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Peripheral obestatin has no effect on feeding behavior and brain Fos expression in rodents

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Abstract

Obestatin is produced in the stomach from proghrelin by post-translational cleavage. The initial report claimed anorexigenic effects of obestatin in mice. Contrasting studies indicated no effect of obestatin on food intake (FI). We investigated influences of metabolic state (fed/fasted), environmental factors (dark/light phase) and brain Fos response to intraperitoneal (ip) obestatin in rats, and used the protocol from the original study assessing obestatin effects in mice. FI was determined in male rats injected ip before onset of dark or light phase, with obestatin (1 or 5 μmol/kg), CCK8S (3.5 nmol/kg) or 0.15 M NaCl, after fasting (16 h, n = 8/group) or *ad libitum* (n = 10-14/group) food intake. Fos expression in hypothalamic and brainstem nuclei was examined in freely fed rats 90 min after obestatin (5 μmol/kg), CCK8S (1.75 nmol/kg) or 0.15 M NaCl (n = 4/group). Additionally, fasted mice were injected ip with obestatin (1 μmol/kg) or urocortin 1 (2 nmol/kg) 15 min before food presentation. No effect on FI was observed after obestatin administration during the light and dark phase under both metabolic conditions while CCK8S reduced FI irrespectively of the conditions. The number of Fos positive neurons was not modified by obestatin while CCK8S increased Fos expression in selective brain nuclei. Obestatin did not influence the refeeding response to a fast in mice, while urocortin was effective. Therefore, peripheral obestatin has no effect on FI under various experimental conditions and did not induce Fos in relevant central neuronal circuitries modulating feeding in rodents.

Keywords

Obestatin; Food intake; CCK; Urocortin 1; Rats; Mice; Dark phase; Light phase

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1. Introduction

Obestatin is a novel 23-amino acid peptide that originates from a post-translational cleavage of proghrelin located in X/A-like cells of the stomach [45]. Ghrelin, a 28-amino acid peptide [10,26] is a well-characterized hormone that stimulates food intake in rodents [1,30,43,47, 49,50] and humans [48]. In contrast to ghrelin, obestatin has been initially reported to elicit anorexigenic effects in mice and rats after peripheral or intracerebroventricular injection [7, 18,27,51]. Furthermore, chronic administration of obestatin reduces body weight gain in mice [27,51]. These findings suggest that obestatin may be involved in the regulation of energy homeostasis in rodents. However, several recent studies performed in rats and mice under various experimental conditions showed that obestatin injected intraperitoneally (ip) has no inhibitory effect on short-term food intake [11,14-17,21,32,41,54] and body weight gain [32, 42]. The responsiveness to exogenous obestatin could be influenced by circadian rhythm and might account for the varying effects of peripheral obestatin on food intake found in recent studies [7,15,18,21,27,32,40-42,51,54].

It has been initially suggested that obestatin could inhibit food intake by stimulating key hypothalamic nuclei involved in feeding control, such as the arcuate nucleus (ARC) [35]. Moreover, it has been reported that peripheral obestatin rapidly crosses the blood brain barrier in mice, thus central pathways might play a role in the modulation of food intake by obestatin [35]. It has not yet been investigated if obestatin induces neuronal activity in relevant brain areas of the hypothalamus and brainstem involved in the regulation of feeding behavior. Over the last years, there is a consistent evidence that food intake alterations in response to peripheral administration of gut peptides (e.g. sulfated cholecystokinin octapeptide, CCK-8S, ghrelin, bombesin or amylin) are mediated at least partly by changes in neuronal activity in distinct brain nuclei [5,20,37,45]. For instance, specific brain pathways activated by intraperitoneally (ip) injected CCK-8S, as revealed by Fos-immunoreactivity (Fos-ir), include the paraventricular nucleus of the hypothalamus (PVN), the dorsomedial hypothalamic nucleus (DMH), the nucleus of the solitary tract (NTS) and the area postrema (AP) [23,25,29,33,38].

Thus, the aim of the present study was first to investigate whether obestatin injected ip influences food intake in male rats under fasting or *ad libitum* feeding conditions during the dark and light phases as conflicting reports may result from differencial responses depending upon the metabolic state of the animals (fed or fasted), and environmental factors (dark and light phase). The effects of obestatin on food intake were compared with those induced by CCK-8S injected ip. Second, we examined the effect on brain neuronal activity induced by the ip administration of obestatin as assessed by Fos-immunohistochemistry in different hypothalamic (PVN, VMH, DMH, ARC), and brainstem nuclei (NTS) which are important for the modulation of feeding behavior. Studies were performed under *ad libitum* feeding conditions at the beginning of the light phase to obtain metabolically stable conditions. Fasting itself causes metabolic stress and induces significant increase in neuronal activity in the PVN [8]. In addition, it is known that orexigenic neuropeptides (e.g. ghrelin, neuropeptide Y) induce Fos expression in hypothalamic brain nuclei namely the ARC nucleus [31,34,47]. Therefore, the activation of the orexigenic systems at the beginning of dark phase could also exert a stimulating effect on neuronal activity in the brain. We administered obestatin at the dose of 1 μmol/kg, and included an additional 5-fold higher dose based on our previous studies that obestatin injected ip in doses ranging from 0.1 to 3 mg/kg (~0.04 to ~1.2 μmol/kg) did not influence food intake in rats [14]. Lastly, in view of recent report by Zhang et al. [52] that a 15 min interval between peptide injection and food replacement is essential to demonstrate the inhibitory effect of obestatin on food intake in fasted mice, additional studies were carried out in mice using similar experimental protocol.

2. Methods

2.1. Animals

Male Sprague-Dawley rats (body weight: 258 ± 26 g; Harlan Winkelmann GmbH, Borchen, Germany) and lean male mice (C57BL/6, 25-30 g; Harlan, San Diego, CA) were housed in groups of 4 rats/cage under conditions of controlled illumination (12 h light:12 h dark cycle, lights on/off: 6:30 a.m./6:30 p.m.), humidity, and temperature (22 ± 2 °C). Animals were fed with a standard rodent diet and tap water *ad libitum*. Rats and mice were accustomed to the experimental conditions for a period of at least 7 days by handling them daily and putting them in the position to mimic the procedure of intraperitoneal (ip) injection. Animal care and experimental procedures followed institutional ethic guidelines and conformed to the requirements of the state authority for animal research conduct.

2.2. Peptide preparation

For rat experiments, rat/mouse obestatin was purchased from Bachem AG (Heidelberg, Germany). For mice studies, rat/mouse obestatin was synthesized by the Dept. of Chemistry, Québec University (Montréal, Canada). Capillary zone electrophoresis and coelution on high pressure liquid chromatography show purity >90% and mass spectrometric data were as expected (calculated 2516.32, found 2516.2) (Peptide Biology Laboratories, Salk Institute, La Jolla, CA). For rat studies, obestatin was dissolved in distilled water and stored at -20 °C. CCK-8S was dissolved in water with 1% (v/v) 1N NH_4OH. Immediately before starting the experiments, peptides were diluted in sterile 0.15 M NaCl (Braun, Melsungen, Germany) to reach the final concentration of 1 or 5 μmol/kg for obestatin and 1.75 and 3.5 nmol/kg (2 and 4 μg/kg) for CCK-8S. In mice experiments, obestatin as well as urocortin 1 (Peptide Biology Laboratories) were stored at -80 °C in powder form and dissolved in sterile saline (0.15 M NaCl) before use. Peptide doses in mice were based on the study by Zhang et al. [51] and in rats from our previous studies [14,23].

2.3. Procedures

2.3.1. Effects of obestatin injected ip on food intake in rats fed ad libitum or fasted for 16 h—Groups of randomized rats either deprived of food but not water for 16 h (n = 8 per group in both experiments) or freely fed (n = 10 per group in the light phase experiment, and n = 14 per group in the dark phase experiment) were injected ip (0.5 ml) with obestatin (1 or 5 μmol/kg), CCK-8S (3.5 nmol/kg; n = 4 in all experiments) or vehicle (0.15 M NaCl) 15 min before the dark or light phase started. Two minutes before the dark or light phase started, weighed rat chow was made available to the animals. Food intake was calculated as the difference between the food weights before and after the feeding period at each time interval (30 min, 1, 2, 3, 4, 5 and 12 h) and cumulative food intake was calculated by summating the values of the different time periods.

2.3.2. Effect of obestatin injected ip on food intake in mice fasted for 16 h—Mice housed singly 7 days prior each experiment. Mice were fasted for 16 h, then randomly injected ip (0.1 ml) with either obestatin (1 μmol/kg, n = 7), urocortin 1 (2 nmol/kg, n = 5) or saline (NaCl 0.9%, n = 6). Rodent weighed chow was given *ad libitum* 15 min after the ip injection, accordingly to the previous experimental protocol used to assess the effects of obestatin [52]. Food intake was recorded at 1, 3, and 5 h after food presentation by weighing (±0.01 g) the food and correcting for spillage, which was collected on papers placed at the bottom of the animal cages. Food intake was calculated as the difference between the food weights before and after the feeding period at each time interval and cumulative food intake was calculated by summating the values of the different time periods.

2.3.3. Effects of obestatin injected ip on Fos-ir in the hypothalamus and brainstem in rats fed ad libitum—Freely fed rats were injected ip (0.5 ml) with vehicle (0.15 M NaCl; $n = 4$), obestatin (5 μmol/kg; $n = 4$) or CCK-8S (1.75 nmol/kg; $n = 4$) and 90 min later deeply anesthetized with ip injections of ketamine (100 mg/kg Ketanest®, Curamed, Karlsruhe, Germany) and xylazine (10 mg/kg, Rompun® 2%, Bayer, Leverkusen, Germany) and heparinized with 2,500 U heparin ip (Liquemin®, Hoffmann-La Roche, Grenzach-Whylen, Germany). Transcardial perfusion, brain processing, and Fos immunochemistry were performed as described before [24].

2.4. Staining for Fos-immunoreactivity (Fos-ir)

Free-floating brain sections (thickness 25 μm) were pretreated with 1% (w/v) sodium borohydride (in phosphate buffer solution, PBS) for 15 min. Subsequently, sections were incubated in a solution containing 5% (w/v) bovine serum albumin (BSA) and 0.3% (v/v) Triton X-100 in PBS for 60 min to block unspecific antibody binding. Thereafter, the diluted primary antibody (rabbit anti-rat c-Fos protein; Oncogene Research Products, Boston, USA; 1:4000 in a solution of 5%, w/v BSA and 0.1%, w/v sodium azide in PBS) was applied for 42 h at room temperature. After rinsing sections in PBS three times and incubation in a solution containing 5% (w/v) BSA for 60 min, FITC-labeled goat-anti-rabbit IgG (Sigma, St. Louis, USA) was applied for 12 h at room temperature in an appropriate dilution (1:600 in 5%, w/v BSA in PBS). Sections were rinsed in PBS three times again and stained with propidium iodide (2.5 μg/ml in PBS) for 15 min to counterstain cell chromatin. Tissue sections were finally embedded in 10 μl anti-fading solution (100 mg/ml 1,4-diazabicyclo[2.2.2]octan (Sigma) in 90%, v/v glycerin, 10%, v/v PBS, pH 7.4) and analyzed using a confocal laser scanning microscope (cLSM 510, Carl Zeiss, Germany).

2.5. Data analysis

All data are expressed as mean ± S.E.M. Food intake data in rats were analyzed by one way repeated measures ANOVA followed by the Fisher LSD post hoc test and in mice by ANOVA followed by the Fisher LSD test. $p < 0.05$ was considered significant. Semi-quantitative assessment of Fos-ir was achieved by counting the number of Fos-ir positive cells as described before [25]. Briefly, neurons with green nuclear staining were considered Fos-ir positive. Every third of all consecutive coronal 25 μm sections was counted bilaterally for Fos-ir positive staining in the hypothalamic (ARC, PVN, VMH, and DMH) and brainstem (NTS) nuclei throughout their rostrocaudal extent. Fos-ir positive cells were counted in 10 sections per rat of the PVN and NTS, and 15 sections per rat in the ARC, VMH and DMH. Anatomic correlations were made according to landmarks given in Paxinos and Watson's stereotaxic atlas [29]. The investigator counting the number of Fos-ir positive cells was blinded to treatments received by the animals. Data were analyzed by ANOVA followed by Fisher LSD test. $p < 0.05$ was considered significant.

3. Results

3.1. Effects of obestatin injected ip on cumulative food intake under ad libitum feeding or fasting conditions in rats

CCK-8S (3.5 nmol/kg body wt) injected ip induced temporary satiety signaling (Figs. 1AB and 2B; Tables 1 and 2). During the light phase in *ad libitum* fed rats, CCK-8S injected ip decreased food intake during the first 30, 60, and 120 min compared to the vehicle group although this did not reach statistical significance due to smaller and more variable amounts of food eaten by the vehicle group under these conditions (Fig. 2A; Tables 1 and 2). In contrast to CCK-8S, obestatin at both doses (1 and 5 μmol/kg body wt, ip) did not suppress the cumulative food intake at any time point throughout the 12 h experiment compared to the

vehicle-treated rats when injected 15 min before the dark or light phase in 16-h fasted or fed rats (Figs.1AB and 2AB; Tables 1 and 2).

3.2. Effects of obestatin injected ip on cumulative food intake in fasted mice

Likewise, 16 h fasted mice injected with obestatin (1 μmol/kg body wt) 15 min before exposure to food had similar food intake as the vehicle group while ip urocortin 1 (2 nmol/kg body wt) inhibited cumulative food intake by 72, 50 and 40% at 1, 3 and 5 h, respectively, post-injection (Fig. 3).

3.3. Effects of obestatin and CCK-8S administered ip on the number of Fos-ir positive neurons in hypothalamic and brainstem nuclei in ad libitum fed rats

CCK-8S injected ip (1.75 nmol/rat) increased the number (mean ± S.E.M.) of Fos-ir positive neurons/section in the PVN (136.7 ± 7.2 *vs.* 39.8 ± 6.8, $p < 0.001$; Figs. 4 and 5), DMH (122.9 ± 5.8 *vs.* 67.8 ± 9.4, $p = 0.03$; Figs. 4 and 6), and NTS (135.1 ± 10.1 *vs.* 60.2 ± 6.9, $p < 0.002$; Figs. 4 and 6) compared to vehicle-treated animals. Obestatin (5 μmol/kg body wt, ip) had no effect on Fos expression in the PVN (44.2 ± 13.0 *vs.* 39.8 ± 6.8, $p > 0.05$; Figs. 4 and 5), ARC (15.8 ± 1.0 *vs.* 21.5 ± 3.7, $p > 0.05$; Figs. 4 and 5), VMH (38.2 ± 9.0 *vs.* 35.9 ± 2.8, $p > 0.05$; Figs. 4 and 5), DMH (67.6 ± 22.6 *vs.* 67.8 ± 9.4, $p > 0.05$; Figs. 4 and 6) and NTS (67.8 ± 16.5 *vs.* 60.2 ± 6.9, $p > 0.05$; Figs. 4 and 6) compared to vehicle treatment.

4. Discussion

The present experiments show that obestatin administered peripherally at 1 and 5 μmol/kg has no satiating effect in rats under *ad libitum* feeding or 16 h fasting/refed conditions, neither during the light phase nor the dark phase for the 12 h post-injection period. Similarly, obestatin injected ip 15 min before food exposure in 16-h fasted mice did not alter the 5-h refeeding period. In contrast, in rats, there is a significant rapid in onset decrease in food intake after peripheral injection of 3.5 nmol/kg of CCK-8S during the dark and light phase under fasted/refed conditions, and at the beginning of the dark phase in *ad libitum* fed rats. During the light phase under *ad libitum* conditions, the ~95% reduction of food intake induced by ip CCK-8S did not reach statistical significance due to the reduced and variable amounts of food ingested in the control non-fasted group (Table 2) along with the small number of animals ($n = 4$) in the CCK-8S-treated group. In fasted/refed mice, we also showed that ip urocortin 1 resulted in a 70-40% suppression over the 5-h post-injection consistent with previous reports [2,46]. Moreover, after obestatin administration (5 μmol/kg, ip) to freely fed rats, no effect on Fos expression pattern was observed in the PVN, ARC, VMH, DMH, and NTS while CCK-8S (1.75 nmol/kg, ip) increased Fos expression in the PVN, DMH, and NTS which is in agreement with previous studies [23,25].

Our observation that obestatin has no inhibitory effect on 16-h fasted rats during the light phase is consistent with our previous results [14,15]. Several other research groups were also unable to demonstrate an inhibitory effect of the peptide on food intake in rodents at this dose [21, 32]. All in all, data concerning obestatin's influence on feeding behavior when injected peripherally are conflicting. There are four studies describing a significant inhibitory effect on food intake [7,18,27,51], three investigations showing a non-significant trend towards a reduction [21,40,42], and seven reports of no effect on feeding behavior after obestatin administration [11,14,15,32,41,54, and present study]. The results of these studies seem to be independent from the investigated species (mice or rats), the metabolic status (fasted or fed *ad libitum*) and the route of delivery (ip, intravenous, intracerebroventricular and intracisternal) [16 and present study]. Although a recent study points to the dose of obestatin administered to the animals as the decisive factor. Indeed, Lagaud et al. found that only amounts between 100 and 300 nmol/kg caused a significant decrease of food intake while higher (1 or 3 μmol/kg) or

lower (10 or 30 nmol/kg) doses had no suppressive effect [27]. However, even these findings are conflicting with several studies that showed significant effects after injection of 1 μmol/kg obestatin [18,51] while in some other reports, no inhibition of food ingestion was observed with similar doses [15,21]. Recently, Zhang et al. raised the issue of the experimental conditions that are essential to detect obestatin inhibitory effects [52]. While most of the studies did not observe any effect of obestatin injected at the same time that the food was presented in fasted animals, Zhang et al. found that ip obestatin-induced inhibition of feeding when injected at 15 min, but not at 0 or 30 min before food presentation in 16-h fasted mice. In our hands, even using the critical 15-min time interval, the ip injection of obestatin did not result in any inhibition of food intake neither in rats nor in mice. Moreover, our data reveal that the effect of peripherally administered obestatin on food intake in rats depends neither on time in the circadian rhythm when the experiment is conducted, nor on the metabolic conditions of the experimental animals. The present findings are also consistent with the lack of effect of peripherally administered obestatin on gastric motor function as assessed by monitoring changes in gastric motility or emptying in both rats and mice [3,11,14,16,17] contrasting with the initial report [51].

Recently, it has been demonstrated that peripheral obestatin crosses the blood brain barrier in mice, indicating that central pathways may be involved in the regulation of food intake by obestatin [35]. In addition, obestatin was initially suggested to inhibit feeding through activation of brain nuclei, particularly the ARC. This was supported by the fact that obestatin was originally claimed to bind the GPR-39, which is highly expressed in the hypothalamus, as detected by Northern blot and reverse transcriptase polymerase chain reaction (RT-PCR) [51]. However, using *in situ* hybridization and RT-PCR, subsequent studies did not find expression of this receptor in the hypothalamus [21,22,32]. More importantly, recent studies and the retraction of initial findings by Zhang clearly established that obestatin is not the endogenous ligand that binds to GRP39 receptor [9,21,22,32,52]. In the present study, we found that ip injection of obestatin has no effect on the Fos expression pattern in the hypothalamus and brainstem. This contrast with most other gut peptides that influence feeding behavior by modulating neuronal activity in hypothalamic and brainstem nuclei. CCK-8S increases Fos expression in the PVN, DMH and NTS [23,25,29,33,38] while systemic or icv application of the long-term satiety signal leptin induces neuronal activation in PVN and DMH neurons [12,44], and peptide YY increases Fos expression in NTS, AP [6], and ARC [4]. Glucagon-like peptide 1 modulates the number of Fos-ir positive neurons in ARC [44], PVN, NTS and AP [28,39], and peripheral amylin leads to increased neuronal activation in the NTS and AP [39]. Another puzzling fact is the lack of evidence for *in vivo* synthesis of obestatin [13]. Using different prohormone convertases, Zhu et al. were not able to observe the formation of obestatin from proghrelin [53]. In addition, plasma obestatin levels measured by radioimmunoassay (RIA) led to varying results which might indicate that the low specificity of these tests could be due to lacking standardization and imprecise values in previous studies [19,36].

In conclusion, our data show that independently from the metabolic status (fed or fasted) and the experimental setting during dark or light phase, and in contrast to CCK-8S or urocortin 1, the ip injection of obestatin does not influence food intake in rodents and Fos expression pattern in rat hypothalamic and brainstem nuclei. Therefore, the present work does not support the assumption that obestatin influences food intake in rodents and the potential biological actions along with the receptors on which the peptide interact remain to be established.

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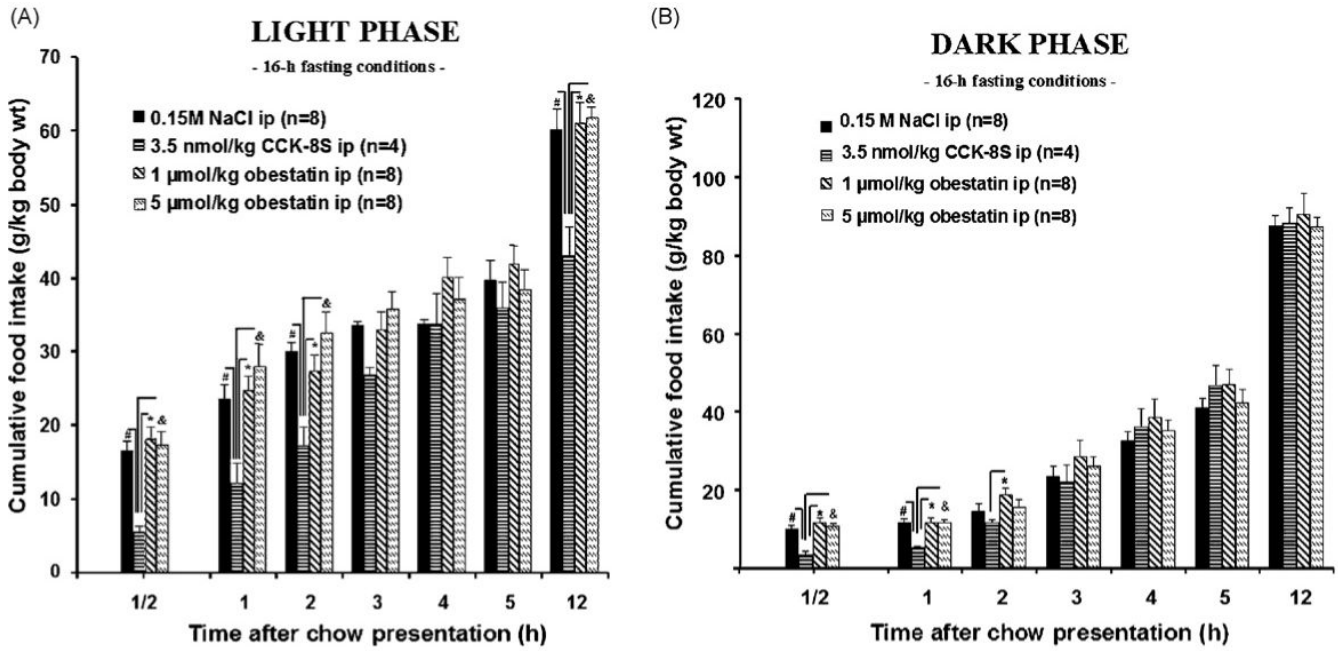

Fig. 1.

Cumulative food intake in 16-h fasted rats after intraperitoneal (ip) injection of 1 or 5 µmol/kg obestatin, 3.5 nmol/kg CCK-8S or 0.15 M NaCl 15 min before food exposure in the dark (B) or light (A) phase. While CCK-8S leads to a significant reduction of food intake, obestatin does not influence feeding behavior. The data are expressed as mean ± S.E.M. $^{\#}p < 0.05$ *vs*. vehicle, $^{*}p < 0.05$ *vs*. 1 µmol/kg obestatin, and $^{\&}p < 0.05$ *vs*. 5 µmol/kg obestatin.

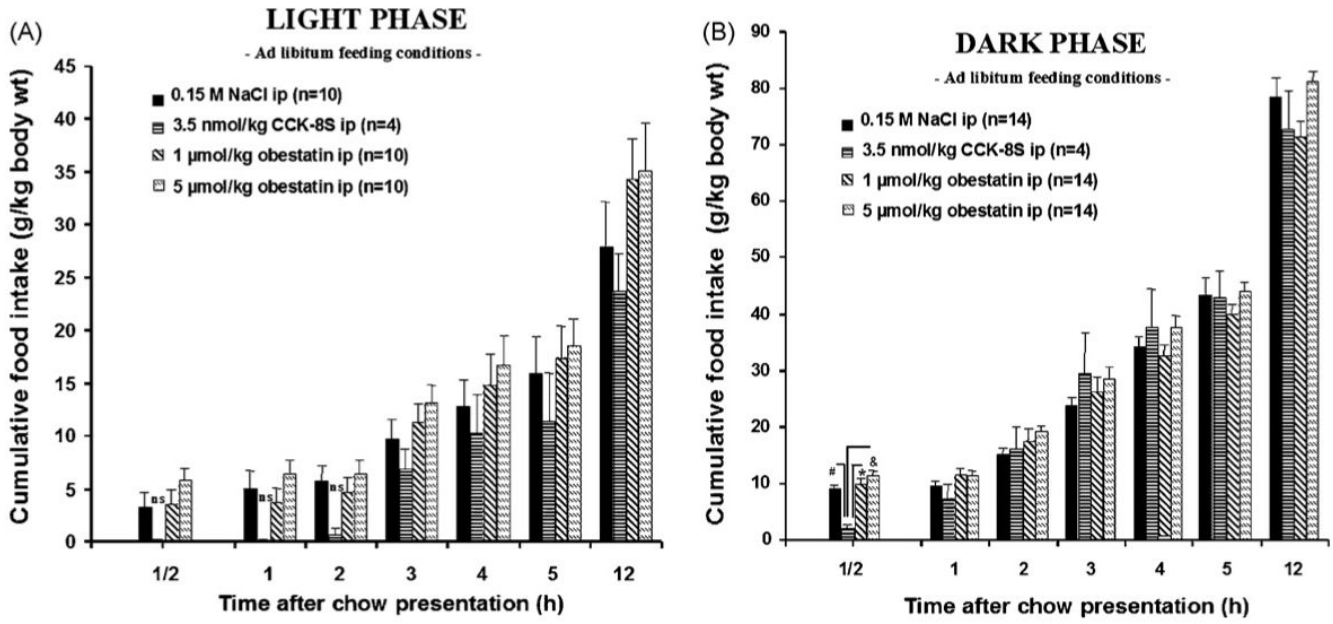

Fig. 2.

Cumulative food intake in *ad libitum* fed rats after intraperitoneal (ip) injection of 1 or 5 µmol/kg obestatin, 3.5 nmol/kg CCK-8S or 0.15 M NaCl 15 min before food exposure before the dark (B) or light (A) phase. CCK-8S significantly reduces food intake, while obestatin does not influence feeding behavior. The data are expressed as mean ± S.E.M. $^{\#}p < 0.05$ *vs*. vehicle, $^{*}p < 0.05$ *vs*. 1 µmol/kg obestatin, and $^{\&}p < 0.05$ *vs*. 5 µmol/kg obestatin.

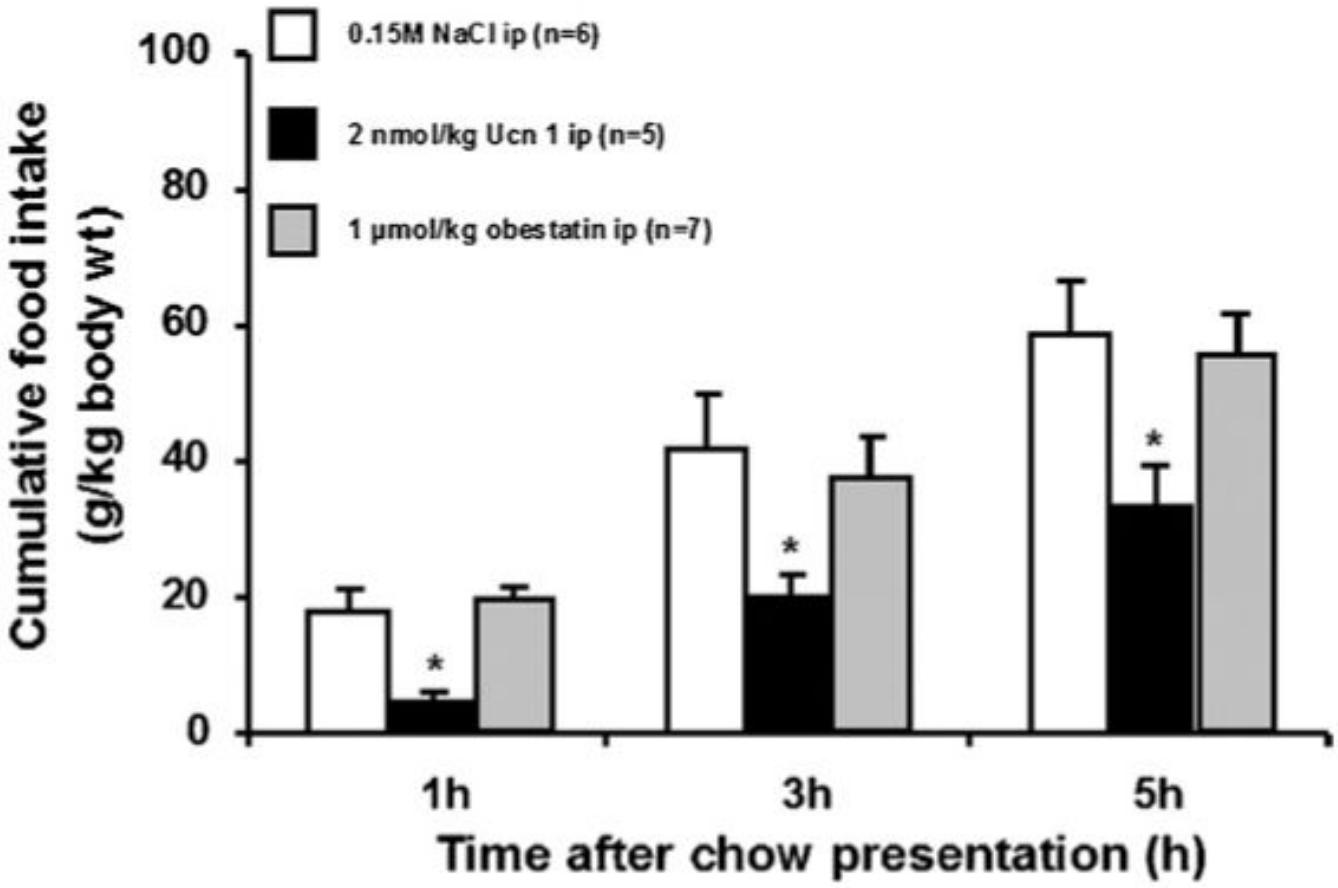

Fig. 3.

Cumulative food intake in fasted mice after intraperitoneal (ip) injection of 1 μmol/kg obestatin, 2 nmol/kg urocortin 1 or 0.15 M NaCl 15 min before exposure to food in the light phase. Obestatin and vehicle have similar food intake while urocortin 1 inhibits cumulative food intake by 72, 50 and 40% at 1, 3 and 5 h post-injection. The data are expressed as mean ± S.E.M. *$p < 0.05$ *vs*. vehicle, and *$p < 0.05$ *vs*. 1 μmol/kg obestatin.

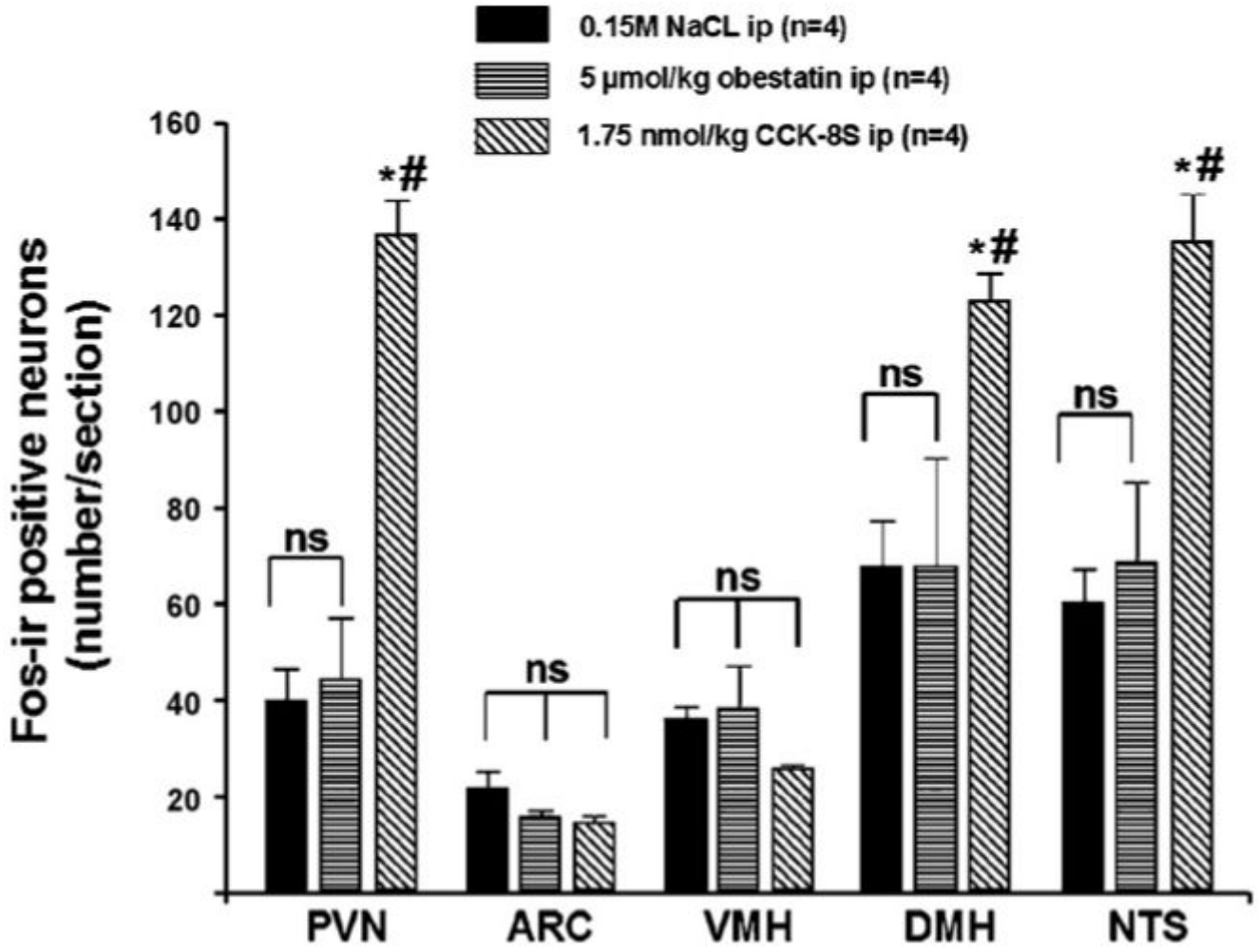

Fig. 4.

Fos expression pattern (number of Fos-ir positive neurons/section) after intraperitoneal (ip) injection of 5 µmol/kg obestatin, 1.75 nmol/kg CCK-8S or 0.15 M NaCl. Obestatin does not change neuronal activity while CCK-8S induces increased Fos expression in the PVN, DMH and NTS. The data are expressed as mean ± S.E.M. $^{\#}p < 0.05$ *vs*. vehicle, $*p < 0.05$ *vs*. 5 µmol/kg obestatin, ns = not significant.

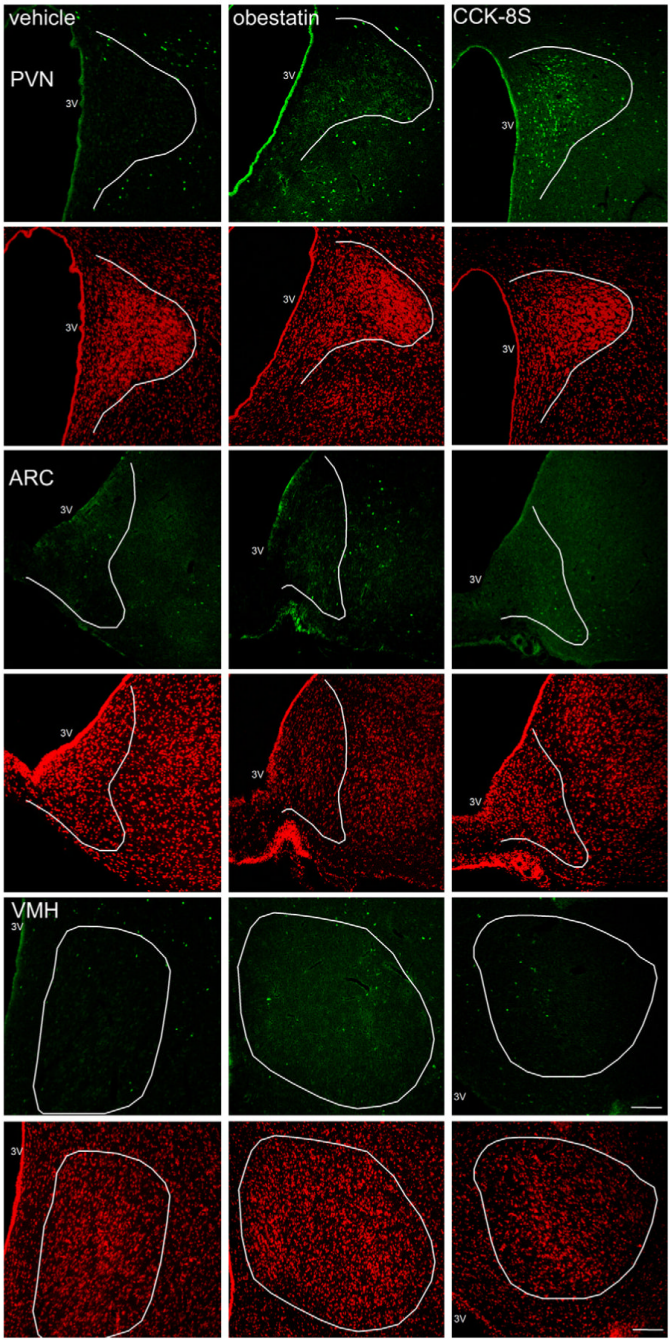

Fig. 5.

Representative images of the PVN, ARC and VMH after ip-injection of 0.15 M NaCl, 5 μmol/kg obestatin and 1.75 nmol/kg CCK-8S. Obestatin does not change neuronal activity in these hypothalamic nuclei while CCK-8S induces increased Fos expression (green staining) in the PVN. Cell nuclei are stained red as a result of the counterstaining with propidium iodide. The white outer line delineates the area of the PVN, ARC and VMH. The white scale bar represents 100 μm. 3V, third ventricle; PVN, paraventricular nucleus of the hypothalamus; ARC, arcuate nucleus of the hypothalamus; VMH, ventromedial nucleus of the hypothalamus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

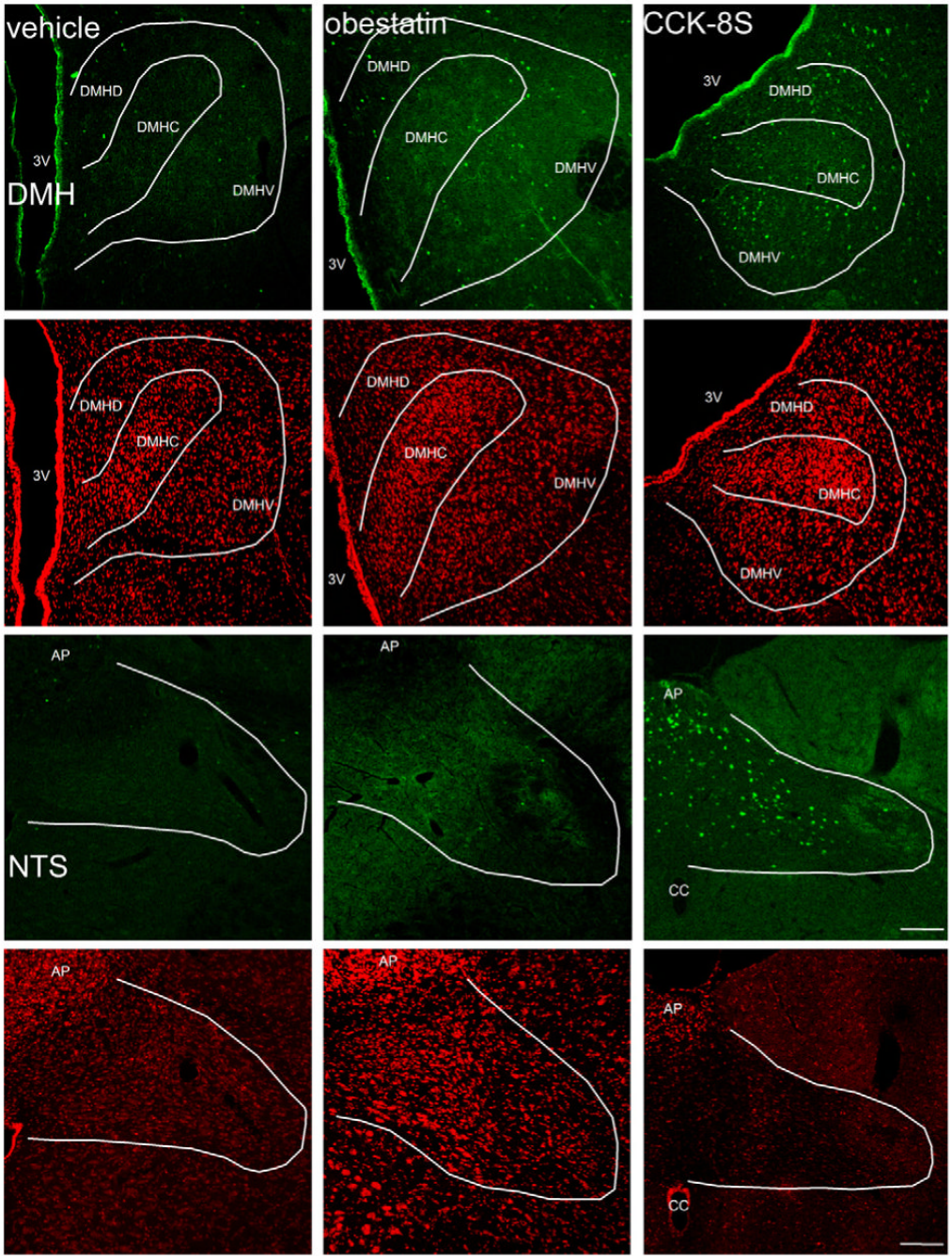

Fig. 6.

Representative images of the DMH and the NTS after ip administration of 0.15 M NaCl, 5 μmol/kg obestatin and 1.75 nmol/kg CCK-8S. Obestatin does not change neuronal activity in these nuclei while CCK-8S induces increased Fos expression (green staining) in both of them. The white outer line delineates the area of the DMH and NTS. Cell nuclei are stained red as a result of the counterstaining with propidium iodide. The white scale bar represents 100 μm. 3V, third ventricle; DMH, dorsomedial hypothalamic nucleus; DMHD, dorsomedial hypothalamic nucleus, dorsal part; DMHC, dorsomedial hypothalamic nucleus, compact part; DMHV, dorsomedial hypothalamic nucleus, ventral part; NTS, nucleus of the solitary tract;

AP, area postrema; cc, canalis centralis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1

Summary of the one way repeated measures ANOVAs on food intake experiments in rats

Experiment	Time (h)	Degrees of freedom	Sum of squares	Mean squares	*F* statistic	*p*-Value
16 h Fasting, light phase	1/2	3	518.432	172.811	9.883	<0.001
	1	3	844.55	281.517	6.144	0.005
	2	3	822.384	274.128	6.572	0.004
	3	3	302.995	100.998	4.427	0.018
	4	3	229.448	76.483	2.17	0.129
	5	3	90.446	30.149	0.633	0.604
	12	3	1079.397	359.799	7.521	0.002
16 h Fasting, dark phase	1/2	3	155.768	51.923	9.511	0.001
	1	3	99.956	33.319	6.874	0.005
	2	3	95.452	31.817	5.142	0.015
	3	3	107.385	35.795	0.919	0.459
	4	3	142.622	47.541	0.766	0.533
	5	3	221.443	73.814	1.598	0.238
	12	3	54.194	18.065	0.254	0.857
Ad libitum feeding, light phase	1/2	3	49.534	16.511	1.386	0.275
	1	3	53.368	17.789	1.243	0.319
	2	3	37.701	12.567	0.837	0.489
	3	3	148.855	49.618	1.416	0.266
	4	3	150.595	50.198	0.646	0.594
	5	3	225.491	75.164	0.764	0.527
	12	3	436.287	145.429	0.822	0.496
Ad libitum feeding, dark phase	1/2	3	150.028	50.009	4.687	0.009
	1	3	41.504	13.835	0.814	0.497
	2	3	123.74	41.247	1.242	0.313
	3	3	315.215	105.072	1.56	0.22
	4	3	347.73	115.91	1.927	0.147
	5	3	140.395	46.798	0.717	0.55
	12	3	721.642	240.547	2.722	0.062

Table 2

Comparison of food intake data between rats treated with CCK-8S, obestatin or saline solution during the first 2 h after ip administration under different conditions

0.15 M NaCl			3.5 nmol/kg CCK-8S			1 µmol/kg Obestatin		5 µmol/kg Obestatin	
Experiment	**Time (h)**	**FI (g/kg)**	**FI (g/kg)**	***p*-Value FI-reduction LSD-test to NaCl (%) *vs.* NaCl**		**FI (g/kg)**	**FI-reduction to NaCl (%)**	**FI (g/kg)**	**FI-reduction to NaCl (%)**
16 h Fasting, light phase	1/2	16.52 ± 1.30	5.50 ± 0.91	<0.001	-66.70	18.14 ± 1.69	+9.80	17.44 ± 1.69	+5.56
	1	23.56 ± 2.02	12.23 ± 2.67	=0.005	-48.09	24.66 ± 1.98	+4.66	28.08 ± 2.93	+19.18
	2	30.03 ± 1.26	17.16 ± 2.76	=0.007	-42.85	27.33 ± 2.16	-8.99	32.56 ± 2.93	+8.42
16 h Fasting, dark phase	1/2	10.10 ± 0.87	3.41 ± 1.10	=0.002	-66.23	11.72 ± 1.21.	+16.03	10.87 ± 0.51	+7.62
	1	11.61 ± 1.21	5.28 ± 0.21	=0.002	-54.52	11.72 ± 1.21	+0.94	11.68 ± 0.77	+0.60
	2	14.58 ± 1.92	11.83 ± 0.65	ns	-18.86	18.74 ± 1.84	+28.53	15.66 ± 2.03	+7.40
Ad libitum feeding, light phase	1/2	3.31 ± 1.33	0.1 ± 0.1	ns	-96.97	3.48 ± 1.41	+5.13	5.77 ± 1.25	+74.32
	1	5.07 ± 1.59	0.1 ± 01	ns	-98.02	3.67 ± 1.42	-27.61	6,42 ± 1.32	+26.62
	2	5.67 ± 1.54	0.67 ± 1.1	ns	-88.18	4.70 ± 1.31	-17.10	6.42 ± 1.32	+13.22
Ad libitum feeding, dark phase	1/2	8.99 ± 0.81	2.06 ± 0.67	=0.017	-77.08	10.02 ± 0.94	+11.45	11.23 ± 0.93	+24.91
	1	9.63 ± 0.78	7.22 ± 2.70	ns	-25.02	11.54 ± 1.24	+19.83	11.41 ± 0.93	+18.48
	2	15.07 ± 1.30	16.10 ± 3.75	ns	+6.83	17.48 ± 2.27	+15.99	19.17 ± 1.04	+27.20

FI = Food intake, values are mean ± S.E.M. of n = 4-14 rats/group; ns = not significant.