

## Central effects of ghrelin on the adrenal cortex: a morphological and hormonal study

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**Abstract.** Ghrelin, a growth hormone secretagogue that exerts an important role in appetite and weight regulation, participates in the activation of the hypothalamo-pituitary-adrenal (HPA) axis. Male Wistar rats (5/group) received daily for 5 days, *via* an ICV (intracerebroventricular) cannula, 5 µl phosphate buffered saline with or without 1 µg of rat ghrelin. Two hours after the last injection, blood and adrenal glands were collected from decapitated rats for blood hormone analyses and histologic and morphometric processing. Ghrelin treatment resulted in increased ( $p < 0.05$ ) body weight (13%), absolute whole adrenal gland weight (18%) and whole adrenal gland volume (20%). The absolute volumes of the entire adrenal cortex, ZG, ZF, and ZR also increased ( $p < 0.05$ ) after ghrelin by 20%, 21%, 21% and 11%, respectively. Ghrelin-treated rats had elevated ( $p < 0.05$ ) blood concentrations of ACTH, aldosterone and corticosterone (68%, 32% and 67%, respectively). The data clearly provide both morphological and hormonal status that ghrelin acts centrally to exert a global stimulatory effect on the adrenal cortex. Clarifying of the ghrelin precise role in the multiple networks affecting the stress hormone release, besides its well known energy and metabolic disbalance effects, remains a very important research goal.

**Key words:** Ghrelin — Adrenal cortex — Aldosterone — Corticosterone — Male rats

**Abbreviations:** HPA, hypothalamo-pituitary-adrenal; CRH, corticotrophin-releasing hormone; TRH, thyrotrophin-releasing hormone; ACTH, adrenocorticotrophic hormone; AVP, arginine vassopresin; ICV, intracerebroventricular; GHS, growth hormone secretagogues; GHS-R, ghrelin receptors; NPY, neuropeptide Y; AgRP, agouti-related protein; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis

### Introduction

Feeding behavior and energy expenditure are controlled by complex neuroendocrine systems to ensure sufficient energy stores for periods of food scarcity. The hypothalamo-pituitary-adrenal (HPA) axis and its associated steroid hormones are among the most important endocrine regulators of energy balance, feeding and energy storage. Diseases of

adrenal insufficiency (e.g. Addison's disease) suppress food intake and energy storage and replacement with exogenous glucocorticoids corrects these deficiencies (Dallman and Bhatnagar 2001; Nieuwenhuizen and Rutters 2008). Feeding under stress, when glucocorticoid levels are high stimulates insulin secretion and results in remodeling of energy stores from muscle to fat, especially in the abdominal region (Follenius et al. 1982; Le Fleur et al. 2004).

The HPA axis is highly sensitive to states of energy balance. Fasting/starvation activates the HPA axis to increase circulating glucocorticoid levels (Dallman et al. 1999), whereas constant feeding dampens the normal diurnal peak of the axis (Saito et al. 1989). Many neurotransmitters

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and neuropeptides influence HPA axis activity *via* actions at hypothalamic and/or suprahypothalamic levels. Among these, growth hormone secretagogues (GHS)-receptor systems have been shown to exert a clear stimulatory effect on corticotrope secretion (Giordano et al. 2006).

Ghrelin is an acylated 28-residue brain-gut peptide that exhibits both growth hormone-stimulatory and appetite-inducing effects (Kojima et al. 1999; Korbonits et al. 2004). Although it is mainly produced by the stomach mucosa, ghrelin and its receptors (GHS-R type 1a) are also expressed in the small intestine, hypothalamus, pituitary, adrenal and other tissues where it may have both endocrine and paracrine effects (Gnanapavan et al. 2002; Ghelardoni et al. 2006). Recent attention has been given to ghrelin activity, as it is one of the most important peripheral physiological signals of hunger, an important component of energy balance (Horvath et al. 2001; Zigman and Elmquist 2003). The proposed growth hormone independent mechanism of ghrelin's orexigenic action is by activation of hypothalamic neuropeptide Y (NPY)/agouti-related protein (AgRP) systems in arcuate neurons (Toshinai et al. 2003; Chen et al. 2004). These neurons were also shown to affect the function of paraventricular hypophysiotropic neurons, namely corticotrophin-releasing hormone (CRH)- and thyrotrophin-releasing hormone (TRH)-producing neurons (Tschöp et al. 2002).

Ghrelin stimulates the HPA axis in the rat directly and by stimulation of both CRH, and arginine vasopressin (AVP) release from the hypothalamus (Mozid et al. 2003). At the pituitary level, we have demonstrated (Stevanović et al. 2006), in agreement with others (Wren et al. 2000; Arvat et al. 2001), that centrally administered ghrelin stimulated both pituitary adrenocorticotrophic hormone (ACTH) and corticosterone secretion. ACTH is known to induce changes in structure of normal adrenal cortex. In this study we explicitly examined changes in structure and secretory activity of all adrenocortical zones i.e., the zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) after central ghrelin administration in adult male rats and correlated these with changes in blood-borne steroids.

The results obtained re-enforce prior research showing that the peptide stimulates ACTH-producing pituitary cells. Collectively, they provide clear evidence that exposure of the brain of normal rats to sub-nanomolar amounts of ghrelin results in an overall activation of ACTH pituitary cells, all zones of the adrenal cortex as well as the hormones associated with these cells.

## Materials and Methods

### Animals

The study was performed on adult male Wistar rats (200 ± 20 g) bred at the Institute of Biomedical Research "Galenika"

in Belgrade. They were kept individually in metabolic cages under constant laboratory conditions of room temperature (22 ± 2°C) and lighting (12 h light : 12 h dark), and were fed with standard pelleted rat food ("D.D.Veterinarski zavod Subotica", Subotica, Serbia). Food and water were available *ad libitum*. The experimental protocols were approved by the Animal Care Committee of the Institute for Biological Research (Belgrade, Serbia) in conformity with the recommendation provided in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123, Appendix A).

### Surgical procedures

At the age of 8 weeks animals were implanted a headset, which consisted of silastic-sealed 20-gauge cannula, introduced into a lateral cerebral ventricle, 1 mm posterior and 1.5 mm lateral to the bregma, and 3 mm below the cortical surface (Starčević et al. 1988). The surgical procedure was performed under deep anesthesia (Thiopental-Sodium, 45 mg/kg, *i.p.*, Rotexmedica, Trittau, Germany). A small stainless steel anchor screw was placed at a remote site on the skull. The cannula and screw were cemented to the skull with dental acrylic (Simgal; ICN Galenika, Belgrade, Serbia). The headset was later used for intracerebroventricular (ICV) injections.

### Experimental protocol

Five days after the surgery, the rats were randomly divided in two groups ( $n = 5$  each). One group (ghrelin) was ICV-treated daily with 1 µg of ghrelin (Global Peptide Services, CO, USA) dissolved in 5 µl phosphate buffer saline (PBS) for 5 consecutive days. The control rats received only 5 µl of PBS daily. The ICV injections were made at 10:00 h. During treatment body weight and food intake were obtained daily, just before the next ICV injection. Two hours after the last ICV injection, all rats were decapitated under deep *i.p.* thiopental sodium anesthesia. The pituitaries and adrenal glands were excised and weighed. The relative organ weights were calculated from the ratio of the measured organ weight and body weight for each animal.

### Light microscopy and histological analyses

Left adrenal glands were excised, fixed in Bouin's solution, embedded in paraffin and serially cut into 5 µm thick sections and stained with hematoxylin-eosin for examination under a light microscope (Leica, Germany).

Digital images of adrenal glands were made using a Leica DM RB Photo Microscope (Leica, Wetzlar, Germany), a JVC TK 1280E Video Camera (Leica) and the Qwin program (Leica).

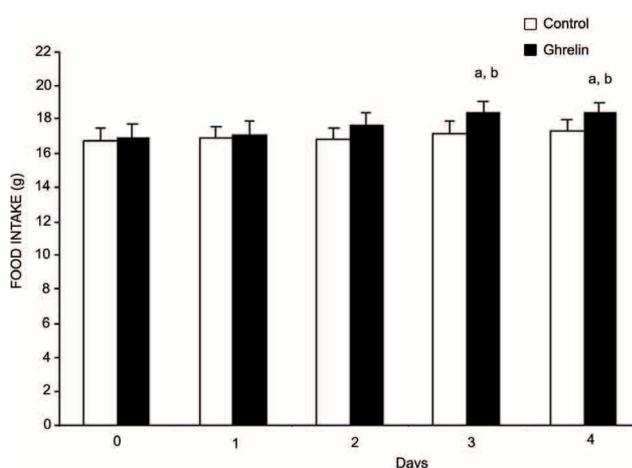
### Morphometric analyses

Stage 1. Zonation of the adrenal gland. In order to evaluate the volume densities of the adrenocortical zones, every 10th section of the gland was analyzed using the multipurpose test system  $M_{42}$  (Weibel, 1979) at magnification 125 $\times$ . The absolute volume of the glands was calculated on the basis of their weight, assuming an average specific gravity of the adrenal of 1.039 g $\cdot$ cm $^{-3}$  (Swinyard 1938).

Stage 2. Size and number of adrenocortical cells. The volume densities of both the nuclei and the cytoplasm of parenchymal cells were estimated on a screen using the multipurpose test system  $M_{42}$  (Weibel and Gomez, 1962) at magnification 1000 $\times$ . For each adrenal gland, a single paraffin section containing zona medullaris was chosen and 30 test areas of ZG and 50 test areas of ZF and ZR were analyzed. Based on earlier karyometric studies (Malendowicz 1974), the shape coefficient  $\beta$  was assumed to be 1.382 for the ZF and 1.500 for the ZG. It relates  $N_v$  (number of cells counted *per unit volume*) to  $N_a$  (number of cells counted *per mm $^2$* ) and  $V_v$  (volume density) and depends on the axial ratio of estimated nuclei. The number of adrenocortical cell nuclei *per mm $^3$*  was calculated according to the method of Weibel and Gomez (1962). The rat adrenocortical cells are mononuclear, therefore the numerical density of the nuclei corresponds to the number of cells *per mm $^3$* .

### Hormonal analyses

Plasma concentrations of ACTH in control and experimental rats were measured by the Immulite method (DPC, USA) for human usage. The serum levels of aldosterone and cor-



**Figure 1.** 24 h food (g) intake prior to and following the 1st, 2nd, 3rd and 4th ICV ghrelin or PBS injection in male rats. All data are expressed as mean values  $\pm$  SD;  $n = 5$  animals *per* group; <sup>a</sup>  $p < 0.05$  vs. pre-ghrelin treatment (0), <sup>b</sup>  $p < 0.05$  vs. 1st ICV ghrelin day.

ticosterone were determined by enzyme immunoassay (Aldosterone ELISA, IBL, Hamburg and R&D systems, GmbH, Wiesbaden, Germany).

### Statistical analyses

Daily body weight and food intake for each rat were averaged and the standard deviation of the mean (SD) was calculated. A one-way analysis of variance (ANOVA) was used for statistical comparisons between the groups: day "0" starting 24 h before the first ICV injection and daily after the 1st, 2nd, 3rd and 4th ICV injections. Averaged morphometric and hormonal data, as well as daily body weight and food intake were calculated using Student's *t*-test, and probability value of 5% or less was considered statistically significant.

## Results

### Body weight, absolute and relative weight of the adrenal gland and food intake

Body weight and absolute weight of the adrenal gland from rats treated with ICV ghrelin were increased ( $p < 0.05$ ) by 13.0% and 19.7%, respectively, compared to control animals. Relative adrenal weight was unchanged ( $p > 0.05$ ) in comparison with corresponding controls (Table 1).

Data summarizing the effects of repetitive ICV administration of 1  $\mu$ g/5  $\mu$ l of ghrelin or solvent on 24 h food intake are shown in Fig. 1. Food intake was significantly higher ( $p < 0.05$ ) after the 3rd and 4th dose of ICV ghrelin compared to pre-ghrelin treatment (time 0) and those after the 1st ICV ghrelin injection. In control rats, there was no significant ( $p > 0.05$ ) change in food intake over the course of the experiment.

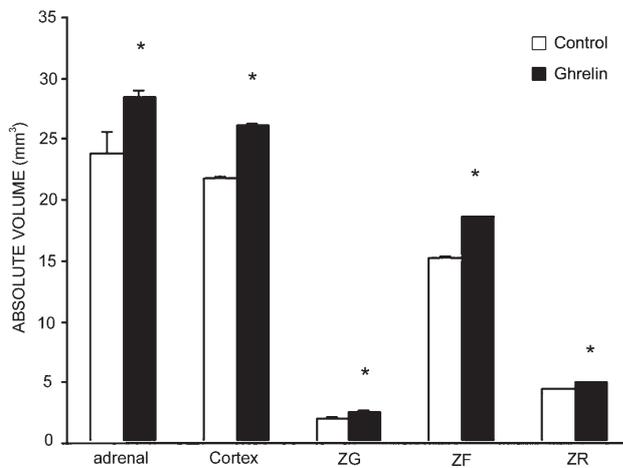
### Adrenal cortex

The three cortical zones of the adrenal gland, i.e., ZG, ZR and ZF, were clearly visible in all examined preparations. The absolute volume of the adrenal gland and the absolute

**Table 1.** The effects of ICV administered ghrelin on body weight, absolute and relative adrenal weights in adult male rats

	Body weight (g)	Absolute adrenal weight (mg)	Relative adrenal weight (mg/%)
Control	212.0 $\pm$ 4.5	24.7 $\pm$ 2.6	11.9 $\pm$ 1.6
Ghrelin	240 $\pm$ 5.9*	29.6 $\pm$ 1.9*	12.3 $\pm$ 0.9

The values are the means  $\pm$  SD for five animals. \*  $p < 0.05$  vs. control.



**Figure 2.** Absolute volumes of adrenal gland, cortex, ZG, ZF, and ZR after ICV administration of ghrelin to male rats. Values are means  $\pm$  SD ( $n = 5$ ), \*  $p < 0.05$  vs. control.

and relative volume of the adrenal cortex in ghrelin-treated rats were all significantly increased ( $p < 0.05$ ) when compared to rats receiving only the PBS solvent (see Fig. 2 and 3).

#### Histological analysis of adrenal cortex

The ZG is arranged in closely packed ovoid clusters. ZG cells are relatively small and columnar or pyramidal. The nuclei are round or oval with an evident nucleolus. The shape of ZG cells in animals ICV-treated with ghrelin was not markedly affected but the cytoplasm in these cells was lighter than controls (Fig. 4A,B). The ZF was large with polyhedral cells. The cells were arranged in long straight cords, one or two cells thick, that are separated by sinusoidal capillaries. In control rats this zone has both, light and dark stained cells (Fig. 4C). In ICV ghrelin-treated rats the ZF has longer cells with lighter cytoplasm (Fig. 4D). ZR cells are noticeably smaller than those of the ZF, and their nuclei are more deeply stained (Fig. 4E). They are arranged in anastomosing cords with few lipid droplets. Both light and dark cells are seen. Dark cells have abundant large pigment granules and deeply staining nuclei are evident. The cells in this zone are smaller as they have less cytoplasm than ZF cells. Thus the nuclei appear more closely packed. The longer cells with lighter cytoplasm are seen in the ZR in ICV ghrelin-treated rats (Fig. 4F).

#### Morphometric parameters of adrenal cortex

The absolute volumes of ZG, ZF and ZR all increased ( $p < 0.05$ ) by 20.9%, 21.4% and 11.1%, respectively, compared to control rats (Fig. 2). The relative volumes of adrenal cortex

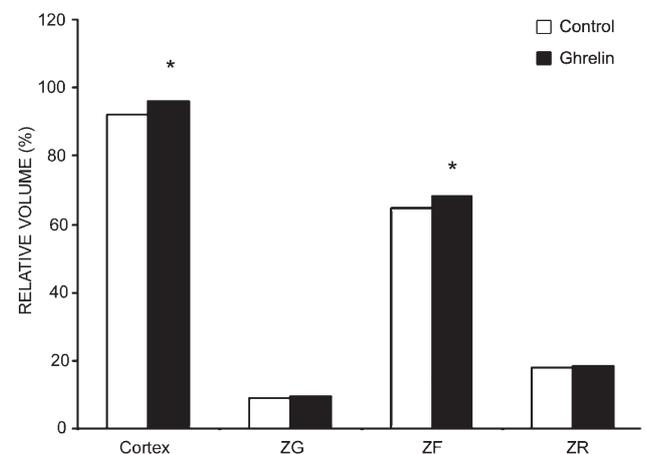
and ZF were significantly increased ( $p < 0.05$ ) by 4.3% and 5.2%, respectively, in comparison with control rats (Fig. 3). The relative volumes of ZG and ZR were unchanged ( $p > 0.05$ , Fig. 3). The volumes of ZG, ZF and ZR cells were significantly increased ( $p < 0.05$ ) by 14.1%, 66.9%, and 30.3%, respectively, in comparison with controls (Fig. 5A). The nuclei of ZG, ZF and ZR cells were also significantly increased ( $p < 0.05$ ) by 36.1%, 50.6% and 47.8%, respectively, compared to corresponding controls (Fig. 5B).

#### Hormonal analyses

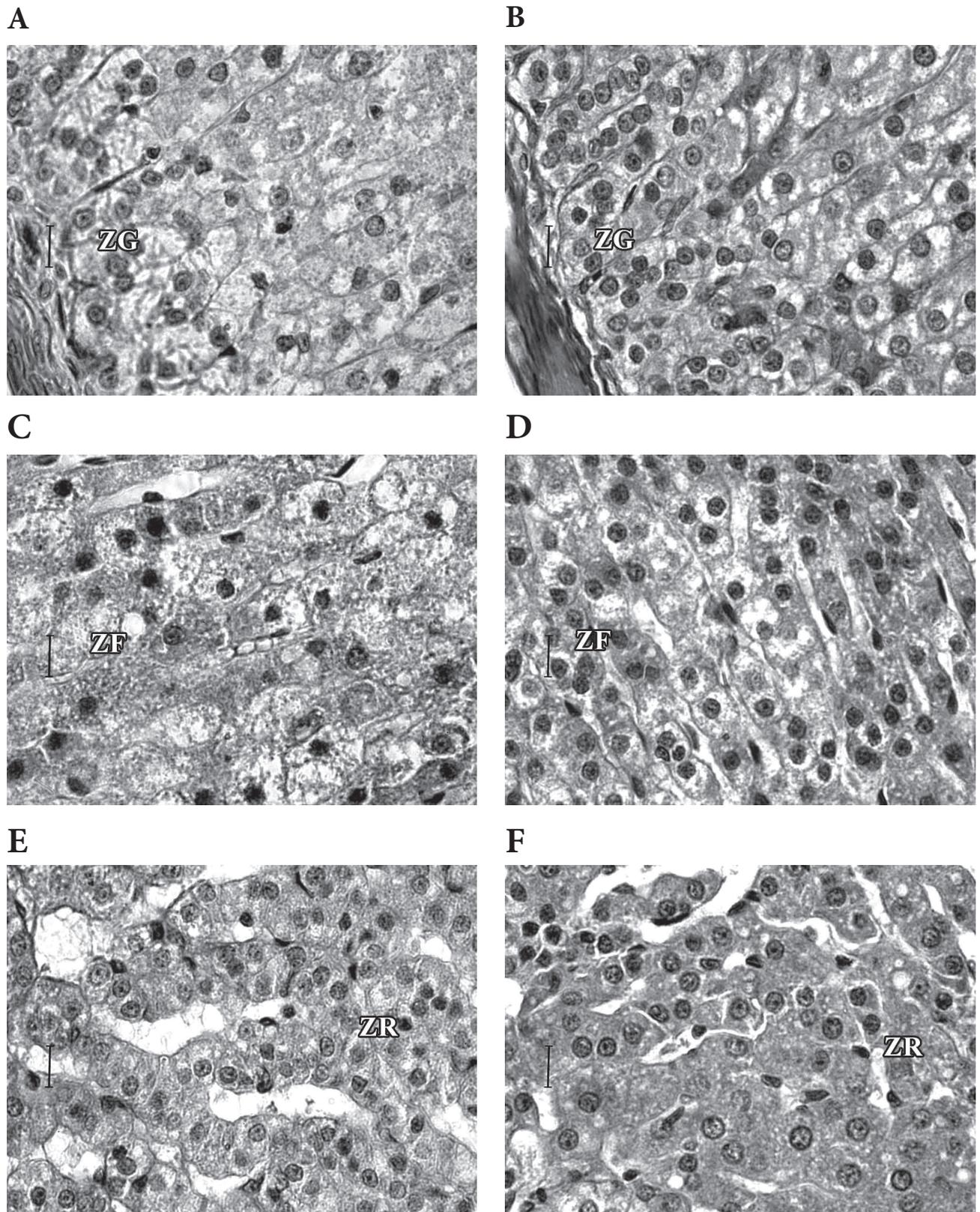
The plasma concentration of ACTH and serum concentrations of aldosterone and corticosterone were all significantly increased ( $p < 0.05$ ) by 68.2%, 32.3% and 66.5%, respectively, in ghrelin-treated animals compared to PBS-treated rats (Fig. 6A,B,C).

#### Discussion

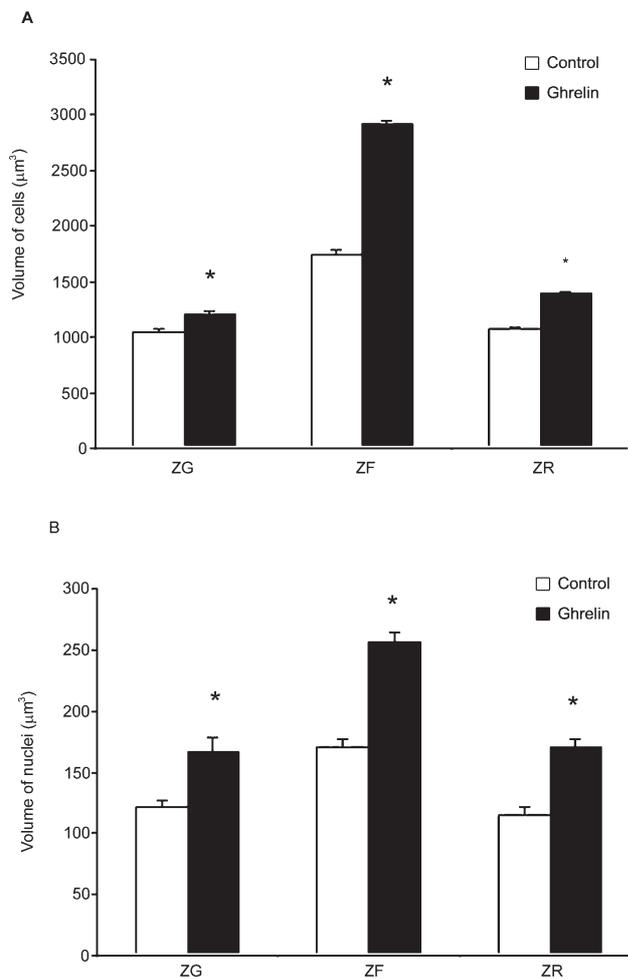
In the case of body weight and food intake Kamegai et al. (2001) demonstrated that chronic central ghrelin treatment every 12 h for 3 days increased food intake and body weight, which supports our findings. The results presented in this study clearly demonstrate a marked central effect of ghrelin on the adrenal cortex. The absolute volume of the adrenal cortex and the three subzones all were significantly increased. This was most apparent in the ZF, where the relative volume also increased. These zonal volume increases were all accompanied by a corresponding increase in nuclear volume of the cells. A clear demarcation of the 3 zones is not observable in some species, however, the



**Figure 3.** Relative volumes of adrenal cortex, ZG, ZF and ZR after ICV administration of ghrelin to male rats. Values are means  $\pm$  SD ( $n = 5$ ), \*  $p < 0.05$  vs. control.

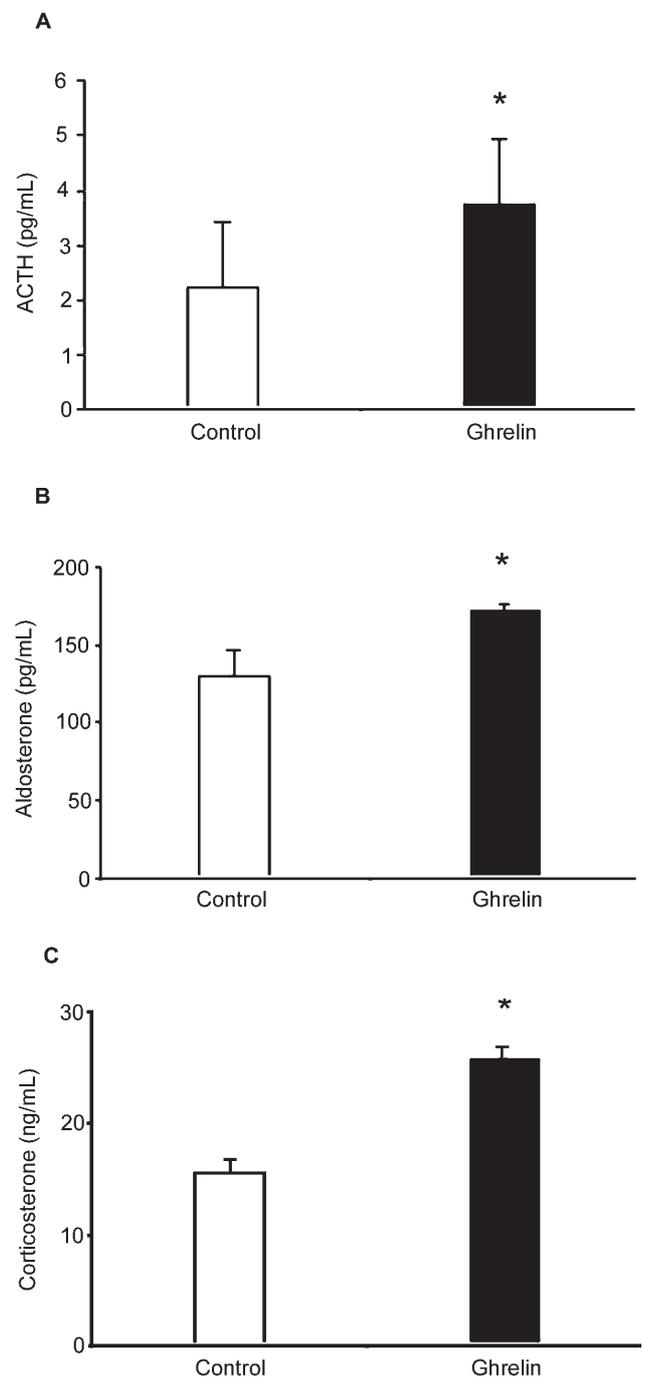


**Figure 4.** Histochemically labelled ZG, ZF and ZR cells in adrenal cortex of male rats in control (A, C, E) and ICV ghrelin-treated rats (B, D, F). (H&E, bar 25  $\mu$ m).



**Figure 5.** Effects of ICV-administered ghrelin on the morphometric parameters of adrenal cortex zones in adult male rats. **A.** Cellular volume ( $\mu\text{m}^3$ ) of ZG, ZF and ZR cells. **B.** Nuclear volume ( $\mu\text{m}^3$ ) of ZG, ZF and ZR cells. Values are means  $\pm$  SD ( $n = 5$ ), \*  $p < 0.05$  vs. control.

zonation in the Wistar rat adrenal cortex appears reasonably well as seen in Fig. 4. The histological demarcation and differences between the ZG and ZF were more distinct than those between ZF and ZR, although still clear. It is widely accepted that for functional purposes, zonation of the rat adrenal cortex can be separated into the ZG and ZF/ZR. This differentiation is associated with the exclusive presence of aldosterone synthase (CYP11B2) in the glomerulosa whereas 11 $\beta$ -hydroxylase (CYP11B1) is exclusively present in the rat ZF/ZR (Ogishima et al. 1992; Mitani et al. 1994). Enzymes that catalyze those steroidogenic pathways are biochemically essential to partitioning mineralocorticoid-active steroids of the glomerulosa from the glucocorticoid-producing steroids of the inner zones (Vinson 2003). The hormone assays from blood obtained after the



**Figure 6.** Hormonal concentrations in adult male rats. **A.** Plasma concentrations of ACTH (pg/ml). **B.** Serum concentrations of aldosterone (pg/ml). **C.** Serum concentrations of corticosterone (ng/ml). Data are expressed as mean values  $\pm$  SD ( $n = 5$ ), \*  $p < 0.05$  vs. control.

last ICV ghrelin dose showed a significant elevation in the principal ZG related mineralocorticoid, aldosterone, and an even more pronounced increased in corticosterone,

the principal steroid with glucocorticoid activity usually associated with ZF/ZR.

Thus, our results clearly document that the stimulatory changes in the morphological features of the ZG and ZF/ZR was accompanied by a physiological response of adrenal cortex after central ghrelin. Other studies showed that ghrelin is potent stimulator of growth in many tissues (Stevanovic et al. 2006, 2007; Warzecha et al. 2006; Yuzuriha et al. 2007). An *in vitro* study by Andreis et al. (2003) showed that ghrelin, expressed in rat adrenal cortex, stimulates proliferation of cultured ZG cells and therefore is involved in autocrine-paracrine regulation of adrenal cortex. However, this study did not report any changes in secretory activity of adrenocortical cells.

In contrast, our prior study using the same *in vivo* ICV treatment protocol with ghrelin documented increased morphometric parameters of the ACTH-positive pituitary cells, accompanied by elevated ACTH and corticosterone blood levels (Stevanovic et al. 2007). Collectively, that study and the present work document a clear concordance between the morphological stimulation observed in ACTH-producing pituitocytes and all three zones of the adrenal cortex with the hormonal observation of elevated blood concentrations of ACTH, aldosterone and corticosterone.

It is important to note that the ICV protocol with ghrelin has the following characteristics: 1) a low (subnanomolar) dose of ghrelin was used; 2) treatment was administered daily for five days; 3) terminal blood samples for hormone assays were obtained two hours after the animals were killed; and 4) multiple half-lives of circulating ghrelin would have passed after 2 h even if the entire small ICV dose was absorbed (Tolle et al. 2002). Therefore, the morphological and hormonal changes observed likely reflect a central effect of ghrelin that shifts, over time, the systemic physiology of conscious animals towards a state of mineralocorticoid and glucocorticoid excess.

It is generally accepted that the steroidogenic potential of ZG is equivalent to the inner zones. Also, all of the steroidogenic enzymes can be induced in the glomerulosa under various conditions including elevations in ACTH (Vinson 2003). The ZG contains the transactivating factor SF-1 as does the fasciculata and this factor is required for the induction of a wide range of cellular components associated with steroidogenesis. This includes various enzymes, with the possible single exception of the glomerulosa specific CYP11B2 (Bassett et al. 2002). Based on the results with the pattern of ghrelin dosing used herein, we posit that ICV ghrelin increases aldosterone secretion *via* ACTH release. Since the ZG is at least partly pituitary (and therefore ACTH) independent, other autocrine-paracrine and nonpituitary regulatory mechanisms may be involved in the observed morphological features and hormonal status of ZG in response to ghrelin.

In conclusion, centrally and repetitively applied ghrelin in relatively small doses increased body weight and absolute adrenal weight. Morphometric examination of tissue revealed increased volumes of ZG, ZF and ZR cells and their nuclei, and these changes were accompanied by significant elevations in ACTH, aldosterone and corticosterone blood levels. These adrenal cortical changes are mostly due to activation of pituitary-adrenal axis evoked by centrally applied ghrelin. Further examination of intracellular and biochemical mechanisms that control those changes should clarify the precise role of ghrelin in stress axis physiology, besides its other well known effects *i.e.* obesity promotion as well as energy and metabolic disbalance.

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## References

- Andreis P. G., Malendowicz L. K., Trejter M., Neri G., Spinazzi R., Rossi G. P., Nussdorfer G. (2003): Ghrelin and growth hormone secretagogue receptor are expressed in the rat adrenal cortex: Evidence that ghrelin stimulates the growth, but not the secretory activity of adrenal cells. *FEBS Lett.* **536**, 173–179; doi:10.1016/S0014-5793(03)00051-6
- Arvat E., Maccario M., Di Vito L., Broglio F., Benso A., Gottero C., Papotti M., Muccioli G., Dieguez C., Casanueva F. F., Deghenghi R., Camanni F., Ghigo E. (2001): Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. *J. Clin. Endocrinol. Metab.* **86**, 1169–1174; doi:10.1210/jc.86.3.1169
- Bassett M. H., Zhang Y., Clyne C., White P. C., Rainey W. E. (2002): Differential regulation of aldosterone synthase and 11 $\beta$ -hydroxylase transcription by steroidogenic factor-1. *J. Mol. Endocrinol.* **28**, 125–135; doi:10.1677/jme.0.0280125
- Chen H. Y., Trumbauer M. E., Chen A. S., Weingarth D. T., Adams J. R., Frazier E. G., Shen Z., Marsh D. J., Feighner S.D., Guan X. M., Ye Z., Nargund R. P., Smith R.G., Van der Ploeg L. H., Howard A. D., MacNeil D. J., Qian S. (2004): Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology* **145**, 2607–2612; doi:10.1210/en.2003-1596
- Dallman M. F., Akana S. F., Bhatnagar S., Bell M. E., Choi S., Chu A., Horsley C., Levin N., Meijer O., Soriano L. R., Strack A. M., Viau V. (1999): Starvation: early signals, sensors and sequelae. *Endocrinology* **140**, 4015–4023; doi:10.1210/en.140.9.4015

- Dallman M. F., Bhatnagar S. (2001): Chronic Stress and Energy Balance: Role of the Hypothalamo-Pituitary-Adrenal Axis. Vol. 4. Coping with the environment. Chap. 10, pp. 179–210, Oxford University Press, New York
- Follenius M., Brandenburger G., Hetter B. (1982): Diurnal cortisol peaks and their relationship to meals. *J. Clin. Endocrinol. Metab.* **55**, 757–761; doi:10.1210/jcem-55-4-757
- Ghelardoni S., Carnicelli V., Frascarelli S., Ronca-Testoni S., Zucchi R. (2006): Ghrelin tissue distribution: comparison between gene and protein expression. *J. Endocrinol. Invest.* **29**, 115–121
- Giordano R., Pellegrino M., Picu A., Bonelli L., Balbo M., Bernardelli R., Lanfranco F., Ghigo E., Arvat E., (2006): Neuroregulation of the hypothalamus-pituitary-adrenal (HPA) axis in humans: effects of GABA-, mineralocorticoid- and GH-secretagogue-receptor modulation. *Scientific World Journal* **6**, 1–11; doi:10.1100/tsw.2006.09
- Gnanapavan S., Kola B., Bustin S. A., Morris D. G., Mc Gee P., Fairclough P., Bhattacharya S., Carpenter R., Grossman A. B., Korbons M. (2002): The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J. Clin. Endocrinol. Metab.* **87**, 2988–2991; doi:10.1210/jc.87.6.2988
- Horvath T. L., Diano S., Sotonyi P., Heiman M., Tschöp M. (2001): Ghrelin and the regulation of energy balance – a hypothalamic perspective. *Endocrinology* **142**, 4163–4169; doi:10.1210/en.142.10.4163
- Kamegai J., Tamura H., Shimizu T., Ishii S., Sugihara H., Wakabayashi I. (2001) Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and agouti related protein mRNA levels and body weight in rats. *Diabetes* **50**, 2438–2443; doi:10.2337/diabetes.50.11.2438
- Kojima M., Hosoda H., Date Y., Nakazato M., Matsuo H., Kangawa K. (1999): Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**, 656–660; doi:10.1038/45230
- Korbons M., Goldstone A. P., Gueorguiev M., Grossman A. B. (2004): Ghrelin – a hormone with multiple functions. *Front. Neuroendocrinol.* **25**, 27–68; doi:10.1016/j.yfrne.2004.03.002
- La Fleur S. E., Akana S. F., Manalo S. L., Dallman M. F. (2004): Interaction between corticosterone and insulin in obesity: regulation of lard intake and fat stores. *Endocrinology* **145**, 2174–2185; doi:10.1210/en.2003-1359
- Malendowicz L., (1974): Sex differences in adrenocortical structure and function. I. The effects of postpubertal gonadectomy and gonadal hormone replacement on nuclear volume of adrenocortical cells in the rat. *Cell Tissue Res.* **151**, 525–536; doi:10.1007/BF00222997
- Mitani F., Suzuki H., Hata J., Ogishima T., Shimada H., Ishimura Y. (1994): A novel cell layer without corticosteroid-synthesizing enzymes in rat adrenal cortex: histochemical detection and possible physiological role. *Endocrinology* **135**, 431–438; doi:10.1210/en.135.1.431
- Mozid A. M., Tringali G., Forsling M. L., Hendricks M. S., Ajodha S., Edwards R., Navarra P., Grossman A. B., Korbons M. (2003): Ghrelin is released from rat hypothalamic explants and stimulates corticotrophin-releasing hormone and arginine-vasopressin. *Horm. Metab. Res.* **35**, 455–459; doi:10.1055/s-2003-41801
- Nieuwenhuizen A. G., Rutters F. (2008): The hypothalamic-pituitary-adrenal-axis in the regulation of energy balance. *Physiol. Behav.* **94**, 169–177; doi:10.1016/j.physbeh.2007.12.011
- Ogishima T., Suzuki H., Hata J., Mitani F., Ishimura Y. (1992): Zone-specific expression of aldosterone synthase cytochrome P-450 and cytochrome P-45011 beta in rat adrenal cortex: histochemical basis for the functional zonation. *Endocrinology* **130**, 2971–2977; doi:10.1210/en.130.5.2971
- Saito M., Nishimura K., Kato H., (1989): Modifications of circadian cortisol rhythm by cyclic and continuous total enteral nutrition. *J. Nutr. Sci. Vitaminol. (Tokyo)* **35**, 639–647
- Starčević V., Morrow B. A., Farner L. A., Keil L. C., Severs W. B. (1988): Long-term recording of cerebrospinal fluid pressure in freely behaving rats. *Brain Res.* **462**, 112–117; doi:10.1016/0006-8993(88)90592-6
- Stevanović D., Milošević V., Nešić D., Ajdžanović V., Starčević V., Severs W. B. (2006): Central effects of ghrelin on serum growth hormone and morphology of pituitary somatotropes in rats. *Exp. Biol. Med.* **231**, 1610–1615
- Stevanović D., Milošević V., Starčević V. P., Severs W. B. (2007): The effect of centrally administered ghrelin on pituitary ACTH cells and circulating ACTH and corticosterone in rats. *Life Sci.* **80**, 867–872; doi:10.41064/j.lfs.2006.11.018
- Swinyard C. (1938): Methods for volumetric determination of fresh endocrine glands. *Anat. Res.* **74**, 71–78; doi:10.1002/ar.1090740108
- Tolle, V. Bassant, M. H., Zkizzari, P., Poindessous-Jazat F., Tomasetto C., Epelbaum J., Bluet-Pajot M. T. (2002): Ultradian rhythmicity of ghrelin secretion in relation with GH, feeding behavior and sleep-wake patterns in rats. *Endocrinology* **143**, 1353–1361; doi:10.1210/en.143.4.1353
- Toshinai K., Date Y., Murakami N., Shimada M., Mondal M. S., Shimbara T., Guan J. L., Wang Q. P., Funahashi H., Sakurai T., Shioda S., Matsukura S., Kangawa K., Nazato M. (2003): Ghrelin-induced food intake is mediated via the orexin pathway. *Endocrinology* **144**, 1506–1512; doi:10.1210/en.2002-220788
- Tschöp M., Flora D. B., Mayer J. P., Heiman M. L. (2002): Hypophysectomy prevents ghrelin-induced adiposity and increases gastric ghrelin secretion in rats. *Obes. Res.* **10**, 991–999; doi:10.1038/oby.2002.135
- Vinson G. P. (2003): Adrenocortical zonation and ACTH. *Microsc. Res. Tech.* **15**, 227–239; doi:10.1002/jemt.10331
- Warzecha Z., Dembiński A., Ceranowicz P., Dembiński M., Cieszkowski J., Konturek S. J., Polus A., Pawlik W. W., Kuwahara A., Kato I., Konturek P. C. (2006): Influence of ghrelin on gastric and duodenal growth and expression of digestive enzymes in young mature rats. *J. Physiol. Pharmacol.* **57**, 425–437
- Weibel E. R. (1979): Stereological methods: Practical Methods of Biological Morphometry. Vol. 1., pp. 1–415, Academic Press, London

- Weibel E. R., Gomez D. M. (1962): A principle for counting tissue structures on random sections. *J. Appl. Physiol.* **17**, 343–348
- Wren A. M., Small C. J., Ward H. L., Murphy K. G., Dakin C. L., Taheri S., Kennedy A. R., Roberts G. H., Morgan D. G., Ghatei M. A., Bloom S. R. (2000): The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* **141**, 4325–4328; doi:10.1210/en.141.11.4325
- Yuzuriha H., Inui A., Asakawa A., Ueno N., Kasuga M., Meguid M. M., Miyazaki J., Ninomiya M., Herzog H., Fujimiya M. (2007): Gastrointestinal hormones (anorexigenic peptide YY and orexigenic ghrelin) influence neural tube development. *FASEB J.* **21**, 2108–2112; doi:10.1096/fj.06-7621.com
- Zigman J. M., Elmquist J. K. (2003): From anorexia to obesity – the yin and yang of body weight control. *Endocrinology* **144**, 3749–3756; doi:10.1210/en.2003-0241

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