graph) of mice IP administered with vehicle or ghrelin and exposed to food at different timepoints. White-filled circles represent individual values. Two-way ANOVA $F_{(5,67)} = 19.62$, $P = 0.0001$; $F_{(1,18)} = 12.10$, $P = 0.0027$; $F_{treatment (1,18)} = 76.32$, $P < 0.0001$] followed by Bonferroni’s multiple comparisons test was performed (*, $P < 0.05$ vs vehicle at each time). b, schematic representation of the experimental design (left timeline) and the quantitative analysis (right bar graph) of the number of c-Fos + cells in the ARH of mice IP injected with vehicle or ghrelin and perfused at different timepoints. White-filled circles represent individual values. Two-way ANOVA $F_{(5,67)} = 15.78$, $P < 0.0001$; $F_{treatment (1,18)} = 19.62$, $P < 0.0001$; $F_{treatment (1,18)} = 50.82$, $P < 0.0001$] followed by Bonferroni’s multiple comparisons test was performed (*, $P < 0.05$ vs vehicle at each time). c representative photomicrographs of a Nissl staining performed in coronal brain sections of ARH-intact (left) and ARH-ablated (right) mice. Scale bar: 100 µm. d representative photomicrographs of a Nissl staining performed in coronal brain sections of ARH-intact (left) and ARH-ablated (right) mice. Scale bar: 100 µm. e quantitative analysis of cumulative food intake in the rapid and delayed time periods of ARH-intact and ARH-ablated mice IP injected with ghrelin. White-filled circles represent individual values. Two-way RM ANOVA $F_{treatment (1,18)} = 9.879$, $P = 0.0056$; $F_{time (1,67)} = 12.10$, $P = 0.0027$; $F_{treatment (1,18)} = 76.32$, $P < 0.0001$] followed by Bonferroni’s multiple comparisons test was performed (*, $P < 0.05$ vs ARH-intact) that ghrelin failed to induce not only rapid but also delayed effects on food intake in ARH$^{AGRP/NPY}$ neurons-ablated mice. Thus, the long-lasting orexigenic effect of ghrelin involves ARH$^{AGRP/NPY}$ neurons. In line with the notion that ghrelin can induce long-term effects via a transient effect on ARH$^{AGRP/NPY}$ neurons, 5-min incubation of hypothalamic slices with ghrelin was shown to increase the stimulatory inputs on ARH$^{AGRP/NPY}$ neurons for even 300 min after exposure [62]. Also, in vivo studies have shown that a brief stimulation of ARH$^{AGRP/NPY}$ neurons induces long-term feeding effects. For instance, 1-min optogenetic stimulation of ARH$^{AGRP/NPY}$ neurons induced voracious feeding in mice that had access to food 30 min later [64]. Also, acute pharmacogenetic activation of ARH$^{AGRP/NPY}$ neurons, using a drug that has a half-life < 60-min, induced a sustained increase of food intake that is observed several days after treatment [65]. Thus, optogenetic and pharmacogenetic studies support the notion that a transient elevation of plasma ghrelin could promote long-term effects on food intake via activation of ARH$^{AGRP/NPY}$ neurons.

Here, we provide additional evidence indicating that the long-term orexigenic effect of ghrelin involves a sustained activation of AgRP signaling. In particular, we found that ghrelin induces a long-term induction of c-Fos in ~ 10% of ARH$^{AGRP/NPY}$ neurons. In this regard, previous studies have also found that ghrelin increases c-Fos in only a small fraction of the ARH$^{AGRP/NPY}$ neurons [55, 66], and optogenetic studies indicated that the stimulation of ~ 8% of ARH$^{AGRP/NPY}$ neurons is sufficient to stimulate food intake [9]. In addition, we found that ghrelin increases AgRP gene expression for, at least, 180 min after treatment, and that the pharmacological blockade of AgRP signaling, after the rapid event of ghrelin-induced food intake, abrogates the delayed effect of ghrelin on food intake. Worth of note, the orexigenic effects of centrally administered NPY and AgRP have been shown to display different dynamics: NPY immediately induces feeding, whereas AgRP displays a delayed effect on food intake that lasts over 24 h [67]. Seminal optogenetic studies showed that activation of the ARH$^{AGRP/NPY}$ neurons rapidly - at ~ 6 min after stimulation-induces a feeding event that involves GABA release in the hypothalamic paraventricular nucleus, and does not depend on the melanocortin system [9, 68]. Similarly, pharmacogenetic studies in genetically modified mice lacking GABA, NPY, AgRP and/or MC4R showed that NPY and GABA mainly mediate the rapid orexigenic effects of ARH$^{AGRP/NPY}$ neurons stimulation, whereas AgRP requires at least 120 min to increase food intake [69]. A subsequent study performing optogenetic activation of the ARH$^{AGRP/NPY}$ neurons confirmed that NPY mediates the initial (tens of minutes) increase of food intake [70]. Notably, it has been shown that the orexigenic effect induced by optogenetic activation of ARH$^{AGRP/NPY}$ neurons can persist even after these neurons are silenced [64]. Future studies will be...
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Fig. 5 Rapid and delayed orexigenic effects of ghrelin involve ARHAgRP/NPY neurons. a Quantitative analysis of cumulative food intake in the rapid and delayed time periods of naïve and ARHAgRP/NPY neurons-ablated mice IP administered with ghrelin. White-filled circles represent individual values. Two-way RM ANOVA [F_int(1,24) = 5.994, P = 0.0220; F_time(1,24) = 15.91, P < 0.0001; F_inttreatment(1,24) = 20.06, P = 0.0002]. In d F_int(2,22) = 6.537, P = 0.0059; F_time(1,22) = 6.348, P = 0.0067; F_treatment(1,22) = 27.43, P < 0.0001] followed by Bonferroni’s multiple comparisons test was performed (*P < 0.05 vs vehicle at each time). b Quantitative analysis of the percentage of GFP-expressing neurons positive for c-Fos at 180 min after IP ghrelin treatment in the ARH of NPY-GFP mice that were allowed, or not, to eat after treatment. One-way ANOVA [F(2,8) = 7.694, P = 0.0137] followed by Dunnett’s multiple comparisons test. *P < 0.05 vs vehicle. c Representative photomicrographs of the anti-c-Fos immunostaining (red signal) in brain sections containing the ARH of NPY-GFP mice (green signal). High-magnification images show the areas delimited with a rectangle in the low-magnification micrographs and arrows point to c-Fos + GFP-expressing cells. Scale bars: 100 µm low- and 10 µm high-magnification images. For a schematic representation of the experimental design (left timeline) and quantitative analysis of the cumulative food intake (right bar graph) in the delayed time period of mice IP administered with ghrelin and ICV injected with vehicle (aCSF) or MT-II. White-filled circles represent individual values. Mann–Whitney U test was performed (U = 6.00, P = 0.0210).

required to determine the extent to which such downstream mechanisms play a role in the long-term orexigenic effect of ghrelin.

The orexigenic effect of ghrelin treatment persisted for ~240-min, but ghrelin induced a biphasic increase of food intake. Thus, we hypothesized that the orexigenic effect of ghrelin was transiently counteracted by post-prandial satiation mechanisms induced by the rapid event of ghrelin-induced food intake. Since food intake transiently elevates plasma levels of glucose, which is a potent satiation signal, we investigated the effect of manipulating post-prandial glucose availability and found that the delayed event of ghrelin-induced food intake was abrogated or enhanced in mice treated with either glucose or 2-DG, respectively. The mechanisms by which glucose availability affects the delayed event of ghrelin-induced food intake remain uncertain. A previous study showed that glucose pre-treatment impairs the effects of ghrelin on both food intake and NPY/AgRP gene expression via a mechanism that involves the energy-sensing enzyme AMP-activated protein kinase in ARHAgRP/NPY neurons [32]. Here, glucose was administered after the rapid effect of ghrelin on food intake and may have also reduced the delayed event of ghrelin-induced food intake by recruiting other satiation signals (e.g., insulin or CCK). Conversely, ghrelin and 2-DG were shown to display an additive effect on food intake that does not require AMP-activated protein kinase in ARHAgRP/NPY neurons [32]. Here, we found that 2-DG treatment enhances the delayed event of ghrelin-induced food intake, independently of its own orexigenic effect, suggesting that glucopenia also down-regulates other satiation signals. Thus, the long-term orexigenic effect of ghrelin seems susceptible to be counteracted by different satiation signals. In line with this possibility, we found that the delayed effect of ghrelin on food intake was abrogated in mice treated, after the first event of ghrelin-induced food intake, with either hyoscine butyl-bromide, which slows gastric emptying [71], leptin or CCK-8S. The molecular mechanisms by which the anorexigenic inputs sculpt the long-term orexigenic effect of ghrelin remains to be determined. Some anorexigenic inputs may directly act on the ARHAgRP/NPY neurons. For instance, intestinal distention inhibits ARHAgRP/NPY neurons via stimulation of intestinal mechanoreceptors [72], and leptin potently abrogates ghrelin-induced activation of ARHAgRP/NPY neurons, which are among the few neuronal types expressing receptors for ghrelin and leptin [73, 74]. In contrast, CCK is thought to mainly inhibit ARHAgRP/NPY neurons and food intake via vagal afferent pathways [75] and also potently reduce gastric emptying that can, in turn, affect ghrelin-induced food intake [76]. Then, further studies are required to elucidate the intricacies of the mechanisms sculpting the long-term orexigenic effect of ghrelin.

Based on our observations, we propose a hypothetical model to describe ghrelin’s effects on energy balance (Fig. 7). Basically, ghrelin primarily induces food intake that has indirect consequences on EE, the RER and satiation signals. The net ghrelin-induced food intake results from the integration of orexigenic mechanisms activated by ghrelin and anorexigenic mechanisms secondary to food intake. We hope this simple model represents a useful tool to facilitate the understanding of the complex mechanisms underlying ghrelin’s actions on energy balance, and has applications for a better use of future pharmacological therapies aimed to manipulate the ghrelin system.
Fig. 6 The delayed orexigenic effect of ghrelin is counteracted by satiety signals. a–e displays a schematic representation of the experimental design (upper timelines) and the quantitative analysis of the cumulative food intake (bottom bar graphs) in the delayed time period of mice IP injected with ghrelin and then IP administered with either vehicle, glucose [a, Mann–Whitney U test, $U=16.00, P=0.0158$], 2-DG [b, unpaired t-test, $t(21)=3.311, P=0.0033$], CCK-8S [c, Mann–Whitney U test, $U=24.00, P=0.0139$], hyoscine butyl-bromide [d, unpaired t-test with Welch’s correction, $t(15.09)=2.319, P=0.0349$] or leptin [e, unpaired t-test, $t(21)=3.014, P=0.0066$].
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Author contributions  MPC, RGPD, GGR, GF, and MR performed the experiments. MPC, MP, RGPD, and SL designed the experiments and wrote the manuscript.

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Availability of data and materials  All data generated or analyzed during this study are included in this published article and are available from the corresponding author on reasonable request.

Declarations

Conflict of interest  The authors have nothing to disclose.

Ethics approval and consent to participate  Not applicable.

Consent for publication  Not applicable.

References

1. Yanagi S, Sato T, Kangawa K, Nakazato M (2018) The homeostatic force of ghrelin. Cell Metab 27:786–804. https://doi.org/10.1016/j.cmet.2018.02.008

2. Fernandez G, Cabral A, Andreoli MF, Labarthe A, M’Kadmi C, Ramos JG et al (2018) Evidence supporting a role for constitutive ghrelin receptor signaling in fasting-induced hyperphagia in male mice. Endocrinology 159:1021–1034. https://doi.org/10.1210/en.2017-03101

3. Zhao T-J, Liang G, Li RL, Xie X, Sleeman MW, Murphy AJ et al (2010) Ghrelin O-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice. Proc Natl Acad Sci 107:7467–7472. https://doi.org/10.1073/pnas.1002271107

4. Cabral A, Valdivia S, Fernandez G, Reynaldo M, Perello M (2014) Divergent neuronal circuitries underlying acute orexigenic effects of peripheral or central ghrelin: critical role of brain accessibility. J Neuroendocrinol 26:542–554. https://doi.org/10.1111/jne.12168

5. Kuo Y-T, Parkinson IRC, Chaudhri OB, Herlihy AH, So P-W, Dhillon WS et al (2007) The temporal sequence of gut peptide–CNS interactions tracked in vivo by magnetic resonance imaging. J Neurosci 27:12341–12348. https://doi.org/10.1523/JNEURON.2391-07.2007

6. McFarlane MR, Brown MS, Goldstein JL, Zhao T-J (2014) Induced ablation of ghrelin cells in adult mice does not decrease food intake, body weight, or response to high-fat diet. Cell Metab 20:54–60. https://doi.org/10.1016/j.cmet.2014.04.007

7. Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG et al (2001) Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab 86:5992–5992. https://doi.org/10.1210/jcem.86.12.8111

8. Andermann ML, Lowell BB (2017) Toward a wiring diagram understanding of appetite control. Neuron 95:757–778. https://doi.org/10.1016/j.neuron.2017.06.014

9. Aponte Y, Atasoy D, Sternson SM (2011) AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nat Neurosci 14:351–355. https://doi.org/10.1038/nn.2739

10. Krashes MJ, Kodag S, Ye C, Rogan SC, Adams AC, Cusher DS et al (2011) Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. J Clin Invest 121:1424–1428. https://doi.org/10.1172/JCI46229

11. Gropp E, Shanabrough M, Borok E, Xu AW, Janoschek R, Buch T et al (2005) Agouti-related peptide-expressing neurons are
mandatory for feeding. Nat Neurosci 8:1289–1291. https://doi.org/10.1038/nn1548
12. Luquet S, Perez FA, Hnasko TS, Palmiter RD (2005) NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. Science 310:683–685. https://doi.org/10.1126/science.1115524
13. Willesen MG, Kristensen P, Rørmer J (1999) Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. GEN 70:306–316. https://doi.org/10.1109/11.800054491
14. Wang Q, Liu C, Uchida A, Chuang J-C, Walker A, Liu T et al (2014) Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin. Mol Metab 3:64–72. https://doi.org/10.1016/j.molmet.2013.10.001
15. Wu C-S, Bongmba O, Yue J, Fehrentz J et al (2019) Growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus neuropeptide Y neurons in a novel transgenic mouse. J Neurosci 29:4622–4639. https://doi.org/10.1523/JNEUROSCI.0131-19.2019
16. Frankenfield DC (2010) On heat, respiration, and calorimetry. Nutrition 26:939–950. https://doi.org/10.1016/j.nut.2010.01.002
17. Tschöp M, Smiley AS, McBride EW, Nguyen M, Al-Haider W, Kopin AS et al (2018) Ghrelin recruits specific subsets of dopamine and GABA neurons of different ventral tegmental area sub-nuclei. Neuroscience 392:107–120. https://doi.org/10.1016/j.neuroscience.2018.09.027
18. Jerlhag E (2008) Systemic administration of ghrelin induces condition place preference and stimulates accumbal dopamine. Addict Biol 13:358–363. https://doi.org/10.1111/j.1369-1600.2008.00125.x
19. Jerlhag E, Egecioglu E, Dickson SL, Engel JA (2011) Glutamatergic regulation of ghrelin-induced activation of the mesolimbic dopaminergic system: mechanisms for ghrelin-induced reinforcement. Addict Biol 16:82–91. https://doi.org/10.1111/j.1369-1600.2010.00231.x
20. Cornejo MP, Barril F, De Francesco PN, Portiansky EL, Reynaldo M, Perello M (2018) Ghrelin recruits specific subsets of dopamine and GABA neurons of different ventral tegmental area sub-nuclei. Neuroscience 392:107–120. https://doi.org/10.1016/j.neuroscience.2018.09.027
21. Kiefer F, Toshinai K, Waise TMZ, Okada T, Sakoda H, Nakazato M (2018) Restoration of metabolic inflammmation-related ghrelin resistance by weight loss. J Mol Endocrinol 60:109–118. https://doi.org/10.1530/JME-17-0192
22. Theander-Carrillo C (2006) Ghrelin action in the brain controls adipocyte metabolism. J Clin Investig 116:1983–1993. https://doi.org/10.1172/JCIC25811
23. van den Pol AN, Yao Y, Fu L-Y, Foo K, Huang H, Coppari R et al (2009) Neuropeptide Y neurons in a novel transgenic mouse expressing strong renilla green fluorescent protein in NPY neurons. J Neurosci 29:4622–4639. https://doi.org/10.1523/JNEUROSCI.3249-08.2009
24. Luquet S, Phillips CT, Palmiter RD (2007) NPY/AgRP neurons are not essential for feeding responses to glucoprivation. Peptides 28:214–225. https://doi.org/10.1016/j.peptides.2006.08.036
25. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington (DC): National Academies Press (US); 2011
26. Cornejo MP, Castrogiovanni D, Schiöth HB, Reynaldo M, Marie J, Fehrentz J et al (2019) Growth hormone secretagogue receptor signalling affects high-fat intake independently of plasma levels of ghrelin and LEAP 2, in a 4-day binge eating model. J Neuroendocrinol. https://doi.org/10.1111/jne.12785
27. Even PC, Nadkarni NA (2012) Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. Am J Physiol-Regul, Integr Comp Physiol 303:R459–R476. https://doi.org/10.1152/ajpregu.00137.2012
28. Weir JBDB (1949) New methods for calculating metabolic rate with special reference to protein metabolism. J Physiol 109:1–9. https://doi.org/10.1113/jphysiol.1949.sp004363
29. Cabral A, Suescun O, Zigman JM, Perello M (2012) Ghrelin indirectly activates hyposyphisostropic CRF neurons in rodents. PLoS ONE 7:e31462. https://doi.org/10.1371/journal.pone.0031462
30. Navarro M, Lermá-Cabrera JM, Carvajal F, Lowery EG, Cubero I, Thiele TE (2011) Assessment of voluntary ethanol consumption and the effects of a melanocortin (MC) receptor agonist on ethanol intake in mutant C57BL/6J mice lacking the MC-4 receptor. Alcohol Clin Exp Res 35:1058–1066. https://doi.org/10.1111/j.1530-0277.2011.01438.x
31. Lockie SH, Stark R, Mequinion M, Ch’ng S, Kong D, Spanswick DC et al (2018) Glucose availability predicts the feeding response to ghrelin in male mice, an effect dependent on AMPK in AgRP neurons. Endocrinology 159:3605–3614. https://doi.org/10.1210/en.2018-00536
32. Bilreiro C, Fernandes FF, Andrade L, Chavarriás C, Simões RV, Matos C, et al. (2020) Hyoscine butylbromide for bowel motion reduction in mouse abdominal MRI. ArXiv. 200704282 [Physics]
33. Padilla SL et al (2015) Palatability can drive feeding independently of leptin, ghrelin, and the effects of a melanocortin (MC) receptor agonist on ethanol intake in mutant C57BL/6J mice lacking the MC-4 receptor. Alcohol Clin Exp Res 35:1058–1066. https://doi.org/10.1111/j.1530-0277.2011.01438.x
34. Enriori PJ, Evans AE, Sinnayah P, Jobst EE, Tonelli-Lemos L, Billes SK et al (2007) Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons. Cell Metab 5:181–194. https://doi.org/10.1016/j.cmet.2007.02.004
35. Kopin AS, Mathes WF, McBride EW, Nguyen M, Al-Haider W, Schmitz F et al (1999) The cholecystokinin-A receptor mediates inhibition of food intake yet is not essential for the maintenance of body weight. J Clin Invest 103:383–391. https://doi.org/10.1172/JCI4901
36. Enriri PI, Evans AE, Sinnayah P, Jobst EE, Tonelli-Lemos L, Billes SK et al (2007) Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons. Cell Metab 5:181–194. https://doi.org/10.1016/j.cmet.2007.02.004
37. Morii A, Ishimura K, Takada M, Ogawa K, Usui T, Okumura Y (1997) Gastric emptying in OLETF rats not expressing CCK-A receptor gene. Dig Dis Sci 42:915–919. https://doi.org/10.1023/a:1011860313674
38. Paxinos G, Franklin KBJ (2001) The mouse brain in stereotaxic coordinates
39. Murai A, Iwamura K, Takada M, Ogawa K, Usui T, Okumura Y (2002) Control of postprandial hyperglycemia by galactosyl maltobionolactone and its novel anti-amylase effect in mice. Life Sci 71:1405–1415. https://doi.org/10.1016/s0024-3205(02)00315-8
40. Page LC, Gaskell J, Smith MA, D’Alessio DA, Tong J (2018) Interaction of GLP-1 and ghrelin on glucose tolerance in healthy humans. Diabetes 67:1976–1985. https://doi.org/10.2337/db18-0451
41. Caixás A, Bashore C, Nash W, Pi-Sunyer F, Laferrère B (2002) Control of postprandial hyperglycemia by galactosyl maltobionolactone and its novel anti-amylase effect in mice. Life Sci 71:1405–1415. https://doi.org/10.1016/s0024-3205(02)00315-8
42. Wells AS, Read NW, Uvnas-Moberg K, Alster P (1997) Influences of fat and carbohydrate on postprandial sleepiness, mood, and hormones. Physiol Behav 61:679–686. https://doi.org/10.1016/s0031-9384(96)00519-7
43. Kobelt P, Tebbe JJ, Tjandra I, Stengel A, Baê H-G, Andreason V et al (2005) CCK inhibits the orexigenic effect of peripheral

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44. Liddle RA, Goldfine ID, Williams JA (1984) Bioassay of plasma cholecystokinin in rats: effects of food, trypsin inhibitor, and alcohol. Gastroenterology 87:542–549

44. Lindén A, Uvnäs-Moberg K, Forsberg B, Bednar I, Södersten P (1989) Plasma concentrations of cholecystokinin octapeptide and food intake in male rats treated with cholecystokinin octapeptide. J Endocrinol 121:59–65. https://doi.org/10.1677/joe.0.1210059

46. Phillips RJ, Powley TL (1996) Gastric volume rather than nutrient content inhibits food intake. Am J Physiol 271:R766-769. https://doi.org/10.1152/ajpregu.1996.271.3.R766

47. Powley TL, Phillips RJ (2004) Gastric satiety is volumetric. Intestinal satiation is nutritive. Physiol Behav 82:69–74. https://doi.org/10.1016/j.physbeh.2004.04.037

48. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N et al (2001) Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology 120:337–345. https://doi.org/10.1015/gast.2001.22158

51. Kim ER, Tong Q (2017) Oxygen consumption rate and energy expenditure following hypothalamic administration of neuropeptide Y and agouti-related protein. Endocrinology 145:2607–2612. https://doi.org/10.1210/en.2003-1596

56. Hassouna R, Zizzari P, Videau C, Culler M, Epelbaum J et al (2013) Actions of agonists and antagonists of the ghrelin/GHS-R pathway on GH secretion, appetite, and cFos expression. Front Endocrinol (Lausanne) 4:25. https://doi.org/10.3389/fendo.2013.00025

67. Pomeroy AR, Rand MJ (1969) Anticholinergic effects and passage through the intestinal wall of N-butylhyoscine bromide. J Pharm Pharmacol 21:180–187. https://doi.org/10.1111/j.2042-7158.1969.tb08224.x

68. Atasoy D, Betley JN, Su HH, Sternson SM (2012) Deconstruction of a neural circuit for hunger. Nature 488:172–177. https://doi.org/10.1038/nature11270

69. Krashes MJ, Shah BP, Koda S, Lowell BB (2013) Rapid versus delayed stimulation of feeding by the endogenously released AgRP neuron mediators GABA, NPY, and AgRP. Cell Metab 18:588–595. https://doi.org/10.1016/j.cmet.2013.09.009

70. Chen Y, Essner RA, Kosar S, Miller OH, Lin Y-C, Mesgarzadeh S et al (2019) Sustained NPY signaling enables AgRP neurons to drive feeding. Elife 8:e46348. https://doi.org/10.7554/eLife.46348

71. Pomeroy AR, Rand MJ (1969) Anticholinergic effects and passage through the intestinal wall of N-butylhyoscine bromide. J Pharm Pharmacol 21:180–187. https://doi.org/10.1111/j.1112-1127.1969.tb08224.x

72. Bai L, Mesgarzadeh S, Ramesh KS, Huey EL, Liu Y, Gray LA et al (2019) Genetic identification of vagal sensory neurons that control feeding. Cell 179:1129-1143.e23. https://doi.org/10.1016/j.cell.2019.10.031

73. Kohno D, Nakata M, Maekawa F, Fujiwara K, Maejima Y, Kuramochi M et al (2007) Leptin suppresses ghrelin-induced activation of neuropeptide Y neurons in the arcuate nucleus via phosphatidylinositol 3-kinase- and phosphodiesterase 3-mediated pathway. Endocrinology 148:2251–2263. https://doi.org/10.1210/en.2006-1240

74. Perello M, Scott MM, Sakata I, Lee CE, Chuang J-C, Osborne-Lawrence S et al (2012) Functional implications of limited leptin receptor and ghrelin receptor coexpression in the brain. J Comp Neurol 520:281–294. https://doi.org/10.1002/cne.22690

75. Beutler LR, Chen Y, Ahn JS, Lin Y-C, Essner RA, Knight ZA (2017) Dynamics of gut-brain communication underlying hunger.
Neuron 96:461-475.e5. https://doi.org/10.1016/j.neuron.2017.09.043

76. Andreoli MF, De Francesco PN, Perello M (2018) Gastrointestinal hormones controlling energy homeostasis and their potential role in obesity. In: Nillni EA (ed) Textbook of energy balance, neuropeptide hormones, and neuroendocrine function. Springer International Publishing, Cham, pp 183–203. https://doi.org/10.1007/978-3-319-89506-2_7

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