

Histomorphometric features of ventral prostate in different aged rats after central ghrelin treatment

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Abstract. Ghrelin, the endogenous ligand of growth hormone secretagogue receptor type 1a (GHS-R1a), has emerged as pleiotropic modulator of diverse biological functions, including energy homeostasis and recently, reproduction. The influence of intracerebroventricularly (ICV) administered ghrelin (1 µg/day/rat for 5 days) to rats of different ages, i.e., peripubertal (38 days), adult (60 days) and middle-aged (180 days) on the ventral prostate size and morphology, serum testosterone levels and testis weight was examined. Ghrelin treatment significantly increased ($p < 0.05$) absolute ventral prostate weight in peripubertal and middle-aged rats, by 27% and 37% respectively, due to enhancement of epithelial and/or luminal compartment of the gland. In adult rats, both absolute and relative volumes of the acinar lumen were significantly decreased ($p < 0.05$), by 38% and 44% respectively, which was associated with significant increases ($p < 0.05$) in relative and absolute volumes of interacinar stroma, whereas ventral prostate weight was unchanged. Irrespective of animal age, ghrelin did not affect serum testosterone levels. These are the first results of ghrelin treatment effects on healthy prostate appearance, which allow us to conclude that the rat ventral prostate response to ghrelin depends on the developmental stage of animals. Our results merit further investigations and may have clinical implications, especially in the light of data on possible role of ghrelin in prostate hypertrophy and adenomas.

Key words: Ghrelin — Ventral prostate — Rats

Introduction

Ghrelin was identified as a novel neuroendocrine signal involved in the physiological control of growth hormone (GH) secretion and energy homeostasis (Kojima et al. 1999; Takaya et al. 2000; Tschop et al. 2000; Wren et al. 2000). In the last years an "unexpected" reproductive facet of ghrelin emerged and indicated that circulating ghrelin might contribute to the functional control of the reproductive axis and its integration with energy balance (Barreiro and Tena-Sempere 2004; Lorenzi et al. 2009). Significant amount of data gathered so far indicate that ghrelin par-

ticipates in the regulation of reproductive physiology by two distinct, probably overlapping pathways: (i) through systemic release of the stomach-derived peptide, which acts at all levels of hypothalamic-pituitary-gonadal (HPG) axis, and (ii) through biological actions of locally expressed ghrelin on reproductive organs (Tanaka et al. 2001; Lorenzi et al. 2009).

Data available in literature strongly suggest that ghrelin predominantly acts as an inhibitory signal for the pituitary-gonadal axis in different mammalian species including rodents, sheep, non-human primates and humans; a phenomenon that might contribute to the well known suppression of gonadotropin levels in conditions of persistent negative energy balance, in which ghrelin levels are commonly elevated (Tena-Sempere 2008; Roa et al. 2010). Thus, it have been reported that systemic administration of ghrelin to pubertal male rats significantly increased gonadotropin

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releasing hormone (GnRH) interpulse interval, whereas *in vitro*, when the peptide was incubated with hypothalamic explants, a reduction of interpulse interval was observed (Lebrethon et al. 2007). The effects of ghrelin on the pituitary function are dependent upon the nature of experimental model used (Lim et al. 2011). Chronic administration of ghrelin consistently decreased luteinizing hormone (LH) secretion in pubertal and adult male rats (Martini et al. 2006). Similarly, a 20-day ghrelin treatment of young adult male rats reduced plasma LH and testosterone, but only in animals exposed to food restriction (Sirotkin et al. 2008). Central application of ghrelin significantly decreased serum levels of LH in intact and orchidectomized peripubertal rats, while *in vitro* ghrelin stimulated the secretion of both gonadotropins (Fernandez-Fernandez et al. 2004). Studies on the influence of ghrelin on puberty onset in rats suggest that elevated ghrelin levels exert inhibitory actions in both males and females, primary *via* reduced gonadotropin level, and that males appear to be more sensitive (Martini et al. 2006). Ghrelin and its receptor GHS R1a are expressed in rat and human gonads (Tena-Sempere et al. 2002; Gaytan et al. 2004). Finally, ghrelin is shown to affect testis size and structure (Sirotkin et al. 2008; Kheradmand et al. 2009) and to modulate testosterone secretion, androgen receptor expression, Leydig cell proliferation and seminiferous tubule gene expression (Barreiro et al. 2004; Kheradmand et al. 2009; Wang et al. 2011).

In normal human prostate, both ghrelin and GHS-R1a are present at the level of mRNA expression (Gnanapavan et al. 2002), but any cellular confirmatory assessment is still lacking, probably due to a very low intracellular concentrations. In addition, a low activity of the newly identified ghrelin acylation enzyme, ghrelin *o*-acyltransferase was detected in human prostate (Lim et al. 2011). In clear contrast to healthy prostate tissue, ghrelin and its receptor are highly expressed in prostate carcinomas (Jeffery et al. 2002, 2003) and the peptide has been shown to increase the proliferation of prostate cancer cell line *in vitro* by 33% (Jeffery et al. 2002). In light of these data and the lack of results concerning the response of healthy prostate tissue to ghrelin treatment, the aim of our work was to examine whether the repetitive ICV administration of ghrelin to male rats of different ages affects the ventral prostate size and structure.

Material and Methods

Experimental animals

Wistar male rats of different age, i.e. peripubertal, adult and middle-aged, bred at the Institute of Biomedical Research "Galenika" (Belgrade, Serbia) were used in the study. They were kept individually in metabolic cages under constant

laboratory conditions of room temperature ($22 \pm 2^\circ\text{C}$) 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* and the animals were accustomed to daily handling.

Surgical procedure

The surgical procedure was performed under deep anesthesia (Thiopental-Sodium, 45 mg/kg, i.p., Rotexmedica, Trittau, Germany) and equipped with headset for ICV injection, consisting of a silastic-sealed 20-gauge cannula positioned in the right lateral cerebral ventricle (1 mm posterior and 1.5 mm lateral to the bregma, and 3 mm below the cortical surface) (Starcevic et al. 1988). 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 standard dental acrylic (Sigma; ICN Galenika, Belgrade, Serbia). Following surgery, the animals received a single dose of s.c. 0.28 mg/kg buprenorphin (Buprenex; Reckitt Benckiser Healthcare) and 5 days of recovery was allowed before experiments.

Treatment

The animals of the same age were randomly divided in 2 groups ($n = 5$): experimental and control. Body weights were obtained daily, just before the intracerebroventricularly (ICV) injections. The mean \pm SEM pre-treatment body weight of peripubertal (38-day old), adult (60-day old) and middle-aged (180-day old) rats, were 123.0 ± 2.0 g; 202.0 ± 7.0 g and 420.0 ± 11.0 g. Experimental group was treated ICV with 1 μg of ghrelin (Global Peptide Services, Fort Collins, CO, USA) dissolved in 5 μl phosphate-buffered saline (PBS)/day over 5 consecutive days. The dose of ghrelin used in this experiment was based on our previous studies related to ICV ghrelin actions on the anterior pituitary secretion (Stevanovic et al. 2006; Milosevic et al. 2010). The control group received 5 μl of PBS according to the same schedule. The ICV injections were made at 10:00 h. The rats were decapitated under deep i.p. thiopental sodium anesthesia 1 h after the last ICV injection. The blood was collected for testosterone concentration analysis and the rat tissues (ventral prostate, the left testis and pituitary) were dissected out and weighed. Experimental protocol was approved by the Local Animal Care Committee, School of Medicine, University of Belgrade and the animals received human care during experimental period.

Testosterone assay

The sera were separated after centrifugation of trunk blood at 3000 rpm for 20 min and stored at -20°C until assayed.

Testosterone levels were measured using a commercial ELAgen Testosterone Kit (Adaltis, Italia). The sensitivity of this method, calculated as two times the standard deviation from standard value (Bo), is 0.01 ng/ml when the value of measured value (B)/Bo (%) is approx 90%. The inter- and intra-run precision had a coefficient of variation of 3.9% and 6.2%, respectively.

Morphometric analysis

The ventral prostates were carefully removed, weighed and immediately fixed in Bouin's solution for 48 h. The tissue specimens were then dehydrated through a graded series of ethyl alcohol, cleared in xylene, and embedded in paraffin. They were serially cut into 5- μ m-thick sections and the intermediate and distal sections of the gland, identified according to Banerjee et al. (1998), were stained with hematoxylin and eosin.

Morphometric analysis was performed by a simple point counting method (Weibel and Gomez 1962; Plecas et al. 1992, 1997) using Olympus BX 50 microscope (Olympus Optical Co., GmbH, Hamburg, Germany) and image analysis software (Micro Image Version 4.0, Olympus). For each of 5 sections *per* animal images of 15 to 20 non-overlapping fields, depending on the size of specimen, were captured separately at total magnification of 100 \times and each digitalized image was saved. The prostate tissue was divided into stromal and glandular compartment and the latter was subdivided into epithelium and lumen. Each captured image was overlaid with A₁₀₀ test grid (the test frame containing 100 intersection points) and the test points hitting the particular tissue component was scored. The area percentage, which is equivalent to volume percentage (density) of each component, was calculated. The volume (absolute volume) of each compartment was determined by multiplying the volume density by the prostate weight based on determination that 1 mg of fresh rat ventral prostate tissue had a volume of approximately 1 mm³ (DeKlerk and Coffey 1978).

Statistical analysis

The data are presented as group means \pm SEM for 5 animals in each group. Two-group (ghrelin-treated and age-matched control) comparisons were analyzed by the nonparametric Mann-Whitney test; serum testosterone concentrations in control groups of different ages were compared with the Kruskal-Wallis ANOVA followed by the Dunn's multiple comparison test. The statistical package GraphPad Prism 5.0 was used. Probability value of 5% or less was considered statistically significant.

Results

Body weight gain and organ weights

The influence of 5 consecutive days ICV ghrelin injections on body weight gain, ventral prostate, testis and pituitary weights in rats of different ages (38-, 60- and 180-day old) at the end of the treatment are summarized in Table 1.

Ghrelin treatment significantly increased body weight gain in rats of all ages. Compared with age-matched control groups, the increases were 37% in peripubertal, 67% in adult and 41% in middle-aged rats ($p < 0.05$). The treatment significantly ($p < 0.05$) augmented ventral prostate absolute weight in peripubertal and middle-aged rats (28% and 37%, respectively), whereas testis absolute weight was only increased in peripubertal animals (17%). However, relative weights of these organs were unaffected by ghrelin treatment. In addition, ghrelin administration significantly ($p < 0.05$) increased both the absolute and relative pituitary weights in peripubertal (35% and 18%, respectively) and middle-aged (43% and 32%, respectively) rats when compared to corresponding controls.

Histomorphometry of ventral prostate

Ghrelin administration resulted in marked changes of the ventral prostate morphometrical parameters at all three

Table 1. The effects of ICV-administered ghrelin on body weight gain, ventral prostate, testis, and pituitary relative and absolute weights in rats of different ages: peripubertal (38-day old), adult (60-day old) and middle-aged (180-day old)

Group		BW gain (g)	Absolute weight (mg)			Relative weight (mg/100 g BW)		
			Ventral prostate	Testis	Pituitary	Ventral prostate	Testis	Pituitary
Peripubertal	Control	33.2 \pm 0.2	36.0 \pm 2.0	645.0 \pm 9.0	3.50 \pm 0.1	23.7 \pm 0.9	424.0 \pm 3.0	2.3 \pm 0.1
	Ghrelin	45.6 \pm 1.3*	46.0 \pm 2.0*	755.0 \pm 21.0**	4.7 \pm 0.2*	26.6 \pm 0.7	436.0 \pm 7.0	2.7 \pm 0.1**
Adult	Control	19.8 \pm 1.4	165.0 \pm 7.0	1261.0 \pm 39.0	8.0 \pm 0.4	70.2 \pm 2.4	535.0 \pm 11.0	3.4 \pm 0.1
	Ghrelin	33.0 \pm 1.5**	154.0 \pm 4.0	1199.0 \pm 41.0	7.1 \pm 0.5	70.4 \pm 4.3	544.0 \pm 13.0	3.2 \pm 0.1
Middle-aged	Control	35.2 \pm 1.0	552.0 \pm 36.0	1349.0 \pm 49.0	8.9 \pm 0.2	125.0 \pm 10.0	304.0 \pm 13.0	2.0 \pm 0.1
	Ghrelin	49.6 \pm 3.0*	754.0 \pm 48.0**	1611.0 \pm 66.0	12.7 \pm 0.3*	157.0 \pm 11.0	336.0 \pm 18.0	2.6 \pm 0.1**

The values are expressed as means \pm SEM for 5 animals; * $p < 0.05$; ** $p < 0.01$ vs. corresponding controls. BW, body weight.

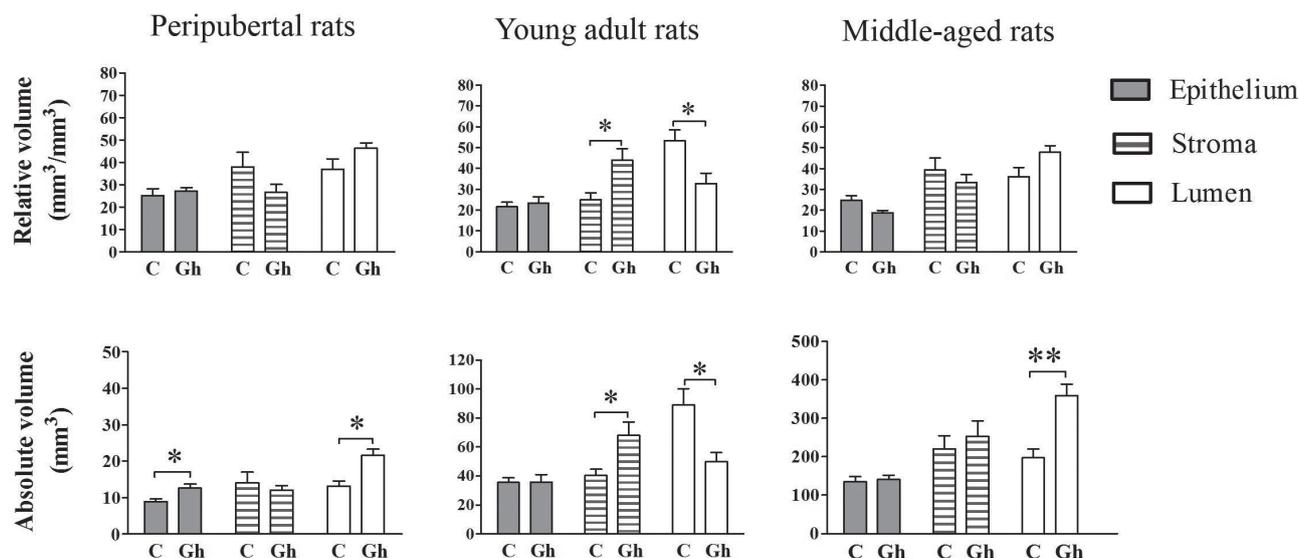


Figure 1. Morphometric parameters of the ventral prostate of rats ICV-injected with ghrelin (Gh; 1 $\mu\text{g}/\text{day}$) or vehicle (C) for 5 days in relation to age. All data are expressed as mean \pm SEM; $n = 5$ animals per group; * $p < 0.05$; ** $p < 0.01$ vs. age-matched control rats.

postnatal periods examined, but not in the same manner (Fig. 1). No signs of cytologic atypia or abnormal proliferation were observed in ghrelin-treated groups.

In the ventral prostate of ghrelin-treated peripubertal rats absolute volumes of glandular epithelium and lumen were significantly ($p < 0.05$) increased (41% and 64%, respectively), although there were no significant ($p > 0.05$) alterations in the relative volumes of the ventral prostate compartments (Fig. 1). No appreciable qualitative histological alterations were noted in the ventral prostate of ghrelin-treated peripubertal rats comparing to controls of the same age (Fig. 2A and B).

Ventral prostates of ghrelin-treated adult rats responded to treatment by significant changes in both relative and absolute volumes of luminal and stromal compartments comparing to age-matched controls (Fig. 1), although the glandular weight was not altered (Tab. 1). Thus, both relative and absolute volumes of the luminal compartment were significantly ($p < 0.05$) reduced (38% and 44%, respectively) whereas these parameters of the interacinar stroma were significantly ($p < 0.05$) increased (76% and 67%, respectively). A histological examination of the ghrelin-treated ventral prostates revealed that atrophic acini of irregular shape were accompanied by a stromal hypertrophy (Fig. 2D vs. C).

In middle-age rats, administration of ghrelin did not affect ($p > 0.05$) relative volumes of the ventral prostate compartments, but it significantly ($p < 0.05$) increased (81%) the absolute volume of glandular lumen (Fig. 1). A conspicuous engorgement of the acini, as evidenced by an accumulation in eosinophilic staining in the lumen, with

corresponding distension of the epithelium, which became cuboidal, was observed in the gland from ghrelin-treated rats (Fig. 2F) comparing to corresponding control tissue (Fig. 2E).

Serum testosterone levels

No change ($p > 0.05$) in serum testosterone concentration was evidence in ghrelin-treated rats of any age examined when compared to values found in age-matched controls (Fig. 3). Of note, as expected, among control groups of different ages the highest testosterone concentration was determined in adult rats; its value was significantly (2-fold) higher than in peripubertal as well as in middle-age group, although the latter difference did not reach statistical significance due to a well-known high individual variations in serum testosterone levels (Benerjee et al. 1998).

Discussion

In the present study, the possible influence of repeated ICV ghrelin administration on the ventral prostate size and morphology of rats of different ages was investigated. The obtained results showed for the first time that ghrelin treatment affected the rat prostate gland and that the effect was not mediated by testosterone, but depended on developmental stage of the animals, and hence of their androgen status. In peripubertal (38 days) and middle-aged (180 days) rats, in which initial serum testosterone levels

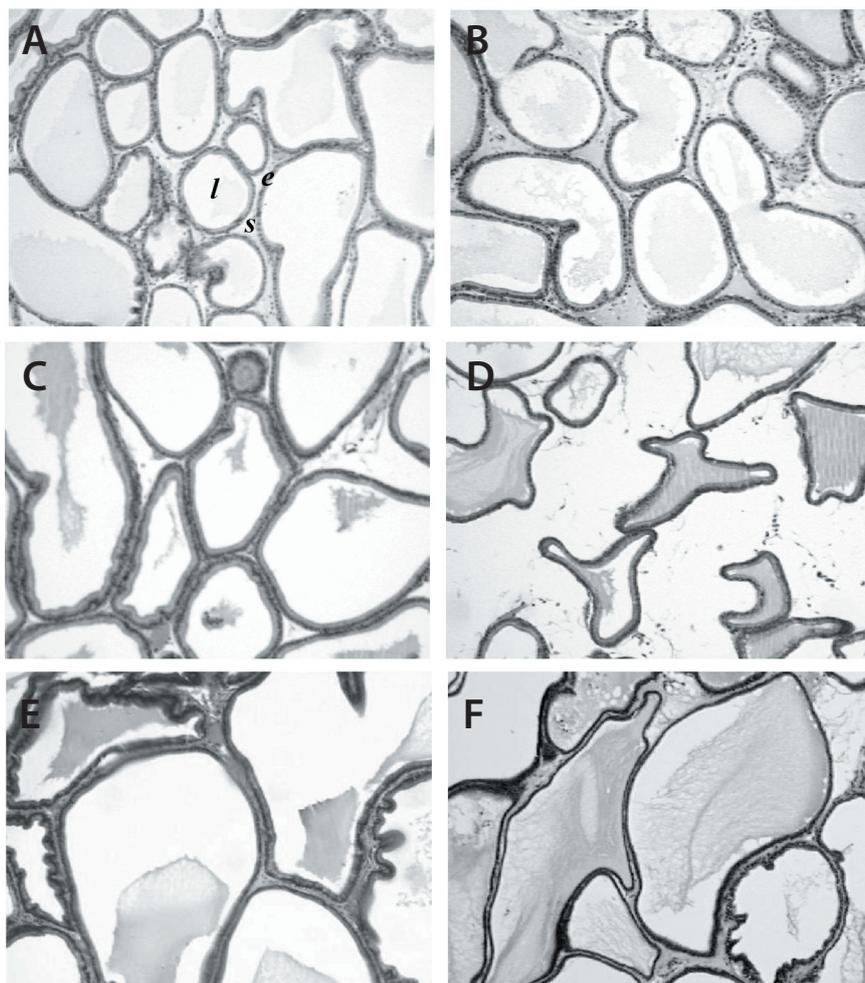


Figure 2. Effect of ICV ghrelin treatment (1 μ g/day for 5 days) on the ventral prostate in relation to age. Representative photomicrographs stained with hematoxylin and eosin, at original magnification 100 \times : controls of different age (A, 38 days; C, 60 days; E, 180 days) and ghrelin-treated rats of corresponding age (B, 38 days; D, 60 days; F, 180 days). *e*, epithelium; *s*, interacinar stroma; *l*, lumen.

were markedly lower than in adult ones, ghrelin treatment resulted in significant increase in ventral prostate weight as a consequence of significant enlargements of epithelial and/or luminal compartments. In adult rats (60 days), with high initial serum testosterone levels, ghrelin application did not affect glandular weight, but evoked a reduction in the volume of luminal compartment with corresponding increase in the volume of fibromuscular stroma. Such age-related differences in the effect of ghrelin treatment were probably due to different physiological levels of ghrelin actions.

Ghrelin treatment did not change serum testosterone levels at the end of the treatment at any age. This finding is in contradiction with a number of data showing mainly inhibitory influence of the peptide on LH and/or testosterone secretion in different experimental conditions (Tena-Sem-

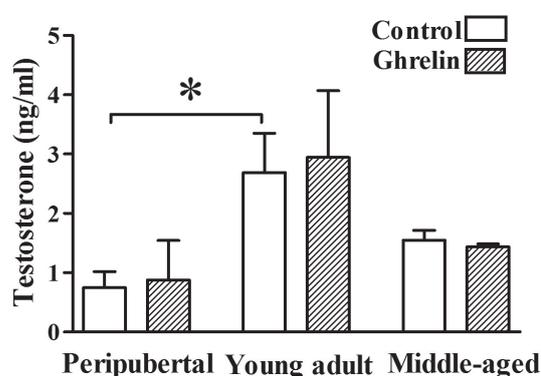


Figure 3. Serum testosterone concentrations in ICV ghrelin-treated rats (1 μ g/day for 5 days) and vehicle-injected control animals in relation to age. All data are expressed as mean \pm SEM; $n = 5$ animals per group; * $p < 0.05$.

pere et al. 2002; Barreiro and Tena-Sempere 2004; Fernandez-Fernandez et al. 2004; Martini et al. 2006; Sirotkin et al. 2008; Wang et al. 2011). However, no effect or stimulatory effect of ghrelin on LH or testosterone secretion were observed as well (Fernandez-Fernandez et al. 2004; Martini et al. 2006; Sirotkin et al. 2008; Wang et al. 2011), depending on the different experimental designs. The lack of ghrelin influence on serum testosterone levels in our experiments could be explained, most likely, by the fact that pituitary-gonadal axis response to this peptide depended on different factors such as developmental stage, strain, doses, and patterns of ghrelin administration (Martini et al. 2006). Importantly, the dose of ghrelin used in our study (1 µg/rat/day) was several (approx. 3 to 10) times lower than those that influenced LH or testosterone secretion *in vivo* in other studies (Fernandez-Fernandez et al. 2004; Wang et al. 2011).

Obviously, the changes in the ventral prostate size and structure observed in this study cannot be ascribed to alterations in serum testosterone, the hormone which plays major role in the regulation of prostate growth and function. However, other hormones, including GH, are involved in prostate physiology and pathology (Ruan et al. 1999; Un-no et al. 2007; Simanainen et al. 2011). Since ghrelin is a potent physiological stimulator of GH secretion both *in vivo* and *in vitro* (Kojima et al. 1999; Seoane et al. 2000; Stevanovic et al. 2006), there is strong evidence that GH and its peripheral mediator of action, insulin-like growth factor I (IGF I) are implicated in the prostate growth and function. In prostate, GH has been shown to stimulate expression of several mRNAs: for androgen receptor, GH receptor, IGF I, IGF I receptor and 5- α reductases (Reiter et al. 1992, 1995). In addition, elevated plasma concentrations of IGF I is linked to a higher risk for prostate cancer (Ryan and Goss 2008).

Previously, we demonstrated that ghrelin, given according to the same dosing protocol as in this study, stimulated rat somatotrope proliferation and significantly increased serum GH (Stevanovic et al. 2006). Therefore, we consider that, in peripubertal and middle-aged rats used in this study, GH secretion was also stimulated by ghrelin. Significantly increased body weight gain and the weights of all organs measured are in a good agreement with this finding.

As for the ventral prostate, the absolute weights were significantly increased in both peripubertal and middle-aged ghrelin-treated rats. Since there were no significant changes in the relative weights of the gland, we concluded that ventral prostate size increased proportionally to overall body weight increase, which indicated a somatotropic regulation. Such regulation was manifested as a proliferative-secretory response in the ventral prostates of peripubertal group in which the volumes of epithelial and luminal compartments were significantly enhanced. These findings are in accordance with the data showing that, like testosterone, GH/IGF I is essential for normal development of prostate epithelial

and luminal compartments (Ruan et al. 1999). In middle-aged rats, only the luminal absolute volume was significantly increased. An engorgement of the lumen with corresponding distension of epithelium resulted in a predominant contribution of this compartment to the ventral prostate weight. Besides, a negative effect of accumulated secretion on the epithelium is proposed (Vilamaior et al. 2006), which is in agreement with a flattened epithelium observed in the middle-age rat ventral prostates. Similar responses of peripubertal and middle-aged rat ventral prostates to GH, elicited by ghrelin treatment, is possible since the sensitivity of the rat pituitary gland to exogenous ghrelin, in terms of GH secretion, appeared to be unaffected by aging (Sun et al. 2007).

Adult rats responded to ghrelin treatment quite differently; their body weight gain was significantly increased, but no changes in the ventral prostate, testis and pituitary weights were found. Accordingly, there are no compelling confirmations in favor of stimulated GH secretion in these animals. In addition, morphological changes in the ventral prostates are not compatible with the well-known GH/IGF I action on the gland. In spite of unchanged weight, an atrophy of acini was observed and both relative and absolute volumes of luminal compartment were reduced. At the same, increased relative and absolute volumes of the fibromuscular stroma indicated a hypertrophy of this compartment. Such histological manifestations are indicative of an inhibitory influence on the ventral prostate. The mechanism of this action remains unclear. We can assume that ghrelin treatment in adult rats affected the secretion of some other one or more hormones that act on the prostate. It is even possible that ghrelin by itself influence at prostate level, since the B peptide crosses the mouse blood-brain barrier predominantly in the brain-to-blood direction (Banks et al. 2002) and ICV ghrelin administration to rats elicits multiple increase in peripheral plasma ghrelin levels (Theander-Carillo et al. 2006; Sangiao-Alvarellos et al. 2009).

In conclusion, these results report the influence of ghrelin treatment on the prostate size and structure, and show that the effect of ICV ghrelin application on the rat ventral prostate is developmental stage dependent. In animals with low initial levels of testosterone, i.e., peripubertal and middle aged, stimulation of the ventral prostate growth was found, most likely due to ghrelin-stimulated GH secretion. In adult rats, with high serum testosterone levels, an inhibitory effect of ghrelin treatment on the ventral prostate was expressed, but the mechanism of this action remains unknown. However, the results merit further investigations and may have clinical implications, especially in the light of data on possible role of ghrelin in prostate hypertrophy and adenomas.

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