

Serotonin Receptors are Involved in the Vagal Afferent Transmission of Exogenous Ghrelin-Evoked Appetite Sensation Mediated Through C-Fibers

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Ghrelin, an endogenous appetite hormone, is secreted from the gastro-intestinal tract, and the ghrelin-induced appetite is conveyed through the afferent vagus nerves. However, it has not been elucidated how the orexigenic sensation produced by ghrelin is transmitted to the afferent vagus nerves that innervate gastro-intestinal tract. To address this issue, ghrelin-induced food intake in Wistar rats was evaluated in the presence or absence of vagal denervation or the administration of a type-3 serotonin receptor antagonist ramosetron. Cumulative food intake was significantly increased 1 hr and 2 hr after the intraperitoneally-applied ghrelin (20 µg/kg). Desensitization of C-fiber of the afferent vagus nerves by intraperitoneally-applied capsaicin completely abolished the increases in the ghrelin-induced food intake. Surgical vagotomy of the hepatic or gastric branches markedly suppressed the ghrelin-evoked feeding. Furthermore, pretreatment with ramosetron also significantly suppressed the ghrelin-evoked feeding. Stimulatory effects of ghrelin on the appetite were transient and the overall food intake was comparable after 24 hr among all groups. These data suggest that 1) exogenous ghrelin rapidly promotes feeding, but such feeding lasts no more than 2 hr; 2) afferent C-fiber of the hepatic or gastric vagus nerves appear to convey ghrelin-induced hungry sensation to the brain; 3) type-3 serotonin receptors at the nerve endings of the C-fiber of the gastric or hepatic afferent vagus nerves may involve in transmitting the ghrelin-evoked hungry sensation.

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Introduction

The neural and humoral signals produced in the gastrointestinal tract promote or suppress feeding through the integration of the hypothalamus. Distension of the stomach and influx of nutrients into the gastrointestinal tract terminate feeding with satiety.¹ On the other hand, empty of the stomach and fall of the nutrient, such as glucose, in the blood evokes feeding with hungry sensation.² An ascending neural network within the vagus nerves conveys such visceral information to the brainstem that ultimately reaches the hypothalamus.³ Condition under negative energy balance containing fasting evokes to increase orexigenic substances, e.g., ghrelin, in the peripheral circulation.^{4,5}

Ghrelin is a peptide that is predominantly secreted from the oxyntic glands of the stomach.^{2,6,7} Plasma level of endogenous ghrelin rises shortly before the onset of a meal and falls abruptly to basal level after food intake.^{2,6,8,9} An administration of exogenous ghrelin promotes initiation of feeding with a short time latency in animals and human.^{2,4,5,10,11} In rats, desensitization of afferent C-fiber of the sensory nerves projecting from cell bodies located in the nodose ganglia by capsaicin abolishes the ghrelin-induced promotion of food intake.^{5,6,12-14} In addition, surgical resection of the gastric branches of the vagus nerves suppresses such stimulatory effect of exogenous ghrelin on food intake.¹⁵ Furthermore, ghrelin given exogenously suppresses discharge activities of the gastric afferent nerves in the vagus nerves.^{2,12,13,15,16}

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These data show that the afferent vagus nerves play a pivotal role in triggering promotion of food intake evoked by endogenous ghrelin. Growth hormone secretagogue receptors (GHS-R), so called ghrelin receptors, were found in neuronal cell bodies in the nodose ganglion.^{13,15-17} When the afferent vagus nerve was ligated by a thread, GHS-R was accumulated in the axonal segments projecting from such cell bodies proximal to the ligature.¹⁵ However, ghrelin receptor has not been found within or around the terminal of the C-fiber innervating the gastric mucosal layer.^{13,15} These observations suggest that 1) ghrelin may not act to the endings of the afferent C-fiber of the gastric vagus nerves but act indirectly on some receptors mediating appetite sensation and 2) such receptors might contain receptors receiving neurotransmitters released from the basal-granulated cells, e.g., serotonin, located close to the endings of the gastric afferent vagus nerves.

Serotonin (5-HT), an important neurotransmitter in the gastrointestinal tract, is formed predominantly in enterochromaffin (EC) cells resided throughout the gastrointestinal mucosa.^{1,18,19} Recent studies suggest that 5-HT acts as a neurotransmitter mediating sensation resulted from testing nutrients or meeting with nociceptive stimulations within lumen and/or mucosa of the gastrointestinal tract.¹ Indeed, 5-HT participates in transmission of mucosal or luminal sensations evoked by digestive consequences (e.g., changes in nutrition, osmolality or mechanical distention etc.) as well as many detrimental stresses (e.g., anaphylaxis, microbial toxins, anti-cancer drugs etc.).^{3,20,22-24} Such visceral information was mediated through the type-3 serotonin (5-HT₃) receptors located at the nerve terminals of the afferent C-fibers of the vagus nerves.²² It has been demonstrated that ondansetron, a selective 5-HT₃ receptor antagonist, markedly attenuates the feeding suppression induced by the endogenous satiety hormone CCK in rats.^{1,23,24} These results suggest that endogenous CCK after meal evokes satiation resulting from release of 5-HT from the EC cells which in turn activates 5-HT₃ receptors in the afferent C-fiber of the vagus nerves.

Based on these observations, it was assumed that the preprandially released ghrelin may evoke appetite through the afferent gastric vagus nerves by the mechanisms of either 5-HT released from the EC cells and/or 5-HT₃ receptors located at the endings of the C-fiber. To prove such hypothesis, it was examined whether ghrelin-induced feeding is suppressed by the following pretreatments in rats: 1) desensitization of C-fiber of the afferent vagus nerves by capsaicin; 2) selective or combined surgical denervation of the hepatic or gastric branches of the vagus nerves; 3) pharmacologic antagonization of the 5-HT₃ receptors by ramosetron.

Materials and Methods

Animals: Male Wistar rats weighting from 300 g to 450 g were housed individually in a room temperature maintained at 25 ± 1 °C and in a 12:12-h light-dark cycles (light on at 8:00). All rats had free access to food and water. The protocols used in this study conformed to the *Guiding Principles for the Care and Use of Animals in the Fields of Physiological Sciences* (approved by Physiological Society of Japan, revised 2002). To habituate animals to the present experimental condition, all animals were handled for around 5 minutes and given intraperitoneal injection (ip) of saline (1 ml/kg) daily for 7 successive days before each experiment.

Chemical or surgical vagotomy: To examine whether the vagus nerves participate in the ghrelin-evoked food intake, chemical and surgical elimination of the vagus nerves were employed. Chemical deafferentation of the C-fiber of the afferent vagus nerves (so called capsaicin-desensitization) were carried out by capsaicin (first day; 6 mg/kg and second day; 30 mg/kg, ip) two weeks before the ghrelin test. Atropine sulphate (10 mg/kg, ip) was given 30 min before capsaicin challenge under anesthesia with sodium pentobarbital (60 mg/kg). Capsaicin (Sigma, Aldrich, Japan) was freshly dissolved in physiological saline containing 10 % Tween-80 and 10 % ethanol.²⁵ Following the first injection of capsaicin, all animals exhibited respiratory arrest for 1 to 5 min. Assistance for manually massaging the chest or artificial respiration was induced until the spontaneous breathing is restored. Surgical vagotomy was underwent at least three weeks before the ghrelin test, and the animals were divided into four groups; 1) hepatic branch vagotomy (HVX), 2) bilateral gastric branch vagotomy (GVX), 3) hepatic plus bilateral gastric branch vagotomy (H&GVX), and 4) sham operation. Briefly, a traverse laparotomy (crossing the midline line between the diaphragm and upper corner of the stomach) was performed under anesthesia with sodium pentobarbital (60 mg/kg, ip).¹⁹ Selective vagotomy was performed followed by protection of the stomach and intestine covered with saline-moistened gauze. In HVX, the hepatic branch of the vagus nerve (ventral site) was resected at 2 mm distal from the junction of the vagal trunks. In GVX, the gastric branches of the vagus nerves (ventral and dorsal site) were resected at 2 mm distal from their junction of the each trunk. In H&GVX, the hepatic branch plus bilateral gastric branches of the vagus nerves were resected at 2 mm distal from their junction of the each trunk.

Experimental protocol: Lyophilized rat ghrelin (Peptide Institute, Inc., Osaka, Japan) was freshly dissolved in saline

and was given ip at 10:00 (two hours after lighting) to intact rats or rats with chemical or surgical vagotomy, or pharmacologic pretreatment with Ramosetoron. Cumulative food intake during 1 hr, 2 hr, 3 hr, and 24 hr after an administration of ghrelin or saline was measured at 11:00, 12:00, 13:00, and 10:00 on the following day, respectively. Ramosetoron (Nasea, Asteras Pharm. Co. LTD, Osaka, Japan) was freshly diluted to 45 $\mu\text{g}/\text{ml}$ with saline and given ip at 20 min prior to the challenge of ghrelin.

Statistical analysis: All data are presented as means \pm S.E. Data were analyzed by one-way or two-way ANOVA with correction for repeated measures and with an appropriate post hoc test (Fisher's protected least significant difference) for multiple comparisons.

Results

Ghrelin-evoked feeding is rapid and transient

Figure 1A shows the time course of cumulative food intakes after the challenge of two different doses of ghrelin (10 or 20 $\mu\text{g}/\text{kg}$). Low dose ghrelin (10 $\mu\text{g}/\text{kg}$) showed a tendency to increase the cumulative food intake time-dependently, but there was no statistical significance. Higher dose of ghrelin (20 $\mu\text{g}/\text{kg}$) significantly increased the cumulative food intake during 1 to 3 hr after the administration. However, the overall cumulative food intake after 24 hrs was comparable regardless of the ghrelin doses (Figure 1B). Hour rate of food intake for the first 1 hr (Table 1) was increased by 7.5 fold by the administration of 20 $\mu\text{g}/\text{kg}$ ghrelin (control: 0.4 ± 0.2 g/h, ghrelin: 3.0 ± 0.2 g/h). Does dependent experiments showed that the minimum effective dose was 15 $\mu\text{g}/\text{kg}$ (data not shown). These data show ghrelin-evoked appetite response is very rapid rather transient.

Capsaicin-desensitization completely abolishes the increase in ghrelin-evoked feeding.

To examine whether the afferent C-fiber of the vagus nerves involved in the increase in ghrelin-evoked feeding, ghrelin was administered intact rats or rats with the pre treatment capsaicin-desensitization. In rats desensitized with capsaicin, cumulative food intake was significantly suppressed both 1hr ($46.7 \pm 13.6\%$, $p < 0.05$) and 2hrs ($39.4 \pm 10.5\%$, $p < 0.05$) after the administration of ghrelin (Figure 2A). Cumulative overall intake of 24 hrs after ghrelin administration (Figure 2B) and hour rate of food intake for the first 2 hrs (data not shown) were comparable among all three groups.

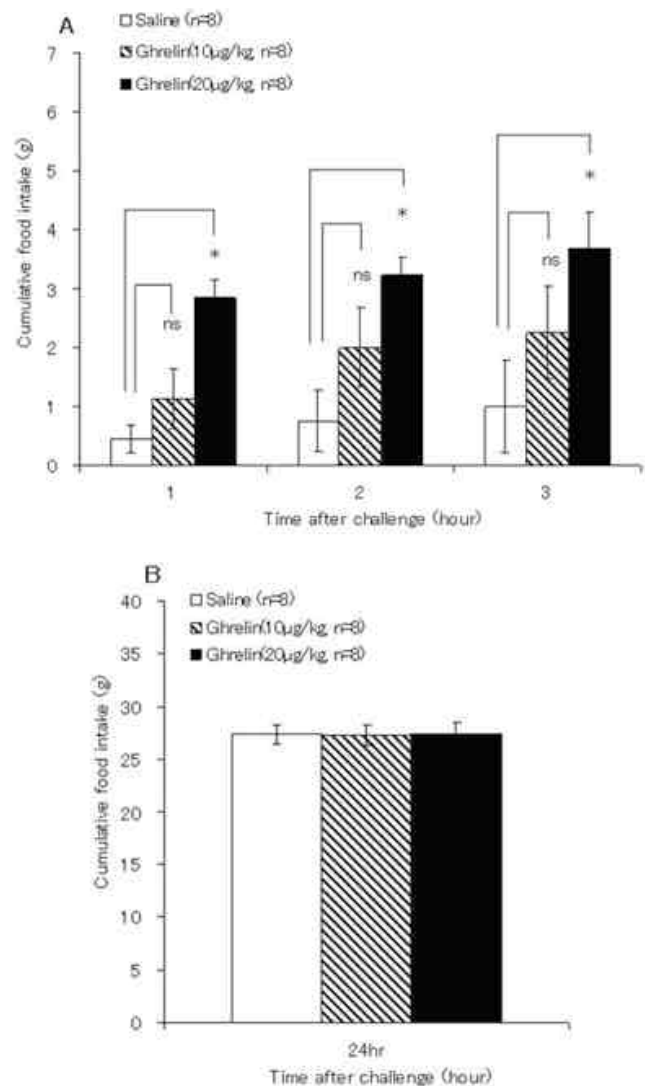


Figure 1. Effect of various doses of ghrelin given intraperitoneally on cumulative food intake in intact rats.

A. 1 hr- or 2 hr-cumulative food intake measured at 1 hr or 2 hr after a challenge of ghrelin. An animal was administered alternatively with a different dose of ghrelin (10 or 20 $\mu\text{g}/\text{kg}$) or saline vehicle into the peritoneal cavity at 10:00 (2 hr after light on).

B. 24 hr-cumulative food intake measured at 10:00 on the following day after the injection of ghrelin. Numbers in parentheses are number of animals. Vertical bars represent S.E. *, $P < 0.05$ vs. saline control; ns: no significant.

Table 1. Net-food intakes every 1hr during 3hr after ghrelin challenge

	1hr	2hr	3hr
Saline (n=8)	0.4 ± 0.2	0.4 ± 0.3	0.4 ± 0.3
Ghrelin (10 $\mu\text{g}/\text{kg}$, n=8)	1.1 ± 0.5	0.9 ± 0.3	0.3 ± 0.3
Ghrelin (20 $\mu\text{g}/\text{kg}$, n=21)	$3.0 \pm 0.2^*$	1.4 ± 0.3	0.3 ± 0.1

Means \pm SE, *, $p < 0.01$ vs. saline control.

These results suggest that the capsaicin desensitization of the C-fiber completely abolished the ghrelin-evoked feeding.

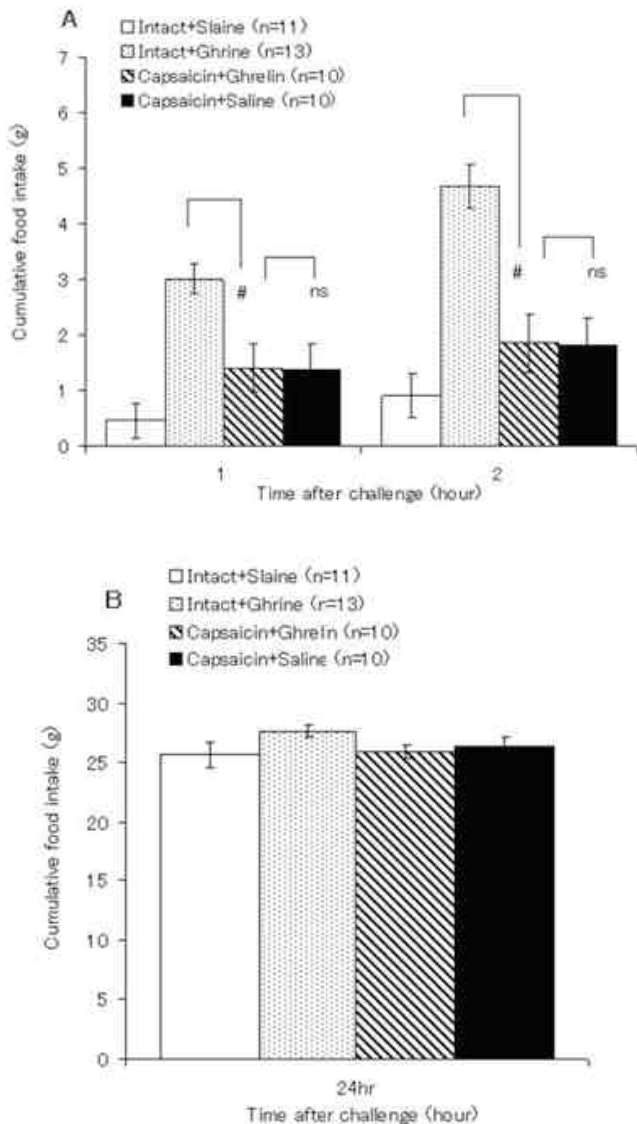


Figure 2. Effects of capsaicin-desensitization of the afferent C-fiber on increases in ghrelin-induced food intake.

A. Rats were given ghrelin (20 μ g/kg, ip) at 10:00. Capsaicin-desensitized rats were given alternatively 20 μ g/kg ghrelin or saline at 10:00. Cumulative food intake was measured at 1 hr or 2 hr after the challenge of ghrelin.

B. 24 hr-cumulative food intake measured at 10:00 on the following day after the injection of ghrelin or saline. Vertical bars represent S.E. #, $P < 0.05$ vs. intact animal given ghrelin; ns: no significant.

Hepatic and gastric branch of the vagus nerves participate increase in the ghrelin-evoked feeding.

To define the vagus nerve branches involved in conveying the ghrelin-evoked appetite, surgical resection of the gastric (GVX) or hepatic (HVX) vagus nerves or their

combination were carried out. GVX markedly suppressed such cumulative food intakes to 31.7 ± 12.3 %, and 39.2 ± 15.2 % of that in sham operated animals during 1 hr and 2 hr after the ghrelin-challenge, respectively (Figure 3A). Similarly, HVX significantly suppressed cumulative food

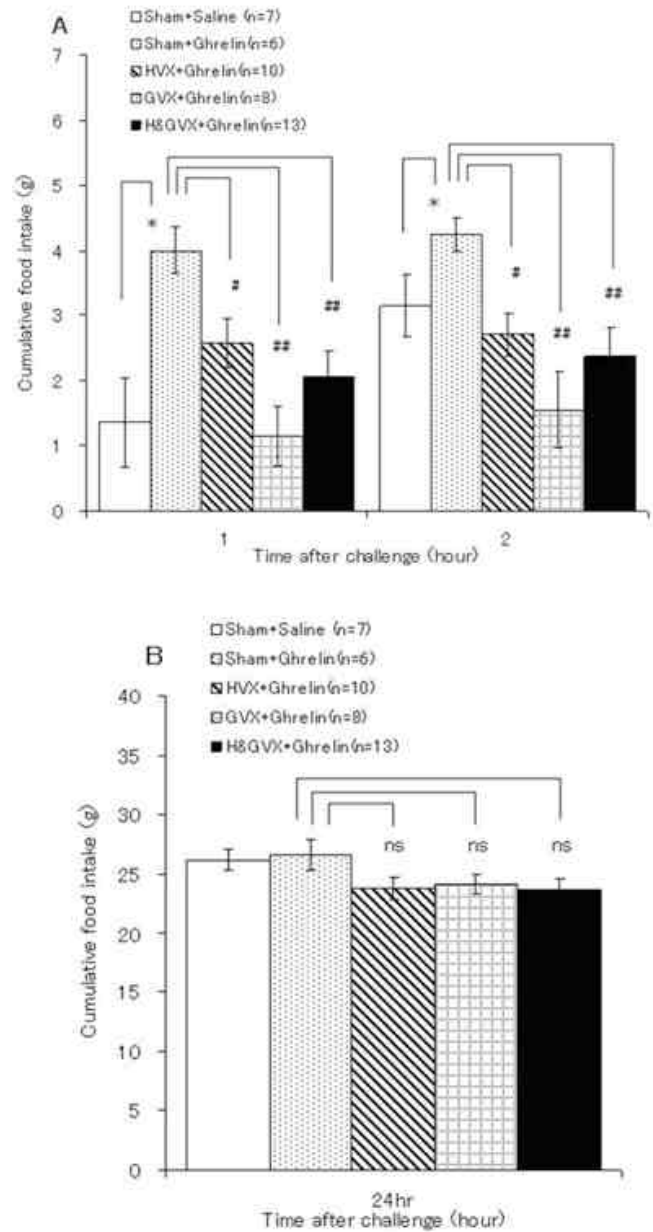


Figure 3. Effects of selective or combined resection of the visceral branch of the vagus nerves on the ghrelin-evoked food intake.

A. Cumulative food intake during 1hr or 2hr after start of injection of ghrelin in rats with sham vagotomy or in rats with surgical vagotomy. HVX: rats resected the hepato-portal branch of the vagus nerve; GVX: rats resected the bilateral gastric branches; H&GVX: rats resected the hepato-portal branch plus bilateral gastric branches.

B. Cumulative food intake during 24 hr after the challenge of ghrelin. Vertical bars represent S.E. *, $P < 0.05$ vs. sham operation + saline control; #, $P < 0.05$; ##, $P < 0.001$ vs. sham operation + ghrelin.

intakes to 64.2 ± 9.4 %, or 63.5 ± 7.8 % during 1 hr and 2 hr, respectively. Combination of HVX and GVX significantly suppressed the cumulative ingestion to 51.6 ± 9.9 % or 55.9 ± 10.2 % during 1 hr and 2 hr, respectively, but failed to show additional suppression over GVX or HVX. Although GVX showed stronger suppressive effect than HVX or H&GVX for the first 2 hrs, the overall food intake was comparable 24 hrs after ghrelin administration (Figure 3B).

5-HT3 receptor antagonist attenuates the increase in ghrelin-evoked feeding.

To explore whether 5-HT plays a role in the transmission between exogenous ghrelin and the nerve ending of the afferent vagus nerves, 5-HT3 receptor was blocked by ramosetron. Pretreatment with ramosetron significantly suppressed the ghrelin-evoked increases in cumulative food intake during

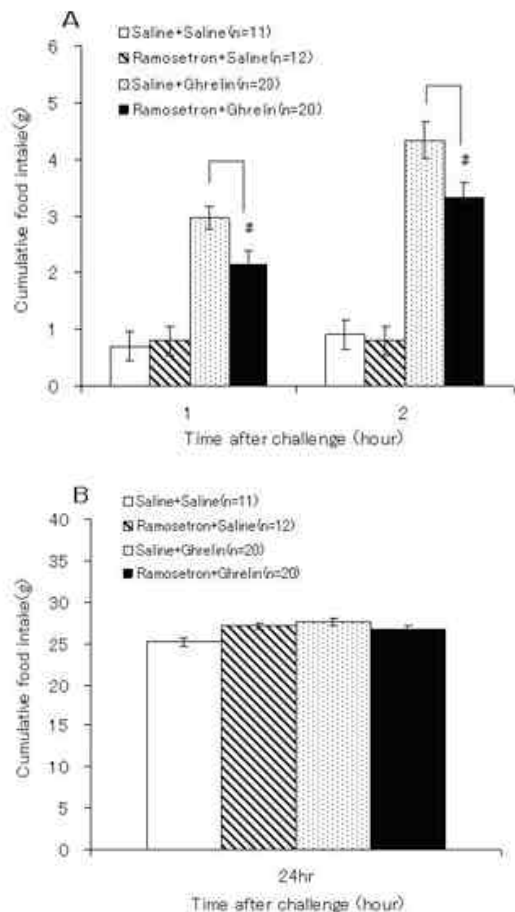


Figure 4. Effects of ramosetron on the ghrelin-evoked food intake. **A.** Cumulative food intake during 1 hr, 2 hr after a challenge of ghrelin. Animals were given a bolus injection of ramosetron (45 μ g/kg, ip) or saline at 20 min before a challenge of 20 μ g/kg ghrelin. **B.** Cumulative food intake during 24 hr after a challenge of ghrelin. Vertical bars represent S.E. #, $P < 0.05$ vs. saline + ghrelin.

1 hr (76.6 ± 6.7 %) and 2 hr (71.8 ± 9.3 %) compared to rats with ghrelin alone (Figure 4A). Cumulative food intake during 24 hr after the challenge was unchanged by ramosetron pretreatment (Figure 4B).

Discussion

Present study refined the transmitting mechanisms between visceral hormone and afferent neuron underlying the appetite promotion by ghrelin. The main observations are that 1) 5HT-3 receptors are involved in the transmission of exogenously-applied ghrelin through the C-fibers of the vagal afferent nerves, 2) hepatic branches as well as gastric branch of the vagus are involved in the ghrelin-evoked appetite promotion.

In rats, nocturnal food intake reached more than 80% of 24 hr-food intake and diurnal food intake (residual 20%) occurred with subdivided feeding by several times.^{26,27} In the present study, rats intake a small amount of food (less than 0.4 ± 0.3 g/hr) during 2 hr (10:00 ~ 12:00) after start of light on (8:00). Under these conditions, exogenously-applied ghrelin enhanced feeding with a dose-dependent manner. Exogenous ghrelin (20 μ g/kg) rapidly stimulated the food intake which peaked at 1 hr, followed by gradual decrease, but did not last more than 24 hr. In the present study, capsaicin-desensitization of afferent C-fiber completely abolished the increase in ghrelin-induced feeding not only during 1 hr but also 2 hr after the challenge of ghrelin. This observation consist with the previous data found by preceding studies^{5,6,12-14} and these data confirmed that ghrelin-induced orexigenic sensation should be conveyed to the brainstem via the afferent C-fiber of the vagus nerves.

Based on the observations that gastrectomy eliminates approximately 80% of the circulating ghrelin,^{28,29} endogenous ghrelin is thought to be produced predominantly by the stomach. It has been shown that exogenous ghrelin decreased firing rates of the afferent gastric vagus nerves and selective resection of gastric branches of the vagus suppressed ghrelin-induced food intake,³⁰ suggesting the primary roles of gastric branches in the neural transmission of ghrelin signaling. Indeed, in the present study, a strong suppression of the increase in ghrelin-evoked food intake occurred in rats with gastric vagotomy. Such abolishment of the ghrelin-induced feeding by GVX consists with the previous studies performed by preceding workers.^{2,15} Furthermore, present study demonstrate for the first time that vagotomy of hepatic branches as well as the that of gastric branches

suppressed ghrelin-evoked food intake, implicating the hepatic branches in this signaling. There are several explanations for these observations. Although endogenous ghrelin is produced predominantly in the stomach (75-80%), the remainder is secreted from the proximal small intestine.³¹⁻³³ Therefore, most of endogenous ghrelin flow ultimately into the portal circulation. Suppose the hepatic afferent vagus nerves have a sensor monitoring the concentration of ghrelin in the portal circulation which is secreted from the gastrointestinal tract, hepatic vagotomy may disrupt the appetite signals by the exogenously-applied ghrelin. Another possibility could explain such discrepancy as follows. Supposing that endogenous ghrelin act on the nerve terminals with a paracrine mode alone before outflowing to the blood stream, the increase in feeding evoked by exogenous ghrelin might be caused via the different ways from that induced by endogenous ghrelin released in response to real hunger sensation. Anyway, exogenous ghrelin-evoked appetite signals may convey to the brainstem via the hepatic and gastric afferent vagus nerves.

Ghrelin receptors have been identified in the nodose ganglion that contains cell bodies of the afferent vagus nerves. Previous studies have suggested that endogenous ghrelin acts directly on the ghrelin receptors in the stomach,³⁴ suggesting that the ghrelin receptors is located within or nearby the nerve terminals of the C-fiber of the afferent gastric vagus nerves. Despite the large extent of efforts, ghrelin receptors have not been yet identified within these regions,^{13,17} suggesting ghrelin might be transmitted indirectly to the afferent vagus nerves mediated through some neurotransmitters. Serotonin is one of these candidate neurotransmitters which plays a pivotal role in gastrointestinal afferent neural networks mediating various gastrointestinal events to the central nervous system.²⁴ In the present study, pretreatment with the 5-HT₃ receptor antagonist ramosetron suppressed ghrelin-induced food intake. However, weaker potency of ramosetron than the chemical or surgical vagotomy may be due to too small dose of ramosetron employed to antagonize serotonergic action induced by 20 µg/kg ghrelin. Alternatively, some other neurotransmitter, e.g., histamine^{35,36} may be involved in the setting of ghrelin/C-fiber-evoked appetite. However, the weak suppressive effect of ramosetron would not exclude a possibility that a set of serotonin/5-HT₃ receptors, in a partly at least, participates the ghrelin evoked-appetite sensation conveyed by C-fiber of the afferent vagus nerves. 5-HT₃ receptor antagonist might attenuate the exogenous ghrelin-induced feeding by the following hypothetic three explanations. The first explanation: exogenous ghrelin might act indirectly on the terminals of the

afferent C-fiber of the vagus nerves via 5-HT released from the EC cells. The second: although exogenous ghrelin might act directly on such nerve terminals, it's information was modulated by the signals mediated by 5-HT₃ receptors located in the same nerve terminals of the C-fibers. The third: exogenous ghrelin might act directly on the nerve terminals of the C-fibers. However, the signals were integrated by the other 5-HT₃ receptor-sensitive neurons of the afferent C-fibers within the nodose ganglion. In the present times, all of them are speculative. Thus, mechanisms for the attenuation of 5-HT₃ receptor antagonist against exogenous ghrelin-induced feeding remain unsolved. Furthermore, ramosetron employed in this experiment has been thought to hardly pass over the blood-brain-barrier.^{20,21} However, a possibility that ramosetron penetrated the barrier might act on the 5-HT₃ receptor located in the central nervous system cannot exclude. Further investigation needs.

Although previous studies have showed that exogenous ghrelin promotes feeding within 4 hr after the challenge,² present study clearly shows that effect of bolus injection of ghrelin on feeding manifests rapidly but does not last over 2 hr. Moreover, chemical or surgical deafferentation or pharmacologic antagonization did not affect the 24 hr-cumulative food intake, suggesting that other humoral or neuronal networks other than such ghrelin/vagal afferent system may be involved in the regulation for the long term energy balance.

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