Brain Struct Funct (2017) 222:341–363
DOI 10.1007/s00429-016-1221-1

ORIGINAL ARTICLE

Distribution of growth hormone-responsive cells in the mouse brain

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Received: 2 September 2015 / Accepted: 30 March 2016 / Published online: 12 April 2016

Abstract Growth hormone (GH) exerts important biological effects primarily related to growth and metabolism. However, the role of GH signaling in the brain is still elusive. To better understand GH functions in the brain, we mapped the distribution of GH-responsive cells and identified the receptors involved in GH central effects. For this purpose, mice received an acute intraperitoneal challenge with specific ligands of the GH receptor (mouse GH), prolactin receptor (prolactin) or both receptors (human GH), and their brains were subsequently processed immunohistochemically to detect the phosphorylated form of STAT5 (pSTAT5). GH induced pSTAT5 immunoreactivity in neurons, but not in astroglial cells of numerous brain regions, including the cerebral cortex, nucleus accumbens, hippocampus, septum and amygdala. The most prominent populations of GH-responsive neurons were located in hypothalamic areas, including several preoptic divisions, and the supraoptic, paraventricular, suprachiasmatic, periventricular, arcuate, ventromedial, dorsomedial, tuberal, posterior and ventral premammillary nuclei. Interestingly, many brainstem structures also exhibited GH-responsive cells. Experiments combining immunohistochemistry for pSTAT5 and in situ hybridization for GH and prolactin receptors revealed that human GH induced pSTAT5 in most, but not all, brain regions through both prolactin and GH receptors. Additionally, males and females exhibited a similar number of GH-responsive cells in forebrain structures known to be sexually dimorphic. In summary, we found GH-responsive cells primarily distributed in brain regions implicated in neurovegetative, emotional/motivational and cognitive functions. Our findings deepen the understanding of GH signaling in the brain and suggest that central GH signaling is likely more ample and complex than formerly recognized.

Keywords GH · STAT5 · Hypothalamus · Cytokine · Hormone receptors · Prolactin

Introduction

Growth hormone (GH) is an important growth factor involved in the regulation of multiple biological functions related to somatic growth, metabolism and cellular processes such as cell division and regeneration (Lichanska and Waters 2008; Moller and Jorgensen 2009). The GH receptor (GHR) is a member of the type I cytokine receptor family and therefore activates multiple signaling pathways (Moutoussamy et al. 1998; Herrington and Carter-Su 2001; Kelly et al. 1991). The signal transducer and activator of transcription 5 (STAT5) is considered the most relevant intracellular signaling pathway recruited by GHR (Teglund et al. 1998). STAT5 is a transcription factor and becomes phosphorylated after GHR activation, followed by its translocation to the nucleus to control the transcription of genes directly regulated by GH (Moutoussamy et al. 1998; Herrington and Carter-Su 2001).

Pioneer studies have indicated that GHR is expressed in the central nervous system (Burton et al. 1992; Kastrup et al. 2005; Walsh et al. 1990; Nyberg 2000). Using in vitro autoradiographic analysis, Walsh et al. (1990) showed GH

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binding sites in several hypothalamic nuclei of rabbits. In another study, GHR-positive cells were identified by the in situ hybridization in the rat brain, indicating the presence of GHR mRNA in the thalamus, septal region, hippocampus, dentate gyrus, amygdala and hypothalamus (Burton et al. 1992). Among the known effects of GH in the brain, hypothalamic neurons responsible for regulating the pituitary secretion of GH are directly affected by GH signaling (Burton et al. 1992). This negative feedback mechanism is important to control GH secretion (Nass et al. 2000). However, there is increasing evidence that the effects of GH on brain functions might be much more ample (Nyberg 2000). For example, GH deficiency produces several neurological symptoms such as depression, social withdrawal, poor memory and impaired cognition (Waters and Blackmore 2011; Nyberg and Hallberg 2013). Moreover, decreased GH secretion during aging has been suggested as a factor that contributes to age-related decline in cognitive functions (Ashpole et al. 2015; Molina et al. 2013; Aberg et al. 2006).

In primates, GH can activate both the GHR and the prolactin receptor (PrlR) with high affinity (Bartke and Kopchick 2015; Forsyth et al. 1965; Brem et al. 1989; Cunningham et al. 1990). Consequently, primate growth hormones, including human GH (hGH), has prolactin-like effects such as stimulation of mammary gland development, lactation, maternal behavior, mammary tumorigenesis and reproductive disorders (Bartke and Kopchick 2015; Milton et al. 1992). Such ability to activate PrlR and cause lactogenic effects is not observed for growth hormones from non-primate species, including mouse GH (mGH) which activates only the GHR.

In the present study, we sought to map in detail the distribution of GH-responsive cells in the mouse brain. For this purpose, mice received an acute intraperitoneal challenge with specific ligands of GHR (mGH), PrlR (prolactin) or both receptors (hGH), and their brains were subsequently processed immunohistochemically to detect the phosphorylated form of STAT5 (pSTAT5). This procedure yielded in a high-resolution staining, superior to that obtained in the aforementioned classical autoradiographic and in situ hybridization studies, allowing us to give a detailed account of the distribution of GH-responsive cells. In addition, we investigated by double immunofluorescence methods whether hGH-induced pSTAT5 immunoreactivity (pSTAT5-ir) was primarily present in neurons or glial cells. We also combined in situ hybridization for GHR and PrlR mRNA with immunohistochemical detection of pSTAT5 to identify the specific receptors activated by hGH in its target brain structures. Finally, we compared the number of mGH- and hGH-responsive cells between males and females in several forebrain structures known to show some kind of sexual dimorphism. Our findings might contribute to a better understanding of the central effects of GH signaling.

Materials and methods

Animals

Adult male and female C57BL/6 mice obtained from a local breeding facility at the Institute of Biomedical Sciences, University of São Paulo, were used in all experiments. The animals were kept in group cages under controlled temperature (23 °C) and illumination (12 h cycle), with water and food ad libitum. All experiments were carried out in compliance with NIH guidelines for the care and use of laboratory animals (NIH publications No. 80-23, revised 1996) and were approved by the Institutional Animal Ethics Committees of the Institute for Biomedical Sciences at the University of São Paulo (Protocols 12/2013). All efforts were made to minimize the number of animals used and their suffering.

Experimental design

To determine the distribution of prolactin-, mGH- and hGH-responsive cells in the mouse brain, adult mice received an acute intraperitoneal injection of ovine prolactin (2 males and 2 females; 20 µg/g b.w.; Sigma), mouse recombinant GH (3 males and 2 females; 20 µg/g b.w.; from Dr. A.F. Parlow, National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases) or human recombinant GH (12 males and 8 females; 20 µg/g b.w.; Dong-A Pharmaceutical Co., Dalsung-Kun, Republic of Korea), respectively. The mice were perfused, as described later, 90 min after the injection. We considered prolactin-, mGH- and hGH-responsive cells to be those that expressed nuclear pSTAT5-ir. Mice that were injected with vehicle solution (sterile PBS; 2 males and 2 females) served as a control to evaluate the basal expression of pSTAT5. Basal pSTAT5-ir indicates cells that do not require any hormonal (exogenous) stimulus to have this signaling pathway activated.

Perfusion and tissue processing

Mice were deeply anesthetized and perfused transcardially with saline followed by a 10 % buffered formalin solution (150–200 mL per mouse). Brains were collected and post-fixed in the same fixative for 1 h and then cryoprotected overnight at 4 °C in 0.1 M PBS containing 20 % sucrose, pH 7.4. Brains were cut (30-µm-thick sections) in the frontal plane using a freezing microtome. Four series of

tissue were collected in antifreeze solution and stored at −20 °C.

pSTAT5 immunohistochemistry

Immunoperoxidase staining was carried out on free-floating sections at room temperature unless otherwise stated. Brain sections of vehicle-, prolactin-, mGH- and hGH-treated mice were rinsed in 0.02 M potassium PBS, pH 7.4 (KPBS), 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 (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 antibodies (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. Finally, 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).

Double immunofluorescence

Free-floating brain sections of hGH-treated mice were subjected to a double immunofluorescence staining protocol to check a possible co-localization of pSTAT5 and markers for neuronal nuclei (NeuN) or astrocytes (glial fibrillary acidic protein; GFAP). Brain sections were rinsed in KPBS, followed by pretreatment in water solution containing 1 % hydrogen peroxide and 1 % sodium hydroxide for 20 min. 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 for 40 h at 4 °C in cocktails of either rabbit anti-pSTAT5^{Tyr694} (Cell Signaling Technology, Beverly, MA; 1:1000) and mouse anti-NeuN (Millipore, Temecula, CA; 1:5000) or mouse anti-GFAP (Millipore; 1:1000) primary antibodies. Subsequently, sections were rinsed in KPBS and incubated for 2 h in AlexaFluor^{594}-conjugated anti-rabbit IgG and AlexaFluor^{488}-conjugated anti-mouse IgG antibodies (1:1500, Jackson Laboratories). The slides were coverslipped with Fluoromount G containing DAPI (Electron Microscopic Sciences, Hatfield, PA).

In situ hybridization

To determine whether hGH-induced pSTAT5-ir co-localizes with GHR or PrlR mRNA, free-floating brain sections of hGH-treated mice were rinsed with DEPC-treated PBS, pH 7.0, for 1 h, followed by incubation in 0.1 % sodium borohydride (Sigma) in DEPC-PBS for 15 min. Sections were then incubated for 10 min in 0.25 % acetic anhydride (Merck) in 0.1 M triethanolamine. GHR^{35}S- and PrlR^{35}S-labeled riboprobes aiming to detect all isoforms of each receptor were generated from cDNA templates as described previously (Donato et al. 2010). The GHR riboprobe corresponds to positions 1217–1565 of GenBank accession number NM_010284.3, comprising exons 9–11 of the mouse *Ghr* gene. The PrlR riboprobe corresponds to positions 2135–2541 of GenBank accession number NM_011169.5. cRNA probes were diluted in hybridization buffer which consisted of 50 % formamide, 10 mM Tris–HCl (Fisher Scientific), 0.01 % sheared salmon sperm DNA (Sigma), 0.01 % yeast tRNA (Sigma), 0.05 % total yeast RNA (Sigma), 10 mM dithiothreitol (Amresco), 10 % dextran sulfate (Amresco), 0.3 M NaCl, 1 mM EDTA (pH 8.0) and 1× Denhardt's solution (Amresco). Sections were hybridized overnight at 56 °C. The following day, tissue was rinsed four times in 4× sodium chloride/sodium citrate (SSC) and incubated in 0.002 % RNase A (Roche Diagnostics GmbH, Mannheim, Germany) diluted in 0.5 M NaCl, 10 mM Tris–HCl, pH 8.0 and 1 mM EDTA for 30 min at 37 °C. Sections were submitted to stringent washes in 2× SSC and 0.2× SSC at 60 °C for 30 min per incubation. Subsequently, sections were processed for immunoperoxidase staining as described above, except that nickel sulfate was omitted from the peroxidase reaction. Tissue was 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 in desiccant-containing, foil-wrapped slide boxes 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.

Data analysis

Photomicrographs were acquired with a Zeiss Axiocam HRc camera coupled to a Zeiss Axioimager A1 microscope (Zeiss, Munich, Germany). Images were digitized using

Axiovision software (Zeiss). Photoshop image-editing software was used to combine photomicrographs into plates. Only sharpness, contrast and brightness were adjusted. The Allen Mouse Brain Reference Atlas (http://mouse.brain-map.org/) was used for anatomical reference and to obtain the approximate bregma coordinates of the sections illustrated in our figures. To determine the relative expression level of prolactin-, mGH- and hGH-responsive cells in the mouse brain, each brain area was classified based on the relative density of responsive cells and the extent of the structure area containing pSTAT5-immunoreactive cells, GHR mRNA or PrlR mRNA. Thus, brain areas were classified as exhibiting very high expression (++++, high density of cells covering the whole extension of the nucleus), high expression (+++, high density of cells covering the most of the nucleus), moderate expression (++, moderate density of cells covering only parts of the nucleus), low expression (+, low density of cells covering only parts of the nucleus) and very low or virtually absent (−). Additionally, we performed a semi-quantification of mGH- or hGH-responsive cells in brain regions known to show some kind of sexual dimorphism. Identification of pSTAT5-positive cells was performed on images captured in a 1388 × 1040 pixel format with a 10× objective. Images were centered on the medial preoptic area, ventromedial nucleus of the hypothalamus, arcuate nucleus, posterodorsal part of the medial amygdalar nucleus and ventral premammillary nucleus, which, if necessary, were circumscribed on the images. All pSTAT5-positive cells in sharp focus were considered for analysis and plotted electronically with the ImageJ Cell Counter software (http://rsb.info.nih.gov/ij/). One representative level of each area of interest was analyzed by a researcher unaware of the experimental groups. Quantification of pSTAT5-labeled cells containing GHR mRNA or PrlR mRNA was performed in 3 or 4 mice, respectively, in selected forebrain areas that showed a high or very high expression of these receptors. Photomicrographs of these forebrain structures were captured under brightfield illumination with a 20× objective with the focal plane on the tissue surface. If necessary, the structures of interest were circumscribed on the images, and pSTAT5 only and pSTAT5/GHR mRNA or pSTAT5/PrlR mRNA cells were plotted electronically with the ImageJ Cell Counter software. This analysis was performed on one representative level of each area of interest. hGH-responsive cells were defined as double-labeled if the density of silver grains overlying or immediately surrounding their pSTAT5-positive nucleus was three times greater than background levels. Results were expressed as mean ± SEM, and the unpaired two-tailed Student's *t* test was used to evaluate possible differences between males and females.

Results

An acute hGH stimulus induces a large number of pSTAT5-immunoreactive cells in the mouse brain

Initially, we determined the specificity of pSTAT5 staining to identify GH-responsive cells in the mouse brain. Thus, basal distribution of pSTAT5-ir was assessed in mice that received vehicle injection and compared with animals that received an acute hGH stimulus. PBS-injected mice showed very low pSTAT5 expression in practically all brain structures analyzed (Fig. 1a–c). Several periventricular regions, particularly the arcuate nucleus of the hypothalamus (ARH), showed some pSTAT5-immunoreactive cells in PBS-injected mice (Fig. 1c). However, an acute hGH stimulus caused robust nuclear pSTAT5-ir in several brain structures, including the medial preoptic area (MPO; Fig. 1d), paraventricular nucleus of the hypothalamus (PVH; Fig. 1e), ARH (Fig. 1f), ventromedial nucleus of the hypothalamus (VMH; Fig. 1f) and dorsomedial nucleus of the hypothalamus (DMH; Fig. 1f). Therefore, pSTAT5 immunostaining after an acute hormonal stimulus represents a sensitive way to identify GH-responsive cells in the mouse brain.

hGH-responsive cells are primarily composed of neurons

Next, we assessed whether hGH-responsive cells are neurons or glial cells. hGH-induced pSTAT5-ir displayed a robust co-localization with NeuN, a neuronal marker, in several brain nuclei analyzed, including the PVH (Fig. 2a), VMH (Fig. 2c–e) and ventral part of the medial amygdalar nucleus (MEA, Fig. 2f). On the other hand, pSTAT5-ir was virtually absent in glial cells that were positive to GFAP (Fig. 2b, g). These findings indicate that hGH-responsive cells are primarily composed of neurons.

Distribution of GH-responsive neurons in the mouse brain

Next, we performed a comprehensive analysis of the distribution of GH-responsive neurons using an acute stimulus with either mGH or hGH to induce pSTAT5-ir. Since hGH also activates PrlR (Bartke and Kopchick 2015; Forsyth et al. 1965; Brem et al. 1989; Cunningham et al. 1990), we also charted the distribution of prolactin-responsive cells for comparison. Our findings are summarized in Table 1, which documents the relative density of pSTAT5-positive cells induced by each ligand in the mouse brain. Additionally, Figs. 3, 4, 5, 6 and 7 exhibit side-by-side representative photomicrographs of prolactin-, mGH- and hGH-

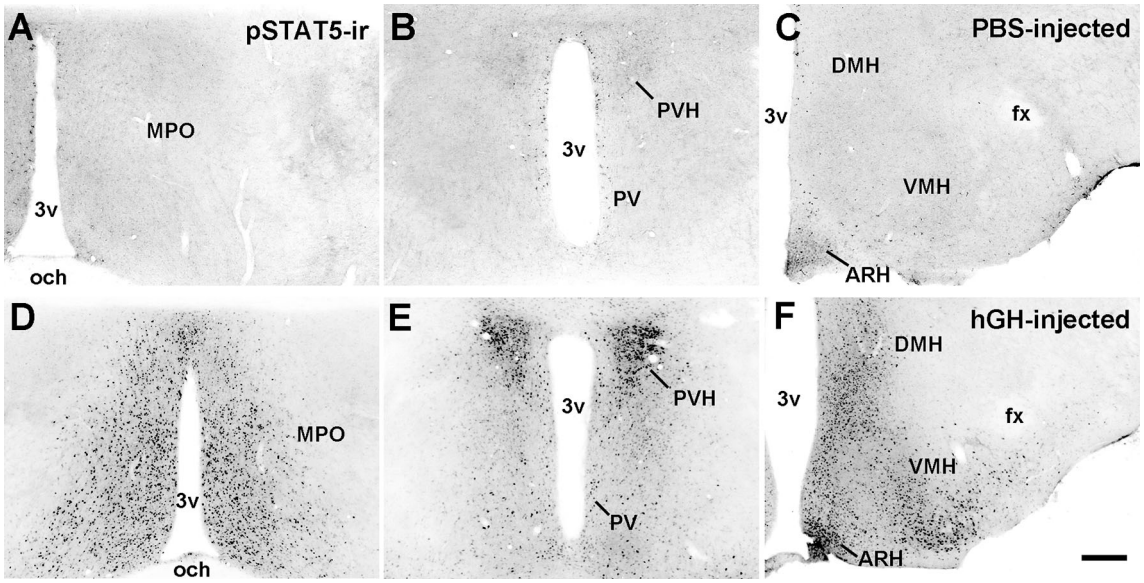


Fig. 1 Basal expression of pSTAT5-ir is very low in vehicle-treated mice. **a–c** Brightfield photomicrographs illustrating pSTAT5-ir in coronal brain sections of mouse that received an acute intraperitoneal injection of sterile PBS. **d–f** Brightfield photomicrographs illustrating pSTAT5-ir in brain sections of hGH-injected mouse. *3v* third ventricle, *fx* fornix, *och* optic chiasm, *PV* periventricular nucleus. *Scale bar* 200 µm

induced pSTAT5-ir in several brain regions. Noteworthy, we included male and female mice in our analysis since we did not observe gross gender-specific differences.

In the septum, we observed moderate expression of mGH- and hGH-responsive cells in the ventral aspects of the lateral septal nucleus (LS; Fig. 3b–c) and in the nucleus of the diagonal band. A lower amount of prolactin-responsive cells was observed in these areas (Fig. 3a). In the cerebral cortex, some mGH- and hGH-responsive cells were primarily found in the prefrontal cortex, piriform cortex (Fig. 3e–f) and hippocampus (Fig. 3h–i), with some additional diffusely distributed cells also observed in other cortical structures. Small populations of mGH- and hGH-induced pSTAT5-immunoreactive cells were identified in the olfactory tubercle and in the caudal aspects of the caudate putamen, whereas a moderate number occupied the vertex region of the shell of the nucleus accumbens. Prolactin-responsive cells were virtually absent in these areas. The anterior and principal subdivisions of the bed nuclei of the stria terminalis (BSTpr) showed neurons responsive to prolactin (Fig. 3j), mGH (Fig. 3k) and hGH (Fig. 3l). Moderate expression and low expression of pSTAT5-ir were induced by mGH or hGH in the anterior part of the paraventricular nucleus of the thalamus (PVT; Fig. 3k, l) and in the lateral habenula, respectively. On the other hand, very few prolactin-responsive cells were found in these structures (Fig. 3j).

In the amygdala, prolactin-responsive cells were confined to the posterodorsal part of the MEA (MEApd; Fig. 3m), whereas mGH- and hGH-responsive cells were present in the central amygdalar nucleus (CEA), as well as in the anterior, posteroventral and posterodorsal aspects of the MEA (Fig. 3n, o). Notably, the distribution pattern of prolactin- and GH-responsive cells was different in the MEApd. Thorough inspection of parallel sections revealed that GH-responsive cells were restricted to sublayer a (close to optic tract) and sublayer c (lateral layer) of the MEApd (Fig. 3n, o), whereas prolactin-responsive cells were localized in all sublayers (a, b and c) of the MEApd (Fig. 3m).

The hypothalamus stood out as the brain region containing the most prominent population of GH-responsive cells. An extensive overlapping distribution of prolactin-, mGH- and hGH-responsive cells was observed along the whole rostrocaudal axis of the preoptic region (Fig. 4a–l), including the MPO (Fig. 4a–i), anteroventral periventricular nucleus (AVPV; Fig. 4d–f), median preoptic nucleus (MEPO; Fig. 4d–f) and medial preoptic nucleus (MPN; Fig. 4g–l). However, while prolactin and hGH induced a larger number of pSTAT5-immunoreactive cells in the MEPO, AVPV and medial aspects of MPO (close to vascular organ of the lamina terminalis, OV), mGH induced pSTAT5-ir predominantly in the lateral aspects of MPO (Fig. 4a–f). Prolactin-responsive cells were found in the medial and lateral parts of the MPN (Fig. 4g). Conversely, mGH and hGH induced pSTAT5-ir predominantly in the medial part of the MPN (Fig. 4h, i). Only few prolactin-responsive cells were found in the suprachiasmatic nucleus (SCH; Fig. 4j), supraoptic nucleus (SO; Fig. 4m) and PVH (Fig. 5a). Conversely, mGH and hGH induced elevated

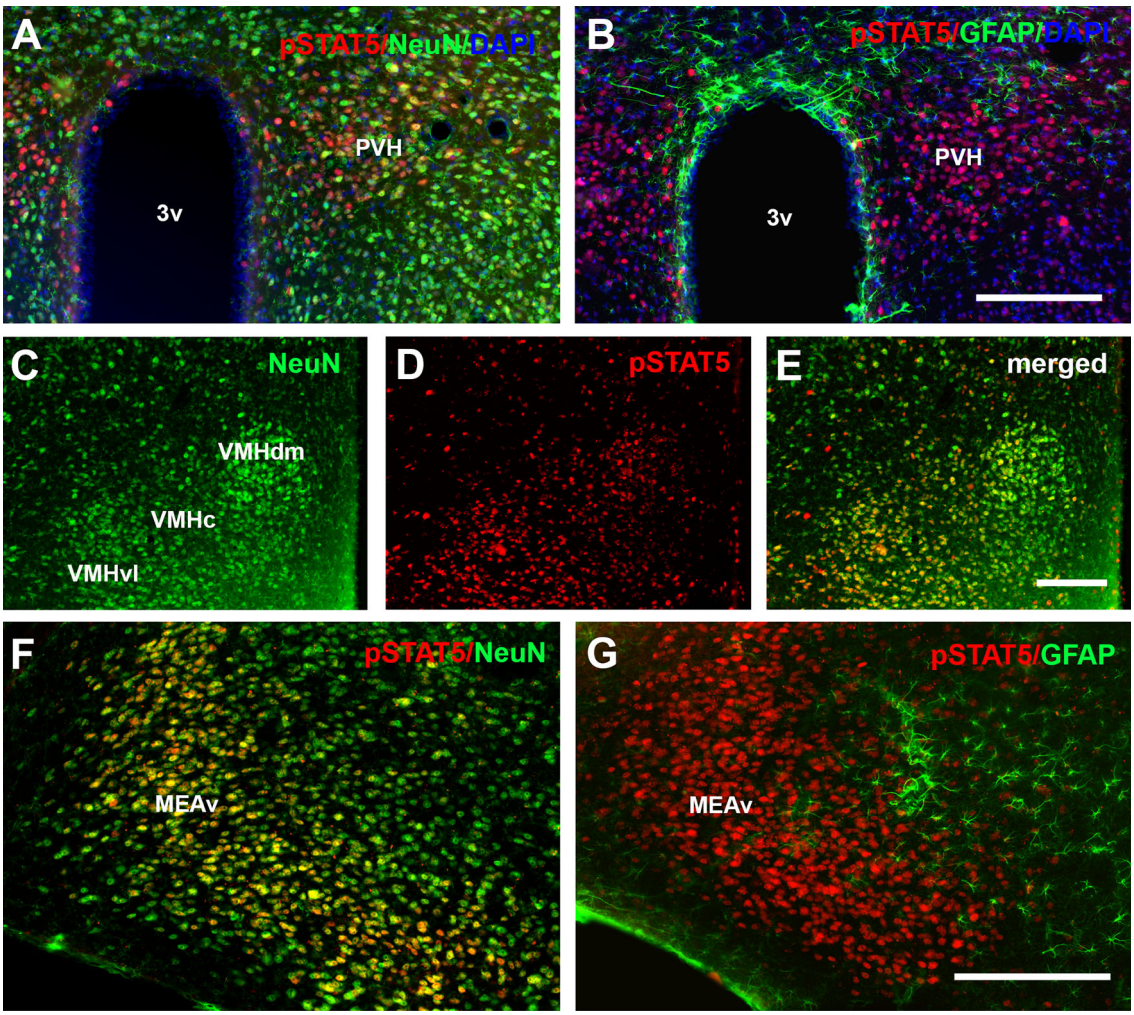


Fig. 2 Acute hGH injection induces nuclear pSTAT5-ir in neurons. **a** Epifluorescence photomicrograph illustrating the co-expression among hGH-induced pSTAT5-ir, NeuN (neuronal marker) and DAPI (nuclear marker). **b** Epifluorescence photomicrograph illustrating the co-expression among hGH-induced pSTAT5-ir, GFAP (astroglial marker) and DAPI. **c–f** hGH-induced pSTAT5-ir shows a robust co-expression with NeuN in the VMH and MEAv. **g** hGH-induced pSTAT5-ir is not observed in GFAP-positive cells. *Scale bar* **a**, **b** 100 μm; **c–e** 50 μm; **f–g** 200 μm

numbers of pSTAT5-immunoreactive cells in these areas (Figs. 4k, l, n, o, 5b, c). Although the distribution pattern of prolactin-, mGH- and hGH-responsive cells slightly differed along the rostrocaudal axis of the periventricular nucleus of the hypothalamus (PV), all these hormones were able to induce pSTAT5-ir in this structure (Fig. 5a–c).

Prolactin-responsive cells prevailed in the ventrolateral part of the VMH (VMHvl), although scattered prolactin-responsive cells were also observed in its dorsomedial part (VMHdm; Fig. 5d, g). Many mGH-responsive cells occupied both VMHvl and VMHdm, with the highest densities of mGH-responsive cells found in the VMHdm (Fig. 5e, h). hGH induced robust pSTAT5-ir in all VMH subdivisions (Fig. 5f, i). The ARH contained elevated numbers of prolactin-, mGH- and hGH-responsive cells (Fig. 5d–l). Prolactin-responsive cells were more prominently distributed in the dorsal part of the tuberal ARH (Fig. 5d, g), whereas mGH-responsive cells were primarily found close to the median eminence (Fig. 5e, h). hGH-responsive cells were diffusely distributed in the tuberal ARH (Fig. 5f, i). In the caudal ARH, prolactin- and hGH-responsive cells formed a dense band occupying its ventrolateral part (Fig. 5j, l). This band was absent in mGH-challenged mice, in which mGH-responsive cells were mostly restricted to the caudomedial ARH (Fig. 5k). Many prolactin-, mGH- and hGH-responsive cells were present in the DMH, although the distribution pattern differed among the hormones (Fig. 5g–i). The tuberal nucleus (TU) contained a moderate number of mGH- and hGH-responsive cells, but fewer prolactin-responsive neurons (Fig. 5d–i). Small numbers of cells expressing pSTAT5-ir were observed in the lateral hypothalamic area (LHA) of prolactin-treated animals

Table 1 Relative distribution of prolactin-, mGH- and hGH-induced pSTAT5-immunoreactive cells in the mouse brain

Brain structure	Prolactin-responsive cells	mGH-responsive cells	hGH-responsive cells
Cerebral cortex			
Prefrontal cortex	−	+	+
Piriform cortex	−	+	+
Hippocampus	±	+	+
Other cortical regions	−	±	±
Basal ganglia			
Striatum (caudate and putamen), caudal aspects	±	+	+
Olfactory tubercle	−	+	+
Septum and amygdala			
Lateral septum, ventral part	+	++	++
Nucleus of the horizontal limb of the diagonal band	+	++	++
Bed nucleus of the stria terminalis, lateral part	+	++	++
Bed nucleus of the stria terminalis, principal part	+	++	+++
Anterior amygdaloid area	±	+	+
Central nucleus of amygdala	±	+++	+++
Medial nucleus of amygdala, anterior part	±	+++	+++
Medial nucleus of amygdala, posterodorsal part	++	++	++
Thalamus and epithalamus			
Paraventricular nucleus of the thalamus, anterior part	±	++	+++
Lateral habenula	−	+	+
Hypothalamus			
Medial preoptic area	+++	+	++++
Anteroventral periventricular nucleus	++++	+	++++
Median preoptic nucleus	+++	+	++++
Medial preoptic nucleus	+++	++	+++
Suprachiasmatic nucleus	−	++	++
Retrochiasmatic area	++	++	++
Supraoptic nucleus	−	+++	+++
Paraventricular nucleus of the hypothalamus	+	+++	++++
Arcuate nucleus of the hypothalamus	+++	++	+++
Ventromedial nucleus of the hypothalamus, ventrolateral part	++++	++	++++
Ventromedial nucleus of the hypothalamus, dorsomedial part	++	++++	++++
Dorsomedial nucleus of the hypothalamus	++	+++	+++
Lateral hypothalamic area	−	+	+
Dorsal hypothalamic area	−	+	+
Tuberal nucleus	+	++	++
Posterior hypothalamic area	++	+++	+++
Ventral premammillary nucleus	+++	+++	++++
Dorsal premammillary nucleus	±	+	+
Supramammillary nucleus	−	++	++
Medial mammillary nucleus	−	+	+
Midbrain, pons and medulla oblongata			
Substantia nigra, reticular part	−	+	+
Ventral tegmental area	−	++	+
Superior colliculus	±	±	+
Periaqueductal gray	+	+++	+++
Ventral nucleus of lateral lemniscus	−	+	+

Table 1 continued

Brain structure	Prolactin-responsive cells	mGH-responsive cells	hGH-responsive cells
Dorsal raphe nucleus	+	++	++
Nucleus raphe pallidus	+	+	++
Locus coeruleus	−	+++	+++
Pontine nuclei	±	+	+
Facial motor nucleus	−	+	+
Lateral reticular nucleus	−	+	+
Spinal trigeminal nucleus, caudal part	−	++	++
Nucleus of the solitary tract	+	++	++
Dorsal motor nucleus of the vagus	−	+++	+++
Hypoglossal nucleus	−	±	+
Cerebellum			
Cerebellar cortex	±	±	±

++++, high density of cells covering the whole extension of the nucleus; +++, high density of cells covering most of the nucleus; ++, moderate density of cells covering only parts of the nucleus; +, low density of cells covering only parts of the nucleus; ±, very low expression; -, virtually absent

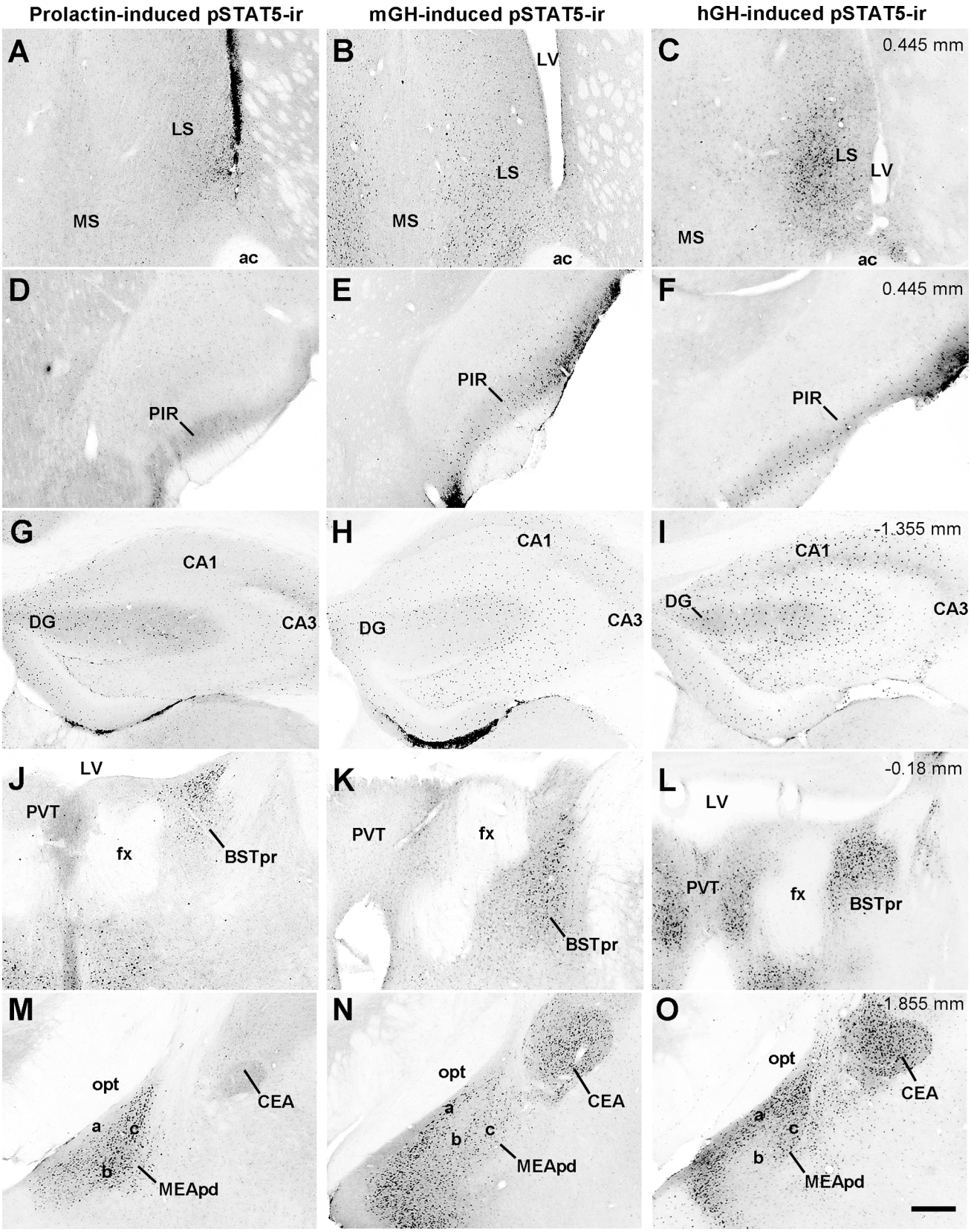
(Fig. 5d, g), whereas mGH and hGH induced higher numbers of pSTAT5-immunoreactive cells (Fig. 5e, f). In the premammillary area, the ventral premammillary nucleus (PMv) contained expressive populations of prolactin-, mGH- and hGH-responsive cells (Fig. 5j–l). Additionally, mGH and hGH induced a higher number of pSTAT5-immunoreactive cells in the posterior hypothalamic nucleus (PH; Fig. 5n, o) compared to prolactin-injected mice (Fig. 5m). The supramammillary nucleus (SUM) and medial mammillary nucleus (MM) showed low-to-moderate expression of mGH- and hGH-responsive cells (Fig. 6b, c), but virtually no prolactin-responsive cells (Fig. 6a).

Interestingly, many mid- and hindbrain structures also contained significant amounts of GH-responsive cells. Low expression of mGH- and hGH-induced pSTAT5-immunoreactive neurons was observed in the reticular part of the substantia nigra (SNr; Fig. 6e, f), ventral tegmental area (VTA; Fig. 6h, i), superior colliculus, ventral nucleus of the lateral lemniscus, pontine nuclei, facial motor nucleus, inferior olivary complex and lateral reticular nucleus (LRN; Fig. 7e, f). The nucleus raphe pallidus, the dorsal nucleus raphe (DR; Fig. 6n, o), the caudal part of the spinal nucleus of the trigeminal nerve (SPV; Fig. 7h, i) and the nucleus of the solitary tract (NTS; Fig. 7k, l) exhibited a moderate expression of either mGH- or hGH-responsive cells. A higher density of mGH- or hGH-responsive cells was observed in the periaqueductal gray (PAG; Fig. 6k, l), locus ceruleus (LC; Fig. 7b, c) and dorsal motor nucleus of the vagus (DMX; Fig. 7k, l). However, prolactin-responsive cells were confined to a much smaller number of brainstem structures. Thus, low expression of prolactin-induced pSTAT5-ir was detected in the PAG (Fig. 6j), DR (Fig. 6m) and NTS (Fig. 7j), whereas the remainder brainstem structures that showed GH-responsive cells were virtually devoid of prolactin-responsive cells. All peptides induced a very high pSTAT5-ir in the choroid plexus (CP). Finally, moderate numbers of mGH- or hGH-responsive cells were also detected in the cerebellar cortex (CBX; Fig. 7n, o), in which only few prolactin-responsive cells could be observed (Fig. 7m).

Fig. 3 Distribution of GH-responsive cells in extra-hypothalamic forebrain structures. **a–o** Brightfield photomicrographs illustrating pSTAT5-ir in side-by-side sections of prolactin-, mGH- and hGH-injected mice. In *panels M*, *N* and *O*, the letters *a*, *b* and *c* refer, respectively, to sublayer a, sublayer b and sublayer c of the MEApd. The approximate bregma coordinates of each rostrocaudal level were obtained from the Allen Mouse Brain Reference Atlas. *ac* anterior commissure, *LV* lateral ventricle, *MS* medial septal nucleus, *opt* optic tract, *PIR* piriform cortex. *Scale bar* 200 μm

Co-expression between hGH-induced pSTAT5-ir and GHR mRNA in the mouse brain

Several brain nuclei showed cells responsive to both mGH and hGH suggesting the presence of GHR in these areas. However, in some brain structures, we found a much lower response to mGH than to hGH. Consequently, hGH probably activated the PrlR in these areas to induce pSTAT5. To elucidate the brain targets of mGH and hGH and the receptors involved in these responses, we examined a possible co-localization of hGH-responsive cells and the GHR by combining pSTAT5 immunohistochemistry with the in situ hybridization for GHR mRNA (Table 2). Double-labeled cells exhibited a brown nucleus indicating hGH-induced pSTAT5-ir and clusters of silver grains in black


Prolactin-induced pSTAT5-ir
mGH-induced pSTAT5-ir
hGH-induced pSTAT5-ir
A
LS
MS
ac
B
LV
LS
MS
ac
C
0.445 mm
LS
LV
MS
ac
D
PIR
E
PIR
F
0.445 mm
PIR
G
CA1
DG
CA3
H
CA1
DG
CA3
I
-1.355 mm
CA1
DG
CA3
J
LV
PVT
fx
BSTpr
K
PVT
fx
BSTpr
L
-0.18 mm
LV
PVT
fx
BSTpr
M
opt
CEA
a
c
b
MEApd
N
opt
CEA
a
b
c
MEApd
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CEA
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b
MEApd

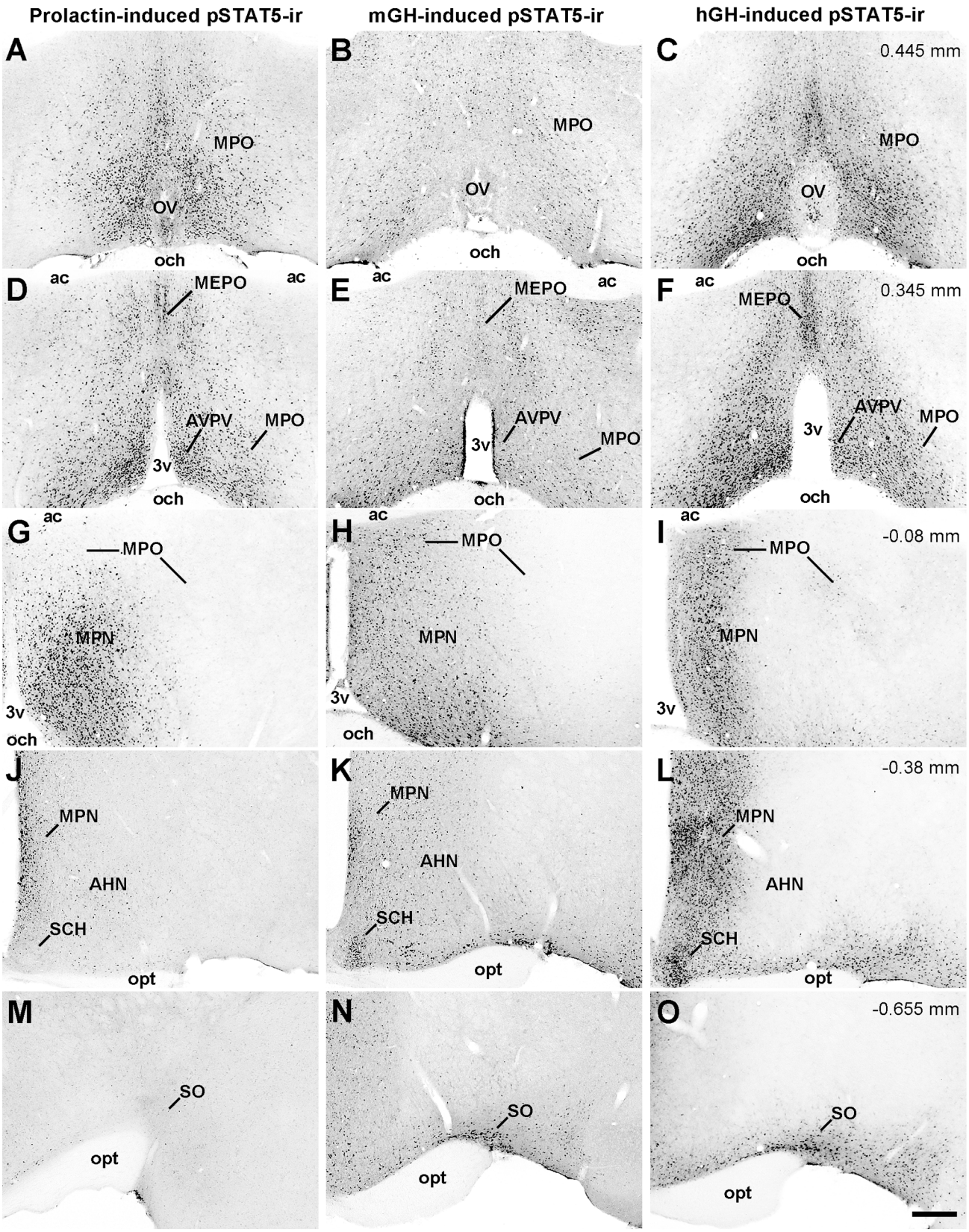

Fig. 4 Distribution of GH-responsive cells in the hypothalamus. **a–o** Brightfield photomicrographs illustrating pSTAT5-ir in side-by-side sections of prolactin-, mGH- and hGH-injected mice. *AHN* anterior hypothalamic nucleus. *Scale bar* 200 μm

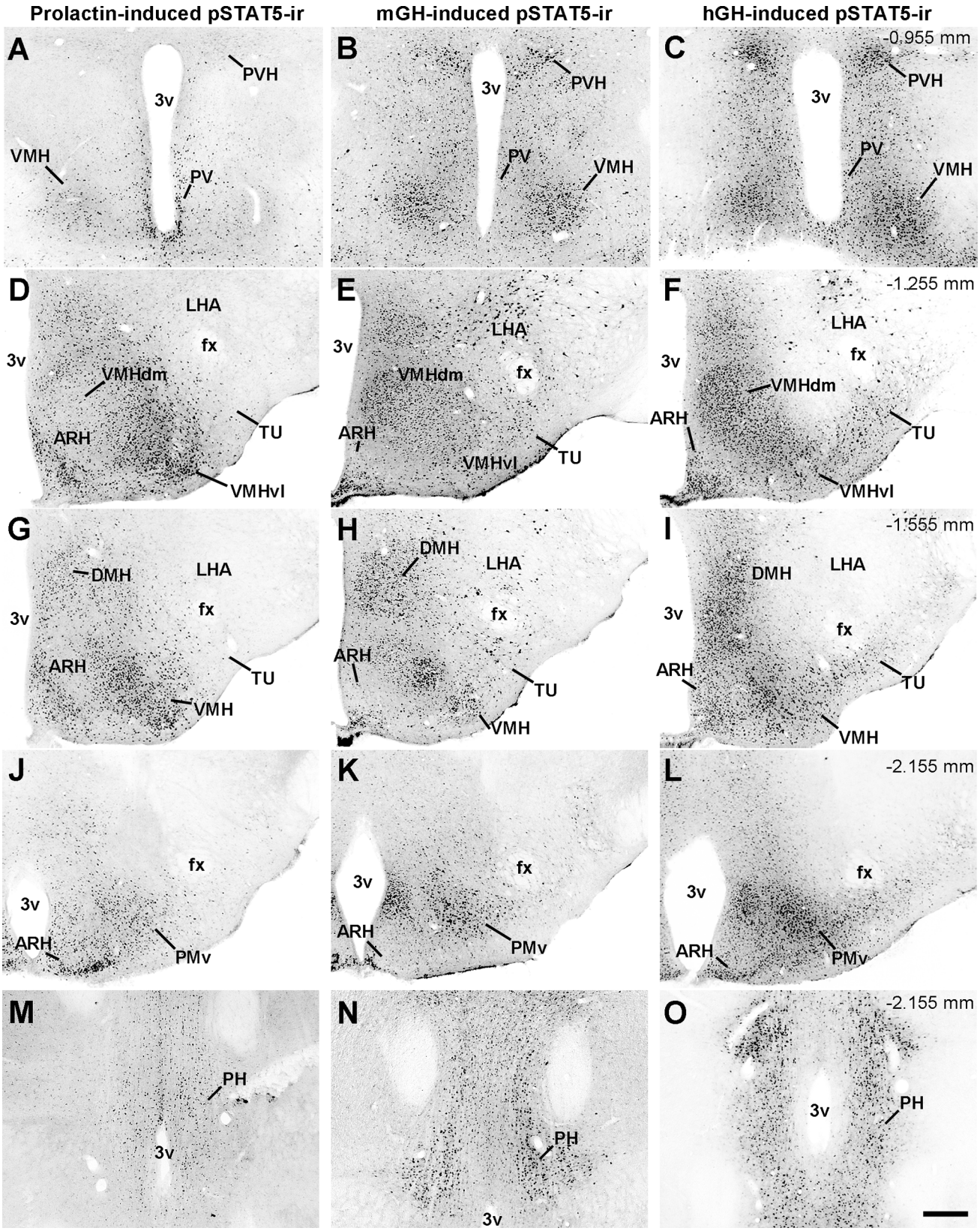

Fig. 5 Distribution of GH-responsive cells in the hypothalamus. **a–o** Brightfield photomicrographs illustrating pSTAT5-ir in side-by-side sections of prolactin-, mGH- and hGH-injected mice. *Scale bar* 200 μm

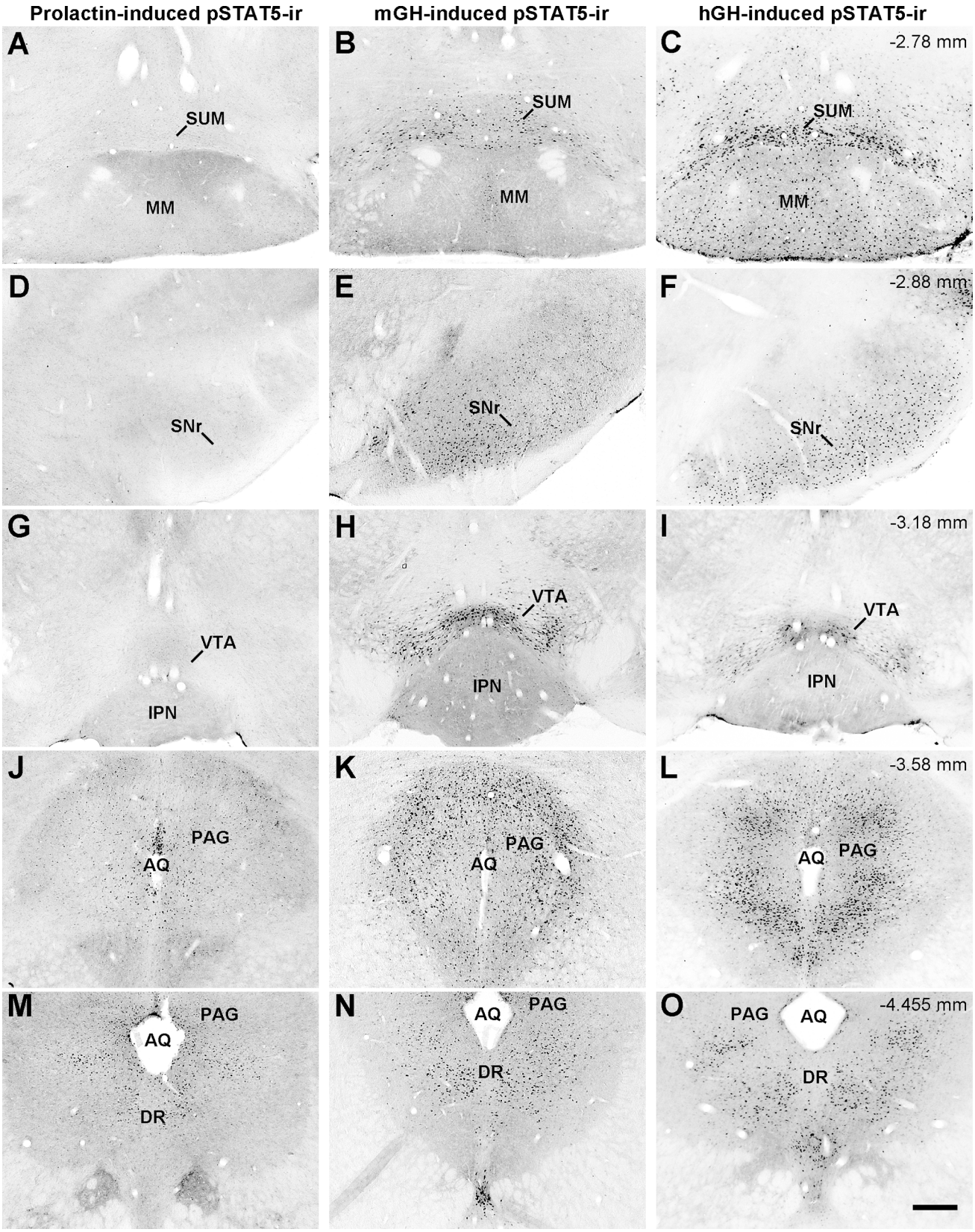

Fig. 6 Distribution of GH-responsive cells in the brainstem. **a–o** Brightfield photomicrographs illustrating pSTAT5-ir in side-by-side sections of prolactin-, mGH- and hGH-injected mice. *4v* fourth ventricle, *AQ* cerebral aqueduct, *IPN* interpeduncular nucleus. *Scale bar* 200 μm

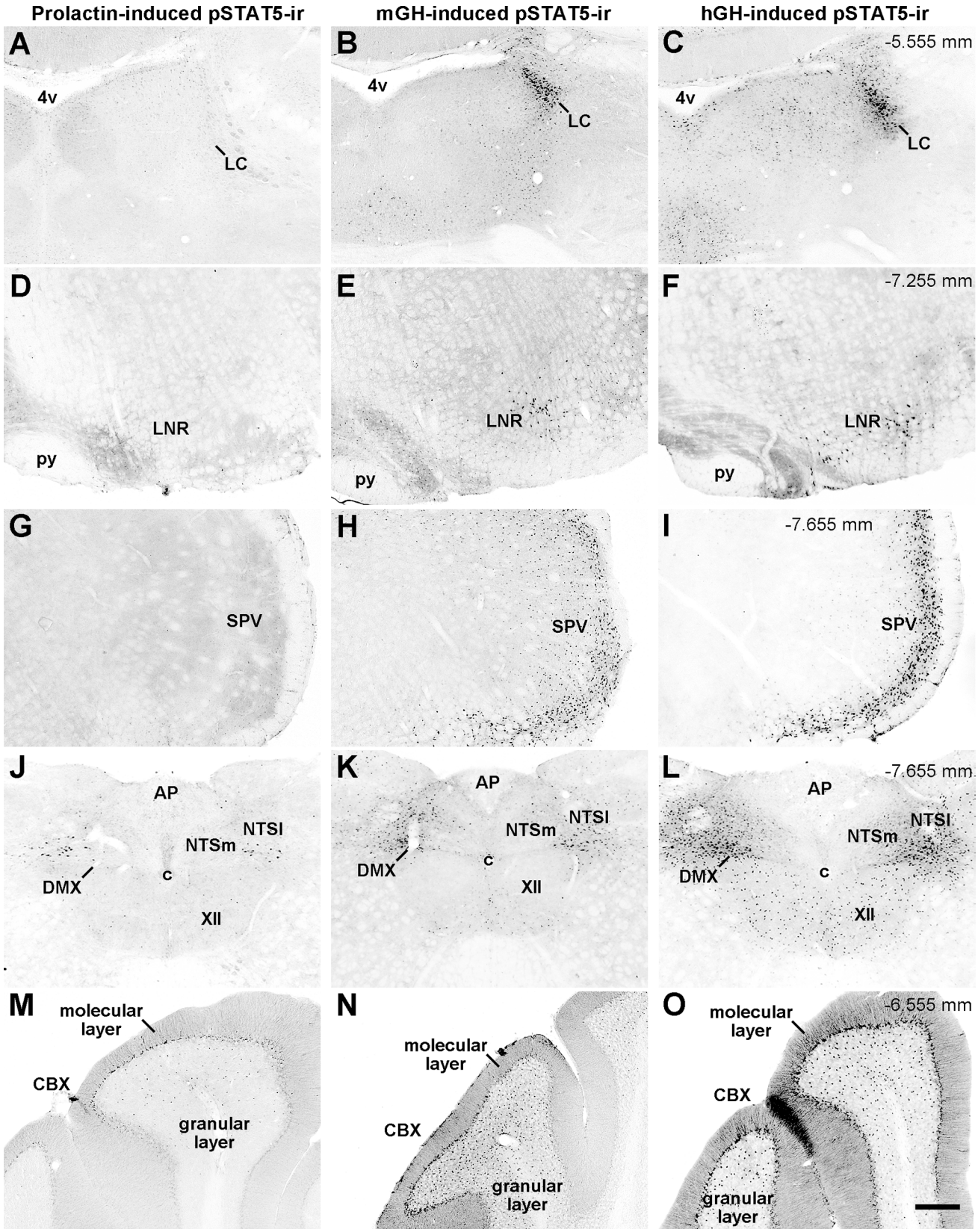

Fig. 7 Distribution of GH-responsive cells in the brainstem and cerebellum. **a–o** Brightfield photomicrographs illustrating pSTAT5-ir in side-by-side sections of prolactin-, mGH- and hGH-injected mice. *XII* hypoglossal nucleus, *AP* area postrema, *c* central channel, *py* pyramid. *Scale bar* 200 μm

Table 2 Qualitative description of neurons expressing hGH-induced pSTAT-5-ir, PrlR mRNA, GHR mRNA and double-labeled cells in the mouse brain

Brain structure	hGH-induced pSTAT5-ir	GHR mRNA	Co-expression pSTAT5 and GHR mRNA	PrlR mRNA	Co-expression pSTAT5 and PrlR mRNA
BSTpr	+++	++	++	+++	+++
CEA	+++	+++	+++	−	−
MEA, anterior part	+++	++	+	−	−
MEApv	++	+	+	+	+
MEApd	+++	++	++	+++	+++
CP	++++	+++	+++	++++	++++
MPO	+++	+	+	+++	++
AVPV	++++	++	++	+++	+++
MEPO	++++	++	++	++	+++
MPN, medial	+++	++	+	+++	+++
MPN, lateral	+	++	+	+++	+
SCH	++	+	+	−	−
SO	++	++	+++	−	−
PVH	++++	+++	+++	+	+
PV	++	++	+++	++	++++
LHA	+	++	++++	−	−
ARH ventral	+++	++++	+++	++	++
ARH dorsal	+++	++	++	+++	+++
VMHvl	++++	++	++	+++	+++
VMHdm	++++	++	++	+	+
DMH	+++	++	+	++	+
PH	+++	+	+	+	+
PMv	++++	++	++	+	+
SNr	++	+	+	−	−
VTA	+	+	+	−	−
PAG	+++	++	++	+	+
DR	++	+	++	+	+
LC	++++	++	+++	−	−
LNR	++	+	+	−	−
SPV	++	+	+	−	−
NTS	++	++	++	+	+++
DMX	++	+++	+++	−	−

++++, very high expression; +++, high expression; ++, moderate expression; +, low expression; −, very low expression or virtually absent

overlying or immediately surrounding the nucleus, indicative of GHR mRNA expression (Figs. 8, 9). Although hGH induced a large number of pSTAT5-immunoreactive neurons in the preoptic region, only 28.1 ± 3.8 % of cells in the MPO (Fig. 8a) and 47.1 ± 3.1 % of cells in the AVPV (Fig. 8b) co-expressed GHR mRNA. On the other hand, moderate levels of GHR mRNA were found in the PVH, and most of hGH-responsive cells expressed GHR mRNA (77.0 ± 6.8 %; Fig. 8c). In the ARH, high GHR mRNA levels were found in its ventromedial aspects, whereas the dorsal ARH showed a moderate GHR expression (Fig. 8d). Consequently, the majority of hGH-responsive cells in the ventromedial ARH expressed GHR (75.3 ± 4.5 %), while less double-labeled cells were observed in the dorsal ARH (35.2 ± 3.6 %; Fig. 8d). In the VMHdm, we observed 71.5 ± 3.6 % of hGH-responsive cells co-expressing GHR mRNA, whereas 71.6 ± 2.3 % of co-localizations were found in the VMHvl (Fig. 9). Only part of hGH-induced pSTAT5 cells co-expressed GHR mRNA in the MEApd (41.7 ± 9.7 %; Fig. 9). In the brainstem, moderate GHR mRNA levels were found in the LC (Fig. 8e), DMX (Fig. 8f) and NTS (Fig. 8f). Importantly, most of the hGH-responsive cells observed in these areas co-expressed the GHR.

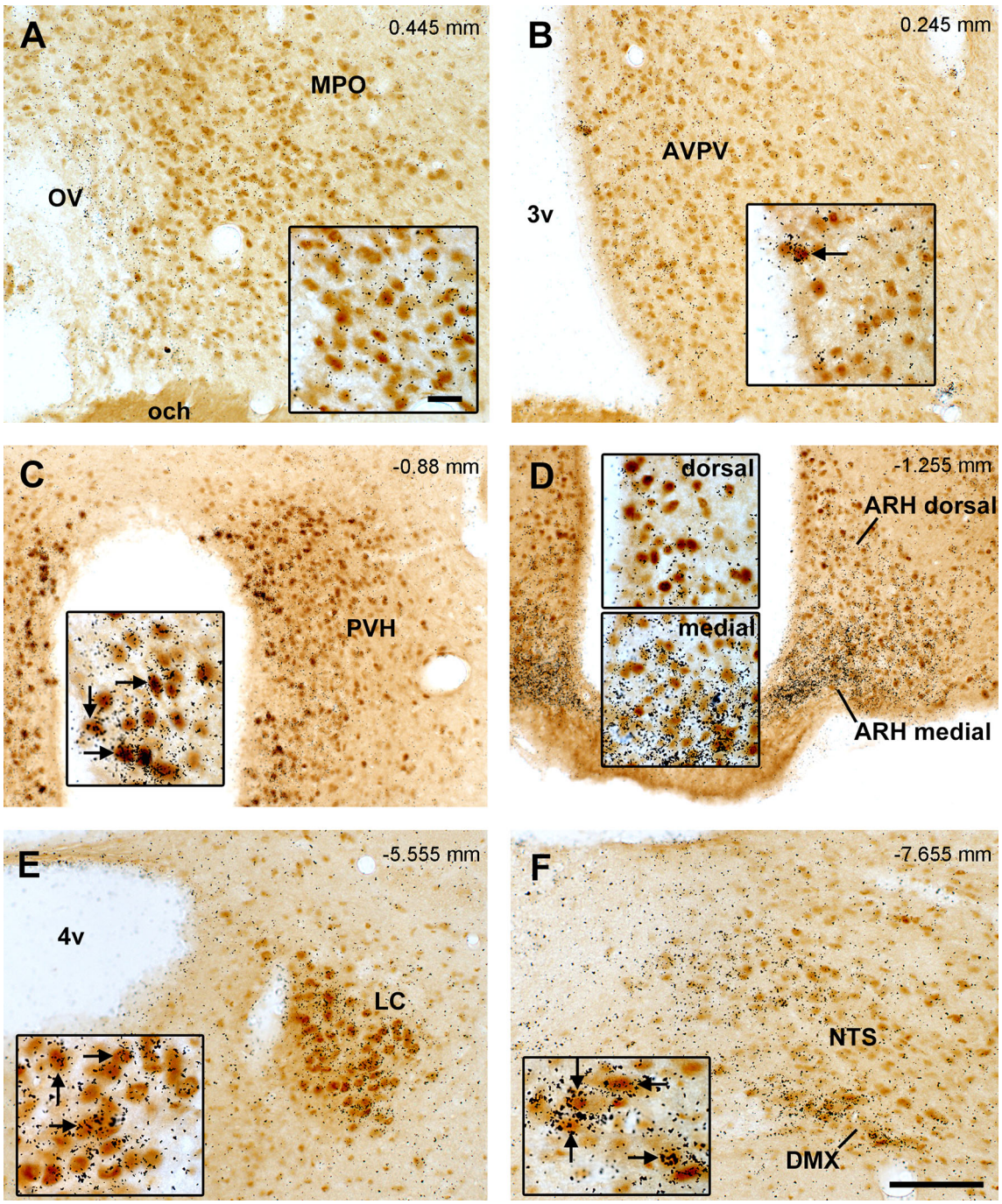

Fig. 8 Co-expression between hGH-induced pSTAT5-ir and GHR mRNA in the mouse brain. **a–f** Brightfield photomicrographs illustrating the co-localization between hGH-induced pSTAT5-ir (brown nucleus) and GHR mRNA (clusters of silver grains in *black*). *Arrows* indicate examples of double-labeled cells which exhibit clusters of silver grains overlying or immediately surrounding the nucleus. The *insets* represent higher magnification photomicrographs of each respective brain region. *Scale bar*: **a–b**, **e–f** 75 µm; **c–d** 100 µm; *insets* 20 µm

Co-expression between hGH-induced pSTAT5-ir and PrlR mRNA in the mouse brain

The aforementioned similarities and differences between the distribution pattern of prolactin-, mGH- and hGH-responsive cells indicate that PrlR and GHR are co-expressed in some areas, but not in others. To further clarify the receptor types involved in central effects of GH, we mapped the distribution of hGH-responsive cells that co-expressed PrlR mRNA (Table 2; Fig. 9). We observed a

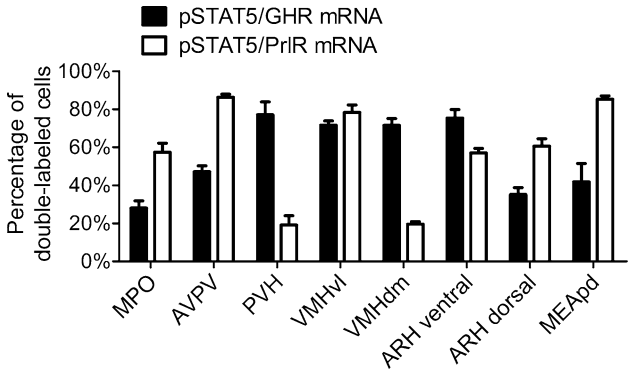

Fig. 9 Percentage of hGH-responsive cells co-expressing GHR or PrlR mRNA in several forebrain structures. Results are expressed as mean ± SEM

moderate proportion of double-labeled cells in the MPO (57.4 ± 4.7 %; Figs. 9, 10a). The AVPV exhibited a high percentage of double-labeled cells (86.3 ± 1.6 %; Fig. 10b). Notably, the medial part of the MPN exhibited a high expression of pSTAT5-immunoreactive cells, PrlR mRNA and co-localizations, whereas the lateral part of the MPN expressed significantly more PrlR mRNA compared to pSTAT5-ir and, consequently, exhibited only few double-labeled cells (Fig. 10c, d). The CP expressed a very high density of pSTAT5-ir and also PrlR mRNA (Fig. 10e). Although the SO and PVH contained hGH-responsive cells, these areas exhibited low PrlR mRNA levels resulting in few double-labeled cells (Figs. 9, 10f). The majority of hGH-induced pSTAT5-immunoreactive cells co-expressed the PrlR in the PV (Fig. 10g). The BSTpr presented high expression of hGH-induced pSTAT5-immunoreactive cells, PrlR mRNA and also elevated proportions of double-labeled cells (Fig. 10h).

In the amygdala, the anterior part of the MEA and the posteroventral aspects of the MEA (MEApv) were characterized by very low PrlR mRNA expression and virtually no double-labeled cells (Table 2). The MEApd showed a high proportion of double-labeled cells (85.3 ± 1.8 %; Figs. 9, 11a, b). Only pSTAT5-ir was observed in the CEA (Fig. 11c). The LHA displayed some hGH-responsive cells but no co-localization with the PrlR (Fig. 11d). The ARH exhibited through all its rostro-caudal extension a high expression of hGH-induced pSTAT5-immunoreactive cells, PrlR mRNA and double-labeled cells (Figs. 9, 11e, f, h). VMHvl showed a high proportion of double-labeled cells (78.3 ± 3.9 %), while fewer co-localizations were found in the VMHdm (19.6 ± 1.3 %; Figs. 9; 11f). Although the DMH contained both hGH- and prolactin-responsive cells, we only observed few co-localizations, most of them in the lateral aspects of the DMH (Fig. 11g). The PH and PMv showed more hGH-induced pSTAT5-ir compared to PrlR mRNA resulting in a low proportion of double-labeled cells (Fig. 11h). Finally, the few cells expressing PrlR mRNA in the NTS were also responsive to hGH (Fig. 11i). No co-localization was observed in the DMX (Fig. 11i).

Males and females exhibit a similar number of GH-responsive cells in several forebrain structures

Since there is a sex dimorphism in the pattern of GH secretion (Jansson et al. 1985; MacLeod et al. 1991; Jaffe et al. 1998; Gatford et al. 1998), we investigated possible gender differences in GH responsiveness by evaluating the absolute numbers of pSTAT5-immunoreactive cells in sections through different forebrain structures of male and female mice. This analysis was conducted in mice challenged either with mGH or with hGH (Fig. 12). We observed that males and females exhibited similar amounts of GH-responsive cells in the MPO, VMH, tuberal ARH, MEApd and PMv. This comparable GH responsiveness was observed either for the mGH (Fig. 12a) or for the hGH (Fig. 12b) stimulus.

Discussion

Although previous studies have reported the expression of GHR in few selected brain regions (Burton et al. 1992; Kastrup et al. 2005; Walsh et al. 1990; Nyberg 2000), a detailed analysis on the distribution of GH-responsive cells in the mouse brain has never been undertaken. In the present study, we demonstrated that neurons, but not astroglial cells, are responsive to GH, and GH-responsive neurons are abundantly distributed in numerous brain regions. In all, our findings confirm a relatively similar distribution of GH-responsive cells in the mouse brain compared to the pattern previously observed in the brain of rats and rabbits (Walsh et al. 1990; Burton et al. 1992; Kastrup et al. 2005). Importantly, our strategy to identify GH-responsive cells resulted in a high-resolution staining, allowing the realization of detailed mapping and co-localization studies, leading to the identification of many additional brain regions, hitherto not known to contain GH-responsive cells.

Identification of GH-responsive cells through pSTAT5 immunostaining

In the present study, we used either mGH or hGH to map the brain distribution of GH-responsive cells. While mGH-responsive cells provide a detailed account of the presence of functional GHR in the mouse brain, hGH-responsive cells represent the combined activation of GHR and PrlR. Consequently, hGH-responsive cells

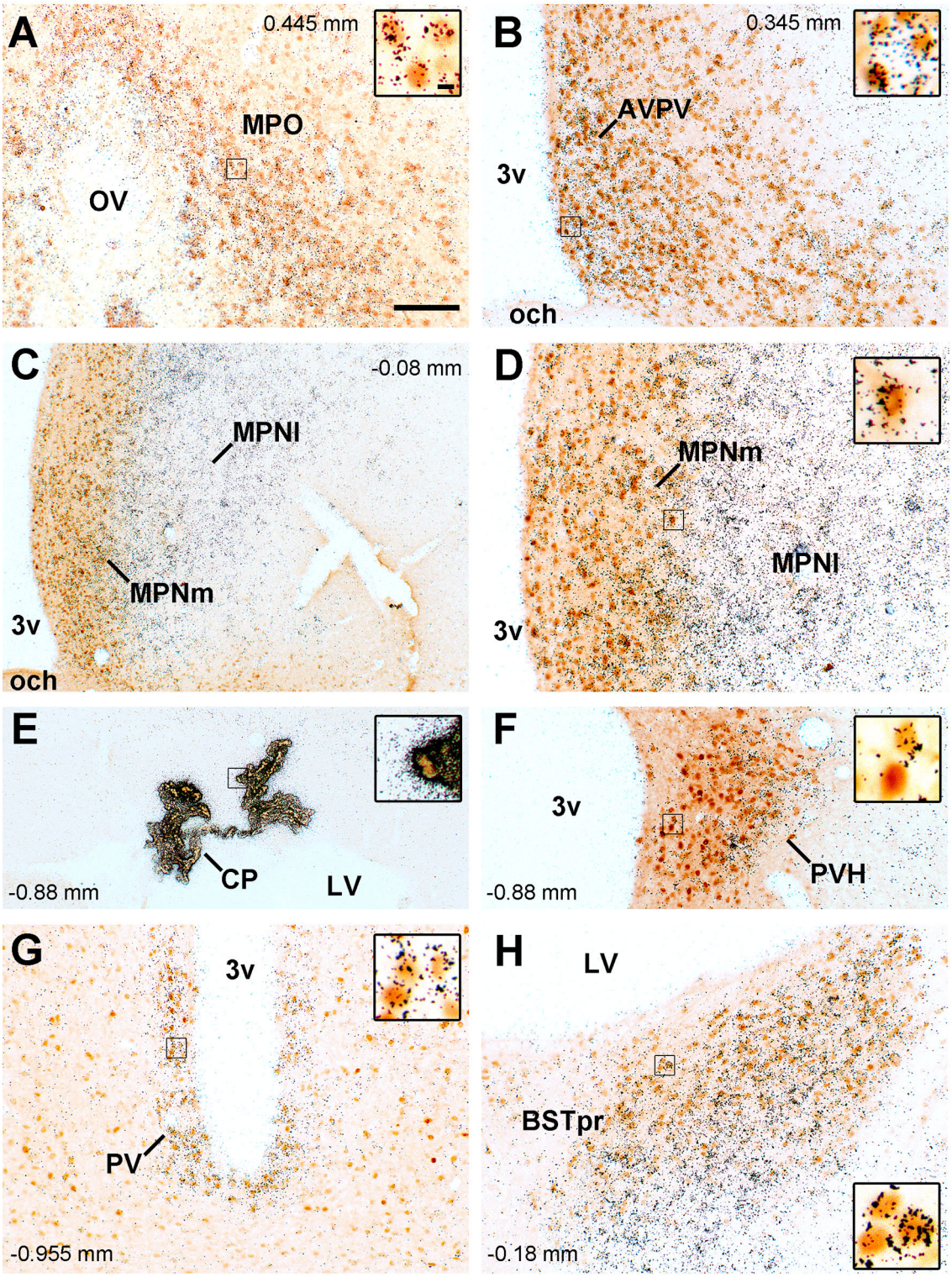

Fig. 10 Co-expression between hGH-induced pSTAT5-ir and PrlR mRNA. **a**–**h** Brightfield photomicrographs illustrating hGH-induced pSTAT5-immunoreactive cells (brown nucleus) and PrlR mRNA expression (clusters of silver grains in *black*). *Panel D* represents a higher magnification picture than that presented in *panel C*. The *insets* represent a zoom of the boxed areas in the low-power micrographs and illustrate the prevalence of double-labeled cells in the respective brain structures. *Scale bar* **a**, **b**, **d**–**h** 100 µm; **c** 200 µm; *insets* 5 µm

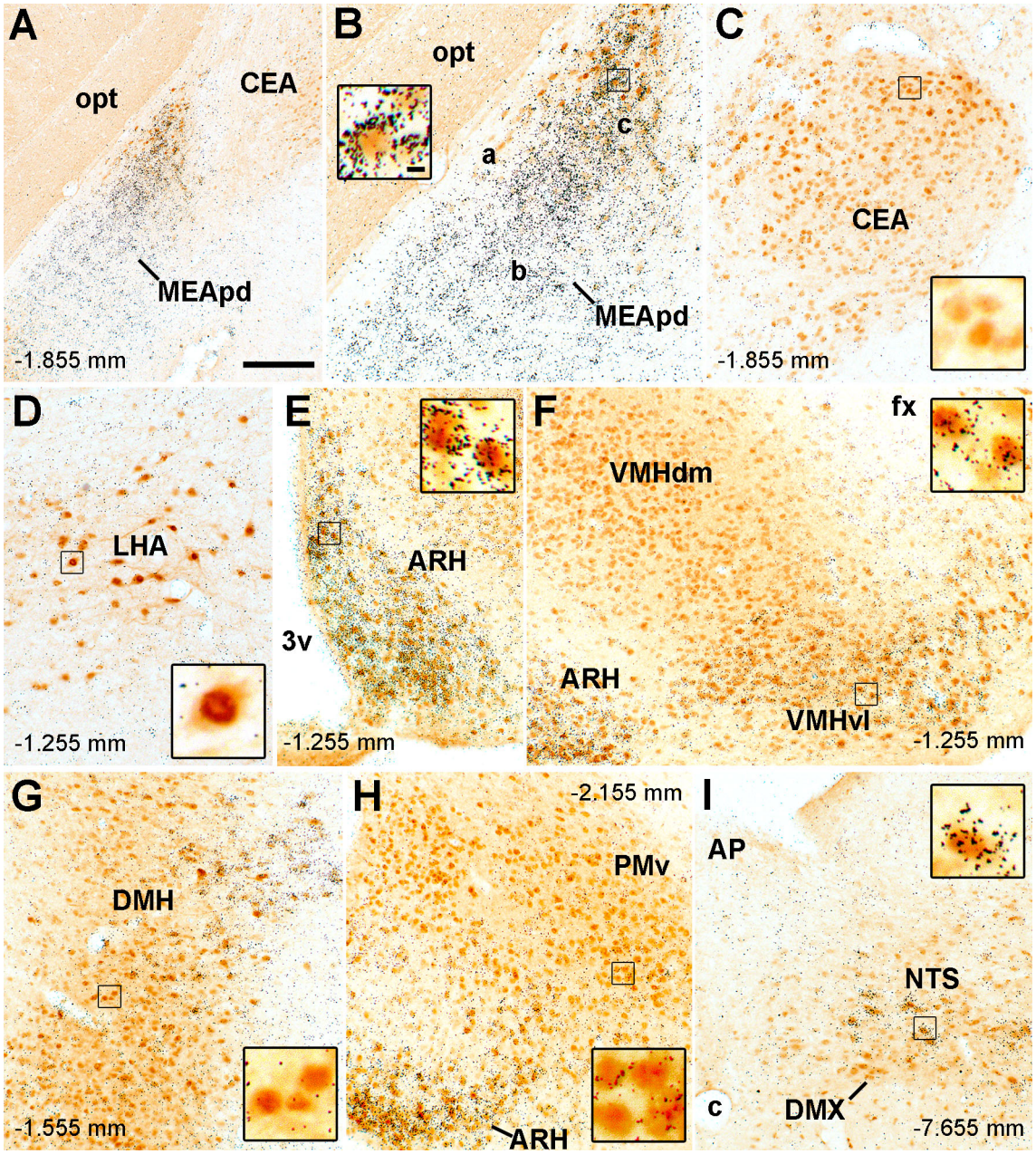

Fig. 11 Co-expression between hGH-induced pSTAT5-ir and PrlR mRNA. **a–h** Brightfield photomicrographs illustrating hGH-induced pSTAT5-immunoreactive cells and PrlR mRNA expression. *Panel B* represents a higher magnification picture than that presented in *panel A*. The *insets* represent a zoom of the boxed areas in the low-power micrographs and highlight the prevalence or absence of double-labeled cells in the respective brain structures. *Scale bar* **a** 200 µm; **b–i** 100 µm; *insets* 5 µm

appeared to represent the sum of mGH- and prolactin-responsive cells in practically all brain areas. Furthermore, the comparison among the distribution of prolactin-, mGH- and hGH-responsive neurons, together with experiments that investigated the co-localization between hGH-responsive cells and GHR or PrlR mRNA, may provide valuable information about the interaction between prolactin and GH in specific cell populations. Thus, our semi-quantitative data indicate that the major response to hGH probably depended on GHR activation in several structures of the hypothalamus (e.g., SCH, SO, PVH, LHA, VMHdm and ARH ventral) and brainstem (e.g., SNr, VTA, LC, LNR and SPV). On the other hand, the higher degree of co-localization between hGH-induced pSTAT5-ir and PrlR mRNA observed in other brain structures (e.g., MPO, AVPV, VMHvl, ARH dorsal and MEApd) suggests that PrlR are more relevant for the observed response in these areas.

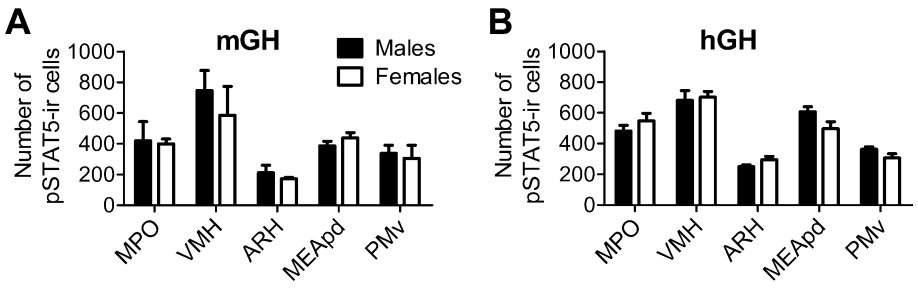

Fig. 12 Quantification of the number of GH-responsive cells in several forebrain structure in male and female mice. **a**–**b** *Bar graphs* showing the number of pSTAT5-immunoreactive (pSTAT5-ir) induced by an acute injection of mGH (**a**; 2 males and 3 females) or hGH (**b**; 12 males and 8 females) in one representative level of the MPO, VMH, tuberal ARH, MEApd and PMv. Results are expressed as mean ± SEM

Previous mapping studies relied on traditional methods, such as immunohistochemistry to stain GHR protein, in situ hybridization to label GHR mRNA, or radiolabeled hormones to identify GH-responsive cells in the brain (Burton et al. 1992; Kastrup et al. 2005; Walsh et al. 1990; Nyberg 2000; Lobie et al. 1993). Although these methods are widely accepted and employed, they have important drawbacks. For example, reliable antibodies against certain membrane receptors, such as the GHR, may be difficult to obtain. Regarding in situ hybridization techniques, many cytokine receptors show low mRNA expression in the brain, often resulting in low signal to background ratios, poor resolution and difficulties in performing co-localization experiments. The same shortfalls exist in autoradiographic studies. Due to these limitations, we decided to employ an alternative strategy to identify GH-responsive cells. As the activation of GHR induces STAT5 phosphorylation (Moutoussamy et al. 1998), cells that exhibited pSTAT5-ir after an acute GH stimulus were considered to be GH responsive. Such strategy has never been performed before to map GH-responsive cells in the brain. Interestingly, our findings revealed an elevated capacity of peripherally administered GH to access virtually the whole brain. The choroid plexus exhibits a high GHR expression and GH binding capacity, suggesting a possible involvement of this structure in the transport of GH from the periphery to the central nervous system (Coculescu 1999; Nyberg 2000; Walsh et al. 1990). However, Pan et al. (2005) found no evidence of a specific GH transport system and suggested a brain influx of GH through the blood–brain barrier by simple diffusion. This extensive central GH penetrance appears to be at least equivalent to that of other cytokines, including leptin (Munzberg et al. 2003; Scott et al. 2009; Donato et al. 2010; Caron et al. 2010) and prolactin (Brown et al. 2010; Nagaishi et al. 2014; Furigo et al. 2014), whose receptors have been mapped using similar strategies than those employed in the present study. Although we observed in our experiments that GH-responsive cells are widely distributed throughout the brain, we cannot rule out that some cells that express GHR are not responsive or cannot recruit STAT5 at a certain moment when the stimulus occurs. Under these conditions, we will not be able to visualize those cells, even though they can be activated by GH in other physiological circumstances or recruit different intracellular signaling pathways. Accordingly, it was reported in a previous study that hormones, such as prolactin, can recruit different combinations of electrical and transcriptional responses in neurons depending upon their anatomical location and the actual physiological state (Brown et al. 2012). These distinct effects may be critical in establishing appropriate responses under different physiological conditions (Brown et al. 2012). Despite this potential drawback, the here observed extensive distribution of GH-responsive cells indicates that these potential false-negative cells may represent a small proportion compared to the total population. Additionally, the very low pSTAT5-ir detected in vehicle-injected mice, the relatively small inter-individual variation observed in the hormone-induced responses and, importantly, the broad co-localization observed among hGH-induced pSTAT5-ir, GHR mRNA and PrlR mRNA indicate that our method to identify GH-responsive neurons is highly reliable and does not produce significant amounts of neither false-negative nor false-positive cells.

Intriguingly, a few areas exhibited prominent PrlR mRNA expression, but virtually no hGH-induced pSTAT5-immunoreactive cells. These structures, in which hGH was unable to induce pSTAT5 through PrlR, include the lateral MPN and sublayer b of the MEApd. The reasons for this unresponsiveness are unknown, but may be due to differences in the ligand levels required to activate the PrlR. Previous studies reported that despite the fact that BSTpr exhibits high PrlR mRNA levels, an acute prolactin stimulus was unable to induce pSTAT5-ir in this structure (Furigo et al. 2014; Brown et al. 2010). In the present study, we were able to induce pSTAT5-ir in the BSTpr using a dose 2 to fourfold higher than that employed in previous studies (Furigo et al. 2014; Brown et al. 2010),

suggesting that BSTpr requires an elevated dose of prolactin to exhibit pSTAT5.

Possible functional implications of GH-responsive cells in the mouse brain

The specific functions of GH signaling in the brain still need to be clarified. One important new finding of our study is that the vast majority of GH-responsive cells are neurons and not glial cells. Previous investigations that described the presence of GHR in the brain did not specify the cell types containing GHR (Walsh et al. 1990; Kastrup et al. 2005). The hypothalamus contained the most prominent population of GH-responsive cells, suggesting that central GH signaling is involved in neurovegetative and neuroendocrine functions. For example, the PV, PVH and ARH exhibited many GH-responsive cells and a high degree of co-localization with PrlR or GHR mRNA. Therefore, it is very likely that GH regulates the release of prolactin and controls its own secretion by a negative feedback loop via hypothalamic neurons. In accordance, previous studies reported GHR expression in somatostatin neurons located in the PV and PVH (Burton et al. 1992). However, further detailed co-localization studies are needed to unravel the specific neuroendocrine systems that are actually influenced by central GH signaling. Interestingly, the abundance of GH-responsive cells in several hypothalamic nuclei involved in the control of metabolism, such as the ARH, VMH and DMH (Morton et al. 2014; Zhang et al. 2011; Williams and Elmquist 2012), suggests that central GH signaling may also affect ingestive behaviors, as well as energy expenditure and glucose homeostasis through a central mechanism. In line with this, GH administration leads to glucose intolerance and insulin resistance (Marcus et al. 1990; Borst 2004; Pell and Bates 1990), and GH-deficient individuals exhibit important metabolic imbalances (Carroll et al. 1998; Nyberg 2000).

We firstly describe here that many brainstem structures also contained prominent populations of GH-responsive cells. Although the specific consequences of GH signaling in the brainstem clearly await more profound investigations, several of these GH-responsive areas are related to neurovegetative and autonomic functions, including the facial motor nucleus, raphe nuclei, LC, spinal nucleus of the trigeminal nerve, LRN, NTS and DMV. For example, the DR and LC displayed significant amounts of GH-responsive cells and are regions well known to be involved in regulating sleep and arousal (Szymusiak and McGinty 2008). Since GH has been shown to participate in sleep regulation (Tanriverdi et al. 2014), direct GH actions on the DR and LC may provide a neuroanatomical basis to explain the effects of GH on sleep regulation.

GH deficiency also causes social withdrawal, poor memory and impaired cognition (Blackmore et al. 2012; Carroll et al. 1998; Waters and Blackmore 2011; Nyberg and Hallberg 2013). Notably, several brain regions associated with learning, memory, decision making and the regulation of behaviors and emotions contain GH-responsive cells. Thus, neurons in the prefrontal cortex, nucleus accumbens, hippocampus, amygdala, striatum, septum, BST and several hypothalamic (i.e., preoptic region, VMH, LHA and mammillary region), as well as important mesopontine modulatory centers (i.e., substantia nigra, VTA, PAG and raphe nuclei), seem to be directly influenced by circulating GH levels. Of note, GH deficiency produces a complex phenotype which includes low circulating levels of insulin-like growth factor-1 (IGF-1), changes in the secretion of several hormones, growth failure and metabolic disorders (Alatzoglou et al. 2014). Therefore, it is conceivable that secondary effects of GH deficiency are more important for the cognitive deficits than impaired brain GHR signaling per se. Several studies have shown that IGF-1 signaling has neuroprotective and neurodevelopmental effects and also influences cognitive processes (Miltiadous et al. 2011; O'Kusky and Ye 2012; Aberg et al. 2006; Witty et al. 2013; Le Greves et al. 2005; Molina et al. 2013; Sonntag et al. 2013; Ramis et al. 2013). However, the wide distribution of GH-responsive cells in areas involved in mechanisms of learning and memory, as well as in emotional/motivational processes, provides neuroanatomical evidence that GH signaling may directly affect cognitive processes and the expression of behaviors, independently of changes in IGF-1 circulating levels.

GH secretion patterns have been shown to be distinct between males and females in several species, including humans, mice and rats (Jansson et al. 1985; MacLeod et al. 1991; Jaffe et al. 1998; Gatford et al. 1998). This sexual dimorphism is thought to be mainly mediated by differential activity patterns and functional roles of hypothalamic neurons that control pituitary GH secretion (Jansson et al. 1985; Argente et al. 1991; Painson and Tannenbaum 1991; Jaffe et al. 1998; Gatford et al. 1998; Low et al. 2001; Jessup et al. 2003). Since subsets of these neurons are responsive to GH (Burton et al. 1992; Nass et al. 2000), it is conceivable that GH effects in the brain are sexually dimorphic. To determine potential sex differences in the brain responsiveness to GH, we semi-quantified GH-responsive cells in brain structures known to exhibit sexual dimorphism. Nonetheless, no significant differences were observed comparing the absolute numbers of GH-responsive cells in males and females. Although this finding suggest the absence of sexual dimorphism regarding the response to GH in the analyzed brain regions, it is important to take into account that our analysis was exclusively based on the absolute number of pSTAT5-immunoreactive cells. However, GH could have caused additional effects that were not evaluated here, including changes in resting membrane potential, firing rate

or the set of genes induced by GH signaling. Of note, all structures analyzed exhibit neurons responsive to sex hormones (Simerly et al. 1990), and it is unknown whether STAT5 can interact with different combinations of transcription factors, including estrogen and androgen receptors, to induce a different transcription program in neurons of males and females. Additionally, we did not evaluate specific subsets of neurons that are important for the sexual dimorphic secretion of GH, such as hypothalamic growth hormone–releasing hormone (GHRH)- and/or somatostatin-positive neurons (Bouyer et al. 2006; Argente et al. 1991; Jansson et al. 1985; Painson and Tannenbaum 1991; Jaffe et al. 1998; Low et al. 2001; Jessup et al. 2003). Therefore, although our study provides additional information about the effects of GH in the brain of male and female mice, we cannot rule out that a sexually dimorphic response may exist for other parameters or specific neuronal populations that were not investigated in the present study.

Concluding remarks

Our results revealed a broad distribution of GH-responsive neurons in the mouse brain. In particular, areas primarily involved in the regulation of neurovegetative, emotional/motivational and cognitive functions were found to be affected by GH signaling. Overall, our findings deepen the understanding of GH signaling in the brain and suggest that central GH signaling is likely more ample and complex than formerly recognized. In addition, the validation of our indirect method to label GH-responsive cells might aid targeting specific neuronal populations directly affected by GH signaling in future studies.

Acknowledgments We thank Ana Maria P. Campos for the technical assistance and the São Paulo Research Foundation (FAPESP-Brazil) for the financial support as grants [2010/18086-0 (J.D.), 2012/24345-4 (C.R.J.S.), 2012/02388-3 (M.M.)] and fellowships [2013/21722-4 (I.C.F.)].

Compliance with ethical standards

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Funding This study was funded by São Paulo Research Foundation (FAPESP-Brazil; Grants Nos: 2010/18086-0, 2012/24345-4, 2012/02388-3 and 2013/21722-4).

Conflict of interest The authors declare that they have no conflict of interest.

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