

Research report

Intraperitoneal injection of ghrelin induces Fos expression in the paraventricular nucleus of the hypothalamus in rats

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Abstract

Ghrelin is a 28-amino acid peptide hormone secreted from the stomach that acts as a gut–brain peptide with potent stimulatory effects on food intake. The aim of the present study was to investigate the effects of peripheral ghrelin (1 and 10 nmol/rat) injected intraperitoneally (i.p.) on food intake and neuronal activity in the hypothalamus and brain stem, as assessed by c-Fos-like-immunoreactivity (c-FLI), using a confocal laser scanning microscope (cLSM) as a sensitive microscopic technique to detect c-FLI-positive neurons. Cumulative food intake was significantly increased 5.3- and 3.7-fold for the 4-h period after i.p. injection of ghrelin at both doses. The number of c-FLI-positive neurons in the paraventricular nucleus of the hypothalamus (PVN) was significantly increased after peripheral administration of ghrelin (1 nmol i.p.; median: 41.8) compared with i.p. saline (median: 17.5). As described before, *c-fos* expression was increased in the arcuate nucleus of the hypothalamus (ARC). In the nucleus of the solitary tract (NTS) or the area postrema (AP), there was no significant change in the density of c-FLI-positive neurons. Our data suggest that an activation of the arcuate–paraventricular axis may be part of the brain circuits involved in the orexigenic effect of peripheral ghrelin.

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Abbreviations: AP, area postrema; ARC, arcuate nucleus of the hypothalamus; c-FLI, c-Fos-like-immunoreactivity; cLSM, confocal laser scanning microscope; CSF, cerebrospinal fluid; DMH, dorsomedial nucleus of the hypothalamus; VMH, ventromedial nucleus of the hypothalamus; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus of the hypothalamus; i.c.v., intracerebroventricular; i.p., intraperitoneal; i.v., intravenous; NPY, Neuropeptide Y; GHS, growth hormone secretagogue

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1. Introduction

Ghrelin is a 28-amino acid peptide hormone that was primarily identified in the rat stomach as an endogenous ligand of the growth hormone secretagogue (GHS) receptor [25]. The principal gastric source of ghrelin synthesis is the A-like endocrine cells of the oxyntic mucosa [16], which contribute about 80% to the circulating concentrations of ghrelin [12,25]. Independent from its effect as a growth hormone secretagogue in the pituitary gland [13,32,36], peripheral ghrelin has been shown to act as a gut–brain peptide with potent stimulatory effects on food intake and to positively influence energy balance in rats [27,39,44,45], mice [1,42], and humans [46].

Compelling evidence showed that ghrelin levels in the circulation are determined by the feeding and metabolic state of the body. Fasting increases plasma ghrelin levels and there is a sharp decrease of circulating ghrelin after food consumption in rats [39] and humans [11,40,41]. The importance of ghrelin in influencing short-term food ingestion is underlined by functional studies demonstrating an enhanced food intake after central (intracerebroventricular, i.c.v.) [27,39] and peripheral (intraperitoneal, i.p. and intravenous, i.v.) [44,45] administration in rats, i.p. injection in mice [1,42], and i.v. injection in humans [46].

Furthermore, it has been shown that ghrelin activates specific brain areas in the hypothalamus which are part of central networks involved in the regulation of feeding and metabolism, as shown by the expression of c-Fos, the product of the immediate-early-gene *c-fos*, in rats [14,22,26,38] and mice [42]. Interestingly, there is an important difference in the pattern of c-Fos-like-immunoreactivity (c-FLI) depending on the route of ghrelin administration. Injection of the peptide into the cerebrospinal fluid (CSF) induces *c-fos* expression in the paraventricular (PVN), dorsomedial (DMH), ventromedial (VMH) and arcuate (ARC) nucleus of the hypothalamus, as well as in the nucleus of the solitary tract (NTS) and area postrema (AP) of the brain stem [13,26]. On the other hand, *c-fos* expression seems to be confined to the ARC after peripheral injection of ghrelin in rats [15,22,38] and mice [42]. However, previous studies in rats have not explored whether the activity of other hypothalamic and brain stem nuclei may be influenced by peripheral administration of ghrelin [22]. The PVN exerts a pivotal role in the putative network of brain nuclei involved in the control of satiety and energy balance [24,43]. Furthermore, peripheral ghrelin induces c-FLI in neuropeptide Y (NPY)-synthesizing neurons of the ARC [42], and NPY-containing fibers originating from the ARC have been shown to innervate neurons in the PVN [3,4].

There is also convincing evidence that brain stem areas, in particular the NTS, play a crucial role in the control of food intake by conveying viscerosensory information about the gastrointestinal food content to higher regulatory brain centers [6,18,29]. Recent studies indicate that vagal afferents dependent mechanisms may be involved in peripheral ghrelin-induced increase in food intake and c-FLI in the ARC [15]. As the NTS is the termination area of afferent vagal fibers from the gastrointestinal tract [30], an involvement of the brain stem in the CNS mediation of the effects of peripheral ghrelin on energy balance can be proposed.

The aim of the present study was to further elucidate whether the activity of PVN and brain stem nuclei is influenced by peripheral injection of ghrelin by mapping c-FLI in the PVN, the NTS, and the AP after peptide injection at doses that influenced food intake in freely fed rats.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Harlan-Winkelmann, Borchon, Germany) weighing 300–350 g were housed in groups of four rats per cage under conditions of controlled illumination (12:12 h light/dark cycle, lights on/off: 6:30 a.m./6:30 p.m.), humidity, and temperature (22 ± 2 °C) for at least 5 days prior to the experiments. They were fed a standard rat diet (Altromin®, Lage, Germany) and tap water ad libitum and maintained in colony cages until the start of the experiments. 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

Rat ghrelin [GSS(octanoyl)FLSPEHQKAQQRKES-KKPPAKLQPR] (Bachem, Heidelberg, Germany) was dissolved in distilled water (1 mg/ml) and stored at -20 °C. Immediately before the experiments, the peptide was diluted in vehicle solution consisting of sterile 0.15 M NaCl solution (Braun, Melsungen, Germany) to reach the final concentration of 1 or 10 nmol/ml. The peptide solution was kept on ice for the duration of the experiments.

2.3. Experimental design

2.3.1. Effects of peripheral ghrelin on food intake

All experiments were started at the same time of the day (between 10:00 and 10:30 h, at 3.5 to 4 h after the start of the light cycle) to achieve maximal consistency of any conditions associated with circadian rhythms of ghrelin secretion [37]. To minimize stress associated with handling, rats were handled daily for at least 3 days before the experiment.

Table 1
Effect of i.p. ghrelin (1 and 10 nmol) on the cumulative 4 h food intake

Time (h)	Group					
	Vehicle		Ghrelin 1 nmol		Ghrelin 10 nmol	
	Median	Median	Median	Median	Median	Median
	25th	75th	25th	75th	25th	75th
1	0.00		0.25		0.02	
	0.00	0.07	0.08	1.74	0.00	1.73
2	0.19		2.40*		0.46	
	0.03	0.63	0.38	3.25	0.01	4.17
3	0.19		4.20*		4.13*	
	0.03	2.05	3.31	5.04	2.03	4.87
4	0.19		4.23*		4.13*	
	0.03	2.07	3.32	7.55	2.08	7.12

Data are given as median, 25th, and the 75th percentile.

* $p < 0.05$ of respective ghrelin group vs. control.

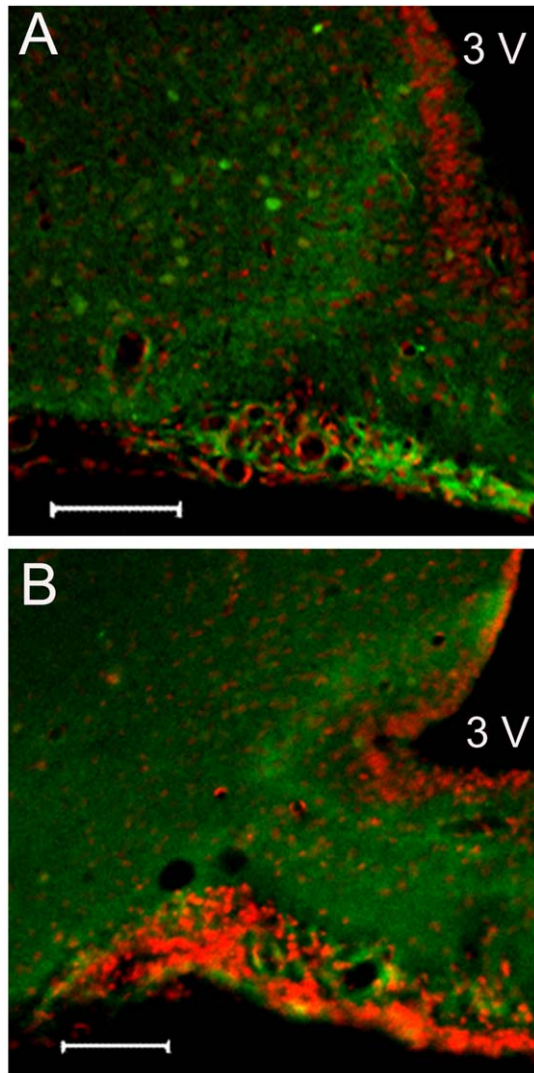


Fig. 1. Effect of peripheral ghrelin on c-Fos-like-immunoreactivity in the arcuate nucleus of the hypothalamus. The arcuate nucleus of the hypothalamus showed c-FLI (green staining) 90 min after i.p. injection of ghrelin (1 nmol/rat) in freely fed rats ($n=4$ /group) (A). After saline injection, none or low c-FLI was observed (B). Cell nuclei are stained red as a result of the counterstaining with propidium iodide. The white scale bar represents 100 μm . 3V=third ventricle.

On the day of the experiment, freely fed animals were injected i.p. (1 ml) with vehicle (0.15 M NaCl) or ghrelin at a dose of 1 or 10 nmol/rat and were housed singly immediately after the injection. Thereafter, preweighed rat chow was given and food intake was determined by measuring the difference between the preweighed standard chow at the beginning and end of every hour during a 4-h observation period.

2.3.2. Effects of peripheral ghrelin on c-Fos-like-immunoreactivity in the hypothalamus and brain stem

Freely fed rats were injected intraperitoneally (1 ml) with ghrelin (1 nmol/rat) or vehicle solution. Immediately after the injection, animals were deprived of food, but had

ad libitum access to water. At 90 min after the injection, animals were deeply anesthetized with 50 mg/kg ketamine (Ketanest[®], Curamed, Karlsruhe, Germany) and 30 mg/kg xylazine (Rompun[®] 2%, Bayer, Leverkusen, Germany), and heparinized with 2500 U heparin (Liquemin[®], Hoffmann-La Roche, Grenzach-Whylen, Germany). Transcardial perfusion was performed as described before [19]. It was started with a 10-s flush of a plasma substitute (Longasteril[®] 70; Fresenius, Bad Homburg, Germany), followed by a mixture of 4% w/v paraformaldehyde, 0.05% v/v glutaraldehyde, and 0.2% v/v picric acid in 0.1 M phosphate buffer, pH 7.4, for 30 min followed by a 5% w/v sucrose solution for 5 min. After dissection, brains were kept in a 5% w/v sucrose solution overnight and then cut into 1.0 to 4.5 mm coronal blocks enclosing the respective hypothalamic and brain stem regions using a plexiglas brain matrix. For cryoprotection, the blocks were moved through a sucrose gradient (15% and 27.3%), then shock-frozen in hexane at $-70\text{ }^{\circ}\text{C}$, and stored at $-80\text{ }^{\circ}\text{C}$ until further processing.

2.4. Immunohistochemistry

2.4.1. Staining for c-Fos-like immunoreactivity (c-FLI)

First, 25 μm free-floating sections were pretreated with 1% w/v sodium borohydride (in PBS) for 15 min.

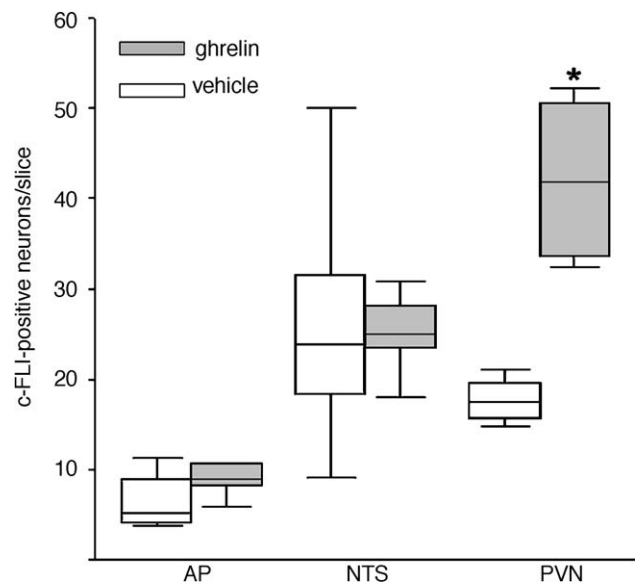


Fig. 2. Effect of i.p. injection of ghrelin on the number of c-FLI positive neurons in the paraventricular nucleus of the hypothalamus, the nucleus of the solitary tract, and the area postrema. Freely fed rats ($n=4$) were injected i.p. with vehicle or ghrelin (1 nmol/rat) and 90 min later were euthanized to process brains for c-FLI. The data are presented using “box and whiskers” plots. The upper hinge of the box represents the 75th and the lower one the 25th percentile. The median is shown as a line across the box. The “whiskers” above and below the boxes represent the largest and smallest observed scores that are less than 1.5 box lengths from the end of the box. * $p < 0.05$ vs. vehicle; AP: area postrema, NTS: nucleus of the solitary tract, PVN: paraventricular nucleus of the hypothalamus.

Subsequently, sections were incubated in a solution containing 10% w/v BSA and 0.3% v/v Triton X-100 in PBS for 60 min for blockade of unspecific antibody binding. Thereafter, the diluted primary antibody solution (rabbit anti-rat c-Fos protein; Oncogene Research Products, Boston, USA; 1:10,000 in a solution of 10% w/v BSA, 0.3% v/v Triton X-100, and 0.1% v/v sodium azide in PBS) was applied for 24 h at room temperature.

After rinsing sections in PBS three times and incubation in a solution containing 10% w/v BSA and 0.3% v/v Triton X-100 for 60 min, FITC-labeled goat-anti-rabbit IgG (Sigma, St. Louis, USA) was applied for 12 h at room temperature and in the dark in an appropriate dilution (1:800 in 10% 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. The tissue sections were finally embedded in 15 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 and statistical analysis

2.5.1. Immunohistochemistry

Semiquantitative assessment of c-FLI was achieved by counting the number of c-FLI positive cells. Cells with bright green nuclear staining were considered c-FLI-positive. Every second of all consecutive coronal 25 µm sections was counted for c-FLI positive staining bilaterally in the ARC, PVN and NTS, and unilaterally in the AP throughout their rostrocaudal extent. Anatomic correlations were made according to landmarks given in a stereotaxic atlas [28]. C-FLI-positive cells were counted in 10 sections per rat of the PVN, and 15 sections per rat of the AP and NTS. Identical numbers of brains ($n=4$) were processed for all groups investigated to accomplish maximal consistency of the results. The investigator counting the number of c-fos positive cells was blinded as to which treatment the animals had received.

The average number of c-FLI-positive cells per section for the brain nuclei mentioned above was calculated for each rat. Data are expressed as median and interquartile ranges of the average number of cells/section of four rats per group. As normal distribution of the values could not be detected,

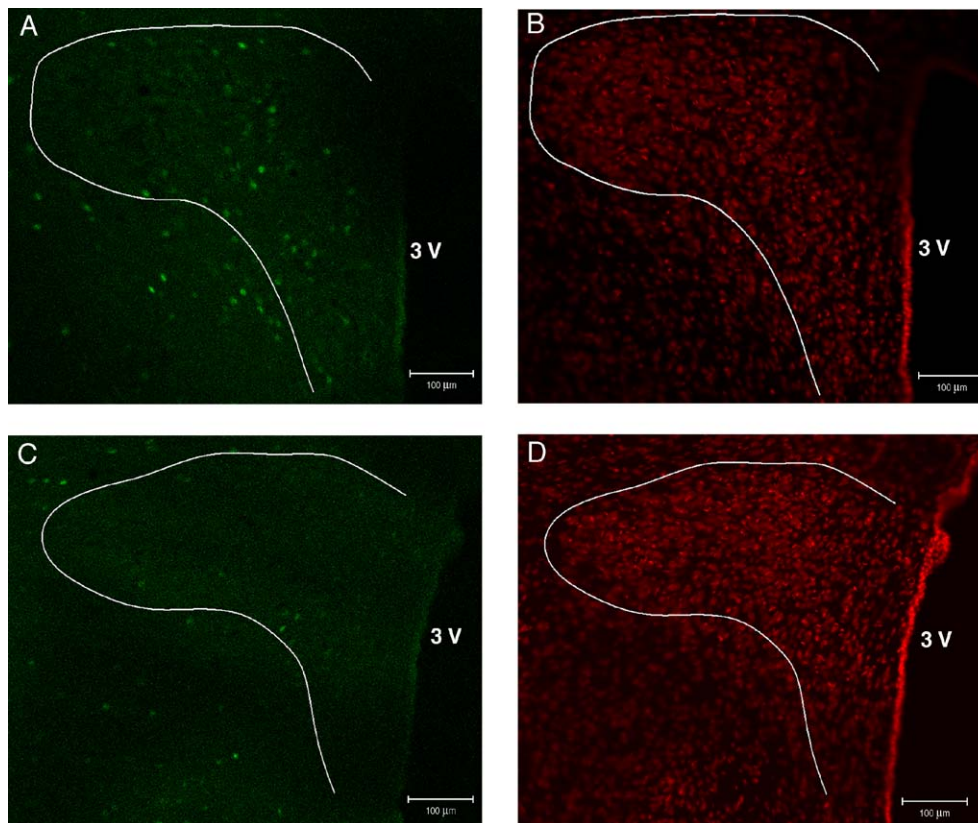


Fig. 3. Intraperitoneal injection of ghrelin-induced c-Fos-like-immunoreactivity in the paraventricular nucleus of the hypothalamus. The PVN showed increased c-FLI (green staining) 90 min after i.p. injection of ghrelin (1 nmol/rat; $n=4$) (A). Low expression of c-Fos was observed after i.p. saline injection (C). Cell nuclei are stained red as a result of the counterstaining with propidium iodide in the same slice of ghrelin- (B) and saline-treated (D) animals. The white line delineates the area of the paraventricular nucleus in accordance with landmarks given in the Paxinos and Watson [28] rat brain atlas.

differences between groups were evaluated by the non-parametric Kruskal–Wallis and Mann–Whitney *U*-tests; $p < 0.05$ was considered significant.

2.5.2. Feeding experiments

The food intake was calculated after every hour following peptide or vehicle injection as food intake (g)/body weight (kg) ($n=5$). Data are expressed as median and interquartile ranges. Difference between groups were evaluated by the Kruskal–Wallis and Mann–Whitney *U*-tests; $p < 0.05$ was considered significant.

3. Results

3.1. Effects of ghrelin injected intraperitoneally on food intake

Compared to the control group, rat ghrelin (1 nmol/rat, i.p.) caused a non-significant ($p=0.053$) increase in the 1 h food intake post injection, and a significant increase in cumulative 2, 3, and 4 h food intake (Table 1). At a 10-fold higher dose, ghrelin (10 nmol/rat, i.p.) also significantly increased the 3 and 4 h food intake compared to the control group (Table 1). The food intake increase induced by i.p. ghrelin was not significantly different between the two doses.

3.2. Effects of ghrelin injected intraperitoneally on c-fos expression in hypothalamic and medullary nuclei

Based on the similar food intake response to ghrelin injected i.p. at 1 and 10 nmol/rat (Table 1), further studies on c-FLI were conducted using the lower peptide dose. Ghrelin (1 nmol/rat, i.p.) induced a robust increase in the density of c-FLI-positive neurons predominantly in the ventromedial part of the ARC compared to vehicle injection where only a few c-FLI-positive nuclei were found (Fig. 1). In the PVN, the number of Fos positive neurons/section was significantly increased after i.p. injection of ghrelin compared with the i.p. saline injected group (median: 41.8 vs. 17.5, respectively, Fig. 2). According to the PVN nomenclature by Swanson and Kuypers [33], c-FLI labeled cells were mainly located within the medioparvocellular division of the PVN while there were only few c-FLI positive cells in the magnocellular part (Fig. 3A).

By contrast, there was no change in the density of c-FLI in the NTS and the AP in response to i.p. injection of ghrelin as compared with the i.p. vehicle group (Fig. 2).

4. Discussion

Ghrelin injected i.p. exerted an orexigenic effect in freely fed rats with the maximal response achieved at 1 nmol/rat.

This was shown by the progressive similar increase in the cumulative food intake per hour during the entire 4-h observation period after ghrelin injected i.p. at either 1 or 10 nmol/rat. These results are consistent with previous reports showing that ghrelin at 1 nmol/rat i.p. is the lowest dose to cause a significant stimulation of food intake in rats and to induce fasting plasma levels of ghrelin 15 min after the i.p. injection [45].

It has been suggested that ghrelin, after secretion into the blood, exerts its effect on energy balance primarily by binding to the growth hormone secretagogue (GHS)-receptor located on ARC neurons [2,42]. This is supported by the demonstration that the ARC is the only hypothalamic structure located outside the blood–brain barrier bearing the GHS-receptor [20,34]. In addition, previous reports showed that ghrelin injected i.v. at 1.5 or 3 nmol/rat induces *c-fos* expression in the ARC in freely fed rats [15,22] and i.p. injection of ghrelin also activated neurons in the ARC in rats and mice [38,42]. Likewise, in the present study we showed that i.p. injection of ghrelin at 1 nmol/rat in non-fasted animals induced c-FLI in the ARC. In addition, we observed that the number of c-FLI-positive neurons in the PVN was increased by 2.4-fold compared with the control group 90 min after the i.p. injection of ghrelin at 1 nmol/rat. The induction of *c-fos* expression was mainly located in the medioparvocellular part of the PVN as shown by confocal laser microscopy. The activation of these hypothalamic nuclei by i.p. ghrelin is site selective, since under the same conditions there were no significant changes in the number of c-FLI-positive neurons in the NTS and AP.

The activation of PVN neurons by i.p. injection of ghrelin at an orexigenic dose contrasts with a previous report where the ARC was the only hypothalamic structure to show a Fos response to i.v. injection of ghrelin in rats [22]. Whether such a difference may be related to the i.v. [22] and i.p. (present study) route of ghrelin injection and/or different methodological approaches used needs further investigation. Here, we used confocal laser microscopy (cLSM) to detect c-FLI-positive neurons, whereas all other groups used conventional light microscopy. As the cLSM has a much higher resolution power, it is well possible that we detected signals that we would have missed had we used conventional light microscopy.

It has been postulated that the PVN plays a pivotal role in the putative brain network involved in the control of satiety and energy balance [24,43]. Information of both anorexigenic systems containing mainly melanocortin-derived peptides and orexigenic peptidergic systems containing neuropeptide Y (NPY) and agouti-related protein (AgrP) seem to converge in the PVN [7,21]. We were able to show here that peripheral ghrelin activates neurons in the PVN, as assessed by c-FLI, beyond its activating effect on neurons in the ARC [42]. It has been shown before that NPY-positive neurons from the ARC project to the medioparvocellular part of the PVN (mpPVN) [3,4] and ghrelin

injected peripherally induces *c-fos* expression in NPY-positive neurons in this hypothalamic area [42]. Thus, it seems reasonable to assume that peripheral ghrelin induces c-FLI in the PVN via NPY-positive projections from the ARC. Further morphological studies will have to be performed to support this hypothesis of an interaction between NPY-positive fibers and c-FLI-positive neurons in the PVN.

While an increased neuronal activation in the PVN after peripheral ghrelin administration fits well into the concept of satiety regulation by the arcuate–paraventricular hypothalamic axis, there are other possible reasons for the increased *c-fos* expression in this brain nucleus. Behavioral studies have shown that centrally administered ghrelin participates in the expression of anxiety-like behavior in rats [8]. As the PVN is involved in a neuronal network mediating the autonomic, neuroendocrine, and skeletal-motor responses of fear and anxiety [10], an increased density of c-FLI-positive neurons in the PVN after peripheral ghrelin administration could also be the result of ghrelin transport into the brain and an activation of the hippocampal–amygdalic–hypothalamic network involved in the regulation of fear and anxiety rather than an activation of hunger/satiety pathways along the arcuate–paraventricular axis. In fact, several rat models of anxiety are associated with an increased density of c-FLI in the medioparvocellular region of the PVN [17]. However, in these anxiety models an increased density of c-FLI was also observed in various other brain nuclei [17], which we did not observe in the present study. Thus, it seems unlikely that the increased c-FLI in the PVN is part of a widespread neuronal activation due to increased anxiety. Furthermore, we did not perceive any gross changes in behavior related to fear or anxiety in the experiments.

Besides activating neurons in the PVN via projections from other brain nuclei, it is also possible that ghrelin directly activates areas inside the blood–brain barrier (BBB) such as the PVN, as the mRNA of the GHS-receptor is expressed in the PVN as well as in the ARC [20]. However, it is unclear at this point if rat ghrelin can cross the BBB, as pharmacokinetic studies have only been performed for mouse and human ghrelin in mice [5].

Apart from humoral pathways, peptides from the gastrointestinal tract may also influence energy balance by acting on vagal afferents [31]. Indeed, it has been shown that the effect of ghrelin on food intake and on *c-fos* expression in the ARC can be abolished by destruction of sensory vagal afferents through perivagal capsaicin treatment and by subdiaphragmatic or gastric vagotomy in rats [15] and mice [1]. Since the NTS in the brainstem is the termination area of the vagus nerve receiving viscerosensory information from the gastrointestinal tract [9,35], it seemed reasonable to assume that peripheral ghrelin induces *c-fos* expression in the NTS. Our data do not support this hypothesis, as we were not able to detect any

increased density of c-FLI-positive neurons in the dorsal vagal complex as previously observed in mice [42]. This may be explained by the fact that ghrelin has been shown to decrease the afferent discharge activity of a gastric vagal nerve filament [15]. Thus, ghrelin seems to have an inhibitory rather than an activating effect on afferent vagal neurotransmission, which may not be associated with an increased c-FLI in brain stem neurons [23].

In conclusion, in the present study we have provided evidence that the PVN is activated in response to peripheral injection of ghrelin at a dose increasing food intake in freely fed rats as shown by the 2.4-fold increase in Fos expression in the medioparvocellular part of the PVN. Based on previous demonstrations that peripheral ghrelin activates NPY-positive neurons in the ARC, it is suggested that ghrelin activates neurons in the PVN indirectly via NPY-positive projections from the ARC. Further histochemical and tracing studies on the possible connections between the ARC and the PVN via NPY-positive projections will have to be performed to strengthen this hypothesis. In addition, future studies will have to clarify the role of vagal afferents in mediating the effects of peripheral ghrelin on food intake. From the data presented here, the action of peripheral ghrelin on central nervous system control of energy balance does not seem to involve an activation of neurons in the NTS, as assessed by c-FLI.

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