Differential effects of GH and GH-releasing peptide-6 on astrocytes

Eva Baquedano^{1,3}, Julie A Chowen^{2,3}, Jesús Argente^{1,2,3} and Laura M Frago^{1,3}

¹Department of Pediatrics, Universidad Autónoma de Madrid, Madrid, Spain

²Department of Endocrinology, Hospital Infantil Universitario Niño Jesús-UAM, Instituto de Investigación La Princesa, Avenida Menéndez Pelayo 65, 28009 Madrid, Spain

³Centro de Investigación Biomédica en Red de fisiopatología de la obesidad y nutrición (CIBERObn), Instituto de Salud Carlos III, Madrid, Spain

Correspondence should be addressed to L M Frago
Email
laura.frago@uam.es

Abstract

GH and GH secretagogues (GHSs) are involved in many cellular activities such as stimulation of mitosis, proliferation and differentiation. As astrocytes are involved in developmental and protective functions, our aim was to analyse the effects of GH and GH-releasing hexapeptide on astrocyte proliferation and differentiation in the hypothalamus and hippocampus.

Treatment of adult male Wistar rats with GH (i.v., 100 µg/day) for 1 week increased the levels of glial fibrillary acidic protein (GFAP) and decreased the levels of vimentin in the hypothalamus and hippocampus. These changes were not accompanied by increased proliferation.

By contrast, GH-releasing hexapeptide (i.v., 150 µg/day) did not affect GFAP levels but increased proliferation in the areas studied. To further study the intracellular mechanisms involved in these effects, we treated C6 astrocytoma cells with GH or GH-releasing hexapeptide and the phosphatidylinositol 3'-kinase (PI3K) inhibitor, LY294002, and observed that the presence of this inhibitor reverted the increase in GFAP levels induced by GH and the proliferation induced by GH-releasing hexapeptide. We conclude that although GH-releasing hexapeptide is a GHS, it may exert GH-independent effects centrally on astrocytes when administered i.v., although the effects of both substances appear to be mediated by the PI3K/Akt pathway.

Key Words

- ▶ GH
- ▶ GHRP-6
- ▶ hippocampus
- ▶ hypothalamus
- ▶ proliferation
- ▶ differentiation

Journal of Endocrinology
(2013) **218**, 263–274

Introduction

It is well established that GH, one of the main regulators of growth and metabolism (Conway-Campbell *et al.* 2007, Lichanska & Waters 2008), also has profound effects in the CNS (Harvey *et al.* 1993, Nyberg 2000, Donahue *et al.* 2006), being involved in the regulation of emotion (Burman & Deijen 1998), cognitive functions, memory (Deijen *et al.* 2011), appetite (Stoving *et al.* 1999) and neuroprotection (Scheepens *et al.* 1999, Frago *et al.* 2002, Lyuh *et al.* 2007). Indeed, the GH receptor (GHR) is expressed in diverse areas throughout the CNS (Fraser *et al.* 1990, Le Greves *et al.* 2005) where this hormone has been

shown to be involved in numerous cellular activities such as stimulation of mitosis, cell proliferation and differentiation (Frago *et al.* 2002, Ajo *et al.* 2003). Binding of GH to its receptor mediates many of its effects through activation of the JAK/STAT signalling pathway (Lanning & Carter-Su 2006); however, GHR is also able to signal through additional pathways including the MAPK and the phosphatidylinositol 3'-kinase (PI3K; Brooks *et al.* 2008) pathways.

Release of GH from the anterior pituitary can be stimulated by ghrelin, as well as by its analogues the GH

secretagogues (GHSs), which include GH-releasing peptides (GHRP)-1, -2 and -6. Both ghrelin and GHSs bind to GHS receptors not only expressed in the pituitary but also widely distributed in the CNS (Ghigo *et al.* 2001, Zigman *et al.* 2006, Frago *et al.* 2011). Upon binding to the GHS receptor, which has been renamed ghrelin receptor (GRLN-R1a; Davenport *et al.* 2005), GHSs activate pathways that regulate the activation of downstream MAPK, protein kinase B (PKB/Akt), nitric oxide synthase and adenosine monophosphate-activated protein kinase cascades in different cellular systems (Petersenn 2002). GRLN-R1a activation leads to an increase in intracellular levels of Ca^{2+} via phosphatidylinositol-specific phospholipase C (Camiña *et al.* 2003, Muccioli *et al.* 2007). Ghrelin and GHRP-6 stimulate proliferation of different cell types (Dieguez & Casanueva 2000, Pettersson *et al.* 2002, Thompson *et al.* 2004, Rossi *et al.* 2009) and may also affect the CNS by protecting neurons from apoptosis (Frago *et al.* 2002, 2011, Delgado-Rubin de Céliz *et al.* 2006, Delgado-Rubin *et al.* 2009).

Astrocytes comprise a heterogeneous family of morphologically and functionally distinct cells whose structural plasticity is mostly maintained by a filamentous network mainly consisting of vimentin and glial fibrillary acidic protein (GFAP; Gomes *et al.* 1999a, Järlestedt *et al.* 2010). Astrocytes have a variety of active roles in maintaining normal brain physiology (Barres 1991, Perea & Araque 2005). Proliferation and differentiation of astrocytes are related in part to the modulation of GFAP expression during CNS development by the regulation of *Gfap* gene promoter (Gomes *et al.* 1999b).

As glial cells express GHR (McLenachan *et al.* 2009) and astrocytes in GHR-deficient mice are smaller and less abundant (List *et al.* 2011), our aim was to analyse the effects of GH and GHRP-6 on astrocyte proliferation and differentiation. In addition, as rats treated with GH or GHRP-6 showed increased levels of phosphorylated Akt in the hypothalamus, hippocampus and cerebellum, but not of ERKs1/2 (Frago *et al.* 2002), and Akt is a central regulator of survival, growth and proliferation (Pascual & Guerri 2007), we evaluated the possible involvement of Akt in these processes.

Materials and methods

Materials

All chemicals were purchased from Sigma Chemical unless otherwise noted. Antibodies to GFAP were from Sigma. The antibody to pan-phospho-Ser was from Calbiochem

(La Jolla, CA, USA), and antibodies to phospho-STAT5 and MAPK 1/2 were from Upstate (Lake Placid, NY, USA). The antibody to STAT5 was from Thermo Fisher Scientific (Waltham, MA, USA), to proliferating cell nuclear antigen (PCNA) from Signet (Dedham, MA, USA), to vimentin from Dakocytomation (Glostrup, Denmark), to β III-tubulin (Tuj1) from R&D Systems (Minneapolis, MN, USA), to phospho-Akt and phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204) from Cell Signalling Technology (Danvers, MA, USA), and to Akt, GHR and GHS-R1a (GRLN-R1a) from Santa Cruz Biotechnology, Inc. Secondary antibodies conjugated to peroxidase were from Thermo Fisher Scientific.

Methods

Animals Male Wistar rats (Charles River, Margate, UK) weighing 200–250 g were used for all experiments. The animals were treated according to the European Community laws for animal care, and studies were approved by the institutional ethics committee. Rats were infused with GH (Novo Nordisk, Bagsvaerd, Denmark; 100 $\mu\text{g}/\text{day}$) or GHRP-6 (Bachem, Bubendorf, Switzerland; 150 $\mu\text{g}/\text{day}$) using Alzet minipumps (1 $\mu\text{l}/\text{h}$, 7d) connected to the jugular vein. Control animals received a minipump-delivering vehicle (saline) at the same infusion rate. Rats ($n=6$ in each group) were killed by decapitation and brains were immediately removed and frozen on dry ice.

For immunofluorescence studies, three control rats were perfused transcardially with phosphate-buffered 4% paraformaldehyde (pH 7.4) under pentobarbital anaesthesia (1 mg/kg). The brains were postfixed overnight at 4 °C and then stored in cryoprotection solution (30% (w/v) sucrose and 30% (v/v) ethylene glycol in phosphate buffer) at -20 °C.

Cell culture All cell culture material was purchased from Gibco (Invitrogen Co.). GH was from Pharmacia, GHRP-6 and D-Lys3-GHRP-6 were from Bachem, antibody to GHR was from R&D Systems and LY294002 was from Calbiochem.

Stock cultures of the astrocyte cell line C6 were routinely grown in 100 mm culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in DMEM (Invitrogen) with 4.5 g/l D-glucose without phenol red supplemented with 10% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin–streptomycin and glutamine (200 mol/l³). The cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Hormonal treatment of astrocyte cell line For all experiments, cells were plated in 100 mm dishes, 60 mm culture dishes or 24-well plates for 72 h in DMEM containing 10% (v/v) FBS and then in DMEM without FBS but containing 1 mol/l³ cAMP. Cultures were maintained for 48 h in this differentiation medium without FBS. Subsequently, the cells were treated with 5, 10, 25 or 50 µg/ml GH (226.24 mol/l⁹, 452.49 mol/l⁹, 1.13 mol/l⁶ and 2.26 mol/l⁶ respectively); 10, 25 or 50 µg/ml GHRP-6 (11.45, 28.63 and 57.27 mol/l⁶ respectively); antibody to GHR (0.3 µg/ml); D-Lys3-GHRP-6 (50 mol/l⁶) and/or LY294002 (50 mol/l⁶) for 24 h. Three different experiments were performed in duplicate, except crystal violet assay where four different experiments were performed in quadruplicate.

Protein extraction and quantification For western blotting, ~100 mg hypothalamus or hippocampus were homogenised in 500 µl radioimmunoprecipitation assay lysis buffer with an EDTA-free protease inhibitor cocktail (Roche Diagnostics). After homogenisation, the samples were centrifuged at 12 000 g for 5 min at 4 °C to remove the insoluble material. Clear supernatants were transferred to a new tube to measure protein content.

Protein of cell culture was extracted in a different way. Cells were collected in 100 µl Laemmli buffer with 10 µl phenylmethylsulphonyl fluoride and then were sonicated. Protein concentration was estimated by the method of Bradford (Bio-Rad Laboratories, Inc.).

Immunoblotting Thirty or 60 µg protein obtained from hypothalamus or hippocampus or 20 µl cells' proteins were resolved using 10% (w/v) SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). Filters were blocked with Tris-buffered saline (TBS) containing 5% (w/v) non-fat dried milk, except for phosphorylated proteins where TBS containing 5% (w/v) BSA was used and incubated with the primary specific antibody at a dilution of 1:1000. Filters were subsequently washed and incubated with the corresponding secondary antibody conjugated with peroxidase at a dilution of 1:2000. Bound peroxidase activity was visualised by chemiluminescence using an Immun-Star WesternC kit (Bio-Rad). Quantification of the bands obtained was carried out by densitometry using a Kodak Gel Logic 1500 Image Analysis system and Molecular Imaging Software version 4.0 (Rochester, NY, USA). All blots were re-blotted with actin to normalise each sample for gel-loading variability.

Immunofluorescence Immunohistochemistry was performed on vibratome 50 µm free-floating sections of paraformaldehyde-perfused control rats. Sections were washed in 0.1 mol/l PB (K₂HPO₄ and NaH₂PO₄, pH 7.4) for 10 min and then washed four times in PBT containing 0.3% v/v Triton X-100 and 0.3% w/v BSA in 0.1 mol/l PB for 10 min; afterwards, sections were left for 48 h at 4 °C with the primary antibody for GFAP (1:500, mouse) and GHR or GRLN1a (1:500, rabbit) in blocking solution. Afterwards, sections were washed and incubated with Alexa-633-conjugated anti-mouse and Alexa-488-conjugated anti-rabbit antibodies (Thermo Fisher Scientific; 1:500 dilution) for 90 min at RT and mounted with crystal mount. Signal was visualised using a confocal microscope (Leica Corp., Madrid, Spain).

Immunoassay for determination of IGF1 The quantitative determination of serum IGF1 was performed with the OCEIA immunoassay (IEMA) from IDS, Immunodiagnostic Systems Limited (Bordon, Tyne & Wear, UK), developed in association with GroPep Limited (Adelaide, South Australia). The method was performed according to the manufacturer's instructions. Briefly, serum samples were incubated with a reagent to inactivate binding proteins (10 min, 25 °C) and then diluted for assay. In the OCEIA rat/mouse IGF1 kit, a purified monoclonal anti-rat IGF1 is coated onto the inner surface of polystyrene microtitre wells (the solid phase or capture antibody). The pretreated, diluted samples were then incubated with biotinylated polyclonal rabbit anti-rat IGF1 in antibody-coated wells and shaken for 2 h at room temperature (25 °C). The wells were washed and enzyme (HRP) labelled avidin, which binds to the biotin complex, was added (30 min, 25 °C). After washing, a single-component chromogenic substrate (a formulation of tetramethylbenzidine) was added to develop colour (30 min, 25 °C). The absorbance of the stopped reaction mixture was read (450 nm; reference 650 nm) in a microtitre plate reader, with colour intensity being directly proportional to the amount of rat IGF1 present in the sample. This assay has a sensitivity limit of 63 ng/ml. The intra- and interassay coefficients of variation were 6.8 and 7.3 respectively.

Crystal violet assay Cells were grown and differentiated in 24-well culture dishes, and after 24 h of exposure to different treatments, the medium was removed and the cells were fixed with 1% (v/v) glutaraldehyde for 20 min at 25 °C. After washing with PBS, 0.1% crystal violet was added to each well for 20 min at 25 °C. The wells were then

washed under running water for 20 min. After drying, 2 ml 8% (v/v) acetic acid was added to each well. The intensity of the resulting colour was measured at 590 nm on an automatic microplate analyser (TECAN Infinite M200, Grödig, Austria).

RNA extraction Total RNA was extracted from cultured astrocytes after 24 h of exposure to GH (5, 10, 25 and 50 µg/ml) or GHRP-6 (10, 25 and 50 µg/ml) following the instructions of TriReagent (Invitrogen). Briefly, cells were homogenised in 1 ml TriReagent and incubated for 5 min at 25 °C to dissociate nucleoprotein complexes. Chloroform (0.2 ml) was added and the samples were shaken vigorously for 15 s and incubated for 15 min at 25 °C. Tubes were centrifuged at 12 000 *g* for 15 min at 4 °C. The aqueous phase was transferred to new tubes and isopropanol (0.5 ml) was added to precipitate RNA. Samples were incubated for 10 min at 25 °C and then centrifuged at 12 000 *g* for 10 min at 4 °C. The supernatants were removed and the pellets were washed in 1 ml of 75% (v/v) ethanol. After vortexing, samples were centrifuged at 7500 *g* for 5 min at 4 °C. Pellets were air-dried, dissolved in RNase-free water and absorbance at 260 nm was measured to determine concentrations.

Real-time RT-PCR cDNA was synthesised from 2 µg total RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCR was performed using assay-on-demand kits (Applied Biosystems) for GHR (Rn00567298_m1), GRLN-R1a (Rn00821417_m1) and IGF1 (Rn99999087_m1) and TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with conventional Applied Biosystems cycling parameters (40 cycles for 95 °C, 15 s; 60 °C, 1 min). Values were normalised to the housekeeping gene *Gapdh* (Rn99999916-s1). According to the manufacturer's guidelines, the $\Delta\Delta C_T$ method was used to determine relative expression levels. Statistical analyses were performed using $\Delta\Delta C_T$ values.

Statistical analysis Protein samples from each animal were analysed in three different assays and the mean value was used for statistical analysis (n =number of animals in each group). Individual culture dishes or wells were analysed separately (no pooling of samples was used). In each experiment, a minimum of two wells of each treatment were used. Each *in vitro* experiment was repeated a minimum of three times. In each experiment,

the mean value of the repetitions was calculated, and this value was used in the statistical analysis. All data were normalised to control values of each assay and are presented as mean $\pm$ S.E.M. Data were analysed by one-way ANOVA followed by a Bonferroni test using the statistical program GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). Significance was chosen as $P < 0.05$.

Results

GH increases GFAP levels in the hypothalamus and hippocampus of rats

Immunoblots for GFAP were prepared from hypothalamus and hippocampus homogenates of rats treated with GH or GHRP-6. GH increased GFAP levels in the hypothalamus (150% of control levels) and hippocampus (228% of control levels, Fig. 1A). A shift in the electrophoretic mobility of GFAP bands was observed in samples from GH-treated rats. No significant changes were observed in response to GHRP-6.

Figure 1

Relative levels of GFAP (A) and phospho-serine (B) proteins in the hypothalamus and hippocampus of control rats, rats treated with GH and rats treated with GHRP-6. Statistical significance by ANOVA: * $P < 0.05$ and *** $P < 0.001$ vs control; $n = 6$ /group.

GH increased the phosphorylation of serine residues in the hypothalamus and hippocampus

In order to analyse the shift in electrophoretic mobility observed in samples from GH-treated rats, the serine phosphorylation pattern was studied using an antibody that recognised phosphorylations on serine residues. Increased labelling in phospho-Ser was coincident with GFAP shifting observed in GH-treated samples. Phospho-Ser levels increased 143% in the hypothalamus and 190% in the hippocampus compared with control levels. No significant changes were observed in response to GHRP-6. (Fig. 1B).

Figure 2

Co-immunostaining for GFAP and GHR and for GFAP and GRLN-R1a in hypothalamus (A) and hippocampus (B) of control rats. Scale bar: 50 μ m (a and c) or 100 μ m (b and d). Panels b and d are a higher magnifications of a and c respectively and indicated by dotted squares. Arrows indicate astrocytes expressing GHR or GRLN-R1a.

GFAP and GH or GRLN-R1a receptor immunohistochemistry

To confirm the presence of GHR and GRLN-R1a in astrocytes of the hypothalamus and hippocampus, double-immunofluorescence labelling studies were performed. Co-localisation of GHR and GFAP and co-localisation of GRLN-R1a and GFAP were observed in the arcuate nucleus of the hypothalamus (Fig. 2A). Labelling for GRLN-R1 was observed in tanycytes, in the specialised glial cells lining the third ventricle (Fig. 2A, photomicrograph c) and in neurons throughout the periventricular areas. The median eminence showed labelling for GFAP but not for GHR or GRLN-R1a (data not shown). In the hippocampus, GFAP labelling was observed for both GHR and GRLN-R1a in the dentate gyrus (Fig. 2B). Neurons of the granular layer showed weak labelling for GHR and GRLN-R1a (Fig. 2B, microphotographs a and c).

GH increases STAT5 levels in the hypothalamus and hippocampus

Immunoblots of STAT5 and activated STAT5 (phosphorylated on Ser726/731) were prepared from hypothalamus and hippocampus homogenates of rats treated with GH and GHRP-6. Treatment with GHRP-6 did not activate STAT5 in any of the areas studied and treatment with GH resulted in activation of STAT5 in the hypothalamus (control: 100 ± 14.3 , GH: 167.2 ± 10.5 and GHRP-6: 102.8 ± 7.8 ; $P < 0.05$) and hippocampus (control: 100 ± 9.9 , GH: 152.1 ± 15.04 and GHRP-6: 102.8 ± 7.8 ; $P < 0.05$). The amount of total STAT5a and -b was not altered by any of the treatments.

Role of GH and GHRP-6 in cell proliferation and differentiation

Cell proliferation was assayed by western blotting against the PCNA. Treatment with GH did not alter the amount of PCNA; however, treatment with GHRP-6 increased PCNA levels in the hypothalamus (318% of control levels) and in the hippocampus (247% of control levels) (Fig. 3A).

To study the possible role of GH and GHRP-6 in astrocyte differentiation, we measured vimentin levels. GH-treated rats had lower levels of vimentin in the hippocampus (58% of control values) and in the hypothalamus, although not statistically significant (75% of control levels). Treatment with GHRP-6 did not alter the levels of vimentin in these areas (Fig. 3B).

Figure 3 Relative levels of PCNA (A), vimentin (B) and Tuj1 (C) proteins in the hypothalamus and hippocampus of control rats, rats treated with GH and rats treated with GHRP-6. Statistical significance by ANOVA: * $P < 0.05$ and *** $P < 0.001$ vs control; $n = 6$ /group.

To study the effects of GH and GHRP-6 on neurons, we measured Tuj1 levels. Treatment with GH or GHRP-6 did not alter the levels of Tuj1 in the hypothalamus or hippocampus (Fig. 3C).

GH and GHRP actions are not mediated by serum IGF1

To assess whether the effects of GH and GHRP-6 are not mediated by increased levels of circulating IGF1, we measured serum IGF1 levels in all experimental groups. IGF1 levels were not different from control levels in the rats treated with GH or GHRP-6 (controls: 1735 ± 161 ng/ml; GH: 1589 ± 89 ng/ml and GHRP-6: 1809 ± 63 ng/ml; $P = 0.413$).

Role of GH and GHRP-6 in C6 glioblastoma cell line

To further investigate the actions of GH and GHRP-6 on astrocytes, we used the glioblastoma cell line C6. Doses–response curves were performed to detect the expression of GHR and GRLN-R1a by real-time RT-PCR and to determine the concentration to use. Twenty-five or 50 $\mu\text{g/ml}$ GH increased (162 and 181% of control values respectively) the expression of GHR mRNA (Fig. 4A). As 25 $\mu\text{g/ml}$ is the lowest dose that increased GHR expression, the subsequent assays were performed at this dose. Treatment with 25 $\mu\text{g/ml}$ GH resulted in statistically

Figure 4 Relative levels of GHR mRNA in C6 cells treated with different doses (5–50 $\mu\text{g/ml}$) of GH (A). Relative levels of GFAP protein in cells treated with GH (25 $\mu\text{g/ml}$) or with GHRP-6 (25 $\mu\text{g/ml}$) (B). Relative levels of GFAP protein in cells treated with GH (25 $\mu\text{g/ml}$) or with a blocking antibody to GHR (0.3 $\mu\text{g/ml}$) (C). The mean of three independent experiments performed in duplicate is shown. Statistical significance by ANOVA: ** $P < 0.01$ and *** $P < 0.001$ vs control; # $P < 0.05$ vs GH.

Figure 5

Quantification of cell viability assayed by crystal violet in C6 cells treated with GH (25 µg/ml) or with GHRP-6 (25 µg/ml) (A). Quantification of cell viability assayed by crystal violet in C6 cells treated with GHRP-6 (25 µg/ml) or with D-Lys3-GHRP-6 (50 µM) (B). Relative vimentin protein levels in C6 cells treated with GH (25 µg/ml) or GHRP-6 (25 µg/ml) (C). The average of three (C) or four (A and B) independent experiments performed in duplicate (C) or in quadruplicate (A and B) is shown. Statistical significance by ANOVA: *** $P < 0.001$ vs control; # $P < 0.05$ vs GHRP-6.

significant activation of STAT5 (control: 100 ± 2.1 and GH: 155.4 ± 21.8 ; $P < 0.05$).

The levels of GRLN-R1a mRNA were undetectable at baseline; however, its expression increased after stimulation with 25 or 50 µg/ml of GHRP-6 (data not shown).

GH increased the levels of GFAP in C6 cells To further study the effect of GH on astrocytes, cells were

treated with 25 µg/ml GH for 24 h. GH increased the levels of GFAP assayed by western blotting (146% of control values). GHRP-6 did not affect the levels of GFAP (Fig. 4B).

To determine the specificity of GH acting through its specific receptor, C6 cells were treated with a blocking antibody specific to GHR. Co-treatment with GH and a blocking antibody to GHR reduced levels of GFAP (Fig. 4C).

GHRP-6 increased proliferation of C6 cells The crystal violet assay, used as a proliferation index, revealed an increase in cell number in response to 25 µg/ml GHRP-6 (135% of control values). By contrast, treatment with GH had no effect on proliferation (Fig. 5A).

To determine the specificity of GHRP-6 acting through its specific receptor, C6 cells were treated with D-Lys3-GHRP-6, an antagonist of the ghrelin receptor. Co-treatment with GHRP-6 and D-Lys3-GHRP-6 reversed the increase in cell number stimulated by GHRP-6 (Fig. 5B). Interestingly, treatment of cells with the antagonist alone increased the number of cells.

GH and GHRP-6 had no effect on vimentin levels in C6 cells Levels of vimentin (Fig. 6A) in C6 cells did not change in response to 25 µg/ml GH or GHRP-6.

Signalling pathways involved GH and GHRP-6 increased the levels of IGF1 mRNA (196 and 235% of control levels respectively $P < 0.01$) in C6 cells. GH and GHRP-6 increased the levels of p-Akt (144 and 133% of control values respectively) (Fig. 6A) but did not activate ERK1/2 (data not shown).

To confirm the involvement of the PI3K/Akt pathway, C6 cells were treated with LY294002, an inhibitor of PI3K, co-treatment of C6 cells with GH+LY294002 reduced the GFAP levels induced by GH (Fig. 6B) and co-treatment of C6 with GHRP-6+LY294002 reversed the increase in cell number induced by GHRP-6 (Fig. 6C).

Discussion

GH and ghrelin are important factors involved in the hypothalamic regulation of energy homeostasis (Nyberg 2000, Nakazato *et al.* 2001) and in functions ascribed to the hippocampus such as learning and memory processes (Diano *et al.* 2006, Donahue *et al.* 2006). These two brain areas express GHR mRNA (Bennett *et al.* 1996, Minami *et al.* 2006) in different cells types including astrocytes (Lobie *et al.* 1993, Blackmore *et al.* 2012). GRLN-R1a is also located in both the CNS and the periphery (Zigman *et al.* 2006). It is also present in astrocytomas where it is

Figure 6

Relative p-Akt protein levels in C6 cells treated with GH (25 µg/ml) or GHRP-6 (25 µg/ml) (A). Relative GFAP protein levels in C6 cells treated with GH (25 µg/ml) or LY294002 (LY; 50 µM) (B). Quantification of cell viability assayed by crystal violet in C6 cells treated with GHRP-6 (25 µg/ml) or LY294002 (50 µM). The average of three (A and B) or four (C) independent experiments performed in duplicate (A and B) or in quadruplicate (C) is shown. Statistical significance by ANOVA: ** $P < 0.01$ and *** $P < 0.001$ vs control; # $P < 0.05$ vs GH or GHRP-6.

involved in cell motility (Dixit *et al.* 2006, Chen *et al.* 2011). In our study, we observed GHR and GRLN-R1a immunolabelling in astrocytes of the hypothalamic arcuate nucleus and of the dentate gyrus in the hippocampus. Although the role of GH and ghrelin in astrocytes remains unclear, it is known that hormonal actions in the brain are exerted through both neurons and glial cells. Glial cells express many of the same hormone receptors as found on neurons (García-Segura & McCarthy 2004). In addition, labelling for GRLN-R1a was observed in tanycytes and in cells throughout the periventricular area.

In this study, we show that treatment with GH but not GHRP-6 increases the levels of GFAP in the hypothalamus and hippocampus of male rats. GFAP has been widely recognised as an astrocyte differentiation marker constituting the major intermediate filament protein of mature astrocyte (Galou *et al.* 1996, Freeman 2010). Modulators of GFAP expression include steroids, cytokines and growth factors (Laping *et al.* 1994, García-Segura & McCarthy 2004). Overexpression of GH in transgenic mice increases GFAP levels (Miller *et al.* 1995). In our *in vivo* model, the observed increased expression of GFAP induced by GH was accompanied by a shift in electrophoretic mobility probably due to an increase in the levels of the phosphorylated forms of GFAP. GFAP phosphorylation contributes to stabilisation of GFAP and protects it from proteolytic degradation (Inagaki *et al.* 1990, 2008). Hence, phosphorylation of GFAP induced by GH may play important roles in astrocyte remodelling and could potentially contribute to the plasticity of the CNS (Takemura *et al.* 2002, Sullivan *et al.* 2012).

Here, we show that GH does not induce cell proliferation markers in the hypothalamus or hippocampus of adult rats. In fact, activation of the *Gfap* gene promoter has been related to decreased astrocyte proliferation (Gomes *et al.* 1999b). Although GH promotes the proliferation of neural precursors, neurogenesis and gliogenesis during brain development (Ajo *et al.* 2003), in our adult rats, GH did not induce proliferation but may induce astrocyte differentiation or activation. To further characterise the cellular differentiation stimulated by GH, we studied the levels of another protein of the cytoskeleton, vimentin. This protein is expressed in cells from mesenchymal origin and poorly differentiated (Mukhina *et al.* 2004), as well as activated astrocytes. In normal adult hypothalamus, the majority of vimentin is in the tanycytes. Here, we showed that the levels of vimentin were decreased in the hypothalamus and hippocampus of rats treated with GH, suggesting that the increase in GFAP is not due to astrocytic activation, while GHRP-6 had no effect on vimentin. An increase in GFAP levels (marker of mature astrocytes) and a decrease in vimentin levels (marker of immature astrocytes) after treatment with GH might indicate a change in the proteins forming the intermediate filaments of the astrocyte cytoskeleton, and, hence, GH might induce astrocyte maturation in the hypothalamus and in the hippocampus. Other hormones are also involved in similar cellular processes; for example, during development, T3 accelerates the transition from vimentin-positive to GFAP-positive cells in the forebrain and hippocampus with an increase in the intensity of

GFAP immunoreactivity (Gould *et al.* 1990, Ghosh *et al.* 2005). Moreover, T3 can induce GFAP synthesis and astrocyte differentiation (Lima *et al.* 1997). Oestradiol also promotes differentiation of glial cells with a redistribution of GFAP in hypothalamic astrocytes (Torres-Aleman *et al.* 1992, Mong & Blutstein 2006). In addition, the transforming growth factor β has been reported to inhibit astrocyte proliferation and to induce the expression of GFAP *in vivo* (Reilly *et al.* 1998, Sousa *et al.* 2004) and *in vitro* (Laping *et al.* 1994).

Treatment with GHRP-6 did not increase GFAP levels but stimulated proliferation in the hypothalamus and hippocampus. GHRP-6 and ghrelin stimulate the proliferation of a wide number of cells including osteoblasts (Kim *et al.* 2005), cardiomyocytes (Pettersson *et al.* 2002), somatotrophs (Dieguez & Casanueva 2000), endothelial cells (Rossi *et al.* 2009) and adipocytes (Thompson *et al.* 2004) and may also affect the CNS by protecting neurons from apoptosis (Frago *et al.* 2002, 2011, Delgado-Rubin de Céliz *et al.* 2006, Delgado-Rubin *et al.* 2009). We also evaluated the possible effect of GH and GHRP-6 on neuron number and did not observed any changes in the levels of Tuj1 in the hypothalamus or hippocampus.

In previous studies, we demonstrated that rats treated with GH or GHRP-6 showed increased levels of phosphorylated Akt but not of ERK1/2 in the hypothalamus, hippocampus and cerebellum (Frago *et al.* 2002); although in some circumstances, GH and ghrelin have been shown to activate not only the PI3K/Akt signalling pathway but also the MAPK pathway (Chung *et al.* 2007, Brooks *et al.* 2008, Andrews 2011). Thus, GH after binding to its receptor activates the PI3K/Akt signalling pathway (Frago *et al.* 2002), which could induce the activation and differentiation of astrocytes in the hypothalamus and hippocampus. By contrast, GHRP-6 binds to GRLN-R1a activating the PI3k/Akt pathway to promote proliferation in the hypothalamus and in the hippocampus confirming the paradigm largely established that Akt is a central regulator of survival, growth and proliferation (Pascual & Guerri 2007). Many of the PI3K-dependent events are mediated by further downstream signalling pathways. Akt has been linked to the phosphorylation of the transcription factor *Creb*, which has been demonstrated to be activated by GH (Yarwood *et al.* 1998, Lobie *et al.* 2000, Zhu *et al.* 2001). Also ghrelin has been demonstrated to stimulate CREB in the hippocampus (Cuellar & Isokawa 2011) and the hypothalamus (Petersen *et al.* 2009). Hence, CREB may be another key molecule involved in the roles of GH and GHRP-6.

The actions of GH and GHRP-6 on proliferation and differentiation could be mediated by an increase in IGF1, which has been demonstrated to affect these processes (Åberg *et al.* 2000). To explore this question, we measured the levels of circulating IGF1 and we found that serum IGF1 levels did not increase in response to GH or GHRP-6 treatment. Some studies on healthy rats demonstrate that i.v. infusion of GH inhibits endogenous GH secretory pulses (Clark *et al.* 1988, Chan *et al.* 2006), possibly explaining why the plasma levels of IGF1 do not increase after a 7-day continuous infusion of GH. Similarly, Thompson *et al.* (2003) reported that a 7-day continuous infusion of GHRP-6 suppressed the amplitude of spontaneous GH secretory episodes and hence plasma levels of IGF1. However, we have previously reported that this same experimental protocol results in an increase in IGF1 mRNA levels in hypothalamus and hippocampus (Frago *et al.* 2002). Hence, increased local production of IGF1 could be involved in activation of these intracellular pathways.

The observed changes in glial proteins in response to i.v. treatments with GH and GHRP-6 may not be the result of direct actions of these factors on astrocytes; hence, we performed studies on C6 astrocytes to support that at least

Figure 7

Diagram representing the effect of GH and GHRP-6 on astrocytes. GH is proposed to induce differentiation acting through GHR and GHRP-6 to induce proliferation by binding GRLN-R1a on astrocytes. GH and GHRP-6 may act through activation of the PI3K/Akt pathway.

some of these effects may indeed be mediated directly on astrocytes. It must be taken into consideration that the cells used are C6 astrocytes that derive from a tumour induced by *N*-nitrosomethylurea. We demonstrated that these astrocytes responded to GH and GHRP-6 by increasing the expression of their respective receptors. Moreover, GH increased the levels of GFAP and GHRP-6 increased proliferation of C6 cells, and these effects were specific as the presence of a blocking antibody against GH reversed the increase in GFAP levels induced by GH and D-Lys3-GHRP-6, an antagonist of GRLN-R1a, reduced the increase in cell number induced by GHRP-6. Interestingly, treatment of cells with the antagonist alone increased the number of cells. This is a paradoxical effect of the antagonist. In some studies using cell lines, D-Lys-GHRP-6 evoked a biphasic effect on proliferation, being inhibitory at 10^{-4} M and stimulatory at 10^{-5} and 10^{-6} M (Mucha & Stepień 2002). In this study, D-Lys3-GHRP-6 was added at a dose of 27×10^{-6} M, a concentration that could have a stimulatory effect. These observed paradoxical effects of D-Lys3-GHRP-6 suggest unknown underlying regulations of the ghrelin system whose understanding requires further investigation.

Similar to that observed *in vivo* (Frago *et al.* 2002), both treatments increased the mRNA levels of IGF1, activated Akt and had no effect on ERKs activation in C6 cells, and, more importantly, the treatment of C6 cells with the PI3K inhibitor, LY294002, reduced the GH-induced rise in GFAP levels and reversed the proliferation induced by GHRP-6. It is possible that the observed effects are mediated by IGF1, as both GH and GHRP-6 increase the levels of IGF1 mRNA *in vivo* (Frago *et al.* 2002) and in C6 astrocytes. Hence, a local increase in IGF1 levels could underlie the increase in Akt activation (Kulik & Weber 1998).

In summary, this study demonstrates that the PI3K/Akt pathway has an important role in regulating cell proliferation and differentiation of astrocytes with GH inducing astrocyte differentiation and GHRP-6 stimulating their proliferation through this signalling pathway (Fig. 7).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was funded by grants from Ministerio de Economía y Competitividad (BFU2008-02950 C03-3 and BFU2011-27492), Universidad Autónoma de Madrid-Comunidad de Madrid (CCG2008-UAM/SAL4451),

Fondo de Investigación Sanitaria (PI100747), CIBER de Fisiopatología de Obesidad y Nutrición (CIBEROBN) Instituto de Salud Carlos III and Fundación de Endocrinología y Nutrición. E B is supported by predoctoral fellowship from the Ministerio de Educación y Ciencia.

Acknowledgements

The authors thank Sandra Canelles and Francisca Díaz for excellent technical assistance.

References

- Åberg MA, Åberg ND, Hedbäck H, Oscarsson J & Eriksson PS 2000 Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. *Journal of Neuroscience* **20** 2896–2903.
- Ajo R, Cacicedo L, Navarro C & Sánchez-Franco F 2003 Growth hormone action on proliferation and differentiation of cerebral cortical cells from fetal rat. *Endocrinology* **144** 1086–1097. (doi:10.1210/en.2002-220667)
- Andrews ZB 2011 The extra-hypothalamic actions of ghrelin on neuronal function. *Trends in Neuroscience* **34** 31–40. (doi:10.1016/j.tins.2010.10.001)
- Barres BA 1991 New roles for glia. *Journal of Neuroscience* **11** 3685–3694.
- Bennett PA, Levy A, Carmignac DF, Robinson IC & Lightman SL 1996 Differential regulation of the growth hormone receptor gene: effects of dexamethasone and estradiol. *Endocrinology* **137** 3891–3896. (doi:10.1210/en.137.9.3891)
- Blackmore DG, Reynolds BA, Golmohammadi MG, Large B, Aguilar RM, Haro L, Waters MJ & Rietze RL 2012 Growth hormone responsive neural precursor cells reside within the adult mammalian brain. *Scientific Reports* **2** 250. (doi:10.1038/srep00250)
- Brooks AJ, Wooh JW, Tunny KA & Waters MJ 2008 Growth hormone receptor; mechanism of action. *International Journal of Biochemistry & Cell Biology* **40** 1984–1989. (doi:10.1016/j.biocel.2007.07.008)
- Burman P & Deijen J 1998 Quality of life and cognitive function in patients with pituitary insufficiency. *Psychotherapy and Psychosomatics* **67** 154–167. (doi:10.1159/000012276)
- Camiña JP, Carreira MC, Micic D, Pombo M, Kalestimur F, Dieguez C & Casanueva FF 2003 Regulation of ghrelin secretion and action. *Endocrine* **22** 5–12. (doi:10.1385/ENDO:22:1:5)
- Chan CB, Tse MC & Cheng CH 2006 Regulation and mechanism of growth hormone and insulin-like growth factor-I biosynthesis and secretion. In *The Somatotrophic Axis in Brain Function*, pp 7–23. Ed. Fred Nyberg. Burlington: Elsevier Academic Press.
- Chen JH, Huang SM, Chen CC, Tsai CF, Yeh WL, Chou SJ, Hsieh WT & Lu DY 2011 Ghrelin induces cell migration through GHS-R, CaMKII, AMPK and NF-κB signaling pathway in glioma cells. *Journal of Cellular Biochemistry* **112** 2931–2941. (doi:10.1002/jcb.23209)
- Chung H, Kim E, Lee DH, Seo S, Ju S, Lee D, Kim H & Park S 2007 Ghrelin inhibits apoptosis in hypothalamic neuronal cells during oxygen–glucose deprivation. *Endocrinology* **148** 148–159. (doi:10.1210/en.2006-0991)
- Clark RG, Carlsson LMS & Robinson ICAF 1988 Growth hormone (GH) secretion in the conscious rat: negative feedback of GH on its own release. *Journal of Endocrinology* **119** 201–209. (doi:10.1677/joe.0.1190201)
- Conway-Campbell BL, Wooh JW, Brooks AJ, Gordon D, Brown RJ, Lichanska AM, Chin HS, Barton CL, Boyle GM, Parsons PG *et al.* 2007 Nuclear targeting of the growth hormone receptor results in dysregulation of cell proliferation and tumorigenesis. *PNAS* **104** 13331–13336. (doi:10.1073/pnas.0600181104)
- Cuellar JN & Isokawa M 2011 Ghrelin-induced activation of cAMP signal transduction and its negative regulation by endocannabinoids in the

- hippocampus. *Neuropharmacology* **60** 842–851. (doi:10.1016/j.neuropharm.2010.12.024)
- Davenport AP, Bonner TI, Foord SM, Harmar AJ, Neubig RR, Pin JP, Spedding M, Kojima M & Kangawa K 2005 International Union of Pharmacology. LVI: ghrelin receptor nomenclature, distribution, and function. *Pharmacological Reviews* **57** 541–546. (doi:10.1124/pr.57.4.1)
- Deijen B, Arwert LI & Drent ML 2011 The GH/IGF-I axis and cognitive changes across a 4-year period in healthy adults. *ISRN Endocrinology* **2011** 249421. (doi:10.5402/2011/249421)
- Delgado-Rubin A, Chowen JA, Argente J & Frago LM 2009 Growth hormone releasing peptide-6 protection of hypothalamic neurons from glutamate excitotoxicity is caspase independent and mediated by insulin-like growth factor I. *European Journal of Neuroscience* **29** 2115–2124. (doi:10.1111/j.1460-9568.2009.06770.x)
- Delgado-Rubin de Célis A, Chowen JA, Argente J & Frago LM 2006 Growth hormone releasing peptide-6 acts as a survival factor in glutamate-induced excitotoxicity. *Journal of Neurochemistry* **99** 839–849. (doi:10.1111/j.1471-4159.2006.04122.x)
- Diano S, Farr SA, Benoit S, McNay EC, da Silva I, Horvath B, Gaskin FS, Nonaka N, Jaeger LB, Banks WA *et al.* 2006 Ghrelin controls hippocampal spine synapse density and memory performance. *Nature Neuroscience* **9** 381–388. (doi:10.1038/nn1656)
- Dieguez C & Casanueva FF 2000 Ghrelin: a step forward in the understanding of somatroph cell function and growth regulation. *European Journal of Endocrinology* **142** 413–417. (doi:10.1530/eje.0.1420413)
- Dixit VD, Weeraratna AT, Tang H, Bertak D, Cooper-Jenkins A, Riggins GJ, Eberhart CG & Taub DD 2006 Ghrelin and the growth hormone secretagogue receptor constitute a novel autocrine pathway in astrocytoma motility. *Journal of Biological Chemistry* **281** 16681–16690. (doi:10.1074/jbc.M600223200)
- Donahue CP, Kosik KS & Shors TJ 2006 Growth hormone is produced within the hippocampus where it responds to age, sex and stress. *PNAS* **103** 6031–6036. (doi:10.1073/pnas.0507761103)
- Frago LM, Pañeda C, Dickson SL, Hewson AK, Argente J & Chowen JA 2002 Growth hormone (GH) and GH-releasing peptide-6 increase brain insulin-like growth factor-I expression and activate intracellular signalling pathways involved in neuroprotection. *Endocrinology* **143** 4113–4122. (doi:10.1210/en.2002-220261)
- Frago LM, Baquedano E, Argente J & Chowen JA 2011 Neuroprotective actions of ghrelin and growth hormone secretagogues. *Frontiers in Molecular Neuroscience* **4** 23. (doi:10.3389/fnmol.2011.00023)
- Fraser RA, Attardo D & Harvey S 1990 Growth hormone receptors in hypothalamic and extra-hypothalamic tissues. *Journal of Molecular Endocrinology* **5** 231–238. (doi:10.1677/jme.0.0050231)
- Freeman MR 2010 Specification and morphogenesis of astrocytes. *Science* **330** 774–778. (doi:10.1126/science.1190928)
- Galou M, Colucci-Guyon E, Ensergueix D, Ridet JL, Gimenez y Ribotta M, Privat A, Babinet C & Dupouey P 1996 Disrupted glial fibrillary acidic protein network in astrocytes from vimentin knockout mice. *Journal of Cell Biology* **133** 853–863. (doi:10.1083/jcb.133.4.853)
- García-Segura LM & McCarthy MM 2004 Minireview: role of glia in neuroendocrine function. *Endocrinology* **145** 1082–1086. (doi:10.1210/en.2003-1383)
- Ghigo E, Arvat E, Giordano R, Broglio F, Gianotti L, Maccario M, Bisi G, Graziani A, Papotti M, Muccioli G *et al.* 2001 Biological activities of growth hormone secretagogues in humans. *Endocrine* **14** 87–93. (doi:10.1385/ENDO:14:1:087)
- Ghosh M, Gharami K, Paul S & Das S 2005 Thyroid hormone-induced morphological differentiation and maturation of astrocytes involves activation of protein kinase A and ERK signaling pathway. *European Journal of Neuroscience* **22** 1609–1617. (doi:10.1111/j.1460-9568.2005.04351.x)
- Gomes FCA, Paulin D & Moura-Neto V 1999a Glial fibrillary acidic protein (GFAP): modulation by growth factors and its implication in astrocyte differentiation. *Brazilian Journal of Medical and Biological Research* **32** 619–631. (doi:10.1590/S0100-879X1999000500016)
- Gomes FCA, Garcia-Abreu J, Galou M, Paulin D & Moura Neto V 1999b Neurons induce GFAP gene promoter of cultured astrocytes from transgenic mice. *Glia* **26** 97–108. (doi:10.1002/(SICI)1098-1136(199904)26:2<97::AID-GLIA1>3.0.CO;2-Z)
- Gould E, Frankfurt M, Westlind-Danielsson A & McEwen BS 1990 Developing forebrain astrocytes are sensitive to thyroid hormone. *Glia* **3** 283–292. (doi:10.1002/glia.440030408)
- Harvey S, Hull KL & Fraser RA 1993 Growth hormone: neurocrine and neuroendocrine perspectives. *Growth Regulation* **3** 161–171.
- Inagaki M, Gonda Y, Nishizawa K, Kitamura S, Sato C, Ando S, Tanabe K, Kikuchi K, Tsuiki S & Nishi Y 1990 Phosphorylation sites linked to glial filament disassembly *in vitro* locate in a non-alpha-helical head domain. *Journal of Biological Chemistry* **265** 4722–4729.
- Inagaki M, Imakamura Y, Takeda M, Nishimura T & Inagaki N 2008 Glial fibrillary acidic protein: dynamic property and regulation by phosphorylation. *Brain Pathology* **4** 239–243. (doi:10.1111/j.1750-3639.1994.tb00839.x)
- Järlestedt K, Rousset CI, Faiz M, Wilhelmsson U, Ståhlberg A, Sourkova H, Pekna M, Mallard C, Hagberg H & Pekny M 2010 Attenuation of reactive gliosis does not affect infarct volume in neonatal hypoxic–ischemic brain injury in mice. *PLoS ONE* **5** e10397. (doi:10.1371/journal.pone.0010397)
- Kim SW, Her SJ, Park SJ, Kim D, Park KS, Lee HK, Han BH, Kim MS, Shin CS & Kim SY 2005 Ghrelin stimulates proliferation and differentiation and inhibits apoptosis in osteoblastic MC3T3-E1 cells. *Bone* **37** 359–369. (doi:10.1016/j.bone.2005.04.020)
- Kulik G & Weber MJ 1998 Akt-dependent and independent survival signalling pathways utilized by insulin-like growth factor. *Molecular and Cellular Biology* **18** 6711–6718.
- Lanning NJ & Carter-Su C 2006 Recent advances in growth hormone signalling. *Reviews in Endocrine & Metabolic Disorders* **7** 225–235. (doi:10.1007/s11554-007-9025-5)
- Laping NJ, Teter B, Nichols NR, Rozovsky I & Finch CE 1994 Glial fibrillary acidic protein: regulation by hormones, cytokines and growth factors. *Brain Pathology* **4** 259–275. (doi:10.1111/j.1750-3639.1994.tb00841.x)
- Le Greves M, Le Greves P & Nyberg F 2005 Age-related effects of IGF-1 on the NMDA-, GH- and IGF-1-receptor mRNA transcript in the rat hippocampus. *Brain Research Bulletin* **15** 369–374. (doi:10.1016/j.brainresbull.2005.01.012)
- Lichanska AM & Waters MJ 2008 New insights into growth hormone receptor function and clinical implications. *Hormone Research* **69** 138–145. (doi:10.1159/000112586)
- Lima FRS, Trentin AG, Rosenthal D, Chagas C & Moura Neto V 1997 Thyroid hormone induces protein secretion and morphological changes in astroglial cells with an increase in expression of glial fibrillary acidic protein. *Journal of Endocrinology* **154** 167–175. (doi:10.1677/joe.0.1540167)
- List EO, Sackmann-Sala L, Berryman DE, Funk K, Kelder B, Gosney ES, Okada S, Ding J, Cruz-Topete D & Kopchick JJ 2011 Endocrine parameters and phenotypes of the growth hormone receptor gene disrupted (GHR^{−/−}) mouse. *Endocrine Reviews* **32** 356–386. (doi:10.1210/er.2010-0009)
- Lobie PE, García-Aragón J, Lincoln DT, Barnard R, Wilcox JN & Waters MJ 1993 Localization and ontogeny of growth hormone receptor gene expression in the central nervous system. *Developmental Brain Research* **74** 225–233. (doi:10.1016/0165-3806(93)90008-X)
- Lobie PE, Zhu T, Graichen R & Goh ELK 2000 Growth hormone, insulin-like growth factor I and the CNS: localization, function and mechanism of action. *Growth Hormone & IGF Research* **10** S51–S56. (doi:10.1016/S1096-6374(00)80010-6)
- Lyuh E, Kim HJ, Kim M, Lee JK, Park KS, Yoo KY, Lee KW & Ahn YO 2007 Dose-specific or dose-dependent effect of growth hormone treatment on the proliferation and differentiation of cultured neuronal cells. *Growth Hormone & IGF Research* **17** 315–322. (doi:10.1016/j.ghir.2007.03.002)
- McLenachan S, Gum M-G, Waters MJ & Turnley AM 2009 Growth hormone promotes proliferation of adult neurosphere cultures. *Growth Hormone & IGF Research* **19** 212–218. (doi:10.1016/j.ghir.2008.09.003)

- Miller DB, Bartke A & O'Callaghan JP 1995 Increased glial fibrillary acidic protein (GFAP) levels in the brains of transgenic mice expressing the bovine growth hormone (bGH) gene. *Experimental Gerontology* **30** 383–400. (doi:10.1016/0531-5565(94)00064-A)
- Minami S, Kamegai J, Hasegawa O, Sugihara H, Okada K & Wakabayashi I 2006 Expression of growth hormone receptor gene in rat hypothalamus. *Journal of Neurochemistry* **5** 691–696.
- Mong JA & Blustein T 2006 Estradiol modulation of astrocytic form and function: implication for hormonal control of synaptic communication. *Neuroscience* **138** 967–975. (doi:10.1016/j.neuroscience.2005.10.017)
- Muccioli G, Baragli A, Granata A, Papotti M & Ghigo E 2007 Heterogeneity of ghrelin/growth hormone secretagogue receptors. *Neuroendocrinology* **86** 147–164. (doi:10.1159/000105141)
- Mucha S & Stepien H 2002 Modulation of ghrelin axis influences the growth of colonic and prostatic cancer cells *in vitro*. *Pharmacological Reports* **64** 951–959.
- Mukhina S, Mertani HC, Guo K, Lee KO, Gluckman PD & Lobie PE 2004 Phenotypic conversion of human mammary carcinoma cells by autocrine human growth hormone. *PNAS* **101** 15166–15171. (doi:10.1073/pnas.0405881101)
- Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K & Matsukura S 2001 A role for ghrelin in the central regulation of feeding. *Nature* **409** 194–198. (doi:10.1038/35051587)
- Nyberg F 2000 Growth hormone in the brain: characteristics of specific brain targets for the hormone and their functional significance. *Frontiers in Neuroendocrinology* **21** 330–348. (doi:10.1006/fme.2000.0200)
- Pascual M & Guerri C 2007 The peptide NAP promotes neuronal growth and differentiation through extracellular signal-regulated protein kinase and Akt pathways, and protects neurons co-cultured with astrocytes damaged by ethanol. *Journal of Neurochemistry* **103** 557–568. (doi:10.1111/j.1471-4159.2007.04761.x)
- Perea G & Araque A 2005 Synaptic regulation of the astrocyte calcium signal. *Journal of Neural Transmission* **112** 127–135. (doi:10.1007/s00702-004-0170-7)
- Petersen PS, Woldbye DP, Madsen AN, Egerod KL, Jin C, Lang M & Holst B 2009 *In vivo* characterization of high basal signalling from the ghrelin receptor. *Endocrinology* **150** 4920–4930. (doi:10.1210/en.2008-1638)
- Petersenn S 2002 Structure and regulation of the growth hormone secretagogue receptor. *Minerva Endocrinologica* **27** 243–256.
- Petterson I, Muccioli G, Granata R, Deghenghi R, Ghigo E, Ohlsson C & Isgaard J 2002 Natural (ghrelin) and synthetic (hexalein) GH secretagogues stimulate H9c2 cardiomyocyte cell proliferation. *Journal of Endocrinology* **175** 201–209. (doi:10.1677/joe.0.1750201)
- Reilly JF, Maher PA & Kumari VG 1998 Regulation of astrocyte GFAP expression by TGF- β 1 and FGF-2. *Glia* **22** 202–210. (doi:10.1002/(SICI)1098-1136(199802)22:2<202::AID-GLIA11>3.0.CO;2-1)
- Rossi F, Castelli A, Bianco MJ, Bertone C, Brama M & Santemma V 2009 Ghrelin inhibits contraction and proliferation of human aortic smooth muscle cells by cAMP/PKA pathway activation. *Atherosclerosis* **203** 97–104. (doi:10.1016/j.atherosclerosis.2008.06.015)
- Scheepens A, Sirimanne E, Beilharz E, Breier BH, Waters MJ, Gluckman PD & Williams CE 1999 Alterations in the neural growth hormone axis following hypoxic-ischemic brain injury. *Molecular Brain Research* **68** 88–110. (doi:10.1016/S0169-328X(99)00051-0)
- Sousa V de O, Romao L, Neto VM & Gomes FC 2004 Glial fibrillary acidic protein gene promoter is differently modulated by transforming growth factor- β 1 in astrocytes from distinct brain regions. *European Journal of Neuroscience* **19** 1721–1730. (doi:10.1111/j.1460-9568.2004.03249.x)
- Stoving RK, Veldhuis JD, Flyvbjerg A, Vinten J, Hangaard J, Koldjaer OG, Kristiansen J & Hagen C 1999 Jointly amplified basal and pulsatile growth hormone secretion and increased process irregularity in women within the GH-insulin-like growth factor I axis. *Journal of Clinical Endocrinology and Metabolism* **84** 2056–2063. (doi:10.1210/jc.84.6.2056)
- Sullivan SM, Sullivan RKP, Miller SM, Ireland Z, Björkman T, Pow DV & Colditz PB 2012 Phosphorylation of GFAP is associated with injury in the neonatal pig hypoxic-ischemic brain. *Neurochemical Research* **37** 2364–2378. (doi:10.1007/s11064-012-0774-5)
- Takemura M, Gomi H, Colucci-Guyon E & Itohara S 2002 Protective role of phosphorylation in turnover of glial fibrillary acidic protein in mice. *Journal of Neuroscience* **22** 6972–6979.
- Thompson NM, Davies JS, Mode A, Houston PA & Wells T 2003 Pattern-dependent suppression of growth hormone (GH) pulsatility by ghrelin and GH-releasing peptide-6 in moderately GH-deficient rats. *Endocrinology* **144** 4859–4867. (doi:10.1210/en.2003-0423)
- Thompson NM, Gill DAS, Davies R, Loveridge N, Houston PA, Robinson LCAF & Wells T 2004 Ghrelin and des-octanoyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* **145** 234–242. (doi:10.1210/en.2003-0899)
- Torres-Aleman I, Rejas MT, Pons S & Garcica-Segura LM 1992 Estradiol promotes cell shape changes and glial fibrillary acidic protein redistribution in hypothalamic astrocytes *in vitro*: a neuronal-mediated effect. *Glia* **6** 180–187. (doi:10.1002/glia.440060305)
- Yarwood SJ, Kilgour E & Anderson NG 1998 Cyclic AMP potentiates growth hormone-dependent differentiation of 3T3-F442A preadipocytes: possible involvement of the transcription factor CREB. *Molecular and Cellular Endocrinology* **138** 41. (doi:10.1016/S0303-7207(98)00049-5)
- Zhu T, Goh EL, Graichen R, Ling L & Lobie PE 2001 Signal transduction via the growth hormone receptor. *Cellular Signalling* **13** 599–616. (doi:10.1016/S0898-6568(01)00186-3)
- Zigman JM, Jones JE, Lee CE, Saper CB & Elmquist JK 2006 Expression of ghrelin receptor mRNA in the rat and the mouse brain. *Journal of Comparative Neurology* **494** 528–548. (doi:10.1002/cne.20823)

Received in final form 31 May 2013

Accepted 21 June 2013

Accepted Preprint published online 21 June 2013