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Ghrelin in the lateral parabrachial nucleus influences the excitability of glucosensing neurons, increases food intake and body weight

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Abstract

Ghrelin plays a pivotal role in the regulation of food intake, body weight and energy metabolism. However, these effects of ghrelin in the lateral parabrachial nucleus (LPBN) are unexplored. C57BL/6J mice and GHSR^{-/-} mice were implanted with cannula above the right LPBN and ghrelin was microinjected via the cannula to investigate effect of ghrelin in the LPBN. *In vivo* electrophysiological technique was used to record LPBN glucose-sensitive neurons to explore potential underlying mechanisms. Microinjection of ghrelin in LPBN significantly increased food intake in the first 3 h, while such effect was blocked by [D-Lys3]-GHRP-6 and abolished in GHSR^{-/-} mice. LPBN ghrelin microinjection also significantly increased the firing rate of glucose-excited (GE) neurons and decreased the firing rate of glucose-inhibited (GI) neurons. Additionally, LPBN ghrelin microinjection also significantly increased c-fos expression. Chronic ghrelin administration in the LPBN resulted in significantly increased body weight gain. Meanwhile, no significant changes were observed in both mRNA and protein expression levels of UCP-1 in BAT. These results demonstrated that microinjection of ghrelin in LPBN could increase food intake through the interaction with growth hormone secretagogue receptor (GHSR) in C57BL/6J mice, and its chronic administration could also increase body weight gain. These effects might be associated with altered firing rate in the GE and GI neurons.

Key Words

- ▶ ghrelin
- ▶ lateral parabrachial nucleus
- ▶ food intake
- ▶ glucose-sensitive neurons
- ▶ energy metabolism

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Introduction

Ghrelin is a 28 amino acids acylated peptide, which was first discovered as an endogenous natural ligand for the growth hormone secretagogue receptor (GHSR) 1 α by Kojima *et al.* in 1999 (1). Ghrelin was first described to stimulate the release of growth hormone from the pituitary (1, 2, 3). Then it was reported to be also involved in the regulation of feeding (4), body weight (5), and

energy metabolism (6). A key role had been established for ghrelin in energy metabolism regulation. Ghrelin acts on its receptor GHSR, a G-protein-coupled receptor in diverse central and peripheral areas to exert multiple effects (7). In peripheral, circulatory ghrelin is primarily produced by X-A cells in stomach (8) and its main orexigenic effects are exerted in the brain. In central, ghrelin-producing

neurons are located in the hypothalamus (1, 9, 10), while GHSR were expressed dispersedly and widely in forebrain, hypothalamus and hindbrain (11, 12), indicating that ghrelin might act as a neuroregulatory transmitter in various brain regions. Previous studies have shown that ghrelin injection into hypothalamic nuclei such as arcuate nucleus (ARC), paraventricular nucleus (PVN) and lateral hypothalamic nucleus (LH) could induce polyphagia and body weight gain (13). These effects were also observed following ghrelin injection into the fourth ventricle and dorsal vagal complex (DVC) in brainstem (14). Additionally, GHSR mRNA has been detected in the lateral parabrachial nucleus (LPBN) (11, 15). However, whether LPBN ghrelin-signaling mediates the regulation of energy balance remains unknown.

LPBN is a brain structure located in the dorsolateral pons that surrounds the superior cerebellar peduncle (16), and the latter has become an increasingly recognized region involved in the control of food intake and energy balance. Abundant direct nerve projections are present between LPBN and hypothalamus or brainstem. Moreover, many feeding relevant peptides including PYY (17), leptin (18) and glucagon-like peptide-1 (GLP-1) (19) are involved in the control of food intake in the LPBN. Besides, our recent research showed that administration of anorexia peptide nesfatin-1 in rats' LPBN could modulate food intake, body weight and enhance uncoupling protein1 (UCP-1) expression in brown adipose tissue (BAT) (20). However, the acute and chronic effects and mechanisms of ghrelin in the LPBN on feeding behavior and energy expenditure are yet to be elucidated.

Glucose is crucial to the central control of energy homeostasis (21). The neuronal glucosensation was first defined by Mayer in 1953 as that certain neurons could regulate food intake by transducing glucose level fluctuations into neural signals (22). Glucose-sensitive (GS) neurons possess the ability to alter their firing rates in response to fluctuating ambient glucose concentrations (23) as well as participating in food intake regulation (24, 25). GS neurons are classified as glucose-excited (GE) neurons and glucose-inhibited (GI) neurons. The GI neurons play especially important roles in the counterregulatory response to hypoglycemia (25, 26). A recent study showed that ghrelin is permissive for the counterregulatory response (27), indicating that ghrelin signaling might be closely related to the physiological function of GS neurons. In fact, these specific neurons distribute widely in the central nervous system (CNS), and our previous studies have demonstrated that they were involved in the mechanisms of feeding regulations in PVN,

LH and ventromedial nucleus (VMH) (24), as well as DVC in brainstem (25). Recently, LPBN was proposed to serve as a novel glucose-sensitive territory in the brain, in addition to the well-known hypothalamic and hindbrain centers (28, 29), which aroused our interest. Our previous studies proposed that ghrelin could influence the excitability of GS neurons in rats and take part in regulating food intake in hypothalamic nucleus (PVN, VMH, LH) (30). With the addition of our recent finding that GS neurons were associated with the feeding and energy regulation in LPBN (20).

In the current study, we investigated the influence of LPBN ghrelin in the firing rate of GS neurons by the *in vivo* electrophysiological technique. Then we explored whether LPBN ghrelin could increase the cumulative food intake and body weight and whether GS neurons were involved in the mechanism of ghrelin's effects mentioned previously.

Materials and methods

Animals

Adult male wild-type (GHSR^{+/+}) and GHSR-knockout (GHSR^{-/-}) mice (on a C57BL/6J background) were housed in standard rodent cages. The original GHSR^{-/-} mice were purchased from Shanghai Research Center For Model Organisms Of China, and the GHSR^{-/-} mice were obtained from crosses between heterozygous and homozygous knockout animals that were backcrossed over 10 generations (31). Animals (ages 8 weeks) were housed in a temperature-controlled (23 ± 2°C) animal room (illumination from 7:00 to 19:00), with free access to standard food and tap water for at least 1 week to adapt to their surroundings. The experimental protocols were approved by the Qingdao University Animal Care and Use Committee and Animal Welfare Committee (No. QYFY WzLL25734) in accordance with the National Institutes of Health guidelines.

Reagents

Ghrelin was purchased from Tocris Bioscience (No. 1463/1). [D-Lys3]-GHRP-6 (GHSR antagonist) was purchased from APEXBio (No. B5234). Pontamine sky blue (C8679) and glucose (G7528) were purchased from Sigma-Aldrich (for electrophysiology experiment). 0.9% NaCl and glucose were obtained from Qingdao University hospital. The chloral hydrate was purchased from Tianjin

Ruijinte chemical company. All other chemicals are of the highest grade obtainable.

Surgery

C57BL/6J mice and $GHSR^{-/-}$ mice were anesthetized with a combination of chloral hydrate (400 mg/kg, i.p.) and ketorolac (1 mg/kg, i.m.) (32) and then positioned in a stereotaxic apparatus (SN-3; Narishige, Tokyo, Japan) for implantation of a 26-gauge chronic guide cannula above the right LPBN without damaging the target area. The placement coordinates for the LPBN were: anteroposterior 5.0 mm, lateral 1.2 mm and ventral 3.3 mm. After surgery, mice were individually housed and allowed to recover for 7 days before further experiments.

Experimental procedures

Experiment 1: Effects of LPBN ghrelin microinjection on nocturnal food intake in C57BL/6J mice and $GHSR^{-/-}$ mice

To measure the effects of LPBN ghrelin microinjection on nocturnal food intake in C57BL/6J mice, mice ($n = 13$) were randomly assigned into two groups: NS (0.5 μ L normal saline, $n = 7$) or ghrelin (0.5 μ L, 300 pmol/mouse, $n = 6$) (33). On the day of experiment, mice were fasted for 2 h, from 16:00 to 18:00. At 18:00, mice were administered NS or ghrelin with a finer injector via a microsyringe extended below the guide cannula. Then, 12 h food intake after refeeding was measured using an electronic scale (TE412-L; 110 Sartorius, Gottingen Germany). Schematic diagram of catheterization and method for determination of injection site were both as described in our previous study (20).

To investigate whether these effects of LPBN ghrelin on nocturnal food intake were mediated through $GHSR$, C57BL/6J mice ($n = 22$) were randomly assigned into four groups: NS (0.5 μ L normal saline, $n = 5$) or ghrelin (0.5 μ L, 300 pmol/mouse, $n = 7$) or [D-Lys3]-GHRP-6 ($GHSR$ antagonist, 0.5 μ L, 10 nmol/mouse, $n = 5$) or co-injection of ghrelin and [D-Lys3]-GHRP-6 (0.5 μ L, $n = 5$). $GHSR^{-/-}$ mice ($n = 7$) were randomly assigned into two groups: NS (0.5 μ L normal saline, $n = 3$) or ghrelin (0.5 μ L, 300 pmol/mouse, $n = 4$). The experimental treatment is the same as described previously, 3 h food intake after refeeding was measured.

Experiment 2: Effects of LPBN ghrelin microinjection on long-term body weight gain in C57BL/6J mice

To investigate the effects of chronic LPBN ghrelin administration on long-term body weight gain, two

groups—C57BL/6J mice (NS (0.5 μ L normal saline, $n = 7$) as control group and ghrelin (0.5 μ L, 300 pmol/mouse/day, $n = 6$)) as treatment group were injected with NS or ghrelin (13). Mice were fasted for 2 h before daily monitor of their body weight before the injection. Ghrelin injections were administered at 18:00 daily from day 1 and lasted for 7 days. On the 8th day's morning, animals were killed.

Experiment 3: Effects of ghrelin microinjection on glucosensitive neurons in the LPBN

C57BL/6J mice ($n = 44$) were anesthetized with urethane (1 g/kg body weight, i.p.) and positioned on a stereotaxic apparatus. The placement coordinates for the LPBN were: anteroposterior 5.0–5.2 mm, lateral 1.0–1.2 mm and ventral 3.3–3.4 mm.

Four-barrel glass microelectrodes (total tip diameter 3–10 μ m, resistance 5–20 MV) were used for electrophysiological recording and micro-pressure injection (20, 30): 5 mM glucose solution, 0.9% NaCl, 1.5×10^{-8} M ghrelin and 0.5 M sodium acetate in 2% pontamine sky blue for recording. During the electrophysiological recording, the first three microelectrodes were connected to a four-channel pressure injector (PM2000B; Micro Data Instrument, Inc., USA) to inject drugs by gas pressure (30). Drugs were directly ejected on the surface of firing cells with short pulse gas pressure (1500 ms, 5.0–15.0 psi) (34). The recorded electrical signals were amplified and displayed on a Memory Oscilloscope (VC-11, Nihon Kohden) as described in our previous study (35). The acquired analog signals were fed into a signal analyzer and then relayed to computer for analysis with Histo software. Although the firing frequencies of neurons vary, the sampling threshold was set to twice the background noise level, signals below which were considered as background noises and therefore not recorded to improve the accuracy of the measurements.

After recording the firing frequencies of neurons in the LPBN, we injected 5 mM glucose solution to identify whether they were glucose-sensitive neurons. After the firing frequencies returned to baseline, 0.9% NaCl was given as a control. Next, the 1.5×10^{-8} M ghrelin was administered to investigate the effects of ghrelin on glucose-sensitive neurons in the LPBN. At the end of each experiment, pontamine sky blue was injected through the electrode to confirm positions of the recording electrode.

To distinguish between GE and GI neurons, the spontaneous firing frequency before and after injection of 5 mM glucose solution were recorded and analyzed. Neurons with an elevation of firing rate greater than

20% were identified as GE neurons, while those with a reduction of firing rate greater than 20% were identified as GI neurons. Cells with firing rate changes less than 20% were classified as glucose-insensitive neurons.

After the spontaneous firing frequency of identified GS neurons had returned to the baseline, ghrelin was injected. If more than 20% changes in spontaneous firing frequency were observed (increased or decreased), the neurons were recorded and analyzed.

Experiment 4: Effects of chronic LPBN ghrelin administration on UCP-1 expression in brown adipose tissue

In order to assess the UCP-1 expression at both mRNA and protein levels, BATs from interscapular region were collected soon after the mice were killed in experiment 2.

Western blot

The BATs were homogenized in RIPA buffer (P0013B, Beyotime, Beijing, China) supplemented with 1:100 protease inhibitors (P1005, Beyotime, Beijing, China) and centrifuged at 14,000 *g*, 10 min. Protein concentrations were adjusted with BCA assay (P0012, Beyotime, Beijing, China), denatured in SDS sampling buffer (95°C, 5 min) and then subjected to 12% SDS-PAGE. Protein samples were then electrotransferred to a polyvinylidene fluoride membrane (Millipore Corp.) for 2 h. After blocking with 5% fetal bovine serum (FBS) for 2 h at room temperature, membranes were incubated with primary antibodies: UCP-1 (ab10983, 1:2000; Abcam); β -actin (#4967, 1:4000 CST, Danvers, MA, USA) at 4°C overnight and with secondary antibodies (goat anti-rabbit IgG H&L (HRP (ab6721, Abcam, 1:2000)) at room temperature for 1 h. Bands were visualized with Immobilon Western chemiluminescent substrate (Millipore, cat. no. WBKLS0100, 200 μ L) and UVP 810 gel-imager (UVP, Upland, CA, USA), and the intensities were analyzed using Image J software (NIH, USA).

Real-time PCR

TRIzol reagents (135306; Ambion) were used to extract RNA from BATs. We used 4 \times gDNA Wiper Mix to rinse genomic DNA, and used 5 \times HisScript II qRT SuperMix II form RNA reverse-transcription. Relative mRNA levels were determined using the SYBR Green RT-PCR Kit (Q311; Vazyme Biotech Co., Ltd., Nanjing, China) and the Realplex Real Time PCR Thermocycle Instrument

(Realplex 4; Eppendorf, Westbury, NY, USA). Primer sequences we used in this study were as follows: UCP-1: 5'-A C T G C C A C A C C T C C A G T C A T T-3', 5'-C T T T G C C T C A C T C A G G A T T G G-3'; β -actin: 5'-A G G C C C A G A G C A A G A G A G G T A-3', 5'-G G G G T G T T G A A G G T C T C A A A C A-3' (32). Real-time PCR data were analyzed using the 2^{- $\Delta\Delta$ Ct} method. Ribosomal L32 mRNA levels were used as the internal control.

Experiment 5: Effects of LPBN ghrelin microinjection on c-fos expression in LPBN.

C57BL/6J mice ($n = 6$) were killed 2 h after injection of NS ($n = 3$) or ghrelin ($n = 3$) into the LPBN. Then brains were carefully dissected for subsequent immunohistochemistry.

Immunohistochemistry

Brains samples were fixed with 4% paraformaldehyde for 24 h and were transferred into 30% sucrose at 4°C for 12 h. Samples were then cryo-sectioned into 15 μ m sections by microtome (CM1860; Leica) and dried at 60°C in an incubator (DHG-9101; SANFA, Yangzhou, China) for 4 h. Sections were then hydrated in 0.01 M PBS for 15 min, antigen retrieval was performed following manufacturer's instructions (P0083, Beyotime, Beijing, China). Sections were further washed with 0.01 M PBS for three times, blocked with 0.1% FBS for 1 h at 37°C, and then incubated with a c-fos primary antibody (rabbit antimouse, ab190289, 1:1000; Abcam) for 2 h at 37°C and HRP (horse radish peroxidase) secondary antibody (PV-6001, 1:200; Zsbio, Tianjin, China) for 20 min at 37°C. Between incubation by antibody and after incubation by secondary antibody, sections were washed as described previously. A DAB kit (ZLI-9018; Zsbio) was used to develop the staining. Morphology was assessed using a light microscope (CX31; OLYMPUS). According to the mouse brain in stereotaxic coordinates (36), LPBN is located in the dorsolateral pons that surrounds the superior cerebellar peduncle (scp), which we considered as the neuroanatomical landmark to delimit LPBN in this work. By using ImagePro Plus 6 (Media Cybernetics, Inc. USA), the number of c-fos immunopositive neurons within LPBN were counted.

Statistical analysis

Data are expressed as means \pm s.e.m. Statistical analyses were performed using a commercially available statistical package (SPSS 17.0). Normal distribution was confirmed with Shapiro–Wilk test, and homogeneity of variances was determined with Levene's test. We applied Student's *t*-test

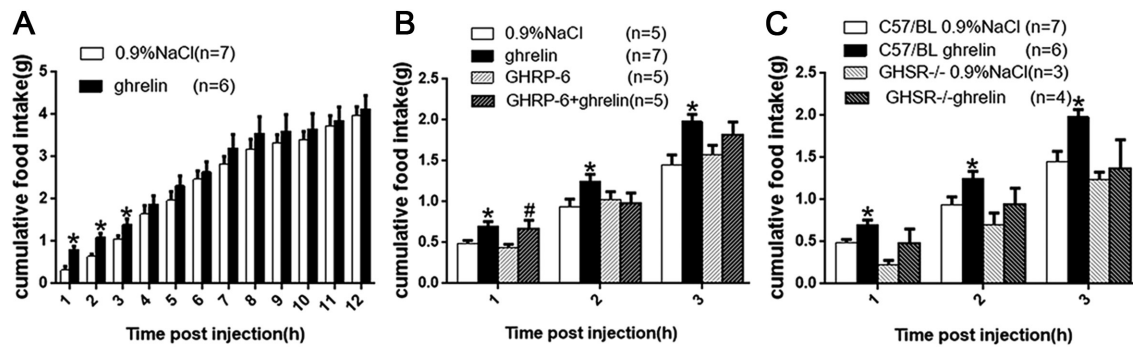


Figure 1

Effects of ghrelin injection in LPBN on nocturnal feeding in mice. (A) Ghrelin increased food intake in 1, 2, 3 h after injection of ghrelin in LPBN. (B) [D-Lys3]-GHRP-6 blocked the effects of LPBN ghrelin on food intake in 2, 3 h after injection of ghrelin in LPBN. (C) Ghrelin receptor knockout abolished the effects of LPBN ghrelin on food intake in 1, 2, 3 h after injection of ghrelin in LPBN. * $P < 0.05$ ghrelin vs 0.9% NaCl, # $P < 0.05$ GHRP-6+ghrelin vs GHRP-6.

to analyze statistical differences between two groups, to compare firing rates before and after drug treatment, and one-way ANOVA for multiple groups. Data were analyzed using repeated measures analyses of variance where appropriate. Degree of freedom and the statistic's value were also reported. In all cases, $P < 0.05$ was considered to be significant.

Results

Effects of ghrelin injection in the LPBN on nocturnal feeding in mice

Comparing with the 0.9% NaCl group, ghrelin (300 pmol) injection into the LPBN significantly increased the cumulative food intake at 1st, 2nd and 3rd h (1st h 0.81 ± 0.07 vs 0.31 ± 0.09 , $P < 0.05$; 2nd h 1.10 ± 0.09 vs 0.64 ± 0.05 , $P < 0.05$; 3rd h 1.40 ± 0.13 vs 1.04 ± 0.09 , $P < 0.05$; Fig. 1A).

The orexigenic effect of ghrelin was blocked by [D-Lys3]-GHRP-6 when comparing co-injection group with ghrelin only group (1st h 0.67 ± 0.10 vs 0.43 ± 0.05 , $P < 0.05$; 2nd h 0.98 ± 0.12 vs 1.03 ± 0.10 , $P > 0.05$; 3rd h 1.82 ± 0.15 vs 1.57 ± 0.12 , $P > 0.05$; Fig. 1B). And it was further abolished in GHSR^{-/-} mice when comparing GHSR^{-/-} mice injected with ghrelin to GHSR^{-/-} mice injected with NaCl (1st h 0.48 ± 0.17 vs 0.23 ± 0.05 , $P > 0.05$; 2nd h 0.94 ± 0.20 vs 0.70 ± 0.14 , $P > 0.05$; 3rd h 1.37 ± 0.34 vs 1.24 ± 0.08 , $P > 0.05$; Fig. 1C).

Effects of ghrelin injection in LPBN on chronic body weight gain in mice

For mice treated with 300 pmol ghrelin daily for 7 days, their body weight gain was significantly higher relative to

mice treated with 0.9% NaCl injection starting from the 4th day (4th day 1.45 ± 0.15 vs 0.82 ± 0.22 , $P < 0.05$; 5th day 1.97 ± 0.26 vs 1.17 ± 0.25 , $P < 0.05$; 6th day 2.19 ± 0.28 vs 1.12 ± 0.20 , $P < 0.05$; Fig. 2).

Effects of ghrelin on the LPBN glucosensitive neurons *in vivo*

Forty-four GS neurons were investigated in the LPBN, among which 40.9% were GE neurons and 38.6% were GI neurons (Table 1). The general average spike frequency of GE neurons in the absence and presence of glucose were 3.74 and 8.16 Hz, respectively, and the data of GI neurons were 4.40 and 1.88 Hz. Treatment with ghrelin (1.5×10^{-8} M) significantly increased the firing rates of GE neurons (3.19 ± 0.64 to 6.56 ± 1.21 Hz, $P < 0.05$; Fig. 3A) and decreased the firing rates of GI neurons relative to the control group (3.66 ± 0.77 to 1.51 ± 0.34 Hz, $P < 0.05$; Fig. 3B).

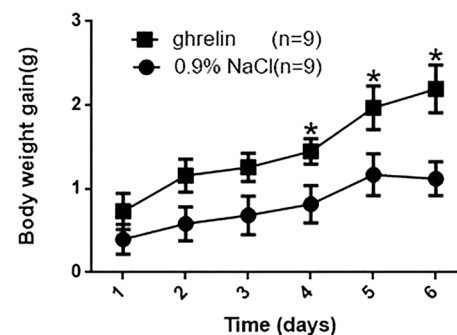


Figure 2

Effects of ghrelin injection in LPBN on long-time body weight gain in mice. After the injection of ghrelin, body weight gain was increased from the 4th day. * $P < 0.05$ ghrelin vs 0.9% NaCl.

Table 1 Numbers of LPBN neurons responsive to glucose (5 mM) and ghrelin (1.5×10^{-8} M).

LPBN neurons (<i>n</i> = 44)	Ghrelin responsive		Ghrelin nonresponsive
	Excited	Inhibited	
GE neurons (<i>n</i> = 18; 40.9%)	10 (55.6%)	4 (22.2%)	4 (22.2%)
GI neurons (<i>n</i> = 17; 38.6%)	3 (17.6%)	11 (64.7%)	3 (17.6%)
Glucose-insensitive neurons (<i>n</i> = 9; 20.4%)	3 (33.3%)	1 (11.1%)	5 (55.6%)

GE neurons, glucose-excited neurons; GI neurons, glucose-inhibited neurons.

Effects of long-term LPBN ghrelin on UCP-1 expression in BAT

After the administration of 300 pmol ghrelin in LPBN for 7 days, no remarkable differences were observed in the BAT expression of UCP-1 at either protein level (1.97 ± 0.24 vs 2.18 ± 0.32 , $P > 0.05$; Fig. 4A) or mRNA level (2.12 ± 0.44 vs 1.00 ± 0.69 , $P > 0.05$; Fig. 4B) relative to those of 0.9% NaCl injection.

Effects of LPBN ghrelin on c-fos expression in LPBN

Comparing with the 0.9% NaCl injection group, injection of 300 pmol ghrelin significantly increased c-fos expression in the LPBN (179.64 ± 10.76 vs 129.88 ± 14.67 , $P < 0.05$; Fig. 5).

Discussion

The underlying mechanism for regulation of feeding and body weight is crucial in understanding obesity pathogenesis. Since 2000, when ghrelin was found to promote food intake and body weight gain in rodents (37), numerous studies have corroborated these findings by administering ghrelin to rodents either peripherally or centrally (13, 37, 38).

Microinjection minimized drug diffusion in brain, thus facilitates the investigation of the drug effects on a specific nucleus. In the current study, ghrelin injection in LPBN significantly increased nocturnal cumulative food intake in the first 3 h, which was blocked by [D-Lys3]-GHRP-6 and abolished in GHSR^{-/-} mice. Therefore, our results demonstrated that ghrelin acted through GHSR to play orexigenic effect in the LPBN, which was consistent with the previously observed effects in the forebrain (13). An interesting phenomenon was presented in Fig. 1C that GHSR^{-/-} mice tend to have a small size food intake compared with the wide type C57 mice though the difference was not significant. This may result from the truth that GHSR was knocked out, which prevented endogenous ghrelin from orexigenic effect. However, it is worth noting that the sensation of forebrain and pons to ghrelin was different. Wren *et al.* (13) used various doses of ghrelin, and reported that the lowest dose of ghrelin to significantly stimulate food intake was 30 pmol, but they indeed resorted to 300 pmol to confirm whether nuclei were really nonresponsive or just requires a higher dose. In our preliminary experiments, we took the 300 pmol to confirm whether LPBN should be considered nonresponsive or just needs stronger stimulation. Interestingly, as reported, PVN, a nucleus that projects LPBN is among the nucleus in hypothalamus that

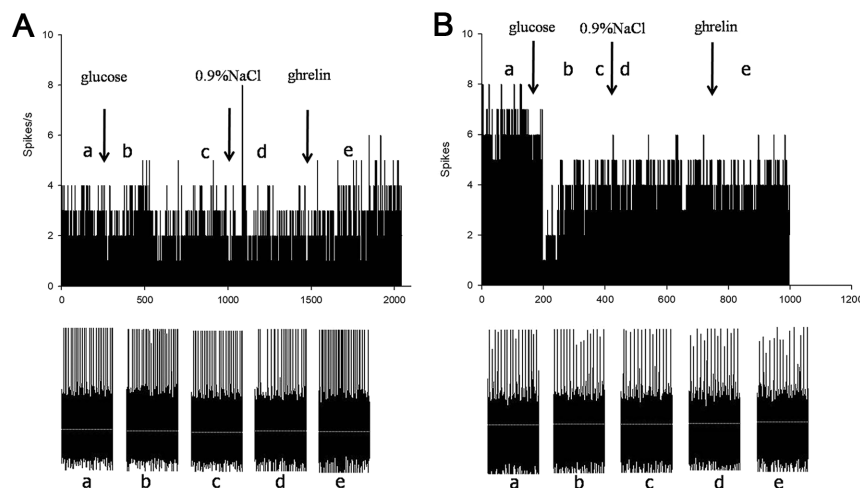


Figure 3

Effects of ghrelin on LPBN glucose-sensitive neurons *in vivo*. (A) Ghrelin of LPBN affects the activity of glucose-excited neurons. (B) Ghrelin of LPBN affects the activity of glucose-inhibited neurons. The first arrow indicates the addition of 5 mM glucose, the second arrow indicates the 0.9% NaCl-treated control, and the third arrow indicates ghrelin (1.5×10^{-8} M) application. (a) Baseline before glucose application. (b) Firing rate after glucose application. (c) Firing rate recovered to baseline before 0.9% NaCl application. (d) Firing rate after 0.9% NaCl application and the baseline before ghrelin application. (e) Firing rate after ghrelin application.

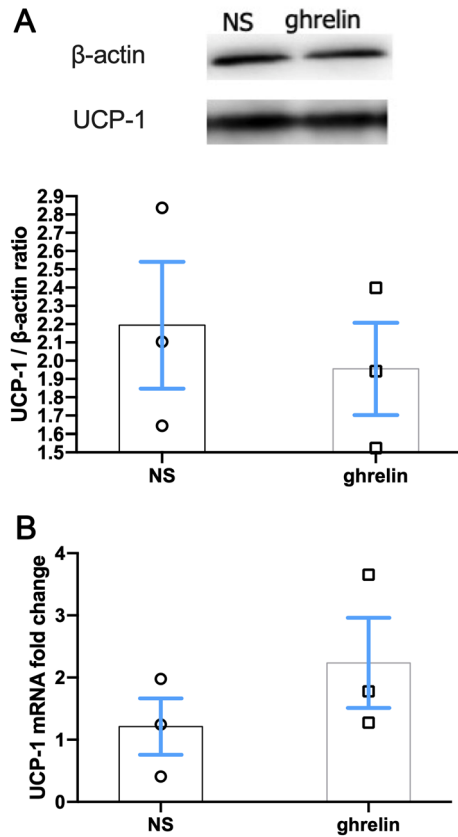


Figure 4 Effects of long-term LPBN ghrelin on UCP-1 expression in BAT. UCP-1 protein expression in BAT had no change in ghrelin group ($n = 7$) compared to 0.9% NaCl group ($n = 6$) (A), UCP-1 mRNA expression in BAT had no change in ghrelin group ($n = 5$) compared to 0.9% NaCl group ($n = 4$) (B) ($P > 0.05$).

only responded to 300 pmol but not 30 pmol ghrelin, suggesting that ghrelin signaling in LPBN probably is associated with PVN.

Moreover, the present study demonstrated that LPBN ghrelin injection increased the number of Fos-positive neurons in the LPBN for the first time, corroborated that ghrelin could increase food intake and body weight through GHSR in the LPBN. To the best of our knowledge, some of the GHSR-expressing sites, such as the Arc, have direct access to ghrelin circulating in the blood stream, as peripheral ghrelin and ghrelin mimetic administration induce c-fos expression there (39, 40, 41). However, the GHSR expression in CNS is fairly widespread. One possible explanation for GHSR expression in those sites without immediate access to the blood-brain barrier comes from the observations of ghrelin-producing central neurons (1, 42, 43). Those neurons were observed to produce ghrelin centrally, which would be readily available for those GHSR-

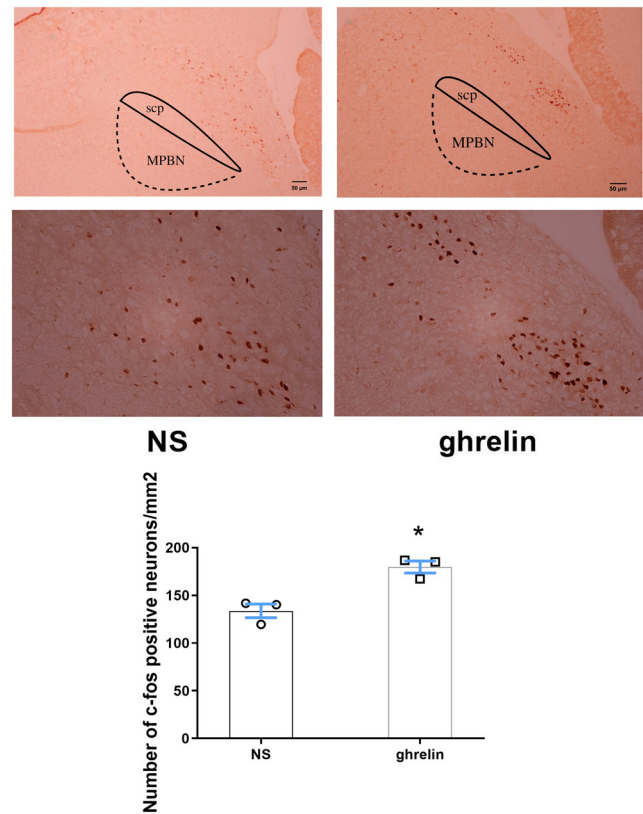


Figure 5 Effects of LPBN ghrelin on c-fos expression in LPBN. Microinjection of ghrelin in LPBN increased c-fos expression in LPBN, ghrelin group ($n = 3$), 0.9% NaCl group ($n = 3$). * $P < 0.05$ ghrelin vs 0.9% NaCl. scp, superior cerebellar peduncle; MPBN, medial parabrachial nucleus.

expressing nuclei without access to circulating ghrelin. Our experiments provided evidences of central ghrelin's effect in energy metabolism regulation as a neurotransmitter. However, the source(s) and physiological significance of central nerves system ghrelin remain to be determined.

In the current study, we focused on a group of specialized neurons in LPBN called GS neurons. Brain glucose regulation is crucial for the maintenance of cell activities, neurotransmitter synthesis, and nerve synapses (44, 45), in which the GS neurons play major roles. Significant increase in the firing rate of GE neurons and decrease in the firing rate of GI neurons were observed following administration of ghrelin in LPBN, along with increased food intake. These findings suggest that GS neurons in the LPBN might be involved in regulation of food intake. Our findings revealed that ghrelin's effect is at least partially associated with GS neurons in LPBN. Our finding expanded the knowledge about the neuronal mechanism of LPBN mediated food intake regulation as well as the area/function of ghrelin. While the route of

administration used in electrophysiology experiment achieved ghrelin treatment on single targeted neuron and measured responses, further evidences, such as utilization of knockout mice as control and/or investigate specific current with *in vitro* patch clamp technique are necessary before a conclusion may be reached regarding whether such effect is a direct effect or indirect through the release of other peptides. Further investigations are planned.

Maintaining energy balance is about reaching the balance between energy intake and expenditure. The changes in body weight result from changes in such balance (46). In the current study, chronic administration of ghrelin in the LPBN did not result in remarkable differences in either protein level or mRNA level of UCP-1 in brown adipose tissue, implying that the ghrelin treatment for 7 days was not enough to translate into a robust expression change of UCP-1 in BAT. Notably, the mean mRNA levels seemed to be elevated in ghrelin-treated samples (not statistically significant). This effect will be further pursued. Studies have raised that ghrelin administration increased body weight gain through promoting a positive energy balance which led to high food intake and low energy expenditure (4, 38). Additionally, ghrelin was found to promote adipose accumulation, reduce storage of lipids and then reduce energy expenditure (47, 48). The next step could be studying its effect on fat mass.

Conclusion

Ghrelin in the LPBN could increase nocturnal cumulative food intake through the interaction with GHSR in C57BL/6J mice, and influence the firing rate of GS neurons in the LPBN. Long term ghrelin administration increased the body weight gain. It was also found to promote c-fos expression in the LPBN.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J D, Z J, C Z and J Y conceived and designed the experiments. C Z, X C, J Y, Q L, M L, L W, R W, K Z and X L C performed the experiments. C Z and J Y analyzed the data. C Z, J Y and J D wrote the paper. J D, C Z, J Y and B W edited the manuscript. All authors read and approved the final manuscript.

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