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Research report

Acute effects of somatomammotropin hormones on neuronal components of the hypothalamic-pituitary-gonadal axis

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HIGHLIGHTS

- GH induces pSTAT5 in a subpopulation of AVPV/PeN kisspeptin neurons.
- GH does not acutely modulate AVPV/PeN kisspeptin neurons RMP.
- ARH kisspeptin neurons are not directly responsive to GH.
- PMv contains neurons responsive to both GH and PRL.
- PRL, but not GH depolarizes the resting membrane potential of PMv neurons.

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ABSTRACT

Growth hormone (GH) and prolactin (PRL) are known as pleiotropic hormones. Accordingly, the distribution of their receptors comprises several organs and tissues, including the central nervous system. The appropriate secretion of both hormones is essential for sexual maturation and maintenance of reproductive functions, while defects in their secretion affect puberty onset and can cause infertility. Conversely, GH therapy at a prepubertal age may accelerate puberty. On the other hand, hyperprolactinemia is a frequent cause of infertility. While the action of PRL in some central components of the Hypothalamic-Pituitary-Gonadal (HPG) axis, such as the kisspeptin neurons, has been well documented, the possible effects of GH in the hypothalamus are still elusive. Thus, the present study was designed to investigate whether somatomammotropin hormones are able to modulate the activity of critical neuronal components of the HPG axis, including kisspeptin neurons and cells of the ventral premammillary nucleus (PMv). Our results revealed that GH effects in kisspeptin neurons of the anteroventral periventricular and rostral periventricular nuclei or in PMv neurons relies predominantly on the recruitment of the signal transducer and activator of transcription 5 (STAT5) rather than through acute changes in resting membrane potential. Importantly, kisspeptin neurons located at the arcuate nucleus were not directly responsive to GH. Additionally, our findings further identified PMv neurons as potential targets of PRL, since PRL induces the phosphorylation of STAT5 and depolarizes PMv neurons. Combined, our data provide evidence that GH and PRL may affect the HPG axis via specific hypothalamic neurons.

Abbreviations: 3v, third ventricle; aCSF, artificial cerebrospinal fluid; APs, action potentials; ARH, arcuate nucleus of the hypothalamus; AVPV, anteroventral periventricular nucleu; *f*APs, frequency of action potentials; GH, growth hormone; GHR, growth hormone receptor; GnRH, gonadotropin-releasing hormone; hGH, human recombinant growth hormone; HPG, Hypothalamic-Pituitary-Gonadal; IGF-1, insulin-like growth factor-1; JAK/STAT, Janus kinase/signal transducers and activators of transcription; KPBS, 0.02 M potassium phosphate buffer saline; LH, luteinizing hormone; oPRL, ovine prolactin; PeN, rostral periventricular nucleus; PBS, phosphate buffer saline; pGH, porcine growth hormone; PMv, ventral premammillary nucleus; PRL, prolactin; PRLR, prolactin receptor; pSTAT5, STAT5 phosphorylation; RMP, resting membrane potential; STAT5, signal transducer and activator of transcription 5

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1. Introduction

The growth hormone (GH) family, which includes the GH itself, prolactin (PRL) and placental lactogens, share residual similarities in their amino acid sequences suggesting that these molecules were developed from a common ancestral peptide (Niall et al., 1971). Despite the classical actions of GH, related to growth and metabolism, and of PRL, stimulating mammary gland development and lactogenesis, these hormones are also known as pleiotropic (Bole-Feysot et al., 1998; Donato and Frazão, 2016; Grattan and Kokay, 2008). Accordingly, the distribution of GH and PRL receptors, both belonging to the family of type I cytokine receptors, are found in several organs and tissues. The activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is considered to be the main signaling mechanism for a wide array of cytokines (Rawlings et al., 2004). Specifically, the transcription factors STAT5a/b are major intracellular proteins recruited by GH and PRL. For this reason, the phosphorylation of STAT5 (pSTAT5) has been used as an important marker to identify GH- or PRL-responsive cells (Brown et al., 2010; Furigo et al., 2017; Silveira et al., 2017), although GH or PRL may also recruit other signaling pathways (Freeman et al., 2000; Mahmoud and Grover, 2006). These alternative signaling pathways may promote, for example, the opening/closure of plasma membrane ion channels and therefore induce short-term effects in the electrical activity of target cells (Buonfiglio et al., 2015; Lyons et al., 2010; Lyons et al., 2012; Mahmoud and Grover, 2006; Silveira et al., 2017).

Interestingly, acute systemic injections of GH or PRL can induce pSTAT5 in several brain nuclei involved in the regulation of the Hypothalamic-Pituitary-Gonadal (HPG) axis, including the anteroventral periventricular and rostral periventricular (AVPV/PeN) nuclei, the arcuate nucleus (ARH) and the ventral premammillary nucleus (PMv) (Brown et al., 2010; Furigo et al., 2017). The AVPV/PeN and ARH contain kisspeptin neurons (Gottsch et al., 2004), which are considered the most important activators of gonadotropin-releasing hormone (GnRH) neurons (d'Anglemont de Tassigny et al., 2008; Han et al., 2005). Accordingly, loss-of-function mutations in the genes encoding kisspeptin or the kisspeptin receptor lead to disruption of puberty and infertility in both humans and animal models (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003). On the other hand, the PMv is a component of the neural circuit that integrates sensory and circulating hormone signals to modulate neuroendocrine reproductive functions (Donato et al., 2010; Leshan and Pfaff, 2014). Consequently, bilateral PMv lesions impair reproductive behaviors, reduce luteinizing hormone (LH) and estradiol levels and decrease the activation of AVPV and GnRH neurons at the time of the preovulatory LH surge (Beltramino and Taleisnik, 1985; Donato et al., 2009, 2013).

Our group and others revealed that kisspeptin neurons express the prolactin receptor (PRLR) and PRL effects in this neuronal population depend predominantly on STAT5, rather than the modulation of ionic membrane channels (Araujo-Lopes et al., 2014; Brown et al., 2014; Kokay et al., 2011; Silveira et al., 2017; Sjoeholm et al., 2011). In addition, systemic or intracerebroventricular infusion of PRL suppresses hypothalamic expression of *Kiss1* mRNA and LH plasma levels, inhibiting the HPG axis (Araujo-Lopes et al., 2014). However, while kisspeptin neurons are known to be important mediators of PRL reproductive effects (Ribeiro et al., 2015; Szawka et al., 2010), the importance of GH signaling on kisspeptin or PMv neurons is unknown.

Considering that in humans and animal models GH deficiency or resistance, as in the Laron Syndrome, correlates with late puberty onset, absent sexual maturation and infertility (Albanese and Stanhope, 1994; De Boer et al., 1997; Laron et al., 1966; Smuel et al., 2015; Zhou et al., 1997), and given the well-known influence of PRL disturbances on the timing of puberty and maintenance of reproductive functions (Glezer and Bronstein, 2015; Newey et al., 2013; Singtripop et al., 1991), the present study was designed to investigate whether somatomammotropin hormones are able to modulate the activity of critical neuronal components of the HPG axis, such as kisspeptin and PMv neurons.

2. Results

2.1. GH induces pSTAT5 in a subpopulation of AVPV/PeN kisspeptin neurons

To determine whether kisspeptin neurons express functional GH receptors (GHR) and further confirm that kisspeptin neurons are responsive to PRL (Silveira et al., 2017), Kiss1-Cre/GFP mice received acute injections of saline, human recombinant GH (hGH), porcine GH (pGH) or ovine PRL (oPRL) and their brain sections were processed to detect pSTAT5 immunoreactivity, as previously described (Brown et al., 2010; Furigo et al., 2017). As a control, saline-treated mice showed no pSTAT5 in AVPV/PeN (Fig. 1). Injection of hGH, which is capable of activating both GHR and PRLR, induced pSTAT5 in an average of 6 cells (range: 5–7 cells per section), representing approximately 19% (14–29%) of AVPV/PeN kisspeptin neurons (Fig. 1B). To determine whether hGH-induced pSTAT5 was caused by the activation of GHR or PRLR, mice received injections of either pGH (selective agonist of GHR) or oPRL (selective agonist of PRLR). Interestingly, both pGH and oPRL were capable of inducing pSTAT5 in an average of 11 cells (9–13 cells per section) or in 22 cells (9–35 cells per section), corresponding to 32% (24–39%) and 42% (32–58%) of AVPV/PeN kisspeptin neurons, respectively (Fig. 1C and D). These results indicate functional expression of GHR and PRLR in AVPV/PeN kisspeptin neurons. Importantly, the

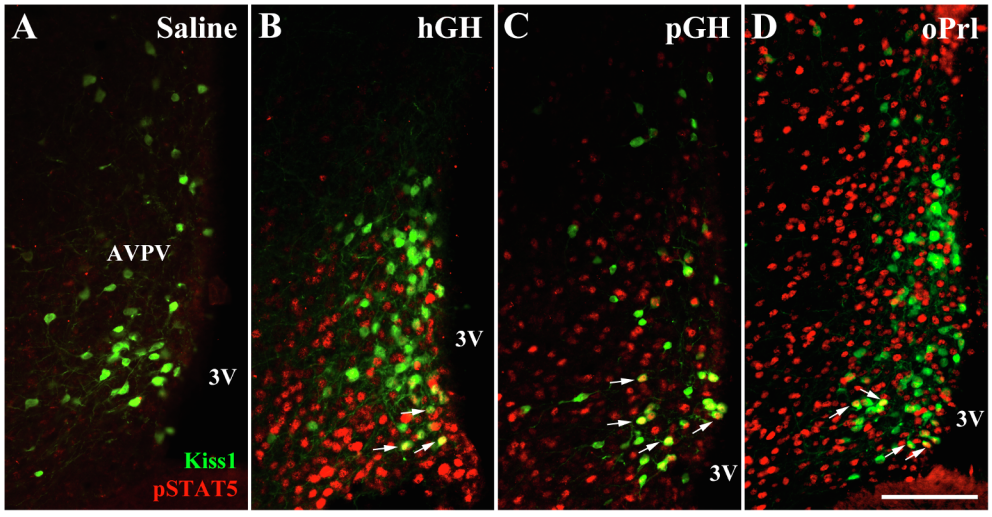

Fig. 1. Responsiveness to GH or PRL in AVPV kisspeptin neurons. A–D. Epifluorescence photomicrographs showing the distribution of pSTAT5-positive (red nuclear staining) and GFP-positive (green cytoplasmic staining) cells in the AVPV of Kiss1-Cre/GFP mice treated with saline (A), human recombinant growth hormone (hGH, B), porcine GH (pGH, C) or ovine prolactin (oPRL, D). Double-labeled cells appear as yellowish/orange (arrows). Abbreviations: 3 V, third ventricle. Scale bar = 100 µm.

Table 1
Absence of growth hormone effects on kisspeptin neurons membrane excitability.

Porcine GH	Baseline	Drug	Washout
AVPV/PeN kisspeptin cells (n = 8)			
RMP (mV)	−59.8 ± 2.8	−59.3 ± 2.8	−60.6 ± 3.2
Input resistance (GΩ)	1.8 ± 0.3	1.7 ± 0.3	1.6 ± 0.3
*f*APS (Hz; n = 5 out of 8)	1.3 ± 0.7	1.2 ± 0.6	1.0 ± 0.5
ARH kisspeptin cells (n = 11)			
RMP (mV)	−51.4 ± 1.5	−51.6 ± 1.4	−50.4 ± 1.5
Input resistance (GΩ)	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.2
*f*APS (Hz; n = 1 out of 11)	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0

Mean ± SEM.

percentage of – AVPV/PeN kisspeptin neurons that were responsive to hGH, pGH or oPRL was not statistically different among the treatments ($P > 0.05$).

Next, we assessed the effects of pGH in the resting membrane potential (RMP) of AVPV/PeN kisspeptin neurons, identified by the GFP expression as previously described (Frazao et al., 2013). In current-clamp mode, neurons were recorded under zero current injection (I = 0) in whole-cell patch-clamp configuration. The average RMP of AVPV/PeN kisspeptin neurons recorded was −59.8 ± 2.8 mV (range: −76.0 to −48 mV) and the average input resistance was 1.8 ± 0.3 GΩ (8 cells from 7 animals). Approximately 60% of the AVPV/PeN recorded neurons showed spontaneous discharge of action potentials (APs) at rest (1.3 ± 0.7 Hz, n = 5 of 8 recorded cells). No significant changes in the RMP, input resistance or frequency of action potentials (*f*APs) were caused by pGH administration to the bath (Table 1). Therefore, despite the fact that pGH induces STAT5 phosphorylation in AVPV/PeN neurons, our results suggest that GH does not acutely modulate membrane ion channels of AVPV/PeN kisspeptin neurons.

2.2. ARH kisspeptin neurons are not directly responsive to GH

In the ARH, while saline injection did not induce the phosphorylation of STAT5 in kisspeptin neurons (Fig. 2A), hGH induced pSTAT5 in an average of 29 cells (20–39 per section), corresponding to approximately 61% (47–72%) of the ARH kisspeptin neurons (Fig. 2B). Interestingly, pGH induced pSTAT5 only in very few ARH kisspeptin neurons (< 1 cell per section in the average, Fig. 2C), whereas oPRL injection induced pSTAT5 in an average of 36 ARH kisspeptin neurons (22–55 cells per section), corresponding to approximately 71% (57–96%) of these cells, suggesting that ARH kisspeptin neurons are responsive to PRL but not to GH ($P = 0.002$).

We also assessed the potential effects of pGH on the electrical activity of ARH kisspeptin neurons. The average RMP of ARH kisspeptin neurons recorded was −51.4 ± 1.5 mV (range: −45 mV to −60 mV) and the average input resistance 1.7 ± 0.1 GΩ (11 cells from 8 animals). As previously reported, most of ARH kisspeptin neurons were quiescent at rest (Frazao et al., 2013), and few ARH kisspeptin neurons showed APs at rest (0.07 ± 0.2 Hz, n = 1 out of 11 recorded cells). Similarly to AVPV/PeN kisspeptin cells, pGH induced no change in the RMP, input resistance or *f*APs of ARH kisspeptin neurons (Table 1), suggesting that ARH kisspeptin neurons are not directly responsive to GH.

2.3. PMv contains neurons responsive to both GH and PRL

To identify neurons responsive to GH or PRL in the PMv, we first determined whether this area express GHR or PRLR mRNA via *in situ* hybridization. We observed that PMv express both GHR and PRLR mRNA (Fig. 3A, B). To further demonstrate the functional expression of GHR or PRLR in the PMv, wild-type mice received acute injections of saline, hGH, pGH or oPRL. No STAT5 phosphorylation was detected in the PMv after saline injection (Fig. 3C). However, hGH, pGH and oPRL induced a robust STAT5 phosphorylation in the PMv (Fig. 3D–F), demonstrating that PMv neurons express functional GH and PRL receptors.

2.4. Prolactin, but not GH depolarizes the resting membrane potential of PMv neurons

Next, to determine whether GH or PRL may induce acute responses in the RMP of PMv neurons, we performed whole-cell patch-clamp recordings in brain slices of wild-type mice. Recorded neurons in the PMv (26 cells from 20 animals) were determined according to anatomical references (Fig. 4A). The recorded neurons were located at ≈390–600 µm laterally from the third ventricle (bregma −2.055 to −2.255). The average RMP of all PMv neurons recorded was −59.6 ± 1.2 mV (range: −47 mV to −74 mV) and average input resistance was 0.9 ± 0.06 GΩ. Spontaneous discharge of APs were observed in 15% of the recorded neurons (0.9 ± 0.3 Hz, n = 4 from 26 recorded cells). Using a selective agonist of GHR, we observed that administration of pGH induced no effect on the average RMP, input resistance or *f*APs of PMv neurons (13 cells out of 9 animals, Table 2; Fig. 4B).

On the other hand, 75% of PMv recorded cells (9 out 12 neurons from 11 mice) were depolarized after application of oPRL to the bath

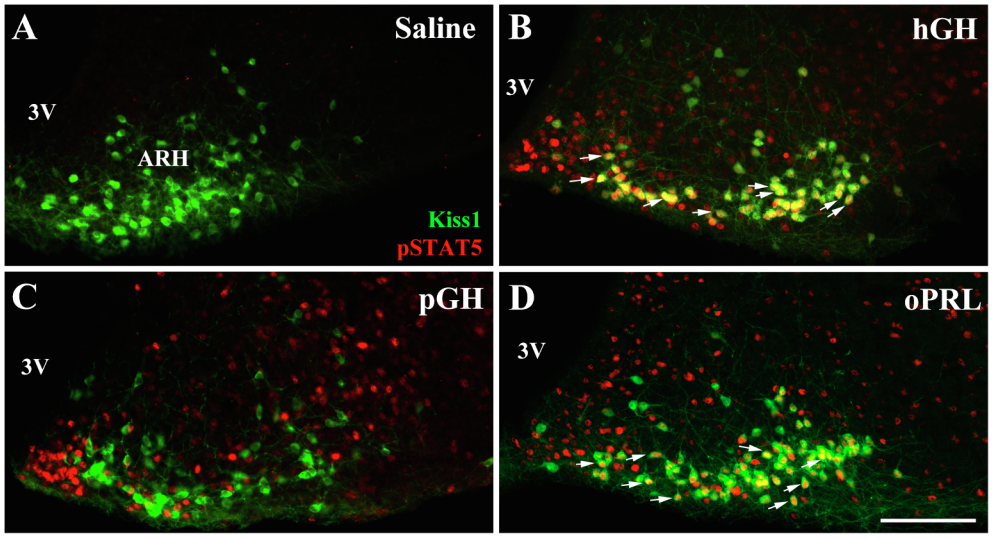

Fig. 2. Responsiveness to GH or PRL in ARH kisspeptin neurons. A–D. Epifluorescence photomicrographs showing the localization of pSTAT5-positive (red nuclear staining) and GFP-positive (green cytoplasmic staining) cells in the caudal ARH of Kiss1-Cre/GFP mice treated with saline (A), human recombinant growth hormone (hGH, B), porcine GH (pGH, C) or ovine prolactin (oPRL, D). Double-labeled cells appear as yellowish/orange (arrows). Abbreviations: 3 V, third ventricle. Scale bar = 100 µm.

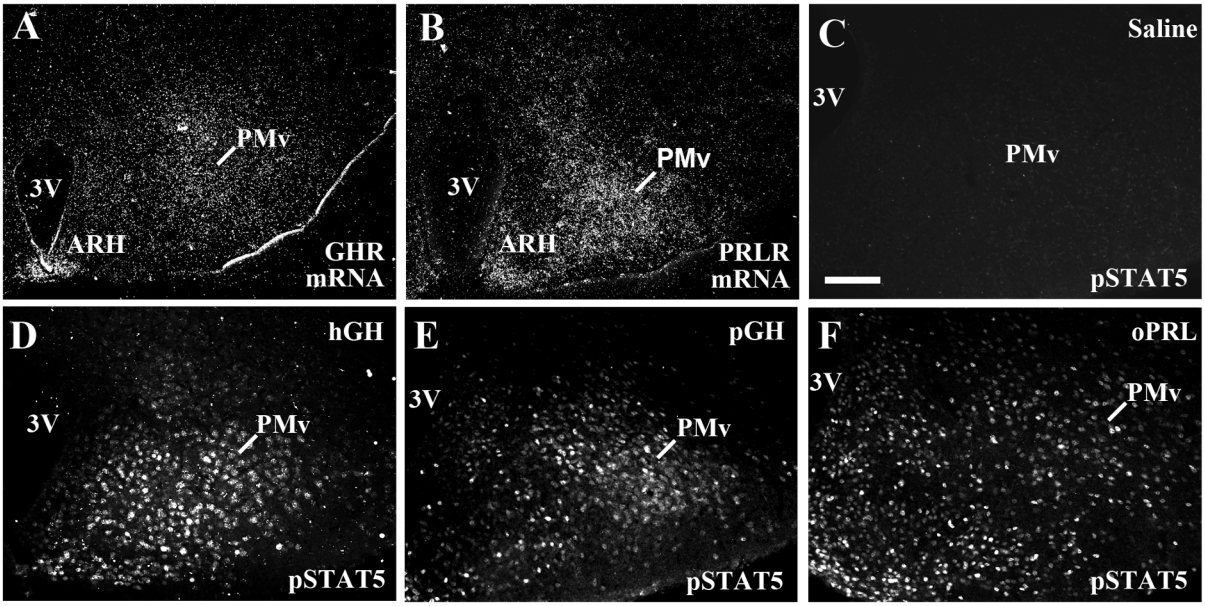

Fig. 3. Distribution of GH- and PRL-responsive neurons in the ventral premammillary nucleus. A and B. Dark-field photomicrographs showing the distribution of growth hormone receptor (GHR) mRNA (A) and prolactin receptor (PRLR) mRNA (B) in the ventral premammillary nucleus (PMv; mRNA is represented by clusters of silver grains in white). C–F. Photomicrographs showing pSTAT5- positive cells (white nuclear staining) in the PMv of mice treated with saline (C), human recombinant growth hormone (hGH, D), porcine GH (pGH, E) or ovine prolactin (oPRL, F). Abbreviations: 3 V, third ventricle; ARH, arcuate nucleus. Scale bar = 100 μm.

(Fig. 4C). PRL-activated cells showed a +6.7 ± 0.9 mV change in the RMP. The depolarization was accompanied by a significantly increase in the input resistance ($P = 0.0012$; Table 2) and *f*APs ($P = 0.008$; Table 2). After washout (up to 20 min), the effects of oPRL on RMP and input resistance were not completely reversed. However, the *f*APs was restored to baseline values (Table 2). The RMP, input resistance and *f*APs of PRL-unresponsive cells (n = 3 out of 12 cells) were not affected by the administration of oPRL to the bath (Table 2).

3. Discussion

In the present study, we confirmed that systemically injected GH or PRL access the central nervous system and that AVPV/PeN kisspeptin and PMv neurons are direct targets of these hormones, while ARH kisspeptin neurons were unresponsive to GH. Interestingly, GH causes no acute electrophysiological responses in AVPV/PeN and PMv neurons, although GH recruits the STAT5 transcription factor in these cells. Conversely, PRL recruits both transcriptional and electrical responses in PMv neurons. Regardless of the mechanism of action, our findings indicate that GH and PRL may affect the HPG axis via hypothalamic neurons.

Our understanding on the effects of GH in the brain is still limited. Substantial information on the importance of GH on brain functions is based on data collected from GH-deficient individuals who exhibit important neurological symptoms. However, GH deficiency causes a complex phenotype, which involves changes in several hormones, including PRL (Zvi, 2011) and insulin-like growth factor-1 (IGF-1). IGF-1 is an important mediator of GH's actions. Accordingly, IGF-1 and GH deficient mice present, in general, a similar phenotype, including growth failure and metabolic disorders (Carroll et al., 1998; Powell-Braxton et al., 1993). Thus, secondary problems caused by GH deficiency could explain possible reproductive deficiencies commonly associated with the lack of adequate GH signaling (Albanese and Stanhope, 1994; Smuel et al., 2015). Therefore, it is important to note that our findings do not rule out a possible action of IGF-1 on kisspeptin or PMv neurons, but clearly demonstrate a direct effect of GH on these cells.

Similar to our electrophysiological data, a previous study demonstrated that GH induces no effect in the RMP or spontaneous postsynaptic current of GnRH neurons (Bhattarai et al., 2010). On the other hand, the activation of GHR can modulate AMPA- and NMDA-receptor-mediated excitatory postsynaptic potentials and cause hyperpolarization in hippocampal cells (Mahmoud and Grover, 2006). The effects of GH on the hippocampus require the activation of JAK2, mitogen-activated protein kinase and phophatidylinositol-3 kinase signaling pathways, as well as the synthesis of new proteins (Mahmoud and Grover, 2006). Therefore, our results suggest that GH preferentially recruits the JAK/STAT signaling pathway in the AVPV/PeN and PMv. However, since we did not study all possible signaling pathways that can be recruited upon GHR activation, we recognize that other mechanisms, that were not explored in the present study, may mediate GH action in these hypothalamic neurons, including ARH kisspeptin cells. Interestingly, in ewes, intracerebroventricular infusion of kisspeptin stimulates GH secretion, an effect observed only in fasted animals (Foradori et al., 2017; Smith et al., 2018). In mice, the AVPV/PeN and ARH nuclei express the ghrelin receptor (Frazao et al., 2014). Considering that fasting stimulates ghrelin secretion and ghrelin is a powerful GH secretagogue (Kojima et al., 1999; Zhao et al., 2010) future studies could determine whether kisspeptin is able to stimulate GH secretion in rodents as well.

In the present study, we confirmed that AVPV/PeN and ARH kisspeptin neurons are PRL-responsive, as previously reported (Araujo-Lopes et al., 2014; Brown et al., 2014; Kokay et al., 2011; Silveira et al., 2017; Sjoeholm et al., 2011). Additionally, we further demonstrated that PRL can affect PMv neuronal activity by at least two distinct mechanisms: the recruitment of the JAK/STAT5 signaling pathway and through changes in the activity of unidentified membrane ion channels, which lead to cell depolarization. The observed PRL effects in input resistance indicate lower membrane permeability, probably via closure of ion channels. Considering the equilibrium potential for the ions in our experimental conditions, it is likely that PRL modulates potassium conductance, rather than chloride conductance, to promote its depolarizing effects. Some potassium channels are known to be rapidly influenced by metabotropic signals, and previous reports have shown that cytokine receptors (e.g., leptin receptor) can activate potassium

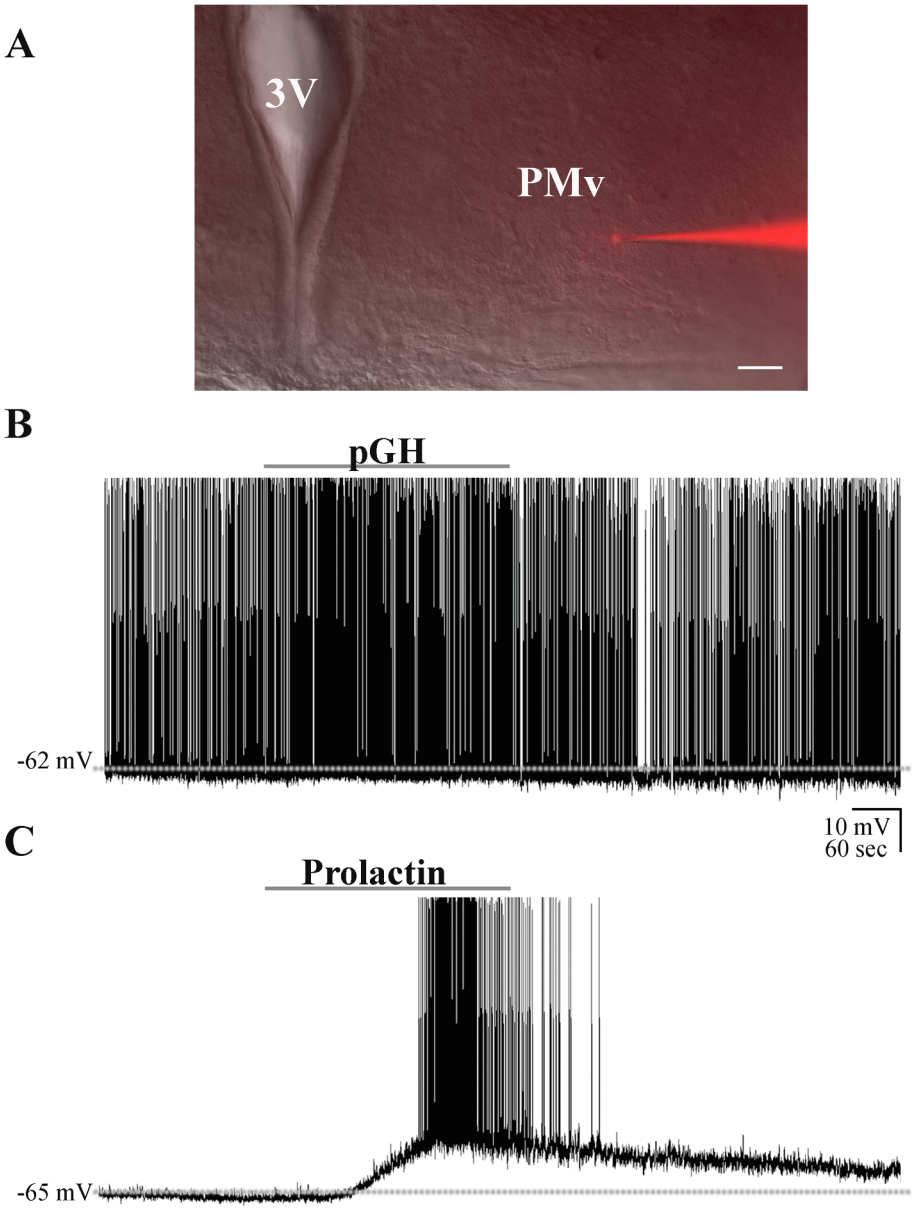

Fig. 4. Acute effects of somatomammotropin hormones on ventral premammillary nucleus resting membrane potential. A. Photomicrograph illustrating a recorded neuron in the ventral premammillary nucleus (PMv). B and C. Representative current-clamp recordings demonstrating that porcine growth hormone (pGH) induced no effect on the resting membrane potential (RMP) of PMv neurons (B), whereas prolactin (PRL) may depolarize PMv neurons (C). The dashed line indicates the RMP. Abbreviation: 3v, third ventricle. Scale bar = 50 μm.

channels and thus modulate the RMP of specific hypothalamic neurons (Spanswick et al., 1997; Williams et al., 2011). While additional studies will be required to identify the ion channels involved in PRL-mediated depolarization of PMv neurons, the observed responses may suggest that PMv neurons are involved in the control of PRL secretion. It is known that PMv cells send projections to AVPV/PeN and ARH nuclei and that PMv neurons can modulate the activity of kisspeptin neurons (Canteras et al., 1992; Ross et al., 2018). Additionally, kisspeptin neurons are known to inhibit PRL secretion (Ribeiro et al., 2015; Szawka et al., 2010), and PMv lesions induce a 2-fold increase in PRL secretion in female rats after a sexual behavior paradigm (Donato et al., 2013). Therefore, PRL signaling in PMv neurons possibly provides a feedback signal to regulate its secretion, a hypothesis that needs to be further explored.

In summary, we showed evidence for a possible action of GH regulating PMv and kisspeptin neurons via STAT5, rather than through acute changes in the RMP. These findings provide further support to the concept of the brain as a potentially important GH-target tissue, and encourage new studies to investigate the physiological relevance of GH signaling in specific hypothalamic populations. Additionally, our findings identified PMv neurons as potential targets of PRL action.

4. Experimental procedure

4.1. Animals

To determine the effects of GH and PRL in PMv neurons we used wild-type C57BL/6 male mice (8–12 week-old). The effect of pGH and oPRL in kisspeptin neurons were investigated using male or female Kiss1-Cre GFP mice (Frazao et al., 2013). Mice were weaned at 3 weeks of age and genotyped via PCR using DNA extracted from the tail tip (REDExtract-N-Amp™ Tissue PCR Kit, Sigma). Mice were housed in the animal care facility of the Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo. Animals were maintained in

Table 2
Effects of somatomammotropin hormones on the membrane excitability of ventral premammillary nucleus (PMv) neurons.

Porcine GH	Baseline	Drug	Washout
PMv cells (n = 13)			
RMP (mV)	−60.1 ± 1.8	−60.6 ± 1.8	−60.4 ± 2.1
Input resistance (GΩ)	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
*f*APS (Hz; n = 4 out of 13)	0.9 ± 0.5	0.6 ± 0.4	0.7 ± 0.5
Ovine Prolactin	Baseline	Drug	Washout
PMv cells (responsive, n = 9)			
RMP (mV)	−60.7 ± 1.1***	−54 ± 1.4	−56.6 ± 1.5
Input resistance (GΩ)	0.7 ± 0.02**	0.8 ± 0.03	0.8 ± 0.03
*f*APS (Hz; n = 8 out of 9)	0.1 ± 0.1	0.7 ± 0.2**	0.3 ± 0.1
PMv cells (n = 3)			
RMP (mV)	−55.3 ± 6.8	−55.7 ± 6.7	−57.0 ± 6.0
Input resistance (GΩ)	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3
*f*APS (Hz; n = 3)	0.7 ± 0.3	1.8 ± 0.1	1.2 ± 0.6

Mean ± SEM, ** $P \leq 0.008$, *** $P < 0.0005$.

microisolator cages (32 cm × 20 cm × 21 cm) in an environment with controlled light (12 h on/12 h off; lights on at 6:00 am) and temperature (21–23 °C). All experiments and procedures were performed in accordance with the guidelines established by the National Institute of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Committee on the Care and Use of Laboratory Animals of the Institute of Biomedical Sciences, University of São Paulo.

4.2. Identification of pSTAT5 positive cells in the mouse brain

Free floating *in situ* hybridization to identify GHR and PRLR mRNA was performed as previously described (Furigo et al., 2017). GHR^{35}S- and PRLR^{35}S-labeled riboprobes were able to detect all isoforms of each receptor. After the reaction, brain sections containing the PMv were mounted onto SuperFrost Plus slides (Fisher Scientific, Fair Lawn, NY), dehydrated in increasing concentrations of ethanol and cleared in xylene for 5 min. Slides were then dipped in NTB2 photographic emulsion (Kodak, Rochester, NY), dried, and stored at 4 °C for approximately 30 days. Slides were developed with D-19 developer (Kodak), dehydrated in increasing concentrations of ethanol, cleared in xylenes and coverslipped with DPX.

To identify pSTAT5 positive cells, we used adult wild-type C57BL/6 mice or Kiss1-Cre/GFP animals. The animals received a single intraperitoneal injection of sterile saline (n = 3 mice), hGH; (20 μg/g; Dong-A Pharmaceutical Co., Dalsung-Kun, Republic of Korea; n = 3 mice), pGH (20 μg/g; from Dr. A.F. Parlow, National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases; n = 3 mice) or oPRL (20 μg/g; Sigma; n = 3 mice). After 90 min, mice were deeply anesthetized and transcardially perfused with saline followed by a 10% buffered formalin solution. Brains were collected and post-fixed in the same fixative for 1 h and cryoprotected overnight at 4 °C in 0.1 M phosphate buffer saline (PBS) containing 20% sucrose, pH 7.4. Brains were cut in 30-μm thick sections using a freezing microtome. Four series of tissue were collected in antifreeze solution and stored at −20 °C.

4.3. Immunohistochemistry

Brain series were rinsed in 0.02 M potassium PBS (KPBS) pH 7.4, followed by pretreatment in an alkaline (pH > 13) 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, sections were blocked in 3% normal donkey serum (Jackson Laboratories, West Grove, PA) for 1 h, followed by incubation in anti-pSTAT5^{Tyr694} primary antibody (#9351; Cell Signaling Technology, Beverly, MA) diluted 1:1000 in KPBS containing 0.25% Triton X-100 for 40 h at 4 °C. Sections were subsequently rinsed in KPBS and incubated for 1 h in biotin-conjugated donkey anti-rabbit secondary antibody (1:1000, Jackson). Sections were rinsed again followed by 1 h incubation in an avidin-biotin complex (1:500, Vector Laboratories, Burlingame, CA,). After thorough rinsing in KPBS, the peroxidase reaction was performed using 0.05% 3,3′-diaminobenzidine, 0.25% nickel sulfate and 0.03% hydrogen peroxide. Sections were rinsed again in KPBS, mounted on gelatin-coated slides, dehydrated through a series of ascending concentrations of ethanol, transferred into xylene, and coverslipped with DPX mounting medium (Sigma, St. Louis, MO).

To determine the co-localization between pSTAT5 and Kiss1 neurons, we performed a double-labeled fluorescent reaction. For this purpose, brain series of Kiss1-Cre/GFP mice were pre-treated as previously described. Next, sections were incubated simultaneously in anti-pSTAT5^{Tyr694} raised in rabbit (1:1000) and in anti-GFP raised in chicken (#GFP-1020; 1:5000; Aves Labs) primary antibodies for 40 h at 4 °C. Then, sections were rinsed in KPBS and incubated for 90 min in AlexaFluor^{488}-conjugated IgG donkey anti-chicken antibody and AlexaFluor^{594}-conjugated IgG donkey anti-rabbit antibody (1:500, Jackson Laboratories). Sections were mounted onto gelatin-coated slides and the slides were coverslipped with Fluoromount G (Electron Microscopic Sciences, Hatfield, PA).

4.4. Image acquisition and analysis

We acquired the photomicrographs with a Zeiss Axiocam HRc camera coupled to a Zeiss Axioimager A1 microscope (Zeiss, Munich, Germany). Images were digitized using Axiovision software (Zeiss). The number of Kiss1-Cre expressing neurons were quantified in two rostrocaudal levels of the AVPV/PeN (relative to bregma: 0.26 and 0.02) and two rostrocaudal levels of the ARH (relative to bregma: −1.94 and −2.30). The quantification was performed in one side of representative rostrocaudal level of each area analyzed. We manually counted the number of single- (GFP) or double-labeled cells (GFP/pSTAT5) in the areas of interest using the ImageJ Cell Counter software (http://rsb.info.nih.gov/ij/).

4.5. Whole-cell recordings

We performed standard whole-cell patch-clamp recordings in hypothalamic neurons using brain slices of male mice (8–12 week-old). Mice were decapitated, their brains were collected and immediately submerged in ice-cold, carbogen-saturated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF, 124 mM NaCl, 2.8 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 5 mM glucose and 2.5 mM $CaCl_2$). Hypothalamic slices were prepared as previously described (Pedroso et al., 2016; Silveira et al., 2017). Once in the recording chamber, slices were allowed to equilibrate for 10–20 min before recording. The slices were bathed in oxygenated ACSF (30 °C) at a flow rate of 2 mL/min. The pipette solution for whole-cell recording contained: 120 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 5 mM EGTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 2 mM (Mg)-ATP, pH 7.3. Infrared differential interference contrast was used to target and obtain neuronal whole-cell recording (Leica DM6000 FS equipped with a fixed stage and a Leica DFC360 FX high-speed monochrome fluorescence digital camera). The electrophysiological signals were recorded using an Axopatch 700B amplifier (Molecular Devices), low-pass-filtered at 2–4 kHz, and analyzed offline on a PC using pCLAMP software (Molecular Devices). The recording electrodes had resistances of 5–7 MΩ when filled with the K-gluconate internal solution. In current-clamp mode, neurons were recorded under zero current injection (I = 0). The input resistance was assessed by measuring the voltage deflection at the end of the response to a hyperpolarizing rectangular current pulse (500 ms of −10 to −50 pA). The RMP was monitored for at least 10 min (basal), followed by the addition of drugs to the bath. Solutions

containing pGH (5 μg/mL) or oPRL (250 nM) were typically perfused for 5 min. The RMP values were compensated to account for the junction potential (−8 mV). Alexa Fluor 488 hydrazide dye was added to the pipette solution to determine the position of the PMv recorded cells relative to the third ventricle and other anatomical references. The *fAPs* was determined offline by analyzing 2 min before, during and after the administration of the drug to the bath.

4.6. Statistical analysis

We performed the statistical analyses using the GraphPad Prism software. We expressed the results as the mean ± SEM. We used the one-way ANOVA and Newman-Keuls Multiple Comparison Test to compare the number of kisspeptin cells and the percentage of kisspeptin/pSTAT5 double-labeled cells. Repeated Measures ANOVA and Newman-Keuls Multiple Comparison Test were used to compare the data before, during and after drug administration to the bath. We considered P values < 0.05 to be statistically significant.

Disclosure summary

The authors have nothing to disclose.

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