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Fasting and prolonged food restriction differentially affect GH secretion independently of GH receptor signaling in AgRP neurons

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Abstract

Growth hormone (GH) receptor (GHR) is abundantly expressed in neurons that co-release the agouti-related protein (AgRP) and neuropeptide Y (NPY) in the arcuate nucleus of the hypothalamus (ARH). Since $ARH^{AgRP/NPY}$ neurons regulate several hypothalamic–pituitary-endocrine axes, this neuronal population possibly modulates GH secretion via a negative feedback loop, particularly during food restriction, when $ARH^{AgRP/NPY}$ neurons are highly active. The present study aims to determine the importance of GHR signaling in $ARH^{AgRP/NPY}$ neurons on the pattern of GH secretion in fed and food-deprived male mice. Additionally, we compared the effect of two distinct situations of food deprivation: 16 h of fasting or four days of food restriction (40% of usual food intake). Overnight fasting strongly suppressed both basal and pulsatile GH secretion. Animals lacking GHR in $ARH^{AgRP/NPY}$ neurons ($AgRP^{\Delta GHR}$ mice) did not exhibit differences in GH secretion either in the fed or fasted state, compared to control mice. In contrast, four days of food restriction increased GH pulse frequency, basal GH secretion, and pulse irregularity/complexity (measured by sample entropy), whereas pulsatile GH secretion was not affected in both control and $AgRP^{\Delta GHR}$ mice. Hypothalamic *Ghrh* mRNA levels were unaffected by fasting or food restriction, but *Sst* expression increased in acutely fasted mice, but decreased after prolonged food restriction in both control and $AgRP^{\Delta GHR}$ mice. Our findings indicate that short-term fasting and prolonged food restriction differentially affect the pattern of GH secretion, independently of GHR signaling in $ARH^{AgRP/NPY}$ neurons.

KEYWORDS
calorie restriction, GH, GHRH, hypothalamus, IGF-1

1 | INTRODUCTION

Growth hormone (GH) is secreted by somatotropic cells of the anterior pituitary gland and plays a key role in regulating cell proliferation, body growth and carbohydrate, protein, and lipid metabolism.[1–3] Additionally, hepatic gene expression and circulating concentration of insulin-like growth factor-1 (IGF-1), which is a critical mediator of the physiological effects of GH, are robustly influenced by the pattern of GH secretion.[1–3]

Pituitary GH secretion is mainly controlled by hypothalamic neuropeptides, including somatostatin (SST) and GH-releasing hormone (GHRH), circulating hormones (e.g., ghrelin and insulin), and

metabolites, like glucose.[4,5] This regulation includes negative feedback loops, in which hypothalamic neurons and somatotropic cells are able to sense variations in GH or IGF-1 levels to regulate GH secretion.[4,5] Accordingly, GH receptor (GHR) or IGF-1 receptor (IGF1R) ablation in SST neurons, GHRH neurons, or somatotropic cells can cause the loss of GH negative feedback and consequently GH oversecretion.[6–9] However, the multiple redundant mechanisms involved in the control of the somatotropic axis may partially compensate for specific failures in GH or IGF-1 feedback.[6–8]

GHR expression is particularly enriched in the arcuate nucleus of the hypothalamus (ARH).[10–14] Notably, this expression is predominantly found in neurons that coexpress the agouti-related protein (AgRP) and neuropeptide Y (NPY), whereas the presence of GHR in other ARH neuronal populations is less abundant.[13,14] In situ hybridization experiments have shown that *Ghr* mRNA is detected in approximately 95% of ARH^{NPY} neurons.[15,16] Using the capacity of GHR to induce the phosphorylation of the signal transducer and activator of transcription 5 (pSTAT5), 95% of ARH^{AgRP} neurons exhibit pSTAT5 immunoreactivity after a pharmacological dose of GH.[17] A systemic GH injection also increases the hypothalamic expression of *Agrp* and *Npy* mRNA.[17] Furthermore, one-third of ARH$^{AgRP/NPY}$ neurons are directly depolarized by GH[17] and between 50 to 65% of ARH$^{AgRP/NPY}$ neurons exhibit c-fos expression, a marker of neuronal activation, after administration of GH or a GH secretagogue.[18,19] Thus, it is clear that ARH$^{AgRP/NPY}$ neurons represent a neuronal population highly responsive to GH.

ARH$^{AgRP/NPY}$ neurons are major regulators of food intake and other metabolic aspects.[20–22] Activation of ARH$^{AgRP/NPY}$ neurons increases feeding and suppresses energy expenditure, leading to weight gain.[23,24] In addition, ARH$^{AgRP/NPY}$ neurons are well-known targets of several hormones that regulate metabolism, like leptin, ghrelin, and insulin.[20–22] Thus, GH may represent another endocrine factor that regulates metabolism via AgRP neurons.[25] In accordance with this hypothesis, GHR ablation in ARH^{AgRP} neurons prevents the activation of these neurons in food-deprived mice.[17,26] Additionally, neuroendocrine and metabolic responses to food restriction are blunted in mice lacking GHR in ARH^{AgRP} neurons, which ultimately precludes the normal suppression of energy expenditure observed during prolonged food deprivation.[17] GHR expression in ARH^{AgRP} neurons also has trophic effects, affecting the formation of axonal projections to postsynaptic targets, including the paraventricular, dorsomedial, and lateral hypothalamic nuclei.[27]

Not only are ARH$^{AgRP/NPY}$ neurons involved in the regulation of metabolism, but also in the control of different hypothalamic–pituitary-endocrine axes.[28–33] The enriched expression of GHR in ARH$^{AgRP/NPY}$ neurons has led authors from different laboratories to suggest that ARH$^{AgRP/NPY}$ neurons possibly play a physiological role in regulating GH secretion via a negative feedback loop.[4,34,35] This suggestion is supported by the fact that NPY administration suppresses the GH/IGF-1 axis.[36–38] Additionally, hypophysiotropic neurons that control GH secretion receive synaptic connections from NPY immunoreactive fibers.[39–41] Of note, although several brain nuclei contain NPY-expressing cells, GHR expression is exclusively found in ARH$^{AgRP/NPY}$ neurons.[15] In a seminal study, Huang et al.[40] demonstrated that fasting suppresses the pulsatile GH secretion in male mice, and this inhibition is prevented in *Npy* knockout mice, even though GH secretion is normal in fed *Npy* knockout mice. This effect is probably mediated by NPY receptor Y1 since the germline absence of this receptor also blunts fasting-induced suppression of GH secretion, whereas the null mutation of Y2 receptor reduces basal GH secretion and consequently body growth in fed mice, without affecting GH secretion in fasted mice.[40] Taken together, there is robust evidence indicating that NPY neurotransmission, likely through ARH$^{AgRP/NPY}$ neurons, controls GH secretion.

However, despite these findings, it is still unknown whether GHR expression in ARH$^{AgRP/NPY}$ neurons is required for the regulation of the GH/IGF-1 axis. Moreover, there are divergent data in the literature indicating that food deprivation can either suppress[40,42–44] or increase[17,45–54] GH secretion in rodents and humans, depending on the protocol used. Most of the studies found increased GH secretion using a starvation protocol induced by chronic food restriction (40% of usual food intake for 5 to 11 days) in mice[17,46–49,51,52] or after fasting (one, three or 6 days) in humans.[45,50] In contrast, decreased GH secretion was observed in short-term fasted mice[40,55] or in rats subjected to a milder food deprivation protocol.[42,43] Thus, the present study aims to (1) compare the effects of two distinct situations of food deprivation (16 h of fasting or 4 days of food restriction) on the pattern of GH secretion in adult male mice, and (2) determine the importance of GHR signaling in ARH$^{AgRP/NPY}$ neurons on the GH/IGF-1 axis of fed and food-deprived mice.

2 | MATERIALS AND METHODS

2.1 | Animals

Selective ablation of GHR in ARH^{AgRP} neurons was achieved by breeding AgRP^{Cre} mice (The Jackson Laboratory, Bar Harbor, ME; RRID: IMSR_JAX:012899) with GHR$^{flox/flox}$ animals.[56] All mice used in the experiments were homozygous for the GHR^{flox} allele, whereas AgRP$^{\Delta GHR}$ mice also carried the AgRP^{Cre} gene in heterozygosity. The control group was exclusively composed of littermates (Cre negative mice). Mice were in the C57BL/6J background and only males were used in the experiments. The visualization of AgRP neurons in the histological experiment was achieved by incorporating a Cre-dependent enhanced green fluorescent protein (eGFP) reporter gene into the colony (The Jackson Laboratory, Bar Harbor, ME; RRID: IMSR_JAX:026175). The experimental procedures were approved by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of São Paulo.

2.2 | Immunofluorescence staining

GH-responsive neurons were analyzed in adult control and AgRP$^{\Delta GHR}$ mice (n = 3/group) that received an intraperitoneal injection of

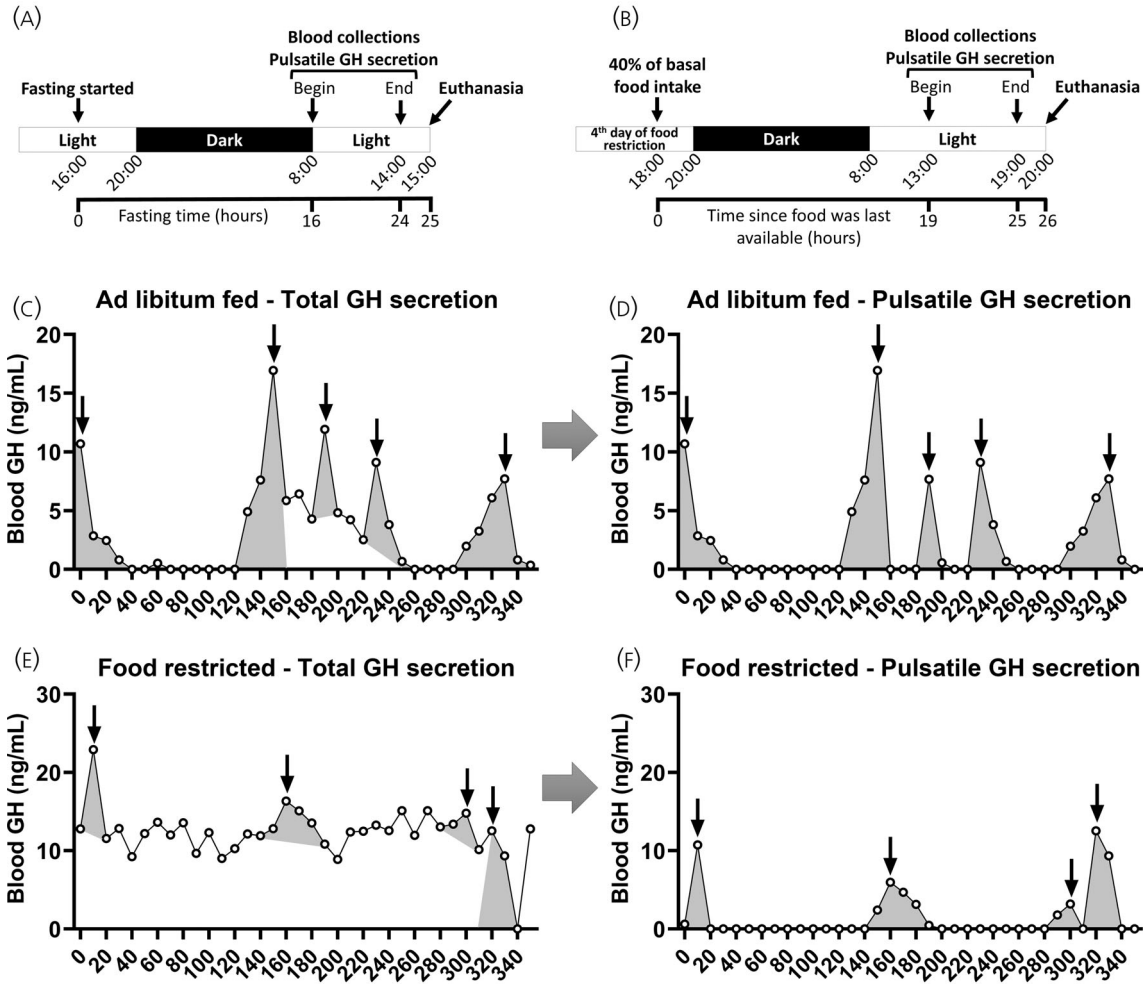


FIGURE 1 Experimental design and representation of the analysis of pulsatile GH secretion. Schemes illustrating the experimental design to determine the pulsatile GH secretion in 16-h fasted mice (A) and in mice after four days of food restriction (B). Representative examples of the pattern of GH secretion in an ad libitum fed mouse (C, D) and in the same animal after four days of food restriction (E, F). GH pulses were identified using the DynPeak pulse detection algorithm (indicated by arrows). The left figures indicate the total GH secretion which represents the blood GH levels obtained in our ELISA (C and E). On the right (D and F), only the values considered as GH pulses were added to the graphs (represented as the highlighted areas). Using this classification, the following parameters were determined for the analysis of the pattern of GH secretion: mean GH levels (ng/ml), GH pulse frequency, pulsatile GH secretion (ng/ml × 6 h), mean GH secretion per pulse, basal GH secretion (ng/ml × 6 h) and contribution of basal secretion to total GH secretion.

20 μg/g of GH purified from porcine pituitary (National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program) and were perfused 30 min later. Mice were anesthetized with isoflurane and perfused transcardially with saline, followed by a 10% buffered formalin solution. Brains were collected and post-fixed in the same fixative for 45 min and cryoprotected overnight at 4°C in 0.1 M phosphate-buffered saline (PBS) containing 20% sucrose. Brains were cut in 30-μm thick sections using a freezing microtome. Brain slices were rinsed in 0.02 M potassium PBS, pH 7.4 (KPBS), followed by pretreatment in a water solution containing 1% hydrogen peroxide and 1% sodium hydroxide for 20 min. After rinsing in KPBS, sections were incubated in 0.3% glycine and 0.03% lauryl sulfate for 10 min each. Next, slices were blocked in 3% normal donkey serum for 1 h, followed by incubation in a primary antibody cocktail containing anti-phospho^{Tyr694}-STAT5 (1:1000; Cell Signaling Technology; cat no. 9351; RRID: AB_2315225) and anti-eGFP (1:5000; Aves Laboratories, Inc.; cat no. GFP-1020; RRID: AB_10000240) for 40 h. Subsequently, sections were rinsed in KPBS and incubated for 90 min in Alexa Fluor-conjugated secondary antibodies (1:500, Jackson ImmunoResearch). After rinses in KPBS, sections were mounted onto gelatin-coated slides and covered with Fluoromount G mounting medium (Electron Microscopic Sciences). A Zeiss Axiocam 512 color camera adapted to an Axioimager A1 microscope (Zeiss) was used to obtain the photomicrographs. Two or three brain sections corresponding to the tuberal ARH were analyzed in each mouse. Single- and double-labeled cells were manually counted using the counting tool available in the Adobe Photoshop software and the data shown represent the average percentage of colocalization.

2.3 | Fasting and food restriction protocols

Mice were acclimated daily to the tail-tip blood sampling procedure for at least 30 days. Then, approximately 12-week-old control and AgRP$^{\Delta GHR}$ mice were subjected to 36 sequential tail-tip blood collections at 10 min intervals (see the details in the next section), beginning at 8:00 h (immediately after lights on). After one week of recovery, control and AgRP$^{\Delta GHR}$ mice were single-housed and subjected to overnight fasting (n = 6–7/group) by removing the food at 16:00 h (lights off at 20:00 h). Serial blood collections were repeated, but now after 16 h of fasting, beginning at 8:00 h (Figure 1A). Bodyweight and body composition, using the LF50 body composition mice analyzer (Bruker, Germany), were determined on blood collection days (in fed and fasted conditions). Mice were euthanized approximately at 15:00 h (after the evaluation of the pulsatile GH secretion) to collect serum to analyze IGF-1 levels and the hypothalamus for gene expression analysis.

In the food restriction group, the procedures were similar, except that instead of fasting, control and AgRP$^{\Delta GHR}$ mice were subjected to four days of food restriction, in which each mouse received 40% of their basal food intake 2 h before lights off for four consecutive days (n = 7–10/group). Basal food intake was determined for 2 days prior the food deprivation protocol by recording the amount of food ingested per day in animals that were already used to staying in individual cages. The average of these days was used to calculate the amount of chow that was offered daily to the animals during the food restriction. Bodyweight was recorded daily. Serial blood collection started on the fourth day of food restriction at 13:00 h, and mice were euthanized after the evaluation of the pulsatile GH secretion, at approximately at 20:00 h (Figure 1B). Another group of control and AgRP$^{\Delta GHR}$ mice (n = 6–9/group) was euthanized in ad libitum fed conditions to determine the effects of fasting or food restriction on serum IGF-1 levels and hypothalamic gene expression.

2.4 | Evaluation of the pattern of GH secretion

Serial blood collection began with the removal of a small portion of the tail tip (1 mm) using a surgical blade. Each blood sample (5 µl) was transferred to a tube containing 105 µL of PBS with 0.05% tween-20. Samples were immediately placed on dry ice and then stored at −80°C. After each blood collection, fingertip pressure was gently applied to the tail tip to stop bleeding. Mice were allowed to move freely in their home cages with ad libitum access to water, but they remained food-deprived during the 6 h of collection (except the ad libitum fed group). An "in-house" enzyme-linked immunosorbent assay (ELISA) was used to determine blood GH levels (1:22 dilution), as described.[6,9,57–59] GH pulses were identified using the DynPeak pulse detection algorithm, which employs a multiscale and multicriteria algorithm to detect discrete peak events using local (on the data point level), semi-local (on the level of possibly moving windows of consecutive data points), and global (on the whole series level) amplitude criteria.[60] Mean GH was calculated by averaging all GH values from each mouse. The number of GH pulses per hour (frequency) was calculated for the period of 6 h. To calculate pulsatile GH secretion, we initially identify the start and end of each pulse by taking into account its nadir and GH half-life. Figure 1C,E show blood GH levels of representative mice with low and high basal GH secretion, respectively. Note that GH pulses are represented by the highlighted areas. Subsequently, we differentiated between basal and pulsatile secretion. For this purpose, if the GH pulse started and ended with values above zero, the average of the initial and final values was subtracted from each point of the GH pulse to remove the values considered as basal secretion. If only the initial or final value of the GH pulse was, respectively, preceded or followed by values above zero, that value was subtracted from this point in the pulse. Points that did not belong to GH pulses were set as zero. The graphic representation of the pulsatile secretion (after excluding basal secretion) can be found in the two representative examples shown in Figure 1D,F. Pulsatile GH secretion was obtained by calculating the area under the curve (AUC) of the values considered only as pulsatile secretion using the Prism software version 8.4.3 (GraphPad). Mean GH secretion per pulse was calculated by dividing the AUC of pulsatile secretion by the number of pulses. Basal GH secretion was calculated by subtracting the AUC of pulsatile secretion from the AUC of total (blood) GH levels. Pattern irregularity, or complexity, was obtained by Sample Entropy (SaEn) (m = 1, r = 20%), as previously described.[61,62]

2.5 | IGF-1 assay and hypothalamic gene expression

Serum IGF-1 levels were determined using a commercially available ELISA (no. MG100; RRID: AB_2827989; R&D Systems). Quantitative real-time PCR was used to determine the hypothalamic gene expression. RNA was extracted from the entire hypothalamus using TRIzol (Invitrogen), followed by incubation in DNase I RNase-free (MilliporeSigma). cDNA was synthesized by reverse transcription using 2 µg of total RNA, SuperScript II reverse transcriptase (Invitrogen), and random primers p(dN)6 (MilliporeSigma). Real-time PCR was performed using the 7500TM Real-Time PCR System (Applied Biosystems), SYBR Green Gene Expression PCR Master Mix (Applied Biosystems) and specific primers for target genes: *Actb* (forward: gctccggcatgtgcaaag; reverse: catcacaccctggtgccta), *Agrp* (forward: ctttggcggaggtgctagat; reverse: aggactcgtgcagccttacac), *Ghrh* (forward: tatgcccggaaagtgatccag; reverse: atccttgggaatccctgcaaga), *Npy* (forward: ccgcccgccatgatgctaggta; reverse: ccctcagccagaatgcccaa), *Pomc* (forward: atagacgtgtggagctggtgc; reverse: gcaagccagcaggttgct), *Ppia* (forward: tatctgcactgccaagactgagt; reverse: cttcttgctggtcttgccattcc) and *Sst* (forward: ctgtcctgccgtctccagt; reverse: ctgcagaaactgacggagtct). Data were normalized to the geometric average of *Actb* and *Ppia*. Relative quantification of mRNA was calculated by $2^{-\Delta\Delta Ct}$.

2.6 | Statistical analysis

Differences of absolute and loss caused by overnight fasting on bodyweight, lean mass and fat mass between control and AgRP$^{\Delta GHR}$ mice

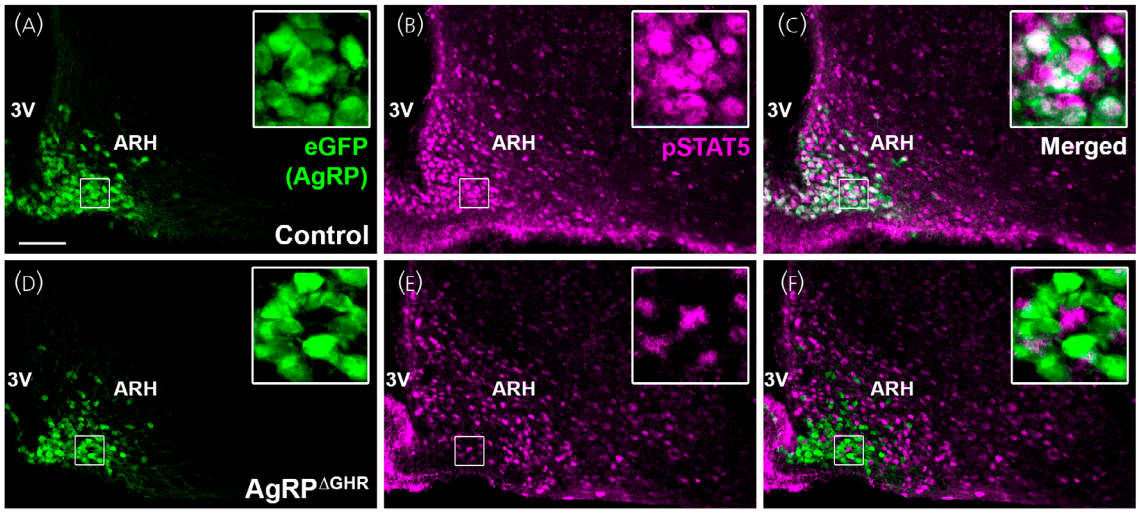


FIGURE 2 GHR inactivation in ARH^{AgRP} neurons. Representative images showing the ARH^{AgRP} neurons (green staining) and the colocalization with pSTAT5 (magenta staining) in control (A–C) and AgRP$^{\Delta GHR}$ (D–F) mice that received an intraperitoneal injection of 20 μg/g of porcine GH and were perfused 30 min later. Scale bar = 100 μm. 3 V, third ventricle; ARH, arcuate nucleus of the hypothalamus.

were analyzed by the unpaired two-tailed student's *t*-test. Normality was confirmed by the Shapiro–Wilk test and homogeneity of variance was assumed. Scheirer-Ray-Hare test was used to analyze the independent effects of fasting/food restriction or GHR ablation, and their interaction, on GH mean levels, pulse frequency, pulsatile secretion, basal secretion, and sample entropy. Differences of bodweight loss induced by food restriction between Control and AgRP$^{\Delta GHR}$ mice were analyzed by a Scheirer-Ray-Hare test. Scheirer-Ray-Hare test was also applied to analyze the changes in IGF-1 and hypothalamic mRNA levels (*Agrp*, *Npy*, *Pomc*, *Ghrh*, and *Sst*) between the treatments. Differences between the groups were identified by Mann–Whitney U test and Bonferroni correction for multiple comparisons. Prism version 8.4.3, Python version 3.11 and R version 4.2.2 were employed for the statistical analyses and generation of graphs. All results were expressed as mean ± standard error of the mean, and only *p*-values < .05 were considered statistically significant.

3 | RESULTS

3.1 | GHR inactivation in ARH^{AgRP} neurons

As previously demonstrated,[14–17,27,63,64] the vast majority of ARH$^{AgRP/NPY}$ neurons express functional GHR since a systemic GH injection induced pSTAT5 in 97 ± 1% of eGFP cells, whose expression was conditioned to the *Agrp* gene promoters (Figure 2A–C). To investigate the possible role of GHR signaling in ARH^{AgRP} neurons for the control of GH secretion, we generated mice lacking GHR specifically in this neuronal population. In contrast to control animals, AgRP$^{\Delta GHR}$ mice exhibited very few ARH^{AgRP} neurons expressing pSTAT5 (0.3 ± 0.3% of colocalization) after systemic GH injection, whereas GH-induced pSTAT5 remained intact in surrounding areas (Figure 2D–F). Thus, we were able to generate mice lacking GHR specifically in ARH^{AgRP} neurons.

3.2 | GHR ablation in ARH^{AgRP} neurons does not affect bodyweight or fasting-induced weight loss

Confirming the results of previous studies,[17,58,64] adult AgRP$^{\Delta GHR}$ male mice exhibited no differences in bodyweight, lean mass, and body adiposity, compared to littermate control mice (Figure 3A–C). Then, the effects of 16 h of fasting were evaluated. Fasting caused weight loss, but no difference was observed when control and AgRP$^{\Delta GHR}$ mice were compared (Figure 3D). Lean body mass and fat mass were also similarly decreased after fasting in control and AgRP$^{\Delta GHR}$ mice (Figure 3E,F).

3.3 | Overnight fasting suppresses basal and pulsatile GH secretion in both control and AgRP$^{\Delta GHR}$ mice

Now, the effect of overnight fasting on GH secretion was determined in control and AgRP$^{\Delta GHR}$ male mice. GH secretion was determined twice in each mouse: in a fed state and then after 16 h of fasting. Representative examples of the pulsatile pattern of GH secretion in fed and fasted states were provided in Figure 4. While mice in the fed state showed the normal pulsatile pattern of GH secretion during the 6 h of serial blood collection, overnight fasting drastically suppressed GH secretion (Figures 4 and 5). Mean GH levels (main effect of fasting [$H_{(1,N\ =\ 25)} = 16.45$, $p < .0001$]; Figure 5A), GH pulse frequency (main effect of fasting [$H_{(1,N\ =\ 24)} = 12.16$, $p < .0001$]; Figure 5B), pulsatile GH secretion (main effect of fasting [$H_{(1,N\ =\ 25)} = 16.54$, $p < .0001$]; Figure 5C), mean GH secretion per pulse (main effect of fasting [$H_{(1,N\ =\ 25)} = 16.54$, $p < .0001$]; Figure 5D), basal GH secretion (main effect of fasting [$H_{(1,N\ =\ 24)} = 16.10$, $p < .0001$]; Figure 5E), and the percentage of basal secretion to total GH secretion (main effect of fasting [$H_{(1,N\ =\ 23)} = 16.20$, $p < .0001$]; Figure 5F) were all decreased by fasting.

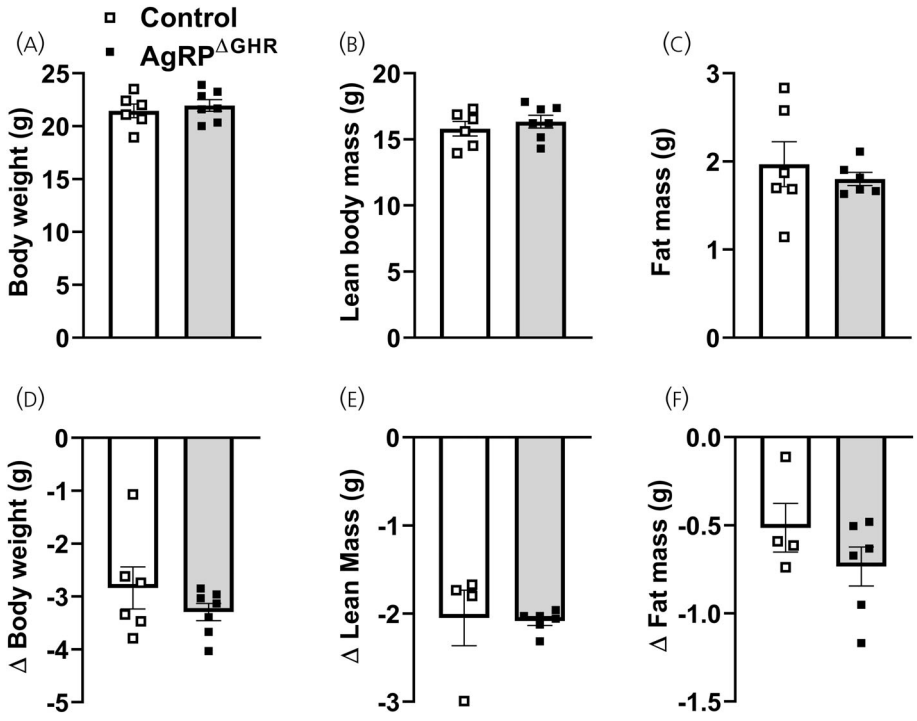


FIGURE 3 GHR ablation in ARH^{AgRP} neurons does not affect bodyweight or fasting-induced weight loss. Bodyweight (A), lean body mass (B) and fat mass (C) in adult control ($n = 6$) and AgRP$^{\Delta GHR}$ ($n = 7$) male mice. Overnight (16 h) fasting reduces the bodyweight (D), lean body mass (E) and fat mass (F) of control ($n = 6$) and AgRP$^{\Delta GHR}$ ($n = 7$) mice. Differences between control and AgRP$^{\Delta GHR}$ mice were analyzed by the unpaired two-tailed student's *t*-test.

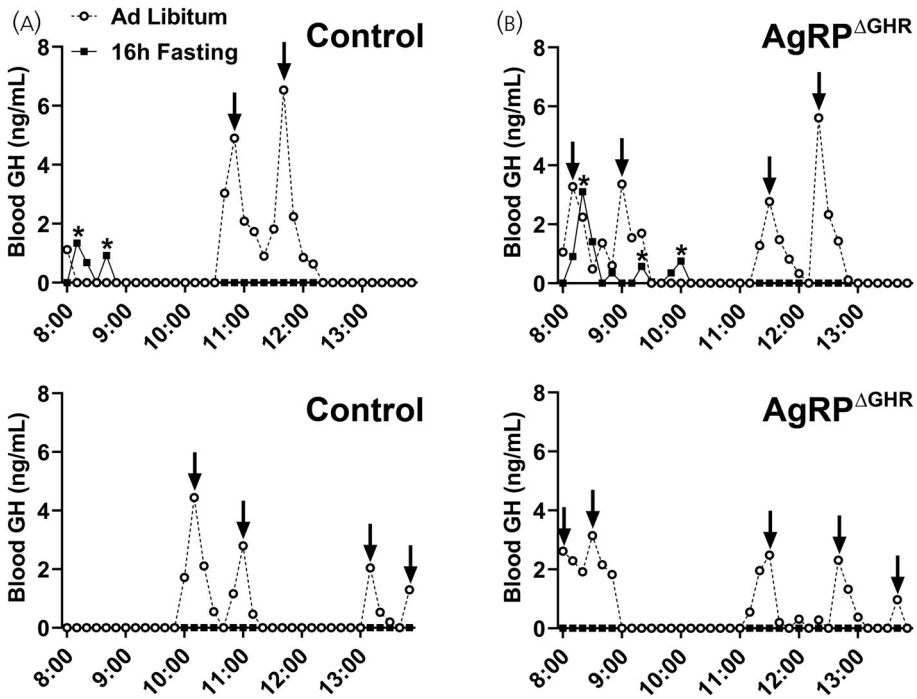


FIGURE 4 Pulsatile GH secretion in fed and fasted male mice. Representative examples of the pattern of GH secretion in two control (A) and AgRP$^{\Delta GHR}$ (B) mice. Each figure shows GH secretion from the same animal in both the ad libitum fed and fasted (16 h) conditions. Blood GH levels were determined in 36 samples collected from tail tip at 10 min intervals, beginning at 8:00 h (immediately after lights on). GH pulses are indicated by arrows in fed mice or asterisks during fasting.

No differences between control and AgRP$^{\Delta GHR}$ mice were observed in GH secretion in both fed and fasting state (Figures 4 and 5). SaEn, which was used as measure of the pattern irregularity of GH secretion,[61,62] was also reduced in fasted mice either when considering total GH secretion (main effect of fasting [$H_{(1,N\ =\ 23)} = 12.0$, $p < .001$]; Figure 5G) or only pulsatile secretion (main effect of fasting [$H_{(1,N\ =\ 23)} = 13.41$, $p < .001$]; Figure 5H). Thus, overnight fasting suppresses both basal and pulsatile GH secretion, and disruption of GHR signaling in ARH^{AgRP} neurons does not affect GH secretion in fed or fasted mice.

3.4 | Prolonged food restriction increases basal GH secretion, independently of GHR signaling in ARH^{AgRP} neurons

In this experiment, control and AgRP$^{\Delta GHR}$ mice were subjected to four days of food restriction (40% of usual food intake). Prolonged food restriction caused a progressive weight loss, without significant differences between the groups, regardless of whether the weight loss is represented in grams (main effect of group [$H_{(3,N\ =\ 68)} = 0.10$,

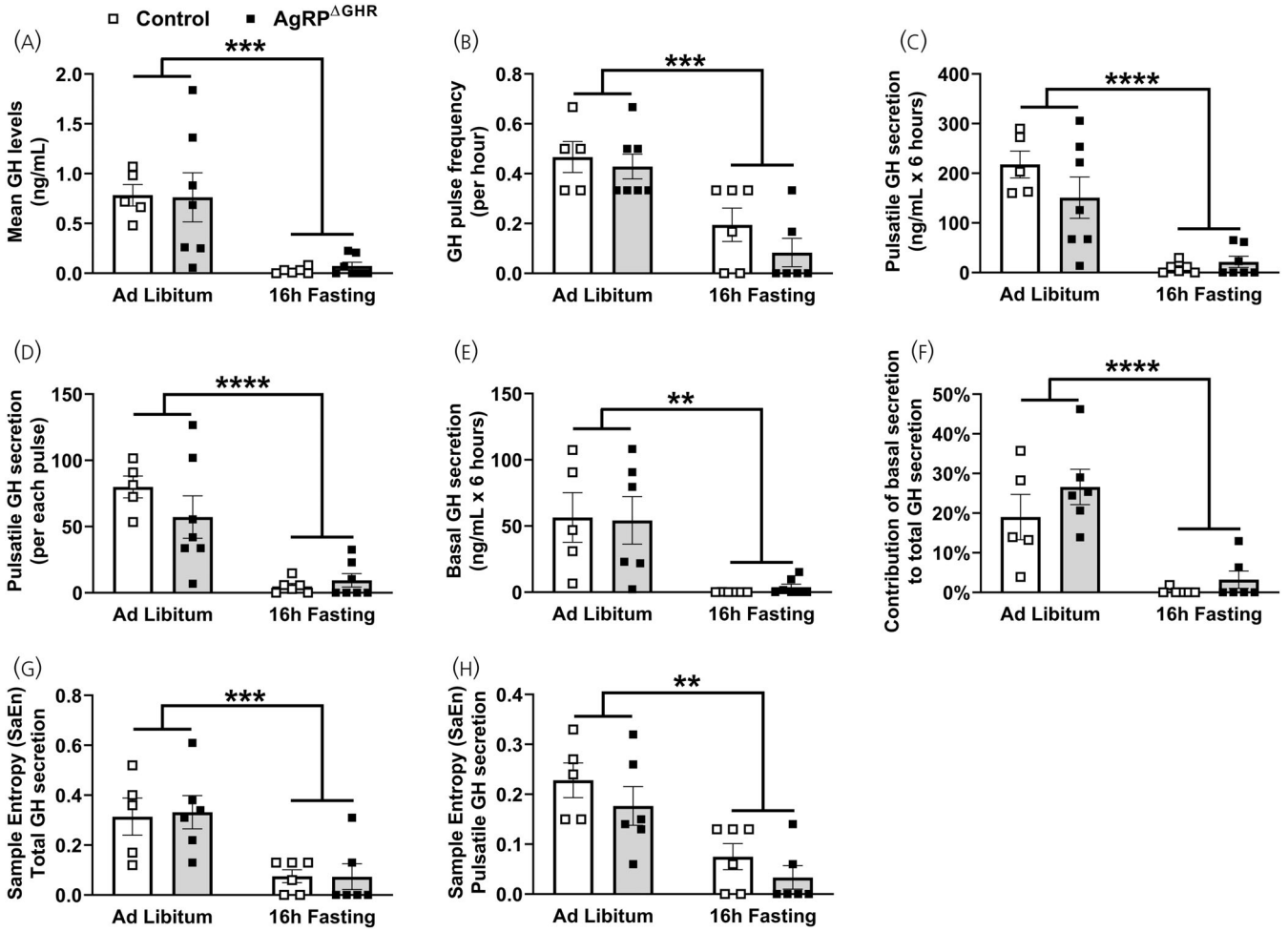
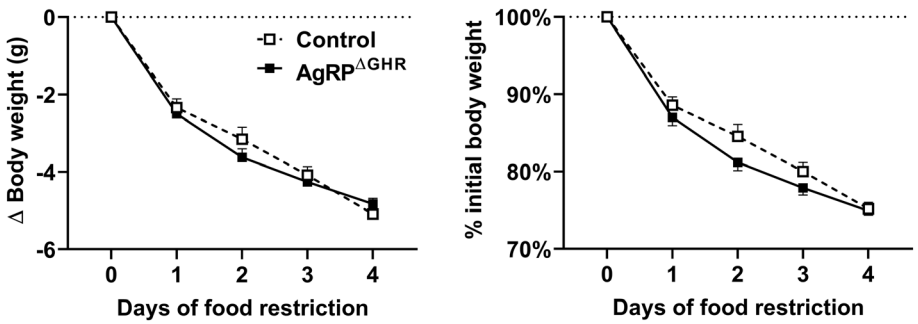


FIGURE 5 Overnight fasting suppresses GH secretion in control and AgRP$^{\Delta GHR}$ mice. Mean GH levels (A), GH pulse frequency (B), pulsatile GH secretion (C), mean GH secretion per pulse (D), basal GH secretion (E), contribution of basal secretion to total GH secretion (F), SaEn of total GH secretion (G) and SaEn of pulsatile GH secretion (H) of control ($n = 7$) and AgRP$^{\Delta GHR}$ ($n = 6$) mice, both in the ad libitum fed and fasted (16 h) conditions. **, $p < .01$; ***, $p < .001$; ****, $p < .0001$ effect of fasting (Scheirer-Ray-Hare test).

FIGURE 6 Weight loss during four days of food restriction. Control ($n = 10$) and AgRP$^{\Delta GHR}$ ($n = 7$) mice received 40% of their basal food intake 2 h before lights off for 4 consecutive days. Differences between control and AgRP$^{\Delta GHR}$ mice were analyzed by the Scheirer-Ray-Hare test.



$p = .75$]) or as percentage of the initial bodyweight (main effect of group [$H_{(3,N\ =\ 68)} = 1.372$, $p = .24$]; Figure 6). Then, the pulsatile pattern of GH secretion was determined on the fourth day of food restriction and representative examples were shown in Figure 7. In contrast to the findings observed after fasting, prolonged food restriction caused a tonic (constitutive) rise in GH secretion (Figure 7), which led to increases in GH pulse frequency (main effect of food restriction [$H_{(1,N\ =\ 26)} = 3.87$, $p = .049$]; Figure 8B) and basal GH secretion (main effect of food restriction [$H_{(1,N\ =\ 26)} = 3.698$, $p = .054$]; Figure 8E), whereas pulsatile GH secretion was not significantly affected. Consequently, prolonged food restriction significantly increased the percentage that basal GH secretion contributed to the total GH secreted in the analyzed period [$H_{(1,N\ =\ 26)} = 9.62$, $p = .002$]; Figure 8F). Prolonged food restriction also increased irregularity of the total GH secretion (main effect of food restriction [$H_{(1,N\ =\ 26)} = 7.81$, $p = .005$]; Figure 8G) or pulsatile GH secretion (main effect of food restriction

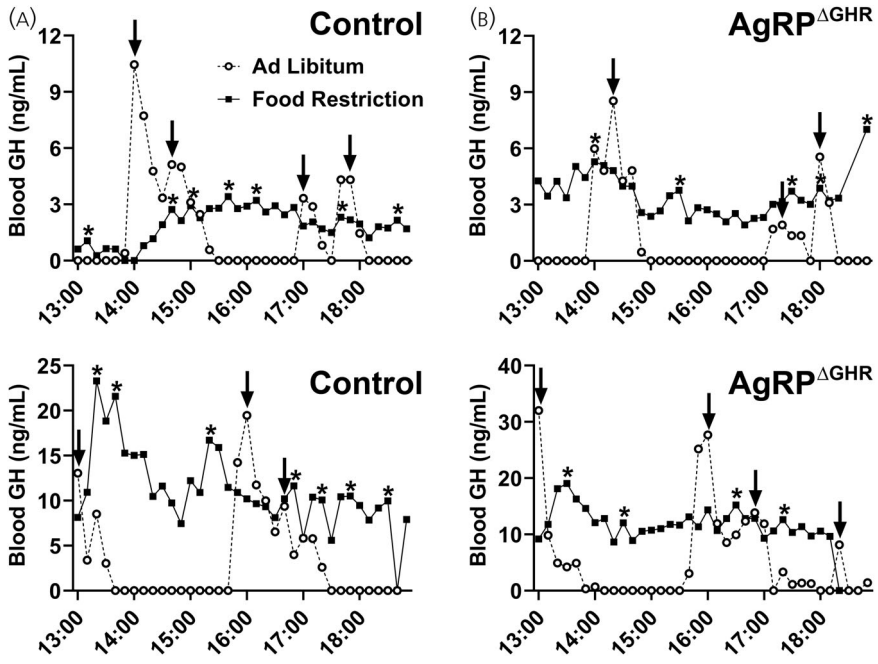


FIGURE 7 Pulsatile GH secretion in fed mice and after four days of food restriction. Representative examples of the pattern of GH secretion in two control (A) and AgRP$^{\Delta GHR}$ (B) mice. Each figure shows GH secretion from the same animal, both in the ad libitum fed condition and after four days of food restriction (40% of their basal food intake). Blood GH levels were determined in 36 samples collected from tail tip at 10 min intervals, beginning at 13:00 h (lights on at 8:00 h). GH pulses are indicated by arrows in fed mice or asterisks in food-deprived mice.

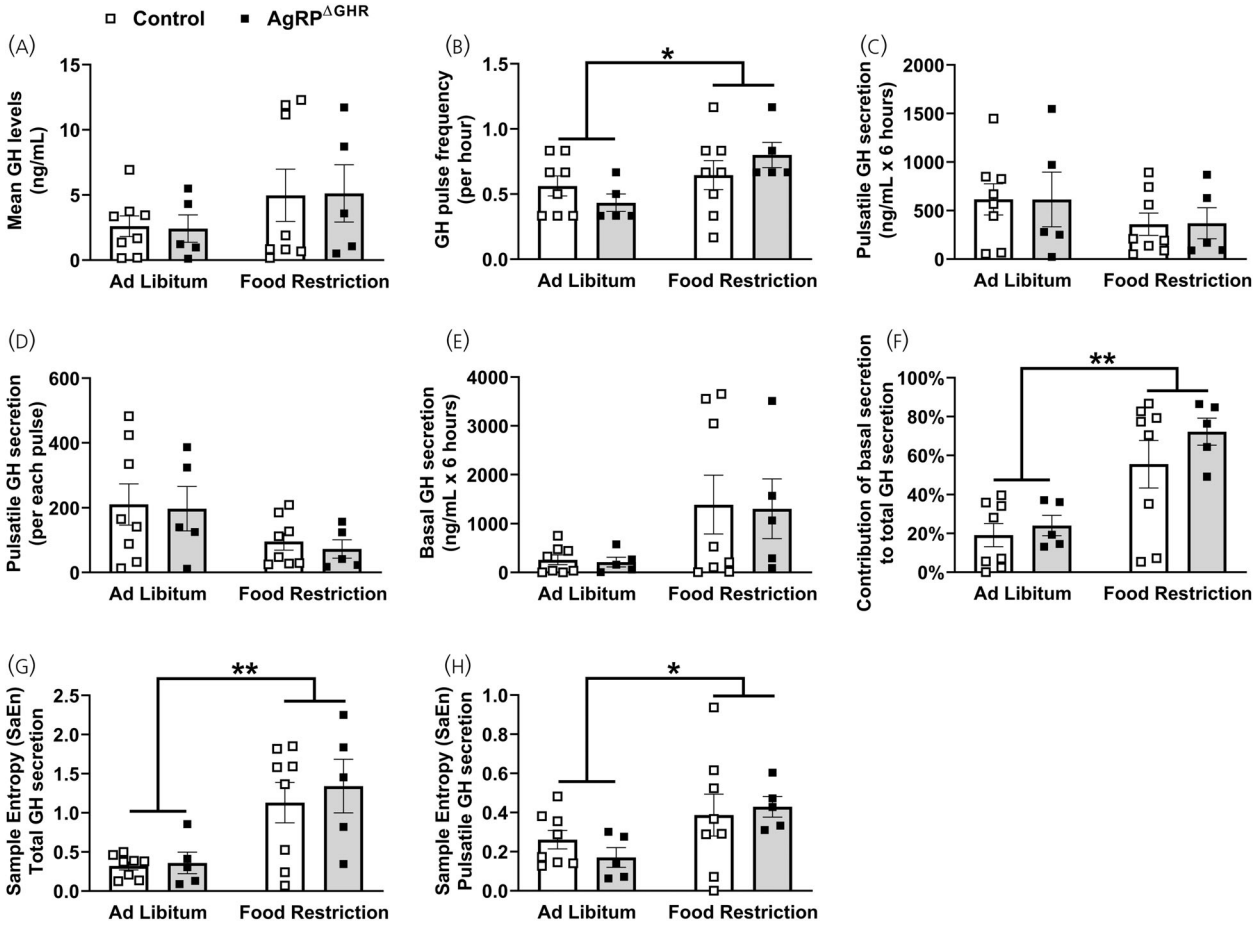


FIGURE 8 Prolonged food restriction causes a tonic (basal) increase in GH secretion. Mean GH levels (A), GH pulse frequency (B), pulsatile GH secretion (C), mean GH secretion per pulse (D), basal GH secretion (E), contribution of basal secretion to total GH secretion (F), SaEn of total GH secretion (G) and SaEn of pulsatile GH secretion (H) of control ($n = 8$) and AgRP$^{\Delta GHR}$ ($n = 6$) mice, both in the ad libitum fed condition and after four days of food restriction (40% of their basal food intake). *, $p < .05$; **, $p < .01$ effect of food restriction (Scheirer-Ray-Hare test).

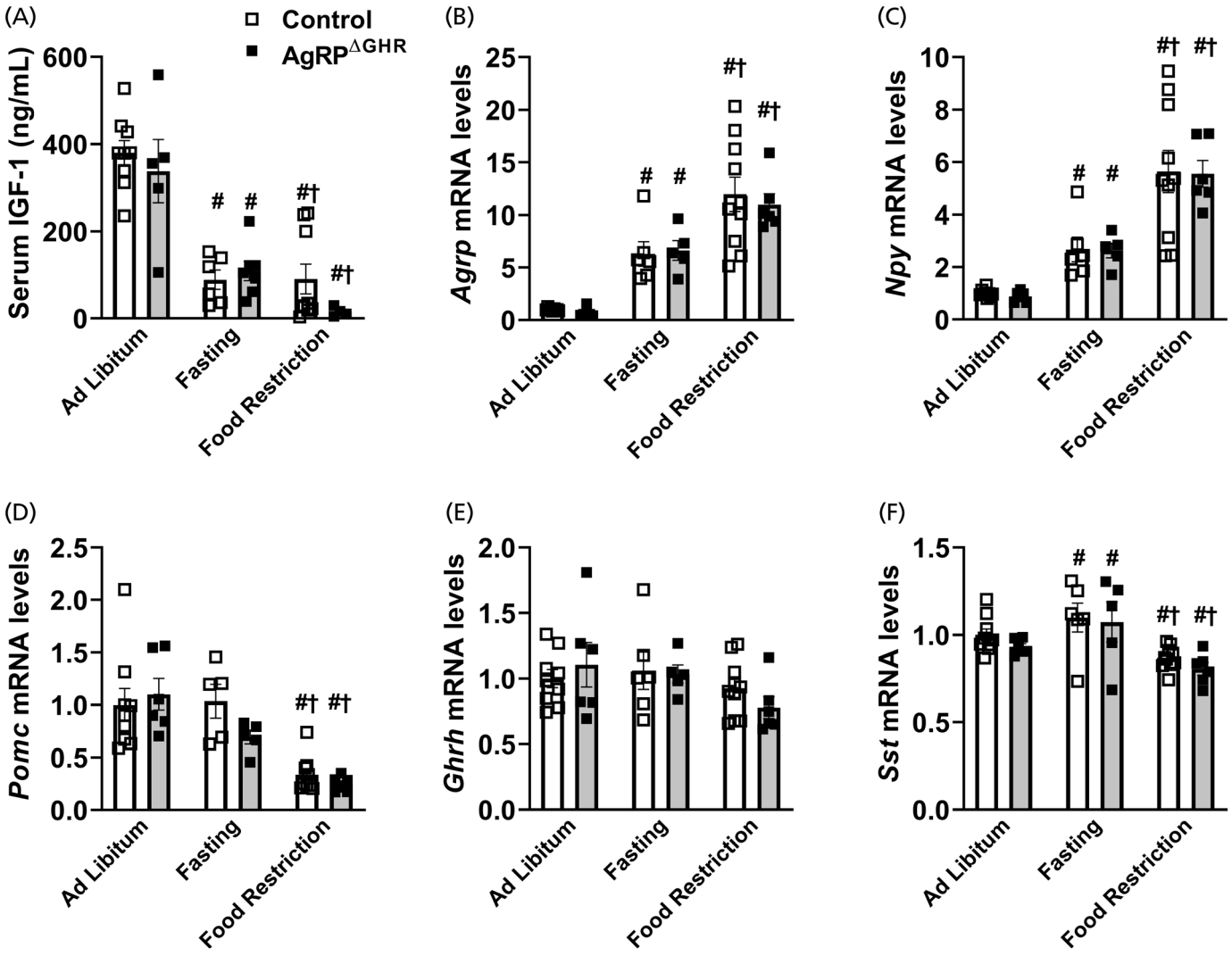


FIGURE 9 Serum IGF-1 levels and hypothalamic gene expression in fed, fasted and food-restricted mice. Serum IGF-1 concentration in fed, fasted and food-restricted control ($n = 6–9$/group) and AgRP$^{\Delta GHR}$ ($n = 4–7$/group) mice (A). Hypothalamic mRNA levels of different transcripts in fed, fasted and food-restricted control ($n = 5–9$/group) and AgRP$^{\Delta GHR}$ ($n = 5–6$/group) mice (B–F). #, $p < .05$ different from ad libitum fed mice. †, $p < .05$ different from fasted mice. Differences between control and AgRP$^{\Delta GHR}$ mice were analyzed by the Scheirer-Ray-Hare test. Differences between the groups were identified by the Mann–Whitney U test and Bonferroni correction for multiple comparisons.

[$H_{(1,N\ =\ 26)} = 4.64$, $p = .031$]; Figure 8H). These effects were observed in both control and AgRP$^{\Delta GHR}$ mice, without differences between the groups. Therefore, fasting suppresses GH secretion, whereas prolonged food restriction leads to a tonic increase in GH levels, which is explained by a higher basal GH secretion. Importantly, GHR signaling in ARH^{AgRP} neurons is not required for these changes.

3.5 | Hypothalamic *Sst* expression increases in acutely fasted mice and decreases after prolonged food restriction

Since circulating IGF-1 levels are marked affected by changes in the pattern of GH secretion,[46,48,51,53] we analyzed serum IGF-1 concentration in fed, fasted and food-restricted mice. Both fasting and food restriction decreased serum IGF-1 levels in control and AgRP$^{\Delta GHR}$ mice, compared to ad libitum fed mice (main effect of food deprivation [$H_{(2,N\ =\ 40)} = 25.89$, $p < .0001$]; Figure 9A). Fasting increased the hypothalamic expression of *Agrp* mRNA in both groups (main effect of food deprivation [$H_{(2,N\ =\ 42)} = 32.21$, $p < .0001$]; Figure 9B), and this increase was even greater in animals on food restriction compared to fasted mice ($p = .002$). Hypothalamic *Npy* mRNA levels were increased by fasting and even more by prolonged food restriction, compared to fed mice, without differences between control and AgRP$^{\Delta GHR}$ mice (main effect of food deprivation [$H_{(2,N\ =\ 42)} = 33.66$, $p < .0001$]; Figure 9C). In contrast, food restriction suppressed hypothalamic *Pomc* mRNA expression in control and AgRP$^{\Delta GHR}$ mice, compared to ad libitum fed and fasted mice (main effect of food deprivation [$H_{(2,N\ =\ 40)} = 25.63$, $p < .0001$]; Figure 9D). Neither fasting nor food restriction affected hypothalamic *Ghrh* mRNA expression (Figure 9E). On the other hand, *Sst* mRNA expression was increased in the hypothalamus of fasted mice, but decreased in food-restricted

mice, compared to fed and fasted animals without differences between control and AgRP$^{\Delta GHR}$ mice (main effect of food deprivation [$H_{(2,N\ =\ 42)} = 16.28$, $p < .0001$]; Figure 9F).

4 | DISCUSSION

In the present study, we investigated the role played by GHR signaling in ARH$^{AgRP/NPY}$ neurons on the pattern of GH secretion, either in fed or food-deprived mice. Furthermore, we compared how different situations of food deprivation affect GH secretion. We observed that short-term fasting robustly suppresses basal and pulsatile GH secretion. In contrast, prolonged food restriction causes a tonic/constitutive increase in GH secretion, which is explained by a higher basal GH secretion. GHR deletion in ARH$^{AgRP/NPY}$ neurons did not affect GH secretion.

GHR ablation in GABAergic or tyrosine hydroxylase-expressing cells induces increases in GH secretion and body growth,[57,59] whereas disruption of GHR signaling in other neuronal populations, including cells that express somatostatin, steroidogenic factor-1, proopiomelanocortin, kisspeptin, choline acetyltransferase, dopamine transporter, dopamine β-hydroxylase, corticotropin-releasing hormone, and vesicular glutamate transporter 2 does not affect body growth or the GH/IGF-1 axis.[6,57,59,65–69] We confirmed the results of previous studies showing that AgRP$^{\Delta GHR}$ male mice exhibit normal body growth.[17,58,64] The present findings further indicate that disruption of GHR signaling in ARH$^{AgRP/NPY}$ neurons is insufficient to cause significant effects on the pattern of GH secretion. Therefore, despite the notable responsiveness of ARH$^{AgRP/NPY}$ neurons to GH, this neuronal population is not involved in the negative feedback control of the GH/IGF-1 axis.

The lack of sufficient food robustly activates ARH$^{AgRP/NPY}$ neurons.[17,20,21,70,71] Accordingly, a strong increase in the hypothalamic expression of *Agrp* and *Npy* mRNA was observed in fasted and food-deprived mice. The greatest expression of *Agrp* and *Npy* mRNA in food-deprived mice confirms that prolonged food restriction induces a more severe catabolic state, as compared to 16 h fasting, representing a starvation protocol.[46–49] Interestingly, hypothalamic *Pomc* expression was only affected by prolonged food restriction, suggesting that POMC neurons require a higher energy deficit to become affected.

Numerous studies have investigated the effects of food deprivation on GH secretion in humans and animal models. However, probably due to differences in the experimental protocols used, conflicting data indicate that situations of negative energy balance can either suppress[40,42–44] or increase[17,45–54] GH secretion. Several studies used a single blood sample to assess GH levels, and this represents a limitation, as the results are more vulnerable to random variations in circulating GH levels caused by the natural pulsatile secretion pattern of this hormone. We believe that the present study reconciliates these conflicting results because our findings clearly indicate that different protocols of food deprivation can have opposite effects on the pattern of GH secretion. The mechanisms behind these differences remain unknown, but we can speculate that since ARH$^{AgRP/NPY}$ neurons are already activated by short-term fasting, the increased NPY neurotransmission may inhibit GH secretion via Y1 receptors, as previously demonstrated.[40] On the other hand, the stronger catabolic state produced by prolonged food restriction can overcome this inhibitory effect and stimulate GH secretion via other mechanisms, including the secretagogue effect of ghrelin, by increasing the pituitary response to factors that stimulate GH secretion and via suppression of hypothalamic SST. In favor of the role of ghrelin in stimulating GH secretion during prolonged food restriction, several studies have found that disruption in ghrelin or ghrelin receptor signaling blunts the increase in GH secretion observed in food-restricted animals.[46–48,52] On the other hand, plasma ghrelin levels (active form) increase after 12 h of fasting and longer periods of fasting do not cause further increases in circulating ghrelin levels.[53] Thus, increased GH secretion during prolonged food restriction is likely not driven by additional increases in circulating levels of ghrelin. Rather, increases in ghrelin responsiveness may play a role in stimulating GH secretion in prolonged food-deprived mice. In line with this idea, the longer the fasting period, the greater the expression of ghrelin receptor in the pituitary gland.[53] Furthermore, pituitary expression of somatostatin receptors is reduced in food-deprived mice,[53] which decreases the inhibitory tone on GH secretion. The combination of these changes in the pituitary gland may be behind the increase in GH secretion in starved mice.

Reduced hypothalamic expression of *Sst* mRNA in prolonged food-deprived mice may also lead to disinhibition in pituitary GH production, possibly contributing to the constitutive increase in GH secretion observed in prolonged food-deprived mice. In accordance with our findings, Thomas et al.[54] observed increased GH secretion and SST release into the hypophysial portal blood of chronically food-deprived ewes, without changes in portal levels of GHRH. Reduction in SST release may not only increase overall GH secretion, but also switch the pattern of GH secretion, favoring a continued increase in basal GH levels because of the potential role of SST as a GH pulse initiator.[72–74] However, other studies did not find evidence that SST acts as a GH pulse generator.[54,75–79]

Fasting increased the regularity (lack of complexity) of GH secretion (measured by a decrease in the SaEn), whereas prolonged food restriction led to a more irregular GH secretion. The reduction in the irregularity (SaEn) during fasting may be a result of greatly reduced GH secretion, particularly basal secretion. On the other hand, several factors may have contributed to the elevated entropy (irregularity in GH secretion) in starved mice. Ghrelin infusion increases the irregularity in the GH secretion pattern,[79] and the food restriction protocol used in the present study is known to increase ghrelin secretion.[46,47,52] Prolonged food restriction reduces circulating testosterone levels in mice[17] and a previous study has shown that testosterone increases the regularity in GH secretion.[80] Furthermore, SST is also involved in the regulation of the regularity of GH secretion.[73]

In our study, the hypothalamus was collected after the 6 h of bleeding. Thus, we cannot rule out a possible influence of stress on the results of the hypothalamic gene expression. However, this equally influenced all experimental groups. In addition, gene expression was analyzed in the whole hypothalamus. This limitation is particularly critical to SST since several distinct hypothalamic neuronal populations express *Sst* mRNA and we were not able to specifically

assess the group of SST neurons involved in GH secretion, which is located in the periventricular nucleus.

Notably, the increased GH secretion observed in food-deprived mice did not lead to higher circulating IGF-1 levels. Actually, serum IGF-1 concentration was suppressed either by fasting or prolonged food restriction. This result can be explained by a state of GH resistance that develops in the liver of food-deprived animals.[80] Fibroblast growth factor 21 (FGF21) is a hormone induced by fasting. FGF21 increases the hepatic expression of the suppressor of cytokine signaling 2 and IGF-1 binding protein 1, which in turn reduce the capacity of hepatic GHR signaling to induce pSTAT5 and the expression of downstream genes, like the *Igf1*.[81] Thus, FGF21 is behind the reduced circulating levels of IGF-1, despite the increased GH secretion in food-restricted mice. Other mechanisms were also proposed to be involved in the fasting-induced hepatic GH resistance,[80] including the role played by sirtuin 1[82] and the leptin receptor overlapping transcript,[83] in which both proteins negatively regulate GH-dependent hepatic IGF-1 production, and are activated by fasting.

Previous studies have shown that AgRP$^{\Delta GHR}$ mice exhibit blunted neuroendocrine adaptations to food restriction, leading to increased weight loss.[17,26,63] In the present study, we found that neither fasting nor four days of food restriction significantly reduced the bodyweight of AgRP$^{\Delta GHR}$ mice, beyond that observed in control animals. We believe that the reduced sample size in the current work, as compared to previous studies,[17,63] prevented the observation of a significant difference between the groups, considering the expected effect size in this variable. We observed that AgRP$^{\Delta GHR}$ mice lost, respectively, 15% and 40% more body and fat mass after fasting, compared to control mice. In the food restriction protocol, AgRP$^{\Delta GHR}$ mice tended to lose more weight after two days of food restriction, but the overall results indicate a non-significant effect compared to control mice. It is also important to note that independently of the role of GHR signaling in ARH$^{AgRP/NPY}$ neurons regulating metabolism, the increased basal GH secretion in starved mice probably helps the organism to survive prolonged food deprivation.[84] Among the metabolic actions of GH, one can highlight that this hormone increases fat mobilization, spares the use of glucose, prevents hypoglycemia, and stimulates feeding.[84] Thus, the shift from the inhibition of GH secretion in acute fasting to the increase of its constitutive secretion during prolonged food deprivation is likely necessary to increase the chances of survival during such severe metabolic stress.

In conclusion, short-term fasting and prolonged food restriction differentially affect the pattern of GH secretion. This result help to understand the apparent divergent findings shown by studies that investigated GH secretion after different protocols of food restriction since our work demonstrates that GH secretion is initially inhibited by lack of food and switches to a pattern of increased basal GH secretion in starved mice. Furthermore, our findings highlight the importance to determine the pulsatile pattern of GH secretion as a single assessment is subject to the inherent randomness in the pattern of GH secretion. Moreover, our study disclosed that the absence of GHR signaling in ARH$^{AgRP/NPY}$ neurons did not lead to the loss of GH negative feedback sufficiently to affect the pattern of GH secretion in any of the situations studied. Finally, our study was entirely performed in males. Considering the important differences in the pattern of GH secretion between the sexes,[85–87] caution is necessary for extrapolating our findings to females.

AUTHOR CONTRIBUTIONS

Maria E de Sousa: Formal analysis; investigation. **Daniela O Gusmao:** Formal analysis; investigation. **Willian O dos Santos:** Formal analysis; investigation. **Henrique T Moriya:** Formal analysis. **Felipe F de Lima:** Formal analysis. **Edward O List:** Resources. **John J Kopchick:** Resources. **Jose Donato:** Conceptualization; data curation; funding acquisition; project administration; writing – original draft.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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