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Growth hormone receptor contributes to the activation of STAT5 in the hypothalamus of pregnant mice

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Abstract

Growth hormone (GH) receptor (GHR) signaling induces the phosphorylation of the signal transducer and activator of transcription 5 (pSTAT5) in the cells of several tissues including in the hypothalamus. During pregnancy, several STAT5-recruiting hormones (e.g., prolactin, GH and placental lactogens) are highly secreted. However, the precise contribution of GHR signaling to the surge of pSTAT5 immunoreactive neurons that occurs in the hypothalamus of pregnant mice is currently unknown. Thus, the objective of the present study was to determine whether GHR expression in neurons is required for inducing pSTAT5 expression in several hypothalamic nuclei during pregnancy. Initially, we demonstrated that late pregnant C57BL/6 mice (gestational day 14 to 18) exhibited increased pulsatile GH secretion compared to virgin females. Next, we confirmed that neuron-specific GHR ablation robustly reduces hypothalamic *Ghr* mRNA levels and prevents GH-induced pSTAT5 in the arcuate, paraventricular and ventromedial hypothalamic nuclei. Subsequently, the number of pSTAT5 immunoreactive cells was determined in the hypothalamus of late pregnant mice. Although neuron-specific GHR ablation did not affect the number of pSTAT5 immunoreactive cells in the paraventricular nucleus of the hypothalamus, reduced pSTAT5 expression was observed in the arcuate and ventromedial nuclei of pregnant neuron-specific GHR knockouts, compared to control pregnant mice. In summary, a subset of hypothalamic neurons requires GHR signaling to express pSTAT5 during pregnancy. These findings contribute to the understanding of the endocrine factors that affect the activation of transcription factors in the brain during pregnancy.

Graphical Abstract

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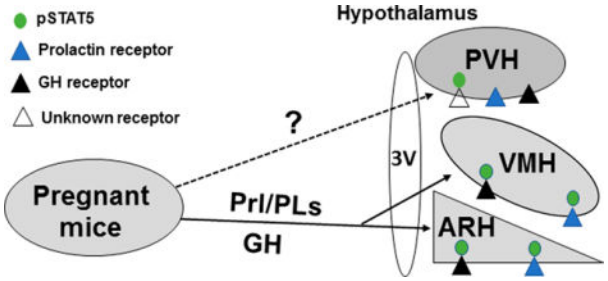
CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Credit authorship statement

Conceptualization: JDJ; Methodology: FW and PDST; Essential reagents: EOL and JJK; Formal Analysis: FW; Data Curation: JDJ; Original Draft Preparation: JDJ; Supervision and Project Administration: JDJ.



Keywords

cytokines; growth hormone; metabolism; neuroendocrinology; pregnancy; transcription factors

1. INTRODUCTION

Pregnancy is a physiological period characterized by critical hormonal alterations. In this regard, the corpus luteum and later the placenta produce high levels of sex steroids. Pituitary hormones or its placental variants also exhibit pronounced increases during pregnancy [1]. These numerous hormones act in practically all tissues leading to the key gestational adaptations. The brain is not an exception since behavioral, neurological and autonomic changes are necessary to maximize the chances of success for fetal development and delivery. Prolactin and placental lactogens are examples of hormones highly secreted during pregnancy [1]. Activation of the prolactin receptor (PrlR) in the hypothalamus triggers gestational metabolic changes and favors maternal behaviors [2–7]. The major intracellular pathway recruited by PrlR signaling involves the signal transducer and activator of transcription 5 (STAT5), which becomes phosphorylated upon PrlR activation and regulates the expression of targeted genes [8, 9]. Systemic prolactin infusion leads to STAT5 phosphorylation (pSTAT5) in several hypothalamic areas, including the paraventricular (PVH), arcuate (ARH) and ventromedial (VMH) nuclei [10–13]. Previous studies have shown that neuronal STAT5 signaling regulates lactation performance, reproductive function and cognitive aspects, like memory formation [9, 14–16]. Furthermore, neuronal STAT5 signaling is likely involved in the long-term metabolic and epigenetic adaptations induced by pregnancy and lactation [17]. Therefore, STAT5-recruiting hormones play a fundamental role in modulating different neuronal processes during pregnancy and lactation.

Growth hormone (GH) secretion also increases during pregnancy. In humans, this increase occurs predominantly through GH placental variants (GH-V), which are encoded by *Gh2* gene, whereas the secretion of GH pituitary variant (GH-N), encoded by *Gh1* gene, is suppressed by negative feedback mechanisms [18–20]. However, rodents do not contain the genes encoding the GH placental variants and the increase in GH secretion during pregnancy relies exclusively on the pituitary gland. Consequently, rodents maintain a pulsatile pattern of GH secretion during gestation [21, 22]. Like the PrlR, activation of GH receptor (GHR) also recruits STAT5 transcription factor as its major intracellular signaling pathway [8]. Furthermore, systemic or intracerebroventricular infusion of GH induces pSTAT5 in practically the same hypothalamic areas that express the PrlR [9, 10, 23, 24].

During pregnancy, there is a natural surge in pSTAT5 immunoreactive cells in several hypothalamic nuclei, including the PVH, ARH and VMH [12, 25, 26]. Importantly, PrlR ablation from forebrain neurons reduces but does not eliminate the number of pSTAT5 immunoreactive cells in the ARH and VMH, whereas pSTAT5 immunoreactive cells in the PVH remain unaltered [25]. Therefore, although PrlR signaling clearly induces the activation of STAT5 pathway in the hypothalamus during pregnancy, other unknown STAT5-recruiting hormone may also recruit these transcription factors in the brain of pregnant animals. The present study was designed to test whether GHR expression in neurons is necessary for inducing pSTAT5 expression in specific hypothalamic nuclei during pregnancy.

2. MATERIALS AND METHODS

2.1. Animals

In the experiment to determine pulsatile GH secretion during pregnancy, adult C57BL/6 female mice were used. To assess whether GHR signaling is necessary to induce pSTAT5 in the brain, nestin-cre mice (The Jackson Laboratory, Bar Harbor, ME; RRID: IMSR_JAX:003771) were bred with mice carrying loxP-flanked *Ghr* alleles. After successive breedings, we were able to produce in the same litters mice homozygous for the loxP-flanked *Ghr* alleles and carrying the nestin-cre transgene (hereafter named as Nestin$^{\Delta GHR}$ mice). Control mice were floxed littermates that did not carry the cre allele. Mice were weaned at 3 weeks of age and their mutations confirmed by polymerase chain reaction (PCR) using the DNA that had been previously extracted from the tail tip (REDExtract-N-Amp™ Tissue PCR Kit, Sigma-Aldrich, St. Louis, MO). Mice had ad libitum access to a regular rodent chow and filtered water and were maintained in standard conditions of light (12-h light/dark cycle). 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 (protocol number: 73/2017).

2.2. Pulsatile GH secretion

Eight-week-old female C57BL/6 mice were handled daily for 30 days to acclimate to the procedure of tail-tip blood sampling and to minimize the stress during the experiment. During the adaptation period, part of the animals were kept in their home cages (virgin group; $n = 4$), whereas another group of female mice were bred with sexually-experienced males (pregnancy group; $n = 3$). Blood collection occurred in late pregnant mice (gestational day 14 to 17) and in the virgin group. Blood collection started at the beginning of the light cycle (approximately 8:00 h) and 36 sequential tail-tip blood samples of 5 μL were collected from each mouse at 10 min intervals [27]. Immediately before the first sample collection, a small portion of the tail tip (1 mm) was cut with a surgical blade to allow the collection of small drops of blood. Mice were allowed to move freely in their home cage with ad libitum access to food and water. For each blood collection, mice were placed inside a cardboard tube and quickly held by the base of the tail. Using a 10 μL pipette, a 5 μL sample of whole blood was collected and transferred to 105 μL of phosphate-buffered saline (PBS) with 0.05% tween-20 (PBS-T). After each blood collection, fingertip pressure was gently applied to the tail tip to stop bleeding. Samples were immediately placed on dry ice and

stored at −80 °C. We used a sensitive sandwich ELISA for GH, according to the instructions previously published [27]. Mean GH levels were calculated by averaging all GH values from each mouse.

2.3. Quantitative real-time PCR

The entire hypothalamus of late pregnant Nestin$^{\Delta GHR}$ (n = 8) and control (n = 7) mice was collected and 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: catcacaccctggtgccta), *Gapdh* (forward: gggtcccagcttaggttcat; reverse: tacggccaaatccgttcaca), *Ghr* (forward: atcaatccaagcctggggac; reverse: acagctgaatagatcctgggg), *Stat5a* (forward: cgctggactccatgcttctc; reverse: gacgtgggctcctcacactga) and *Stat5b* (forward: ggactccgtccttgataccg; reverse: tccatcgtgtcttccagatcg). Data were normalized to the geometric average of *Actb* and *Gapdh*. Relative quantification of mRNA was calculated by $2^{-\Delta\Delta Ct}$.

2.4. Perfusion and tissue processing

The experiment to confirm the absence of GH-induced pSTAT5 in the brain of Nestin$^{\Delta GHR}$ mice was carried out in non-pregnant mice to avoid pregnancy-induced pSTAT5 in the hypothalamus [12, 25, 26]. Thus, non-pregnant Nestin$^{\Delta GHR}$ and control mice (n = 3/group) received an intraperitoneal (i.p.) injection of porcine pituitary GH (20 μg/g of body weight, National Hormone and Pituitary Program) and were perfused 90 min later. To determine the number of pSTAT5 immunoreactive cells in the hypothalamus during pregnancy, control (n = 8) and Nestin$^{\Delta GHR}$ mice (n = 6) were perfused during late gestation (gestational day 14 to 18) without any prior treatment. For the perfusion, mice were deeply anesthetized and perfused transcardially with saline, followed by a 10% buffered formalin solution. Brains were post-fixed for 45 min and cryoprotected overnight at 4 °C in 0.1 M PBS containing 20% sucrose. Brains were cut in 30-μm thick sections using a freezing microtome. Four series of tissues were collected in antifreeze solution and stored at −20°C.

2.5. pSTAT5 staining

Brain slices were rinsed in 0.02 M potassium PBS, pH 7.4 (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 serum for 1 h, followed by incubation in an anti-pSTAT5^{Tyr694} primary antibody (Cell Signaling, Beverly, MA, Cat# 9351; RRID: AB_2315225; 1:1,000). After two days, sections were rinsed in KPBS and incubated for 90 min in AlexaFluor^{488}-conjugated 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).

2.6. Image analysis

A Zeiss Axiocam 512 color camera adapted to an Axioimager A1 microscope (Zeiss, Munich, Germany) was used to obtain the photomicrographs. The ImageJ software (http://rsb.info.nih.gov/ij/) was used to manually count the number of pSTAT5 immunoreactive neurons in two or three rostrocaudal levels of the PVH (Bregma −0.70 and −0.95 mm), ARH (Bregma −1.25 to −1.75 mm) and VMH (Bregma −1.25 to −1.75 mm).

2.7. Statistical analysis

GraphPad Prism software (GraphPad, San Diego, CA) was used for the statistical analyses. Unpaired two-tailed Student's t-test was used in the comparisons between the groups. Data were expressed as mean ± standard error of the mean.

3. RESULTS

3.1. Increased pulsatile GH secretion in late pregnant mice

In accordance with previous studies that have shown augmented GH secretion in pregnant mice [21, 22], we observed increased mean GH levels in late pregnant mice, as compared to virgin group ($t_{(5)} = 3.169$, $P = 0.0249$; Fig. 1). Thus, similarly to other hormones like prolactin and placental lactogens [8, 9] that also induce the activation of the STAT5 signaling pathway, circulating GH levels are also increased in pregnant mice.

3.2. Generation of neuron-specific GHR knockout mice

To investigate the importance of GHR signaling in the brain, we produced a neuron-specific GHR knockout mouse (Nestin$^{\Delta GHR}$ mice). To confirm the efficacy of the targeted deletion, hypothalamic *Ghr* gene expression was determined in late pregnant mice. Compared to control animals, Nestin$^{\Delta GHR}$ mice exhibited a robust reduction in *Ghr* mRNA levels in the hypothalamus ($t_{(13)} = 5.841$, $P < 0.0001$; Fig. 2A). In contrast, no differences in *Stat5a* and *Stat5b* mRNA levels were observed between control and Nestin$^{\Delta GHR}$ mice (Fig. 2A). To determine whether neuron-specific GHR ablation is sufficient to prevent GH-induced activation of the STAT5 signaling pathway in the hypothalamus, non-pregnant control and Nestin$^{\Delta GHR}$ mice received an i.p. GH injection. As expected [23, 24], numerous pSTAT5 immunoreactive cells were observed in the PVH, ARH and VMH of GH-injected control mice (Fig. 2B). On the other hand, virtually no pSTAT5 positive cell was observed in these nuclei of GH-injected Nestin$^{\Delta GHR}$ mice (Fig. 2C).

3.3. Reduced number of pSTAT5 immunoreactive cells in the hypothalamus of pregnant Nestin$^{\Delta GHR}$ mice

Late pregnancy causes the surge of pSTAT5 immunoreactive cells in hypothalamic nuclei that also exhibit GH-induced pSTAT5 [12, 25, 26]. To determine whether GHR signaling is responsible for inducing pSTAT5 in the hypothalamus of pregnant mice, pregnancy-induced pSTAT5 was assessed in the hypothalamus of control and Nestin$^{\Delta GHR}$ mice. As previously shown [12, 25, 26], pregnancy alone is sufficient to induce pSTAT5 in the PVH, ARH and VMH of control mice (Fig. 3). Although the number of pSTAT5 immunoreactive cells in the PVH was not different between control and Nestin$^{\Delta GHR}$ pregnant mice, neuron-specific

GHR ablation reduced the number of pSTAT5 immunoreactive cells in the ARH ($t_{(12)}$ = 2.243, P = 0.0446) and VMH ($t_{(12)}$ = 2.684, P = 0.0199; Fig. 3). Thus, GHR signaling contributes with the activation of the STAT5 intracellular pathway in the hypothalamus of pregnant mice.

4. DISCUSSION

In the present study, we showed evidence that GHR signaling is partially responsible for the expression of pSTAT5 that naturally occurs in the hypothalamus of pregnant mice. Using a mouse model in which the PrlR was deleted from forebrain neurons, Gustafson et al. [25] observed a partial reduction in the number of pSTAT5 immunoreactive cells in several hypothalamic nuclei during pregnancy [25]. Thus, the activation of both PrlR and GHR during pregnancy recruits the STAT5 transcription factor in the brain.

Neuron-specific GHR ablation was achieved by using the nestin-cre mouse model. This strain is known to present neuroendocrine abnormalities like reduced body growth and suppressed pituitary GH secretion [28–30]. These defects are caused by the presence of a GH minigene that was inserted into the transgene construct, whose expression inhibits endogenous GH secretion via negative feedback mechanisms [30]. Importantly, since we induced GHR ablation in neurons, these endocrine dysfunctions were prevented in Nestin$^{\Delta GHR}$ mice. Actually, the absence of GH negative feedback in Nestin$^{\Delta GHR}$ mice leads to GH hypersecretion and consequently increased body growth [27, 31–33]. Therefore, differently than nestin-cre mice that exhibit a GH deficiency phenotype, Nestin$^{\Delta GHR}$ mice are protected from the neuroendocrine abnormalities caused by central transgene expression. We did not determine whether prolactin secretion is normal in Nestin$^{\Delta GHR}$ mice. However, Nestin$^{\Delta GHR}$ mice show normal fertility (data not shown), which is a function regulated by prolactin [34]. Furthermore, Nestin$^{\Delta GHR}$ mice exhibit normal litter size at birth and weaning, unaltered litter growth and intact lactation-induced hyperphagia [31]. Considering that defects in prolactin secretion could lead to infertility, poor maternal behavior and reduced milk production (which affects both litter growth and maternal feeding), it is very unlikely that the lower number of pSTAT5 immunoreactive cells displayed by pregnant Nestin$^{\Delta GHR}$ mice was caused by decreased prolactin secretion. Finally, in late pregnant mice, the activation of PrlR relies on the secretion of placental lactogens and not through pituitary prolactin, whose expression could be affected by neuron-specific transgene expression.

Although GH is not considered a typical gestational hormone, circulating GH levels increase during pregnancy in humans and mice [18–22]. Since the mouse pituitary is responsible to produce GH during pregnancy, GH secretion maintains a pulsatile pattern [21, 22]. In the present study, we confirmed the pulsatile pattern of GH secretion and also the increased mean GH levels during late pregnancy, as compared to virgin mice. Of note, all three pregnant mice evaluated exhibited very distinct patterns of GH secretion during pregnancy. This great variability was not previously reported [21, 22] and its cause is unknown, but it may be related to the range of gestational days used in our experimental sampling. GH possibly plays essential physiological roles during pregnancy, controlling fetus growth, maternal metabolism and protein synthesis [35]. More recently, our group has shown that neuron-specific GHR ablation reduces the pregnancy-induced hyperphagia and body fat

gain, and produces profound improvement in insulin resistance in late pregnant mice [31]. Whether these effects are mediated by STAT5 signaling pathway is currently unknown. Nonetheless, *Stat5a/b* ablation in neurons prevents behavioral, metabolic and epigenetic long-term changes induced by the experience of gestation and lactation [17]. Therefore, the activation of STAT5 signaling pathway in the brain during pregnancy is likely involved in short- and long-term gestational adaptations.

Central ablation of either PrlR or GHR reduces the number of pSTAT5 immunoreactive cells in the ARH and VMH. In the ARH, GHR expression is predominantly observed in neurons that co-express agouti-related peptide (AgRP) and neuropeptide Y [32, 36–38], although other ARH neuronal populations also exhibit a variable degree of responsiveness to GH [27, 38–41]. In contrast, PrlR is mostly expressed in ARH tyrosine hydroxylase- or Rip-expressing neurons, and not in AgRP cells [42, 43]. Thus, central ablation of PrlR or GHR probably prevented STAT5 activation in different ARH neuronal populations during pregnancy. In the VMH, PrlR expression is restricted to its ventrolateral subdivision (VMHvl), whereas GH responsive cells are observed in the entire VMH [23, 44]. During pregnancy, pSTAT5 immunoreactive cells are only observed in the VMHvl, suggesting a prevailing role of PrlR signaling inducing pSTAT5 in this brain region. However, PrlR ablation in forebrain neurons did not completely prevent the pregnancy-induced pSTAT5 expression in the VMH [25], suggesting that other STAT5-recruiting hormones, like GH, are also involved in this activation.

Intriguingly, neither PrlR nor GHR ablation was able to reduce pSTAT5 expression in the PVH [25]. PVH neurons express *Prlr* and *Ghr* mRNA [10, 23]. However, while a systemic GH injection induces a robust pSTAT5 expression in the mouse PVH [45], exogenous prolactin has a limited capacity to induce pSTAT5 in the PVH of virgin animals [10]. However, lactating mice exhibit a prolactin-dependent pSTAT5 expression in the PVH [13]. Furthermore, reproductive experience increases the responsiveness to prolactin in the PVH [46]. In the PVH, prolactin responsive cells are mainly composed of oxytocin neurons [46, 47], whereas just a few PVH oxytocin neurons exhibit GH-induced pSTAT5 [45]. In this regard, GH-induced pSTAT5 in the PVH is majoritarily found in corticotropin-releasing hormone, somatostatin and thyrotropin-releasing hormone neurons [45, 48]. Thus, similarly to the ARH, GH and prolactin act in different neuronal populations of the PVH. Besides prolactin and GH, numerous others cytokines can potentially induce pSTAT5 [8]. Leptin levels increase during pregnancy and leptin can recruit STAT5 signaling pathway in the hypothalamus [49]. However, the mouse PVH exhibits very low leptin receptor expression [50, 51]. Other examples of STAT5-recruiting hormones that increase during pregnancy are erythropoietin [52], thrombopoietin [53], interleukin-4 [54] and interleukin-10 [55]. However, whether PVH neurons express the receptors of these hormones and if they are responsible to induce pregnancy-induced pSTAT5 in this nucleus is currently unknown. In conclusion, a subset of hypothalamic neurons requires GHR signaling to express pSTAT5 during pregnancy. Our findings help to identify the endocrine factors that induce the activation of the STAT5 in the brain during pregnancy, which in turn is likely involved in short- and long-term metabolic gestational adaptations.

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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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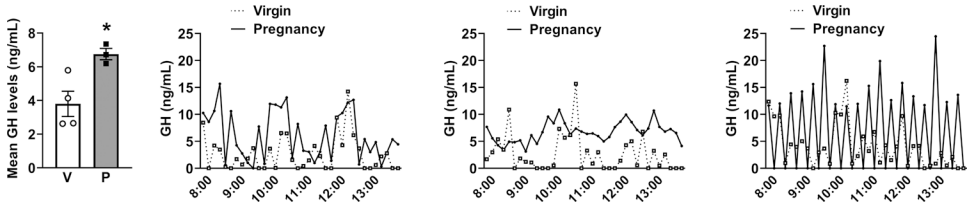

Fig. 1.

Increased pulsatile GH secretion in late pregnant mice. Mean circulating GH levels and three examples of the pulsatile pattern of GH secretion in virgin females (V; $n = 4$) and late pregnant C57BL/6 mice (P; $n = 3$). *, $P = 0.0249$.

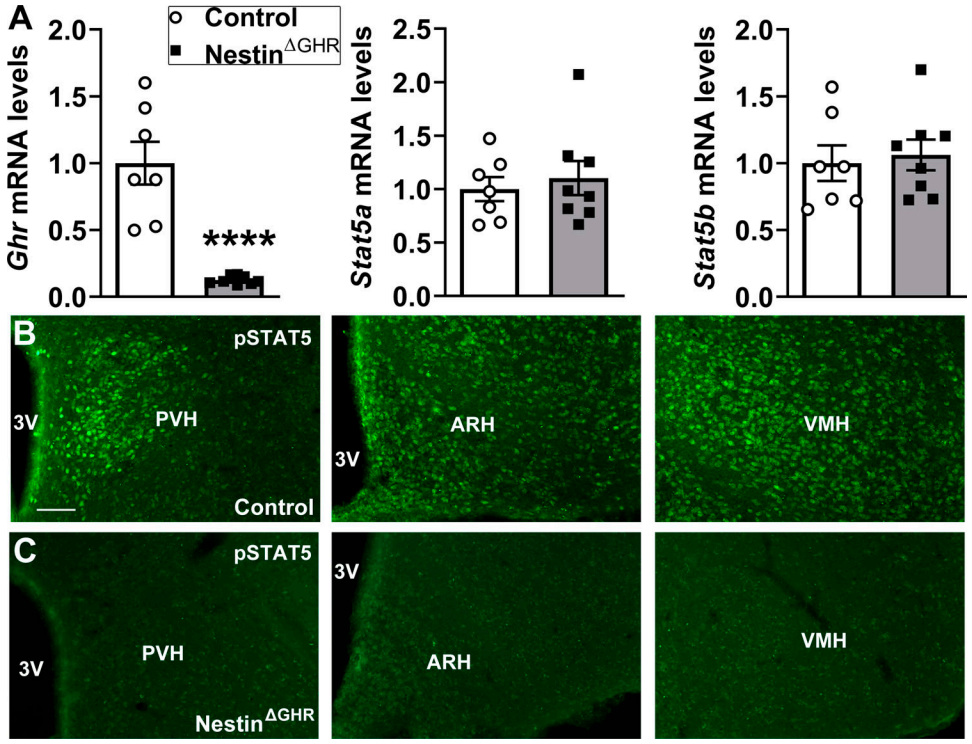

Fig. 2.

Validation of neuron-specific GHR knockout mice. **A**. Hypothalamic gene expression in late pregnant control ($n = 7$) and Nestin$^{\Delta GHR}$ ($n = 8$) mice. ****, $P < 0.0001$. **B**. GH-induced pSTAT5 in hypothalamic nuclei of non-pregnant control mice ($n = 3$). **C**. GH-induced pSTAT5 in hypothalamic nuclei of non-pregnant Nestin$^{\Delta GHR}$ mice ($n = 3$). Abbreviation: 3V, third ventricle. Scale Bar = 100 μm.

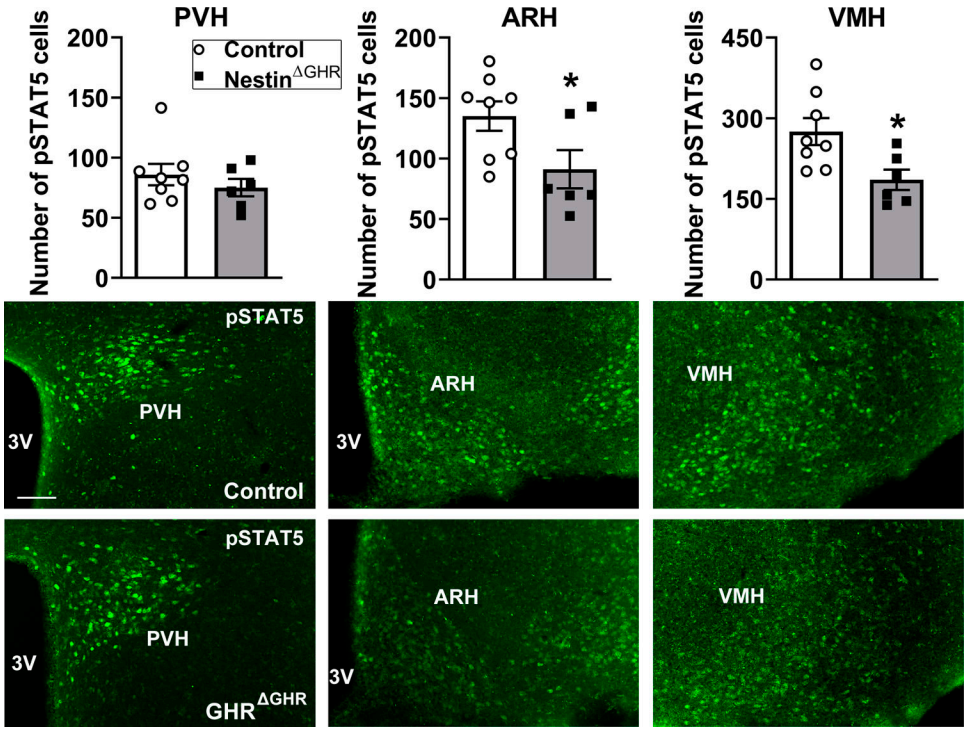

Fig. 3.

Reduced number of pSTAT5 immunoreactive cells in the hypothalamus of pregnant Nestin$^{\Delta GHR}$ mice. Distribution of pSTAT5 immunoreactive cells in hypothalamic nuclei of late pregnant control ($n = 8$) and Nestin$^{\Delta GHR}$ mice ($n = 6$). Abbreviation: 3V, third ventricle. Scale Bar = 100 μm. *, $P < 0.05$.