The opposing effects of ghrelin on hypothalamic and systemic inflammatory processes are modulated by its acylation status and food intake in male rats

Cristina García-Cáceres, Esther Fuente-Martín, Francisca Díaz, Miriam Granado, Pilar Argente-Arizón, Laura M. Frago, Alejandra Freire-Regatillo, Vicente Barrios, Jesús Argente, Julie A. Chowen

Hospital Infantil Universitario Niño Jesús, Department of Endocrinology. Instituto de Investigación La Princesa, University Autónoma of Madrid and Centro de Investigación Biomédica en Red (CIBER) de la Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, Madrid, Spain.

Ghrelin is an endogenous hormone that stimulates appetite and adipose tissue accrual. Both the acylated (AG) and non-acylated (DAG) isoforms of this hormone are also reported to exert anti-inflammatory and protective effects systemically and in the central nervous system. As inflammatory processes have been implicated in obesity-associated secondary complications, we hypothesized that this natural appetite stimulator may protect against negative consequences resulting from excessive food intake. Adult male Wistar rats were treated *icv* (5 μg/day) with AG, DAG, the ghrelin mimetic GH-releasing peptide (GHRP)-6, AG and pair-fed with controls (AG-pf), or saline for 14 days. Regardless of food intake AG increased visceral adipose tissue (VAT) and decreased circulating cytokine levels. However, AG reduced cytokine production in VAT only in rats fed *ad libitum*. Hypothalamic cytokine production was increased in AG treated rats fed *ad libitum* and by DAG, but intracellular inflammatory signaling pathways associated with insulin and leptin resistance were unaffected. Gliosis was not observed in response to any treatment as glial markers were either reduced or unaffected. AG, DAG and GHRP-6 stimulated production of hypothalamic insulin like-growth factor I, which is involved in cell protective mechanisms. In hypothalamic astrocyte cell cultures AG decreased tumor necrosis factorα and DAG decreased interleukin-1β mRNA levels, suggesting direct anti-inflammatory effects on astrocytes. Thus, while ghrelin stimulates food intake and weight gain, it may also induce mechanisms of cell protection that help to detour or delay systemic inflammatory responses and hypothalamic gliosis due to excess weight gain, as well as its associated pathologies.

Obesity and its associated comorbidities are a major health problem in developed countries. Increasing caloric intake and/or reducing energy expenditure induce excessive energy storage resulting in adipose accumulation and weight gain. However, the cellular and molecular mechanisms underlying the response to prolonged positive energy balance require further investigation, especially in light of studies demonstrating that some outcomes are contingent on nutrient composition and not solely caloric content (1). Excess adipose accumulation is often associated with production of inflammatory mediators by several tissues, including adipose tissue (2). Hypothalamic inflammation is reported to occur prior to high fat diet (HFD)-induced weight gain and may act as a determining factor in the onset of obesity-associated secondary complications, such as insulin and leptin resistance (3).

In HFD-induced hypothalamic inflammation there is a rise in inflammatory factors (eg, IL-1β, IL-6 and TNF-α) accompanied by glial activation (3–5). Microglia and astrocytes act as immune cells in the brain releasing cytokines in response to diverse stimuli (6, 7), including in response to fatty acids (8, 9). Thus, some of the hypotha-

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lamic processes that drive obesity-induced inflammation are thought to result from direct dietary effects on glial cells, while hormones or other factors modified by weight gain may also be involved (10–12). As weight gain depends on multiple variables, such as circadian timing of eating (13), genetics (14) and the nutritional, hormonal (15) and stress status of the individual (16), overfeeding does not always induce the same degree of weight gain or obesity-associated pathologies. Indeed, hypothalamic inflammatory and glial responses have recently been shown to differ depending on the underlying cause of weight gain (17, 18).

Ghrelin is an endogenous promoter of appetite and stimulates weight gain that does not depend on HFD intake and adipose accumulation that does not depend on increased caloric intake (19). This orexigenic hormone is mainly produced by the gut (20), but also by other tissues including the hypothalamus (21–24). It circulates as different isoforms with its acylated form (AG) being the endogenous ligand for the GH secretagogue or ghrelin receptor (GHSR)1a (20). Although first thought to be inactive, desacylated ghrelin (DAG) is now purported to have physiological effects that may be similar, opposite or antagonistic to those of AG (25). In addition to stimulating food intake and modulating energy expenditure and glucose homeostasis, AG also participates in processes of cell protection, including in the CNS (26–29). DAG also modulates cell turnover and differentiation (30, 31), as well as triglyceride storage and adipocyte metabolism (32). Ghrelin and its mimetics such as GH-releasing peptide (GHRP)-6 stimulate the GH/IGF-I axis (33) and possibly exert protective and/or proliferative effects on neurons and glial cells, at least in part, through stimulation of IGF-I production in the CNS (34–37). Ghrelin also modulates cytokine production by microglia and oligodendrocytes, inhibits microglial activation and oligodendrocyte apoptosis (38–40) and elevates intracellular calcium levels in astrocytes (41).

Thus, ghrelin participates in physiological metabolic and immune processes and neuroprotection, but little is known regarding the interrelationship between these effects or the mechanisms involved. We hypothesized that ghrelin protects against systemic and central inflammation during weight gain and that this is dependent on its acylation status. To this end, systemic and hypothalamic inflammatory markers were analyzed in rats receiving chronic *icv* treatment with AG, DAG or the GHS-R1a receptor agonist GHRP-6. AG treated pair-fed rats were employed to determine if these anti-inflammatory effects are dependent on food intake and primary astrocyte cultures were used to delineate the direct effects of the two isoforms of ghrelin on these glial cells.

Materials and Methods

Ethical statement

All studies were approved by the local ethics committee and approved by the commission of investigation of the Hospital Infantil Universitario Niño Jesús. Animal usage complied with the Royal Decree 1201/2005 (BOE number 252) pertaining to the protection of experimental animals and with the European Communities Council Directive (86/609/EEC). Special care was taken to reduce animal suffering and to use the minimum number of animals inall studies.

Animals and treatments

Male Wistar rats (250 g; Harlan Interfauna Ibérica S.A., Barcelona, Spain) were acclimated to the new conditions 1 week before surgeries were performed. They were anesthetized with 0.02 ml ketamine/100g body weight (bw) and 0.04 ml xylazine/100g bw. A minipump (Alzet, Durect Co, Canada) that delivered either 5 μg/d (0.208 μg/hr) AG (Anaspec, Fremont, CA, USA), DAG (Anaspec) or GHRP-6 (Bachem, Torrance, CA, USA) or saline for 14 days was connected to a cannula implanted in the left lateral ventricle (0.08 mm anteroposterior, 0.16 mm lateral from Bregma; n = 16/group). Rats were maintained at a constant temperature (21 $\pm$ 1°C) and humidity (50 $\pm$ 1%) in a 12-hour light-dark cycle (lights on 07:30). All rats had free access to food and water except the pair-fed AG group that received the amount of food that the control group consumed the previous day.

Rats were weighed every two days until killed between 0900 and 1100 by decapitation after overnight fasting. Before sacrifice glycemia was measured with a glucometer via venous tail puncture (Optium Plus, Abbot Diabetes Care, Witney Oxon, UK). Trunk blood was collected, centrifuged and the serum separated and stored at –80°C. Brains and fat pads were rapidly dissected, weighed, frozen on dry ice and stored at –80°C.

Determination of serum levels of total and acylated ghrelin, IGF-I and corticosterone by RIA

Total and acylated ghrelin were measured following the manufacturer's instructions (Millipore, Billerica, MA, USA). The sensitivity was 93 pg/ml for both assays and the intra- and interassay coefficients of variation where 6.4 and 16.3% for total ghrelin and 7.4 and13.4% for AG, respectively. To avoid degradation of acylated ghrelin a specific aliquot of untreated serum was rapidly frozen and maintained at –80°C until assayed (42).

Corticosterone levels were measured following the manufacturer's instructions (MP Biomedicals, Orangeburg, NY, USA). The assay sensitivity was 7.7 ng/ml and the intra- and interassay variations were 7.3 and 6.9%, respectively.

IGF-I levels were measured by a double-antibody RIA as previously described (36). The IGF-I antiserum (UB2–495) was a gift from Drs. Underwood and Van Wyk and is distributed through the National Hormone and Pituitary Program. The intra-assay coefficient of variation was 8%.

Samples were run in duplicate in the same RIA for all determinations.

Determination of serum leptin, insulin and adiponectin levels by ELISA

Leptin, insulin and adiponectin levels were measured following the manufacturer's instructions (Millipore). Sensitivities for

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leptin, insulin and adiponectin were 0.04, 0.2 and 0.16ng/ml, respectively. All samples were run in duplicate in the same assay for all analyses. The intra-assay variation was 2.2% for leptin, 1.9% for insulin and 1.3% for adiponectin. Homeostasis model assessment (HOMA) was calculated as fasting glucose (mmol/L) x fasting insulin (mU/L)/22.5.

Determination of serum cholesterol, triglycerides, free fatty acids and total lipids

Total lipids, triglycerides, cholesterol, LDL and HDL were determined with enzymatic colorimetric assays (SpinReact; SantEsteve de Bas, Spain) and free nonesterified fatty acids (FFAs) with a kit from Wako Chemicals (Neuss, Germany) according to the manufacturer's instructions.

Multiplex analysis of serum cytokine levels

Serum IL-1β, IL-6 and TNFα concentrations were measured with a multiplexed bead immunoassay according to the manufacturer's instructions (Millipore) in a Bio-Plex suspension array system 200 (Bio-Rad Laboratories, Hercules, CA, USA). Mean fluorescence intensity was analyzed using Bio-Plex Manager Software 4.1.

Primary hypothalamic astrocyte cultures

Primary astrocyte cultures were prepared from two-day old male Wistar rat diencephalon as previously described (10–12). Cultures were serum starved for 24 hs before treatment with either 100 μM of rat AG or DAG or vehicle. This concentration was chosen after performing a dose response curve (10 μM-500 μM). Cells were collected at 24 hrs. In each experiment treatments were done in duplicate or triplicate and each experiment was performed 4 times (n = 4).

RCA6 neurons cell line

Stock cultures of the hypothalamic neuronal cell line RCA-6(43) (I. Torres-Alemán, Madrid, Spain) were grown as previously described (10, 35) and treated with AG or DAG (100 μM) or vehicle for 24 hrs. In each experiment treatments were done in duplicate or triplicate and each experiment was repeated 4 times (n = 4).

Protein extraction

Hypothalami were isolated on ice by making an anterior cut at the level of the optic chiasm, a posterior coronal section anterior to the mammilary bodies, sagittal cuts parallel to the lateral ventricles and a dorsal horizontal cut at the level of the anterior commissure. Tissue was homogenized on ice in 500 μl RIPA lysis buffer (50 mM NaH_2PO_4, 100 mM $Na_2H_2PO_4$, 0.1% SDS, 0.5% NaCl, 1% Triton X-100) with 5 mg/ml sodium deoxycholate, phenyl-methane-sulfonylfluoride (1 mM) and a cocktail of EDTA-free protease inhibitors (Roche Diagnostics, Mannheim, Germany). Lysates were incubated overnight at –80°C, centrifuged at 14000 g for 10 minutes at 4°C and the supernatant stored at –80°C. Hippocampal and cerebellar tissue was similarly processed.

Primary astrocyte cultures were processed with a lysis buffer containing 25 mM HEPES, 150 mM KCl, 2 mM of EDTA, 0.1% Igepal, 1 mM PMSF, 10 μM benzamidine and leupeptin and 0.5 mM DTT. The samples were stored at –80°C for 24 hrs and then centrifuged for 20 minutes at 14000 rpm (4°C). Supernatants were removed, frozen, lyophilized, resuspended in 40μl sterile water and stored at –80°C.

Total protein concentration was determined by the method of Bradford (Bio-Rad Laboratories).

Western blotting

Protein was resolved on 10% SDS-polyacrylamide gels under denaturing conditions and electro-transferred to PVDF membranes (Bio-Rad). Transfer efficiency was determined by Ponceau red dyeing. Membranes were blocked with Tris-buffered saline (TBS, 20 mM) containing 0.1% Tween 20 and 5% nonfat dried milk or 5% BSA for 2 hrs and incubated overnight at 4°C under agitation with the primary antibody at a concentration of 1:1000 unless otherwise stated. Primary antibodies used were against glial structural proteins and indicators of glial activation: glial acidic fibrillary acidic protein (GFAP), vimentin, anti-ionized calcium binding adapter molecule (Iba1) and F4/80; intracellular signaling proteins: pAKT (1:500), AKT (1:500), pIKB, STAT3, pSTAT3$^{(thy705)}$ (1:500), protein tyrosine phosphatase 1B (PTP1B) and the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Membranes were incubated with the corresponding secondary antibody conjugated with peroxidase (Pierce Biotechnology, Rockford, IL, USA). Peroxidase activity was visualized by chemiluminescence (Perkin Elmer Life Science, Boston, MA, USA) and quantified with a Kodak Gel Logic 1500 Image Analysis system and Molecular Imaging Software, version 4.0 (Rochester, NY, USA). Gel loading variability was normalized with either the nonphosphorylated form of the protein, Ponceau or GAPDH. Data were normalized to control values on each gel.

Quantitative real-time PCR

Total RNA was extracted from hypothalamus, hippocampus, cerebellum, VAT (n = 5–6/group) and astrocyte and RCA6 neuronal cultures (n = 4) according to the Tri-Reagent protocol. cDNA was prepared from 1 or 2 μg of total RNA. Assay-on demand kits with Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were used according to the manufacturer's protocol and analyzed in an ABI PRISM 7900HT (Applied Biosystems). The mRNA levels for the inflammation-associated cytokines IL-6 (Rn01410330), IL1-β (Rn00580432) and TNFα (Rn01525859) were assessed in the hypothalamus, hippocampus, cerebellum, VAT and astrocyte cultures. Leptin mRNA (Rn00565158) levels were determined in VAT. In the hypothalamus mRNA levels for neuropeptide Y (NPY, Rn01410145), pro-opiomelanocortin (POMC, Rn00595020), agouti-related protein (AgRP, Rn01431703), cocaine amphetamine related transcript (CART; Rn00567382), orexin (Rn00565995), IGF-I (Rn99999087), leptin receptor (LepR, Rn01433205), insulin receptor (InsR, Rn00567670), ghrelin receptor (GHSR, Rn00821417), ghrelin (Rn01425835), the enzyme involved in ghrelin acylation ghrelin O-acyltransferase (GOAT, Mm1200389) and suppressor of cytokine signaling 3 (SOCS3, Rn00585674) were also measured. Proteins involved in inflammatory signaling including, toll-like receptor 4 (TLR4, Rn00569848), cluster of differentiation68 (Cd68, Rn0149534), inhibitor of kappa light polypeptide gene enhancer in B-cells kinase beta (Ikbkb, Rn00584379), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (Nfkbia,

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Rn01473657), TRAF-associated NFκB activator (TANK, Rn00595794) and CCAAT/enhancer-binding protein-homologous protein (CHOP, Rn00492098), which is involved in endoplasmic reticulum (ER) stress, were also measured in the hypothalamus. Values were normalized to phosphoglycerate kinase 1 (Pgk-1, Rn00821429), β-actin (Rn00667869) and GAPDH (Rn99999916) in the hypothalamus and 18S (Rn01428915) and GAPDH in adipose tissue and astrocyte cultures. The $\Delta\Delta C_T$ method was used for relative quantification.

Statistical analysis

Data were analyzed by one-way ANOVAs followed by Scheffe's f tests. ANOVA with repeated measures was used for changes in weight and food intake over time. Results are reported as mean ± SEM and $P < .05$ was chosen as the limit for statistical significance. Only physiologically or experimentally relevant comparisons are reported.

Results

Effect of ghrelin on body weight gain depends on food intake and its acylation status

Daily food intake (Figure 1A) was modified by treatment ($P < .007$) and time ($P < .0001$) with the treatment effect changing over time ($P < .05$). Both AG and GHRP-6 increased food intake from study onset until approximately day 11 ($P < .0001$). DAG decreased food intake during the last part of the study, being significant on days 11 and 12. Accumulated food intake was increased by both AG and GHRP-6 ($P < .005$; Figure 1B).

Body weight was not different between groups at study onset (Day 0; Figure 1C). Weight gain was affected by treatment ($P < .0001$) and time ($P < .0001$), with an interaction between these factors ($P < .0001$). Significant weight gain was induced by GHRP-6 and AG after 3 or 5 days, respectively. DAG had a delayed inhibitory effect on weight gain, while the effects of AG and GHRP6 waned at the end of the study (weight gain final 4 days of study: Ct: 12.4 ± 1.5, AG: 10.6 ± 3.3, DAG: 3.8 ± 3.5, GHRP-6: 10.0 ± 1.8, AG-pf: 12.4 ± 5.6 g; $P < .001$). Despite this decline in effect, both AG and GHRP-6 increased total weight gain during the 14 day study period (Figure 1D; $P < .0001$) with the weight promoting effect of AG depending on food intake.

Ghrelin and GHRP-6 cause serum hormonal and lipid imbalances in accordance with body weight changes

Total ghrelin levels were increased by *icv* administration of AG and DAG regardless of food intake, but not by GHRP-6 ($P < .0002$; Table 1). Leptin levels rose in AG and GHRP-6 treated rats ($P < .0001$) in parallel with weight gain, whereas insulin and HOMA were elevated only in AG-treated rats ad libitum ($P < .01$ and $P < .03$, respectively), with a direct correlation between weight gain and HOMA index ($R = 0.65$, $P < .0001$). Adiponectin levels were significantly increased only in GHRP-6-treated rats ($P < .002$).

Triglyceride ($P < .002$) and HDL ($P < .006$) levels increased in ad libitum AG rats. In contrast, LDL levels increased in GHRP-6 and AG-pf rats ($P < .0001$), with AG-pf rats also having increased FFA levels ($P < .03$).

Serum glucose, AG, corticosterone, IGF-I, total lipids and total cholesterol levels were unchanged.

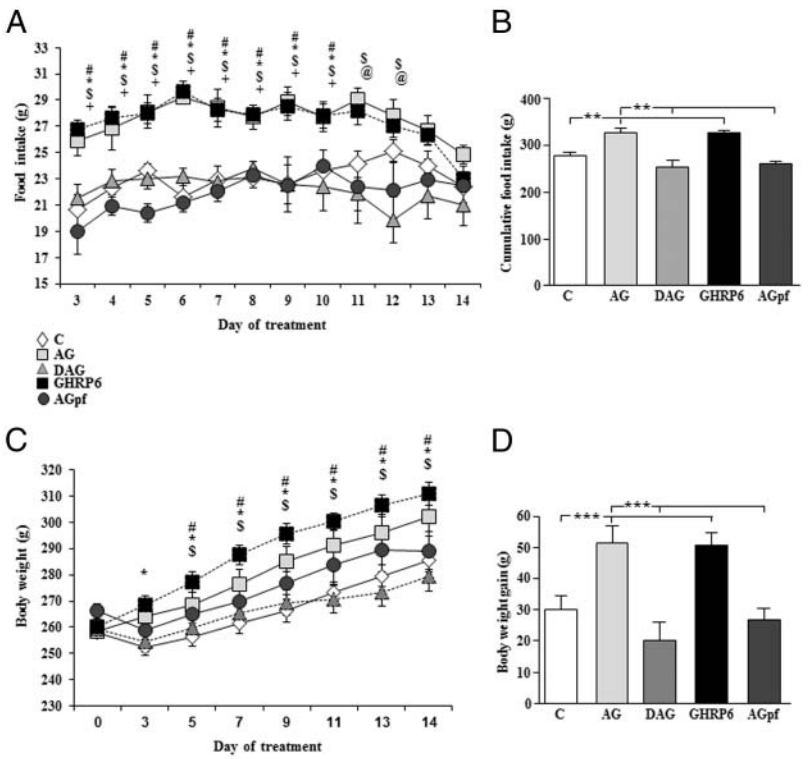

Figure 1. Daily changes in rat chow intake (A) and body weight gain (C) throughout 14 days of treatment with saline (C), acylated ghrelin (5 μg/d; AG), desacylated ghrelin (5 μg/d; DAG), GH-releasing peptide (GHRP)-6 (5 μg/d) or AG and being pair fed (5 μg/d; AGpf). Mean accumulated food intake (B) and total weight gain (D) per rat after 14 days of treatment. Figures A& C ANOVA = $P < .0001$; # AG different from C; *GHRP-6 different from C; @ DAG different from C. $ DAG different from AG; +AG different from AG-pf; Figures B &D: **= $P < .005$; ***=$P < .0001$.

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Table 1. Serum levels of hormones and metabolic factors in rats treated *icv*with saline (control), acylated ghrelin (AG), desaclyghrelin (DAG), growth hormone-releasing peptide (GHRP)-6 or with AG and pairfed (AG-pf) for 14 days. IGF-I: insulin-like growth factor I, FFAs: non-esterified fatty acids, HOMA: homeostasis model assessment, HDL: high density lipoprotein, LDL: low density lipoprotein.

	Control	AG	DAG	GHRP-6	AG-pf	ANOVA
Total ghrelin (pg/ml)	1444.8 ± 128.8	2862.4 ± 472.8[a]	3520.1 ± 478.5[a]	1708.1 ± 175.4[b,c]	3875.6 ± 928.5[a,d]	$P <$ 0.0002
Acyl-ghrelin (pg/ml)	129.6 ± 9.4	168.5 ± 33.6	238.5 ± 52.7	178.9 ± 42.7	169.1 ± 10.8	NS
Glycemia (mg/dl)	92.7 ± 3.0	93.7 ± 2.3	93.5 ± 3.0	98.1 ± 2.4	87.4 ± 3.3	NS
Insulin (ng/ml)	1.8 ± 0.3	3.0 ± 0.4 [a]	1.4 ± 0.1[b]	2.2 ± 0.3	2.1 ± 0.3 [b]	$P <$ 0.01
HOMA	2.6 ± 0.4	4.0 ± 0.6 [a]	1.9 ± 0.2 [b]	3.4 ± 0.5	2.7 ± 0.5	$P <$ 0.03
Leptin (ng/ml)	4.9 ± 0.7	10.3 ± 1.7[a]	3.3 ± 0.7 [b]	7.4 ± 0.7 [a]	5.0 ± 0.6 [b]	$P <$ 0.0001
Adiponectin (ng/ml)	30.8 ± 2.4	43.4 ± 5.2	27.1 ± 2.8 [b]	48.2 ± 2.8[a]	40.0 ± 4.8	$P <$ 0.002
Corticosterone (ng/ml)	198.6 ± 66.3	299.9 ± 100.9	335.5 ± 92.7	274.1 ± 58.3	188.1 ± 69.4	NS
IGF-I (ng/ml)	738.4 ± 75.1	641.8 ± 64.8	509.0 ± 27.9	949.6 ± 142.2	646.6 ± 108.0	NS
FFAs (mmol/liter)	0.44 ± 0.05	0.38 ± 0.05	0.43 ± 0.06	0.44 ± 0.8	0.74 ± 0.07[a,b,c,d]	$P <$ 0.03
Triglycerides (mg/dl)	66.9 ± 10.5	103.5 ± 8.1 [a]	47.9 ± 4.8 [b]	90.0 ± 20.6	62.6 ± 5.2 [b]	$P <$ 0.002
Total lipids (mg/dl)	416.0 ± 20.9	409.1 ± 39.0	407.9 ± 29.8	468.4 ± 47.7	444.9 ± 32.7	NS
Total cholesterol(mg/dl)	34.4 ± 4.4	43.7 ± 4.5	43.9 ± 4.7	43.5 ± 6.0	44.3 ± 4.0	NS
HDL (mg/dl)	23.1 ± 2.2	29.5 ± 2.1 [a]	19.8 ± 1.1 [b]	27.2 ± 3.7	27.2 ± 2.0	$P <$ 0.006
LDL (mg/dl)	19.9 ± 1.4	20.4 ± 1.6	22.3 ± 2.4	39.3 ± 4.8[a,b]	42.8 ± 3.3[a,b]	$P <$ 0.0001

a: different from control, b: different from AG, c: different from DAG, d: different from GHRP-6.

Acylated ghrelin and GHRP-6 induce visceral adipose accumulation without an underlying systemic proinflammatory phenotype

Weight gain was not accompanied by a change in the percentage of subcutaneous adipose tissue (Ct: 0.68 ± 0.01, AG: 0.64 ± 0.02, DG: 0.70 ± 0.02, GHRP-6: 0.64 ± 0.05, AG-pf: 0.69 ± 0.02 g/100 g of bw). VAT was increased in AG and GHRP-6 treated rats (Figure 2A; $P <$ .0001), with the effect of AG being independent of food intake. Leptin mRNA levels were increased by AG, but decreased in DAG and AGpf rats (Figure 2B; $P <$ .0001).

Increases in VAT were not associated with up-regulation of inflammatory markers in the circulation or VAT. Circulating IL-1β (Figure 2C; $P <$ .02) and IL-6 (Figure

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2D; $P < .05$) levels were decreased by AG regardless of food intake, whereas their mRNA levels in VAT were decreased only in rats treated with AG and fed ad libitum (Figures 2E; $P < .02$ and 2F; $P < .05$, respectively). TNFα mRNA levels were decreased in VAT of AG rats (Figure 2G; $P < .05$), but circulating levels of this cytokine were below the limit of detection (3.2 pg/ml) in most rats of all groups (data not shown).

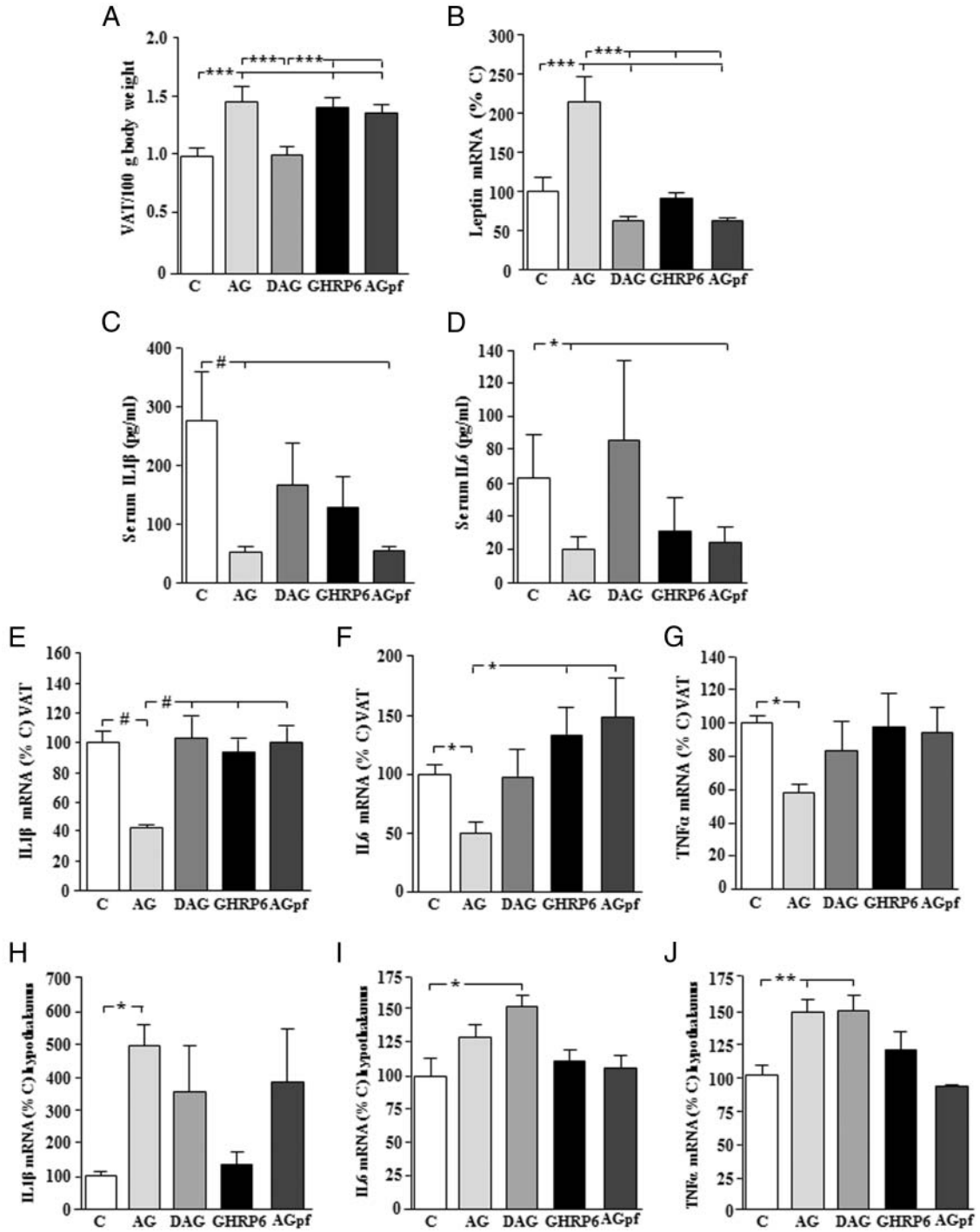

Figure 2. Visceral adipose tissue (VAT) per 100 g of body weight (A), leptin mRNA levels in VAT (B), interleukin (IL)-1β (C, E), IL-6 (D, F) in circulation or mRNA levels in VAT respectively, and tumor necrosis factor (TNF)α mRNA levels in VAT (G) of rats treated centrally with saline (C), acylated ghrelin (5 μg/d; AG), desacylated ghrelin (5 μg/d; DAG), GH-releasing peptide (GHRP)-6 (5 μg/d) or AG and being pair fed (5 μg/d; AGpf) for 14 days. TNFα (H), IL-1β (I) and IL-6 (J) mRNA levels in the hypothalamus of the same rats. *=$P < .05$; #= $P < .02$; **=$P < .002$; ***=$P < .0001$.

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Ghrelin and GHRP-6 modulate cytokine production without gliosis in the hypothalamus

As central inflammation is reported to precede systemic inflammation in obesity (3), we determined hypothalamic cytokine production. Only rats treated with AG and allowed to eat ad libitum increased IL-1β (Figure 2H; $P < .05$). DAG stimulated IL-6 (Figure 2I; $P < .05$), with both isoforms increasing TNFα mRNA levels (Figure 2J; $P < .002$). Factors involved in inflammatory signaling and regulation of IKK kinases (44), such as Ikbkb, NFkbia, TANK and CHOP, as well as TLR4 mRNA levels, were unaffected (Table 2). Likewise, by Western blotting no change in pIκB levels (Ct: 100 ± 1.0; AG: 108.1 ± 17.0; DG: 159.1 ± 31.5; GHRP-6: 129.1 ± 23.3; AG-pf: 139.5 ± 29.4% Ct) was found.

A rise in hypothalamic cytokine production is proposed to induce central leptin and insulin resistance (45, 46). The mRNA levels of NPY, AgRP, POMC, CART and orexin were not statistically different when analyzed by ANOVA. However AG tended to increase the expression of NPY (117%), AgRP (117%), CART (121%) and orexin (125%) (Table 2). LepR mRNA levels were increased by AG and GHRP-6 (Figure 3A; $P < .03$), but this was not associated with changes in proteins involved in leptin signaling such as pSTAT3 or SOCS3 (Supplementary Figure 1A & B).We observed no effect on hypothalamic InsR mRNA levels (Supplementary Figure 1C), but DAG decreased pAKT (Figure 3B; $P < .05$) and increased PTP1B (Figure 3C; $P < .05$), an inhibitor of insulin signaling (47).

Hypothalamic mRNA levels for preproghrelin were decreased in AG-pf rats (Supplementary Figure 2A; $P < .05$), with no significant change in GHSR or GOAT (Supplementary Figure 2 B & C).

To determine whether the rise in cytokine production was associated with hypothalamic gliosis, microglia and astrocyte associated markers were analyzed.CD68 mRNA (Table 2) and Iba1 protein levels (Figure 3D), both expressed in macrophages and microglia, were unchanged. F4/80, which is expressed in activated microglia, was decreased in rats treated with GHRP-6 (Figure 3E; $P < .006$).

Regarding astroglia, GFAP protein levels were decreased in AG rats regardless of food intake (Figure 3F; $P < .05$). Vimentin, a marker of both immature and activated astrocytes and tanycytes, was not significantly affected according to ANOVA, although AG-pf rats had mean levels that were only 50% of control values (Ct: 100 ± 16.1; AG: 130.7 ± 41.2; DAG: 93.2 ± 31.2; GHRP-6: 157.1 ± 63.1; AG-pf: 50.5 ± 19.7% Ct).

Table 2. Hypothalamic mRNA levels in rats treated *icv* for 14 days with saline (control), acylated ghrelin (AG), desacylghrelin (DAG), growth hormone-releasing peptide (GHRP)-6 or with AG and pairfed (AG-pf). Inhibitor of κ light polypeptide gene enhancer in B-cells kinase β (Ikbkb), nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (Nfkbia), TRAF-associated NFκB activator (TANK), CCAAT/enhancer-binding protein-homologous protein (CHOP), toll-like receptor 4 (TLR4), cluster of differentiation 68 (Cd68), neuropeptide Y (NPY), Agouti-related peptide (AgRP), proopiomelanocortin (POMC), cocaine and amphetamine regulated transcript (CART).

	Control	AG	DAG	GHRP-6	AG-pf	ANOVA
Ikbkb	100 ± 9.3	94.8 ± 5.5	104.9 ± 5.4	104.0 ± 5.5	105.8 ± 8.0	NS
Nkkbia	100 ± 17.0	97.3 ± 5.8	95.4 ± 11.3	88.0 ± 5.2	117.7 ± 14.9	NS
TANK	100 ± 5.6	92.9 ± 2.4	91.6 ± 5.7	89.2 ± 9.0	100.8 ± 7.5	NS
CHOP	100 ± 6.1	109.1 ± 5.2	100.9 ± 8.6	112.6 ± 7.5	115.1 ± 12.7	NS
TLR4	100 ± 6.7	118.0 ± 6.8	122.0 ± 19.2	121.9 ± 11.3	169.2 ± 27.6	NS
Cd68	100 ± 44.6	104.7 ± 43.0	78.5 ± 22.9	70.5 ± 23.5	144.6 ± 6.6	NS
NPY	100 ± 6.4	117.7 ± 6.2	80.2 ± 4.9	92.7 ± 11.7	97.5 ± 15.4	NS
AGRP	100 ± 5.8	117.6 ± 10.1	83.1 ± 13.2	95.4 ± 11.5	106.5 ± 16.9	NS
POMC	100 ± 10.1	110.5 ± 9.9	88.1 ± 14.2	87.4 ± 9.5	80.7 ± 14.7	NS
CART	100 ± 6.7	120.9 ± 7.1	90.1 ± 7.0	104.5 ± 11.5	102.9 ± 10.9	NS
orexin	100 ± 10.9	125.3 ± 7.4	117.3 ± 8.4	103.2 ± 7.9	83.6 ± 6.8	NS

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Anatomical specificity of central inflammatory and glial changes

No change in GFAP or vimentin protein levels was found in the hippocampus or cerebellum (Table 3); although AG decreased vimentin (approximately 50% of control values) in the hippocampus this did not reach significance by ANOVA due to variability in the other experimental groups. This observation deserves further investigation. IL-1β and TNFα were unaffected in both areas, while IL-6 mRNA levels were increased by AG and DAG in the cerebellum (Table 3; $P < .04$).

Ghrelin induces hypothalamic IGF-I production that could participate in its central protective effects

Ghrelin mimetics stimulate central production of IGF-I (34, 48), which has both neuroendocrine (49, 50) and protective effects on both astrocytes and neurons (51, 52). Hypothalamic IGF-I mRNA levels were increased by AG, but only in ad libitum fed rats, and by GHRP-6 and DAG (Figure 4A; $P < .05$).

Both AG and DAG have direct effects on hypothalamic astrocytes

We found that astrocytes in vitro express mRNA for GHSR and this was not modulated by either AG or DAG (C: 100, AG: 95.6 ± 11.0, DAG: 118.1 ± 5.0% control). DAG but not AG increased IGF-I expression in astrocytes (Figure 4B; $P < .01$). Both AG and DAG increased GFAP protein (Figure 4C; $P < .05$). No change in vimentin (Figure 4D) was found.

DAG decreased IL-1β (Figure 4E; $P < .05$), while AG increased IL-6 (Figure 4F; $P < .05$) and decreased TNFα mRNA levels (Figure 4G; $P < .05$) in hypothalamic astrocyte cultures. In the hypothalamic neuronal cell line, neither AG nor DAG modified IL-6 (C: 100, AG: 107.6 ± 9.4, DAG: 111.1 ± 5.3% Ct) or TNF-α (C: 100, AG: 95.8 ± 12.9, DAG: 88.0 ± 7.6% Ct) mRNA levels. Expression of IL-1β was undetectable.

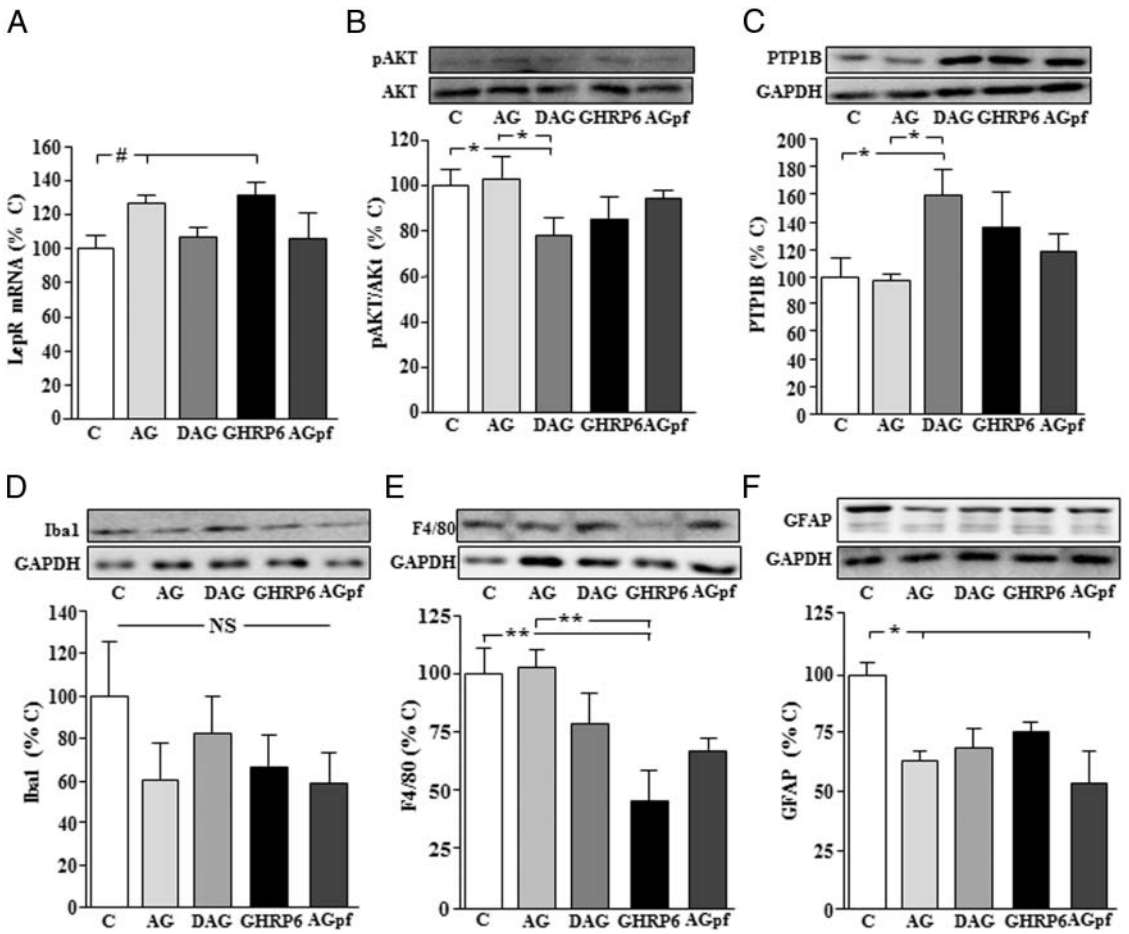

Figure 3. Leptin receptor (LepR) mRNA levels(A) and protein levels of pAKT (B), phosphotyrosine phosphatase 1B (PTP1B; C), anti-ionized calcium-binding adapter molecule 1 (Iba 1; D), F4/80 (E) and glial fibrillary acidic protein (GFAP; F) in the hypothalamus of rats treated centrally with saline (C), acylated ghrelin (5 μg/d; AG), desacylated ghrelin (5 μg/d; DAG), GH-releasing peptide (GHRP)-6 (5 μg/d) or AG and pair fed (5 μg/d; AGpf) for 14 days.*=$P < .05$; **= $P < .006$; NS: not statistically different.

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Table 3. Protein levels of glial filbrillary acidic protein (GFAP) and vimentin and mRNA levels of interleukin (IL)1β, IL6 and TNFα in the hippocampus and cerebellum of rats treated *icv* for 14 days with saline (control), acylated ghrelin (AG), desaclyghrelin (DAG), growth hormone-releasing peptide (GHRP)-6 or with AG and pairfed (AG-pf).

			Hippocampus			
	Control	**AG**	**DAG**	**GHRP-6**	**AG-pf**	**ANOVA**
GFAP	100 ± 10.1	101.5 ± 17.9	104.5 ± 29.1	108.7 ± 14.4	116.3 ± 12.1	NS
Vimentin	100 ± 19.9	48.6 ± 7.9	133.8 ± 29.5	87.6 ± 36.6	105.8 ± 31.7	NS
IL1β mRNA	100 ± 40.0	168.6 ± 62.8	251.7 ± 117.3	79.5 ± 17.4	117.3 ± 16.6	NS
IL6 mRNA	100 ± 20.2	121.5 ± 11.6	132.9 ± 18.2	96.4 ± 5.4	98.2 ± 9.5	NS
TNFα mRNA	100 ± 32.3	108.3 ± 5.2	124.8 ± 19.9	113.3 ± 13.4	120.9 ± 16.9	NS
Cerebellum						
	Control	**AG**	**DAG**	**GHRP-6**	**AG-pf**	**ANOVA**
GFAP	100 ± 10.1	93.7 ± 13.6	102.6 ± 12.4	96.8 ± 9.2	96.9 ± 8.9	NS
Vimentin	100 ± 14.3	119.8 ± 39.9	115.0 ± 17.2	88.4 ± 15.7	158.5 ± 59.3	NS
IL1βmRNA	100 ± 29.9	109.9 ± 28.0	92.4 ± 19.7	53.1 ± 11.6	96.2 ± 26.1	NS
IL6 mRNA	100 ± 15.6	208.5 ± 20.1[a]	185.7 ± 16.6[a]	153.7 ± 14.3	168.1 ± 33.1 [a]	$P < 0.04$
TNFα mRNA	100 ± 21.4	132.9 ± 19.0	99.8 ± 5.7	82.4 ± 10.5	90.3 ± 9.2	NS

a: different from Control.

Discussion

Being overweight or obese is frequently associated with chronic systemic inflammation (53); however, increased weight gain due to AG or GHRP-6-induced food intake was not. A larger increase or longer duration of weight/fat accumulation or increased intake of specific dietary components may be needed for this to ensue. It is also possible that as the physiological drive to eat is preceded by a rise in circulating ghrelin levels (54) and AG and its mimetics inhibit inflammation (55), potentially harmful inflammatory processes may be refrained during physiologically stimulated energy intake and storage. Indeed, VAT mass increased in response to AG, but cytokine production was reduced and this did not depend on food intake. Moreover, although AG and its mimeticGHRP-6 had similar effects on food intake and adipose accrual, their effects on systemic and central cytokine production differed dramatically.

In accordance with previous studies (56, 57), food-intake and weight gain were stimulated by AG and GHRP-6; and, although weight gain and VAT accumulation were not different, only ad libitum fed AG rats had increased insulin, HOMA, triglycerides and HDL and decreased circulating IL-1β and IL-6 levels. This most likely results from differential affinities of AG and GHRP-6 for GHSR-1a and because AG exerts effects that are not mediated through this receptor (25, 26). The reductions in circulating IL-1β and IL-6 were specific to AG and did not depend on food intake or weight gain. Ghrelin and its mimetics have been shown to directly modulate cytokine production both in vivo, by tissues such as liver and muscle (58, 59), and in vitro (60); however, VAT cytokine mRNA levels were only reduced in AG rats fed ad libitum and this

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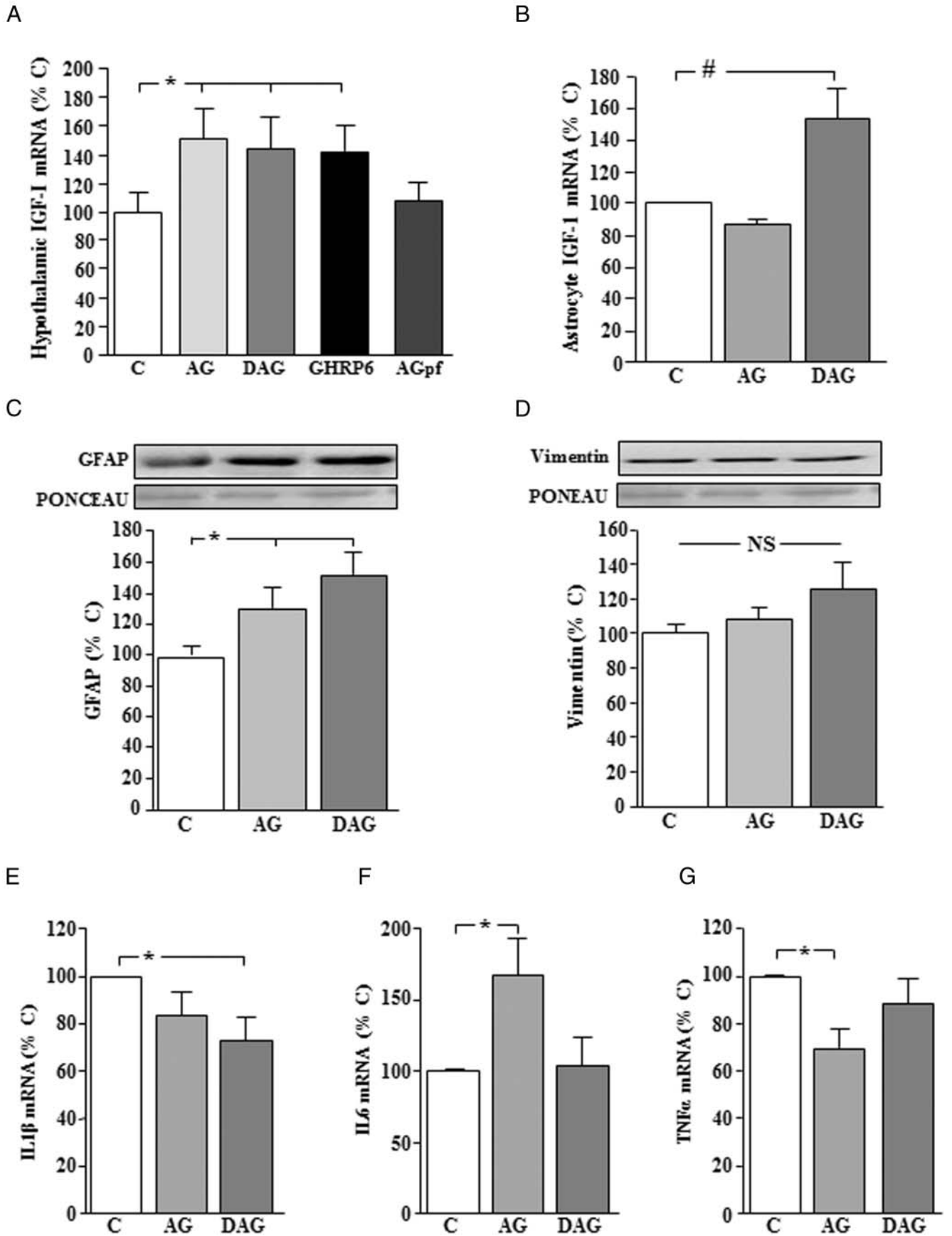

Figure 4. Insulin-like growth factor (IGF)-I mRNA levels (A) in the hypothalamus of rats treated centrally with saline (C), acylated ghrelin (5 μg/d;

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did not correlate with circulating AG levels. This suggests that factors other than the direct action of AG on VAT are involved, including possible central actions (61). Under physiological conditions numerous hormones and metabolic factors determine the final output of adipose tissue. For example, the effects of GHRP-6 on adipose tissue accrual and leptin secretion are dependent on insulin levels (62). Thus, the differences in insulin levels may be involved in the differential responses to AG in ad libitum and pair-fed rats. Likewise, serum triglycerides rose in response to AG only with increased energy intake, as triglyceride accumulation in peripheral tissues is decreased by AG (58, 59). In contrast, FFA levels were higher only in AG pair-fed rats, which may be related to the imbalance between the increased stimulation to eat due to AG treatment and the inability to do so, similar to what is seen in fasting individuals (63).

In contrast to weight gain, AG-stimulated VAT accumulation was not dependent on increased energy intake, in agreement with previously reports (19, 64). Increased VAT mass with no change in bodyweight suggests that lean mass may be decreased in the AG-pf group. The rise in VAT was associated with higher serum leptin levels in AG and GHRP-6 but not AG-pf rats, possibly due to their relative food restriction as fasting decreases leptin biosynthesis and secretion without modifying its mRNA levels (65). Indeed, leptin mRNA levels were only increased in AG ad libitum fed rats, while pair-feed AG-treated rats actually had decreased leptin production in VAT. The rise in circulating FFAs in AG-pf rats could be involved in this inhibition as FFAs decrease leptin synthesis by adipocytes (66). In ad libitum fed AG rats both increased fat mass and leptin expression/g VAT would contribute to the rise in serum levels. In contrast, in GHRP-6 treated rats higher serum leptin levels were not associated with increased VAT mRNA levels, which could be because insulin, which stimulates leptin production (65), was only increased in AG ad libitum fed rats.

Some of the observed systemic effects could be mediated, at least in part, by the rise in circulating total ghrelin levels in DAG and AG treated rats. AG can be transported bidirectionally between the brain and blood (67), such that removal of AG from the brain and deacylation could underlie this increase. However, DAG is reported to only be transported from blood to brain, at least acutely (67). If there is no outward movement of DAG, the rise in serum levels must be due to increased peripheral production or decreased clearance. In contrast, circulating levels of AG were unchanged. Thus, although AG and its mimetics can directly regulate adipocyte proliferation and differentiation (32, 68), the observed effects of AG and GHRP-6 on VAT were most likely mediated centrally or indirectly. One might expect a rise in serum AG in the pair-fed rats as they were not allowed to fully respond to the central orexigenic signal. However, the *icv* infusion of AG could also inhibit this systemic response.

We found no significant change in hypothalamic neuropeptide mRNA levels in response to any of the treatments used here. In contrast, Salome et al (57) report an increase in CART, orexin and NPY expression with *icv* AG treatment. The expression of these genes tended to increase in our AG treated rats, but the changes were not statistically significant. This discrepancy could be due to differences in the dose of AG employed. However, our observation of no change in metabolic neuropeptide expression is in agreement with no effect of AG and GHRP6 on food intake or weight gain during the later stages of the study.

Hypothalamic production of cytokines was stimulated by both AG and DAG. Hypothalamic inflammation occurs before systemic inflammation and insulin/leptin resistance, at least in HFD-induced obesity (3). Here, hypothalamic production of cytokines increased in response to AG, but markers of astrocytes and microglia were either unaffected or decreased, with no indications of central insulin or leptin resistance in rats with AG or GHRP-6 induced weight gain. DAG did not induce weight gain or adipose accumulation, but stimulated hypothalamic cytokine production that was associated with increased PTP1B and decreased pAKT suggesting that central insulin signaling may be decreased by this ghrelin isoform. Although their HOMA index indicates that systemic insulin sensitivity is not reduced, further studies are necessary to determine whether insulin sensitivity in peripheral tissues is affected by central DAG signaling.

During the latter stages of our study DAG decreased food intake. This isoform is reported to abate the stimulatory effects of AG (69) and could thus be inhibiting the endogenous orexigenic drive. DAG stimulated hypothalamic IL-6 expression, which has been shown to protect against weight gain and obesity (70, 71), possibly through stimulation of uncoupling protein 1 expression in brown

Legend to Figure 4 Continued. . .

AG), desacylated ghrelin (5 μg/d; DAG), GH-releasing peptide (GHRP)-6 (5 μg/d) or AG and pair fed (5 μg/d; AGpf) for 14 days. IGF-1 mRNA levels in primary hypothalamic astrocytes (B) treated 24 hours with saline (C), AG (100 μM) or DAG (100 μM). Protein levels of glial fibrillary acidic protein (GFAP; C) and vimentin (D) and mRNA levels of interleukin (IL)-1β (E), IL6 (F) and TNF (TNFα; G) in primary hypothalamic astrocytes cultures. *=$P < .05$; #=$P < .01$; NS: not statistically different.

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adipose tissue (72) or by modulating hypothalamic metabolic neuropeptides (73). Thus, the observed modifications in IL-6 expression could indicate a neuroendocrine vs inflammatory role for this cytokine. We found no effect of DAG on total weight gain or body fat, while Heppner et al report an increase in both parameters (74). These discrepancies may be due to differences in mice vs rats or the doses employed, as the higher dose employed here could be activating a different subset of receptors.

Both AG and DAG stimulated hypothalamic cytokine production and had similar effects on glial markers in vivo. It is possible that DAG is acylated centrally, but it is probable that only some of the effects of AG are mediated through GHS-R1a, while other AG actions and those of DAG are due to activation of another receptor(s). Both ghrelin isoforms stimulated hypothalamic TNFα expression, but their effects on IL-1β and IL-6 differed, indicating distinct underlying mechanisms. Some changes may be indirect and subsequent to metabolic modifications, while specific cell types may also respond differently to these two isoforms. For example, both AG and DAG increased GFAP levels in astrocytes in vitro, but an opposite, and most likely indirect, response was found in vivo. Likewise, the in vivo increase in hypothalamic cytokine production does not appear to result from a direct effect of AG or DAG on astrocytes. The in vivo changes could be due to indirect effects on astrocytes and/or activation of other cell types such as microglia or neurons, although we found no change in cytokine production in a hypothalamic neuronal cell line. Expression of GHSR was detected in astrocyte cultures, but at very low levels, and the doses of AG and DAG were relatively high considering their activities on this receptor. Hence, it is quite possible that they are acting through another receptor and further studies are necessary to clarify this issue.

In addition to protecting neurons against adverse situations, astrocytes can also modify neuronal function (6, 10). IL-6 has anti-inflammatory activity, inhibits ER stress and increases hypothalamic insulin and leptin sensitivity (75) and overexpression of IL-6 in astrocytes protects against HFD-induced weight gain (70). Thus, the AG-induced rise in IL-6 production by astrocytes could participate in metabolic control and protect against secondary complications of weight gain.

IGF-I stimulates neurogenesis and neuroprotection (76); hence, increased central production could be beneficial as HFD-induced obesity is associated with increased hypothalamic cell death (77) and decreased neuronal proliferation (78). Increased hypothalamic synthesis of this growth factor could be in response to cytokine production (79)and/or directly to ghrelin (34, 48). Although hypothalamic neurons increase IGF-I synthesis in response to GHRP-6 (35), DAG increased IGF-I mRNA levels in astrocytes, with no effect of AG. Stimulation of hypothalamic cytokines by AG was not observed in pair-fed rats, nor was increased IGF-I expression. Thus, some responses to AG could be mediated indirectly through weight-gain associated changes, while pair-feeding could possibly induce modifications that inhibit some of the central effects of AG.

One caveat that should be taken into consideration is a possible reduction over time in the biological activity of the peptides stored in the mini-pumps. However, despite that the stabilities of AG and GHRP-6 would be anticipated to differ (80), the temporal changes in food intake for these factors were analogous. This suggests a similar underlying mechanism and could indicate a decrease in sensitivity of the system or activation of a negative feedback mechanism. This does not negate the associations between the increases in weight and/or adipose tissue and inflammatory cytokines and glial changes reported here.

In conclusion, we found AG-induced weight gain to associate with increased hypothalamic cytokine production, but not classical gliosis or activation of inflammatory signaling pathways involved in development of central insulin and leptin resistance. Moreover, unlike its mimetic GHRP-6, AG actually decreased circulating cytokine levels independently of its effect on body weight. These results support and extend recent studies showing that these secondary processes depend on the cause or type of diet inducing weight gain (18). As ghrelin has anti-inflammatory effects (58–60), we propose that this endogenous promoter of food intake may simultaneously stimulate protection against metabolism-associated mechanisms that may be harmful to the cell or organism and that these protective mechanisms include IGF-I. Increases in IGF-I could underlie the lack of hypothalamic glial activation.

Acknowledgments

Address all correspondence and requests for reprints to: Julie A. Chowen or JesúsArgente, Department of Endocrinology, Hospital Infantil Universitario Niño Jesús, Avenida Menéndez Pelayo 65, 28009 Madrid, Spain, Telephone/FAX: +3491 503 5939, Emails: julieann.chowen@salud.madrid.org, jesus.argente@uam.es.

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