

Growth Hormone (GH) and GH-Releasing Peptide-6 Increase Brain Insulin-Like Growth Factor-I Expression and Activate Intracellular Signaling Pathways Involved in Neuroprotection

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Beneficial effects of GH on memory, mental alertness, and motivation have been documented. Many actions of GH are mediated through IGF-I; hence, we investigated whether systemic administration of GH or GH-releasing peptide (GHRP)-6 modulates the brain IGF system. Treatment of adult male rats with GHRP-6 or GH for 1 wk significantly increased IGF-I mRNA levels in the hypothalamus, cerebellum, and hippocampus, with no effect in cerebral cortex. Expression of the IGF receptor and IGF-binding protein (IGFBP)-2 were not affected. Phosphorylation of Akt and Bad was stimulated in areas where IGF-I was increased, with no change in MAPK or glycogen synthase kinase-3 β . This suggests that GH and GHRP-6 activate phosphatidylinositol kinase intracellular pathways involved in cell survival in response to growth factors. Indeed, the antiapoptotic protein Bcl-2 was augmented in these same areas, with no change in the proapoptotic protein Bax. IGFBP-5, also reported to be involved in neuron survival processes, was increased mainly in the hypothalamus, suggesting a possible neuroendocrine role. In conclusion, GH and GHRP-6 modulate IGF-I expression in the central nervous system in an anatomically specific manner. This is coincident with activation of intracellular signaling pathways used by IGF-I and increased expression of proteins involved in cell survival or neuroprotection. (*Endocrinology* 143: 4113–4122, 2002)

THE PROFOUND EFFECTS of GH on the central nervous system (CNS) have become more apparent in the past decade. Not only is it involved in brain growth and development, but its qualities as a neuroprotective factor against injury are now appreciated (1–3). Recent studies by Scheepens *et al.* (1, 2) have demonstrated that GH is involved in neuroprotection during hypoxic-ischemic brain injury. Not only is there an increase in GH-like immunoreactivity on injured brain cells, but GH administered intracerebroventricularly is capable of preventing cell loss in this paradigm. This protection is coincident with the anatomical localization of the GH receptor and does not fully correlate with the neuroprotection exerted by IGF-I in this model, suggesting at least part of the effect is via GH itself.

Decreased GH secretion in some physiological or pathophysiological conditions has been associated with impaired cognitive function or brain activity (4–7). For example, activity of the GH-IGF-I axis undergoes an age-related decline, including decreased spontaneous GH secretion and circulating IGF-I levels, which may approximate the levels found in GH-deficient patients (8, 9). In both the elderly and GH-deficient adults, this decreased GH-IGF-I activity has been associated with changes in body composition and metabolism, altered sleep patterns, and reduced cognitive function

(4–6, 8, 9). Indeed, GH replacement therapy has been found to improve some age-dependent cognitive functions, such as memory, motivation, or mental processing speed, as well as behavioral problems in GH-deficient patients (4–6, 8, 9).

Synthetic peptide analogs that stimulate GH release were first described in 1981 (10). This led to the discovery of a family of related compounds, the GH secretagogues (GHS), that include GH-releasing peptide (GHRP)-6, a potent and safe GHS with activity in humans (11) and orally active nonpeptide GHSs (12–14). It is now known that these GHSs activate receptors for the endogenous peptide ghrelin (12, 13), as well as adenosine (15, 16) and that these receptors are expressed not only in the anterior pituitary and hypothalamus, but also in other brain regions (17–19), suggesting functions independent from the neuroendocrine control of GH secretion.

Direct action of GH on the CNS is supported by the fact that its receptor is expressed in diverse areas of the brain, including those involved with memory and cognitive function (7, 20), and this hormone can cross the blood-brain barrier in specific regions (7, 21). However, many effects of GH are mediated through stimulation of IGF-I production and the neuroprotective effects of this growth factor are evident (22–26). Very elegant studies by Carro *et al.* (23, 24) demonstrate that exercise via a physiological increase in circulating IGF-I levels is neuroprotective against diverse types of brain injury and in various brain regions and when IGF-I uptake into the brain is blocked, this neuroprotection is lost. Hence, GH can have direct effects on brain function or it can

Abbreviations: CNS, Central nervous system; CREB, cAMP-response element binding protein; GHRP, GH-releasing peptide; GHS, GH secretagogues; GSK, glycogen synthase kinase; IGFBP, IGF binding protein; p, phosphorylated; PI3K, phosphatidylinositol kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

act indirectly by increasing circulating IGF-I, which then enters the CNS. Furthermore, as IGF-I is produced in most areas of the brain, GH could also stimulate local IGF-I production in the CNS to promote neuroprotection.

IGF-I promotes cell survival in many tissues and cell proliferation in some, including neurons and oligodendrocytes (27). GH can also promote growth by directly inducing the proliferation of different cell types (28). Surprisingly, a direct effect of GH on cell survival has yet to be reported. The mechanisms by which IGF-I prevents cells from entering a death program have not been completely defined, but both the phosphatidylinositol kinase (PI3K) and the MAPK pathways have been implicated (29). Recent studies link IGF-I neuroprotective actions to the Bcl family (30). Current dogma suggests that cell fate is determined, at least in part, by the balance between the products of antiapoptotic and proapoptotic genes of the Bcl-2 family.

The purpose of these studies was to determine whether systemic administration of GHRP-6 modulates the brain IGF system and to compare this to the effects of GH. In addition, intracellular mechanisms activated in response to IGF-I and involved in neuroprotective processes were also determined.

Materials and Methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO) or Merck (Mollet de Vallés, Barcelona, Spain) unless otherwise noted.

Antibodies to phosphorylated forms of Akt (p-Akt) and glycogen synthase kinase (GSK) (pGSK), and MAPK were purchased from New England Biolabs, Inc. (Beverly, MA). Antibodies to Bcl-2 and Bax were from Neomarkers (Fremont, CA). Antibodies to IGF-IR, IGF-binding protein (IGFBP)-5, IGFBP-2, Akt, Bad and actin were from Santa Cruz Biotechnology, Santa Cruz, CA). GSK-3 β was from Transduction Laboratories, Inc. (Lexington, KY) and pBad from Upstate Biotechnology, Inc. (Lake Placid, NY). Secondary antibodies conjugated with peroxidase were from Pierce Chemical Co. (Rockford, IL).

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 in accordance with the UK Animal (Scientific) Procedures Act 1986. Rats were maintained on a 12-h light, 12-h dark schedule. Rats were infused with GH (Novo Nordisk, Bagsvaerd, Denmark; 100 μ g/d) or GHRP-6 (Bachem, Bubendorf, Switzerland; 150 μ g/d) using Alzet minipumps (1 μ l/h, 7 d) 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 immediately removed and frozen on dry ice.

Immunoblotting. For Western blotting, approximately 100 mg of hypothalamus, hippocampus, cerebellum, and cerebral cortex were homogenized in 500 μ l radioimmunoprecipitation assay lysis buffer with an EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After homogenization, the samples were centrifuged at 12,000 \times 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 concentration was estimated by Bio-Rad Laboratories, Inc. (Hercules, CA) protein assay. Thirty or 60 μ g of protein were resolved using 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). Filters were blocked with Tris-buffered saline containing 5% (wt/vol) nonfat dried milk, except for p-Akt where TBS containing 5% BSA was used, and incubated with the primary specific antibody at a dilution of 1:500. 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 chemiluminescence (NEN Life Science Products, Boston, MA) and quantified by densitometry using Bio-1D (Vilber Lourmat, Marne La Vallée, France). To correct for variations in the starting amount of protein all the blots were reprobed using an anti actin antibody.

RNA extraction. Total RNA was extracted from 100 mg of hypothalamus, hippocampus, cerebellum, and cerebral cortex according to the Tri-Reagent protocol (Sigma). Briefly, the samples were homogenized in 1 ml of Tri-Reagent and then centrifuged at 12,000 \times 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 then added, and the samples were shaken vigorously for 15 sec and then allowed to stand 15 min at room temperature. They were then centrifuged at 12,000 \times 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 12,000 \times 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 7,500 \times g at 4 C for 5 min. The pellets were air-dried and resuspended in diethyl pyrocarbonate-H₂O.

RT-PCR. The RNA (200 or 500 ng) was subjected to RT-PCR by using the Acces RT-PCR system (Promega Corp., Madison, WI) according to the manufacturer's instructions. Oligonucleotide primers for IGF-I, IGF-IR, IGFBP-2, and IGFBP-5 were synthesized by Life Technologies, Inc. (Barcelona, Spain) Custom Primers. The sequences of primers are shown in Table 1.

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 sec, followed by 40 cycles of 94 C for 1 min and 55 C for IGF-I and IGF-I receptor or 60 C for IGFBP-2 and IGFBP-5 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 UV illumination, compared with a known standard ladder (Promega Corp.) and quantified by densitometry using the Bio-1D system (Vilber Lourmat). The PCR primers were designed to obtain products of 253 bp for IGF-I, 321 bp for IGF-I receptor, 346 bp for IGFBP-2, and 643 bp for IGFBP-5. A single amplified band was observed for each reaction.

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 55C 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'.

Cell death assessment. Cell death detection assays were performed following manufacturer's instructions (Roche Molecular Biochemicals, Barcelona, Spain). Briefly, sections were fixed in 4% paraformaldehyde (wt/vol) for 10 min and then incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min. The labeling was done with the terminal deoxynucleotidyl transferase enzyme in the terminal deoxynucleotidyl transferase buffer containing fluorescein-16-deoxyuridine triphosphate, for 1 h at 37 C. Terminal deoxynucleotidyltrans-

TABLE 1. Sequences of primers used for RT-PCR

	Sense	Antisense
IGF-I	GCATTGTGGATGAGTGTTC	GGCTCCTCTACATTCTGTA
IGF-IR	AGGAGAAGCCCATGTGTGA	ATCTGAGTCACTGCTCTCC
IGFBP-2	AGAGACGCGTGGGCCACCC	GAGATGTTCCAGAGGCCCG
IGFBP-5	TCAGCGTGGTCTCCTGC	GTTGGGCAGGTACACGGC

ferase-mediated dUTP nick end labeling (TUNEL) signal was visualized by using a fluorescent microscope DMIL (Leica Corp., Madrid, Spain).

Immunohistochemistry. Immunohistochemistry was performed on frozen 20 μ m sections, fixed in 4% paraformaldehyde (wt/vol) and blocked in TBS containing 3% BSA and 1% Triton X-100 for 1 h. Sections were left overnight in a humid chamber at 4 C with the primary antibody, rabbit polyclonal anti-IGF-I antibody (a gift from Dr. I. Torres-Aleman, Instituto Cajal, Madrid) (1:500) or rabbit polyclonal anti-p-AKT (Ser473) (1:1000), in blocking solution. Afterward, sections were incubated with a biotin-conjugated antirabbit antibody (Pierce Chemical Co., Rockford, IL; 1:1000) for 90 min. Sections were then incubated in streptavidin-Alexa Fluor 594 conjugate (Molecular Probes, Inc., Eugene, OR; 1:2000) for 1 h. Incubation chambers were covered with foil paper to avoid exposure to light. Signal was visualized by using a confocal microscope (Leica Corp.).

Statistical analysis

The protein and mRNA from each animal was analyzed separately (no pooling of samples was used); therefore, the "n" represents the number of animals used in each group. Protein samples from each animal were analyzed twice and RT-PCR was repeated two to three times on each sample. The mean value of each animal was used for statistical analysis. All data were normalized to control values of each assay. Data were analyzed by one-way ANOVA. Significance was chosen as $P < 0.05$.

Results

IGF-I mRNA concentrations

Both GH and GHRP-6 significantly increased IGF-I mRNA levels in the hypothalamus, hippocampus, and cerebellum (Fig. 1). IGF-I mRNA concentrations in the hypothalamus increased to 300% of control levels in GH and 400% in GHRP-6-treated rats. In the hippocampus, IGF-I mRNA concentrations increased to approximately 200% of control levels in both treatment groups. In the cerebellum, the concentrations were 150% and 175% of control values in GH and GHRP-6 treated rats, respectively. No changes were observed in the cerebral cortex with either treatment.

IGF receptor and IGFBP-2 concentrations

There was no significant change in mRNA or proteins levels of either IGFR or IGFBP-2 in the hypothalamus, hip-

pocampus, cerebellum, or cerebral cortex in response to GH or GHRP-6 treatment (data not shown).

Activation of PI3K pathway

Analysis of MAPK by Western blot indicated that neither GH nor GHRP-6 treatment significantly activated this pathway in the brain areas studied (data not shown).

Immunoblots of Akt were prepared from hypothalamus, hippocampus, cerebellum, and cerebral cortex homogenates of rats treated with GH or GHRP-6 (Fig. 2). Activated Akt was assessed by using an antibody that specifically recognizes the form phosphorylated on Ser473 (*upper band*). Little Akt was phosphorylated in the basal state. Treatment with GH or GHRP-6 resulted in marked activation of Akt in the hypothalamus (300%), hippocampus (140% and 170%) and cerebellum (180% for GH). The apparent increase in Akt activation in the cerebellum of GHRP-6-treated rats did not reach statistical significance. Cerebral cortex samples did not show any variation in Akt phosphorylation. Figure 2, *lower band*, shows the same homogenates probed with an antibody that detects both the phosphorylated and nonphosphorylated forms of Akt. The total amount of Akt protein was not altered by any of the treatments.

Bad and Gsk levels

Analysis of Gsk-3 β by Western blot indicated that neither GH nor GHRP-6 treatment significantly increased either basal or phosphorylated forms in the brain areas studied (data not shown).

Immunoblots of Bad were prepared from hypothalamus, hippocampus, cerebellum, and cerebral cortex homogenates of rats treated with GH or GHRP-6 (Fig. 3). Inactivated Bad was assessed by using an antibody that specifically recognizes the phosphorylated form (*upper band*). Treatment with GH or GHRP-6 resulted in increased pBad levels in the hypothalamus and cerebellum. No change was seen in the cortex. Levels of unphosphorylated Bad did not differ between groups. Bad could not be detected in the hippocampus.

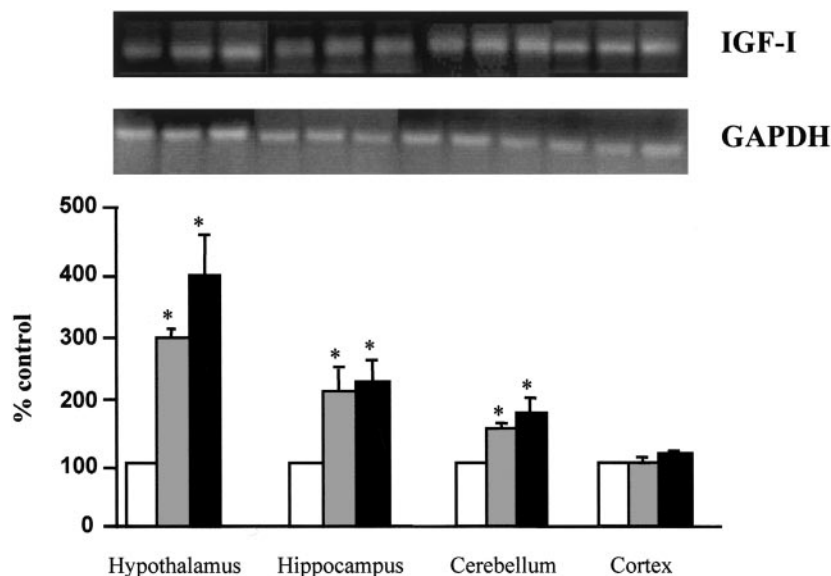


FIG. 1. Relative levels of IGF-I mRNA in different areas of the brain in response to GH or GHRP-6. *, $P < 0.05$ by ANOVA. Control, □, GH, ▨, GHRP-6, ■.

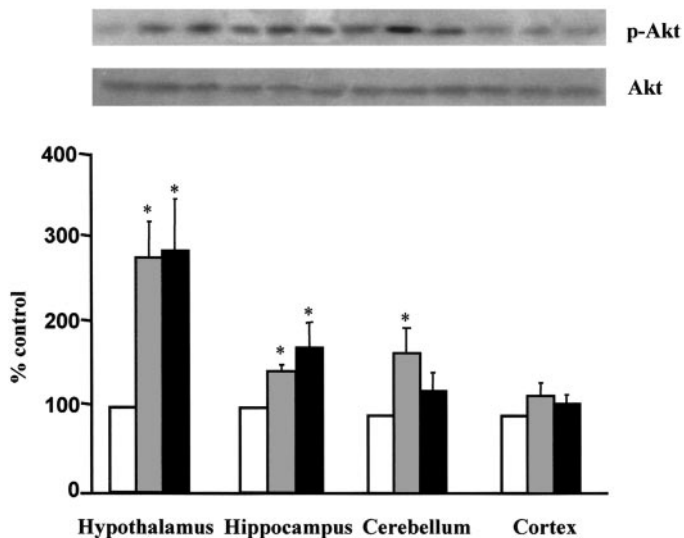


FIG. 2. Relative p-AKT protein levels in different areas of the brain in response to GH or GHRP-6. The upper band represents p-AKT and the lower band total AKT. *, $P < 0.05$ by ANOVA. Control, □, GH, ▨, GHRP-6, ■.

Bcl-2 and Bax protein levels

Hypothalamic Bcl-2 protein levels were increased to 200% of control levels in GH-treated rats and approximately 150% in GHRP-6-treated rats (Fig. 4). In the hippocampus, Bcl-2 protein abundance was increased to 200% of control levels in GH- and 180% in GHRP-6-treated rats. In cerebellum, levels were increased to 270% in GH-treated animals and 150% in GHRP-6-treated rats. In cerebral cortex, Bcl-2 expression was very low, and no differences could be discerned between the three experimental groups.

No changes in Bax protein levels in response to either GH or GHRP-6 were found in any area of the brain studied (data not shown).

IGFBP-5 concentrations

IGFBP-5 mRNA levels were increased in the hypothalamus of GH- and GHRP-6-treated rats and in the hippocampus of GH-treated rats (Fig. 5A). However, in the hippocampus of GHRP-6-treated and in the cerebellum of both GHRP-6- and GH-treated rats, there were no significant changes in IGFBP-5 mRNA levels. In cerebral cortex, expression of IGFBP-5 was low in the three treatment groups, and no differences could be discerned.

The same pattern was found when we studied protein abundance by Western blot. Compared with normal hypothalamus, IGFBP-5 protein abundance was increased 2-fold in GH-treated rats and 1.7-fold in GHRP-6-treated animals. In the hippocampus, IGFBP-5 protein content was increased 1.3-fold in GH-treated rats (Fig. 5B). No other significant changes in IGFBP-5 protein levels were detected.

Cell death detection by TUNEL

Very low levels of TUNEL-positive cells were detected in the brains of normal control rats. Representative examples of TUNEL labeling in the cerebellum and hippocampus are shown in Fig. 6, A and C. This low staining almost completely

disappeared in the equivalent areas of GHRP-6-treated rats (Fig. 6, B and D). Decreased labeling was also seen in GH-treated rats (data not shown).

IGF-I and p-Akt immunohistochemistry

IGF-I and p-Akt immunolabeling was increased in the hypothalamus of rats treated with either GH or GHRP-6. In response to both treatments, labeling for IGF-I was highest in tanycytes, the specialized glial cells lining the third ventricle (Fig. 7A). GHRP-6 treatment also increased p-AKT labeling in tanycytes. GH increased p-AKT immunolabeling in tanycytes, although to a lesser degree, and in cells throughout the periventricular area (Fig. 7A). Increased specific labeling for both IGF-I and p-AKT was observed in the arcuate nucleus of the hypothalamus (Fig. 7B). The median eminence of GH- and GHRP-6-treated rats also showed more intense labeling for both IGF-I and p-Akt compared with control rats (Fig. 7C).

In the cerebellum, there was increased immunostaining for both IGF-I and p-AKT in response to GH and GHRP-6 (Fig. 7D), with the Purkinje cells more strongly labeled after both treatments.

The epithelial cells lining the lateral ventricles were intensely labeled for IGF-I and slightly labeled for p-AKT. In the hippocampus, labeling for both p-AKT and IGF-I was specific, but low and diffuse (data not shown).

Discussion

Many actions of GH are mediated through stimulation of IGF-I synthesis. This growth factor, in turn, promotes cell survival in many tissues and cell proliferation in some. Here we demonstrate that both GH and the synthetic GHS, GHRP-6, induce IGF-I mRNA expression in specific areas of the CNS, such as the hypothalamus, hippocampus, and cerebellum. Furthermore, this increase is coincident with activation of intracellular signaling pathways used by IGF-I and increased expression of cell survival factors.

The survival of certain subsets of neurons can be promoted by activation of a pathway that includes the guanosine triphosphate-binding protein Ras and a series of protein kinases leading to MAPK (31). In addition, a pathway that includes the lipid kinase PI3K/Akt is important for the survival of several cell lines (32) and activation of this pathway is required for growth factor-induced survival (33). In our studies, no activation of MAPK was detected, suggesting that the Ras-MAPK pathway may not be critical for GH- and GHRP-6-promoted processes in these brain areas. The promotion of cell survival by IGF-I has been shown to require Akt activation (34). Likewise, a signaling pathway was delineated in human leukemic cells (HL-60) and Chinese hamster ovary cells by which GH promotes cell survival via GH induction of Akt phosphorylation. Here we show that GH and GHRP-6 increase phosphorylation of Akt in the hypothalamus, hippocampus and cerebellum, areas where IGF-I expression was also increased. Therefore, activation of intracellular signaling mechanisms involving Akt could be directly activated by GH, or via increased IGF-I.

More than one pathway has been suggested for the antiapoptotic effects of IGF-I, including the inactivation of Bad

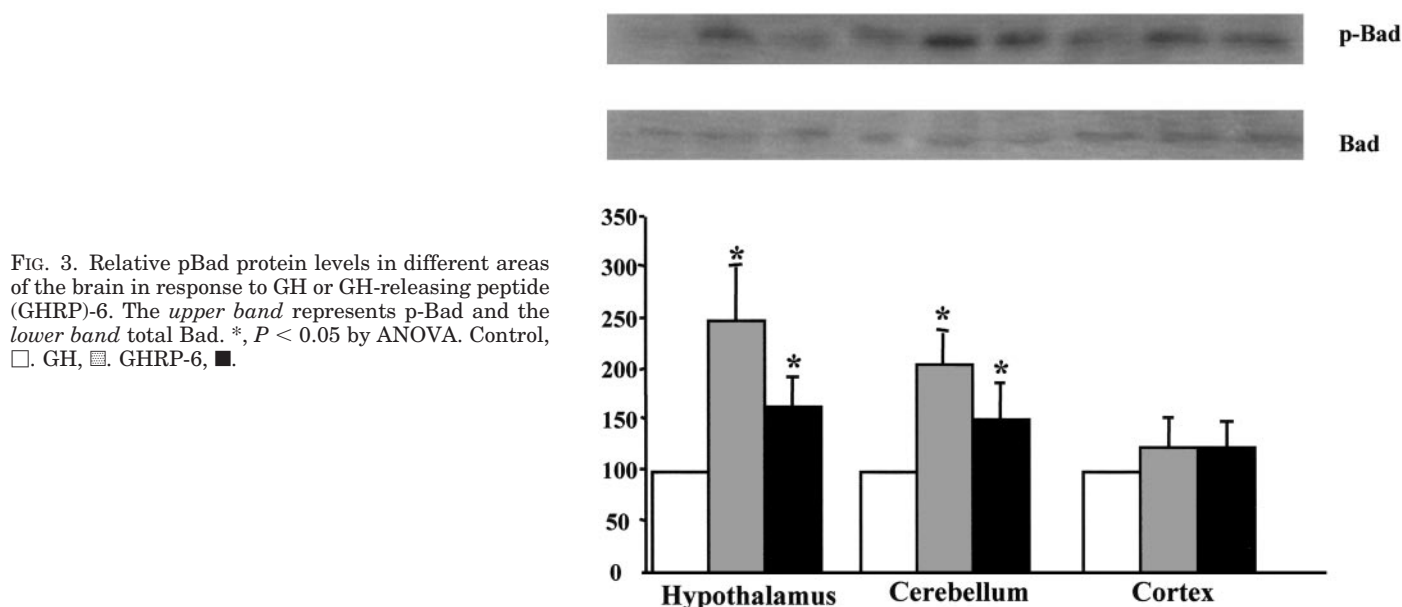


FIG. 3. Relative pBad protein levels in different areas of the brain in response to GH or GH-releasing peptide (GHRP)-6. The upper band represents p-Bad and the lower band total Bad. *, $P < 0.05$ by ANOVA. Control, □. GH, ▒. GHRP-6, ■.

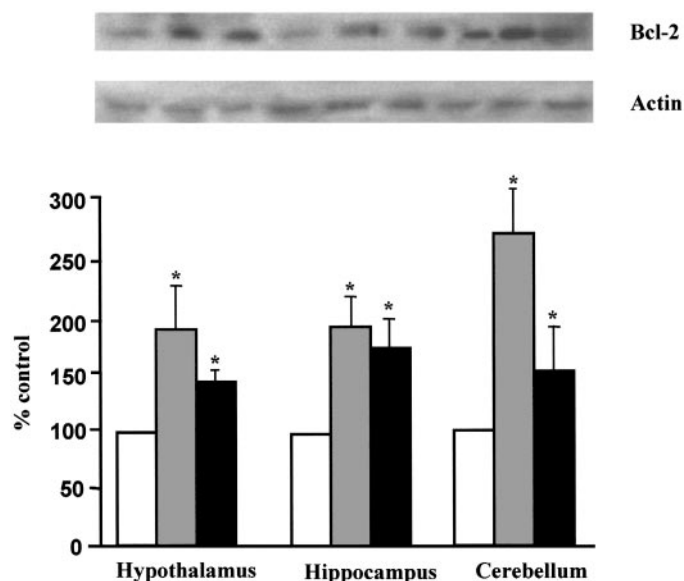


FIG. 4. Relative levels of Bcl-2 protein in different areas of the brain in response to GH or GHRP-6. *, $P < 0.05$ by ANOVA. Control, □. GH, ▒. GHRP-6, ■.

by phosphorylation (35–40). We observed increased Bad phosphorylation in both the hypothalamus and cerebellum in correlation with increased IGF-I levels. Indeed, in the cerebellum IGF-I induced dephosphorylation of Bad is involved in the prevention of neuronal death (36). In contrast, we found no activation of Gsk-3 β , another intracellular protein activated by IGF-I in many cell types. However, this protein appears to be more involved in cellular proliferation (41–43), indicating that in this paradigm IGF-I is most likely involved in neuroprotection and not activation of the cell cycle.

One family of proteins involved in cell death is the Bcl family. Up-regulation of Bcl-2 expression has been identified as a critical step by which growth factors promote cell sur-

vival (44, 45) and IGF-I increases the expression of Bcl-2 in adult rat brain (26). This protein forms homodimers, as well as heterodimers with other Bcl-2 family members and increased dimerization with proapoptotic members, such as Bax increases the susceptibility of a cell to cell death stimuli. Bcl-2 is expressed by neurons in many areas of the brain, where it functions to prevent both natural and induced neuronal death. In addition, it promotes the growth and regeneration of axons, suggesting that Bcl-2 may also be involved in brain repair and neural plasticity (46). Increased Bcl-2 and no change in Bax expression, as reported here, is consistent with conditions for increased cell survival.

The promoter region of Bcl-2 contains a cAMP-response element and the transcription factor cAMP-response element binding protein (CREB) up-regulates Bcl-2 expression (47). Akt, a target of IGF-I signaling, activates CREB (48). These data indicate that IGF-I regulation of Bcl-2 expression may involve a signaling cascade mediated by PI3K/Akt/CREB. Indeed, in PC-12 cells enhanced CREB activity by Akt signaling leads to increased Bcl-2 promoter activity and cell survival (49). We show for the first time that GH and GHRP-6 are capable of increasing Bcl-2 levels in hypothalamus, hippocampus, and cerebellum. Furthermore, this is coincident with increases in IGF-I and activation of Akt, suggesting the possible mechanism involved in this process.

In the adult brain, IGFBP-5 is one of the most highly expressed IGFBPs (50). Expression of this binding protein increased in response to GH and GHRP-6 treatment in some brain areas in coordination with increased IGF-I. This is consistent with data indicating that IGF-I promotes IGFBP-5 gene expression in some brain cells (51, 52) via the PI3K pathway (52). The classical role of the IGFBPs is to regulate the availability and actions of the IGFs (53); however, some of these proteins have also been reported to have IGF independent effects, including on cell survival (54). In situations of hypoxia-ischemia, both IGF-I and IGFBP-5 are reduced immediately in affected neurons (55, 56) and correlated with neuronal death. However, after 72 h of recovery, although

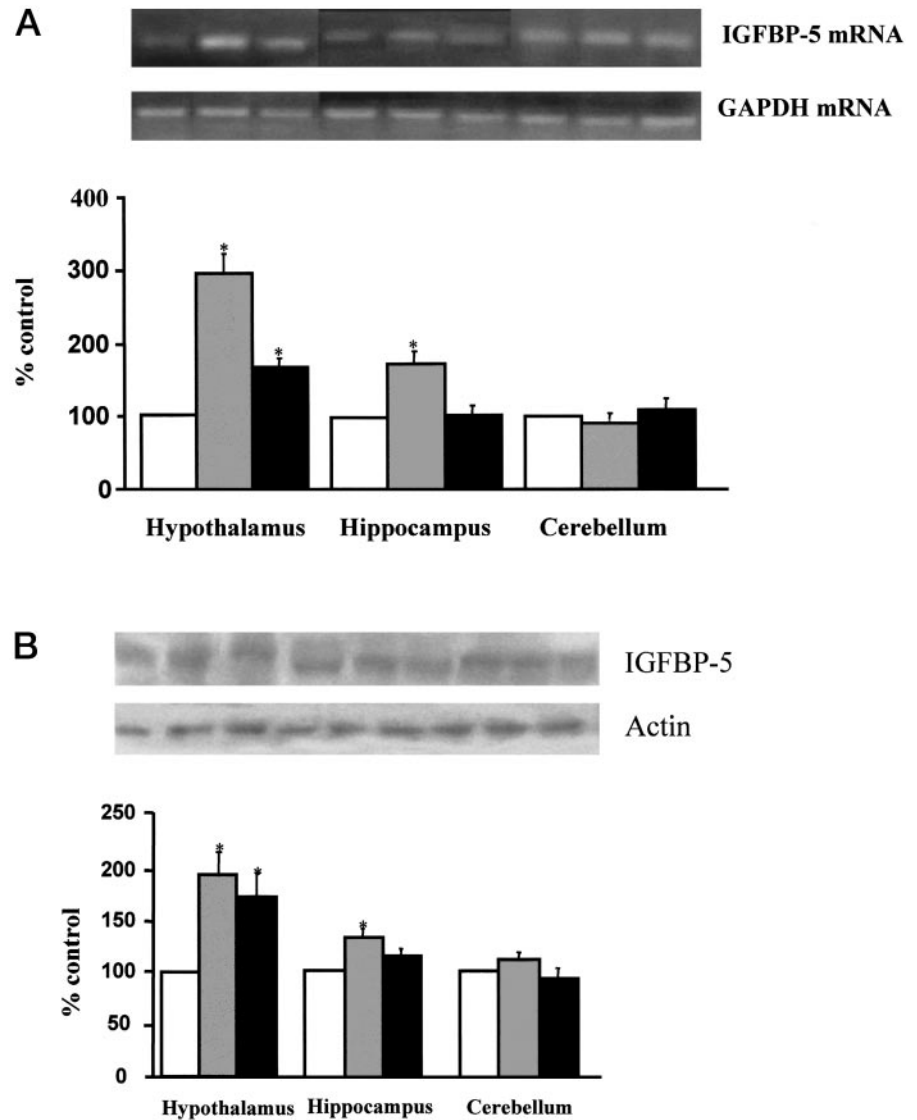


FIG. 5. Relative levels of IGFBP-5 mRNA (A) or protein (B) in different areas of the brain in response to GH or GHRP-6. *, $P < 0.05$ by ANOVA. Control, □. GH, ▨. GHRP-6, ■.

IGF receptor and IGFBP-2 expression remain low, IGF-I and IGFBP-5 levels increase in reactive astrocytes (56) and this increase is correlated with decreased neuronal death. Hence, IGFBP-5 may be involved in the neuroprotective actions of IGF-I in some areas of the brain. However, in our study IGFBP-5 levels were not modulated in all areas where IGF-I was increased. One possible explanation is that, because this protein is expressed in relatively low levels, with the methods employed we were unable to detect some of these changes. It is also possible that IGFBP-5 is selectively activated in some brain areas to participate in neuroprotective processes or is increased to serve other functions. Indeed, because the largest increase was found in the hypothalamus, IGFBP-5 may be involved primarily in neuroendocrine functions in response to GH and GHRP-6 treatment. Because IGFBP-5 can impede the binding of IGF-I to its receptor (57), increased IGFBP-5 could inhibit the actions of IGF-I in this area.

Apoptosis is a regulated process designed to eliminate damaged or aged cells from the body and can be induced by

a wealth of proapoptotic signals and cellular stresses, including withdrawal of survival factors (58). In normal young adult brain, the number of dying cells is low in most areas (59–61). In agreement, we found a very low level of TUNEL-positive cells in control animals. However, even this low basal level of cell death disappeared with GH or GHRP-6 infusion and the consequent increase in IGF-I and cell survival factor expression. Intracerebroventricular infusion of GH conveys neuroprotection against hypoxic-ischemic injury (1, 2); however, it remains to be determined whether systemic treatment is effective in promoting neuroprotection in the face of a noxious assault or other circumstances of increased neuronal death.

In the elderly, the decreased activity of the GH-IGF-I axis has been associated with the decline in age-sensitive cognitive function (4–7). Furthermore, in young adults, GH deficiency can lead to changes similar to those observed in aging, including cognitive impairment, which are clearly improved by GH therapy (9). Some of these effects are most likely due to changes in circulating and central IGF-I. Indeed, IGF-I

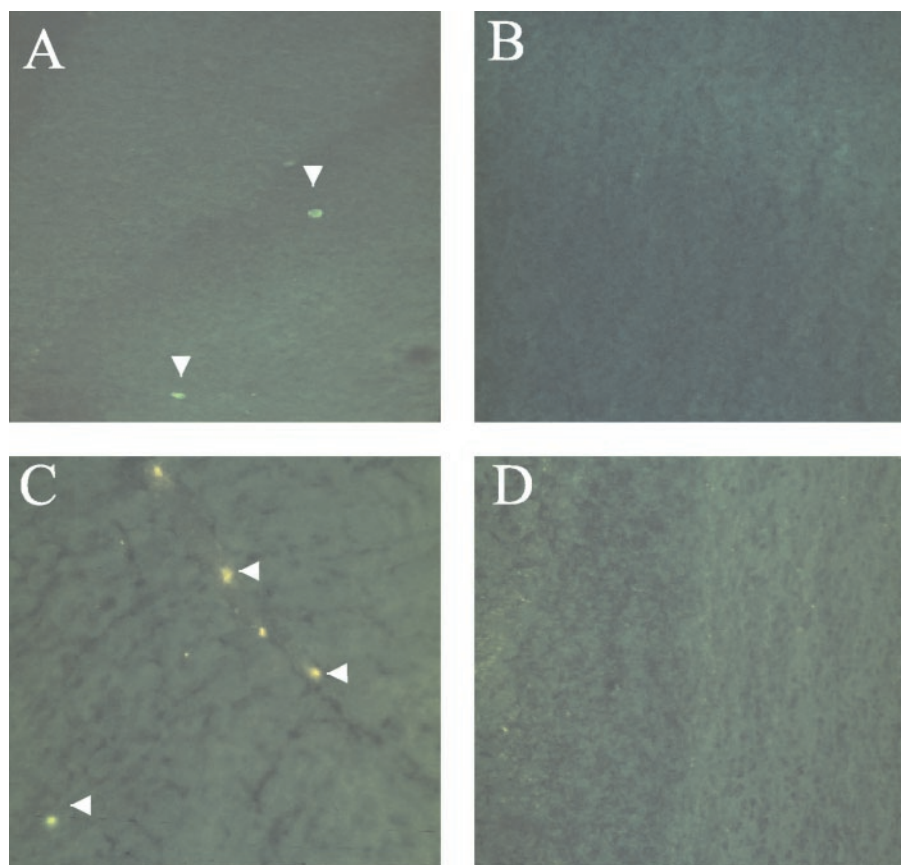


FIG. 6. TUNEL labeling in the control rat brains in the cerebellum (A) and hippocampus (C) and in GHRP-6 treated animals in the cerebellum (B) and hippocampus (D). White arrows indicate TUNEL-positive cells.

treatment can also ameliorate some age-related deficits (62), and recently it was proposed that GH or GHS treatment could be of value in minimizing the health-related consequences associated with the aging process (63). Our data not only support this hypothesis, because both GH and its synthetic GHS can increase IGF-I and activate pathways involved in neuroprotection, but also indicate the possible mechanism involved in this process.

The extrahypothalamic localization of the GH receptor suggests a nonclassical endocrine role for this hormone and that it may have direct effects in these brain areas (7, 20). Although GH could act via stimulation of IGF-I, as suggested by our studies, the neuroprotective effects of GH and IGF-I do not always correlate anatomically (2, 64), indicating a direct action of GH. Indeed, the GH receptor belongs to the superfamily of cytokine receptors and cytokines are one of the best-characterized groups of survival factors (65).

Similar, but not identical, results were obtained with GH and GHRP-6 treatment, suggesting that at least some of the actions of this GHS may be mediated through its ability to increase circulating GH concentrations (11, 66). However, data regarding the increase in circulating GH with chronic GHS treatment in rat are conflicting (67, 68). In addition, the GHS-R is expressed in all areas where a response was observed (14), indicating a possible direct effect. Unfortunately, the physiological function of these extrahypothalamic receptors remains unknown. Both the GHS and GH receptors are expressed in neurons of the arcuate nucleus involved in

growth and metabolic processes (69–72). The arcuate nucleus was one of the areas of the hypothalamus immunostained for both IGF-I and p-Akt, indicating zones where the PI3K/Akt pathway is activated. Increased IGF-I labeling of the tanycytes lining the third ventricle and in the borders of the lateral ventricle in rats treated with GH or GHRP-6 was also found. Because these cells do not appear to produce IGF-I (73), IGF-I uptake from the circulation most likely is increased (74).

Immunocytochemistry for IGF-I and p-Akt indicated that in most areas studied only subsets of cells were labeled, and some of them very intensely. Therefore, although by Western blot or RT-PCR these changes in protein or RNA levels may appear to be small, it is possible that because only a select population of cells are activated and their response is diluted by nonresponding cells when extraction techniques are used. The actual increase per cell may be quite dramatic if only a small number of cells are activated, as indicated by immunocytochemistry.

In recent years, the importance of circulating IGF-I in neuroprotective processes has become apparent (23–31). Systemic IGF-I is taken up into specific areas of the brain, and this process is modulated by different physiological situations such as gonadal steroid levels (74). Experimental (injection) or physiological (*e.g.* exercise) increases in systemic IGF-I stimulate brain IGF-I protein levels and neuroprotection (23–26). Indeed, in some paradigms, this uptake has a fundamental role in neuron protection even though circu-

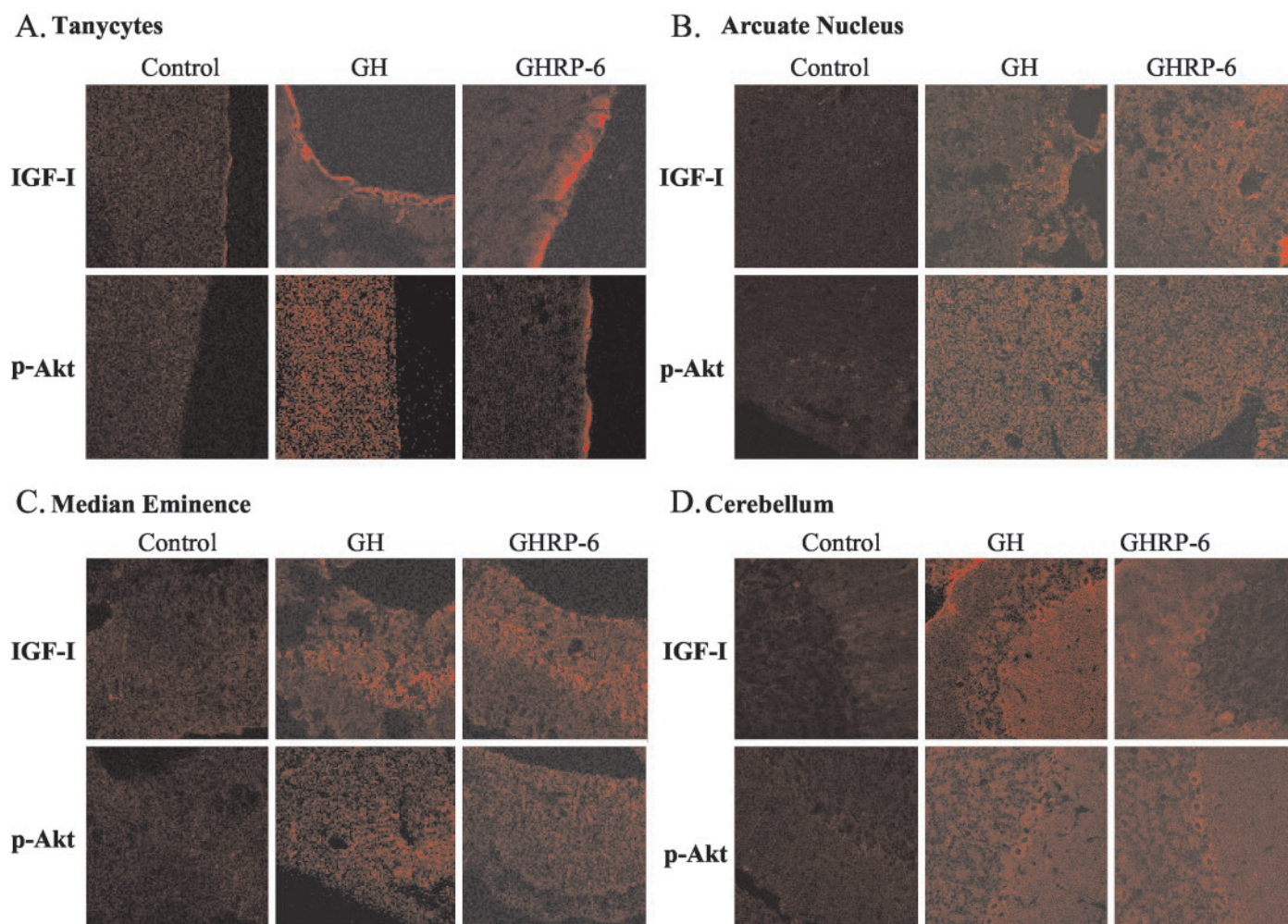


FIG. 7. Immunostaining for IGF-I and p-AKT in control, GH and GHRP-6-treated animals.

lating IGF-I levels are not significantly elevated (23, 24). Thus, it is possible that part of the effect reported here, *i.e.* p-Akt activation or increased Bcl-2, is due to increased uptake of circulating IGF-I. However, we have not detected a sustained increase in circulating IGF-I in response to the chronic GHS treatment paradigm reported here (our unpublished data). Connely *et al.* (68) reported an increase in circulating IGF-I after long-term, but not short-term, continuous hexarelin treatment to adult male rats. However, treatment of aged rats did not stimulate serum IGF-I levels in spite of increased GH secretion (67). Hence, the type of GHS, length of treatment and age of the animal, among other factors, most likely affect the circulating IGF-I response.

Up-take of IGF-I from the circulation could increase even if circulating levels do not increase. However, we did not detect any increase in uptake mechanisms, such as the IGF receptor or IGFBP-2, as has been reported in other studies (75). However, because IGF-I is increased in cells that do not produce this growth factor, increased uptake is a plausible mechanism and the influence of circulating IGF-I on the brain in response to GH or GHSs deserves further investigation as it is obviously important in neuroprotective processes. The observation that IGF-I mRNA levels are increased in specific

brain areas suggests increased local production and that, either alone or in conjunction with increased IGF-I uptake, it may be involved in local processes.

Numerous studies have demonstrated that IGF-I has neuroprotective properties in various brain areas, although the mechanisms underlying these processes are not well understood. Our results show that IGF-I expression in the CNS can be increased by systemic administration of either GH or GHS and that intracellular pathways involved in neuroprotective processes are activated in specific brain regions. These results may help to begin understanding the beneficial effects that GH has on the brain in specific situations, such as in the elderly or GH deficient adults.

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