HHS Public Access

Author manuscript

Neuroscience. Author manuscript; available in PMC 2021 May 10.

Published in final edited form as:

Neuroscience. 2020 May 10; 434: 136–147. doi:10.1016/j.neuroscience.2020.03.037.

Growth hormone receptor deletion reduces the density of axonal projections from hypothalamic arcuate nucleus neurons

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Abstract

The arcuate nucleus (ARH) is an important hypothalamic area for the homeostatic control of feeding and other metabolic functions. In the ARH, proopiomelanocortin- (POMC) and agouti-related peptide (AgRP)-expressing neurons play a key role in the central regulation of metabolism. These neurons are influenced by circulating factors, such as leptin and growth hormone (GH). The objective of the present study was to determine whether a direct action of GH on ARH neurons regulates the density of POMC and AgRP axonal projections to major postsynaptic targets. We studied POMC and AgRP axonal projections to the hypothalamic paraventricular (PVH), lateral (LHA) and dorsomedial (DMH) nuclei in leptin receptor (LepR)-deficient mice (*Lepr*$^{db/db}$), GH-deficient mice (*Ghrhr*$^{lit/lit}$) and in mice carrying specific ablations of GH receptor (GHR) either in LepR- or AgRP-expressing cells. *Lepr*$^{db/db}$ mice presented reduction in the density of POMC innervation to the PVH compared to wild-type and *Ghrhr*$^{lit/lit}$ mice. Additionally, both *Lepr*$^{db/db}$ and *Ghrhr*$^{lit/lit}$ mice showed reduced AgRP fiber density in the PVH, LHA and DMH. LepR GHR knockout mice showed decreased density of POMC innervation in the PVH and DMH, compared to control mice, whereas a reduction in the density of AgRP innervation was observed in all areas analyzed. Conversely, AgRP-specific ablation of GHR led to a significant reduction in AgRP projections to the PVH, LHA and DMH, without affecting POMC innervation. Our findings indicate that GH has direct trophic effects on the formation of POMC and AgRP axonal

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AUTHORS' CONTRIBUTIONS

J.D. designed the study; F.W., I.C.F., P.D.S.T. and A.M.R.L. conducted the research; F.W. and J.D. analyzed the data; C.N.P., P.B., E.O.L. and J.J.K. provided essential equipment, reagents, and expertise. F.W. and J.D. wrote the paper. All authors revised and approved the final manuscript.

COMPETING INTERESTS

The authors declare no conflicts of interest.

projections and provide additional evidence that GH regulates hypothalamic neurocircuits controlling energy homeostasis.

Keywords

AgRP; development; food intake; GH; melanocortin system; POMC

INTRODUCTION

Feeding behavior is regulated by complex neurocircuits that integrate homeostatic and decision-making/reward information. The neurons that control feeding are distributed in different areas of the central nervous system, including the hypothalamus, amygdala, cerebral cortex and brainstem (Andermann and Lowell, 2017; Livneh et al., 2017; Ramos-Lobo and Donato, 2017). Although it is evident that food intake is controlled by neurocircuits and not by isolated neural populations, the arcuate nucleus of the hypothalamus (ARH) plays a particularly important role regulating not only feeding, but different aspects of metabolism. This occurs because ARH neurons are able to detect changes in systemic levels of different hormones, nutrients and metabolites proving critical homeostatic information to the brain (Andermann and Lowell, 2017; Livneh et al., 2017; Ramos-Lobo and Donato, 2017). In this sense, the alterations in food intake induced by different hormones, including leptin (Xu et al., 2018), ghrelin (Wang et al., 2014), insulin (Loh et al., 2017) or glucagon-like peptide-1 (He et al., 2019), depend on ARH neurons.

“Novel” neuronal populations in the ARH have been described to regulate food intake, including tyrosine hydroxylase- or somatostatin-expressing neurons (Zhang and van den Pol, 2016; Campbell et al., 2017; Luo et al., 2018). However, neurons that express either the proopiomelanocortin (POMC) or the agouti-related peptide (AgRP) are classically involved in the control of food intake and energy balance (Andermann and Lowell, 2017; Livneh et al., 2017; Ramos-Lobo and Donato, 2017). Activation of AgRP neurons induces feeding (Luquet et al., 2005; Aponte et al., 2011; Krashes et al., 2011), whereas the release of α-melanocyte-stimulating hormone by POMC neurons and the consequent activation of melanocortin receptor 3 (MC3R) and 4 (MC4R) inhibit food intake and are required for energy homeostasis (Fan et al., 1997; Huszar et al., 1997; Ollmann et al., 1997).

A pronounced expression of GH receptor (GHR) has been described in the ARH (Walsh et al., 1990; Burton et al., 1992; Kamegai et al., 1996; Furigo et al., 2017). In addition, recent studies indicate that GH action in both AgRP and POMC neurons control different aspects of metabolism (Bohlen et al., 2019; Furigo et al., 2019b; Quaresma et al., 2019; Teixeira et al., 2019). For example, Furigo et al. (2019b) showed that GH activates AgRP neurons and increases food intake, whereas AgRP-specific GHR ablation impairs the neuroendocrine adaptions to weight loss. On the other hand, GHR deletion in POMC cells blunts the glucoprivic hyperphagia induced by 2-deoxy-D-glucose administration (Quaresma et al., 2019). Thus, GH emerges as a new circulating factor that regulates metabolism via ARH neurons.

Some hormones that regulate food intake, such as leptin, also exhibit trophic effects on ARH neurons by regulating axonal development (Bouret et al., 2004b; Bouret et al., 2004a) and synaptic plasticity (Pinto et al., 2004). Interestingly, GH deficient or GHR knockout (KO) mice also present reduced formation of AgRP and POMC projections to postsynaptic targets (Sadagurski et al., 2015). However, these mouse models present multiple confounding factors, such as dwarfism, changes in leptin and insulin-like growth factor-1 (IGF-1) levels, increased insulin sensitivity, endocrine defects and developmental alterations (Young et al., 2016; Basu et al., 2018). Thus, whether the formation of ARH neural projections requires a direct action of GH on AgRP and POMC cells or is indirectly influenced by the endocrine abnormalities caused by the complete deficiency of GH action remains to be determined. Thus, the objective of the present study was to investigate if GHR expression in ARH neurons regulates the density of axonal terminals to major hypothalamic projection areas. To investigate the neurotrophic effects of leptin and GH, we studied POMC and AgRP axonal projections in leptin receptor (LepR)-deficient ($Lepr^{db/db}$) and GH-deficient ($Ghrhr^{lit/lit}$) mice, as well as in mice carrying specific ablations of GHR in both LepR- and AgRP-expressing cells.

EXPERIMENTAL PROCEDURES

Mice

All experiments were conducted in 4-month-old male mice in accordance with the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of São Paulo (protocol #73/2017). C57BL/6 wild-type (WT) mice, $Lepr^{db/db}$ mice (Stock No: 000697; The Jackson Laboratory) and $Ghrhr^{lit/lit}$ mice (Stock No: 000533; The Jackson Laboratory) were used in the experiments. Adult $Lepr^{db/db}$ and $Ghrhr^{lit/lit}$ mice exhibit, respectively, morbid obesity and dwarfism. Genetic deletion of the *Ghr* gene either in AgRP cells or LepR-expressing cells were obtained through the breeding of AgRP-Cre mouse (Stock No: 012899; The Jackson Laboratory) or LepR-Cre mouse (Stock No: 008320; The Jackson Laboratory) with animals carrying loxP-flanked *Ghr* alleles (List et al., 2013). AgRP GHR KO and LepR GHR KO mice carried the Cre transgene and were homozygous for the loxP- flanked alleles, whereas their respective controls were littermates negative for the Cre transgene. To visualize AgRP- or LepR-expressing neurons, a group of control, AgRP GHR KO and LepR GHR KO mice also carried a Cre-dependent expression of the tdTomato reporter protein (Stock No: 007909, The Jackson Laboratory). Animals were weaned at 21 days of age and tail tips were collected for DNA extraction. Genomic DNA was amplified by PCR, and PCR products were submitted to electrophoresis in agarose gel for the identification of their mutations. Mice were maintained in standard conditions of 12h light/dark cycles and controlled temperature (21–23 °C), and received potable water and regular chow *ad libitum* (2.99 kcal/g; 9.4% calories from fat; Quimtia, Brazil).

Evaluation of axonal projections from ARH neurons

Adult (4-month-old) male mice were anesthetized with isoflurane and transcardially perfused with saline followed by 10% buffered formalin solution (~180 mL/mouse). Collected brains were maintained for 1 hour in formalin for post-fixation and were transferred to 20% sucrose solution overnight. Brains were cut in a freezing microtome in 30

μm thickness sections and maintained in cryoprotection buffer at −20°C. Brain sections were subjected to immunofluorescence staining to evaluate the innervation of POMC or AgRP fibers in the paraventricular nucleus of the hypothalamus (PVH), lateral hypothalamic area (LHA) and dorsomedial nucleus of the hypothalamus (DMH). Brain sections were rinsed in 0.02 M potassium PBS, pH 7.4 (KPBS), followed by incubation in 3% normal donkey serum for 1 hour. Next, sections were incubated overnight in anti-AgRP antisera (1:2,000, Phoenix Pharmaceuticals, Inc.; Cat# H-003–53) or anti-β-endorphin antisera (1:2,000; Phoenix Pharmaceuticals; Burlingame, CA; Cat# H-022–33), since β-endorphin is a POMC-derived peptide and presents a complete overlapping expression compared to α-melanocyte-stimulating hormone (Lima et al., 2016). Subsequently, sections were incubated for 90 min in Alexa Fluor^{488}-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; Cambridge, MA). After rinses in KPBS, sections were mounted onto gelatin-coated slides and covered with Fluoromount G mounting medium (Electron Microscopic Sciences, Hatfield, PA). One representative rostrocaudal level of the LHA (Bregma −1.22) and DMH (Bregma −1.82), and two levels of the PVH (Bregma −0.70 and −0.94) were analyzed. In the case of PVH, we considered the mean values obtained in the two rostrocaudal levels. Initially, epifluorescence photomicrographs were acquired with an Axio Imager A1 microscope (Carl Zeiss, Munich, Germany), as demonstrated in the representative pictures. Using the ImageJ software (http://rsb.info.nih.gov/ij), we selected the area corresponding to each brain structure (PVH: 0.03 mm^2; LHA: 0.2 mm^2; DMH: 0.08 mm^2). 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, LHA and DMH were subtracted from the IOD determined in adjacent nuclei with low staining (background) in each photomicrograph. In the first set of experiments, we studied WT (n = 5), *Lepr*$^{db/db}$ (n = 6) and *Ghrhr*$^{lit/lit}$ (n = 6) mice. In the second experiment, LepR GHR KO mice (n = 7) and control littermates (n = 7) were compared. In the third set of experiments, AgRP GHR KO mice (n = 6) and control littermates (n = 6) were evaluated.

Evaluation of GH responsive neurons

Control (n = 4–5), AgRP GHR KO (n = 3) and LepR GHR KO (n = 3) mice received an intraperitoneal injection of a saline solution containing GH extracted from porcine pituitary (from Dr. A.F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program, Los Angeles, CA) at a final dose of 20 μg/g body weight. PBS-injected mice (n = 4) were used as a negative control of pSTAT5 staining. Mice were perfused 90 minutes later. To detect the phosphorylated form of the signal transducer and activator of transcription-5 (pSTAT5), brain slices were rinsed in KPBS, followed by pretreatment in 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 anti-pSTAT5^{Tyr694} primary antibody (1:1000; Cell Signaling Technology; Beverly, MA; Cat# 9351) for 40 h. After that, sections were rinsed in KPBS and incubated for 90 min in AlexaFluor^{488}-conjugated secondary antibody (1:500, Jackson ImmunoResearch Laboratories). The visualization of tdTomato fluorescence does not require any reaction. Sections were mounted onto gelatin-coated slides and covered with

Fluoromount G (Electron Microscopic Sciences). Photomicrographs were acquired with an Axio Imager A1 microscope (Carl Zeiss).

Gene expression analysis

For the gene expression analysis, total RNA from the whole hypothalamus was extracted with TRIzol (Invitrogen). Assessment of RNA quantity and quality was determined using an Epoch Microplate Spectrophotometer (Biotek). Total RNA was incubated in DNase I RNase-free (Roche Applied Science), followed by reverse transcription using 2 μg of total RNA with SuperScript II Reverse Transcriptase (Invitrogen) and random primers p(dN)6 (Roche Applied Science). Real-time polymerase chain reaction was performed using the 7500TM Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems). Relative quantification of mRNA was calculated by $2^{-\Delta\Delta Ct}$. Data were normalized to the expression of *Actb* and reported as fold changes compared to values obtained from the control group (set at 1.0). The following primers were used: *Actb* (forward: gctccggcatgtgcaaag; reverse: catcacaccctggtgccta), *Mc3r* (forward: ttgatgaaaacctgctcgca; reverse: tatccgacgctgcctaacct) and *Mc4r* (forward: cttccccagagactcgctggca; reverse: acccaccaccatggcatgta).

Statistical analysis

Data were expressed as mean ± s.e.m. and analyzed with GraphPad Prism software (San Diego, CA). Comparisons between two groups were performed using two-tailed Student's t-test. When three groups were compared simultaneously, the analysis was performed by one-way ANOVA with Tukey's test for multiple comparisons. Differences were considered significant if $P \leq 0.05$.

RESULTS

Development of ARH projections requires GH and leptin signaling

Previous studies indicate that the absence of leptin or GH signaling leads to defects in the development of ARH neuronal projections (Bouret et al., 2004b; Bouret et al., 2004a; Sadagurski et al., 2015; Ramos-Lobo et al., 2019). To confirm the importance of the neurotrophic effects of leptin and GH, AgRP and POMC projections were compared among WT, $Lepr^{db/db}$ and $Ghrhr^{lit/lit}$ mice. As expected, 4-month-old $Lepr^{db/db}$ mice are morbidly obese, whereas the dwarfism of $Ghrhr^{lit/lit}$ mice was confirmed by their reduced body weight, compared to WT animals (Fig. 1A). The number of POMC-expressing cells in the ARH was reduced in $Lepr^{db/db}$ mice, which is in accordance with the stimulatory effect of leptin on ARH POMC neurons that is missing in $Lepr^{db/db}$ mice (Schwartz et al., 1997; Thornton et al., 1997). In contrast, $Ghrhr^{lit/lit}$ mice exhibited a similar number of POMC neurons compared to WT animals (Fig. 1B). Next, ARH projections were evaluated. $Lepr^{db/db}$ mice presented reduced density of POMC innervation in the PVH, compared to both WT and $Ghrhr^{lit/lit}$ mice (Fig. 1C and Fig. 2). However, $Lepr^{db/db}$ mice exhibited no changes in POMC fiber density in the LHA and DMH (Fig. 1D–E and Fig. 2). Additionally, POMC innervation in $Ghrhr^{lit/lit}$ mice was similar to that observed in WT mice (Fig. 1C–E and Fig. 2). Regarding the innervation of AgRP, both $Lepr^{db/db}$ and $Ghrhr^{lit/lit}$ mice showed

reduced AgRP fiber density in the PVH, LHA and DMH, compared to WT animals (Fig. 1F–H and Fig. 3).

Axonal projections of POMC and AgRP neurons are affected by LepR-specific GHR disruption

To determine whether a direct action of GH on ARH neurons is necessary for the formation of POMC and AgRP axonal projections, mice carrying ablation of GHR only in LepR-expressing cells, which include the majority of POMC and AgRP neurons (Lima et al., 2016; Xu et al., 2018), were produced. We determined the expression of pSTAT5 as a marker of GH responsive cells (Furigo et al., 2017; Silveira et al., 2019). PBS-injected mice showed very few pSTAT5 cells in the ARH and PVH and no co-localization with LepR-expressing cells (Fig. 4A–B). In accordance with previous studies (Furigo et al., 2019a; Furigo et al., 2019b; Teixeira et al., 2019), 93.2 ± 0.8% of ARH LepR-expressing neurons co-localized with GH-induced pSTAT5 in control animals (Fig. 4C,F). In contrast, 11.0 ± 2.6% of LepR-expressing cells in the ARH of LepR GHR KO mice remained responsive to GH (Fig. 4D,F), demonstrating the efficacy of our gene deletion. Importantly, other areas that exhibit low LepR expression, such as the PVH, still exhibited a normal distribution of GH responsive cells in LepR GHR KO mice (Fig. 4E). LepR GHR KO mice tended to have more LepR-expressing cells in the ARH compared to control animals ($P = 0.0578$; Fig. 5A), although the number of POMC neurons was similar ($P = 0.1549$; Fig. 5B). Additionally, the hypothalamic expression of *Mc3r* and *Mc4r* were not affected by GHR ablation in LepR-expressing cells (Fig. 5C). Regarding the innervation of ARH neurons, LepR GHR KO mice exhibited a decreased density of POMC innervation in the PVH and DMH compared to control mice (Fig. 5D,F and Fig. 6A–F), whereas no significant differences were observed in POMC innervation of the LHA (Fig. 5E). A significant decrease in the density of AgRP innervation in the PVH, LHA and DMH was also observed in LepR GHR KO mice (Fig. 5G–I and Fig. 6G–L). Thus, GH signaling specifically in LepR neurons is required for the development of ARH axonal projections.

AgRP innervation is impaired in mice carrying AgRP-specific GHR ablation

While PBS-injected control mice showed virtually no pSTAT5 in the ARH or other brain areas (Fig. 7A), we observed that 91.7 ± 0.7% of AgRP neurons in GH-injected control animals contained pSTAT5 (Fig. 7B). As expected, AgRP-specific GHR ablation significantly reduced the expression of pSTAT5 in AgRP neurons (12.3 ± 2.2%), whereas GH responsive cells were still observed in the lateral ARH and surrounding areas (Fig. 7C–D). AgRP GHR KO mice exhibited a similar number of AgRP or POMC neurons compared to control animals (Fig. 8A–B). In addition, AgRP-specific GHR ablation did not cause changes in *Mc3r* and *Mc4r* mRNA levels in the hypothalamus (Fig. 8C). The innervation of POMC neurons was initially investigated and AgRP GHR KO mice showed similar density of POMC fibers in the PVH, LHA and DMH, compared to control animals (Fig. 8D–F and Fig. 9A–F). In contrast, the density of AgRP innervation in these brain areas was significantly reduced in AgRP GHR KO mice (Fig. 8G–I and Fig. 9G–L). Thus, GHR ablation produces a cell-specific effect on the development of ARH neuronal projections.

DISCUSSION

Although the expression of GHR in the ARH was first reported almost 30 years ago (Walsh et al., 1990; Burton et al., 1992; Lobie et al., 1993; Minami et al., 1993; Bennett et al., 1995; Burton et al., 1995; Chan et al., 1996; Kamegai et al., 1996; Pellegrini et al., 1996), the physiological function of GH signaling on ARH neurons is only beginning to be described. Our previous studies have demonstrated that GH is able to regulate food intake, energy expenditure and glucose homeostasis via ARH neurons (Bohlen et al., 2019; Furigo et al., 2019b; Quaresma et al., 2019; Teixeira et al., 2019). Several distinct neurochemically-defined neuronal populations can be found in the ARH (Campbell et al., 2017). Among the different neural populations located in the ARH, we know that GH responsive cells are composed of AgRP- (Furigo et al., 2019b; Teixeira et al., 2019) and POMC-expressing cells (Quaresma et al., 2019). In contrast, ARH kisspeptin-expressing neurons do not contain GH-induced pSTAT5 (Bohlen et al., 2019; Silveira et al., 2019). It is worth mentioning that the majority of AgRP and POMC neurons are also responsive to leptin (Lima et al., 2016; Xu et al., 2018), confirming the large percentage of GH responsive cells in the ARH that co-localize with LepR (Bohlen et al., 2019; Furigo et al., 2019a; Furigo et al., 2019b; Teixeira et al., 2019). In the present study, we revealed that, in addition to regulating metabolism, GH action on ARH neurons leads to direct trophic effects controlling the formation of axonal projections from POMC and AgRP neurons to important postsynaptic targets, including the PVH, DMH and LHA. In this aspect, GH shares with leptin neurotrophic effects on the neurocircuits that regulate energy homeostasis.

In the present study, the axonal projections of AgRP and POMC neurons were investigated through the detection of immunoreactive fibers using antibodies against these peptides. Although this method has been widely used for this purpose (Bouret et al., 2004b; Bouret et al., 2004a; Bouret et al., 2008; Kirk et al., 2009; Vogt et al., 2014; Sadagurski et al., 2015; Kamitakahara et al., 2018; Ramos-Lobo et al., 2018; Ramos-Lobo et al., 2019), the use of neurotracers could produce a more reliable way to detect and visualize axonal projections. In this regard, we cannot guarantee that the peptide content is uniformly distributed along the axon or if its expression is not different between the experimental groups, regardless of the innervation. However, in a previous study we compared AgRP axonal projections visualized by immunofluorescence staining or tdTomato expression using AgRP-specific reporter mice and the distribution of AgRP fibers in the brain was similar (Lima et al., 2019). In addition, LepR-deficient mice exhibit increased hypothalamic AgRP expression (Ramos-Lobo et al., 2019), even though AgRP innervation is drastically reduced, compared to WT mice, indicating that the method we used is efficient to detect axonal terminals, independently of changes in peptide expression. Thus, although our data may be interpreted with caution, our findings are consistent with the literature and indicate that both leptin (Bouret et al., 2004b; Bouret et al., 2004a; Kamitakahara et al., 2018; Ramos-Lobo et al., 2019) and GH (Sadagurski et al., 2015) are required for the normal formation of POMC and AgRP axonal projections.

Although a reduced density of POMC fibers was observed in the PVH of *Lepr*$^{db/db}$ mice, we were unable to detect significant changes in POMC innervation in the LHA and DMH of *Lepr*$^{db/db}$ or *Ghrhr*$^{lit/lit}$ mice. In contrast, AgRP innervation was reduced in all areas

analyzed in both *Lepr*$^{db/db}$ and *Ghrhr*$^{lit/lit}$ mouse models. Rather than a specific effect of leptin or GH signaling on AgRP neurons, we believe that the lower amount of POMC fibers, in comparison with the density of AgRP innervation, reduced the sensitivity of our IOD quantification. Thus, the analysis of AgRP fibers seems to be more accurate than the quantification of POMC fibers. Nevertheless, a reduced density of POMC innervation in the PVH and DMH was detected in LepR GHR KO mice.

After comparing the density of axonal projections of ARH neurons between *Lepr*$^{db/db}$ and *Ghrhr*$^{lit/lit}$ mice, our findings indicate that the absence of leptin signaling caused a greater reduction in fiber density compared to GH-deficient animals, although statistically significant changes were only observed in POMC innervation in the PVH and AgRP innervation in the DMH. Although these findings may indicate that leptin has a dominant neurotrophic role, compared to GH, *Ghrhr*$^{lit/lit}$ mice are not entirely deficient of GH (Godfrey et al., 1993; Cecchi et al., 2014). On the other hand, the long form of LepR is completely non-functional in *Lepr*$^{db/db}$ mouse, which makes this animal irresponsive to leptin (Chen et al., 1996). Thus, the comparison in the density of POMC and AgRP innervation between these models does not allow us to determine whether the neurotrophic action of one hormone prevails over the other.

The first study describing that GH possesses trophic effects on ARH neurons investigated these projections in two models of GH-deficient dwarf mice and in whole-body GHR KO dwarf mice (Sadagurski et al., 2015). In this study, the authors showed that early-life GH treatment rescues POMC and AgRP projections in GH-deficient mice. Furthermore, they also demonstrated that liver-specific GHR KO mice show normal POMC and AgRP innervation (Sadagurski et al., 2015), despite a drastic reduction in serum IGF-1 levels (List et al., 2014). Thus, these previous studies suggest that GH rather than IGF-1 is required for the formation of POMC and AgRP projections. In the present study, we provided further evidence of a direct neurotrophic effect of GH on ARH neurons. Although LepR GHR KO mice have normal body growth and no gross hormonal dysfunctions (Furigo et al., 2019a; Furigo et al., 2019b; Teixeira et al., 2019), ablation of GHR from LepR-expressing cells decreased both POMC and AgRP innervation to major hypothalamic projection areas. Importantly, GHR ablation exclusively from AgRP-expressing cells led to a reduction in AgRP innervation without affecting the projections of POMC neurons. These findings indicate that only cells affected by the conditional GHR deletion exhibit defects in the development of ARH neuronal projections.

We described in a recent study the consequences of inactivating GHR in POMC cells (Quaresma et al., 2019). The density of POMC and AgRP innervation was also determined, but the lack of GH action on POMC cells caused no apparent changes in the projections of ARH neurons (Quaresma et al., 2019). Several possibilities can explain this contrasting result compared to our findings. First, approximately 60% of POMC neurons are responsive to GH (Quaresma et al., 2019), compared to 90 to 95% of AgRP neurons (Furigo et al., 2019b). Thus, only a subset of POMC neurons is affected by GHR ablation. Second, as previously mentioned, the reduced density of POMC innervation, compared to AgRP fibers, decreases the sensitivity to detect slight changes. Third, a small number of POMC-expressing neurons are also found in the nucleus of the solitary tract (NTS), contributing to

the POMC innervation observed in the brain (Huo et al., 2006). However, differently than ARH neurons, POMC neurons in the NTS are not responsive to GH (Quaresma et al., 2019). Additionally, NTS POMC neurons send axons predominantly to the brainstem, although they also innervate the hypothalamus and particularly the PVH (Wang et al., 2015). Finally, POMC neurons may present a distinct plasticity capacity to compensate the absence of the trophic effects of GH.

Some additional points deserve to be discussed. In the present study, we studied exclusively male mice, so we cannot assure that the present findings are also observed in females. Of note, males and females exhibit important differences in GH secretion (Jansson et al., 1985; Maiter et al., 1991; Painson and Tannenbaum, 1991; Chowen et al., 1996; van den Berg et al., 1996; Jaffe et al., 1998). However, male and female mice present similar responsiveness to GH in ARH LepR-expressing cells or AgRP neurons (Bohlen et al., 2019; Furigo et al., 2019b; Teixeira et al., 2019; Furigo et al., 2020). Thus, it is likely that both genders may exhibit similar neurotrophic actions of GH, despite sexually dimorphic changes in the pattern of GH secretion. Additionally, AgRP neurons co-express other neurotransmitters, in special neuropeptide Y (NPY) (Hahn et al., 1998; Cowley et al., 1999). In this sense, NPY transmission is also important for the metabolic effects of ARH AgRP/NPY neurons (Erickson et al., 1996; Hahn et al., 1998). We decided to investigate AgRP rather than NPY innervation because NPY is expressed in numerous brain areas (Morris, 1989), whereas AgRP neurons are exclusively found in the ARH (Hahn et al., 1998). Consequently, while AgRP staining represents a reliable marker of ARH AgRP/NPY-expressing cells, NPY immunoreactive fibers may originate from several neural populations. Nevertheless, defects in the development of AgRP fibers can also lead to changes in NPY neurotransmission of ARH neurons with all associated physiological repercussions.

Until now, several studies described situations in which the innervation of POMC or AgRP neurons to postsynaptic targets is reduced. In this regard, these projections are impaired by the lack of leptin signaling (Bouret et al., 2004a; Bouret et al., 2008; Kamitakahara et al., 2018; Ramos-Lobo et al., 2019) or GH action (Sadagurski et al., 2015), by maternal obesity (Bouret et al., 2008; Kirk et al., 2009; Vogt et al., 2014) and by alterations in gestational metabolic adaptations (Ramos-Lobo et al., 2018). Although defects in these projections may indicate a reduced capacity of ARH neurons to influence the neurocircuits that control energy and glucose homeostasis, which presumably leads to metabolic imbalances, no study to our knowledge demonstrated that the reduced innervation of ARH neurons is the primary cause of these dysfunctions. Regarding GH action on ARH neurons, both AgRP- and POMC-specific GHR KO mice exhibit normal body weight, energy expenditure and food intake in *ad libitum* fed conditions or during refeeding (Furigo et al., 2019b; Quaresma et al., 2019). Therefore, it is essential that future studies investigate the importance of changes in the innervation of POMC or AgRP neurons for the central regulation of metabolism. Nevertheless, our findings clearly demonstrate that ARH neurons are directly affected by GH action. Thus, GH should be included in the set of hormones that modify neurocircuits that control feeding, body weight and glucose homeostasis.

ACKNOWLEDGEMENTS

We thank Ana M.P. Campos for the technical assistance. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP-Brazil; grant numbers: 14/11752-6 to A.M.R.L.; 16/20897-3 to F.W., 16/09679-4 to I.F. and 17/02983-2 to J.D.) and NIH (NIA; grant number: R01AG059779 to E.O.L. and J.J.K.).

Abbreviations:

3	third ventricle
AgRP	agouti-related peptide
ARH	arcuate nucleus of the hypothalamus
DMH	dorsomedial nucleus of the hypothalamus
GH	growth hormone
GHR	growth hormone receptor
IGF-1	insulin-like growth factor-1
IOD	integrated optical density
KO	knockout
LepR	leptin receptor
LHA	lateral hypothalamic area
MC3R	melanocortin receptor 3
MC4R	melanocortin receptor 4
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
POMC	proopiomelanocortin
PVH	paraventricular nucleus of the hypothalamus
STAT5	signal transducer and activator of transcription-5
WT	wild-type

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HIGHLIGHTS

- Formation of POMC and AgRP projections requires GH and leptin signaling.
- POMC and AgRP projections are affected by LepR-specific GH receptor disruption.
- AgRP innervation is impaired in mice carrying AgRP-specific GH receptor ablation.

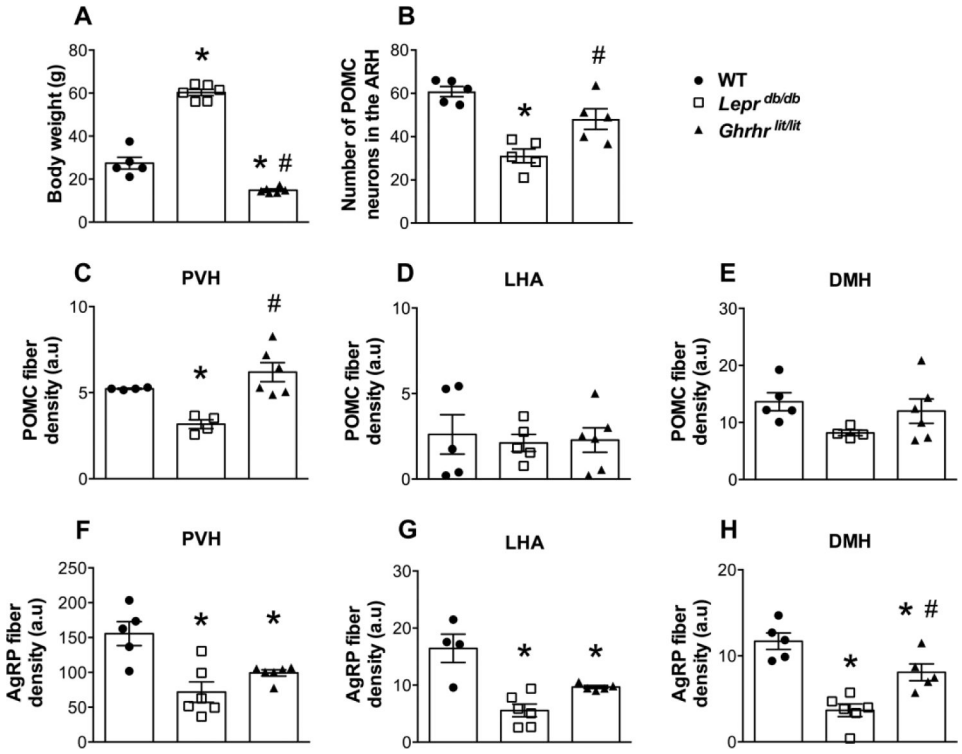

Fig. 1. Axonal projections of POMC and AgRP neurons require GH and leptin signaling.

A. Body weight of 4 month-old control (WT, $n = 5$), *Lepr*$^{db/db}$ ($n = 6$) and *Ghrhr*$^{lit/lit}$ ($n = 6$) male mice. **B.** Number of POMC-expressing cells in the ARH of WT ($n = 5$), *Lepr*$^{db/db}$ ($n = 5$) and *Ghrhr*$^{lit/lit}$ ($n = 5$) mice. **C-E.** Quantification of POMC fiber density in the PVH (B), LHA (C) and DMH (D) of WT ($n = 4$–5), *Lepr*$^{db/db}$ ($n = 4$–5) and *Ghrhr*$^{lit/lit}$ ($n = 6$) mice. **F-H**. Quantification of AgRP fiber density in the PVH (E), LHA (F) and DMH (G) of WT ($n = 4$–5), *Lepr*$^{db/db}$ ($n = 6$) and *Ghrhr*$^{lit/lit}$ ($n = 5$–6) mice. Mean ± s.e.m. One-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$ vs. WT. # $P < 0.05$ vs. *Lepr*$^{db/db}$.

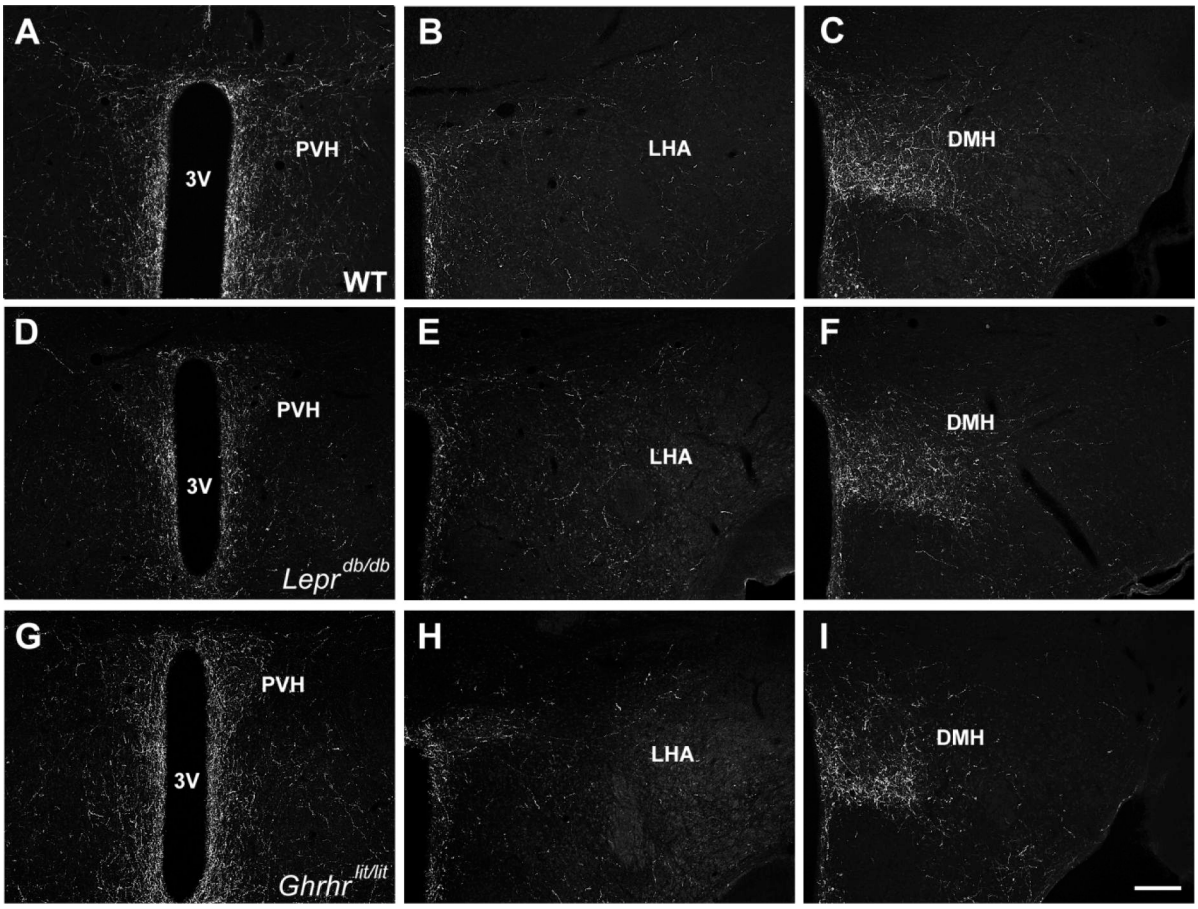

Fig. 2. Projections of POMC neurons in mice deficient of leptin receptor and GH.

A-C. Representative photomicrographs showing POMC innervation in WT mice. **D-F**. Representative photomicrographs showing POMC innervation in *Lepr*$^{db/db}$ mice. **G-I.** Representative photomicrographs showing POMC innervation in *Ghrhr*$^{lit/lit}$ mice. Abbreviations: 3V, third ventricle; DMH, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; PVH, paraventricular nucleus of the hypothalamus. Scale bar = 200 μm.

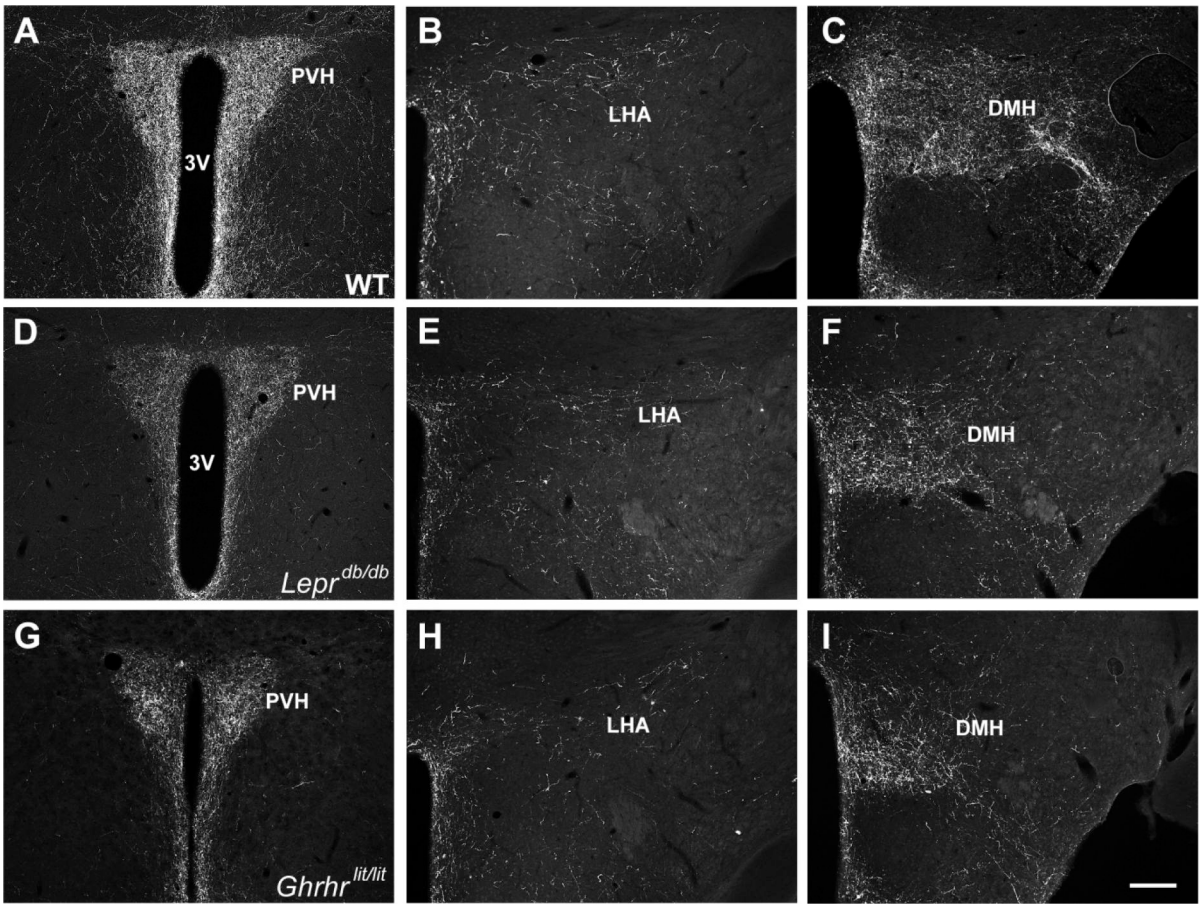

Fig. 3. Projections of AgRP neurons in mice deficient of leptin receptor and GH.

A-C. Representative photomicrographs showing AgRP innervation in WT mice. **D-F**. Representative photomicrographs showing AgRP innervation in *Lepr*$^{db/db}$ mice. **G-I.** Representative photomicrographs showing AgRP innervation in *Ghrhr*$^{lit/lit}$ mice. Abbreviations: 3V, third ventricle; DMH, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; PVH, paraventricular nucleus of the hypothalamus. Scale bar = 200 μm.

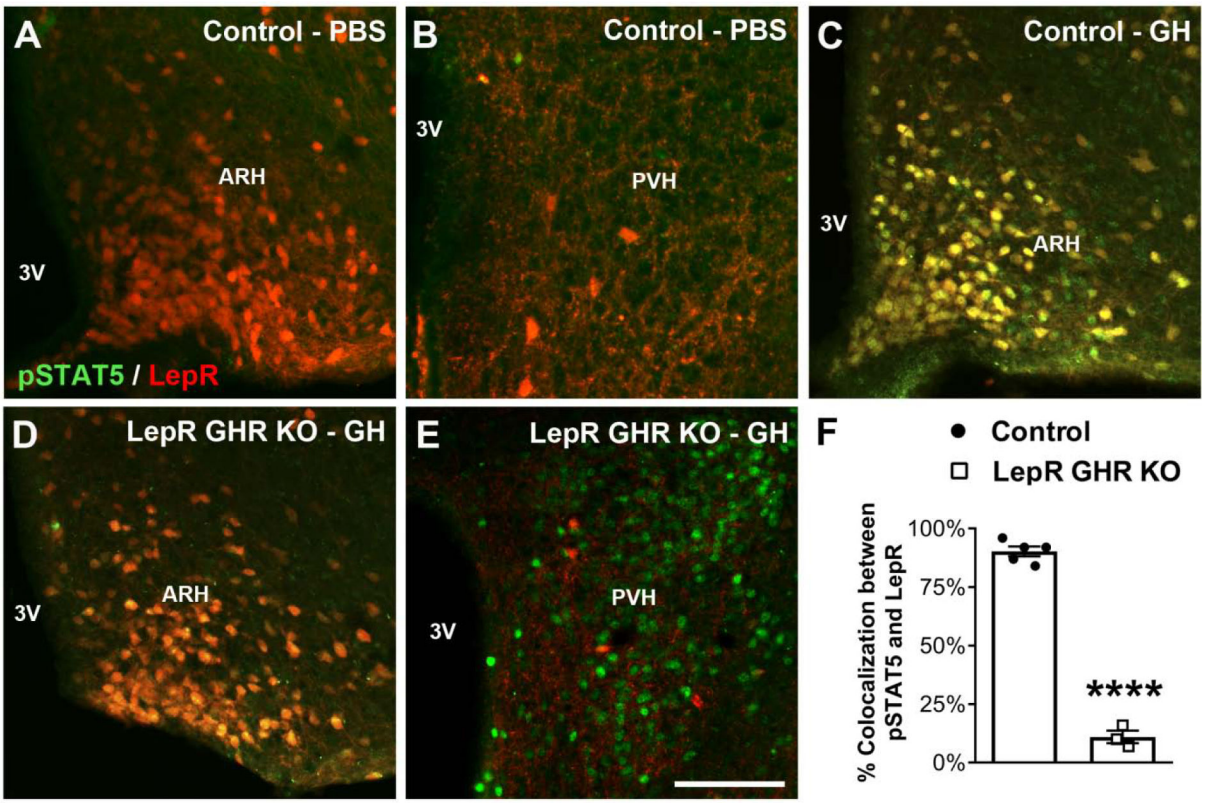

Fig. 4. LepR-expressing neurons in the arcuate nucleus are responsive to GH.

A-E. Epifluorescence photomicrographs showing double-labeling immunofluorescence staining of pSTAT5 (green) and LepR (red tdTomato protein) in the arcuate nucleus (ARH) or paraventricular nucleus of the hypothalamus (PVH) of PBS-injected control mice (A-B), GH-injected control mice (C) and GH-injected LepR GHR KO mice (D-E). Yellow represents double-labeled cells. **F.** Percentage of colocalization between pSTAT5 and LepR in the ARH of GH-injected control mice ($n = 5$) and GH-injected LepR GHR KO mice ($n = 3$). Mean ± s.e.m. Abbreviation: 3V, third ventricle. Scale bar = 100 μm. **** $P < 0.0001$ vs. LepR GHR KO.

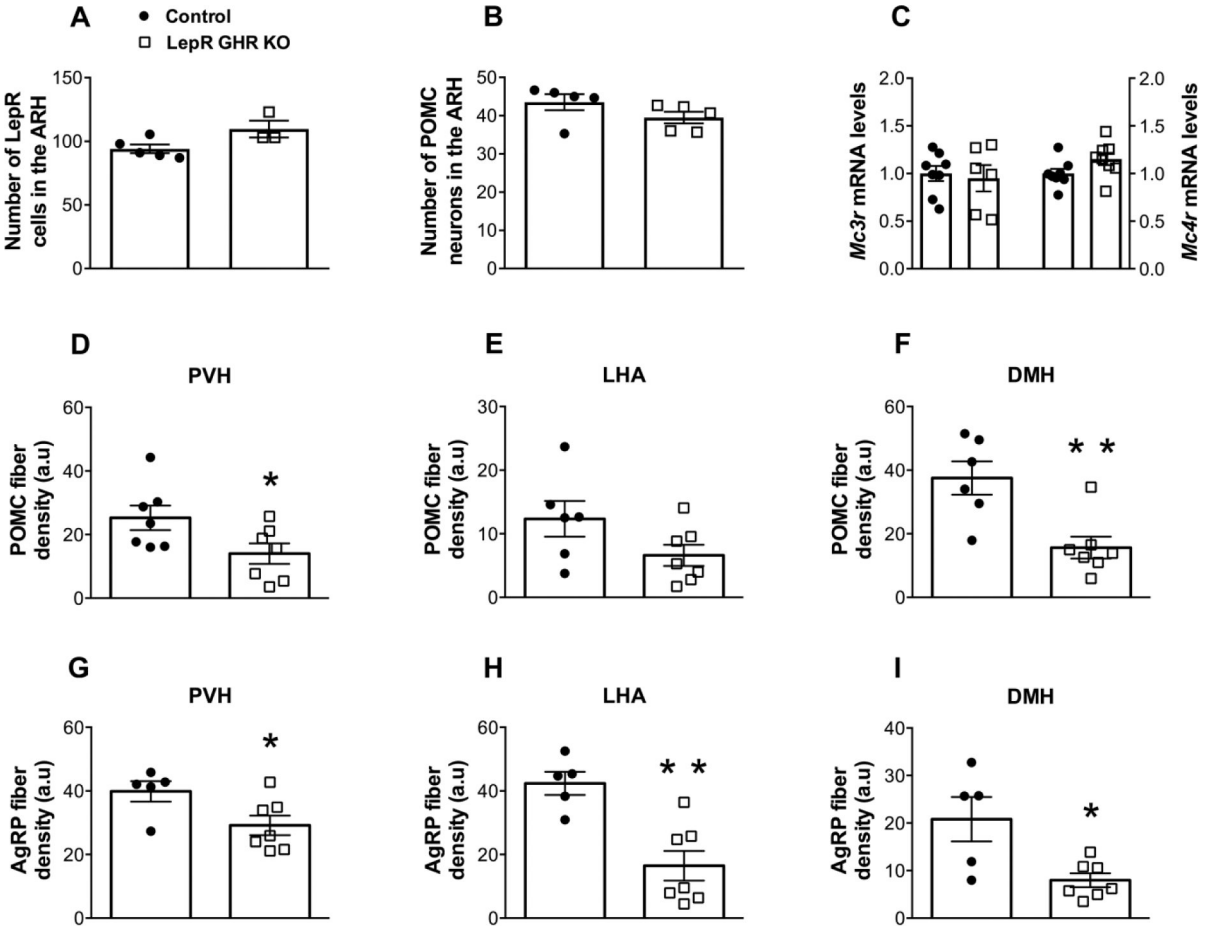

Fig. 5. GHR ablation in LepR-expressing neurons affects the neuronal projections of the arcuate nucleus.

A. Number of LepR-expressing cells in the ARH of control ($n = 5$) and LepR GHR KO ($n = 3$) mice. **B.** Number of POMC neurons in the ARH of control ($n = 5$) and LepR GHR KO ($n = 5$) mice. **C.** Hypothalamic mRNA levels of *Mc3r* and *Mc4r* in control ($n = 8$) and LepR GHR KO ($n = 6–8$) mice. **D-F.** Quantification of POMC fiber density in the PVH (A), LHA (B) and DMH (C) of control ($n = 6–7$) and LepR GHR KO ($n = 7$) mice. **G-I.** Quantification of AgRP fiber density in the PVH (D), LHA (E) and DMH (F) of control ($n = 5$) and LepR GHR KO ($n = 7$) mice. Mean ± s.e.m. Two-tailed Student's t-test. * $P < 0.05$, ** $P < 0.01$ vs. LepR GHR KO.

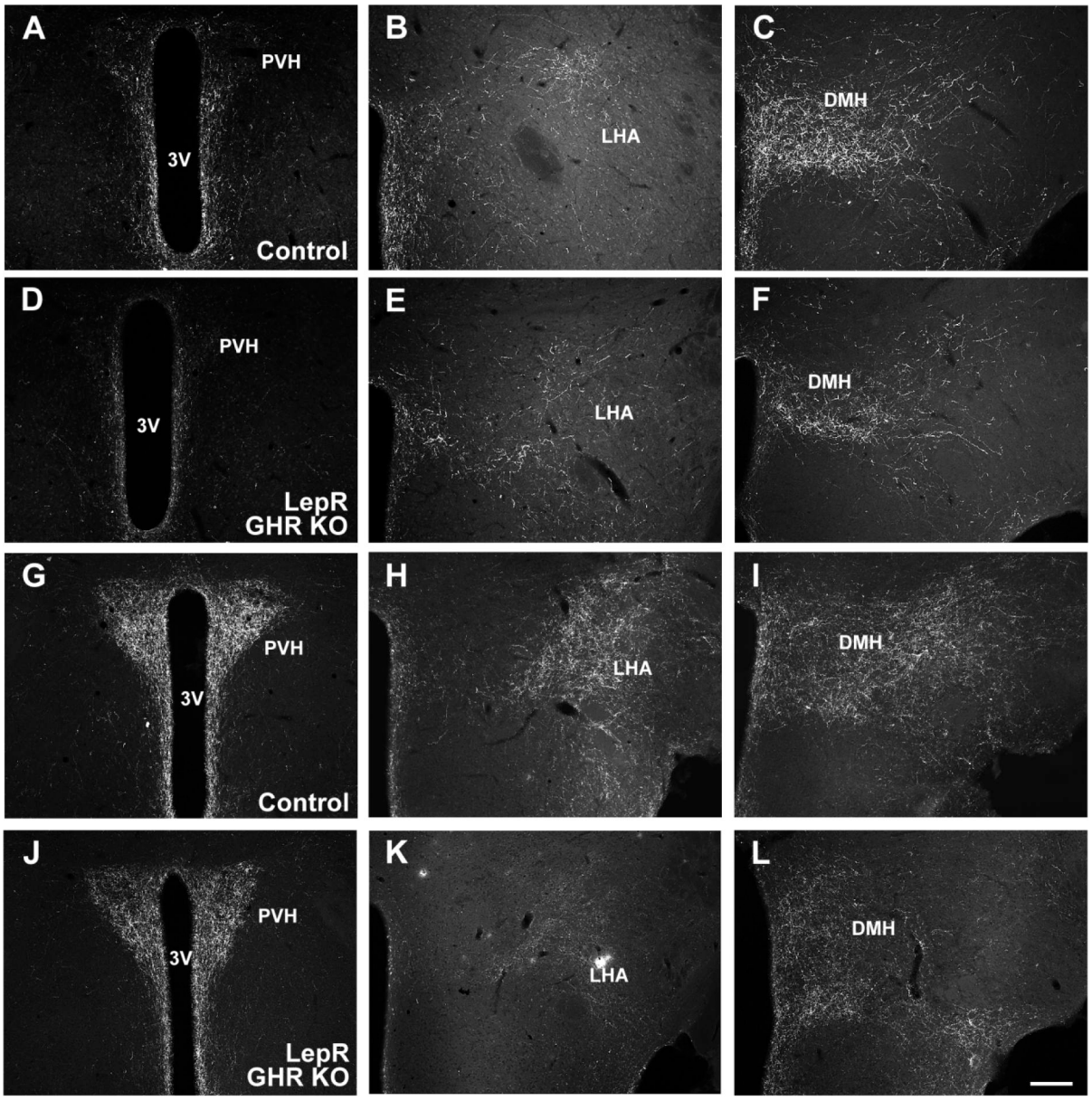

Fig. 6. GHR ablation in LepR-expressing neurons affects the projections of POMC and AgRP neurons.

A-C. Representative photomicrographs showing POMC innervation in control mice. **D-F.** Representative photomicrographs showing POMC innervation in LepR GHR KO mice. **G-I.** Representative photomicrographs showing AgRP innervation in control mice. **J-L.** Representative photomicrographs showing AgRP innervation in LepR GHR KO mice. Abbreviations: 3V, third ventricle; DMH, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; PVH, paraventricular nucleus of the hypothalamus. Scale bar = 200 μm.

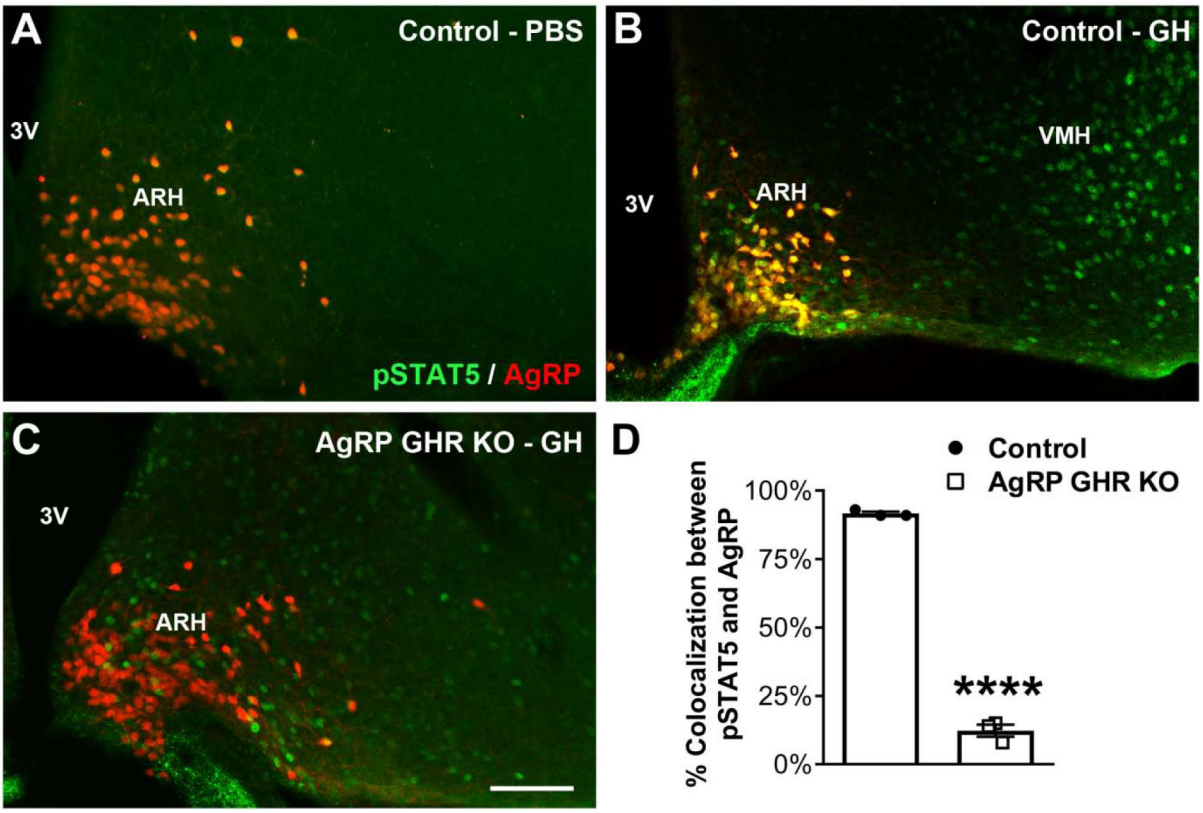

Fig. 7. AgRP-expressing neurons are responsive to GH.

A-C. Epifluorescence photomicrographs showing double-labeling immunofluorescence staining of pSTAT5 (green) and AgRP (red tdTomato protein) in the arcuate nucleus of the hypothalamus (ARH) of PBS-injected control mice (A), GH-injected control mice (B) and GH-injected AgRP GHR KO mice (C). Yellow represents double-labeled cells. **D.** Percentage of colocalization between pSTAT5 and AgRP in the ARH of GH-injected control mice ($n = 3$) and GH-injected AgRP GHR KO mice ($n = 3$). Mean ± s.e.m. Abbreviations: 3V, third ventricle; VMH, ventromedial nucleus of the hypothalamus. Scale bar = 100 μm. **** $P < 0.0001$ vs. AgRP GHR KO.

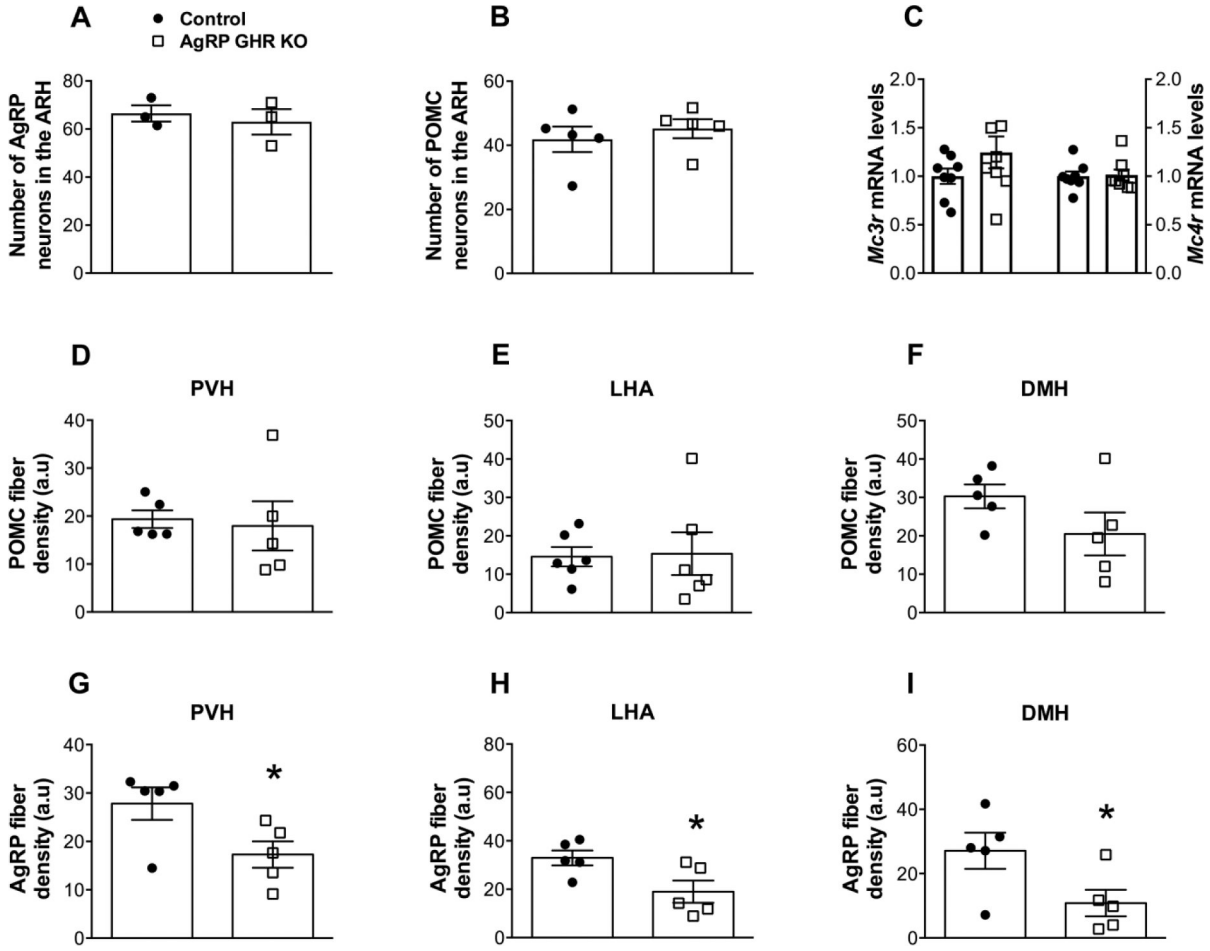

Fig. 8. GHR ablation in AgRP-expressing cells reduces the projections of AgRP neurons without affecting POMC projections.

A. Number of AgRP neurons in the ARH of control ($n = 3$) and AgRP GHR KO ($n = 3$) mice. **B.** Number of POMC neurons in the ARH of control ($n = 5$) and AgRP GHR KO ($n = 5$) mice. **C.** Hypothalamic mRNA levels of *Mc3r* and *Mc4r* in control ($n = 8$) and AgRP GHR KO ($n = 8$) mice. **D-F.** Quantification of POMC fiber density in the PVH (A), LHA (B) and DMH (C) of control ($n = 5–6$) and AgRP GHR KO ($n = 5–6$) mice. **G-I.** Quantification of AgRP fiber density in the PVH (D), LHA (E) and DMH (F) of control ($n = 5$) and AgRP GHR KO ($n = 5$) mice. Mean ± s.e.m. Two-tailed Student's t-test. * $P < 0.05$ vs. AgRP GHR KO.

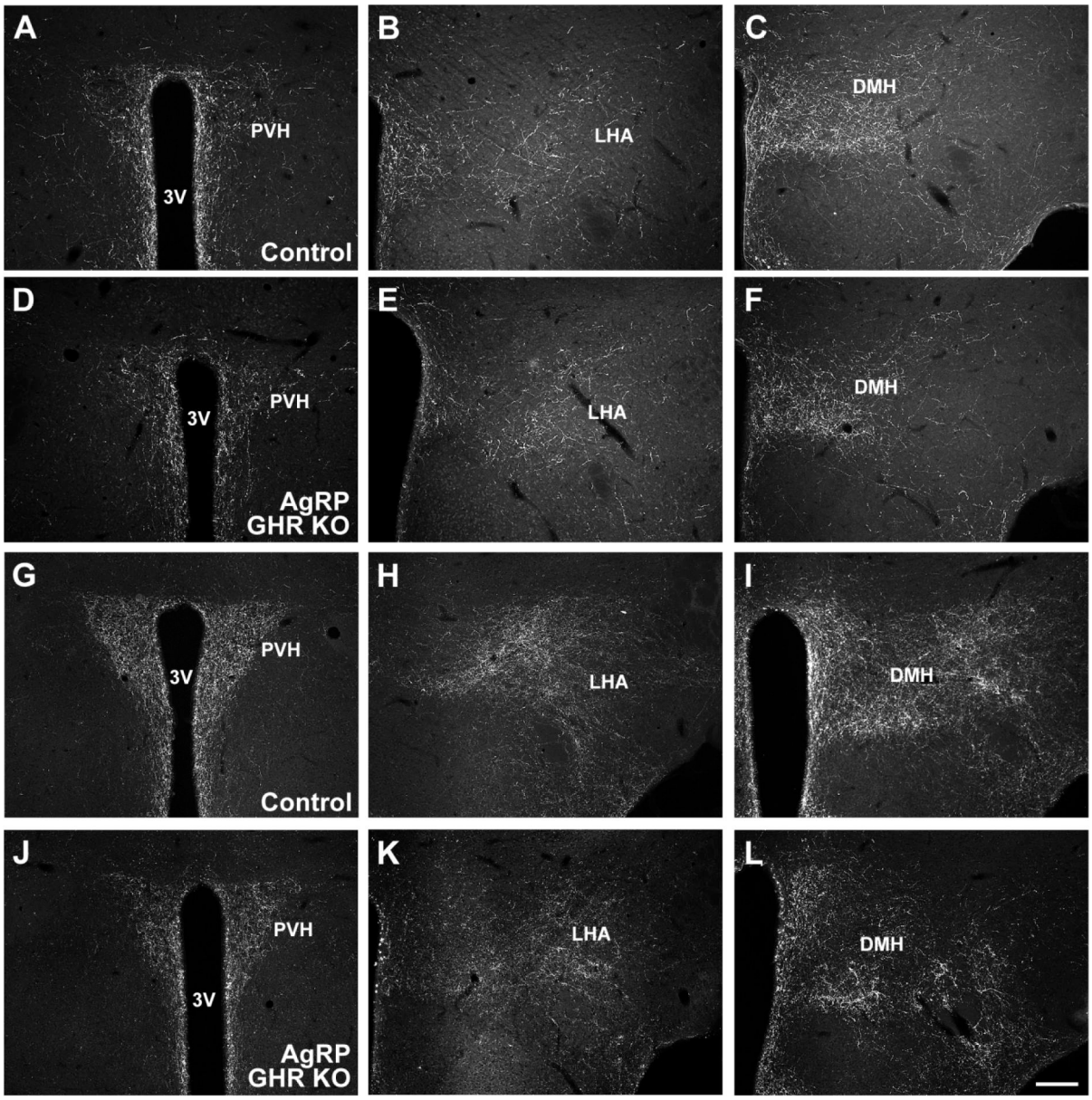

Fig. 9. GHR ablation in AgRP-expressing cells reduces the projections of AgRP neurons.

A-C. Representative photomicrographs showing POMC innervation in control mice. **D-F.** Representative photomicrographs showing POMC innervation in AgRP GHR KO mice. **G-I.** Representative photomicrographs showing AgRP innervation in control mice. **J-L.** Representative photomicrographs showing AgRP innervation in AgRP GHR KO mice. Abbreviations: 3V, third ventricle; DMH, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; PVH, paraventricular nucleus of the hypothalamus. Scale bar = 200 μm.