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# Consummatory behavior and metabolic indicators after central ghrelin injections in rats

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**Abstract**

Ghrelin, an endogenous ligand for the growth-hormone-secretagogue receptor, is a 28-amino acid peptide with a post-translational acyl modification necessary for its activity. It has central nervous system actions that affect appetite, body mass and energy balance. An intracerebroventricular (ICV) injection protocol of sub-nanomolar doses of ghrelin, known to alter the morphology of ACTH and GH producing pituicytes and plasma levels of these hormones, was used to provide an overview of metabolic changes linked to energy metabolism. Variables measured were: food intake (FI), water intake (WI), fecal mass, urine volume, body weight (BW), retroperitoneal (RP) and epididymal (EPI) white adipose tissue (WAT), and changes in serum leptin, insulin, triglycerides, cholesterol, and glucose. Five injections of rat ghrelin or PBS ($n=8$ per group) were given ICV every 24 h (1 μg/5 μL PBS) to adult male rats. Ghrelin had a positive and cumulative effect on FI, WI and BW ($p<0.05$), but not feces mass or urine volume ($p>0.05$). Centrally applied ghrelin clearly increased RP WAT (by 235%, $p<0.001$), EPI WAT (by 85%, $p<0.05$) and serum insulin levels (by 43%, $p<0.05$), and decreased serum leptin levels (by 77%, $p<0.05$) without ($p>0.05$) evoking changes in blood triglyceride cholesterol, or glucose levels.

These data and the available literature clearly document that exposure of the brain of normal rats, over time, to sub-nanomolar doses of ghrelin results in metabolic dysregulation culminating in increased body mass, consummatory behavior, and lipid stores as well as changes in blood leptin/insulin levels. Thus, modulation of central ghrelin receptors may represent a pharmacological approach for controlling multiple factors involved in energy balance and obesity.


*Keywords:* Ghrelin; Central nervous system; Consummatory behavior; Adipose tissue; Leptin; Rats; Insulin; ICV

## 1. Introduction

Energy balance is a physiological equilibrium where total dietary energy intake equals energy expenditure. Normally, it is very well controlled by neuroendocrine circuits within the CNS that regulate appetite and food intake and set metabolic rate.

The discovery of two hormones, leptin and ghrelin, has profoundly advanced the scientific model of energy balance regulation. Leptin, secreted by, and in proportion to white adipose tissue (WAT), signals the status of energy stores through a leptin receptor that is, along with ghrelin and insulin receptors, present in the hypothalamic arcuate (ARC) and ventromedial (VMN) nuclei. Leptin inhibits food intake, increases energy expenditure and influences other neuroendocrine peptides involved in energy balance.

Ghrelin is 28-amino acid brain-gut peptide cleaved from the precursor preproghrelin with the unique post-translational modification of the $Ser^3$ residue to which an octanoyl moiety is esterified [26]. The presence of a hydrophobic group at $Ser^3$ allows the peptide to bind to its receptor, the growth hormone (GH) secretagogue receptor (GHS-R) type 1a [27]. Independent from its role as a GHS, exogenous ghrelin administration caused a positive energy balance and increased body weight when

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administered pharmacologically [57,36,15]. Several studies suggest that ghrelin has an essential role in the regulation of meal initiation. Systemic ghrelin levels increased before meals and decreased after food intake within 1 h of meal initiation [58,11,12,46,47]. In experimental animals, central or systemic ghrelin administration promoted food intake [57,36,64]. Exogenous ghrelin induced weight gain in rodents by increasing food intake and reducing fat utilization [57,64,49]. In the same species, centrally applied ghrelin was relatively more potent than was peripherally administered ghrelin, suggesting important central effects [57]. Intracerebroventricular (ICV) administration of ghrelin to free-feeding rats increased food intake in a dose dependent manner [36]. Continuous ICV administration of ghrelin induced food intake and an increase in fat mass by selectively utilizing carbohydrates, and promoting weight gain [57]. However, it was shown that central ghrelin independently regulated adipocyte metabolism by increasing lipogenesis and inhibiting lipid oxidation. Finally, centrally applied ghrelin exerted orexigenic effects probably through neuropeptide Y (NPY) and agouti-related protein (AgRP) systems [8] and it induced immunoreactivity for c-fos (a marker of neuronal activation) in arcuate hypothalamic neurons [37] suggesting that ghrelin induces food intake via the orexin pathway [55].

Leptin has been shown to be an upstream regulator of ghrelin in rodents [36,25,62]. Also, some studies showed that ghrelin is an upstream regulator of NPY and AgRP that antagonizes leptin's effects on NPY/AgRP neurons [20,21,60,39]. Although leptin and ghrelin have multiple overlapping pathways related to energy homeostasis, the sequences and mechanisms of their physiological integration remains unclear.

Available data suggest a negative association between systemic ghrelin and insulin levels [11,59]. Ghrelin inhibited insulin secretion in vitro and in most human or animal studies [6,7,38,3]. However, it was shown that ghrelin might stimulate insulin secretion in certain paradigms [14]. In addition it is not clear when the peptide's endocrine and paracrine effects physiologically regulate insulin secretion [56]. Thus, further studies are needed to fully elucidate the exact role of ghrelin on glucose homeostasis.

The above effects of ghrelin were often induced by pharmacological doses of the peptide, and it would therefore be useful to determine the effects of centrally applied ghrelin on energy homeostasis after a more "physiological" dosing regimen. This study was specifically designed to evaluate a daily ICV injection regimen in normal rats given PBS with or without sub-nanomolar doses of ghrelin, and measuring multiple variables related to energy metabolism. These measures included body weight, food and water intake, feces and urine elimination, retroperitoneal and epididymal WAT content, and ending blood levels of triglycerides, cholesterol, glucose, leptin and insulin.

## 2. Materials and methods

This study was performed on adult male Wistar rats (200±20 g), bred at the Institute of Biomedical Research "Galenika" in Belgrade. They were kept in individual metabolic cages under a 12:12 h light–dark cycle, at 22±2 °C, and were accustomed to daily handling. They were fed a balanced diet for laboratory rats (prepared by "D. D. Veterinarski zavod Subotica", Subotica, Serbia). Food and water were available to rats *ad libitum*. Experimental protocols were approved by the Local Animal Care Committee and conformed to the recommendations given in "Guide for the Care and Use of Laboratory Animals" (1996 National Academy Press, Washington, D.C.).

### 2.1. Animal preparation

Surgical procedures were performed under ether anesthesia (aether ad narcosis Ph. Iug. III. produced by "Lek", Ljubljana, Slovenia). The rats were implanted with a headset later used for ICV injections. A minimum recovery time of 5 days was permitted before the onset of experiments. The headset consisted of a silastic-sealed 20-gauge cannula [43], implanted into a lateral cerebral ventricle 1 mm posterior and 1.5 mm lateral to the bregma, and 3 mm below the cortical surface. 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, "Galenika", Belgrade, Serbia).

### 2.2. Animal treatment

After recovery, rats were divided into two groups, each containing eight animals. The first group of rats was treated ICV with 1 μg of ghrelin (lot. no. C-et-004; Global Peptide Services, LLC Ft. Collins, CO) dissolved in 5 μL phosphate buffered saline (PBS) every 24 h for five consecutive days. This dose regimen was successfully used in prior experiments to determine ghrelin effects on GH and ACTH-producing pituicytes and their hormones inserum [44,45]. The second group was a control group, comprised of rats treated in the same manner, except that they received only control injections 5 μL of PBS. All ICV treatments were administered between 10:00 and 11:00 h. During treatment, body weight, food and water intake, as well as feces elimination and urine excretion were obtained daily, just before the next ICV injection. All animals were killed by decapitation under deep ether anesthesia 2 h after the last (5th) ICV injection. Retroperitoneal and epididymal adipose tissue pads were dissected, weighed and prepared for light microscopy. Blood was collected from each rat immediately after decapitation. Serum samples were obtained and stored at −20 °C until assayed.

### 2.3. Light microscopy

White adipose tissue pads were excised, fixed in Bouin's solution for 48 h and embedded in paraffin. Serial 5 μm thick tissue sections were deparaffinized in xylol and serial alcohol and visualized using by haematoxylin-eosin staining. Digital images were acquired with a DM RB Photomicroscope Olympus BX51 (Olympus, Tokyo, Japan) with a Digital Camera (Olympus) DP70 for the acquisition and analysis of the images.

### 2.4. Hormone and biochemical assays

Serum concentrations of leptin and insulin in control and experimental rats were measured by the DSL-10-23100

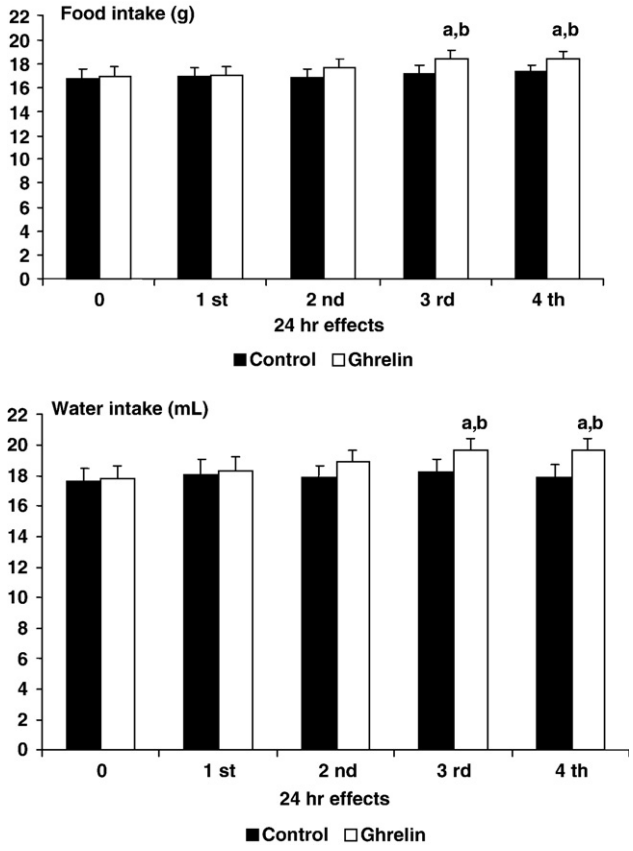


Fig. 1. 24 h food (g) and water (mL) intake prior to and following the 1st, 2nd, 3rd and 4th ICV ghrelin or PBS injection in male rats. All data are expressed as mean values ± standard deviation; n = 8 animals per group; a. $p < 0.05$ vs. before ghrelin treatment (0 time), b. $p < 0.05$ vs. after the 1st ICV ghrelin day.

ACTIVE leptin ELISA (Texas, USA) and DSL-10-1600 ACTIVE Insulin ELISA (Texas, USA) immunoassay, respectively. Biochemical blood analyses (glucose, cholesterol and triglycerides) were obtained by standard peroxidase based clinical assays.

### 2.5. Statistical analyses

Daily body weight, food and water intake, feces elimination and urine excretion, from 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. Blood hormone and chemical concentrations and adipose tissue weight obtained from each rat were averaged per experimental group and standard deviation of the mean (SD) was calculated. Student's *t*-test was used to test the two means (control vs ghrelin). Univariate ANOVA was used to determine if leptin and insulin serum levels as well as retroperitoneal (RP) and epididymal (EPI) WAT contents between controls and ghrelin-treated group remained significant after correction for food intake. Spearman and Pearson correlation test was used to correlate either leptin and insulin levels, as well as both RP and

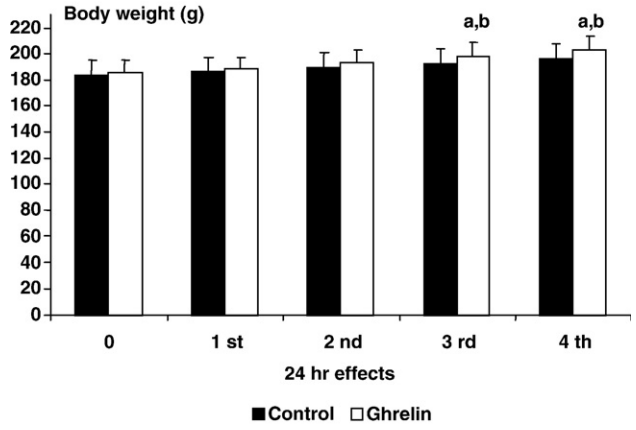


Fig. 2. Daily body weight (g) corresponding to the intake data from Fig. 1. All data are expressed as mean values ± standard deviation; $n = 8$ animals per group; a) $p < 0.05$ vs. before ghrelin treatment (0 time), b) $p < 0.05$ vs. after the 1st ICV ghrelin day.

EPI WAT contents. Probability values of 5% or less were considered statistically significant.

## 3. Results

### 3.1. Consummatory behavior, body weight and excretory functions

Data summarizing the effects of repetitive ICV administration of 1 μg/5 μL of ghrelin or solvent on 24 h food and water intake are shown in Fig. 1. Food intake was significantly higher ($p < 0.05$, ANOVA) after the 3rd and 4th dose of ICV ghrelin

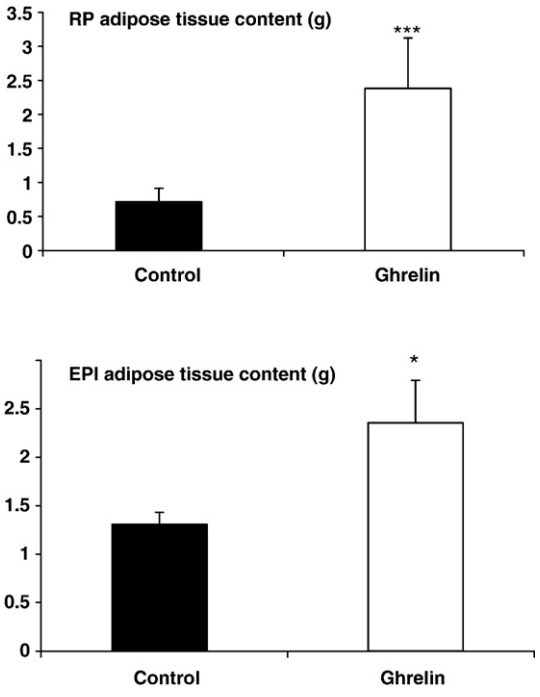


Fig. 3. Retroperitoneal (RP) and epididymal (EPI) adipose tissue content in control and ghrelin-treated adult male rats. All data are expressed as mean values ± standard deviation; $n = 8$ animals per group; $*p < 0.05$, $***p < 0.001$ vs. control.

compared to pre-ghrelin treatment (time 0) and those after the 1st ICV ghrelin injection. In control rats, there was no significant ($p>0.05$, ANOVA) change in food intake over the course of the experiment. Water intake in ghrelin-treated rats increased ($p<0.05$, ANOVA) in the same pattern as food intake (Fig. 1). Water intake of control rats did not show statistically significant changes ($p>0.05$, ANOVA). Urine volume and fecal mass output were stable throughout the experiment and there were no control versus ghrelin differences ($p>0.05$, ANOVA).

Fig. 2 presents the body weight of these animals. Control rats had no significant weight change over the 5 days ($p>0.05$, ANOVA) whereas those treated with ICV ghrelin had a small but statistically significant ($p<0.05$, ANOVA) increase that paralleled the increased consummatory behavior.

### 3.2. White adipose tissue

Repetitive ICV ghrelin treatment increased retroperitoneal (RP) and epididymal (EPI) fat deposits (Fig. 3) in a statistically significant manner. RP of control versus ghrelin-treated rats was 0.72 g±0.2 vs 2.38 g±0.74 ($p<0.001$). The corresponding values for EPI fat deposits were: 1.31 g±0.12 vs 2.36 g±0.44 ($p<0.05$). Fig. 4 shows a photomicrograph of white adipose tissue with its characteristic meshwork in H&E-stained paraffin sections. Each spherical, polyhedral and oval space represents a single large drop of lipid before its dissolution from the cell during tissue preparation. The surrounding eosin-stained material is the cytoplasm of the adjoining cells and intervening connective tissue. The adipocytes' large size is due to the accumulated cellular lipid, and adipocytes from ghrelin-treated rats (Fig. 4B) consistently appeared larger compared to adipocytes from white adipose tissue of control rats (Fig. 4A).

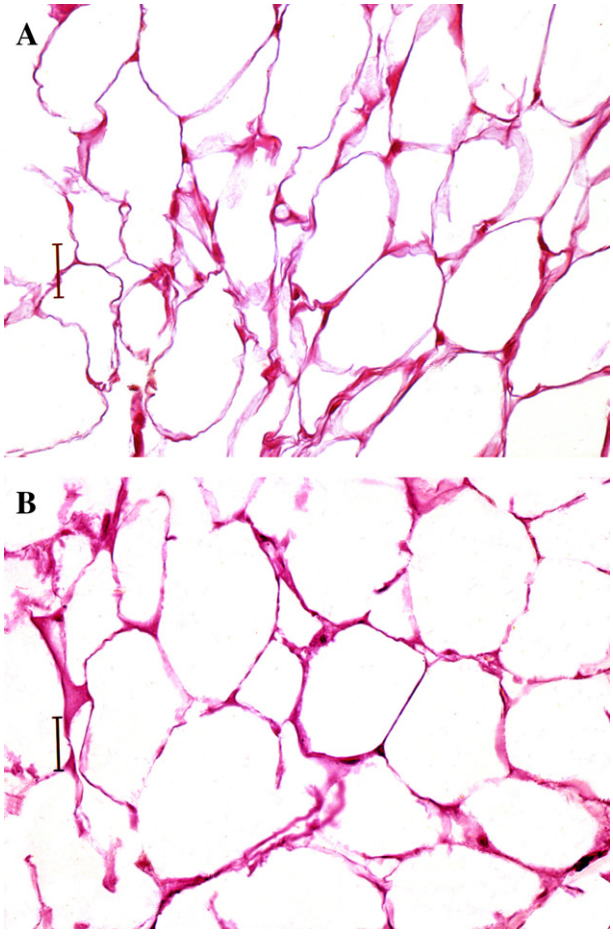


Fig. 4. Visceral adipose tissue: A) Control rats; B) Ghrelin-treated rats (40×, bar 10 μm).

Table 1
The effects of daily ICV injections of ghrelin or PBS on terminal serum levels of glucose, triglycerides, cholesterol, leptin and insulin in adult male rats

| Experimental group | Glucose (mmol/L) | Triglycerides (mmol/L) | Cholesterol (mmol/L) | Leptin (ng/mL) | Insulin (μIU/mL) |
|---|---|---|---|---|---|
| Control | 6.39±0.6 | 0.80±0.2 | 1.21±0.2 | 1.72±0.5 | 1.73±0.6 |
| Ghrelin | 5.85±0.5 (−8%) | 1.00±0.1 (+25%) | 1.31±0.1 (+8%) | 0.40±0.09[a] (−77%) | 2.47±0.8[a] (+43%) |

All data are expressed as mean values±standard deviation; $n=8$ animals per group.
[a] $p<0.05$ vs. control.

### 3.3. Biochemical changes in blood evoked by ICV ghrelin

Blood leptin and insulin levels in control vs ghrelin-treated rats are shown in Table 1. Serum leptin levels decreased by 77% ($p<0.05$). Insulin levels were increased by 43% ($p<0.05$). Serum concentration of triglycerides, cholesterol and glucose were unchanged ($p>0.05$) by ghrelin compared to controls (Table 1), although there was a trend toward higher levels of triglycerides (25%) and cholesterol (8%) after ghrelin. It should be noted that the hormone changes (insulin and leptin) were measured 2 h after the last sub-nanomolar ICV dose of ghrelin. Thus, multiple half-lives of ghrelin, insulin and leptin had all passed since the last ICV injection.

Taking this findings together, the increase in food intake after repetitive ghrelin treatments proves the orexigenic effect of this hormone, even with subpharmacological doses. On the other hand, differences in leptin and insulin serum levels as well as retroperitoneal and epididymal white adipose tissue contents between controls and ghrelin-treated groups remained significant after correction for food intake introduced as covariate in univariate ANOVA. Furthermore, there was no statistically significant connection when food intake was correlated with either leptin or insulin levels, as well as both epididymal and retroperitoneal content ($p>0.05$, Spearman and Pearson correlation test). These statistical analyses prove that ghrelin has direct effect on serum leptin and insulin levels, as well as on visceral white adipose tissue contents, and those effects are independent of its orexigenic effect.

## 4. Discussion

An expanding literature (see below) documents that ghrelin affects multiple, individual parameters related to energy balance. In the present study, our objective was to use a low dose of centrally administered ghrelin and determine, in the same rats, changes in baseline physiology using measurements of

behavior, excretory function, lipid storage, blood-borne hormones, triglycerides, cholesterol and glucose as endpoints. The ICV protocol selected was based on prior quantitative morphology of GH and ACTH producing pituicytes and serum levels of these hormones two hours (i.e., multiple half-lives) after the last ICV ghrelin injection [44,45]. Those experiments documented pituitary activation resulting in increased blood levels of GH and ACTH, changes compatible with a shift in energy metabolism. The effects were considered to be of central origin, considering the low dose of ghrelin vs. the short half-life of the peptide in the circulation (~30 min) [53]. Minimal circulatory effects would be anticipated even if all of the ICV injections were systemically absorbed.

The results of the present investigation collectively show that exposure of the brain to ICV doses of ghrelin less than 1 nM displaces normal rats toward a positive energy balance. The increased body weight, food and water intake without a change in urine/feces output, increased retroperitoneal and epididymal fat stores, increased serum insulin and decreased serum leptin levels, with minimal changes in blood glucose, cholesterol and triglycerides collectively support the concept that ghrelin acts centrally to displase normal rats toward a positive energy balance. These findings are in good agreement with data from multiple other studies.

### *4.1. Consummatory behavior, urine/feces output and body weight*

In the case of food intake, Kamegai et al. [21] demonstrated that chronic central ghrelin treatment every 12 h for 3 days increased food intake and body weight. It was also shown that ghrelin, when administered either ICV or peripherally, stimulated food intake in a dose dependent manner [65]. Six days of ICV ghrelin treatment increased 24 h food intake [30]. ICV ghrelin injection into the third ventricle or direct injection of the peptide into the ARC (30 pmol) increased food intake, which was prevented by pretreatment with ghrelin antibody [4,64]. In experimental animals, ghrelin is more potent in promoting food intake when administered centrally rather than peripherally [57,36,64]. Ghrelin leads to c-fos activation in arcuate hypothalamic neurons that express the GHS receptor and are recognized as a site integrating energy balance [36,21]. The expression of the appetite-stimulating peptides, NPY and AgRP, is also increased by ghrelin in the ARC [21,41]. Korbonits et al. [29] proposed three pathways for the appetite-inducing effects of ghrelin. First, after release into the bloodstream by the stomach, ghrelin may cross the blood-brain barrier and bind to its receptors in the hypothalamus [5,54]. Secondly, ghrelin may affect the brain via afferent vagal fibers and nucleus tractus solitarius activation [62]. Thirdly, ghrelin produced locally in the hypothalamus may directly affect multiple hypothalamic nuclei related to metabolic pathways [9].

Our results also showed significant increase of water intake in ICV ghrelin-treated free-feeding rats. Since drinking usually occurred with feeding, increased water intake could be explained as the following effect of food intake with the purpose of maintaining osmotic homeostasis in the organism. However, it was recently demonstrated that ICV or peripherally applied ghrelin potently inhibits water intake when food was withdrawn to remove the prandial drinking [18]. Their data may suggest an intrinsic effect that couples primary stimuli such as osmoreceptor or volume receptors to the efferent pathways regulating drinking [40]. Further studies are needed to elucidate these findings.

Feces elimination and urine volume were unchanged in the present experiments. This is consistent with the fact that ghrelin is ultimately an anabolic hormone [29]. However, ICV ghrelin has been reported to stimulate arginine vasopressin release, which is both anti-dipsogenic and antidiuretic [33]. A recent study reported that ICV ghrelin reduced urine excretion, while intravenous peptide had no effect on urine volume [18]. Further clarification of ghrelin, vasopressin release, and urine excretion appears necessary.

The present results documented a small, statistically significant gain in body mass at the 72 and 96 h time intervals. This is concordant with the findings of Kamegai et al. [21] who reported that ICV ghrelin, applied every 12 h for 72 h significantly increased body weight. Similar results by Tschop et al. [57] showed that a chronic ICV ghrelin infusion (1.2 nM/kg/day and 12 nM/kg/day) induced a significant, dose dependent increase in body weight and fat content. Elevated body weight also occurred after intraperitoneal [61] and subcutaneous [57] ghrelin administration.

### *4.2. Adipogenic effects of ghrelin*

An adipogenic effect of ghrelin was clearly observed in the present experiments. Retroperitoneal fat increased by 235% and epididymal fat content was elevated by 85% two hours after the last ICV injection. Wren et al. [64] found an increased epididymal fat content after ICV ghrelin whereas Kamegai et al. [21] did not find increased deposits. This disparity may be explained by different dosing and timing patterns. Intraperitoneal ghrelin increased white adipose tissue in rodents [64,61]. This effect also occurred after subcutaneous ghrelin [57]. Increased white adipose content occurred after chronic central [64] or peripheral administration of ghrelin [31] and was linked to food intake and weight gain. Recent results [61] demonstrated specific adipogenic effects of ghrelin after 7 consecutive daily intraperitoneal injections; white adipose tissue increased without affecting food intake or body weight. Wren et al. [66] obtained similar results with continuous, subcutaneous ghrelin infusion. More recent studies proposed a novel CNS-based neuroendocrine circuit that regulates metabolic homeostasis of adipose tissue [51]. In vitro methods also detected a direct adipogenic effect of ghrelin [52].

### *4.3. Ghrelin effects on leptin and insulin*

The present experiments revealed that centrally administered ghrelin reduced serum leptin levels by 77% without significantly affecting circulating triglycerides or cholesterol, although there was a tendency towards higher levels after ghrelin application. These results are consistent with other data. A reciprocal relationship between circulating ghrelin and leptin was observed on a daily basis in rats [19]. Pre-prandial ghreliln hypersecretion at

the onset of dark-phase ingestive behavior and before the time of food availability in a scheduled feeding paradigm was coincident with low circulating leptin levels [10,67]. Also, a gradual rise in leptin preceded the postprandial decline in ghrelin secretion [10,58,59,67]. Exogenous intraperitoneal ghrelin significantly attenuated plasma leptin levels and markedly increased food intake [28]. Pre-treatment with IgG anti-leptin antibody to immunoneutralize plasma leptin increased plasma ghrelin and increased food intake [28]. Collectively, the available data support the hypothesis that plasma ghrelin and leptin are negatively correlated [13]. Possibly, ghrelin effects in all species studied are exactly the opposite of leptin, and these two hormones may act on the same hypothalamic areas [11,2]. Multiple studies indicate that ghrelin upregulates the orexigenic peptides NPY and AgRP, and that it antagonizes leptin effects on NPY/AgRP expressing neurons [20,21,60,39]. By activating NPY/Y1 receptors, ghrelin may be a natural antagonist to leptin [41]. However, ghrelin does not seem to be a direct regulator of leptin secretion, as fasting produced identical decreases in serum leptin in ghrelin-null and wild-type mice [48]. Ghrelin and leptin likely have different effects on hypothalamic neurons producing the various orexigenic and anorexigenic peptides, resulting in more or less opposing effects on energy balance [24].

Insulin has similar central catabolic effects to leptin on energy homeostasis, in that it inhibits feeding, stimulates thermogenesis and induces weight loss. Also, insulin acts directly to inhibit hypothalamic NPY-producing neurons under normal feeding conditions [42,63]. Our data showed that ICV ghrelin significantly increased serum insulin by 43% without a concomitant change in blood glucose. These data are consistent with some other studies. ICV ghrelin significantly increased fasting plasma insulin levels in both free-feeding and pair-fed rats [22]. Lee et al. [32] demonstrated that intravenous ghrelin stimulated insulin secretion. However, Kamegai et al. [21] reported non-significant increases in plasma insulin after ICV ghrelin. Ghrelin evoked insulin release from isolated islet cells from both normal and streptozotocin-induced diabetic rats [1]. It increased the cytosolic free calcium concentration in beta cells and stimulated insulin secretion in isolated rat pancreatic islets [14]. On the other hand, intravenous administration of ghrelin inhibited insulin secretion even in the presence of increased glucose levels in humans [6,50]. In isolated rat pancreas, perfused in situ, ghrelin injection inhibited insulin release [16]. Also, ghrelin inhibited in vivo and in vitro glucose-stimulated insulin secretion in mouse islet cell preparations [38]. Despite variable data related to direct ghrelin effects on insulin release, it has been shown that ghrelin modulates glucose levels via releasing GH, increasing insulin resistance and stimulating gluconeogenesis [34,35].

In summary, the present study has shown that repetitive ICV administration of low doses of ghrelin, in normal rats, increased consummatory behavior and body weight without affecting fecal mass or urine volume. Adipose tissue stores increased and, terminal serum insulin levels rose while serum leptin decreased with no change in blood triglycerides, cholesterol or glucose. These data and the literature discussed strongly support the opinion that ghrelin acts centrally to yield an increase in energy intake and decrease in energy expenditure. The relative contributions of ghrelin, insulin and leptin, and multiple other neurochemicals as well as sequence of events their mechanisms require further study, and have been reviewed by King [23] and Guillemin [17]. But, based on the available data, it is likely that ghrelin may contribute to energy balance problems such as obesity. Manipulation of ghrelin receptors in the brain may provide a pharmacological approach to control obesity and other diseases of energy metabolism.

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