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Research Paper

Ghrelin acts on rat dorsal vagal complex to stimulate feeding via arcuate neuropeptide Y/agouti-related peptide neurons activation

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Abstract: Ghrelin, an endogenous ligand for the growth hormone secretagogue (GHS) receptor, stimulates feeding and increases body weight. The primary action site of ghrelin has been reported to be the neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons in the hypothalamic arcuate nucleus (ARC). In addition to the hypothalamus, the caudal brainstem also appears to be an important mediator for the orexigenic activity of ghrelin. However, it is not clear whether ghrelin applied directly to the caudal brainstem activates forebrain structures. The aim of this study was to determine whether recruitment of forebrain structures was required for hyperphagic responses stimulated by ghrelin delivery within the caudal brainstem. In our experiment, all rats were surgically implanted with indwelling cannulas in the dorsal vagal complex (DVC), and ghrelin (20 pmol in 0.5 μL) was delivered to the DVC. After the injection, the orexigenic response to ghrelin was recorded by Feeding and Activity Analyser, and NPY/AgRP mRNA expressions in rat hypothalamus were detected by real-time PCR. In addition, the NPY immunoreactive neurons in the ARC were assayed by immunohistochemistry. The results showed that ghrelin significantly increased cumulative food intake at 1, 2 and 3 h after ghrelin injection, maximal response occurring at 2 h after injection. NPY/AgRP mRNA levels in ARC treated with ghrelin increased significantly compared with those in control group (injected with saline). The highest levels of NPY and AgRP mRNA were detected at 2 h after injection. The total number and mean optical density of NPY-positive neurons increased in ghrelin treated rats compared with those in control group. Consistently, ghrelin's effect was most pronounced at 2 h after injection. Taken together, we conclude that the activation of NPY/AgRP neurons in the ARC is involved in the mediation of the hyperphagic response to brainstem ghrelin administration in neurologically intact rats.

Key words: ghrelin; food intake; dorsal vagal complex; arcuate nucleus; neuropeptide Y/agouti-related peptide; rat

大鼠延髓背侧迷走复合体注射 ghrelin 激活弓状核神经肽 Y/ 刺鼠色蛋白相关蛋白神经元而诱发多食

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摘 要：Ghrelin 是生长素促分泌受体的内源性配体，刺激摄食并增加体重。已有研究证实 ghrelin 刺激摄食的作用靶点主要是下丘脑弓状核(hypothalamic arcuate nucleus, ARC)内的神经肽 Y (neuropeptide Y, NPY)/ 刺鼠色蛋白相关蛋白(agouti-related peptide, AgRP)神经元。除下丘脑外，脑干尾部迷走复合体具有 ghrelin 受体，是 ghrelin 调控摄食活动的另一靶点。本实验旨在验证 ghrelin 作用于脑干尾部所诱发的摄食增加是否需要下丘脑 NPY/AgRP 神经元参与。在大鼠延髓背侧迷走复合体(dorsal vagal complex, DVC)微量注射 20 pmol 的 ghrelin，用摄食自动分析仪测量大鼠的摄食反应，用荧光定量 PCR 技术测定 ARC 的 NPY/AgRP mRNA 的表达水平，同时利用免疫组化技术测定 ARC 的 NPY 阳性神经元数量及光密度。结果显示，与对照组(DVC 微量注射生理盐水)相比，ghrelin 微注射组大鼠摄食量增加，其累积摄食量在注射后 2 h 达最高峰；ARC 处 NPY/AgRP mRNA 的表达水平、NPY 免疫阳性神经元的数量及光密度也明显增加，且均在 ghrelin 注射后 2 h 增高达到高峰。以上结果提示，大

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鼠 DVC 注射 ghrelin 可能通过上行纤维激活弓状核 NPY/AgRP 神经元，介导大鼠的多食反应。

关键词：ghrelin；摄食；背侧迷走复合体；弓状核；神经肽 Y/ 刺鼠色蛋白相关蛋白；大鼠
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Ghrelin, an acetylated 28-amino acid peptide isolated from mammalian stomach, has been identified as the endogenous ligand for growth hormone secretagogue receptors (GHS-R)[1]. Ghrelin is a potent stimulant of growth hormone release and can independently elicit an increase in food intake and a decrease in fat utilization. Many studies suggest a physiological role of ghrelin in the initiation of feeding[1-5]. There is considerable interest as to the mechanism by which ghrelin exerts its appetite-stimulating effects, and, in particular, the action sites of ghrelin in the central nervous system (CNS). Originally, the primary action site of ghrelin seems to be the arcuate nucleus (ARC) of the hypothalamus[6]. In support, Tamura *et al*.[7] showed that ablation of ARC neurons by neonatal monosodium glutamate (MSG) treatment resulted in a loss of the appetite-stimulating effects of ghrelin, as did double knockout of the potent orexigenic neurotransmitters neuropeptide Y (NPY) and agouti-related peptide (AgRP)[8]. Furthermore, Kohno *et al*.[9] demonstrated that ghrelin directly interacted with NPY neurons in the ARC to elicit an increase in cytosolic Ca^{2+} concentration. Together, these observations support the hypothesis that the NPY/AgRP neurons within the ARC are suggested to be the primary location for the orexigenic activity of ghrelin. However, GHS-R is present in numerous other brain sites in which the effects of ghrelin have been tested[10,11]. Ghrelin administration into the hypothalamic paraventricular nucleus (PVN)[12,13] or the ventral tegmental area (VTA)[14] also stimulated feeding, and ghrelin may stimulate food intake through a direct action at GHS-R in the caudal brainstem[15]. Our previous study demonstrated the the presence of GHS-R1a in the rat nodose ganglion and dorsal root ganglion[16]. However, there are conflicting reports concerning the precise mechanism by which caudal brainstem ghrein administration induces hyperphagia. Kinzig *et al*.[17] have provided evidence that the fourth ventricle ghrelin (1 nmol) injection increases food intake by a mechanism involving an activation of NPY neurons in ARC. More recently, Faulconbridge *et al*.[18] demonstrated that the fourth ventricle ghrelin (150 pmol) injections increased Fos expression only in the nucleus of the solitary tract (NTS), but not in the ARC or the PVN, indicating that the ingestive response to caudal brainstem ghrelin administration does not depend on the activation of neurons in the PVN or ARC. The lack of Fos expression in these forebrain nuclei after caudal brainstem administration of ghrelin is in clear contrast to other studies[19,20] reporting increases of Fos in these structures after peripheral or forebrain delivery. The reason for the aforementioned discrepancies among the various Fos expression studies is unclear, but likely includes differences in doses of ghrelin and the experimental injections used. The anatomical extent of the neural substrates necessary and sufficient for the mediation of these responses, however, is not completely understood, and remains the topic of some controversy. Here we studied the effects of low-dose injections of ghrelin into the dorsal vagal complex (DVC) parenchyma on the hypothalamic NPY/AgRP neurons and the underlying mechanism.

1 MATERIALS AND METHODS

1.1 *Animals*

Healthy, male Sprague-Dawley rats (from Laboratory Animal Center of Shandong University of Traditional Chinese Medicine, license: SCXK Lu 20050015), weighing 250-300 g, were housed in hanging stainless steel cages under 12/12 h light/dark cycle (lights on from 8: 00 am). Rats had *ad libitum* access to water and food. All animal experiments were carried out in accordance with the guidelines of Qingdao University for animal care.

1.2 *Surgery*

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p) and mounted on a stereotaxic apparatus (Narishige SN-3, Tokyo, Japan) in the prone position. All rats were surgically implanted with indwelling DVC cannulas. Using aseptic techniques, the skull was exposed, head screws were inserted, and a 22-gauge stainless steel cannula was implanted into the DVC (AP: 13.24-13.8 mm, ML: 0.4 mm, DV: 8.0 mm) according to the atlas of Paxinos and Watson[21]. After anchoring the cannula and sealing all skull openings with dental acrylic, the incision was sutured, and a 28-gauge obturator was placed in the cannula.

1.3 *Ghrelin preparation and injection*

Rat ghrelin (American Peptide Co. Inc., USA) was dissolved in sterile saline and stored at -20 ºC. Just before injection, ghrelin was diluted in sterile saline with a final

concentration (20 pmol/0.5 μL). This dose is known to be a moderate, suprathreshold dose for the feeding response upon brainstem parenchyma delivery[15]. On day 7 after surgery, ghrelin or saline was delivered to the DVC parenchyma in 2 min with a 28-gauge injector, and the injector was kept in place for an additional 10 min. All injections were delivered at about 12: 00 am[22].

1.4 *Feeding responses to DVC ghrelin injection*

To perform a feeding response test, twenty nine rats were divided into ghrelin group (n=15) and control group (n=14). Ghrelin group received 20 pmol ghrelin in 0.5 μL of volume, and control group were injected with equal volume of saline. Food intake was measured at 1, 2, 3 and 4 h after injection by measuring the weight of the food containers with electronic precision scales (Feeding and Activity Analyser 47552-002, UGO BASILE, Italy). The outputs were continuously monitored by Data Acquisition software 51800 (Feed-Drink Mointoring System Ver 1.31, UGO BASILE, Italy). Cumulative food intakes were recorded. At the end of the test, all the brains of the rats were stained with pontamine sky blue and sectioned in the coronal plane on a freezing microtome (Kryostat 1720, Leica, Germany) at a thickness of 50 μm to verify the location of the cannula. Only data from the rats whose injection sites were located within the DVC were included in the study (Fig. 1).

1.5 *NPY and AgRP gene expression*

1.5.1 *Experimental animals*

The rats were randomly divided into 2 groups, ghrelin group and control group. Each group included three subgroups (each n=12) according to the time (1, 2, 3 h) after the injections into the DVC parenchyma.

1.5.2 *Total RNA extraction*

At 1, 2 and 3 h after ghrelin (or saline) injection, the rats were sacrificed. The rat hypothalamus was isolated and total RNA was extracted by the TRIzol reagent method (Invitrogen, Carlsbad, CA). Confirmation of intact 18S and 28S RNA bands was achieved with agarose gel electrophoresis. The concentration and purity of RNA were determined by Eppendorf Biophotometer (Eppendorf,

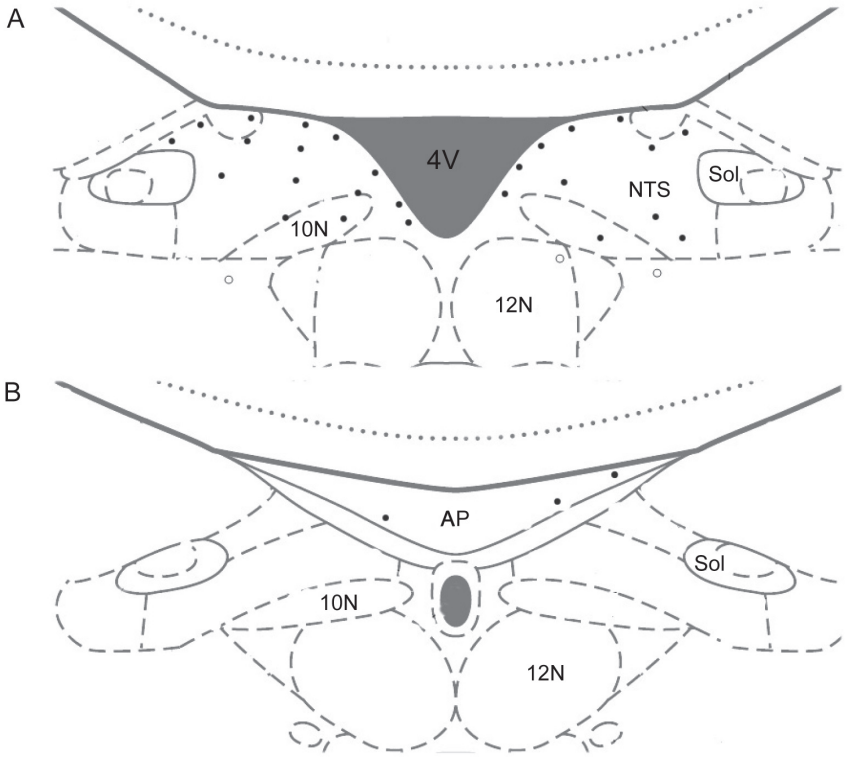

Fig. 1. Reconstruction of dorsal medullary injection sites. Black dots or open circles indicate the location of the injector tip. Of the 29 cannula placements, 26 were located in a position within 1 mm anterior to the AP, i.e. twenty six spots (black dots) were checked to lie within the dorsal vagal complex (DVC), and three spots (open circles) were checked to lie outside the DVC. Placements shown were -13.24 mm from bregma (*A*). The other 3 injection sites were at the level of the AP (*B*), -13.80 mm from bregma. AP, area postrema; Sol, solitary tract; 4V, the fourth ventricle; NTS, nucleus of the solitary tract; 10N, dorsal motor nuclear of the vagus nerve; 12N, hypoglossal nucleus.

Germany).

1.5.3 *Primers and real-time PCR*

Single-stranded cDNA was synthesized with a reverse transcription kit (Fermentas, Lithuania). Rat hypothalamic expressions of NPY and AgRP mRNA were assessed by real-time PCR using the following primers (Shanghai Bioengineering Company): rat NPY forward, 5'-TGTGGACTGACCCTCGCTCTAT-3'; NPY reverse, 5'-TGTAGTGTCGCAGAGCGGAGTA-3'; rat AgRP forward, 5'-AGCTTTGGCAGAGGTGCTAGATC-3'; AgRP reverse, 5'-TGCCAGTACCTAGCTTGCGG-3'; β-actin forward, 5'-CCATCC AGGCTGTGTTGTCC-3', β-actin reverse, 5'-GCTTCTCTTTAATGTCACGCACG-3'. Real-time PCR reaction system contained 2 μL primers, 12.5 μL SYBR Green Mix (TOYOBO, Japan), 2 μL cDNA and 8.5 μL RNase-free water. All reactions were carried out in capillaries in the ABI PRISM® 7700 sequence detection system (Perkin-Elmer applied Biosystem, USA) with the following protocol: 1 min at 95 ºC, 40 cycles for 15 s at 95 ºC, 1 min at 60 ºC. Product purity was confirmed by dissociation curves and random agarose gel electrophoresis. To obtain relatively accurate results, each sample was run in triplicate, and β-actin was used as internal control under the same conditions. The PCR products of NPY, AgRP and β-actin were 139, 173 and 243 bp, respectively. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative levels in NPY and AgRP gene transcription[23] .

1.6 *Hypothalamic NPY neurons immunohistochemistry*

Rats from ghrelin and control groups (each n=8) had *ad libitum* access to water and food. At 1, 2 and 3 h after ghrelin injection, the rats were killed, and then the brains were sectioned in the coronal plane on a freezing microtome (Kryostat 1720, Leica, Germany) at a thickness of 20 μm. For immunohistochemistry staining, brain sections were incubated with rabbit anti-NPY antibody (1:6 000, N-9528, Sigma Company, USA) over night at 4 ºC. In the next day, the sections were rinsed with PBS three times and incubated with biotin-conjugated goat anti-rabbit IgG (1:500, SC-2054, Santa Cruz, CA) for 30 min at room temperature. Then the sections were incubated with horseradish peroxidase conjugated streptavidin complex for 30 min at room temperature. The immunoreaction was visualized by DAB staining (DAB Substrate Kit, Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 5 min and observed under microscope. Negative controls were obtained by omission of the primary antibody. Three representative sections of ARC were selected from each rat. In each section, an area within the ARC nucleus was selected for counting the NPY-positive neurons and optical density analyzing. The amount and mean optical density of NPY-positive neurons were obtained with Olympus BX50 microscope and analyzed with the image analysis software (Compix Inc., USA).

1.7 *Statistical analysis*

Statistical analyses were performed using SPSS 16.0 for Windows. Food intake data were analyzed using two-way repeated-measures ANOVA. The rest of data were all analyzed by one-way ANOVA. Values were presented as means±SEM, and P<0.05 was considered significantly different.

2 RESULTS

2.1 Effects of ghrelin on food intake

Cumulative food intakes in ghrelin group were significantly increased in first 3 h of test period compared with those in control group [1 h: (0.92±0.99) g *vs* (0.61±0.13) g, P< 0.05; 2 h: (2.03±0.10) g *vs* (1.21±0.17) g, P<0.01; 3 h: (2.38±0.11) g *vs* (1.62±0.19) g, P<0.05]. The promoting effect of ghrelin reached the greatest level at 2 h after injection, and the effects continued at 3 h after injection. No significant difference was observed between control and ghrelin groups at 4 h after injection (Fig. 2).

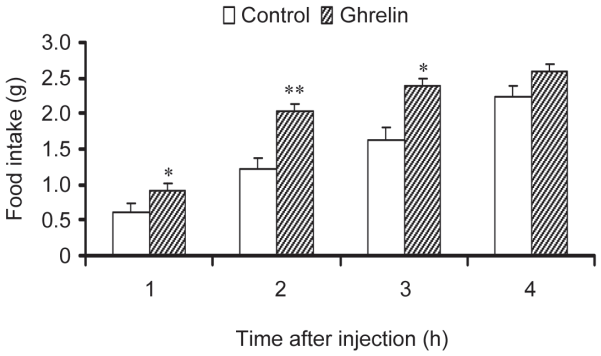

Fig. 2. Effect of DVC parenchymal injections of ghrelin on the cumulative food intake in freely fed rats. All injections were delivered in the middle of the light phase. Food intakes in ghrelin group (n=15) significantly increased at 1, 2 and 3 h after injection compared to those of control group (n=14), but there was no significant difference between ghrelin and control groups at 4 h after injection. The maximal effect of the ghrelin was achieved at 2 h after injection. Values are presented as means±SEM. $^{*}P$<0.05, $^{**}P$<0.01 *vs* Control group.

2.2 Effect of ghrelin on hypothalamic NPY/AgRP mRNA expression

As shown in Fig. 3, hypothalamic NPY and AgRP mRNA

expression levels were significantly higher in ghrelin group than those in control group at 1, 2 and 3 h after injection. Ghrelin administration increased NPY mRNA levels by 238.09%, 648.84% and 156.82% over those in control group at 1, 2 and 3 h after injection, respectively. On the other hand, ghrelin administration increased AgRP mRNA levels by 161.45%, 576.82% and 121.30% over those in control group at 1, 2 and 3 h, respectively. NPY/AgRP mRNA expressions at 2 h after ghrelin injection exhibited higher levels than those at 1 and 3 h after injection. No significant differences were found between ghrelin groups at 1 and 3 h after injection.

2.3 Effects of ghrelin on NPY expression in the ARC

The number of NPY-immunopositive neurons in the ARC nucleus was significantly increased at 1, 2 and 3 h after ghrelin injection compared with that in control group, respectively (1, 3 h, $P<0.05$; 2 h, $P<0.01$) (Fig. 4). Ghrelin's promoting effect was most pronounced at 2 h after pep-

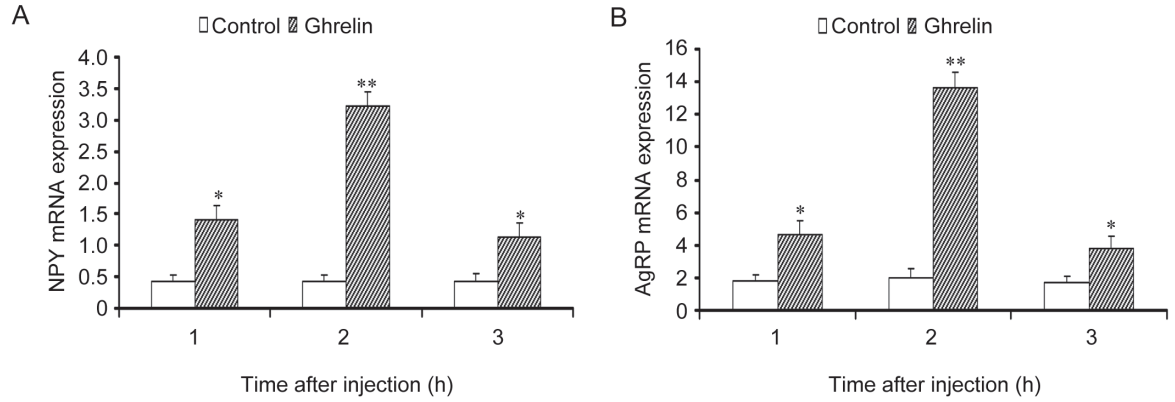

Fig. 3. Expression levels of NPY (*A*) and AgRP (*B*) mRNA in the ARC after DVC injection of ghrelin (20 pmol). Ghrelin administration into the DVC resulted in significant increases in NPY mRNA expression levels compared with those in control group. AgRP mRNA expressions were similarly elevated in ghrelin group compared with those in control group that received saline. Values are expressed as means±SEM. $^{*}P<0.05$, $^{**}P<0.01$ *vs* Control group.

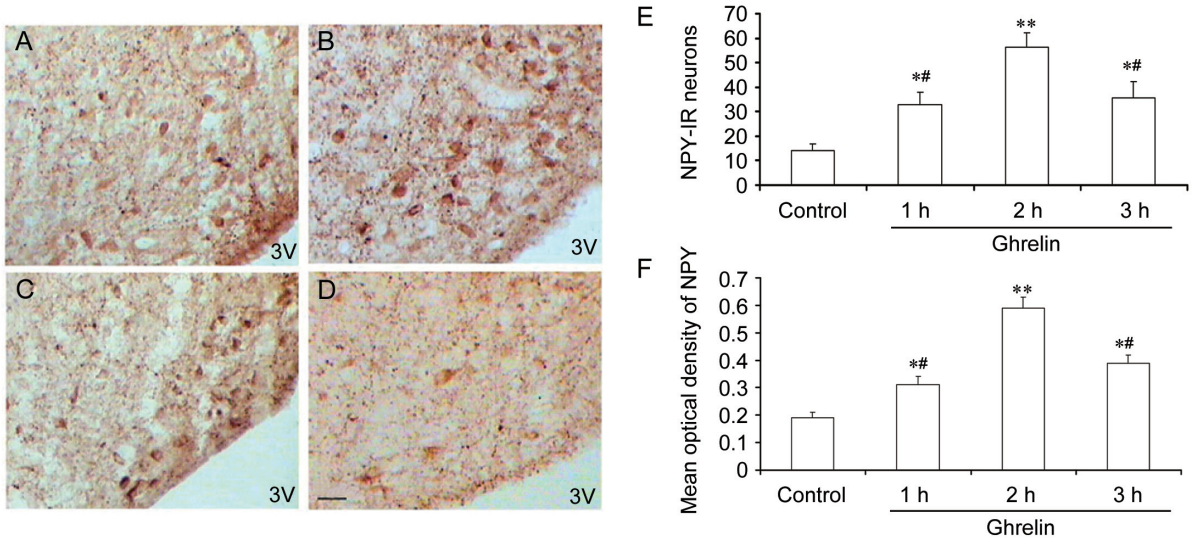

Fig. 4. Effects of ghrelin on NPY expression in ARC. NPY-immunoreactive neurons (brown) in the ARC were identified after DVC parenchyma (mainly in the NTS) injection of ghrelin at 1 (*A*), 2 (*B*) and 3 h (*C*), as well as injection of saline (*D*). Ghrelin resulted in a significant increase in mean cell count (*E*) and mean optical density (*F*) of NPY-positive neurons compared with those in control group. Data are presented as means±SEM. $^{*}P<0.05$, $^{**}P<0.01$ *vs* Control group; $^{\#}P<0.05$ *vs* ghrelin group at 2 h after injection, $n=8$. Scale bar, 10 μm. 3V, the third ventricle.

tide injection. There was no significant difference between the number of NPY-immunopositive neurons at 1 and 3 h in ghrelin group. Quantitative analysis revealed significant increases of mean optical density at 1, 2 and 3 h after ghrelin administration compared with that in control group (1, 3 h, $P<0.05$; 2 h, $P<0.01$) (Fig. 4). In ghrelin group, the mean optical density of NPY-positive neurons at 2 h after injection was significantly higher than those at 1 and 3 h after injection. There was no significant difference between the mean optical densities of NPY-positive neurons at 1 and 3 h after injection in ghrelin group.

3 DISCUSSION

Ghrelin, a recently discovered peptide-hormone, has been portrayed as a "hunger signal". It increases food intake when being injected into either the forebrain or hindbrain ventricles. It is not clear, however, if ghrelin applied directly to the hindbrain activates forebrain structures. In addition to the hypothalamus, functional evidence suggests that the DVC in the caudal brainstem is in a prime position within the brain to make important contributions to the integration of feeding-relevant signals [15,17,18,24]. GHS-Rs are expressed in all three components of the DVC, including the area postrema, NTS, and the dorsal motor nucleus of the vagus[10,11]. Similar responses have been obtained with forebrain and hindbrain administration of leptin[25], as well as with the melanocortins [26] and urocortins[27]. However, it remains to be determined whether the brainstem contains integrative substrates sufficient to mediate behavioral responses, and whether recruitment of forebrain structures are required for the hyperphagic responses stimulated by GHS-R agonist delivery to sensitive sites within the caudal brainstem.

In the present study, we obtained a clear orexigenic effect from delivery of ghrelin into the DVC at a dose of 20 pmol. Our data demonstrating increases in NPY mRNA in the ARC after DVC application of ghrelin extend the findings of Kinzig *et al*.[17], i.e. NPY mRNA expression increased following the more general application of ghrelin into the fourth ventricle. In addition, our study demonstrated a significant increase in AgRP mRNA expression and the amount of NPY immunoreactive neurons in the ARC after the administration of ghrelin to the DVC of the hindbrain. Taken together, these data provide strong support for the hypothesis that central NPY pathways play a key role in mediating the hyperphagic response following brainstem ghrelin administration. In a recent study, however, Faulconbridge *et al*.[18] observed that the fourth ventricle ghrelin delivery increased Fos only in the NTS, but not in the ARC or PVN. Moreover, ghrelin did not increase Fos in tyrosine hydroxylase-positive neurons in the NTS. The reason for the aforementioned discrepancies is unclear, although the possibility remains that a response in the forebrain may be observed with higher doses of ghrelin or with different experimental conditions (e.g., changing the feeding state of the animal or injection timing). For example, Gilg *et al*.[22] showed that ghrelin's effect was most pronounced when injected in the middle of the light phase. The experimental injections used by Faulconbridge *et al*.[18] differed from those of Kinzig *et al*.[17], for Faulconbridge *et al*. treated the rats with a hyperphagic dose of ghrelin in conjunction with aqueduct occlusion. This maneuver would have prevented diffusion of the injected ghrelin into forebrain regions thereby preventing any direct stimulation of forebrain substrates. The caudal flow of cerebrospinal fluid (CSF) would suggest that forebrain activation (e.g., of PVN and ARC) reported after delivery to the fourth ventricle would be indirect, occurring transsynaptically via ascending pathways such as those from the NTS to PVN.

Brainstem parenchyma delivery of 20 pmol ghrelin is known to be a moderate, suprathreshold dose for the feeding response[15]. Yet the dose of 20 pmol ghrelin is much lower than the doses applied to the fourth ventricle[18] and is unlikely to directly activate NPY neurons in the ARC. Nevertheless, the relatively low dose used here produced reliable hyperphagic effects, as well as increases in NPY mRNA, AgRP mRNA and NPY immunopositive neurons in the ARC. Our results would not allow us to rule out the possibility of a forebrain contribution to the hyperphagic response induced by brainstem ghrelin administration. A recent study by Date *et al*.[28] showed that intracerebroventricular administration of ghrelin similarly increased food intake in midbrain transected rats. This finding demonstrates that bilateral midbrain transections specifically blocked peripherally administered intravenous ghrelin-induced feeding, but did not affect centrally administered ghrelin-induced feeding. Centrally and peripherally administered ghrelin may therefore stimulate feeding by distinct mechanisms. Luquet *et al*. [29] reported that ablation of NPY/AgRP neurons in neonatal mice did not affect feeding in response to glucoprivation, whereas the feeding response to the ghrelin receptor agonist was completely abrogated. These findings demonstrate that NPY/AgRP neurons are critical for centrally administered ghrelin-induced feeding.

The neurochemical phenotype of the NTS neurons that respond to low-dose parenchymal injections of ghrelin in

the DVC is still unknown. Ghrelin was observed to modulate electrical activity of area postrema neurons using patch-clamp techniques [30], suggesting that the area postrema is one sensor of ghrelin in the caudal brainstem. Our previous study[31] showed that ghrelin inhibited glucose-sensing neurons in the DVC. Additionally, catecholamine/NPY coexpressing neurons in the hindbrain have been shown to be glucose-responsive[32]. Our results are consistent with the finding by Ritter *et al*.[32] that an NPY/noradrenergic pathway arising from the NTS plays a significant role in mediating the hyperphagic effects of caudal brainstem GHS-R stimulation.

In conclusion, our results demonstrate that recruitment of the forebrain structures are required for the hyperphagic response stimulated by ghrelin delivery to DVC within the caudal brainstem. Additional work is required to evaluate the contrasts between the neurologically intact and chronically maintained decerebrate rats in the pattern of Fos expression after ghrelin treatment via the fourth ventricle.

REFERENCES

1 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999; 402: 656-660.

2 Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR. Ghrelin causes hyperphagia and obesity in rats. Diabetes 2001; 50: 2540-2547.

3 Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2000; 407: 908-913.

4 Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA, Bloom SR. Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab 2001; 86: 5992.

5 Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 2001; 50: 1714-1719.

6 Bagnasco M, Tulipano G, Melis MR, Argiolas A, Cocchi D, Muller EE. Endogenous ghrelin is an orexigenic peptide acting in the arcuate nucleus in response to fasting. Regul Pept 2003; 111: 161-1617.

7 Tamura H, Kamegai J, Shimizu T, Ishii S, Sugihara H, Oikawa S. Ghrelin stimulates GH but not food intake in arcuate nucleus ablated rats. Endocrinology 2002; 143: 3268-3275.

8 Chen HY, Trumbauer ME, Chen AS, Weingarth DT, Adams JR, Frazier EG, Shen Z, Marsh DJ, Feighner SD, Guan XM, Ye Z, Nargund RP, Smith RG, Vander Ploeg LH, Howard AD,MacNeil DJ, Qian S. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. Endocrinology 2004; 145: 2607-2612.

9 Kohno D, Gao HZ, Muroya S, Kikuyama S, Yada T. Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca^{2+} signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. Diabetes 2003; 52: 948-956.

10 Zigman JM, Jones JE, Lee CE, Saper CB, Elmquist JK. Expression of ghrelin receptor mRNA in the rat and the mouse brain. J Comp Neurol 2006; 494: 528-548.

11 Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, Smith RG, Van der Ploeg LH, Howard AD. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res 1997; 48: 23-29.

12 Olszewski PK, Grace MK, Billington CJ, Levine AS. Hypothalamic paraventricular injections of ghrelin: effect on feeding and c-Fos immunoreactivity. Peptides 2003; 24: 919-923.

13 Currie PJ, Mirza A, Fuld R, Park D, Vasselli JR. Ghrelin is an orexigenic and metabolic signaling peptide in the arcuate and paraventricular nuclei. Am J Physiol Regul Integr Comp Physiol 2005; 289: R353-R358.

14 Naleid AM, Grace MK, Cummings DE, Levine AS. Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. Peptides 2005; 26: 2274-2279.

15 Faulconbridge LF, Cummings DE, Kaplan JM, Grill HJ. Hyperphagic effects of brainstem ghrelin administration. Diabetes 2003; 52: 2260-2265.

16 Jia YD (贾云丹), Chen X, Tang M, Jiang ZY. Expression of growth hormone secretagogue receptor type 1a in visceral vagal and spinal afferent parthways. Acta Physiol Sin (生理学报) 2008; 60: 149-155 (Chinese, English abstract).

17 Kinzig KP, Scott KA, Hyun J, Bi S, Moran TH. Lateral ventricular ghrelin and fourth ventricular ghrelin induce similar increases in food intake and patterns of hypothalamic gene expression. Am J Physiol Regul Integr Comp Physiol 2006; 290: R1565- R1569.

18 Faulconbridge LF, Grill HJ, Kaplan JM, Daniels D. Caudal brainstem delivery of ghrelin induces fos expression in the nucleus of the solitary tract, but not in the arcuate or paraventricular nuclei of the hypothalamus. Brain Res 2008; 1218: 151-157.

19 Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. Nature 2001; 409: 194-198.

20 Olszewski PK, Li D, Grace MK, Billington CJ, Kotz CM, Levine AS. Neural basis of orexigenic effects of ghrelin acting within lateral hypothalamus. Peptides 2003; 24: 597-602.

21 Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 5th ed. San Diego: Academic Press, 2005, 98-116.

22 Gilg S, Lutz TA. The orexigenic effect of peripheral ghrelin differs

between rats of different age and with different baseline food intake, and it may in part be mediated by the area postrema. Physiol Behav 2006; 87: 353-359.

23 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001; 25: 402-408.

24 Faulconbridge LF, Grill HJ, Kaplan JM. Distinct forebrain and caudal brainstem contributions to the neuropeptide Y mediation of ghrelin hyperphagia. Diabetes 2005; 54: 1985-1993.

25 Grill HJ, Schwartz MW, Kaplan JM, Foxhall JS, Breininger J, Baskin DG. Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. Endocrinology 2002; 143: 239-246.

26 Grill HJ, Ginsberg AB, Seeley RJ, Kaplan JM. Brainstem application of melanocortin receptor ligands produces long-lasting effects on feeding and body weight. J Neurosci 1998; 18: 10128-10135.

27 Grill HJ, Markison S, Ginsberg A, Kaplan JM. Long-term effects on feeding and body weight after stimulation of forebrain or hindbrain CRH receptors with urocortin. Brain Res 2000; 867: 19-28.

28 Date Y, Shimbara T, Koda S, Toshinai K, Ida T, Murakami N, Miyazato M, Kokame K, Ishizuka Y, Ishida Y, Kageyama H, Shioda S, Kangawa K, Nakazato M. Peripheral ghrelin transmits orexigenic signals through the noradrenergic pathway from the hindbrain to the hypothalamus. Cell Metab 2006; 4: 323-331.

29 Luquet S, Phillips CT, Palmiter RD. NPY/AgRP neurons are not essential for feeding responses to glucoprivation. Peptides 2007; 28: 214-225.

30 Fry M, Ferguson AV. Ghrelin modulates electrical activity of area postrema neurons. Am J Physiol Regul Integr Comp Physiol 2009; 296: R485-R492.

31 Wang WG, Chen X, Jiang H, Jiang ZY. Effects of ghrelin on glucose-sensing and gastric distension sensitive neurons in rat dorsal vagal complex. Regul Pept 2008; 146: 169-175.

32 Ritter S, Bugarith K, Dinh TT. Immunotoxic destruction of distinct catecholamine subgroups produces selective impairment of glucoregulatory responses and neuronal activation. J Comp Neurol 2001; 432: 197-216.