Research Article

Ghrelin-induced Food Intake, but not GH Secretion, Requires the Expression of the GH Receptor in the Brain of Male Mice

Frederick Wasinski,[1] Franco Barrile,[2] João A. B. Pedroso,[1] Paula G. F. Quaresma,[1] Willian O. dos Santos,[1] Edward O. List,[3] John J. Kopchick,[3] Mario Perelló,[2,*] and Jose Donato Jr.[1,*]

[1]Universidade de Sao Paulo, Instituto de Ciencias Biomedicas, Departamento de Fisiologia e Biofísica, São Paulo, SP, 05508-000, Brazil; [2]Laboratory of Neurophysiology, Multidisciplinary Institute of Cell Biology, La Plata, BA, 1900, Argentina; and [3]Edison Biotechnology Institute and Heritage College of Osteopathic Medicine, Ohio University, Athens, OH, 45701, USA

ORCiD numbers: 0000-0001-7310-6760 (F. Wasinski); 0000-0001-5414-2029 (P. G. F. Quaresma); 0000-0002-8230-6331 (E. O. List); 0000-0003-4561-2177 (J. J. Kopchick); 0000-0003-2114-6765 (M. Perelló); 0000-0002-4166-7608 (J. Donato Jr.).

*These authors contributed equally as co-senior authors.

Abbreviations: AgRP, agouti-related protein; ARH, arcuate nucleus of the hypothalamus; GHR, GH receptor; GHSR, GH secretagogue receptor; IOD, integrated optical density; KO, knockout; KPBS, potassium PBS; pSTAT5, phosphorylation of the signal transducer and activator of transcription-5; PVH, paraventricular nucleus of the hypothalamus

Abstract

Ghrelin stimulates both GH secretion and food intake. The orexigenic action of ghrelin is mainly mediated by neurons that coexpress agouti-related protein (AgRP) and neuropeptide Y (NPY) in the arcuate nucleus of the hypothalamus (ARH). GH also stimulates food intake and, importantly, ARH$^{AgRP/NPY}$ neurons express GH receptor (GHR). Thus, ghrelin-induced GH secretion may contribute to the orexigenic effect of ghrelin. Here, we investigated the response to ghrelin in male mice carrying GHR ablation specifically in neurons (brain GHR knockout [KO] mice) or exclusively in ARH$^{AgRP/NPY}$ neurons (AgRP GHR KO mice). Although brain GHR KO mice showed normal ghrelin-induced increase in plasma GH levels, these mutants lacked the expected orexigenic response to ghrelin. Additionally, brain GHR KO mice displayed reduced hypothalamic levels of *Npy* and *Ghsr* mRNA and did not elicit ghrelin-induced c-Fos expression in the ARH. Furthermore, brain GHR KO mice exhibited a prominent reduction in AgRP fiber density in the ARH and paraventricular nucleus of the hypothalamus (PVH). In contrast, AgRP GHR KO mice showed no changes in the hypothalamic *Npy* and *Ghsr* mRNAs and conserved ghrelin-induced food intake and c-Fos expression in the ARH. AgRP GHR KO mice displayed a reduced AgRP fiber density (~16%) in the PVH, but this reduction was less than that observed in brain GHR KO mice (~61%). Our findings indicate that GHR signaling in the brain is

required for the orexigenic effect of ghrelin, independently of GH action on ARH[AgRP/NPY] neurons.

Ghrelin is produced by enteroendocrine cells of the gastro-intestinal tract and was recognized as the endogenous agonist of the GH secretagogue receptor (GHSR) in 1999 [1]. Ghrelin plays a variety of well-established metabolic, neuroendocrine, autonomic, and behavioral effects [2]. Among a plethora of actions, the most evident effects of ghrelin treatment include its capability to increase plasma GH levels and food intake [2]. Regarding to the former, bolus injections or continuous infusions of ghrelin acutely increase plasma GH levels or the pulsatile GH secretion, respectively, in healthy human volunteers [3-6]. In mice, systemically injected ghrelin induces a rapid ~15-fold increase of plasma GH levels, which peak at 10 minutes postinjection and return to basal levels around 40 minutes after treatment [7]. Studies in rodents revealed that ghrelin-induced GH secretion involves the activation of the hypothalamic GHRH neurons, as well as ghrelin's action on somatotropic pituitary cells [8-12]. In terms of food intake regulation, ghrelin is recognized as the most potent orexigenic peptide hormone. Most studies in lean individuals have found that either bolus or continuous ghrelin infusions increase hunger sensation and food intake [6, 13, 14]. Similarly, systemically injected ghrelin induces a rapid and transient increase of food intake, which is observed ~10 minutes after injection and disappears ~45 minutes after treatment, in satiated fed mice [15-17]. Ghrelin-induced food intake in mice mainly involves a subset of neurons located in the hypothalamic arcuate nucleus (ARH) that produce the orexigenic neuropeptides agouti-related protein (AgRP) and neuropeptide Y (NPY), hereafter named ARH[AgRP/NPY] neurons, which highly express GHSR and rapidly sense elevations in plasma ghrelin levels [15, 18-22]. Indeed, ghrelin increases food intake in mice with GHSR expression limited to ARH[AgRP/NPY] neurons [23, 24], and AgRP-specific GHSR knockout (KO) mice fail to increase food intake in response to ghrelin [25]. Thus, ghrelin treatment induces a rapid and simultaneous increase of plasma GH levels and food intake, and these effects have been extensively documented and studied in the past 20 years.

Although less reported, GH also displays orexigenic effects. In particular, GH treatment was shown to increase food intake in children and in GH-deficient patients [26, 27]. GH treatment increases food intake in rats [28], mice [29] and fish [30, 31]. Furthermore, central overexpression of bovine GH induces hyperphagia and obesity in mice [32]. In line with the notion that GH increases food intake, GH receptor (GHR) is amply expressed in brain targets that control appetite, including the ARH [33-35]. In particular, *Ghr* mRNA is expressed in ~95% of ARH[AgRP/NPY] neurons [36, 37] that, in turn, are directly depolarized by GH [29]. Additionally, GH injection induces c-Fos expression, a marker of neuronal activation, or leads to phosphorylation of the signal transducer and activator of transcription-5 (pSTAT5), the main intracellular signaling pathway recruited after GHR activation, in ARH[AgRP/NPY] neurons of rodents [29, 38-41]. Although these observations raise the possibility that the orexigenic effects of ghrelin could depend on the central action of GH, some evidence argues against such notion. For instance, bolus GH injections induce a slower increase of food intake, as compared with ghrelin, that is detected several hours after treatment [29]. In addition, ghrelin treatment rapidly stimulates food intake in GH-deficient rats [42], and endogenous pulses of plasma ghrelin directly correlate to food intake but not to plasma GH levels in rats [43]. Strikingly, however, it has been found that ghrelin fails to increase food intake and induce *Agrp* gene expression in whole-body GHR KO mice, suggesting that the orexigenic effects of ghrelin do require GHR signaling in this species [44]. Also, a blunted ghrelin-induced increase in hypothalamic *Agrp* and *Npy* mRNA levels was observed in GHRH KO mice, another model of decreased GH secretion [45]. These observations must be interpreted with caution because they may be secondary to some metabolic and neuroendocrine disturbances caused by the long-term deficiency of GH or GHR [46]. In particular, whole-body GHR KO mice display hyperphagia, dwarfism, increased body adiposity, and changes in insulin sensitivity, as compared with wild-type littermates. In addition, GHR KO mice show higher plasma levels of leptin, corticosterone, triglycerides, and cholesterol, as well as lower glycemia and insulinemia [46]. Thus, it is currently uncertain if ghrelin-induced food intake requires central GHR signaling itself.

The goal of the current study was to gain insights regarding the requirements of the central GHR signaling for the orexigenic effects of ghrelin. For this purpose, we investigated the effects of ghrelin treatment in mice lacking GHR specifically in the cells of the nervous system or in ARH[AgRP/NPY] neurons. As we have shown in the past [29, 38, 39, 47], these genetically modified mice show minor metabolic and neuroendocrine disturbances in ad libitum–fed conditions and emerge as a more accurate animal model

to investigated the putative requirement of GHR signaling for ghrelin-induced food intake.

Materials and Methods

Mice

Genetic inactivation of GHR was obtained by initially breeding mice carrying loxP-flanked *Ghr* alleles (48) with Nestin-Cre mice (The Jackson Laboratory, Bar Harbor, ME; RRID: IMSR_JAX:006660) or AgRP-Cre mice (The Jackson Laboratory; RRID: IMSR_JAX: 012899). After successive matings to establish a mouse colony homozygous for the loxP-flanked *Ghr* alleles, we generated the Cre-expressing conditional KO mice that were used in the experiments (brain GHR KO or AgRP GHR KO mice) and their respectively littermate controls that did not carry the Cre transgenes. The visualization of AgRP neurons was achieved by incorporating a Cre-dependent reporter protein (tdTomato; The Jackson Laboratory; RRID: IMSR_JAX:007909) in the experimental animals. Mice were in the C57BL/6 background, they were weaned at 3 weeks of life and their mutations were confirmed by PCR using the DNA that had been previously extracted from the tail tip (REDExtract-N-Amp Tissue PCR Kit, Sigma-Aldrich, St. Louis, MO). In all experiments, we used male mice with ad libitum access to a regular rodent chow and filtered water. The experiments were approved by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of São Paulo, and were performed according to the ethical guidelines adopted by the Brazilian College of Animal Experimentation.

Detection of GH-responsive neurons

Adult mice received an IP injection of 20 µg/g body weight of porcine pituitary GH (from Dr. A.F. Parlow, National Hormone and Pituitary Program, Torrance, CA) and were perfused 60 to 90 minutes later. Saline-injected mice were used as negative controls to demonstrate the very low basal pSTAT5 expression observed in mice without GH stimulus (29, 33, 47). Mice were anesthetized with isoflurane and then perfused with saline followed by 10% buffered formalin. Brains were cryoprotected overnight in 20% sucrose and cut in 30-µm thick sections using a freezing microtome. To detect pSTAT5 immunoreactive cells, brain slices were rinsed in 0.02 M potassium PBS (KPBS), pH 7.4, followed by pretreatment in water solution containing 1% hydrogen peroxide and 1% sodium hydroxide for 20 minutes. After rinsing in KPBS, sections were incubated in 0.3% glycine and 0.03% lauryl sulfate for 10 minutes each. Next, slices were blocked in 3% normal serum for 1 hour, followed

by incubation in an anti-pSTAT5^{Tyr694} primary antibody made in rabbit (Cell Signaling, Beverly, MA, catalog #9351; RRID: AB_2315225; 1:1000). After 2 days, sections were rinsed in KPBS and incubated for 90 minutes in AlexaFluor-conjugated anti-rabbit IgG secondary antibody (1:500, Jackson ImmunoResearch Laboratories, Cambridge, MA). Then, sections were rinsed in KPBS, mounted onto gelatin-coated slides, and covered with Fluoromount G mounting medium (Electron Microscopic Sciences, Hatfield, PA).

Evaluation of body weight and food intake

Adult male mice were housed in individual cages for 1 week for acclimation. Subsequently, body weight and food intake were manually measured for approximately 5 consecutive days. Food intake results shown for each animal were the average of the analyzed days. Circadian food intake was determined by measuring the food intake specifically during the light and dark cycles (12 hours each) for 2 consecutive days. Fasting-induced hyperphagia was assessed in adult mice that were fasted for 24 hours. Then, the amount of ingested food was determined 2, 24, and 48 hours during the refeeding period. The food intake was also determined after 5 consecutive days of 60% food restriction, using a protocol previously described (29, 49).

Gene expression analysis

The entire hypothalamus or a liver sample was collected to determine the gene expression by quantitative PCR. Initially, total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA), followed by incubation in DNase I RNase-free (Roche Applied Science) and then reverse transcription using 2 µg of total RNA, SuperScript II Reverse Transcriptase (Invitrogen), and random primers p(dN)6 (Roche Applied Science). Real-time PCR was performed using the 7500TM Real-Time PCR System (Applied Biosystems, Warrington, UK), Power SYBR Green Gene Expression PCR Master Mix (Applied Biosystems), and specific primers for target genes: *Actb* (forward: gctccggcatgtgcaaag; reverse: catcacaccctggtgcca), *Agrp* (forward: ctttggcggaggtgctagat; reverse: aggactcgtgcagccttacac), *Cartpt* (forward: cagtcacacagcttccccgat; reverse: cagatcgaagcgttgcaaga), *Gapdh* (forward: gggtcccagcttaggttcat; reverse: tacggccaaatccgttcaca), *Ghr* (forward: atcaatccaagcctggggac; reverse: acagctgaatagatcctggggg), *Ghsr* (forward: ctcagggaccagaaccacaa; reverse: agccagcagaggatgaaagc), *Igf1* (forward: gtacttccttccttctcctttgc; reverse: ccacactgacatgcccaaga), *Npy* (forward: ccgcccgccatgatgctaggta; reverse: ccctcagccagaatgcccaa), and *Pomc* (forward: atagacgtgtggagctggtgc;

reverse: gcaagccagcaggttgct). Data were normalized to the geometric average of *Actb* and *Gapdh*. The expression of the reference genes was not different between the groups. Relative quantification of mRNA was calculated by $2^{-\Delta\Delta Ct}$.

Evaluation of ghrelin-induced food intake and GH secretion

Ghrelin-induced food intake was analyzed in adult (2- to 3-month-old) male mice that received an IP injection of ghrelin (0.2 µg/g body weight, Global Peptide, catalog #C-et-004) or saline. The amount of food ingested was determined in several time points after the injections. Injections were performed in ad libitum–fed mice, approximately 3 hours after lights on (lights on at 8 AM; 12-hour light/dark cycle). To evaluate ghrelin-induced GH secretion, mice were initially habituated to handling to minimize stress interference during blood collection. Immediately before the injection of saline or ghrelin (0.2 µg/g body weight), a small portion of the tail tip (approximately 1 mm) was cut with a surgical blade to allow the collection of small drops of blood. Using a 10-µL pipette, a 5-µL sample of whole blood was collected and transferred to 105 µL of PBS with 0.05% tween-20. The same blood collection procedure was repeated 10, 20, 30, and 40 minutes after the injections. GH levels in the blood were determined using a mouse GH-sensitive sandwich ELISA, as detailed described in a previous publication (47). The lower limit of detection was 0.04 ng/mL, and the intra-assay and inter-assay coefficients of variation were 2.6% and 9.7%, respectively. The area under the curve of GH secretion was calculated for each animal using the GraphPad Prism software (San Diego, CA).

Assessment of ghrelin-induced c-Fos expression

Adult male mice were perfused 90 minutes after an IP injection of ghrelin (0.2 µg/g body weight) and saline-injected mice were used as negative controls. The brains were processed as described for the detection of GH-responsive neurons. To label c-Fos, brain sections were rinsed in KPBS, pretreated in 0.3% hydrogen peroxide for 30 minutes and, after rinsing in KPBS, sections were incubated in 3% normal donkey serum for 1 hour. Then, brain sections were incubated in anti-c-Fos antibody (1:20 000, Ab5, Millipore, RRID: AB_2314043) for 2 days. Subsequently, sections were incubated for 1 hour in biotin-conjugated secondary antibody (1:1000, Jackson ImmunoResearch Laboratories) and next for 1 hour with an avidin-biotin complex (1:500, Vector Labs,

Burlingame, CA). The peroxidase reaction was performed using 0.05% 3,3′-diaminobenzidine (Sigma), 0.25% nickel sulfate (Sigma), and 0.03% hydrogen peroxide. The slides were covered with DPX mounting medium (Sigma).

Evaluation of axonal projections of ARH neurons

Brain sections of adult male mice were rinsed in KPBS, followed by incubation in 3% normal serum for 1 hour. Next, sections were incubated overnight in anti-AgRP (1:4000, Phoenix Pharmaceuticals, Inc.; catalog # H-003-53) or anti-POMC antisera (1:2000; Phoenix Pharmaceuticals; Burlingame, CA; catalog # H-022-33). Subsequently, sections were incubated for 90 minutes in Alexa Fluor-conjugated secondary antibody (1:500; Jackson ImmunoResearch Laboratories). After rinses in KPBS, sections were mounted onto gelatin-coated slides and covered with Fluoromount G mounting medium.

Image analysis

Photomicrographs were acquired using a Zeiss Axioimager A1 microscope (Zeiss, Munich, Germany). Two or 3 representative levels of the tuberal ARH (Bregma -1.25 to -1.75 mm) or midrostral-caudal paraventricular nucleus of the hypothalamus (PVH; Bregma -0.65 to -0.95 mm) were analyzed in each mouse. The ImageJ Cell Counter software (http://rsb.info.nih.gov/ij/) was used to manually count the number of single- or double-labeled cells, and the Allen Brain Atlas (http://mouse.brain-map.org/) was used as a neuroanatomical reference. To evaluate the axonal projections of AgRP and POMC neurons, we selected the area corresponding to each brain structure (PVH or ARH) using ImageJ software. Then, we used the Measure tool to obtain the integrated optical density (IOD), which represents the average staining intensity in the selected area. The IOD obtained in the PVH and ARH was subtracted from the IOD determined in adjacent nuclei with low staining (background) in each photomicrograph. The values obtained at different rostral-caudal levels were averaged, representing the data for each animal.

Statistical analysis

Data were analyzed using GraphPad Prism software and expressed as mean ± SEM. Comparisons between the groups were performed using unpaired 2-tailed Student *t* test. Two-way ANOVA was used to analyze the effects of ghrelin/saline injections in control and conditional KO mice. Differences were considered significant if $P \le 0.05$.

Results

Characterization of brain GHR KO mice

To assess whether the main effects of ghrelin treatment require the central expression of GHR, we generated mice carrying a brain-specific GHR ablation by crossing GHR^floxed mice and Nestin-Cre mice (nestin promotor drives Cre recombinase expression in the central and peripheral nervous system, including neuronal and some glial cell precursors) (50). Brain GHR KO mice displayed a significant reduction in the number of GH-induced pSTAT5 cells in the ARH ($F(2, 9) = 61.88$, $P < 0.0001$; Fig. 1A-E) and other brain areas (data not shown), compared with GH-injected control mice. Confirming the efficacy of central GHR ablation, brain GHR KO mice also showed a drastic reduction of *Ghr* mRNA levels in the hypothalamus, compared with control mice ($t(12) = 13.38$, $P < 0.0001$; Fig. 1F). In contrast, *Ghr* mRNA levels remained intact in the liver of brain GHR KO mice (Fig. 1G). Of note, hepatic *Igf1* mRNA levels were increased in brain GHR KO mice ($t(9) = 2.661$, $P = 0.026$; Fig. 1G), which is in accordance

with the increased serum IGF-1 concentration previously observed in this mouse model (39). Before evaluating ghrelin's effects, we provided a gross metabolic characterization of the consequences of GHR ablation in the nervous system. As we have shown in the past (29, 39, 47), brain GHR KO mice showed increased body weight ($t(20) = 4.976$, $P < 0.0001$; Fig. 1H), compared with control mice. Furthermore, Brain GHR KO mice showed increased food intake ($t(20) = 2.856$, $P = 0.0098$; Fig. 1I), which did not differ from food intake of control mice when normalized by body weight (Fig. 1J). To determine possible circadian eating alterations, food intake was determined in the dark and light cycles. As expected, approximately two-thirds of the daily food intake occurred at the dark cycle, without differences between the groups (Fig. 1K). Subsequently, fasting-induced hyperphagia was determined. Remarkably, brain GHR KO mice showed reduced food intake 2 hours ($t(7) = 2.53$, $P = 0.0393$) and 24 hours ($t(7) = 3.206$, $P = 0.0149$) after a 24-hour fasting period compared with control mice (Fig. 1L). Likewise, a different cohort of brain GHR KO mice exhibited a reduced refeeding response 24 hours after 5 days of 60%
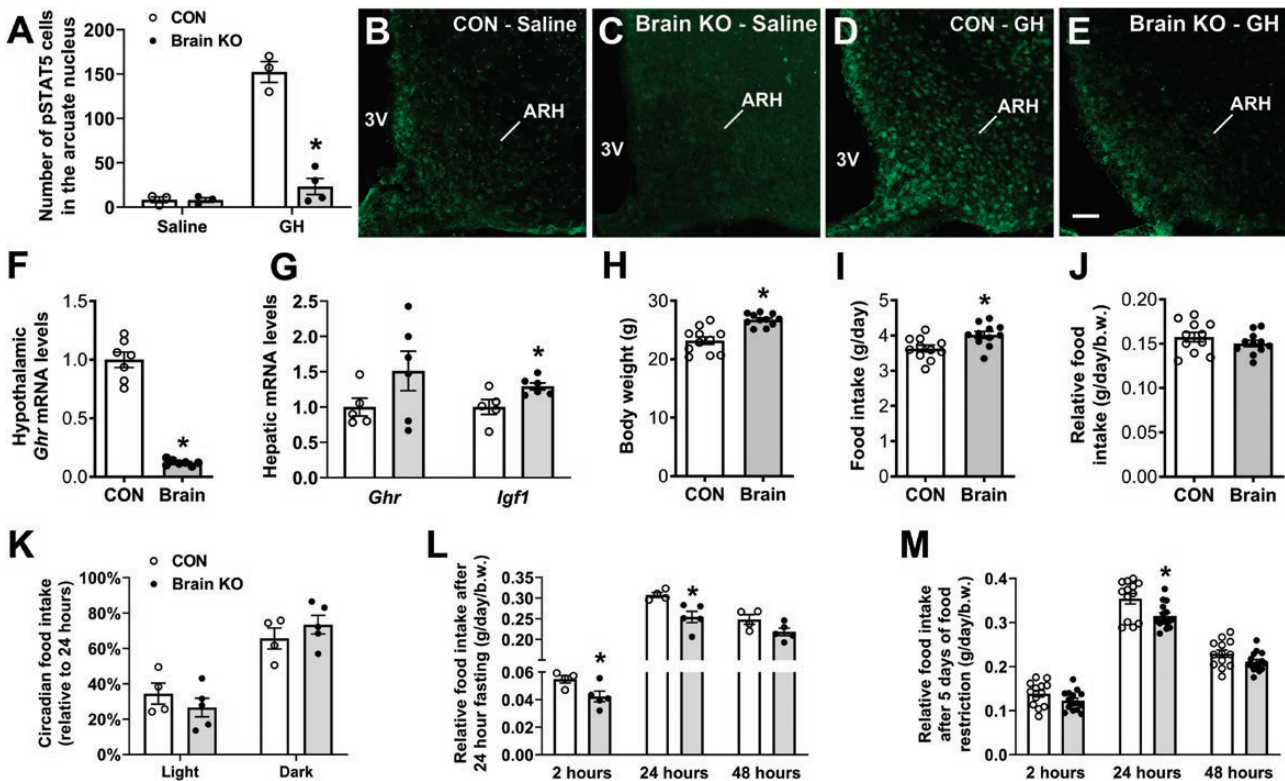

Figure 1. Characterization of Brain GHR KO male mice. (A-E) STAT5 phosphorylation (pSTAT5) in the arcuate nucleus of the hypothalamus (ARH) of saline-injected control mice (B), saline-injected brain GHR KO mice (C), GH-injected control mice (D), and GH-injected brain GHR KO mice (E; n = 3-4/group). Abbreviation: 3V, third ventricle. Scale bar = 50 μm. (F) *Ghr* mRNA levels in the hypothalamus (n = 7/group). (G) *Ghr* and *Igf1* mRNA levels in the liver (n = 5-6/group). (H) Body weight in 3-month-old male mice (n = 11/group). (I-J) Absolute and relative to body weight food intake (n = 11/group). (K) Percentage of food intake relative to 24 hours in the light and dark cycles (n = 4-5/group). (L) Food intake after a 24-hour fasting period (n = 4-5/group). (M) Refeeding response after 5 days of 60% food restriction (n = 13-15/group). *Significantly different compared with control mice ($P < 0.05$).

food restriction ($t(26) = 2.883$, $P = 0.0078$; Fig. 1M). Overall, this new cohort of brain GHR KO mice confirmed our previous observations and provides new evidence indicating that the lack of GHR exclusively in the brain induced some metabolic alterations that are less severe than those reported for whole-body GHR KO mice (46). Additionally, Brain GHR KO mice displayed an attenuated hyperphagia after 2 different protocols of food restriction.

Brain GHR KO mice are unresponsive to ghrelin-induced food intake

Next, we assessed the effects of systemically administered ghrelin. Brain GHR KO mice displayed no differences in ghrelin-induced increase of plasma GH levels when compared with control mice (main effect of ghrelin [$F(1, 22) = 25.66$, $P < 0.0001$]; Fig. 2A). In contrast, brain GHR KO mice did not increase food intake in response to ghrelin (main effect of time [$F(2, 78) = 117.8$, $P < 0.0001$], main effect of GHR ablation [$F(1, 33) = 19.56$, $P < 0.0001$], and interaction [$F_{(6, 198)} = 15.02$, $P < 0.0001$]; Fig. 2B). Because the orexigenic effect of systemically injected ghrelin mainly involves its action at the ARH level (23-25), we assessed ghrelin-induced c-Fos expression in this hypothalamic nucleus. In accordance with the lack of orexigenic response to ghrelin, brain GHR KO mice did not display ghrelin-induced increase of the number of c-Fos positive cells in the ARH, as observed in control mice (main effect of ghrelin [$F(1, 23) = 23.61$, $P < 0.0001$], main effect of GHR ablation [$F(1, 23) = 5.746$, $P = 0.025$], and interaction [$F(1, 23) = 6.318$, $P = 0.0194$]; Fig. 2C-G). Thus, brain GHR KO mice were unresponsive to the orexigenic effects of systemically injected ghrelin and failed to show ghrelin-induced c-Fos expression in the ARH, although ghrelin-induced raise in circulating GH levels remained unaltered.

Reduced expression of NPY and ghrelin receptor in the hypothalamus of brain GHR KO mice

To gain insights of the putative mechanisms that abrogate the capability of brain GHR KO mice to increase food intake in response to ghrelin, we investigated the mRNA levels of different genes expressed by ARH neurons that regulate food intake. Brain GHR KO mice showed significantly less hypothalamic mRNA levels of *Npy* ($t(13) = 3.851$, $P = 0.002$), as compared with control mice, whereas the mRNA levels of *Agrp* and *Pomc* remained unaffected (Fig. 3). On the other hand, brain GHR KO mice showed higher *Cartpt* mRNA levels in the hypothalamus ($t(13) = 2.291$, $P = 0.0393$; Fig. 3). Interestingly, brain GHR KO mice

showed reduced mRNA levels of *Ghsr* in the hypothalamus, as compared with control mice ($t(13) = 2.682$, $P = 0.0188$; Fig. 3).

Reduced AgRP innervation in brain-specific GHR KO mice

Orexigenic effects of systemically injected ghrelin mainly recruit ARH$^{AgRP/NPY}$ neurons (23-25), which send axonal projections to the PVH to regulate feeding and metabolic responses (51-54). Because AgRP is exclusively produced in the ARH, whereas NPY is also produced in other brain areas (55), we used fluorescent immunostaining against AgRP to estimate the levels of this orexigenic ARH$^{AgRP/NPY}$ neuron-derived peptide in the hypothalamus of brain GHR KO mice (Fig. 4A-F). Brain GHR KO mice displayed a significant reduction in the density of the AgRP fluorescent signal in both the ARH ($t(6) = 3.747$, $P = 0.0095$; Fig. 4A-C) and PVH ($t(6) = 6.489$, $P = 0.0006$; Fig. 4D-F). In contrast, levels of POMC fluorescent signal in the PVH were not different between groups (Fig. 4G-I). Thus, the lack of GHR in the brain specifically affects the ARH$^{AgRP/NPY}$ neurons that, in turn, may explain the abrogated capability of brain GHR KO mice to increase food intake in response to ghrelin treatment.

AgRP GHR KO mice display normal orexigenic response to ghrelin treatment

To determine whether the orexigenic effect of ghrelin treatment requires GHR expression in the ARH$^{AgRP/NPY}$ neurons, we generated mice lacking GHR specifically in this neuronal population by crossing mice carrying loxP-flanked *Ghr* alleles and AgRP-Cre mice. Confirming the cell-specific deletion, GH-induced pSTAT5 was virtually absent in the ARH$^{AgRP/NPY}$ neurons of AgRP GHR KO mice, whereas $94.7 \pm 1.1\%$ of NPY/AgRP neurons were responsive to GH in control animals ($t(4) = 44.79$, $P < 0.0001$; Fig. 5A, B, D). Importantly, GH-induced increase of pSTAT5 was intact in other parts of the brain of AgRP GHR KO mice (e.g., in the PVH; Fig. 5C). Additionally, body weight and food intake of AgRP GHR KO mice were not different to control mice (Fig. 5E, F). Furthermore, AgRP GHR KO mice showed a refeeding response to 24-hour fasting or 5 days of 60% food restriction that was not different from the response of control mice, calculated as either absolute values (Fig. 5G, H) or relative to body weight (data not shown).

In contrast to brain GHR KO mice, AgRP GHR KO mice showed mRNA levels of *Npy*, *Agrp*, *Pomc*, *Cartpt*, and *Ghsr* in the hypothalamus that were not different than those measured in control mice (Fig. 6A). Additionally, no difference between the groups was
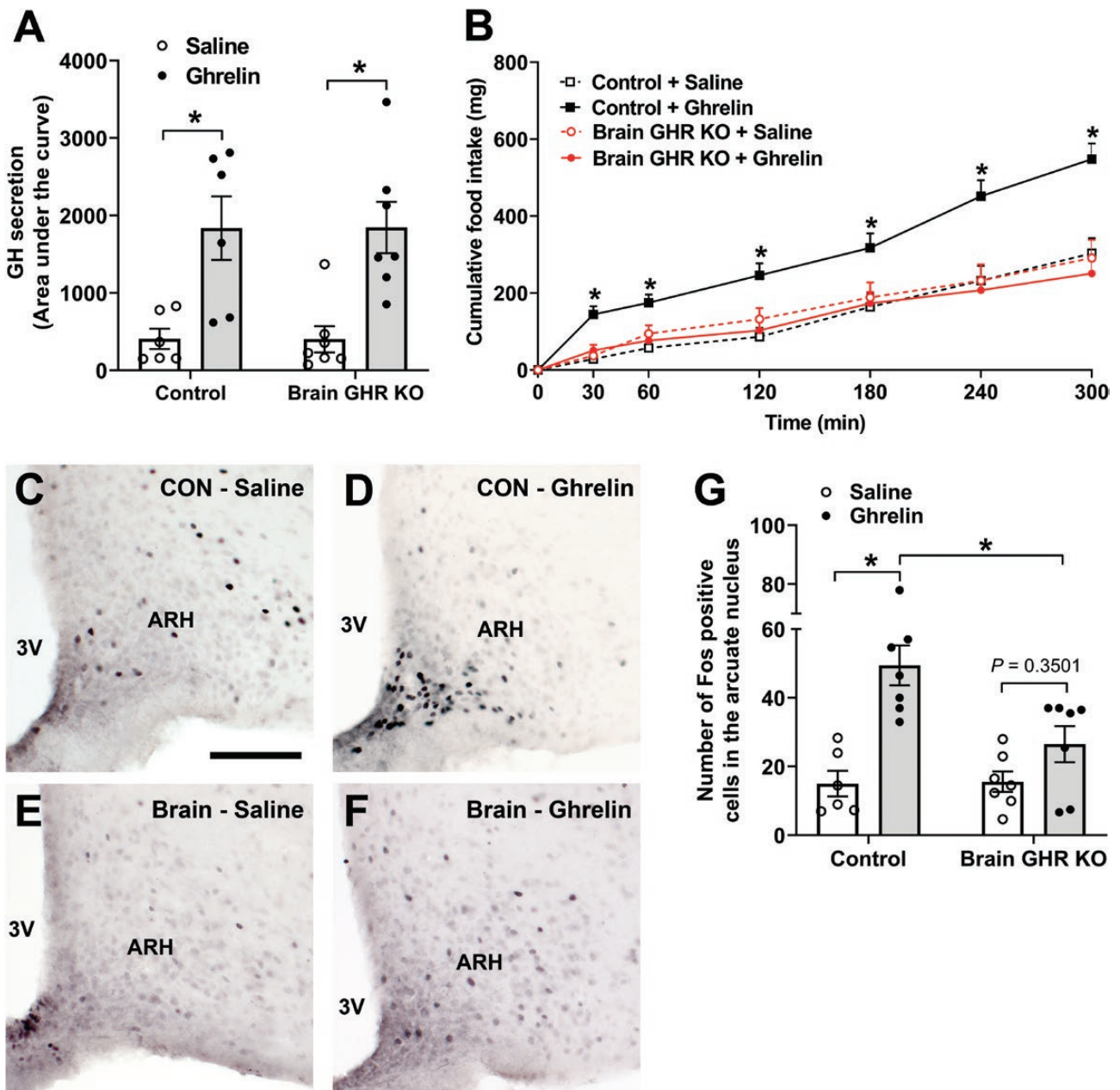
Figure 2. Brain GHR KO mice are unresponsive to ghrelin-induced food intake. (A) Area under the curve of plasma GH levels in control (n = 6) and brain GHR KO (n = 7) mice that received saline or ghrelin IP injections. (B) Cumulative food intake in control (n = 18) and brain GHR KO (n = 17) mice that received saline or ghrelin IP injections. (C-G). c-Fos expression in the ARH of control (n = 6) and brain GHR KO (n = 7) mice that received saline or ghrelin IP injections. Abbreviations: 3V, third ventricle; GHR, GH receptor; KO, knockout. Scale bar = 100 μm. *Significantly different compared with control mice ($P < 0.05$).
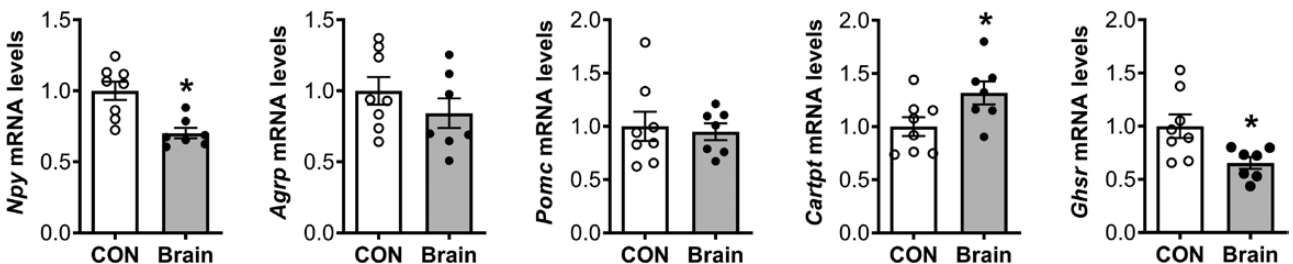

Figure 3. Reduced expression of NPY and ghrelin receptor in the hypothalamus of brain GHR KO mice. Relative mRNA levels in the hypothalamus of control (n = 8) and brain GHR KO (n = 7) mice. *Significantly different compared to control mice ($P < 0.05$). Abbreviations: GHR, GH receptor; KO, knockout; NPY, neuropeptide Y.
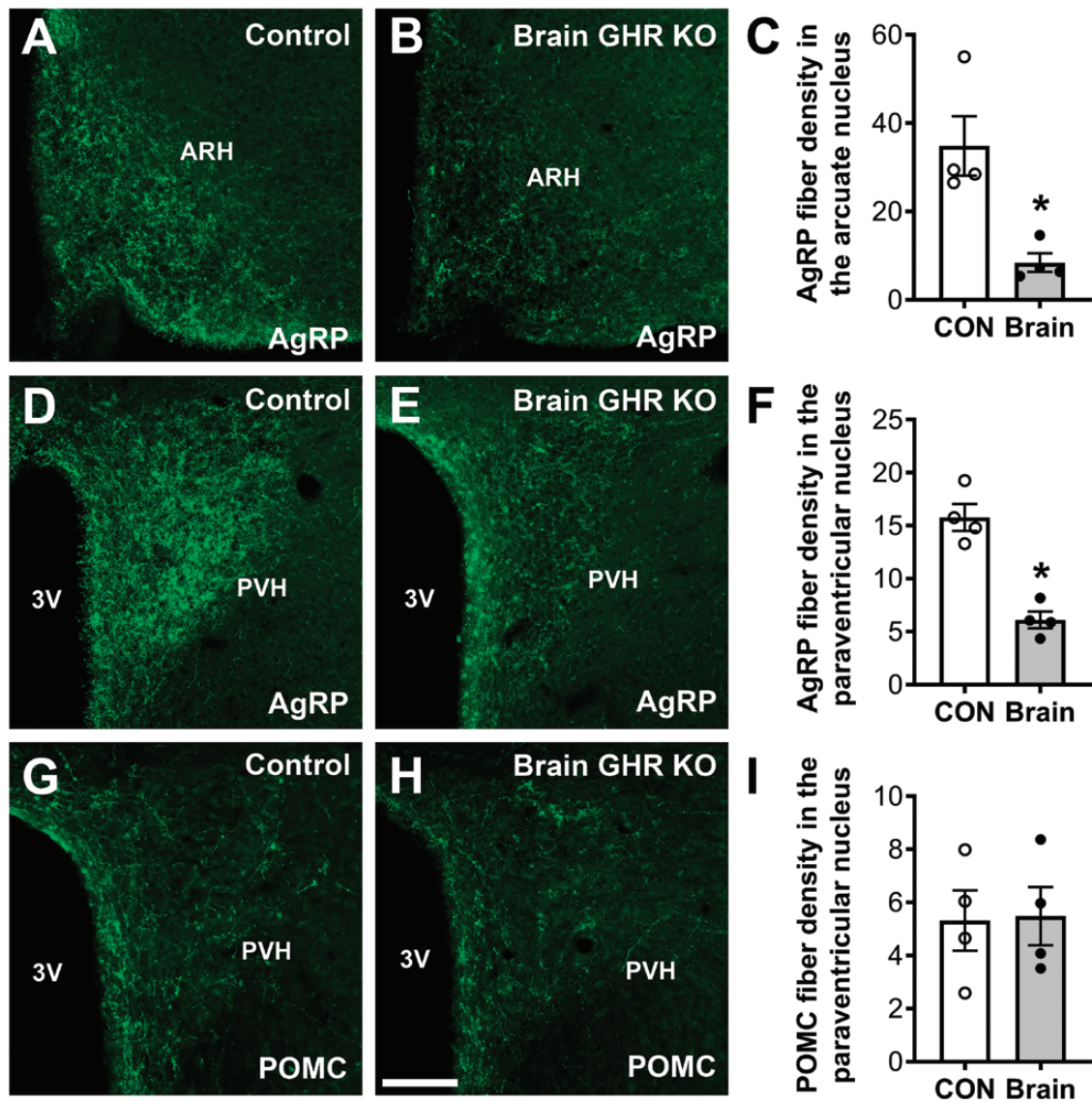
Figure 4. Reduced AgRP innervation in the hypothalamus of brain-specific GHR KO mice. (A-F) Immunostaining and quantification of AgRP fluorescent signal in the ARH and paraventricular nucleus of the hypothalamus (PVH) of control (n = 4) and brain GHR KO (n = 4) mice. (G-I) Immunostaining and quantification of POMC fluorescent signal in the PVH of control (n = 4) and brain GHR KO (n = 4) mice. Abbreviations: 3V, third ventricle: AgRP, agouti-related protein; GHR, GH receptor; KO, knockout. Scale bar = 100 μm. *Significantly different compared to control mice ($P < 0.05$).

observed in the AgRP fluorescent signal in the ARH (Fig. 6B-D). However, AgRP GHR KO mice displayed a significant reduction in the density of AgRP fluorescent signal in the PVH ($t(5) = 2.74$, $P = 0.0408$; Fig. 6E-G), as also seen in brain GHR KO mice.

In response to systemically administered ghrelin, food intake of AgRP GHR KO and control mice was not different (Fig. 7A). In addition, the ghrelin-induced increase in the number of c-Fos positive cells in the ARH of AgRP GHR KO mice and control mice was not different (Fig. 7B-D). Thus, the orexigenic effect of ghrelin treatment and its capability to induce c-Fos activation

in the ARH do not require GHR expression in ARH[AgRP/NPY] neurons.

Discussion

The current study provides functional and neuroanatomical data supporting 2 major findings. First, ghrelin treatment is unable to increase food intake or induce c-Fos expression in the ARH of mice lacking GHR in the peripheral and central nervous systems, despite these mice are fully responsive to ghrelin-induced GH secretion. Second, ghrelin treatment increases food intake and induces c-Fos
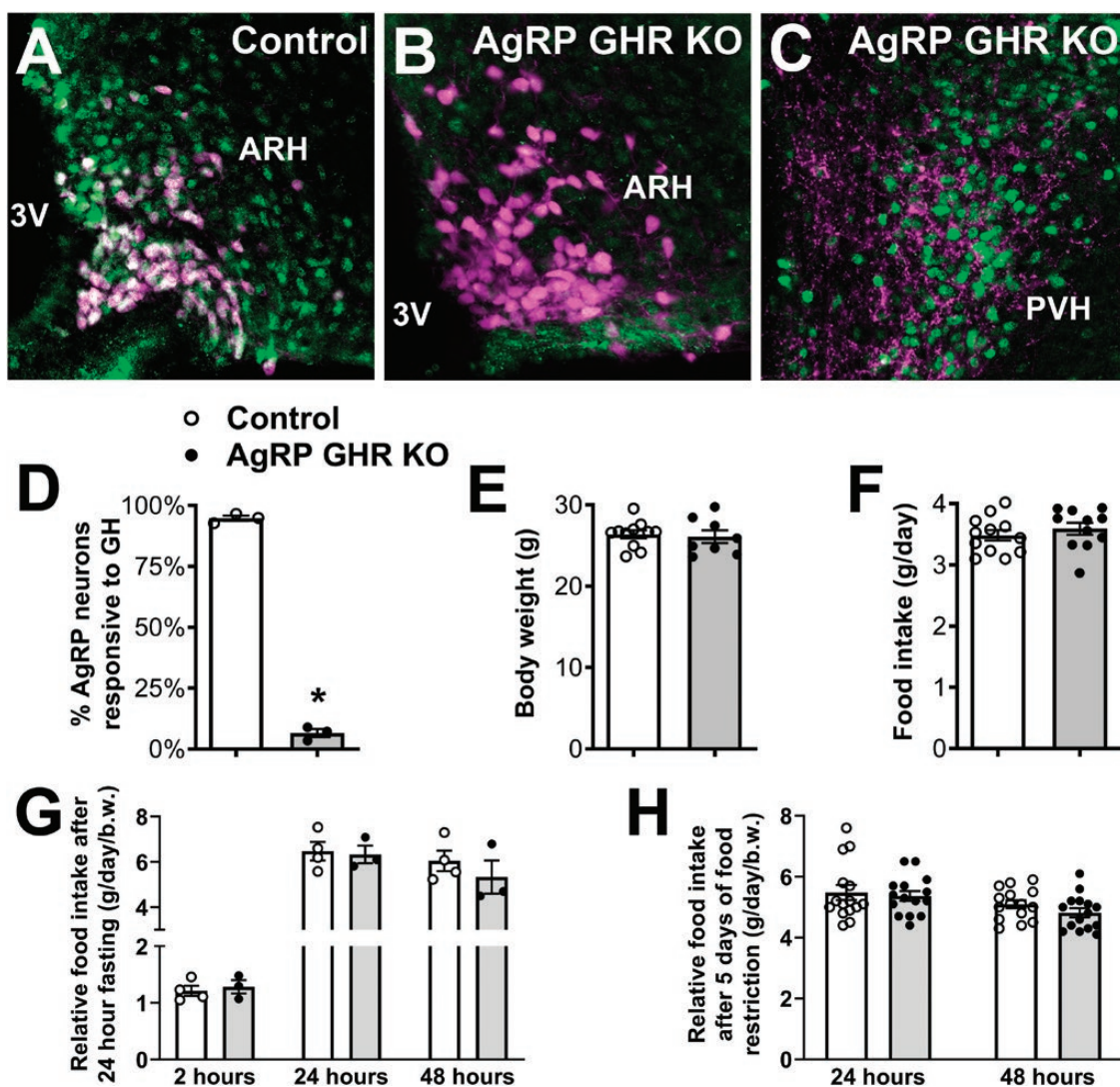
Figure 5. Characterization of AgRP GHR KO male mice. (A-D) Colocalization of STAT5 phosphorylation (green) and AgRP neurons (magenta) in the ARH of GH-injected control and AgRP GHR KO mice (n = 3/group). Double-labeled cells appear in white. Note that AgRP fibers abundantly innervate the PVH. Scale bar = 50 μm. (E) Body weight in control (n = 11) and AgRP GHR KO (n = 8) male mice. (F) Food intake in control (n = 12) and AgRP GHR KO (n = 11) mice. (G) Food intake after a 24-hour fasting period (n = 3-4/group). (H) Refeeding response after 5 days of 60% food restriction (n = 15/group). *Significantly different compared with control mice ($P < 0.05$). Abbreviations: 3V, third ventricle; AgRP, agouti-related protein; ARH, arcuate nucleus of the hypothalamus; GHR, GH receptor; KO, knockout.

expression in the ARH of mice lacking GHR exclusively in ARH$^{AgRP/NPY}$ neurons. Thus, the orexigenic effect of ghrelin seems to require GHR signaling in a subset of cells of the nervous system, which are not ARH$^{AgRP/NPY}$ neurons.

In 2006, Egecioglu and colleagues reported the intriguing observation that ghrelin treatment fails to increase food intake and induce changes in the gene expression of orexigenic neuropeptides in male whole-body GHR KO mice (44). However, the fact that GHR KO mice display a number of severe abnormalities, such as hyperphagia and dwarfism (46), raised some concerns regarding the direct implications of GHR signaling for the orexigenic effect of ghrelin. To clarify the requirement of the GHR signaling for the orexigenic effect of ghrelin, we studied the effects

of systemically injected ghrelin on the food intake of male mice lacking GHR specifically in neurons or exclusively in the ARH$^{AgRP/NPY}$ neurons. As a strategy to simulate the route by which the stomach-derived hormone reaches the brain, we used a single bolus intraperitoneal injection of ghrelin, which induces a transient supraphysiological increase in the plasma ghrelin levels that return to basal levels at ~45 minutes after treatment and mainly acts at brain areas nearby fenestrated capillaries (56, 57). The assessment of ghrelin-induced c-Fos expression as well as the analysis of the accessibility of fluorescent variants of ghrelin into the brain have revealed that systemically injected ghrelin mainly acts at the ARH (20, 58, 59). Indeed, systemically injected ghrelin does not increase food intake
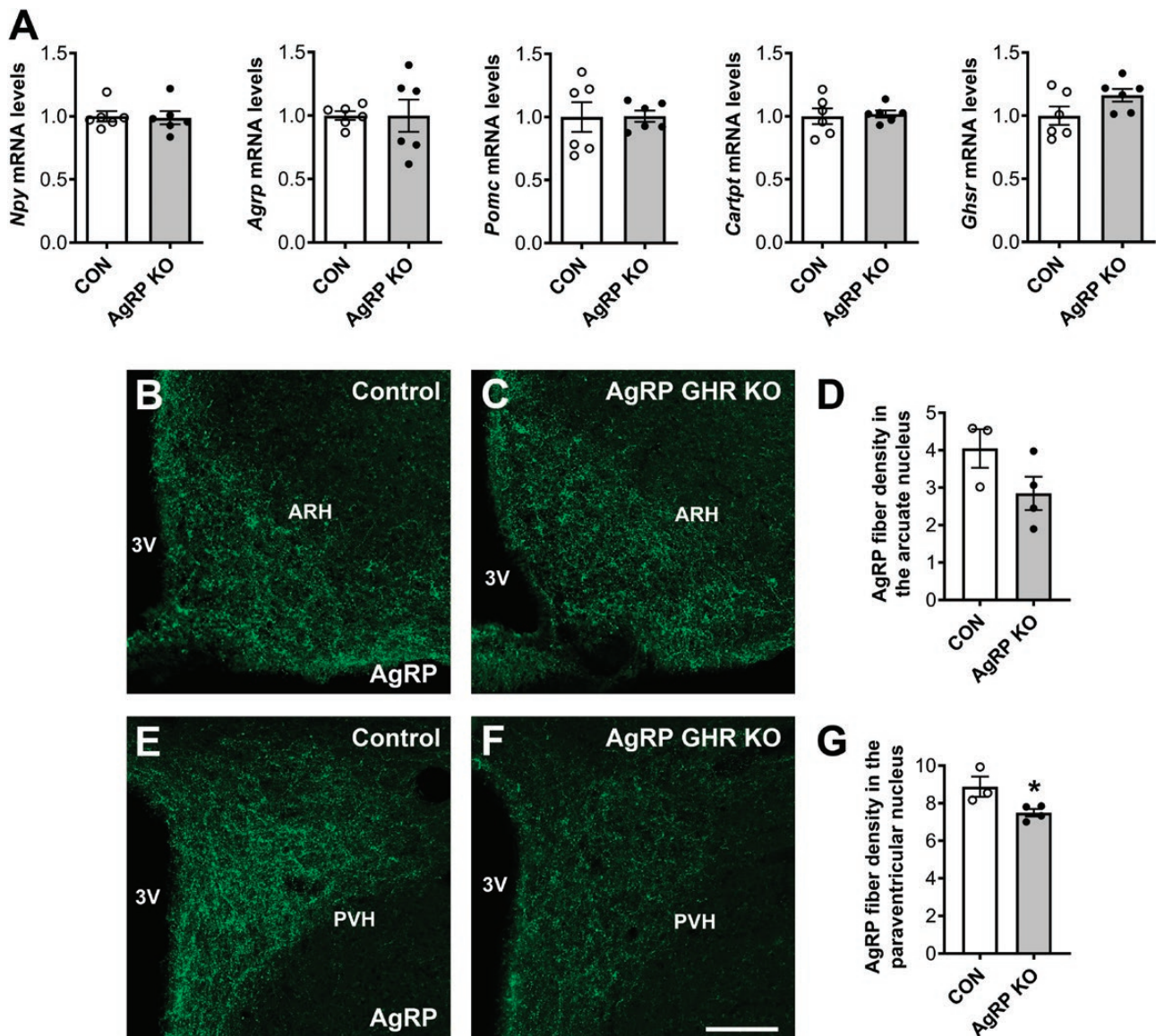
Figure 6. AgRP GHR KO mice exhibit reduced AgRP innervation in the hypothalamus. (A) Relative mRNA levels in the hypothalamus of control and AgRP GHR KO mice (n = 6/group). (B-G) Immunostaining and quantification of AgRP fluorescent signal in the ARH and PVH of control (n = 3) and AgRP GHR KO (n = 4) mice. Abbreviations: 3V, third ventricle; AgRP, agouti-related protein; GHR, GH receptor; KO, knockout. Scale bar = 100 µm. *Significantly different compared with control mice ($P < 0.05$).

in ARH-ablated mice whereas centrally injected ghrelin does so (15). Importantly, most studies have reported that the systemic treatment with similar doses of ghrelin does not affect food seeking or locomotor activity, unlike what is observed when ghrelin is centrally administered or systemically administered at higher doses (as reviewed elsewhere (60)). Thus, the current study was performed using carefully chosen experimental conditions in which injected ghrelin reaches the brain, in a similar fashion as endogenous ghrelin, and mainly regulates food intake.

First, we studied the effects of systemically injected ghrelin in brain GHR KO mice, which were generated crossing GHR^floxed mice and Nestin-Cre mice and had

already been characterized in our laboratory (29, 47). Of note, Nestin-cre mice present hypopituitarism and reduced body weight because a copy of the GH minigene was inserted downstream the Cre sequence leading to the central production of GH that, in turn, induces local GHR activation and suppresses pituitary GH secretion (61). In contrast, young brain GHR KO mice are ~20% heavier than control mice. Such increased body weight has been attributed to the lack of hypothalamic GHR signaling, which not only prevents the action of transgene-derived GH, but also abrogates the GH negative feedback leading to increased GH secretion and serum IGF-1 levels that, consequently, increase body length and lean body mass
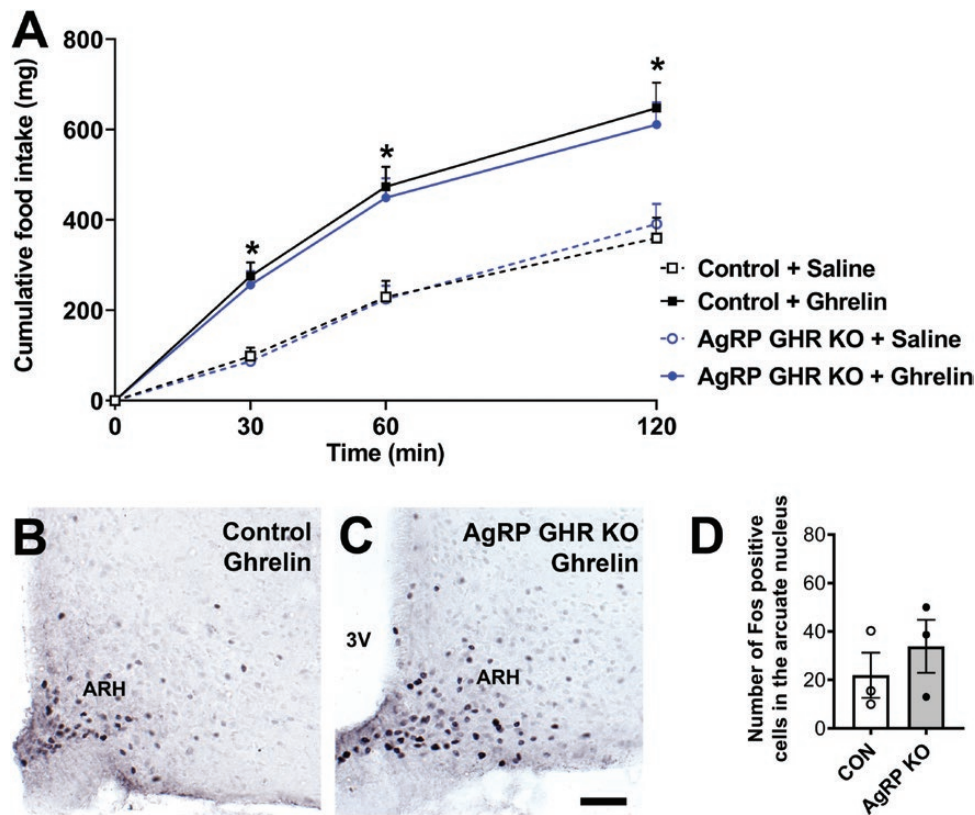
Figure 7. AgRP GHR KO mice display normal orexigenic response to ghrelin. (A) Cumulative food intake in control (n = 19) and AgRP GHR KO (n = 18) mice that received saline or ghrelin IP injections. (B-D) c-Fos expression in the ARH of control and AgRP GHR KO mice that received ghrelin injection (n = 3/group). Abbreviations: 3V, third ventricle; AgRP, agouti-related protein; GHR, GH receptor; KO, knockout. Scale bar = 50 μm. *Significantly different compared with saline-injected mice ($P < 0.05$).

(29, 39, 47). Brain GHR KO mice display unaltered ad libitum food intake, adiposity, plasma leptin levels and leptin responsiveness, glycemia, glucose tolerance, and insulin sensitivity (29, 39). Here, we show that brain GHR KO mice also display unaltered ghrelin-induced GH secretion.

To release GH, ghrelin could act in somatotropic pituitary cells as well as in hypothalamic neurons that, in turn, produce GH-releasing neuropeptides that act at the pituitary (as reviewed previously (11, 62)). GHSR is highly expressed in somatotropic cells, and ghrelin potently releases GH in primary cultures of pituitary cells (63). In the pituitary, ghrelin also enhances GHRH-stimulated release of GH and antagonizes the inhibitory effect of somatostatin. In hypothalamic explants, ghrelin releases GHRH and inhibits the secretion of somatostatin (64, 65). Ghrelin-mediated activation of GHRH neurons involves direct and indirect mechanisms. In particular, ghrelin directly increases the firing rate of ~25% of GHRH neurons, and also decreases the inhibitory gamma-aminobutyric acid neurotransmission on GHRH neurons, whereas it does not affect the excitatory glutamatergic neurotransmission (10,

66, 67). The observation that ghrelin induces similar increase of GH secretion in control and brain GHR KO mice strongly indicates that the action of ghrelin on the pituitary is sufficient to evoke its potent GH secretagogue effect.

Here, we found that ghrelin fails to increase food intake in male brain GHR KO mice indicating that the orexigenic effects of ghrelin requires GHR signaling in the nervous system. Current observations help to narrow the putative mechanisms mediating this phenomenon. For instance, the blunted feeding response to ghrelin of whole-body GHR KO mice had been linked to the basal hyperphagia, which could unmask a ghrelin-induced increase of food intake (44), or the high plasma leptin levels, which acts on GHSR expressing neurons of the ARH and impairs the orexigenic effects of ghrelin (68, 69). However, current results suggest that the inability of ghrelin treatment to increase food intake in brain GHR KO mice is independent of these 2 parameters. Notably, we also found that brain GHR KO mice exposed to fasting-refeeding or food restriction-refeeding protocols show a reduced hyperphagic response. In addition, we found that central GHR deficiency in male brain GHR KO mice results

in a dysfunction of the orexigenic ARH^AgRP/NPY neurons. In particular, brain GHR KO mice display reduced: (1) hypothalamic *Npy* gene expression; (2) hypothalamic *Ghsr* gene expression, which is enriched in ARH^AgRP/NPY neurons (18); (3) ghrelin-induced c-Fos in the ARH, which involves ARH^AgRP/NPY neurons (70, 71); (4) AgRP peptide levels in the ARH; and (5) ARH^AgRP/NPY projections to the PVH, whose selective activation induces feeding, whereas its pharmacological blockade reduces feeding induced by activation of ARH^AgRP/NPY neurons (53, 72-74). In contrast, *Pomc* gene expression in the ARH and the biosynthesis of POMC peptides were unaltered in brain GHR KO mice, suggesting that the lack of GHR signaling mainly affects the ARH^AgRP/NPY neurons. Because ARH^AgRP/NPY neurons are required for ghrelin-induced food intake (23-25), it can be hypothesized that brain GHR KO mice fails to increase food intake in response to ghrelin treatment because the lack of GHR signaling in the mouse brain causes a major disruption in the biology of the ARH^AgRP/NPY neurons. It also likely that the reduced hyperphagic response after energy-deficit conditions in brain GHR KO mice is partially due to their inability to respond to the orexigenic effects of ghrelin, whose plasma levels increase under energy-deficit conditions and contribute to enhance the compensatory hyperphagic response of refed mice (75). Thus, current results strongly highlight the requirement of the GHR signaling in the brain not only for ghrelin-induced food intake but also for other orexigenic responses that involve the ghrelin system.

To test the impact of GHR signaling exclusively in ARH^AgRP/NPY neurons and its consequences on ghrelin-induced food intake, we studied AgRP GHR KO mice. These mice did not show any evident alteration of food intake or body weight in ad libitum–fed conditions. We have recently shown that GH directly activates ARH^AgRP/NPY neurons, and induces *Agrp* and *Npy* gene expression (29); however, AgRP GHR KO mice did not show significant alterations of *Agrp* and *Npy* gene expression in the hypothalamus, in contrast to brain GHR KO mice. Thus, current results suggest that endogenous GH in ad libitum–fed mice is insufficient to activate the ARH^AgRP/NPY neurons and that the indirect action of GH is responsible for the alterations found in the ARH^AgRP/NPY neurons of brain GHR KO mice. Still, AgRP GHR KO mice did show a reduction of the ARH^AgRP/NPY projections to the PVH suggesting that GHR signaling directly affect, in some extent, the biology of these neurons. AgRP GHR KO mice display *Ghsr* mRNA levels in the hypothalamus similar to control mice, in line with the fact that the *Ghsr* promoter lacks pSTAT5 responsive elements (76). However, specific changes of *Ghsr* gene expression in ARH^AgRP/NPY neurons may have been masked in our analysis of the whole hypothalamus. Of relevance

for the current study, AgRP GHR KO mice show unaltered compensatory hyperphagia in response to fasting or calorie restriction as well as conserved ghrelin-induced food intake and c-Fos in the ARH. Thus, GHR signaling in the ARH^AgRP/NPY neurons does affect some cell features that end up disturbing their neuronal projections. Nevertheless, these changes are not sufficient to impair food intake responses to energy deficit conditions or to ghrelin-induced food intake. Thus, ghrelin-induced activation of ARH neurons and ghrelin-induced food intake require GHR signaling in a subset of non-ARH^AgRP/NPY neurons, whose identity still needs to be uncovered.

The neuroanatomical basis for the abrogated capability of brain GHR KO mice to increase food intake in response to ghrelin remain uncertain. Brain GHR KO mice failed to show ghrelin-induced increase of c-Fos expression in the ARH, suggesting that GHR signaling is required for the intrinsic capability of ARH neurons to respond to the hormone. Also, the observation that brain GHR KO mice display a more severe reduction of ARH^AgRP/NPY projections to the PVH, compared with AgRP GHR KO mice (~61% vs ~16%), may impair their response to the orexigenic effect of ghrelin because experiments using optogenetic activation of ARH^AgRP/NPY projections to the PVH have shown that the magnitude of the evoked food intake directly depends on its degree of stimulation (73). Notably, whole-body GHR KO mice exhibit a ~60% reduction in the AgRP fiber density in the ARH and PVH (77), as observed in brain GHR KO mice. GH-deficient mice also display a severe reduction of the ARH^AgRP/NPY projections to the PVH, as well as in other hypothalamic nuclei, and such reduction is reversed by the treatment with GH, starting at 2 weeks of age (77). In contrast, male mice lacking GHR specifically in liver, which show low plasma IGF-1 levels but retain the capability to respond to GH at central level, have no alterations of the ARH^AgRP/NPY projections to the PVH (77). Thus, GH itself seems to play an important neurodevelopmental role regulating the connectivity of the hypothalamic circuits that control food intake, and alterations of such connectivity could affect the capability of brain GHR KO mice to respond to the orexigenic effects of ghrelin. Also, the more drastic reduction of ARH^AgRP/NPY projections to the PVH in brain GHR KO mice may be related to the reduced hypothalamic *Ghsr* gene expression because GHSR signaling in ARH^AgRP/NPY neurons increases the density of these projections (78). Future studies will be required to investigate the specific roles of the GH signaling and its neuronal targets affecting the capability of ghrelin to increase food intake.

In summary, our findings revealed that brain GHR signaling in male mice is required for the stimulatory effect of ghrelin on food intake. Although the central disruption

of GHR signaling significantly affected the biology of ARH$^{AgRP/NPY}$ neurons, a direct GH action on this neuronal population is not sufficient to explain the blunted response to ghrelin observed in brain GHR KO mice. A functional role of the ghrelin-GH axis has already been described for the regulation of glycemia during starvation (49, 79, 80) and for fear learning associated with posttraumatic stress disorder (81, 82), but the extent to which GHR signaling is involved in other actions of ghrelin (e.g., learning or memory formation) remains to be investigated. Finally, it is important to stress that the current study was performed in male mice because seminal research indicating that the orexigenic effect of ghrelin requires the GHR signaling had been conducted in male rodents. Such male bias in the study of the orexigenic effects of ghrelin is likely because ghrelin induces a more pronounced increment of food intake in males than females (83). Still, it is well established that ghrelin/GHSR and GH/GHR systems display sexually dimorphic responses; thus, specific studies will be required to clarify the extent to which brain GHR signaling affects the orexigenic effect of ghrelin in females.

Acknowledgments

Additional Information

Correspondence: Mario Perelló, PhD, Multidisciplinary Institute of Cell Biology, Calle 526 S/N entre 10 y 11, La Plata, Buenos Aires, 1900. Argentina. Email: mperello@imbice.gov.ar and Jose Donato Jr., PhD, Instituto de Ciencias Biomedicas. Av. Prof. Lineu Prestes, 1524, São Paulo, SP, 05508-000, Brazil; Email: jdonato@icb.usp.br.

Disclosures: The authors have nothing to disclose.

Data Availability: The data that support the findings of this study are available from the corresponding authors upon reasonable request.

References

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*. 1999;**402**(6762):656-660.

2. Müller TD, Nogueiras R, Andermann ML, et al. Ghrelin. *Mol Metab*. 2015;**4**(6):437-460.

3. Veldhuis JD, Reynolds GA, Iranmanesh A, Bowers CY. Twenty-four hour continuous ghrelin infusion augments physiologically pulsatile, nycthemeral, and entropic (feedback-regulated) modes of growth hormone secretion. *J Clin Endocrinol Metab*. 2008;**93**(9):3597-3603.

4. Arvat E, Di Vito L, Broglio F, et al. Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. *J Endocrinol Invest*. 2000;**23**(8):493-495.

5. Peino R, Baldelli R, Rodriguez-Garcia J, et al. Ghrelin-induced growth hormone secretion in humans. *Eur J Endocrinol*. 2000;**143**(6):R11-R14.

6. Schmid DA, Held K, Ising M, Uhr M, Weikel JC, Steiger A. Ghrelin stimulates appetite, imagination of food, GH, ACTH, and cortisol, but does not affect leptin in normal controls. *Neuropsychopharmacology*. 2005;**30**(6):1187-1192.

7. Hassouna R, Zizzari P, Viltart O, et al. A natural variant of obestatin, Q90L, inhibits ghrelin's action on food intake and GH secretion and targets NPY and GHRH neurons in mice. *Plos One*. 2012;**7**(12):e51135.

8. Hataya Y, Akamizu T, Takaya K, et al. A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans. *J Clin Endocrinol Metab*. 2001;**86**(9):4552.

9. Popovic V, Miljic D, Micic D, et al. Ghrelin main action on the regulation of growth hormone release is exerted at hypothalamic level. *J Clin Endocrinol Metab*. 2003;**88**(7):3450-3453.

10. Feng DD, Yang SK, Loudes C, et al. Ghrelin and obestatin modulate growth hormone-releasing hormone release and synaptic inputs onto growth hormone-releasing hormone neurons. *Eur J Neurosci*. 2011;**34**(5):732-744.

11. Steyn FJ, Tolle V, Chen C, Epelbaum J. Neuroendocrine regulation of growth hormone secretion. *Compr Physiol*. 2016;**6**(2):687-735.

12. Tannenbaum GS, Epelbaum J, Bowers CY. Interrelationship between the novel peptide ghrelin and somatostatin/growth hormone-releasing hormone in regulation of pulsatile growth hormone secretion. *Endocrinology*. 2003;**144**(3):967-974.

13. Wren AM, Small CJ, Ward HL, et al. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology*. 2000;**141**(11):4325-4328.

14. Levin F, Edholm T, Schmidt PT, et al. Ghrelin stimulates gastric emptying and hunger in normal-weight humans. *J Clin Endocrinol Metab*. 2006;**91**(9):3296-3302.

15. Cabral A, Valdivia S, Fernandez G, Reynaldo M, Perello M. Divergent neuronal circuitries underlying acute orexigenic effects of peripheral or central ghrelin: critical role of brain accessibility. *J Neuroendocrinol*. 2014;**26**(8):542-554.

16. McFarlane MR, Brown MS, Goldstein JL, Zhao TJ. Induced ablation of ghrelin cells in adult mice does not decrease food intake, body weight, or response to high-fat diet. *Cell Metab*. 2014;**20**(1):54-60.

17. Kuo YT, Parkinson JR, Chaudhri OB, et al. The temporal sequence of gut peptide CNS interactions tracked in vivo by magnetic resonance imaging. *J Neurosci*. 2007;**27**(45):12341-12348.

18. Willesen MG, Kristensen P, Rømer J. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology*. 1999;**70**(5):306-316.

19. Cabral A, Fernandez G, Perello M. Analysis of brain nuclei accessible to ghrelin present in the cerebrospinal fluid. *Neuroscience*. 2013;**253**:406-415.

20. Schaeffer M, Langlet F, Lafont C, et al. Rapid sensing of circulating ghrelin by hypothalamic appetite-modifying neurons. *Proc Natl Acad Sci U S A*. 2013;**110**(4):1512-1517.

21. Mani BK, Walker AK, Lopez Soto EJ, et al. Neuroanatomical characterization of a growth hormone secretagogue receptor-green fluorescent protein reporter mouse. *J Comp Neurol*. 2014;**522**(16):3644-3666.

22. Perello M, Sakata I, Birnbaum S, et al. Ghrelin increases the rewarding value of high-fat diet in an orexin-dependent manner. *Biol Psychiatry*. 2010;**67**(9):880-886.

23. Cabral A, Portiansky E, Sánchez-Jaramillo E, Zigman JM, Perello M. Ghrelin activates hypophysiotropic corticotropin-releasing factor neurons independently of the arcuate nucleus. *Psychoneuroendocrinology*. 2016;**67**:27-39.

24. Wang Q, Liu C, Uchida A, et al. Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin. *Mol Metab*. 2014;**3**(1):64-72.

25. Wu CS, Bongmba OYN, Yue J, et al. Suppression of GHS-R in AgRP neurons mitigates diet-induced obesity by activating thermogenesis. *Int J Mol Sci*. 2017;**18**(4):832.

26. Snel YE, Brummer RJ, Doerga ME, Zelissen PM, Koppeschaar HP. Energy and macronutrient intake in growth hormone-deficient adults: the effect of growth hormone replacement. *Eur J Clin Nutr*. 1995;**49**(7):492-500.

27. Blissett J, Harris G, Kirk J. Effect of growth hormone therapy on feeding problems and food intake in children with growth disorders. *Acta Paediatr*. 2000;**89**(6):644-649.

28. Azain MJ, Roberts TJ, Martin RJ, Kasser TR. Comparison of daily versus continuous administration of somatotropin on growth rate, feed intake, and body composition in intact female rats. *J Anim Sci*. 1995;**73**(4):1019-1029.

29. Furigo IC, Teixeira PDS, de Souza GO, et al. Growth hormone regulates neuroendocrine responses to weight loss via AgRP neurons. *Nat Commun*. 2019;**10**(1):662.

30. Zhong C, Song Y, Wang Y, et al. Increased food intake in growth hormone-transgenic common carp (Cyprinus carpio L.) may be mediated by upregulating Agouti-related protein (AgRP). *Gen Comp Endocrinol*. 2013;**192**:81-88.

31. Kim JH, Leggatt RA, Chan M, Volkoff H, Devlin RH. Effects of chronic growth hormone overexpression on appetite-regulating brain gene expression in coho salmon. *Mol Cell Endocrinol*. 2015;**413**:178-188.

32. Bohlooly YM, Olsson B, Bruder CE, et al. Growth hormone overexpression in the central nervous system results in hyperphagia-induced obesity associated with insulin resistance and dyslipidemia. *Diabetes*. 2005;**54**(1):51-62.

33. Furigo IC, Metzger M, Teixeira PD, Soares CR, Donato J Jr. Distribution of growth hormone-responsive cells in the mouse brain. *Brain Struct Funct*. 2017;**222**(1):341-363.

34. Walsh RJ, Mangurian LP, Posner BI. The distribution of lactogen receptors in the mammalian hypothalamus: an in vitro autoradiographic analysis of the rabbit and rat. *Brain Res*. 1990;**530**(1):1-11.

35. Burton KA, Kabigting EB, Clifton DK, Steiner RA. Growth hormone receptor messenger ribonucleic acid distribution in the adult male rat brain and its colocalization in hypothalamic somatostatin neurons. *Endocrinology*. 1992;**131**(2):958-963.

36. Chan YY, Steiner RA, Clifton DK. Regulation of hypothalamic neuropeptide-Y neurons by growth hormone in the rat. *Endocrinology*. 1996;**137**(4):1319-1325.

37. Kamegai J, Minami S, Sugihara H, Hasegawa O, Higuchi H, Wakabayashi I. Growth hormone receptor gene is expressed in neuropeptide Y neurons in hypothalamic arcuate nucleus of rats. *Endocrinology*. 1996;**137**(5):2109-2112.

38. Wasinski F, Furigo IC, Teixeira PDS, et al. Growth hormone receptor deletion reduces the density of axonal projections from hypothalamic arcuate nucleus neurons. *Neuroscience*. 2020;**434**:136-147.

39. Teixeira PDS, Couto GC, Furigo IC, List EO, Kopchick JJ, Donato J Jr. Central growth hormone action regulates metabolism during pregnancy. *Am J Physiol Endocrinol Metab*. 2019;**317**(5):E925-E940.

40. Kamegai J, Minami S, Sugihara H, Higuchi H, Wakabayashi I. Growth hormone induces expression of the c-fos gene on hypothalamic neuropeptide-Y and somatostatin neurons in hypophysectomized rats. *Endocrinology*. 1994;**135**(6):2765-2771.

41. Dickson SL, Luckman SM. Induction of c-fos messenger ribonucleic acid in neuropeptide Y and growth hormone (GH)-releasing factor neurons in the rat arcuate nucleus following systemic injection of the GH secretagogue, GH-releasing peptide-6. *Endocrinology*. 1997;**138**(2):771-777.

42. Nakazato M, Murakami N, Date Y, et al. A role for ghrelin in the central regulation of feeding. *Nature*. 2001;**409**(6817):194-198.

43. Tolle V, Bassant MH, Zizzari P, et al. Ultradian rhythmicity of ghrelin secretion in relation with GH, feeding behavior, and sleep-wake patterns in rats. *Endocrinology*. 2002;**143**(4):1353-1361.

44. Egecioglu E, Bjursell M, Ljungberg A, et al. Growth hormone receptor deficiency results in blunted ghrelin feeding response, obesity, and hypolipidemia in mice. *Am J Physiol Endocrinol Metab*. 2006;**290**(2):E317-E325.

45. Recinella L, Leone S, Ferrante C, et al. Effects of growth hormone-releasing hormone gene targeted ablation on ghrelin-induced feeding. *Growth Horm IGF Res*. 2017;**37**:40-46.

46. List EO, Sackmann-Sala L, Berryman DE, et al. Endocrine parameters and phenotypes of the growth hormone receptor gene disrupted (GHR-/-) mouse. *Endocr Rev*. 2011;**32**(3):356-386.

47. Wasinski F, Pedroso JAB, Dos Santos WO, et al. Tyrosine hydroxylase neurons regulate growth hormone secretion via short-loop negative feedback. *J Neurosci*. 2020;**40**(22):4309-4322.

48. List EO, Berryman DE, Funk K, et al. The role of GH in adipose tissue: lessons from adipose-specific GH receptor gene-disrupted mice. *Mol Endocrinol*. 2013;**27**(3):524-535.

49. Zhao TJ, Liang G, Li RL, et al. Ghrelin O-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice. *Proc Natl Acad Sci U S A*. 2010;**107**(16):7467-7472.

50. Dahlstrand J, Lardelli M, Lendahl U. Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. *Brain Res Dev Brain Res*. 1995;**84**(1):109-129.

51. Balthasar N, Dalgaard LT, Lee CE, et al. Divergence of melanocortin pathways in the control of food intake and energy expenditure. *Cell*. 2005;**123**(3):493-505.

52. Fenselau H, Campbell JN, Verstegen AM, et al. A rapidly acting glutamatergic ARC→PVH satiety circuit postsynaptically regulated by α-MSH. *Nat Neurosci.* 2017;**20**(1):42-51.

53. Atasoy D, Betley JN, Su HH, Sternson SM. Deconstruction of a neural circuit for hunger. *Nature.* 2012;**488**(7410):172-177.

54. Ramos-Lobo AM, Donato J Jr. The role of leptin in health and disease. *Temperature (Austin).* 2017;**4**(3):258-291.

55. Hahn TM, Breininger JF, Baskin DG, Schwartz MW. Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nat Neurosci.* 1998;**1**(4):271-272.

56. Cabral A, De Francesco PN, Perello M. Brain circuits mediating the orexigenic action of peripheral ghrelin: narrow gates for a vast kingdom. *Front Endocrinol (Lausanne).* 2015;**6**:44.

57. Perello M, Cabral A, Cornejo MP, De Francesco PN, Fernandez G, Uriarte M. Brain accessibility delineates the central effects of circulating ghrelin. *J Neuroendocrinol.* 2019;**31**(7):e12677.

58. Cornejo MP, Castrogiovanni D, Schiöth HB, et al. Growth hormone secretagogue receptor signalling affects high-fat intake independently of plasma levels of ghrelin and LEAP2, in a 4-day binge eating model. *J Neuroendocrinol.* 2019;**31**(10):e12785.

59. Uriarte M, De Francesco PN, Fernandez G, et al. Evidence supporting a role for the blood-cerebrospinal fluid barrier transporting circulating ghrelin into the brain. *Mol Neurobiol.* 2019;**56**(6):4120-4134.

60. Cornejo MP, Mustafá ER, Barrile F, et al. The intriguing ligand-dependent and ligand-independent actions of the growth hormone secretagogue receptor on reward-related behaviors. *Neurosci Biobehav Rev.* 2021;**120**:401-416.

61. Declercq J, Brouwers B, Pruniau VP, et al. Metabolic and behavioural phenotypes in nestin-cre mice are caused by hypothalamic expression of human growth hormone. *Plos One.* 2015;**10**(8):e0135502.

62. Khatib N, Gaidhane S, Gaidhane AM, et al. Ghrelin: ghrelin as a regulatory peptide in growth hormone secretion. *J Clin Diagn Res.* 2014;**8**(8):MC13-MC17.

63. Reichenbach A, Steyn FJ, Sleeman MW, Andrews ZB. Ghrelin receptor expression and colocalization with anterior pituitary hormones using a GHSR-GFP mouse line. *Endocrinology.* 2012;**153**(11):5452-5466.

64. Tolle V, Zizzari P, Tomasetto C, Rio MC, Epelbaum J, Bluet-Pajot MT. In vivo and in vitro effects of ghrelin/motilin-related peptide on growth hormone secretion in the rat. *Neuroendocrinology.* 2001;**73**(1):54-61.

65. Wren AM, Small CJ, Fribbens CV, et al. The hypothalamic mechanisms of the hypophysiotropic action of ghrelin. *Neuroendocrinology.* 2002;**76**(5):316-324.

66. Tannenbaum GS, Lapointe M, Beaudet A, Howard AD. Expression of growth hormone secretagogue-receptors by growth hormone-releasing hormone neurons in the mediobasal hypothalamus. *Endocrinology.* 1998;**139**(10):4420-4423.

67. Osterstock G, Escobar P, Mitutsova V, et al. Ghrelin stimulation of growth hormone-releasing hormone neurons is direct in the arcuate nucleus. *Plos One.* 2010;**5**(2):e9159.

68. Perello M, Scott MM, Sakata I, et al. Functional implications of limited leptin receptor and ghrelin receptor coexpression in the brain. *J Comp Neurol.* 2012;**520**(2):281-294.

69. Kohno D, Nakata M, Maekawa F, et al. Leptin suppresses ghrelin-induced activation of neuropeptide Y neurons in the arcuate nucleus via phosphatidylinositol 3-kinase- and phosphodiesterase 3-mediated pathway. *Endocrinology.* 2007;**148**(5):2251-2263.

70. Wang L, Saint-Pierre DH, Taché Y. Peripheral ghrelin selectively increases Fos expression in neuropeptide Y - synthesizing neurons in mouse hypothalamic arcuate nucleus. *Neurosci Lett.* 2002;**325**(1):47-51.

71. Hassouna R, Labarthe A, Zizzari P, et al. Actions of agonists and antagonists of the ghrelin/GHS-R pathway on GH secretion, appetite, and cFos activity. *Front Endocrinol (Lausanne).* 2013;**4**:25.

72. Betley JN, Cao ZF, Ritola KD, Sternson SM. Parallel, redundant circuit organization for homeostatic control of feeding behavior. *Cell.* 2013;**155**(6):1337-1350.

73. Chen Y, Lin YC, Zimmerman CA, Essner RA, Knight ZA. Hunger neurons drive feeding through a sustained, positive reinforcement signal. *eLife.* 2016;**5**:e18640.

74. Padilla SL, Qiu J, Soden ME, et al. Agouti-related peptide neural circuits mediate adaptive behaviors in the starved state. *Nat Neurosci.* 2016;**19**(5):734-741.

75. Fernandez G, Cabral A, Andreoli MF, et al. Evidence supporting a role for constitutive ghrelin receptor signaling in fasting-induced hyperphagia in male mice. *Endocrinology.* 2018;**159**(2):1021-1034.

76. Kaji H, Tai S, Okimura Y, et al. Cloning and characterization of the 5'-flanking region of the human growth hormone secretagogue receptor gene. *J Biol Chem.* 1998;**273**(51):33885-33888.

77. Sadagurski M, Landeryou T, Cady G, et al. Growth hormone modulates hypothalamic inflammation in long-lived pituitary dwarf mice. *Aging Cell.* 2015;**14**(6):1045-1054.

78. Cabral A, Fernandez G, Tolosa MJ, et al. Fasting induces remodeling of the orexigenic projections from the arcuate nucleus to the hypothalamic paraventricular nucleus, in a growth hormone secretagogue receptor-dependent manner. *Mol Metab.* 2020;**32**:69-84.

79. Goldstein JL, Zhao TJ, Li RL, Sherbet DP, Liang G, Brown MS. Surviving starvation: essential role of the ghrelin-growth hormone axis. *Cold Spring Harb Symp Quant Biol.* 2011;**76**:121-127.

80. Li RL, Sherbet DP, Elsbernd BL, Goldstein JL, Brown MS, Zhao TJ. Profound hypoglycemia in starved, ghrelin-deficient mice is caused by decreased gluconeogenesis and reversed by lactate or fatty acids. *J Biol Chem.* 2012;**287**(22):17942-17950.

81. Gisabella B, Farah S, Peng X, Burgos-Robles A, Lim SH, Goosens KA. Growth hormone biases amygdala network activation after fear learning. *Transl Psychiatry.* 2016;**6**(11):e960.

82. Meyer RM, Burgos-Robles A, Liu E, Correia SS, Goosens KA. A ghrelin-growth hormone axis drives stress-induced vulnerability to enhanced fear. *Mol Psychiatry.* 2014;**19**(12):1284-1294.

83. Clegg DJ, Brown LM, Zigman JM, et al. Estradiol-dependent decrease in the orexigenic potency of ghrelin in female rats. *Diabetes.* 2007;**56**(4):1051-1058.