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Growth Hormone-Releasing Peptide-6 Increases Insulin-Like Growth Factor-I mRNA Levels and Activates Akt in RCA-6 Cells as a Model of Neuropeptide Y Neurones

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Key words: neuropeptide Y, IGF-I, intracellular mechanisms, ghrelin, hypothalamus.

Abstract

Chronic systemic administration of growth hormone (GH)-releasing peptide-6 (GHRP-6), an agonist for the ghrelin receptor, to normal adult rats increases insulin-like growth factor (IGF)-I mRNA and phosphorylated Akt (pAkt) levels in various brain regions, including the hypothalamus. Because neuropeptide Y (NPY) neurones of the arcuate nucleus express receptors for ghrelin, we investigated whether these neurones increase their IGF-I and p-Akt levels in response to this agonist. In control rats, immunoreactive pAkt was practically undetectable; however, GHRP-6 increased p-Akt immunoreactivity in the arcuate nucleus, with a subset of neurones also being immunoreactive for NPY. Immunoreactivity for IGF-I was detected in NPY neurones in both experimental groups. To determine if activation of this intracellular pathway is involved in modulation of NPY synthesis RCA-6 cells, an embryonic rat hypothalamic neuronal cell line that expresses NPY was used. We found that GHRP-6 stimulates NPY and IGF-I mRNA synthesis and activates Akt in this cell line. Furthermore, inhibition of Akt activation by LY294002 treatment did not inhibit GHRP-6 induction of NPY or IGF-I synthesis. These results suggest that some of the effects of GHRP-6 may involve stimulation of local IGF-I production and Akt activation in NPY neurones in the arcuate nucleus. However, GHRP-6 stimulation of NPY production does not involve this second messenger pathway.

The hypothalamus, and in particular the arcuate nucleus, is intricately involved in integrating the multitude of signals controlling both growth and metabolism (1–4). Circulating hormones, including ghrelin, leptin, insulin-like growth factor (IGF)-I and growth hormone (GH), amongst others, relay information from systemic tissues to the hypothalamus regarding both the acute and long-term metabolic status of the individual or animal (1–6). However, how these factors interact to obtain the fine-tuned control of growth and metabolism necessary for normal physiological development and function is only now beginning to be deciphered.

We recently reported that chronic systemic treatment with GH-releasing peptide (GHRP)-6, an agonist of the endogenous ghrelin receptor, increases IGF-I levels in various brain regions, including the hypothalamus (7). Furthermore, intracellular signalling cascades normally associated with anti-apoptotic actions in response to IGF-I, such as the PI3/Akt cascade (8–10), are activated in the same areas and this is coincident with a decrease in basal cell death (7). Indeed, GH secretegogues (GHS), as well as ghrelin, have been shown to exert anti-apoptotic or proliferative actions in the brain and other tissues and this may or may not involve IGF-I production or Akt activation (7, 11–13). However, in the hypothalamus, ghrelin has important neuroendocrine actions (1–6, 14–22), suggesting that IGF-I synthesis and Akt activation may also be involved in these processes. Indeed, systemic and/or locally produced IGF-I is known to be involved in the neuroendocrine control of growth, reproduction and metabolism (23, 24, 25, 26).

Neuropeptide Y (NPY) neurones in the hypothalamus are intricately involved in controlling metabolism, growth, reproduction and appetite (17, 19, 20, 22, 24). Because many of these neurones express the GHS or ghrelin receptor (GHSR) (18, 19, 22, 27–29), they are a prime target for the regulatory effect of this hormone on neuroendocrine function. However, little is known regarding the interaction of NPY and IGF-I at the hypothalamic level.

To further understand the effects of GHRP-6 in the hypothalamus, we analysed whether NPY neurones in the arcuate nucleus respond to this secretagogue by increasing IGF-I levels and activating Akt. Furthermore, we have employed a simian virus-40 (SV-40)-transformed embryonic

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rat hypothalamic cell line that expresses GHSR mRNA and protein and responds to GHRP-6 and IGF-I by increasing their expression of NPY mRNA and protein. The RCA-6 cell line represents a new cell-based model for elucidating the actions of GHS in the hypothalamus.

Materials and methods

Materials

All chemicals were purchased from (St Louis, MO, USA) or Merck (Barcelona, Spain) unless otherwise noted.

Antibodies to p-Akt, p-MAPK and pGSK were purchased from Cell Signalling (New England Biolabs, Beverly, MA, USA), to Akt from Santa Cruz Biotechnology (Santa Cruz, CA, USA), to MAPK from Upstate Biotechnology (Lake Placid, NY, USA), to GSK-3β from BD-Transduction Laboratories (Franklin Lakes, NJ, USA), to actin from Sigma and to GHSR from Alpha Diagnostic International (San Antonio, TX, USA). Antibodies to somatostatin, NPY, β-endorphin, GHRH, cocaine- and amphetamine-regulated transcript (CART) and nestin were obtained from Bachem (Penninsula Laboratories Inc, San Carlos, CA, USA). The IGF-I primary antibody was a gift from Dr I. Torres-Alemán (Instituto Ramón y Cajal, CSIC, Madrid, Spain). Secondary antibodies conjugated with peroxidase were from Pierce (Rockford, IL, USA).

Animals

Male Wistar rats weighing 200–250 g were used for all *in vivo* experiments. The animals were treated according to the European Community laws for animal care and were maintained on a 12 : 12 h light/dark cycle. All studies were approved by the corresponding ethical committee. Rats were infused with GHRP-6 (Bachem, 150 μg/day) using Alzet minipumps (Durect Corporation, Cupertino, CA, USA; 1 μl/h for 7 days) connected to the jugular vein. Control animals received a minipump delivering vehicle (saline) at the same infusion rate. For real-time polymerase chain reaction (PCR) studies, rats ($n = 6$ in each group) were killed by decapitation and brains immediately removed and frozen on dry ice. For immunocytochemistry studies, animals were cardiac perfused first with saline and then with 4% paraformaldehyde. The brains were then removed and frozen at −80 °C until processed.

Cell culture

Stock cultures of the cell lines RCA-6 and RCF-8 (30–32), a gift of Dr I. Torres-Alemán (Instituto Ramón y Cajal, Madrid, Spain) were routinely grown in 100-mm culture dishes in Dulbecco's modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) without phenol red supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin-streptomycin and glutamine (200 mM). The cells were grown at 37 °C in 5% CO_2 humidified air.

Hormonal treatment of neuronal cell lines

For all experiments, cells were plated in 60-mm culture dishes (2×10^5 cells/p60) or in 100-mm dishes (6×10^5 cells/p100) for 72 h in DMEM containing 10% FCS and then in DMEM-Ham's F12 (1 : 1) without FCS, but containing 1.2 g/l $NaHCO_3$, 15 mM HEPES and supplemented with 0.1 mg/ml transferrin, 10^{-5} M putrescine, 2×10^{-8} M progesterone, 10^{-7} M corticosterone, 10^{-5} M T_3, 1 μg/ml arachidonic acid and 2×10^{-8} M Na_2SeO_3 (33). Cultures were maintained for 48 h in this differentiation media without FCS. Subsequently, the cells were treated with GHRP-6 at the corresponding concentration and then subjected to protein or RNA extraction and analysis as described below. In experiments using IGF-I, a concentration of 10 ng/ml was used. The PI3 kinase inhibitor LY294002 was purchased from Calbiochem (La Jolla, CA, USA) and was used at a concentration of 25 μM dissolved in dimethylsulphoxide (DMSO) and added 45 min before treatment with GHRP-6. The maximum concentration of DMSO in the culture media was 0.05%, which alone had no effect.

Immunoblotting

For western blotting, cells were homogenized in 500 μl RIPA lysis buffer with an EDTA free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After homogenization, the samples were centrifuged at 12000 ***g*** for 15 min at 4 °C and clear supernatants were transferred to a new tube. Protein concentration was estimated by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Thirty or 60 μg of protein were resolved using 10% SDS-PAGE and then transferred onto PVDF membranes (Bio-Rad). Filters were blocked with Tris-buffered saline containing 5% (w/v) bovine serum albumin (BSA) and incubated with the primary antibody for p-Akt, Akt, p-MAPK, MAPK, p-Gsk-3β, GSK-3β or GHSR 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 visualized by chemiluminiscence (Dupont-NEN, Boston, MA, USA) and quantified by densitometry using a Bio-1D system (Vilber Lourmat, Marne La Vallee, France). To correct for variations in the starting amount of protein, all blots were re-probed using an anti-actin antibody.

RNA extraction

Total RNA was extracted from cultured neurones and 100 mg of hypothalamus according to the Tri-Reagent protocol (Sigma). Briefly, the samples were homogenized in 1 ml of Tri-Reagent and centrifuged at 12000 ***g*** for 10 min at 4 °C to remove the insoluble material. Clear supernatants were transferred to a new tube and incubated at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes. Chloroform was added, the samples shaken vigorously for 15 s, allowed to stand for 15 min at room temperature and then centrifuged at 12000 ***g*** at 4 °C for 15 min. The aqueous phases were transferred to fresh tubes and 0.5 ml of isopropanol was added to precipitate RNA. After 5 min, samples were centrifuged at 12000 ***g*** at 4 °C for 10 min. The supernatants were removed and the pellets washed by adding 1 ml of 75% ethanol. After vortexing, samples were centrifuged at 7500 ***g*** at 4 °C for 5 min. The pellets were air-dried and resuspended in DEPC-H_2O.

Reverse transcriptase (RT)-PCR

The RNA (200 or 500 ng) was subjected to RT-PCR using the Access RT-PCR system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Oligonucleotide primers for IGF-I, were synthesized by Gibco BRL Custom Primers (Life Technologies, Barcelona, Spain). The sequences of IGF-I primers used were GCA TTG TGG ATG AGT GTT GC (sense) and GGC TCC TCC TAC ATT CTG TA (antisense). The sequences of GHSR primers used were CGT GAA GCT GGT CAT CCT T (sense) and CAG ACC AAG GTT CCT CGT T (antisense).

The RT reaction was performed for 45 min at 48 °C and PCR cycling was performed with the following cycle profile: 94 °C for 120 s, followed by 40 cycles of 94 °C for 1 min and 55 °C for 1 min and 68 °C for 2 min. After the last cycle, the elongation step was extended by 7 min at 68 °C. RT-PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, photographed under ultraviolet illumination, compared with a known standard ladder (Promega) and quantified by densitometry using the Bio-1D system (Vilber Lourmat).

To correct for variations in the starting amount of RNA, GAPDH primers that amplified products of 835 bp were added to all reactions. Primers used for reactions at 55 °C were: sense 5′-AGG GCT GCC TTC TCT TGT G-3′ and antisense 5′-CAG CAT CAA AGG TGG AGG A-3′. For reactions at 60 °C primer sense 5′- AGG GCT GCC TTC TCT TGT GG-3′ and antisense 5′-CAG CAT CAA AGG TGG AGG AA-3′.

Real-time RT-PCR

Changes in mRNA levels were determined by using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Madrid, Spain). PCR was carried out in a volume of 50 μl in a reaction containing 100 or 200 ng of total sample RNA (The number of copies of total RNA used in the reaction was chosen so that a signal could be obtained with 25–30 cycles), Sybergreen PCR mastermix (2 ×), multiscribe enzyme (1.25 U), sense and antisense primers 100 nM each, 5.5 mM $MgCl_2$ and RNAse inhibitor (20 U). Each primer pair was optimized at three different temperatures (58, 60 and 62 °C) and at each combination of three concentrations (50, 100, 200 nM). Optimal performance was achieved by selecting the primer concentrations that provide the lowest C_T and highest ΔR_n. A typical reverse transcription cycle of 30 min at 48 °C was carried out followed by 5 min at 95 °C for transcriptase inactivation.

Table 1. Sequences of Primers Used for Real-Time Reverse Transcriptase-Polymerase Chain Reaction.

	Sense	Antisense
IGF-I	CTCTGGGAGATCGCCACTCT	AACGAAGAACTTGCTCGTTGGA
NPY	CCTGTCCCACCCAATGCA	CAACGACAACAAGGGAAATGG
GAPDH	CCAAGTATGACATCAAGAAG	AGGCCAGGATGCCCTTTAGT

IGF-1, insulin-like growth factor-1; NPY, neuropeptide Y.

Because AmpliTaq Gold DNA Polymerase was used, 10 min at 95 °C were necessary for polymerase activation. Then 40 cycles of 95 °C for 15 s (denaturation), 60 °C for 1 min (annealing and extension) were carried-out. Relative gene expression comparisons were carried out by using an invariant endogenous control (GAPDH). The primers were designed with the Primer Express software (Applied Biosystems) and the sequences of the primers are shown in Table 1.

According to the manufacter's guidelines, the $\Delta\Delta C_T$ method was used for relative quantification. Data are expressed as C_T values (the cycle number at which logarithmic PCR plots cross a calculated threshold) and used to determine ΔC_T values ($\Delta C_T = C_T$ of the target gene $- C_T$ of the housekeeping gene). This value is calculated for each sample. The control sample was used as the baseline for each comparison to be made. The last step in quantification is to transform these values to absolute value. The formula for fold changes in gene expression is $2^{-\Delta\Delta C_T}$. Statistics were performed with ΔC_T values.

Each experiment was carried out three times and each sample was measured a minimum of two times for each assay.

NPY enzyme-linked immunosorbent assay

This assay was carried out according to the manufacturer's instructions (PhoenixPeptide, Belmont, CA, USA). This enzyme immunoassay kit (range 0–100 ng/ml) is based on the principle of a competitive enzyme immunoassay. Briefly, cells were lysed in 70 µl of assay buffer and centrifuged at 12000 ***g*** for 15 min at 4 °C and the clear supernatant transferred to a new tube. Each sample (50 µl) was added to their designated wells, followed by 25 µl of rabbit anti-NPY IgG and 25 µl of biotinylated peptide. The plate was then covered and incubated at room temperature for 2 h. Following this incubation, the plate was washed five times with 300 µl of assay buffer and then 100 µl of streptavidin-horseradish peroxidase was added to all wells for 1 h. After washing, the plate six times with 300 µl of assay buffer, 100 µl of substrate solution was added to the wells for 1 h. Finally, to stop the reaction 100 µl of 2 N HCl was added and, 20 min later, the absorbance optic density (OD) at 450 nm was read on an automatic plate reader. Samples were assayed in duplicate. The intra-assay variability was < 5% and the interassay variability was < 14%.

Immunohistochemistry

Immunohistochemistry was performed on frozen 20-µm cryostat sections, fixed in 4% paraformaldehyde (w/v) and blocked in TBS containing 3% BSA and 1% Triton X-100 for 1 h. Sections were left for 24–48 h in a humid chamber at 4 °C with a polyclonal rabbit anti-NPY antibody (1 : 200) and a monoclonal anti-IGF-I antibody (1 : 500) or a monoclonal anti-pAKT (Ser473) antibody (1 : 1000) in blocking solution. Afterwards, sections were incubated with a biotin-conjugated antimouse antibody (Pierce; 1 : 1000) for 90 min, washed and then incubated in streptavidin-Alexa Fluor 633 conjugate and antirabbit Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR, USA; 1 : 2000) for 1 h. Incubation chambers were covered with foil paper to avoid exposure to light. Immunolabelling was visualized by using a confocal microscope (Leica, Madrid, Spain).

For immunohistochemistry on RCA-6 cultures, cells were plated in 24-well culture dish, 2×10^4 cells/well and grown in DMEM supplemented with 10% FCS for 72 h. The media was then changed to differentiation media (described above) for 48 h. The media was changed and the cells were stimulated with GHRP-6 (25 µg/ml) for 4 h. The media was then removed and the cells washed three times with TBS and fixed with 4% paraformaldehyde (15 min). Endogenous peroxidase activity was blocked by incubating the cells in the presence of H_2O_2 (0.3%) in MeOH (30 min). After washing in TBS, cells were blocked in normal blocking buffer for 1 h and then left for 24–48 h in a humid chamber at 4 °C with the corresponding primary antibodies for somatostatin, NPY, β-endorphin, GHRH, CART, IGF-I or nestin (1 : 1000) in TBS solution. After washing, the cells were incubated with a biotinylated universal antibody (Vectastain Universal Elite kit, Vector Laboratories Inc, Burlingame, CA, USA) in blocking serum for 1 h. Cells were then incubated in Vectastain ABC Reagent for 30 min. After washing, a solution of DAB (0.1 mg/ml) with 0.1% H_2O_2 was added to the wells to visualize the reaction that was stopped by the addition of water.

Control experiments omitting the primary antibody were performed in all assays. No labelling and very low background were found in all control slides and tissue cultures.

Statistical analysis

The protein and mRNA from each animal was analysed separately (no pooling of samples was used); therefore, 'n' represents the number of animals used in each group. Real-time RT-PCR was repeated two to three times on each sample. The mean value of each animal was used for statistical analysis. All *in vitro* experiments were repeated a minimum of three times. All data were normalized to control values of each assay. Data were analysed by one-way ANOVA. $P < 0.05$ was considered statistically significanct.

Results

IGF-I mRNA levels in the hypothalamus in response to GHRP-6

Using IGF-I-specific primers, real-time RT-PCR was performed. Adult male rats treated systemically with GHRP-6 had significantly higher IGF-I mRNA levels in the hypothalamus compared to saline treated control rats. After normalization using GAPDH mRNA levels as the calibrator, IGF-I mRNA levels were found to be 3.5-fold higher in GHRP-6 treated rats (351 ± 27% of control values, $P < 0.001$).

IGF-I immunoreactivity in NPY neurones

Cells immunoreactive for IGF-I were found in the arcuate nucleus in both experimental groups (Fig. 1B,E), as well as more generalized IGF-I immunolabelling that most likely represents fibres and possibly interstitial space. Immunoreactive NPY neurones in the hypothalamic arcuate nucleus (Fig. 1A,D) were found to colocalize IGF-I in both groups (Fig. 1C,F).

Akt is activated in a subset of NPY neurones in response to GHRP-6

Neuropeptide Y neurones were found in the arcuate nucleus of both control and GHRP-6 treated rats (Fig. 2A,D, respectively). Although activated Akt levels were practically undetectable by immunohistochemistry in control animals (Fig. 2B), phosphorylated Akt (pAkt) increased in cells of the arcuate nucleus after treatment with GHRP-6 (Fig. 2E). Neurones double-labelled for NPY and pAkt could be found in the arcuate nucleus of rats treated with GHRP-6 (Fig. 2F, but not in the control rat, Fig. 2C).

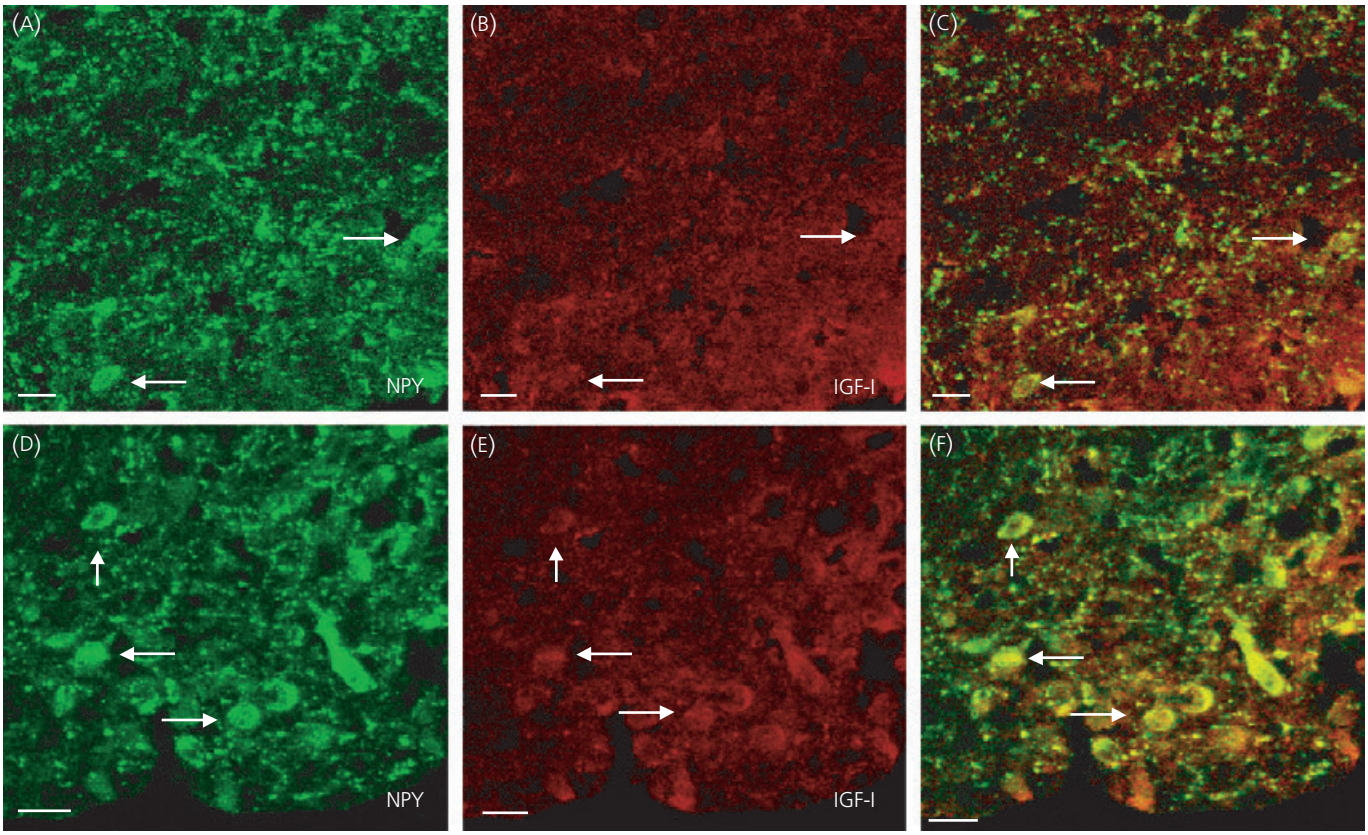

FIG. 1. Immunofluorescent labelling for neuropeptide Y (NPY) (green) and insulin-like growth factor (IGF)-I (red) in the arcuate nucleus of control (A–C) and growth hormone-releasing peptide-6 (D–F) treated rats. Neurones immunopositive for both NPY and IGF-I are indicated by the solid arrows. Omission of the primary antibody resulted in no specific labelling and a very low (black) background (not shown). The white bar represents 30 μm in each photomicrograph.

Characterization of GHSR in RCA-6 and RCF-8 hypothalamic cells

Detection of GHSR

The hypothalamic cell lines, RCA-6 and RCF-8, were obtained by viral transfection of foetal hypothalamic neurones and have been partially characterized previously (30–32).

To determine whether the RCA-6 or RCF-8 cell lines express GHSR mRNA, total RNA was isolated from cells treated with different concentrations of GHRP-6 (15–30 μg/ml) for 24 h. As shown in Fig. 3(A), the RCA-6 cell-line expresses the GHSR mRNA, as detected by RT-PCR. Basal levels of this receptor were very low and were up-regulated in the presence of GHRP-6. This receptor was undetectable in the RCF-8 cell line. To confirm the presence of the receptor protein in RCA-6 cells, western blot analysis was performed. Protein for GHSR was expressed at very low levels in the control cultures and increased 2.6-fold after treatment with 25 μg/ml of GHRP-6.

Immunocytochemical characterization of the cell line RCA-6

To further characterize the RCA-6 foetal hypothalamic cell line, immunocytochemistry was performed for some of the most common neuropeptides expressed in the hypothalamus. These neurones were found to express somatostatin, NPY and GHRH, whereas CART or β-endorphin could not be detected (Fig. 3B). GHRH was only detected when the cells were undifferentiated, as determined by nestin immunolabelling. After 48 h in the above described differentiation media, only a small percentage express nestin (data not shown).

Effects of GHRP-6 on IGF-I mRNA

GHRP-6 stimulated IGF-I mRNA levels in RCA-6 cells in a concentration- (Fig. 4A) and time-dependent (Fig. 4B) manner. The maximum response was obtained at a concentration of 25 μg/ml (Fig. 4A). This response was significant at 2 h and declined by 6 h, although IGF-I mRNA levels remained significantly elevated up to 24 h after treatment (Fig. 4B). IGF-I mRNA levels did not change in the cell line RCF-8 in response to GHRP-6 (data not shown).

IGF-I protein was detected in RCA-6 cells by immunocytochemistry (Fig. 4C,E). Cells with (Fig. 4D) and without (Fig. 4C) GHRP-6 treatment were clearly labelled for IGF-I protein in comparison to the negative control (Fig. 4E; omission of primary antibody).

Effects of GHRP-6 on IGF-I signalling pathways in RCA-6 cells

Increased activation of Akt, but not MAPK, in RCA-6 neurones

Cultures treated with vehicle or 25 μg/ml GHRP-6 for 0.5, 1 or 4 h were analysed by Western blot to determine activation

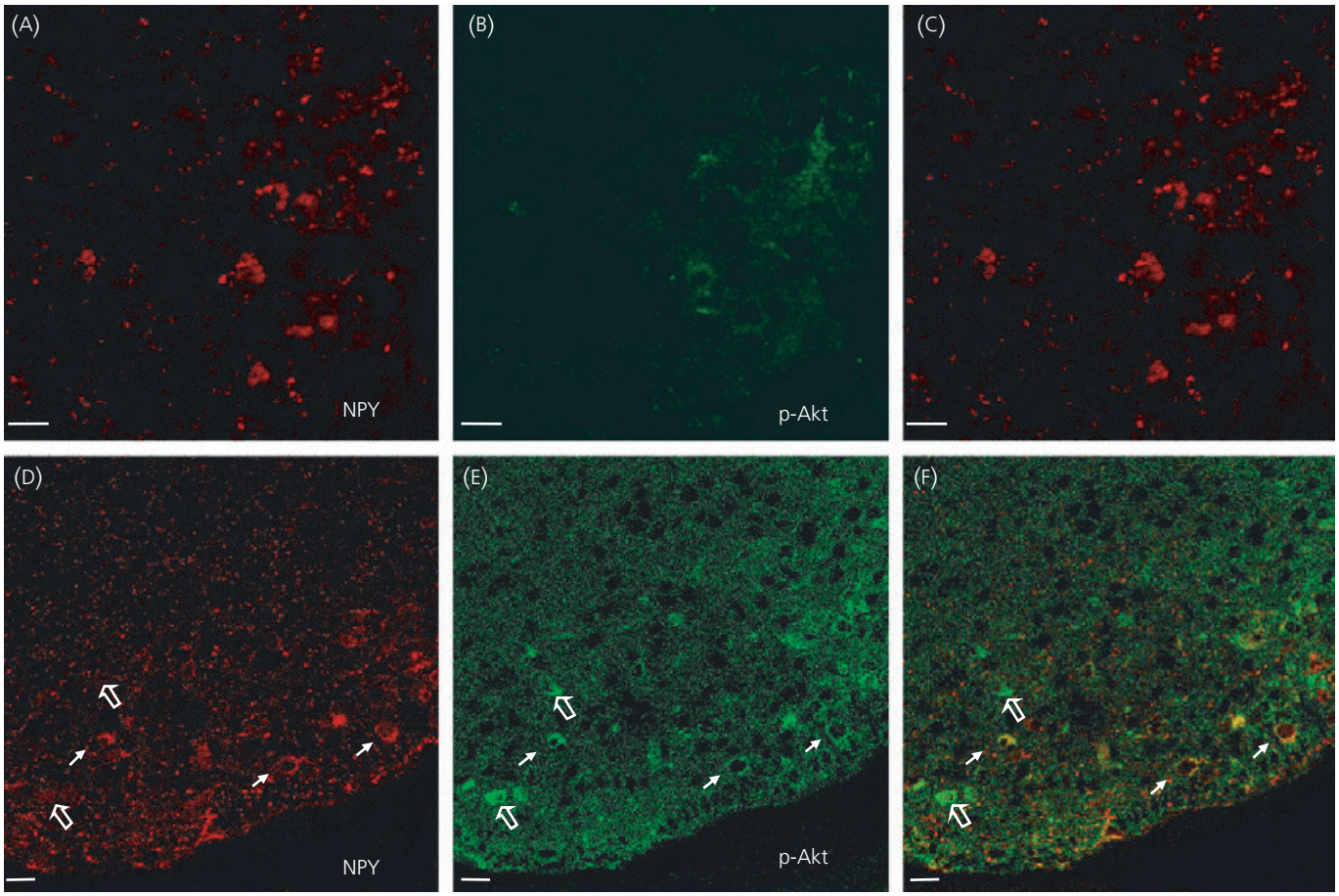

FIG. 2. Immunofluorescent labelling for neuropeptide Y (NPY) (red) and phosphorylated Akt (pAkt, green) in the arcuate nucleus of a control (A–C) or growth hormone-releasing peptide-6 (D–F) treated rat. Neurones immunopositive for both NPY and pAkt are indicated by the solid arrows. Cells immunopositive for only pAkt are indicated by the open arrows. Omission of the primary antibody resulted in no specific labelling and a very low (black) background (not shown). The white bar represents 20 μm in each photomicrograph.

of Akt and MAPK (ERK-1/ERK-2). The results were normalized against the nonphosphorylated forms of Akt and MAPK. Phosphorylated Akt levels were significantly increased by GHRP-6 as early as 0.5 h after treatment whereas, at 4 h, this increase was even greater (Fig. 5). No increase in activation of MAPK or Gsk could be detected in RCA-6 neurones with GHRP-6 treatment at any of the concentrations or time-points studied (data not shown).

This cell-line also responded to IGF-I (10 ng/ml) by increasing pAKT levels (IGF-I treated: 241 ± 13% versus control: 100 ± 21%; $P < 0.001$) and this activation remained significantly elevated until at least 24 h after treatment.

GHRP-6 increases NPY mRNA and protein levels in RCA-6 neurones

As shown in Table 2, both NPY mRNA ($n = 4$) and NPY protein levels ($n = 3$) increase in RCA-6 neurones in response to GHRP-6 treatment. This increase in NPY mRNA concentrations was significant as early as 30 min after GHRP-6 treatment, whereas a significant increase in NPY protein levels could not be detected until 1 h after treatment.

IGF-I increases NPY mRNA levels in RCA-6 neurones

After 2 h of treatment with 10 ng/ml IGF-I, RCA-6 neurones had significantly increased NPY mRNA concentrations (controls 100 ± 12 versus IGF-I: 181 ± 18; $P < 0.001$). No significant change was found at earlier time-points.

Inhibition of Akt activation does not inhibit GHRP-6 induction of NPY synthesis

Akt activation by GHRP-6 was inhibited in RCA-6 neurones by pretreatment with the PI3K inhibitor LY294002 in a concentration-responsive manner (Fig. 6A). GHRP-6 continued to increase NPY mRNA and IGF-I mRNA concentrations in RCA-6 cells even when Akt activation was inhibited (Fig. 6B,C, respectively).

Discussion

Chronic systemic administration of GHRP-6, a synthetic ghrelin receptor agonist, stimulates IGF-I synthesis in different areas of the adult male rat brain, including the hypothalamus. Furthermore, this rise in local IGF-I is associated with

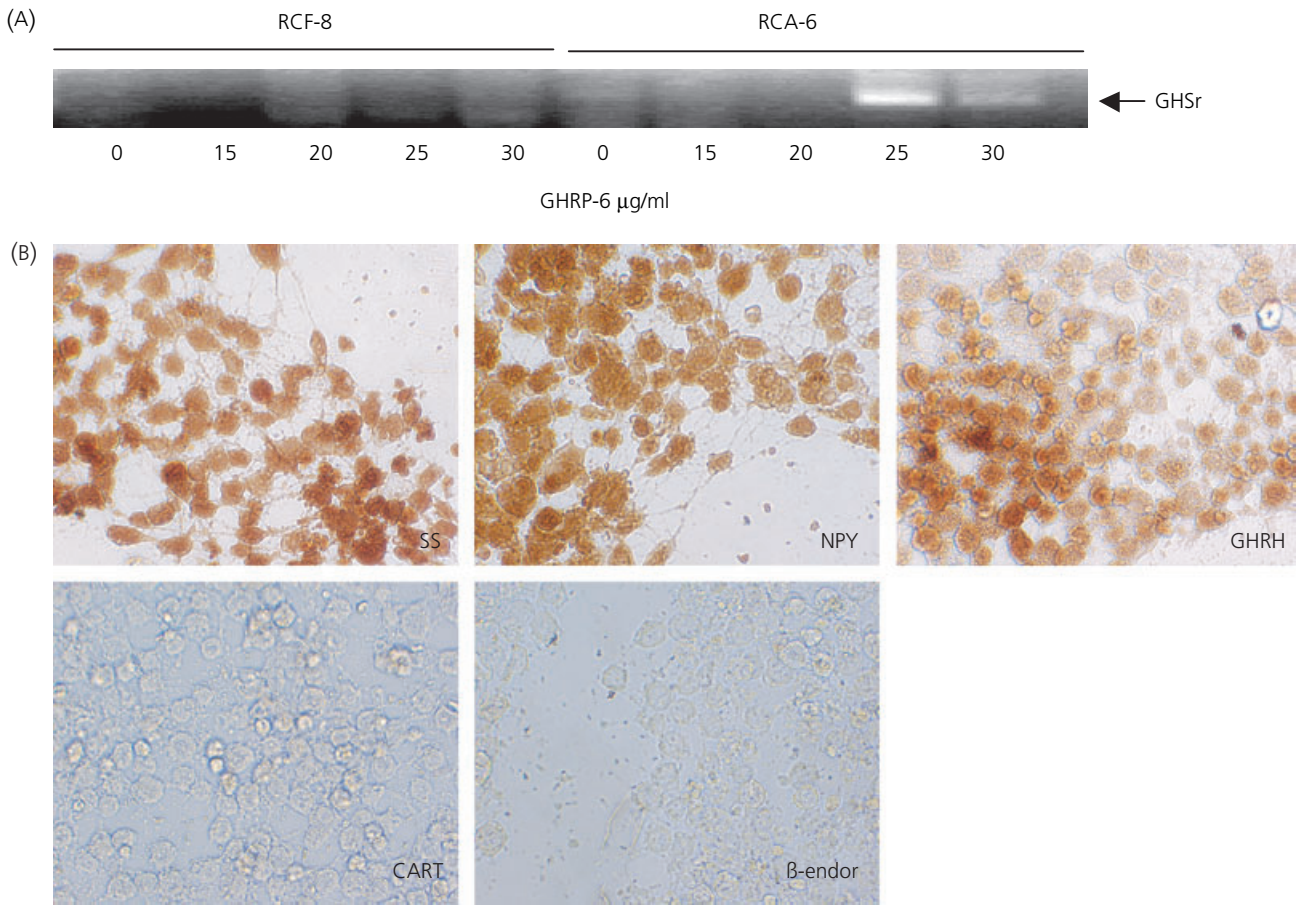

FIG. 3. (A) Detection of growth hormone secretagogue receptor ghrelin receptor (GHSR) mRNA in the foetal hypothalamic cell lines RCF-8 and RCA-6 by reverse transcription-polymerase chain reaction. This receptor was not detected in RCF-8 cells, but was detected at low levels in RCA-6 cells that increased in response to treatment with growth hormone-releasing peptide (GHRP)-6. Results are representative of three independent experiments. (B) Immunocytochemistry for somatostatin (SS), neuropeptide Y (NPY), growth hormone-releasing hormone (GHRH), cocaine- and amphetamine-related transcript (CART) and β-endorphin (β-endor) in the hypothalamic cell line RCA-6.

increased phosphorylation of Akt (7), an intracellular protein activated in response to IGF-I (8–10, 34, 35). NPY neurones in the hypothalamic arcuate nucleus express the GHSR and are fundamental for normal neuroendocrine responses to ghrelin, the endogenous GHSR agonist (1–6, 16–22, 24). Hence, it is plausible that some effects of ghrelin or its agonists on hypothalamic NPY neurones may be mediated via activation of this signalling pathway.

In the present study, we report that NPY neurones in the arcuate nucleus colocalize immunoreactive IGF-I, suggesting that the increase in this growth factor in response to GHRP-6 treatment could involve this neuronal population. An increase in IGF-I protein could be due to increased uptake of circulating IGF-I by hypothalamic cells (36, 37); however, the fact that IGF-I mRNA levels are elevated in the hypothalamus in response to GHRP-6 suggests that local IGF-I synthesis is increased. Furthermore, the NPY producing cell line employed in the studies presented here also increased IGF-I mRNA levels in response to GHRP-6. Taken together, these results suggest that hypothalamic NPY neurones may respond to GHRP-6 by increasing their synthesis of IGF-I. Although data in rats regarding an increase in circulating GH levels in response to chronic GHS treatment are conflicting (38, 39), chronic GH secretagogue treatment as used in these experiments does not significantly alter serum IGF-I levels (38, personal observation), suggesting that the observed effects may be due to GHRP-6 itself.

Most IGF-I effects are mediated via activation of either the MAPK (ERK1/2) or PI3K/Akt intracellular pathways (8–10, 34, 35). In the *in vivo* experimental paradigm used here, and previously (7), increased local IGF-I concentration was accompanied by increased activation of Akt in various brain regions, but no increase in ERK1/2 activation was detected. Activation of Akt in NPY neurones could be a direct response to GHRP-6 because ghrelin has been shown to directly activate the PI3K-Akt pathway in other cell types, or to an autocrine or paracrine action of increased local IGF-I synthesis (11, 12, 40).

To further understand the effect of GHRP-6 on hypothalamic NPY neurones, the hypothalamic cell line RCA-6 was employed. This foetal hypothalamic neuronal line expresses GHSR and produces somatostatin, NPY and GHRH. Coexpression of these neuropeptides is not unexpected because somatostatin and NPY are coexpressed in neurones of various brain regions (41, 42) and GHRH and NPY have been colocalized in neurones of the arcuate nucleus (43). In

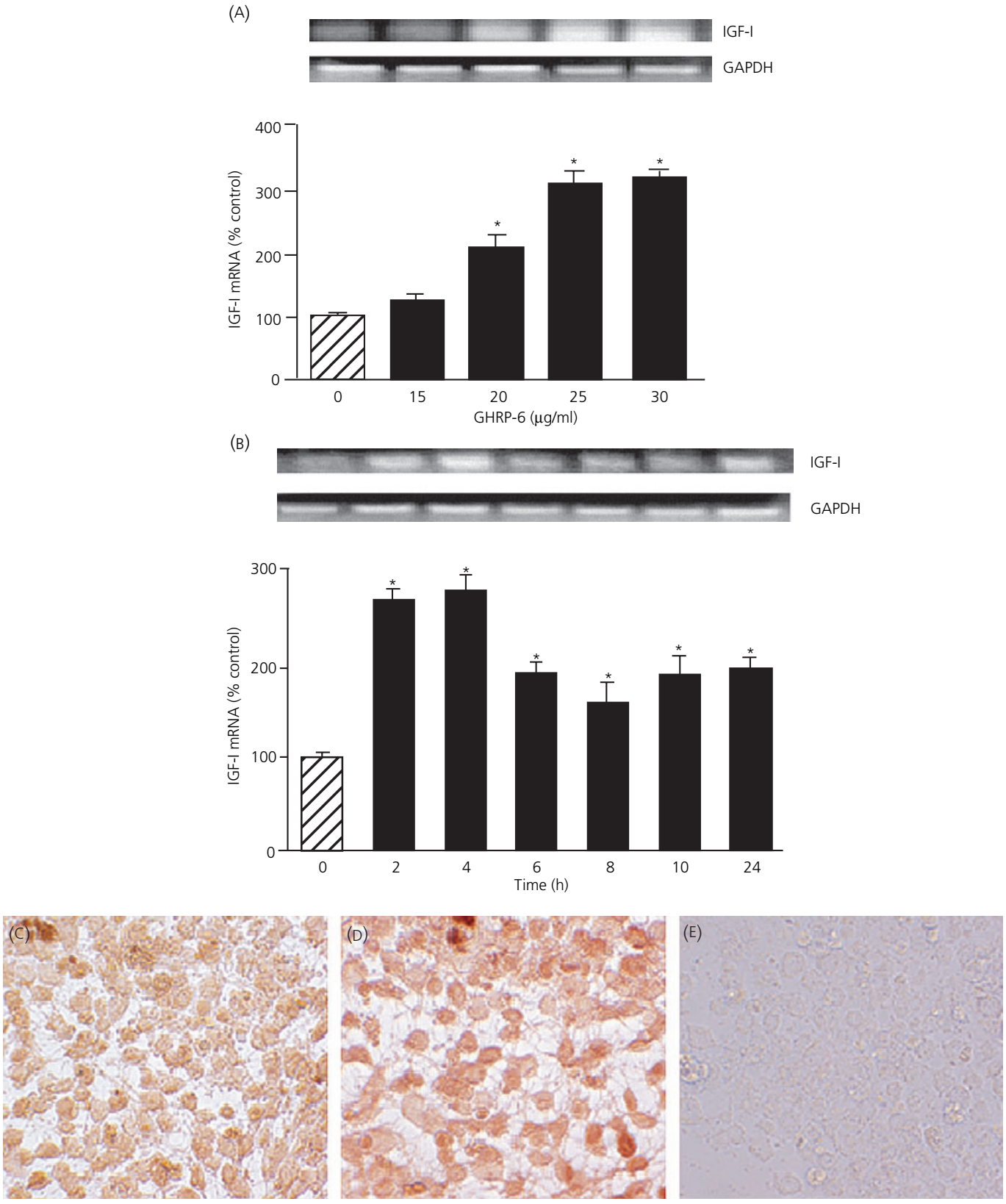

FIG. 4. (A) Relative levels of insulin-like growth factor (IGF)-I mRNA, as detected by real-time polymerase chain reaction, in RCA-6 cells in response to increasing concentrations of growth hormone-releasing peptide (GHRP)-6 after 4 h of treatment. (B) Relative levels of IGF-I mRNA in RCA-6 cells after treatment with 25 μg/ml GHRP-6 for increasing periods of time. *$P < 0.05$ by ANOVA compared to control values. The results are representative of four experiment performed in triplicate ($n = 4$ animals). (C–E) Immunocytochemistry for IGF-I in RCA-6 cells after treatment with vehicle (C) or 25 μg/ml GHRP-6 (D) for 4 h. Omission of the primary antibody resulted in no specific labelling (E).

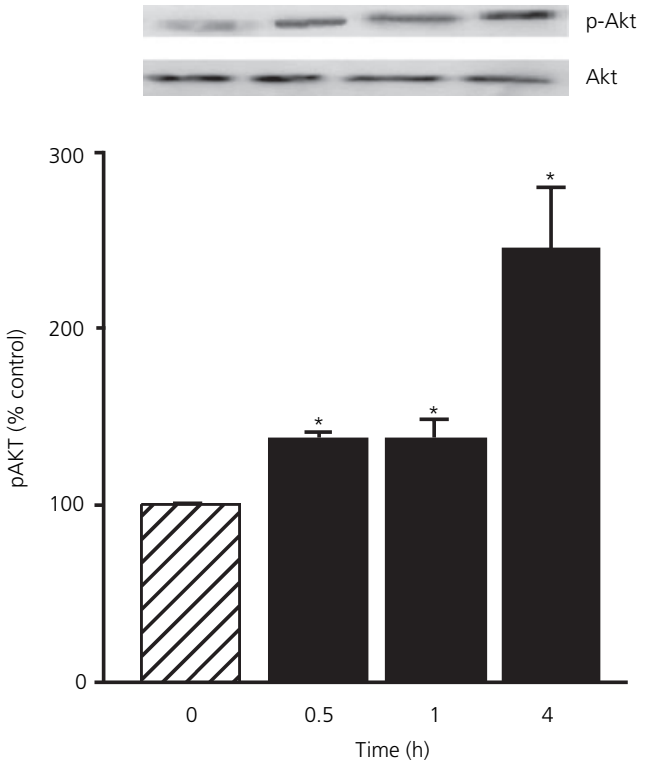

FIG. 5. Relative levels of phosphorylated Akt (pAkt) in RCA-6 cells at 0, 0.5, 1 and 4 h after treatment with 25 μg/ml growth hormone-releasing peptide (GHRP)-6. *P < 0.05 by ANOVA compared to control values. The results are representative of four experiment performed in triplicate (n = 4 animals).

TABLE 2. Neuropeptide Y (NPY) mRNA and Protein Levels in the Hypothalamic Neuronal Cell Line RCA-6 in Response to Treatment with Growth Hormone-Releasing Peptide (GHRP)-6 (25 μg/ml).

	30 min	1 h	2 h	4 h
NPY mRNA	1.80 ± 0.20*	1.60 ± 0.09*	1.55 ± 0.12*	1.30 ± 0.08*
NPY protein	1.35 ± 0.65	1.36 ± 0.04*	1.76 ± 0.60*	1.76 ± 0.03*

Control levels at each time-point were taken to be 1.0 and all data are expressed relative to control levels at their appropriate time-point. *P < 0.05 compared to control levels.

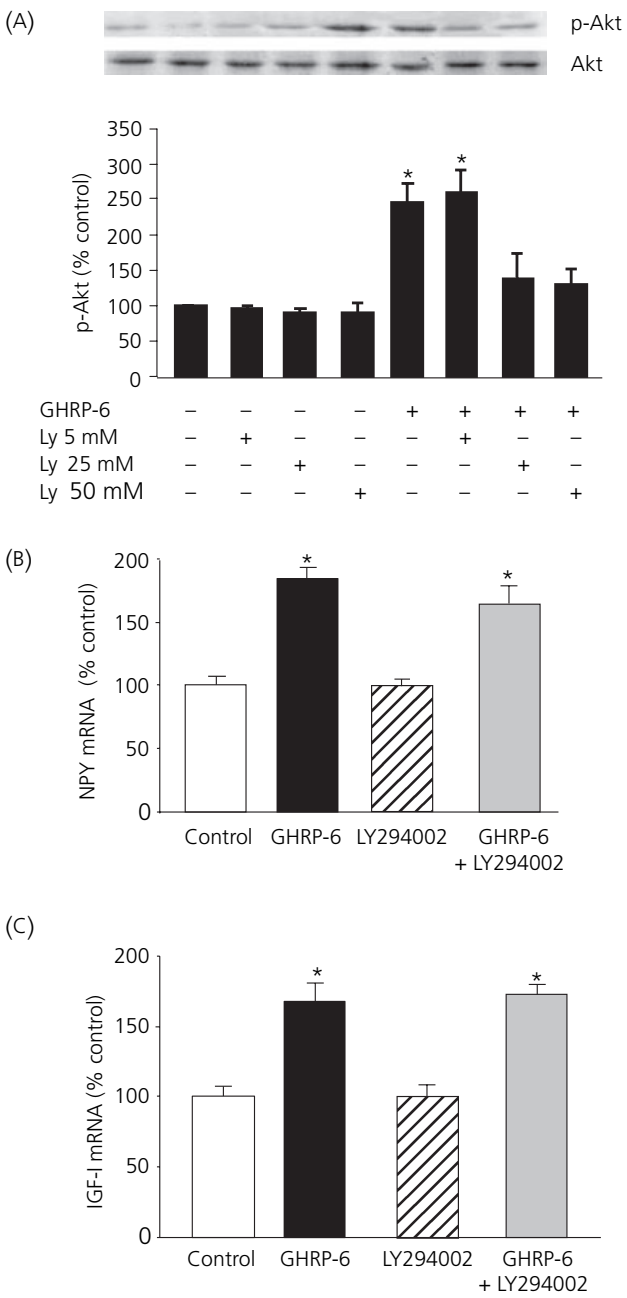

FIG. 6. (A) Relative levels of pAkt in RCA-6 cells in response to growth hormone-releasing peptide (GHRP)-6 in the presence or absence of the PI3/Akt inhibitor, LY294002 (LY) at increasing concentrations. (B) Relative neuropeptide Y (NPY) mRNA levels in RCA-6 cells in response to 25 μg/ml GHRP-6 in the presence or absence of 25 μM LY294002. (C) Relative IGF-I mRNA levels in RCA-6 cells in response to 25 μg/ml GHRP-6 in the presence or absence of 25 μM LY294002. *P < 0.05 by ANOVA in comparison to control values. All results are representative of four experiment performed in triplicate (n = 4 animals).

response to GHRP-6, these cells increase IGF-I production and activate Akt, but not MAPK or Gsk, and the responses to GHRP-6 are both concentration- and time-dependent. This is similar to what is observed in the *in vivo* hypothalamus (7). However, although these neurones have many responses similar to those found in the *in vivo* hypothalamus, they are transformed foetal cells and this should be taken into consideration when interpreting the results reported here.

In RCA-6 neurones, GHRP-6 stimulated NPY synthesis, which is in agreement with studies demonstrating that ghrelin or GH secretagogues stimulate hypothalamic NPY production (17, 19), giving further support to the hypothesis that this effect is mediated directly via GHSRs on NPY neurones. The stimulatory effect of GHRP-6 on NPY production could be mediated through IGF-I because IGF-I also stimulated NPY production in this cell line and GHRP-6 stimulates IGF-I synthesis. However, GHRP-6 resulted in an increase in NPY mRNA concentration as early as 30 min after treatment, whereas no significant increase in response to IGF-I was detected until 2 h after treatment. This delayed response to IGF-I suggests that it most likely is not mediating the

more rapid response to GHRP-6, although the prolonged effect of this secretagogue on NPY neurones could involve IGF-I synthesis. Furthermore, activation of Akt does not appear to be required for stimulation of NPY synthesis. Indeed, when activation of Akt is inhibited in RCA-6 neurones, GHRP-6 continues to stimulate NPY synthesis. The Akt-independent regulation of NPY by GHRP-6 confirms previous studies using the SH-SY5Y neuroblastoma cell line showing that, when IGF-I activation of Akt is blocked by the presence of LY294002, NPY gene expression is only partially depressed (34). Hence, this intracellular signalling pathway is most likely mediating other actions of GHSs, as well as ghrelin.

One well studied function of IGF-I and activation of the intracellular mechanisms involving Akt is the promotion of cell survival (8, 9, 34, 35), and activation of the GHSR has been shown to exert antiapoptotic or neuroprotective effects (7, 11, 12). However, the number of neurones undergoing cell death in the arcuate nucleus of normal young adults is very low (7, 44–46), leading to the speculation that promotion of cell survival is not the primary effect of these hormones in this paradigm. Activation of Akt has also been implicated in the regulation of glucose metabolism via downstream modulation of Gsk (47, 48). However, Gsk levels were not changed *in vivo* or *in vitro* in response to GHRP-6. Growth hormone secretagogues, as well as IGF-I, have been implicated in memory or cognitive function improvement (49, 50), a process that may involve modifications of synaptic strength or inputs and these changes could involve activation of Akt (51). More recently, ghrelin has been implicated in synaptic remodelling in the arcuate nucleus, including on NPY neurones (52), and this is suggested to play a role in the neuroendocrine response to this hormone. Therefore, activation of Akt in response to GHRP-6 may be involved in processes of synaptic remodulation and could underlie some of the neuroendocrine response to these hormones, although this remains to be demonstrated.

In summary, we have confirmed that treatment with GHRP-6 stimulates IGF-I production and activates the Akt pathway in the hypothalamus. NPY neurones in the arcuate nucleus colocalize immunoreactive IGF-I and respond to GHRP-6 by increasing pAkt immunoreactivity. Similar results were found using a hypothalamic cell line that expresses GHSR mRNA. In addition, this cell line also responded to GHRP-6 by increasing IGF-I mRNA levels as well as activating the PI3K/Akt signalling pathway. Furthermore, GHRP-6 stimulates the production of NPY, and the specific PI3K inhibitor LY29400 was incapable of blocking this process. These findings indicate that the PI3K/Akt pathway does not mediate the effect of GHRP-6 on NPY synthesis in the arcuate nucleus or RCA-6 cells.

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