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Insulin and growth hormone-releasing peptide-6 (GHRP-6) have differential beneficial effects on cell turnover in the pituitary, hypothalamus and cerebellum of streptozotocin (STZ)-induced diabetic rats☆

Miriam Granado[a,b,c], Cristina García-Cáceres[a,b], María Tuda[a,b], Laura M. Frago[a,b], Julie A. Chowen[a,b], Jesús Argente[a,b,*]

[a] Department of Endocrinology, Hospital Infantil Universitario Niño Jesús, Madrid, Spain
[b] Department of Pediatrics, Universidad Autónoma de Madrid, Instituto de Investigación Sanitaria La Princesa and CIBER Fisiopatología de Obesidad y Nutrición, Instituto de Salud Carlos III, Madrid, Spain
[c] Department of Physiology, Faculty of Medicine, Universidad Autónoma de Madrid, Spain

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ABSTRACT

Poorly controlled type1 diabetes is associated with hormonal imbalances and increased cell death in different tissues, including the pituitary, hypothalamus and cerebellum. In the pituitary, lactotrophs are the cell population with the greatest increase in cell death, whereas in the hypothalamus and cerebellum astrocytes are most highly affected. Insulin treatment can delay, but does not prevent, diabetic complications. As ghrelin and growth hormone (GH) secretagogues are reported to prevent apoptosis in different tissues, and to modulate glucose homeostasis, a combined hormonal treatment may be beneficial. Hence, we analyzed the effect of insulin and GH-releasing peptide 6 (GHRP-6) on diabetes-induced apoptosis in the pituitary, hypothalamus and cerebellum of diabetic rats. Adult male Wistar rats were made diabetic by streptozotocin injection (65 mg/kg *ip*) and divided into four groups from diabetes onset: those receiving a daily *sc* injection of saline (1 ml/kg/day), GHRP-6 (150 μg/kg/day), insulin (1–8 U/day) or insulin plus GHRP-6 for 8 weeks. Control non-diabetic rats received saline (1 ml/kg/day). Diabetes increased cell death in the pituitary, hypothalamus and cerebellum ($P < 0.05$). In the pituitary, insulin treatment prevented diabetes-induced apoptosis ($P < 0.01$), as well as the decline in prolactin and GH mRNA levels ($P < 0.05$). In the hypothalamus, neither insulin nor GHRP-6 decreased diabetes-induced cell death. However, the combined treatment of insulin + GHRP-6 prevented the diabetes induced-decrease in glial fibrillary acidic protein (GFAP) levels ($P < 0.05$). In the cerebellum, although insulin treatment increased GFAP levels ($P < 0.01$), only the combined treatment of insulin + GHRP-6 decreased diabetes-induced apoptosis ($P < 0.05$). In conclusion, insulin and GHRP-6 exert tissue specific effects in STZ-diabetic rats and act synergistically on some processes. Indeed, insulin treatment does not seem to be effective on preventing some of the diabetes-induced alterations in the central nervous system.

1. Introduction

Diabetes mellitus, the most common chronic disease in childhood, results in long-term complications when poorly controlled over an extended period of time. Indeed, poor glycemic control is not only associated with metabolic and hormonal imbalances (Bestetti et al., 1985; Boujon et al., 1995; Välimäki et al., 1991), but also with an increased risk of disorders in the central nervous system (CNS) as a result of changes in brain metabolism, vascular reactivity, blood–brain barrier integrity and increased oxidative stress (Fouyas et al., 2003; Manschot et al., 2007; Valko et al., 2007). Some of these alterations could be due, at least in part, to increased apoptosis of both neurons and glia cells, as chronic hyperglycemia has been reported to induce cell death of cortical, hippocampal and hypothalamic neurons (Jakobsen et al., 1987; Klein et al., 2004; Li et al., 2002), as well as to induce cell death and decrease cell proliferation of astrocytes both *in vivo* and *in vitro* (Acheampong et al., 2009; García-Cáceres et al., 2008; Lechuga-Sancho et al., 2006a,b; Rungger-Brändle et al., 2000).

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* Corresponding author at: Department of Pediatrics & Pediatric Endocrinology, Hospital Infantil Universitario Niño Jesús, Avenida Menéndez Pelayo, 65, 28009 Madrid, Spain. Tel.: +34 91 5035939; fax: +34 91 5035939.
E-mail addresses: argentefen@terra.es, jesus.argente@uam.es (J. Argente).

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Alterations in the cellular composition of the anterior pituitary could also be involved in some of the diabetes-induced endocrine disruptions, as this gland undergoes increased apoptosis in poorly controlled STZ-diabetic rats (Arroba et al., 2003, 2005; Granado et al., 2009). Moreover, the increased cell death is cell-type specific, with lactotrophs being most highly affected (Arroba et al., 2003) as a result of caspase-8 activation (Arroba et al., 2005; Granado et al., 2009). However, pituitary levels of proteins involved in the intrinsic cell death pathway, including members of the Bcl-2 family, the effector caspase 3 and the anti-apoptotic proteins Hsp-70 and XIAP, are either unchanged or balanced towards cell survival (Arroba et al., 2005; Granado et al., 2009).

Astrocytes play a major role in the homeostatic regulation of the CNS as they are involved in neurotransmitter uptake, neuronal metabolic support, pH regulation, and neural-protection against toxic episodes such as excitotoxicity and oxidative stress (Aschner, 2000; Lamigeon et al., 2001; Montgomery, 1994). In poorly controlled diabetes a decrease in GFAP levels has been reported both in the cerebellum and hypothalamus as a result of increased death of astrocytes (García-Cáceres et al., 2008; Lechuga-Sancho et al., 2006a,b). However the mechanism by which these glial cells undergo apoptosis is different in these two brain areas. In the hypothalamus this process involves nuclear translocation of apoptosis inducing factor (AIF) (García-Cáceres et al., 2008), whereas in the cerebellum activation of the intrinsic cell death pathway occurs (Lechuga-Sancho et al., 2006b).

Many of the diabetes-induced endocrine and CNS disruptions are prevented after insulin replacement, which is due largely to the improvement in glycaemia and metabolism (Biessels et al., 1998; Pérez Díaz et al., 1982). However, insulin also has direct effects on cell survival. Indeed, insulin decreases neuronal death both *in vivo* and *in vitro* (Li et al., 2002; Duarte et al., 2008; Lee-Kwon et al., 1998; Voll and Auer, 1991a,b).

GHRP-6 is a synthetic compound that binds to the ghrelin receptor (GHS-R) and promotes GH secretion (Bowers et al., 1984; Howard et al., 1996). In addition, both ghrelin and GH secretagogues (GHSs) exert GH-independent effects such as stimulation of food intake (Wren et al., 2001), induction of adiposity (Tschöp et al., 2000), anti-inflammatory effects (Dixit et al., 2004; Granado et al., 2005a) and anti-apoptotic actions (Chung et al., 2007; Granata et al., 2006; Miao et al., 2007). Indeed, GHRP-6 protects hypothalamic neurons from glutamate excitotoxicity (Delgado-Rubín et al., 2009), decreases age-induced cell death in the cerebellum (Pañeda et al., 2003) and activates intracellular signaling pathways involved in neuroprotection (Frago et al., 2002). Furthermore ghrelin, the endogenous ligand of GHS-R, prevents diabetes-induced apoptosis of lactotrophs (Granado et al., 2009) and the development of diabetes during adulthood in rats treated neonatally with streptozotocin (Irako et al., 2006). Ghrelin is also involved in the regulation of insulin secretion and glucose metabolism (Dezaki et al., 2004) and the combined treatment of diabetic rats with insulin and GHRP-6 has additive effects on body composition (Granado et al., 2010).

Thus, the aim of this study was to analyze the possible protective effects of GHRP-6 both in the presence and absence of insulin treatment in the development of diabetes induced alterations of the anterior pituitary, the hypothalamus and the cerebellum.

2. Material and methods

2.1. Animals

All experiments were designed according to the European Union laws for animal care and the study was approved by the local institutional ethical committee. Adult male Wistar rats from Harlan Iberica S.A. (Barcelona, Spain) were housed two per cage with free access to food and water, under constant conditions of temperature (20–22 °C) and light/dark cycles (lights on from 07:30 to 19:30). Before diabetes induction, rats were adapted for one week to the new environment and diet.

Table 1
Western blot antibodies.

Antibody	Source	Western blot concentration
GFAP	Sigma–Aldrich	1:1000
PCNA	Signet Laboratories	1:1000
Caspase 3	Becton-Dickinson Biosciences	1:1000
Caspase 6	Medical Biological Laboratories	1:1000
Caspase 8	Neomarkeres	1:1000
Caspase 9	Medical Biological Laboratories	1:1000
Hsp 70	Stressgen Bioreagents	1:1000
XIAP	Becton-Dickinson Biosciences	1:1000
GAPDH	Anaspec	1:1000
Actin	Santa Cruz Biotechnology	1:1000

The rats, weighing approximately 250 g, were injected (*i.p.*) with 65 mg/kg streptozotocin (Sigma, Steinheim, Germany). Controls received vehicle. Blood glucose concentrations were measured via tail puncture (Glucocard Memory 2; Menarini Diagnostic, Florence, Italy) to verify the diabetic state (defined as blood glucose levels > 300 mg/dl). Immediately after the onset of diabetes, the rats were randomly divided into treatment groups.

The treatments consisted in a daily subcutaneous (*sc*) injection of saline (1 ml/kg, $n = 11$), GHRP-6 (Bachem, Bubendorf, Swizerland, 150 μg/kg/day, $n = 11$), insulin (Humulin NPH Pen 100 IU/ml, 1–8 U/day, $n = 12$) or insulin plus GHRP-6 ($n = 12$). The dose of GHRP-6 was selected from previous studies (Granado et al., 2005a,b; Cibrián et al., 2006; Winter et al., 2004). Control rats received saline ($n = 12$). All rats received their treatments between 18.00 and 19.00 just before the lights were turned-off and the animals naturally began their feeding period. After 8 weeks of treatment, all rats were killed by decapitation 15 h after the last injection. Trunk blood was collected in cooled tubes, allowed to clot, and then centrifuged. Serum was stored at −80 °C until hormone levels were measured. The brains and pituitaries were removed, weighed and stored at −80 °C until processed.

2.2. Control of body weight and glycemia levels

Glycemia and body weight were assessed daily before treatment administration. To maintain glucose levels in the most normal physiological levels and in order to avoid hypoglycaemia a slow acting insulin with a maintained effect for at least 12 h was injected and the insulin dose was adjusted depending on glycemia levels according to the following criteria: no insulin if glycemia was <50 mg/dl, 1 U of insulin if glycemia was between 50 and 70 mg/dl, 2 U of insulin if glycemia was between 70 and 100 mg/dl, 4 U of insulin if glycemia was between 100 and 200 mg/dl, 6 U of insulin if glycemia was between 200 and 400 mg/dl, 7 U of insulin if glycemia was between 400 and 500 mg/dl, and 8 U of insulin if glycemia was >500 mg/dl.

2.3. Tissue homogenization and protein quantification

Tissue was homogenized on ice in 200 μl of radioimmunoprecipitation assay lysis buffer with an EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After homogenization, samples were centrifuged at 14,000 rpm for 20 min at 4 °C. Supernatants were transferred to a new tube and protein concentration was estimated by Bradford protein assay.

2.4. ELISA cell death detection

This photometric enzyme immunoassay for the quantification of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) was performed according to the instructions of the manufacturer (Roche Diagnostics). The same amount of total protein was loaded in all wells and each sample was measured in duplicate (Tecan InfiniteM200, Grödig, Austria). The background value was subtracted from the mean value of each sample and all values are referred to the mean value of the control group.

2.5. Immunoblotting

In each assay the same amount of protein was loaded in all wells (1–60 μg) depending on the protein to be detected and resolved by using 12% SDS-acrylamide gels. After electrophoresis proteins were transferred to polyvinylidine difluoride (PVDF) membranes (Bio-Rad) and transfer efficiency was determined by Ponceau red dyeing. Filters were then blocked with Tris-buffered saline (TBS) containing 5% (w/v) non-fat dried milk and incubated with the appropriate primary antibody (for details, see Table 1). Membranes were subsequently washed and incubated with the corresponding secondary antibody conjugated with peroxidase (1:2000; Pierce, Rockford, IL, USA). Bound peroxidase activity was visualized by chemiluminescence and quantified by densitometry using a Kodak Gel Logic 1500 Image Analysis System and Molecular Imaging Software, version 4.0 (Rochester, NY, USA). All blots were rehybridized with actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to normalize each sample for gel-loading variability. All data are normalized to control values on each gel.

Table 2
Pituitary hormones mRNA levels.

	C + saline	Db + saline	Db + GHRP-6	Db + Ins	Db + Ins + GHRP-6
GH	100 ± 10.9	44.1 ± 7*	61.8 ± 11.5*	104.1 ± 14.9	140.1 ± 15.2
PRL	100 ± 6.1	61.9 ± 12.5*	57.7 ± 5.9*	106.8 ± 11.1	101.8 ± 9.4
POMC	100 ± 7.2	75.3 ± 19.6	54.6 ± 14.1*	133.9 ± 16.5	175.9 ± 23.1*
TSH	100 ± 26.1	94.7 ± 31.8	50.1 ± 4.9	66.9 ± 13.1	64.6 ± 4.9
LH	100 ± 13.1	229 ± 17.3*	254.3 ± 28*	139.8 ± 15.4	182.1 ± 26.1
FSH	100 ± 15.2	166.5 ± 10.1*	100.1 ± 17.7	93.7 ± 14.3	120.4 ± 8.4

Gene expression of growth hormone (GH), prolactin (PRL), pro-opiomelanocortin (POMC), thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the pituitary of control rats injected with saline (C + saline) and diabetic rats injected with saline (Db + saline), growth hormone-releasing peptide 6 [GHRP-6 (Db + GHRP-6, 150 μg/kg/day)], insulin (Db + Ins, 0–8 U/day) or insulin plus GHRP-6 (Db + Ins + GHRP-6). Data are presented as mean ± SEM and referred to control values (n = 4–6).

* P-value is lower than 0.05.

2.6. RNA preparation and purification and quantitative real-time PCR

Total RNA was extracted from the pituitaries, hypothalami and cerebelli according to the Tri-Reagent protocol (Chomczynski, 1993). cDNA was then synthesized from 1 μg of total RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA).

2.7. Quantitative real-time PCR

Growth hormone (GH), prolactin (PRL), pro-opiomelanocortin (POMC), thyroid-stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and tumour necrosis factor α (TNF-α) mRNAs were assessed in pituitary samples by quantitative real-time PCR. Insulin-like growth factor I (IGF-I), IGF-I receptor (rIGF-I), GHS receptor (GHS-R), and insulin receptor (INS-R) mRNAs were measured in the pituitaries, hypothalami and cerebelli. Quantitative real-time PCR was performed by using assay-on-demand kits (Applied Biosystems) for each gene: FSH (Rn01484594), GH (Rn01495894), GHS-R (Rn00821417), IGF-I (Rn99999087), rIGF-I (Rn01477918), INS-R (Rn00567670), LH (Rn00563443), POMC (Rn00595020), PRL (Rn00440945), TNF-α (Rn01525859), TSH (Rn00565424). TaqMan Universal PCR Master Mix (Applied Biosystems) was used for amplification according to the manufacturer's protocol in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Values were normalized to the housekeeping gene 18S (Rn01428915). According to manufacturer's guidelines, the ΔΔCT method was used to determine relative expression levels. Statistics were performed using ΔΔCT values (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Statistics were performed using the statistics program GraphPad Prism 4.0. Two-way analysis of variance (ANOVA) was used to determine the effect and interaction of treatments on the measured outcomes in diabetic rats. Differences among experimental groups were analyzed by one-way ANOVA and post hoc comparisons were made using subsequent Bonferroni multiple range tests. Data are presented as mean ± SEM and the values were considered significantly different when the P-value was lower than 0.05.

3. Results

3.1. Glycemia and body weight gain

We have previously reported that the diabetic rats injected with saline or GHRP-6 had decreased bodyweight gain and increased glycaemia compared to control animals (Granado et al., 2010). Insulin treatment resulted in bodyweight gain similar to that observed in control rats and significantly decreased glycaemia compared to saline-injected rats, although their glycaemia levels were still significantly increased compared to non-diabetic animals. Diabetic rats treated with insulin and GHRP-6 gained more weight than rats from all other experimental groups, including control animals. However, their glycaemia levels, although significantly reduced compared to saline-treated diabetic rats were still higher than those of control rats (Granado et al., 2010).

4. Pituitary

4.1. Cell death, total protein content and proliferating cell nuclear antigen (PCNA) levels

Diabetes increased cell death (Fig. 1A; P < 0.05) and decreased total protein content (Fig. 1B; P < 0.001) and proliferating cell nuclear antigen (PCNA) levels (Fig. 1C; P < 0.001) in the pituitary. Insulin, but not GHRP-6, normalized the diabetes-induced increase in cell death and total protein content and the decrease in pituitary PCNA levels, with no interaction between the two factors.

4.2. Pituitary hormone mRNA levels

The mRNA levels of GH, PRL, POMC, TSH, LH and FSH in the pituitary of control and diabetic rats are shown in Table 2. GH mRNA levels were decreased in the pituitary of saline-injected diabetic rats (P < 0.01) and both insulin and GHRP-6 increased the pituitary mRNA levels of this hormone (P < 0.001 and P < 0.05, respectively), with no interaction between the two factors. Likewise, diabetes decreased the expression of PRL (P < 0.01) and insulin, but not GHRP-6, prevented the diabetes-induced decrease of PRL mRNA levels (P < 0.01), with no interaction between insulin and GHRP-6. On the contrary, pituitary LH and FSH mRNA content were increased in diabetic rats treated with saline (P < 0.001 and P < 0.05, respectively). Insulin, but not GHRP-6, decreased the diabetes-induced rise in pituitary LH mRNA to control levels, with no interaction between the two factors. However, both insulin and GHRP-6 treated rats returned pituitary FSH mRNA levels to that of control rats (P < 0.05), with an interaction between the two factors (F = 11.68; P < 0.01). Pituitary TSH mRNA levels were unchanged in response to diabetes as well as the treatments employed. Likewise, diabetes did not modify POMC levels in the pituitary. However, diabetic rats treated with GHRP-6 had decreased POMC pituitary content compared to controls (P < 0.001) whereas the pituitary levels of this hormone were significantly increased in diabetic rats treated with insulin plus GHRP-6 (P < 0.001).

4.3. Pituitary Bcl-2, HSP-70 and XIAP content

Bcl-2 (Fig. 2A; P < 0.01), Hsp-70 (Fig. 2B; P < 0.05) and XIAP (30 kDa, Fig. 2C; P < 0.001) were up-regulated in the pituitary of diabetic rats injected with saline (P < 0.01, P < 0.05 and P < 0.001, respectively) and insulin, but not GHRP-6, prevented diabetes-induced up-regulation of these anti-apoptotic proteins, with no interaction between the two treatments.

4.4. Caspase-8 and TNF-a in the pituitary

Caspase-8 content was increased in the pituitary of diabetic rats injected saline (Fig. 3A; P < 0.01) compared to controls. There was no interaction between insulin and GHRP-6, with GHRP-6 having no effect and insulin preventing the diabetes-induced up-regulation of pituitary caspase-8 (P < 0.001).

Likewise, TNF-α gene expression was increased in the pituitary of diabetic rats injected saline compared to control rats (Fig. 3B; P < 0.05) and only insulin treatment prevented the diabetes-induced increase of TNF α mRNA levels, with no interaction between insulin and GHRP-6.

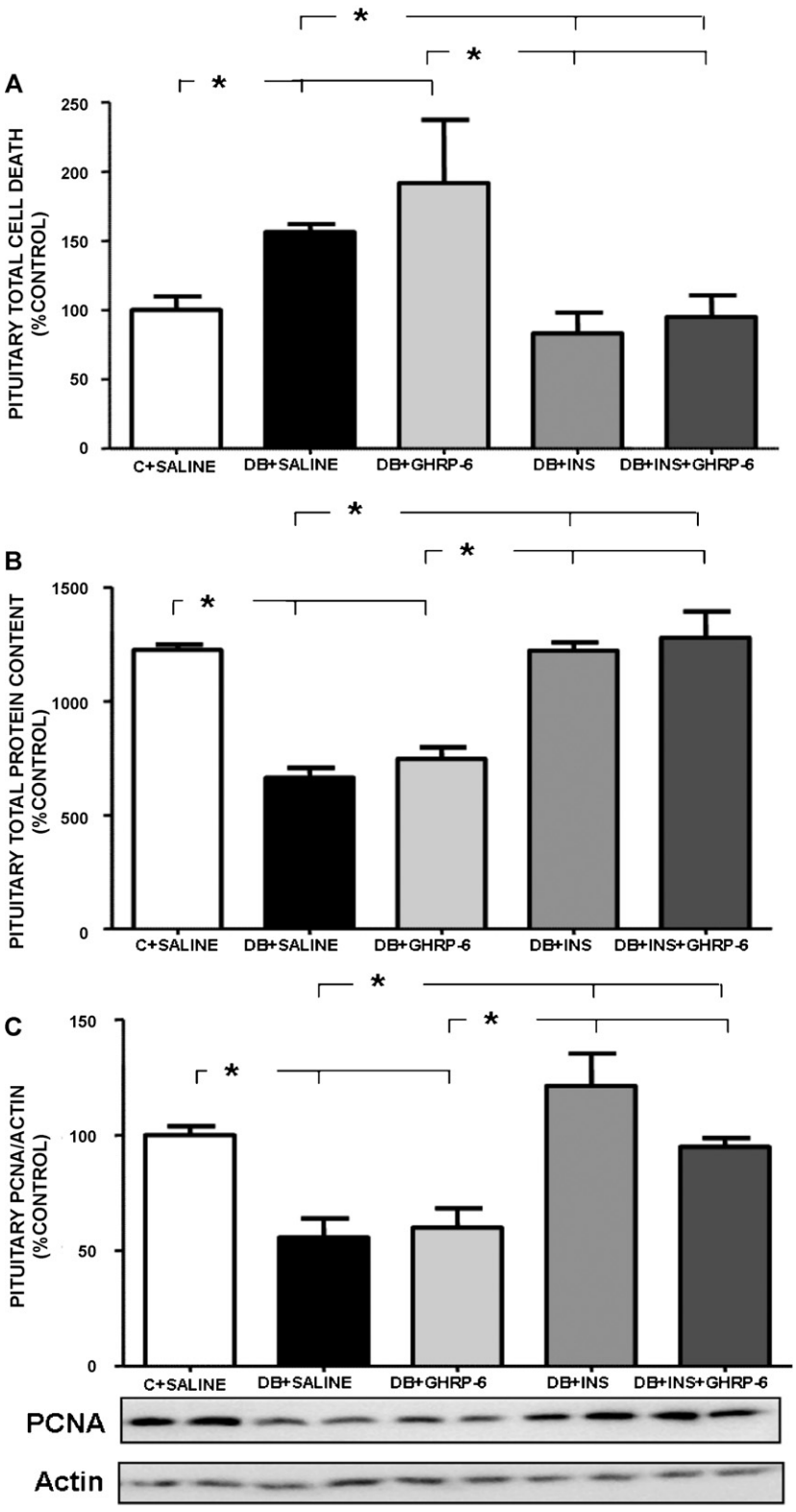

Fig. 1. Cell death (A), total protein content (B) and proliferating cell nuclear antigen (PCNA) levels (C) in the pituitary of control rats injected with saline (C + saline) and diabetic rats injected with saline (Db + saline), growth hormone-releasing peptide 6 [GHRP-6 (Db + GHRP-6, 150 μg/kg/day)], insulin (Db + Ins, 0–8 U/day) or insulin plus GHRP-6 (Db + Ins + GHRP-6). Data are presented as mean ± SEM and referred to control values (n = 4–6). *P-value is lower than 0.05.

4.5. Pituitary IGF-I and rIGF-I mRNA levels

There was no significant effect of diabetes or the treatments on IGF-I (C + saline: 100 ± 6; Db + saline: 163 ± 57; Db + GHRP-6: 76 ± 9; Db + Ins: 105 ± 8; Db + Ins + GHRP-6: 112 ± 9) or rIGF-I mRNA levels (C + saline: 100 ± 5; Db + saline: 185 ± 66; Db + GHRP-6: 153 ± 13; Db + Ins: 100 ± 6; Db + Ins + GHRP-6: 110 ± 7) in the pituitary (Fig. 4A and B; P > 0.05).

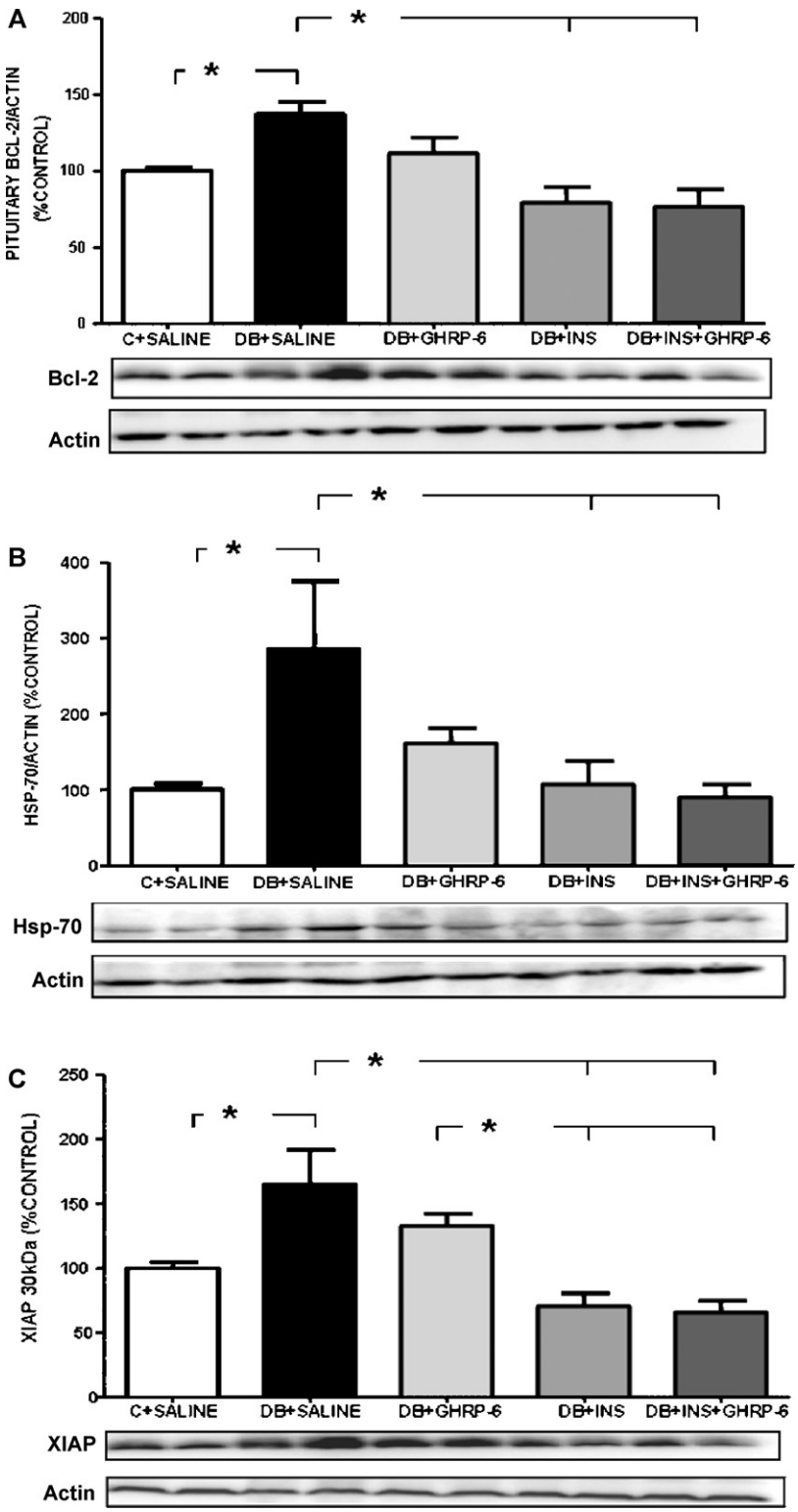

Fig. 2. Pituitary levels of B-cell lymphoma 2 (Bcl-2; A), heat shock protein-70 (Hsp-70; B) and X-linked inhibitor of apoptosis protein (XIAP) 30 kDa (C) in control rats injected with saline (C + saline) and diabetic rats injected with saline (Db + saline), growth hormone-releasing peptide 6 (GHRP-6 (Db + GHRP-6, 150 μg/kg/day), insulin (Db + Ins, 0–8 U/day) or insulin plus GHRP-6 (Db + Ins + GHRP-6). Data are presented as mean ± SEM and referred to control values (n = 4–6). *P-value is lower than 0.05.

5. Hypothalamus

5.1. Cell death, PCNA and GFAP levels in the hypothalamus

Diabetes increased cell death (Fig. 5A; $P < 0.01$) and decreased PCNA levels (Fig. 5B; $P < 0.01$) in the hypothalamus. There was no interaction between insulin and GHRP-6 either on cell death or PCNA levels in the hypothalamus of diabetic rats. Hypothalamic PCNA content was decreased in all diabetic animals compared to controls, regardless of the treatment received ($P < 0.01$). In contrast, cell death was increased in the hypothalamus of all diabetic animals ($P < 0.05$), except for those who received the combined treatment

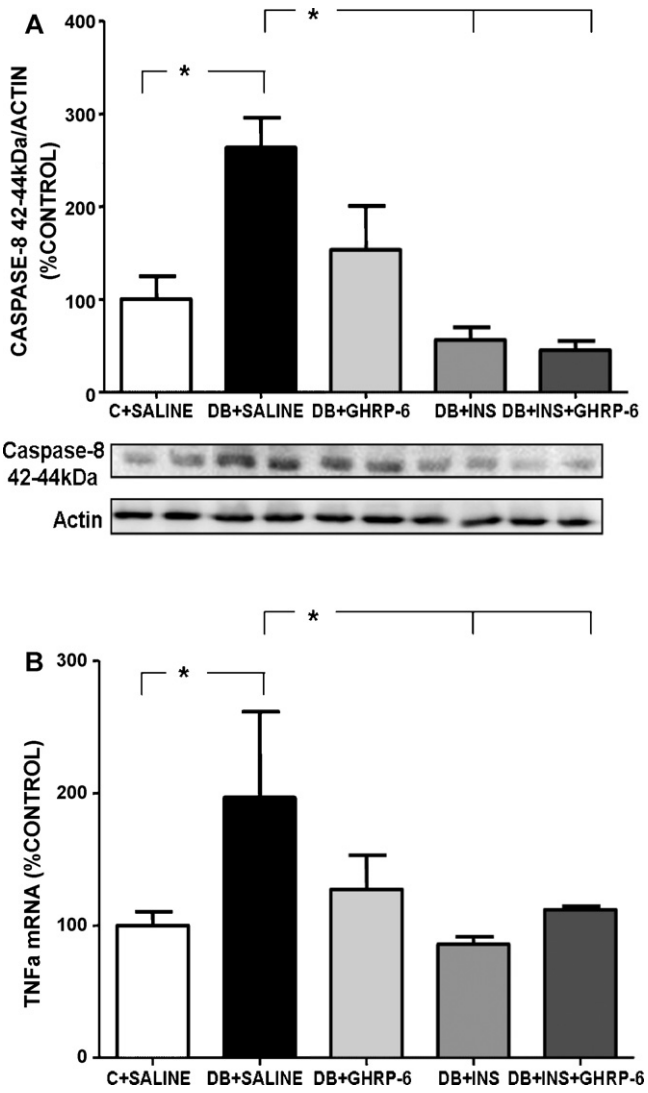

Fig. 3. Active caspase-8 (A) and tumour necrosis factor α (TNF-α) mRNA levels (B) in the pituitary of control rats injected with saline (C + saline) and diabetic rats injected with saline (Db + saline), growth hormone-releasing peptide 6 (GHRP-6 (Db + GHRP-6, 150 μg/kg/day), insulin (Db + Ins, 0–8 U/day) or insulin plus GHRP-6 (Db + Ins + GHRP-6). Data are presented as mean ± SEM and referred to control values (n = 4–6). *P-value is lower than 0.05.

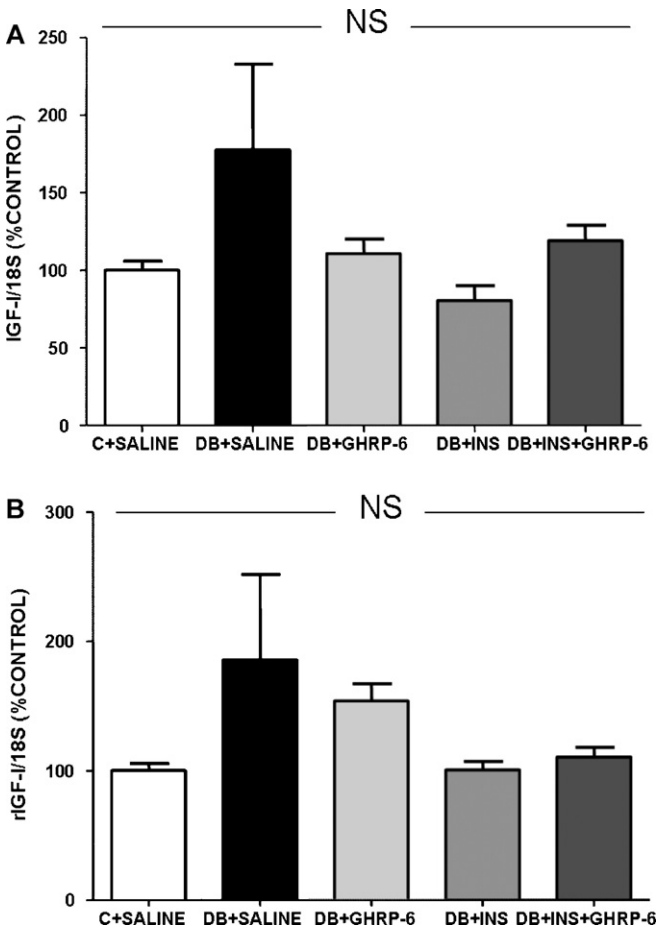

Fig. 4. Insulin-like growth factor I (IGF-I; A) and IGF-I receptor (rIGF-I; B) mRNA levels in the pituitary of control rats injected with saline (C + saline) and diabetic rats injected with saline (Db + saline), growth hormone-releasing peptide 6 (GHRP-6 (Db + GHRP-6, 150 μg/kg/day), insulin (Db + Ins, 0–8 U/day) or insulin plus GHRP-6 (Db + Ins + GHRP-6). Data are presented as mean ± SEM and referred to control values (n = 4–6). *P-value is lower than 0.05.

in which hypothalamic total cell death was not different from that of control rats.

Hypothalamic GFAP levels were decreased in diabetic rats injected saline (Fig. 5C; $P < 0.05$). Treatment with insulin or GHRP-6 partially normalized GFAP levels such that there was no statistical difference from either control rats or diabetics treated with saline, with no interaction between the two factors. On the contrary, the combined treatment of insulin plus GHRP-6 returned hypothalamic GFAP levels to control levels ($P < 0.05$).

5.2. Hypothalamic IGF-I and rIGF-I mRNA levels

All diabetic rats, regardless of the treatment, had decreased IGF-I mRNA levels in the hypothalamus compared to controls (Fig. 6A; $P < 0.001$). Neither insulin nor GHRP-6 prevented this decrease, with no interaction between the two factors. Although diabetes did not modify the hypothalamic gene expression of rIGF-I, both insulin and GHRP-6 treatments decreased rIGF-I mRNA levels in diabetic rats (Fig. 6B; $P < 0.01$), with no interaction between the two factors.

6. Cerebellum

6.1. Cell death, PCNA and GFAP levels in the cerebellum

Diabetes increased cell death in the cerebellum and insulin, but not GHRP-6, prevented this effect with no interaction between the two treatments (Fig. 7A; $P < 0.05$).

Cell proliferation, as indicated by PCNA content, was decreased in the cerebellum in response to diabetes (Fig. 7B; $P < 0.001$). There was an interaction between insulin and GHRP-6 ($F = 46.52$; $P < 0.001$) as both insulin and GHRP-6 when administered alone increased PCNA content in the cerebellum compared to controls and to diabetic rats injected with saline ($P < 0.001$), but when administered together the treatments had no effect.

GFAP levels in the cerebellum were also decreased in diabetic rats injected saline (Fig. 7C; $P < 0.001$) compared to control rats. Insulin increased GFAP levels ($P < 0.05$) and GHRP-6 had no effect, with no interaction between treatments. However, only diabetic rats treated with insulin plus GHRP-6 had significantly higher levels of GFAP in the cerebellum than saline-treated diabetic rats

6.2. Active caspase-9, caspase-6 and caspase-3 levels in the cerebellum

Activation of both caspase-9 and caspase-6 was up-regulated in the cerebellum of diabetic rats treated with saline (Fig. 8A

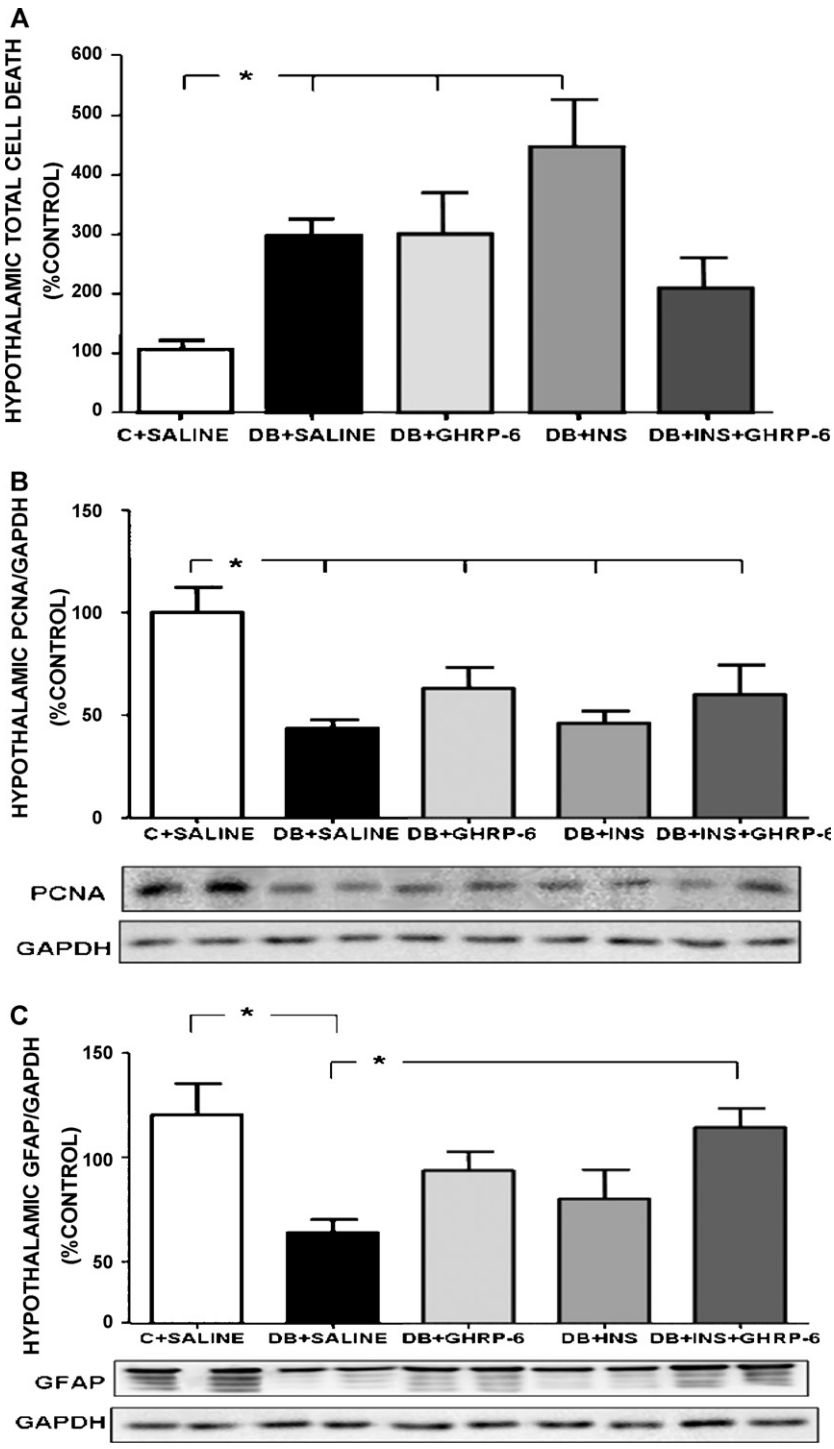

Fig. 5. Cell death (A), proliferating cell nuclear antigen (PCNA; B) and glial fibrillary acidic protein (GFAP; C) levels in the hypothalamus of control rats injected with saline (C + saline) and diabetic rats injected with saline (Db + saline), growth hormone-releasing peptide 6 (GHRP-6 (Db + GHRP-6, 150 μg/kg/day), insulin (Db + Ins, 0–8 U/day) or insulin plus GHRP-6 (Db + Ins + GHRP-6). Data are presented as mean ± SEM and referred to control values ($n = 4–6$). *P-value is lower than 0.05.

and B; $P < 0.05$ and $P < 0.001$, respectively), while caspase-3 levels were unchanged (Fig. 8C). Insulin treatment partially reduced the activation of caspases 9 ($P < 0.05$) and 6 ($P < 0.0001$) such that they were no longer different from control levels, but also not significantly different from saline treated diabetic rats. GHRP-6 exerted no effect. However, insulin plus GHRP-6 treated rats had significantly lower levels of both caspases compared to saline-injected diabetic rats ($P < 0.05$ and $P < 0.0001$, respectively).

6.3. IGF-I and rIGF-I mRNA levels in the cerebellum

IGF-I mRNA levels were decreased in the cerebellum of all diabetic animals compared to controls, regardless of the treatment received (Fig. 9A; $P < 0.001$). However, diabetic rats treated with GHRP-6 had higher IGF-I levels than those injected saline and those treated with the combined treatment of insulin plus GHRP-6 ($P < 0.05$), without an interaction between insulin and GHRP-6. On the contrary, rIGF-I gene expression was unchanged in response

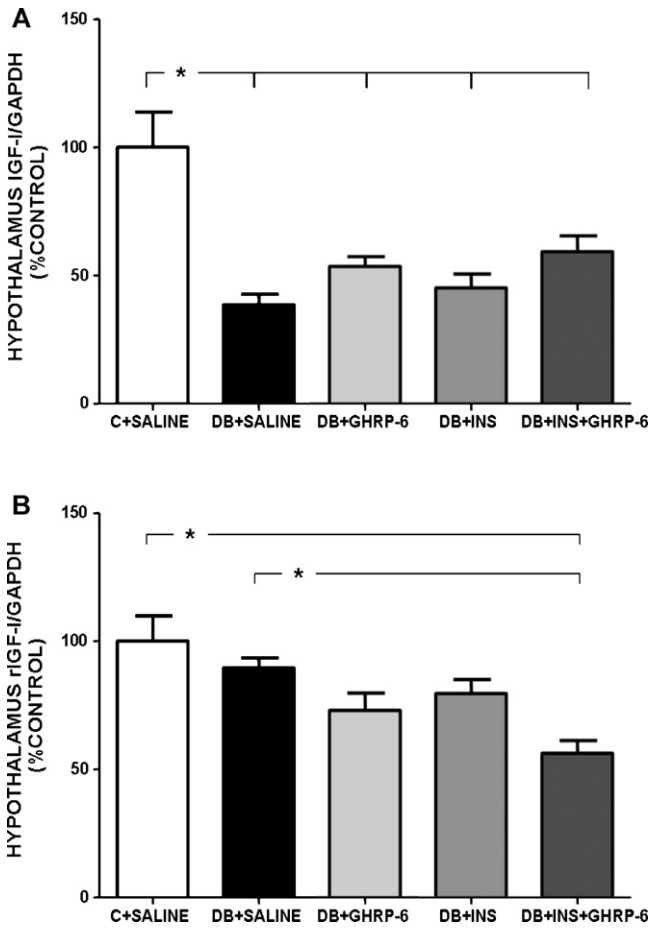

Fig. 6. Insulin-like growth factor I (IGF-I; A) and IGF-I receptor (rIGF-I; B) mRNA levels in the hypothalamus of control rats injected with saline (C + saline) and diabetic rats injected with saline (Db + saline), growth hormone-releasing peptide 6 (GHRP-6 (Db + GHRP-6, 150 μg/kg/day), insulin (Db + Ins, 0–8 U/day) or insulin plus GHRP-6 (Db + Ins + GHRP-6). Data are presented as mean ± SEM and referred to control values (n = 4–6). *P-value is lower than 0.05.

to diabetes (Fig. 9B). There was a significant interaction between insulin and GHRP-6 (F = 38.66, P < 0.0001) as GHRP-6 increased rIGF-I gene expression in the cerebellum when it was administered alone (P < 0.001), but not when it was administered with insulin.

6.4. GHS-R and INS-R mRNA levels in the pituitary, hypothalamus and cerebellum

As different levels of the GHS-R and INS-R in the pituitary, hypothalamus and cerebellum could play a role in the response to the treatments employed, the gene expression of these receptors was measured in the three different tissues.

GHS-R mRNA levels were increased in the pituitary of diabetic rats injected with saline compared to controls (C + saline: 100 ± 34; Db + saline: 277 ± 45; P < 0.05), whereas they were unchanged in the cerebellum (C + saline: 100 ± 37; Db + saline: 57 ± 8) and in the hypothalamus (Granado et al., 2010). In the pituitary GHRP-6 treatment decreased GHS-R mRNA to control levels (C + saline: 100 ± 34; Db + GHRP-6: 105 ± 16), whereas insulin and insulin + GHRP-6 treatments decreased GHS-R mRNA below control levels (C + saline: 100 ± 34; Db + Ins: 53 ± 13; Db + Ins + GHRP-6: 74 ± 12; P < 0.05), with an interaction between insulin and GHRP-6 (F = 11.67; P < 0.01). In the cerebellum neither insulin nor GHRP-6 modified GHS-R mRNA levels, with no interaction between the two factors (C + saline: 100 ± 37; Db + GHRP-6: 168 ± 36; Db + Ins: 151 ± 9; Db + Ins + GHRP-6: 107 ± 70).

In the hypothalamus we have previously reported that treatment with insulin or insulin + GHRP-6 reduced the expression of GHS-R compared to control and saline-injected diabetic rats with an interaction between the two treatments (Granado et al. 2010).

Diabetic rats injected saline had increased INS-R mRNA levels in the pituitary (C + saline: 100 ± 15; Db + saline: 193 ± 33; P < 0.05), and cerebellum (C + saline: 100 ± 9; Db + saline: 134 ± 7; P < 0.05) compared to controls. There was an interaction between insulin and GHRP-6 treatments on INS-R mRNA levels both in the pituitary (F = 8.65, P < 0.05) and cerebellum (F = 5.45, P < 0.05). While in the pituitary both insulin and GHRP-6 decreased the diabetes-induced up-regulation of INS-R mRNA levels (C + saline: 100 ± 15; Db + GHRP-6: 110 ± 15; Db + Ins: 118 ± 11; Db + Ins + GHRP-6: 140 ± 16), none of the treatments prevented the diabetes-induced modifications of INS-R mRNA levels in the cerebellum (C + saline: 100 ± 9; Db + GHRP-6: 177 ± 7; Db + Ins: 173 ± 28; Db + Ins + GHRP-6: 132 ± 7).

We previously reported that gene expression of this receptor was decreased in the hypothalamus and that none of the treatments employed had a significant effect (Granado et al., 2010).

7. Discussion

Type-1 diabetes is associated with structural and functional changes in the CNS and in the pituitary that in many instances can be prevented by insulin replacement (Biessels et al., 1998; Pérez Díaz et al., 1982). The results reported here show that insulin and GHRP-6 treatments exert different protective effects in the pituitary, cerebellum and hypothalamus of STZ-induced diabetic rats and that the combined treatment may be beneficial in some instances.

In a previous study we reported the metabolic and hormonal outcomes of these animals in response to insulin and GHRP-6 treatments (Granado et al., 2010). We found that one daily injection of insulin for eight weeks starting at diabetes onset prevented diabetes-induced hyperphagia, polydipsia and body weight loss and reduced diabetes-induced hyperglycemia in STZ-diabetic rats. On the contrary, GHRP-6 administration for the same period did not attenuate either hyperglycemia or body weight loss in these diabetic rats. However, there was an additive effect of these two hormones on distinct variables, including body weight, suggesting that GHRP-6, and possibly ghrelin, may need the presence of insulin in order to exert its metabolic effects.

In addition to its classical metabolic effects GHRP-6, as well as ghrelin, exerts protective effects on specific cell types, including neurons (Delgado-Rubín et al., 2009; Frago et al., 2002) and pituitary cells (Granado et al., 2009). As poorly controlled diabetes induces cell death in distinct tissues, which is involved in the development of some of the secondary effects, treatment with this hormone could possibly be beneficial in this disease.

Diabetes increased cell death in the pituitary, hypothalamus and cerebellum as previously reported (García-Cáceres et al., 2008; Lechuga-Sancho et al., 2006a,b; Granado et al., 2009). However, while in the pituitary insulin treatment prevented diabetes-induced apoptosis, in the cerebellum and hypothalamus it had no significant effect on cell death. Although there was no significant effect of GHRP-6 alone on cell death in any of the areas studied, the combination of insulin and GHRP-6 resulted in levels not different from those found in control animals, suggesting that the combined treatment may be more beneficial than insulin alone.

The different effects exerted by insulin and GHRP-6 in these tissues could be due, at least in part, to the different levels of expression and regulation of the INS-R and GHS-R. Indeed, INS-R mRNA levels were decreased in the hypothalamus of STZ-diabetic rats (Granado et al., 2010; Grünblatt et al., 2007), but increased in the

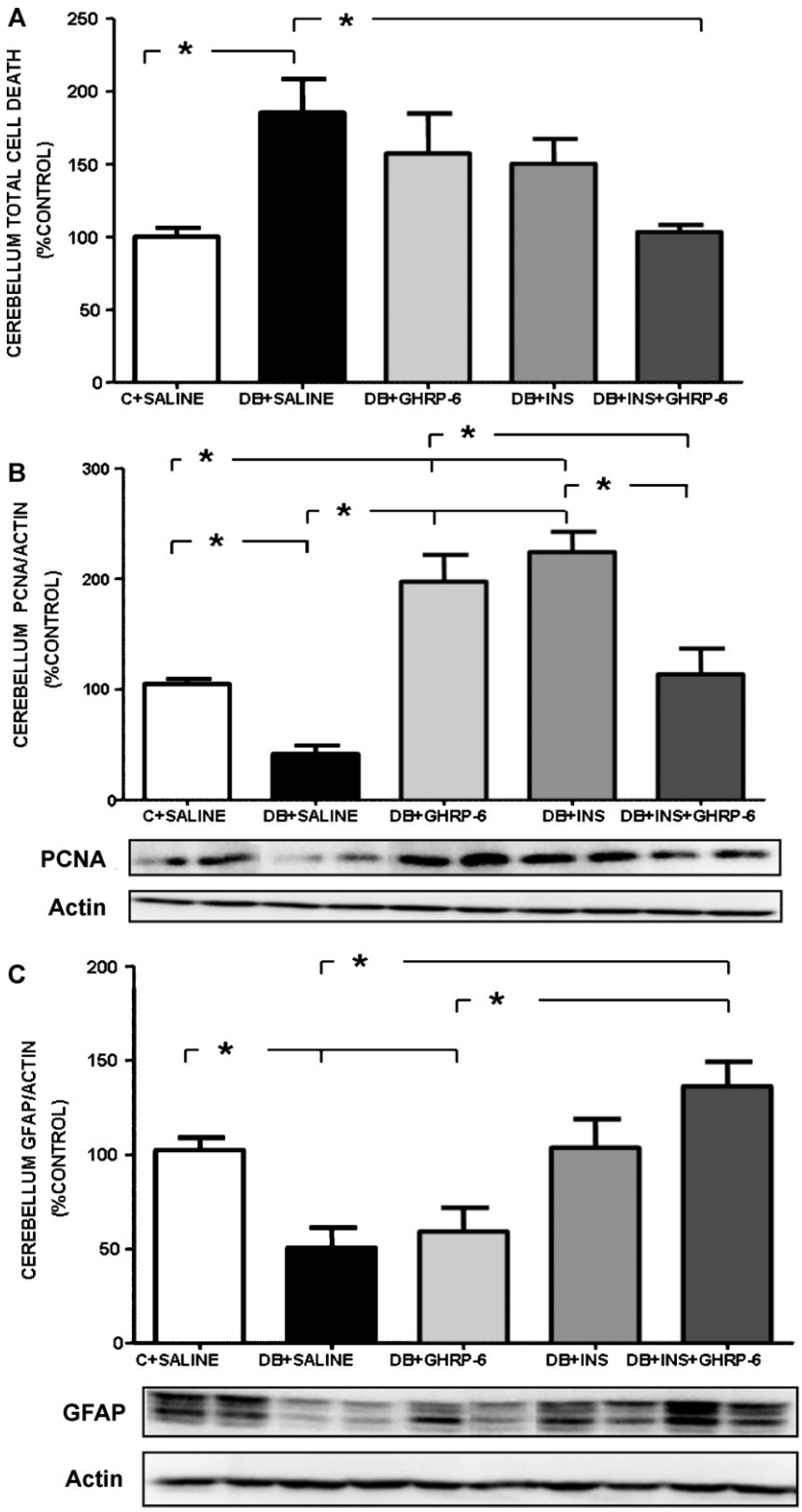

Fig. 7. Cell death (A), proliferating cell nuclear antigen (PCNA) (B) and glial fibrillary acidic protein (GFAP) content (C) in the cerebellum of control rats injected with saline (C + saline) and diabetic rats injected with saline (Db + saline), growth hormone-releasing peptide 6 (GHRP-6 (Db + GHRP-6, 150 μg/kg/day), insulin (Db + Ins, 0–8 U/day) or insulin plus GHRP-6 (Db + Ins + GHRP-6). Data are presented as mean ± SEM and referred to control values (n = 4–6). *P-value is lower than 0.05.

pituitary and cerebellum, as shown here and previously (Peeyush et al., 2009). Moreover, insulin differentially affected its receptor levels in these tissues, as it prevented the diabetes-induced alterations of INS-R mRNA levels in the pituitary, but not in the hypothalamus or cerebellum. Contrary to our results, Peeyush *et al.* found that insulin treatment prevented the diabetes-induced upregulation of INS-R in the cerebellum. However, in their study blood glucose levels were normalized to control levels after two daily injections of insulin whereas in our study glycemia, although significantly decreased compared to saline-injected rats, remained

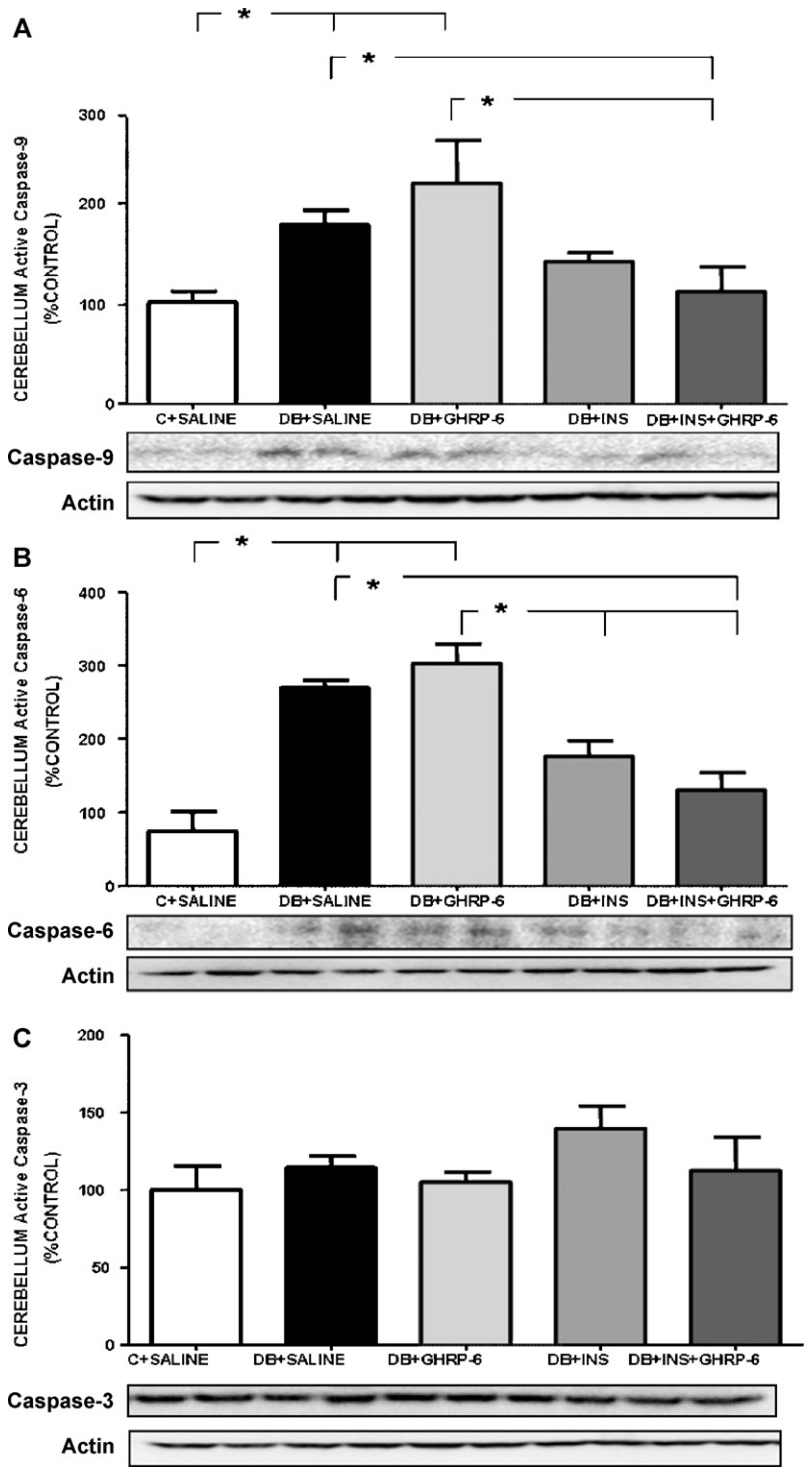

Fig. 8. Active caspase 9 (A), caspase 6 (B) and caspase 3 (C) levels in the cerebellum of control rats injected with saline (C + saline) and diabetic rats injected with saline (Db + saline), growth hormone-releasing peptide 6 (GHRP-6 (Db + GHRP-6, 150 μg/kg/day), insulin (Db + Ins, 0–8 U/day) or insulin plus GHRP-6 (Db + Ins + GHRP-6). Data are presented as mean ± SEM and referred to control values (n = 4–6). *P-value is lower than 0.05.

increased compared to controls (Granado et al., 2010). For this reason we cannot exclude that the lack of effects of insulin in the cerebellum and hypothalamus could be due, at least in part, to the still increased glucose levels or the daily variations in glycemia.

Diabetes also induced differential changes in the gene expression of GHS-R. While diabetes increased GHS-R mRNA levels in the pituitary as previously reported (Park et al., 2005), no change was observed in the hypothalamus or cerebellum. In addition, both insulin and GHRP-6 treatments blunted the up-regulation of GHS-R mRNA in the pituitary and decreased GHS-R mRNA in the hypothalamus, with no significant effects in the cerebellum. Thus, the differential regulation of the INS-R and GHS-R, both in response to diabetes and the treatment protocols, could underlie, at least in part, the different protective effects of insulin and GHRP-6 in these tissues.

Since the insulin regimen used did not totally normalize serum glucose levels, the differential susceptibility of the pituitary and CNS cells to hyperglycemia-induced cell death should be taken into

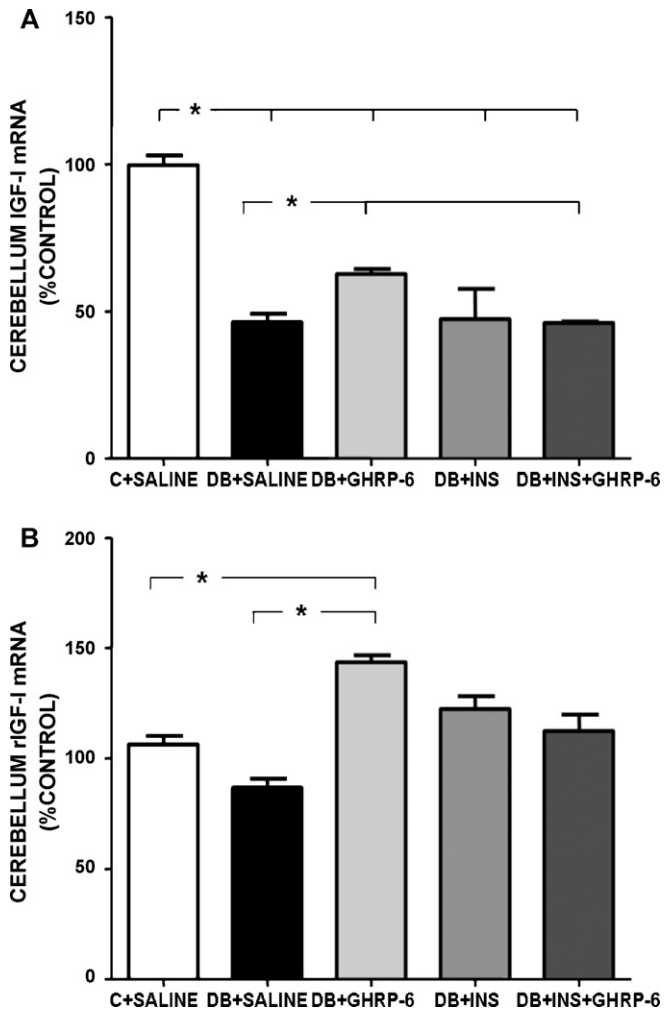

Fig. 9. Insulin-like growth factor I (IGF-I) (A) and IGF-I receptor (rIGF-I; B) mRNA levels in the cerebellum of control rats injected with saline (C + saline) and diabetic rats injected with saline (Db + saline), growth hormone-releasing peptide 6 (GHRP-6 (Db + GHRP-6, 150 μg/kg/day), insulin (Db + Ins, 0–8 U/day) or insulin plus GHRP-6 (Db + Ins + GHRP-6). Data are presented as mean ± SEM and referred to control values (n = 4–6). *P-value is lower than 0.05.

consideration, as well as the distinct mechanisms involved in cell death in the three different tissues. Indeed, we previously reported that in the pituitary diabetes-induced apoptosis is mediated by activation of the extrinsic cell death pathway, with an up-regulation of TNF-α and caspase-8 levels (Arroba et al., 2005; Arroba et al., 2003; Granado et al., 2009). This activation results in decreased PRL content due to increased death of lactotrophs (Arroba et al., 2003). In addition, the decreased gene expression of PRL in saline treated STZ-diabetic rats indicates that the decrease in PRL pituitary content is not only due to a decreased number of lactotrophs, but may also be due to decrease synthesis of this hormone by the existing prolactin producing cells. On the contrary LH and FSH pituitary content are increased in response to diabetes. This effect has already been reported by other authors and seems to be do to increased number of gonadothropes (Bestetti et al., 1985, 1989; Pitton et al., 1987; Rossi and Bestetti, 1981) and decreased LH and FSH secretion (Bestetti et al., 1997; Garibay et al., 1998). In the current study insulin treatment, but not GHRP-6, decreased cell death and caspase-8 activation and increased PRL gene expression in the pituitary, suggesting that insulin replacement protects PRL producing cells from diabetes-induced apoptosis, and/or induces PRL synthesis. Indeed, insulin replacement has been reported to restore the diabetes-induced decrease of PRL levels in STZ-diabetic rats (Pérez Díaz et al., 1982) and to induce PRL secretion in pituitary cell cultures (Yamashita and Melmed, 1986). In addition, insulin administration prevented the diabetes-induced down-regulation of total protein content and PCNA levels and the increase in TNFα gene expression and Bcl-2, Hsp-70 and XIAP protein content in the pituitary. This effect does not seem to be mediated by changes in the local IGF-I system, as both IGF-I and rIGF-I mRNAs are unchanged in the pituitary of untreated and treated diabetic rats, as previously reported by other authors (Olchovsky et al., 1991). Furthermore, insulin decreased the diabetes-induced rise in pituitary FSH and LH mRNA levels. However, although GHRP-6 administration increased both GH mRNA and GH content in the pituitary of diabetic rats (Granado et al., 2010), it did not attenuate the decline in PRL levels (Granado et al., 2010) or in pituitary cell death. These results are in contrast to what we previously observed with ghrelin treatment of diabetic rats from the sixth to eighth week after diabetes onset, where the diabetes-induced pituitary cell death and decrease in PRL pituitary content were reduced (Granado et al., 2009). The discrepancies between these two studies might be explained by the different routes of administration used. In the current study GHRP-6 was injected subcutaneously once daily and in the previous report ghrelin was continuously infused through the jugular vein for two-weeks. The fact that the plasma concentrations of ghrelin were maintained elevated over the two week period of treatment in the previous study and that GHRP-6, which has a short half life, was only injected once daily in this study could also be a cause for the different effects found. Moreover, it is also possible that ghrelin's anti-apoptotic effects are not mediated through the GHS-R1a receptor. Indeed it has been reported that ghrelin induces cell proliferation and inhibits apoptosis in different cell types through a GHSR1a independent mechanism (Granata et al., 2006, 2007; Baldanzi et al., 2002; Filigheddu et al., 2007).

We have previously described a decrease in GFAP content in the hypothalamus as a result of diabetes-induced changes in astrocyte morphology and a decrease in the number of astrocytes due, at least in part, to increased cell death (Lechuga-Sancho et al., 2006a,b; García-Cáceres et al., 2008). Likewise, in this study cell death was increased and GFAP levels decreased in poorly controlled diabetic rats, most likely indicating increased cell death of astrocytes, However, decreased synthesis of GFAP by the existing astrocytes cannot be excluded. Although both insulin and GHRP-6 attenuated the diabetes-induced decrease of hypothalamic GFAP levels, none of these treatments prevented cell death. On the contrary, the combination of the two treatments attenuated cell death and returned GFAP levels to control values. Thus, insulin and GHRP-6 may also be modulating GFAP levels through a process other than cell protection, including stimulation of proliferation or induction of morphological changes. However, although insulin is important for the maintenance of astrocyte function and induces astrocyte differentiation *in vitro* (Aizenman and de Vellis, 1987; Avola et al., 2004; Bramanti et al., 2007a,b; Matsuda et al., 1996), we found no effect of insulin, or GHRP-6, on hypothalamic PCNA levels. Thus, the increased hypothalamic GFAP levels in diabetic rats treated with insulin + GHRP-6 do not appear to be the result of increased proliferation. However, it has recently been proposed that obesity can affect astrocytes, increasing hypothalamic GFAP levels (Horvath et al., 2010; Hsuchou et al., 2009). Hence, as diabetic rats administered insulin and GHRP-6 have increased body weight gain, adiposity and serum leptin levels compared to control rats (Granado et al., 2010), the increased hypothalamic GFAP levels could be related to the change in body composition.

In the cerebellum cell death was increased and GFAP decreased in response to diabetes, as previously described (Lechuga-Sancho et al., 2006b). However, although our group has reported that the decrease in GFAP is due, at least in part, to a decreased number of astrocytes it has also been reported that the number of

astrocytes in the cerebellum is unchanged in response to diabetes (Coleman et al., 2004). Insulin treatment prevented the diabetes induced decrease in GFAP levels in the cerebellum as previously reported (Coleman et al., 2010), but it only partially decreased cell death and the up-regulation of caspases 9 and 6. In contrast, PCNA content was increased in the cerebellum of insulin-treated diabetic rats compared to saline-injected animals, suggesting that the increased GFAP levels could be the result of increased proliferation of glial cells and/or stimulation of GFAP expression in the existing astrocytes. Indeed, insulin has been reported to increase GFAP expression and cell proliferation in astrocyte primary cultures (Avola et al., 2004; Matsuda et al., 1996). However, GHRP-6 treatment of diabetic rats also increased PCNA levels in the cerebellum without increasing GFAP levels, which could indicate that GHRP-6 induces proliferation of other cell types different than astrocytes, which may include neurons. Indeed ghrelin, induces neurogenesis and proliferation of neurons from the dorsal motor nucleus of the vagus (Ammori et al., 2008; Zhang et al., 2004), as well as neurogenesis in the spinal cord (Sato et al., 2006) and the nucleus of the solitary tract (Zhang et al., 2005). Furthermore, GHRP-6 treated diabetic rats had increased rIGF-I and IGF-I mRNA levels in the cerebellum compared to saline-treated rats, which suggests that GHRP-6 might induce proliferation through an IGF-I mediated mechanism.

8. Conclusions

In conclusion insulin and GHRP-6 treatments exert different protective effects in the pituitary, hypothalamus and cerebellum of STZ-diabetic rats. These results could have clinical relevance as they show that insulin treatment, although it prevents most of diabetes-induced alterations in the pituitary, does not prevent some of the alterations occurring at different levels of the CNS. In contrast, the combination of insulin and GHRP-6 prevents some of diabetes-induced alterations in the hypothalamus and the cerebellum.

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