Table 1
Expected molecular forms of isolated ghrelins from feline stomach

Groups	Peaks	Mass [M+H]	Expected molecular form	Yields (pmol)
A	1	3188.16	ghrelin-(1-27)(C8:0)	10.00
	2	3188.81	ghrelin-(1-27)(C8:0)	10.00
	3	3212.65	ghrelin-(1-27)(C10:2)	2.50
	4	3212.65	ghrelin-(1-27)(C10:2)	3.13
B	5	3060.56	des-Gln^{14}-ghrelin-(1-27)(C8:0)	2.50
	6	3060.36	des-Gln^{14}-ghrelin-(1-27)(C8:0)	6.25
	7	3213.89	ghrelin-(1-27)(C10:1)	3.13
	8	3214.35	ghrelin-(1-27)(C10:1)	2.50
	9	3214.92	ghrelin-(1-27)(C10:1)	5.00
C	10	3343.21	ghrelin-(1-28)(C8:1)	18.75
	11	3345.13	ghrelin-(1-28)(C8:0)	2.50
	12	3216.64	ghrelin-(1-27)(C10:0)	3.13
D	13	3344.88	ghrelin-(1-28)(C8:0)	105.00
	14	3367.10	ghrelin-(1-28)(C10:2)	16.25
E	15	3215.90	des-Gln^{14}-ghrelin-(1-28)(C8:0)	8.00
	16	3369.50	ghrelin-(1-28)(C10:2)	5.00
	17	3371.12	ghrelin-(1-28)(C10:1)	3.75
	18	3371.11	ghrelin-(1-28)(C10:1)	11.25
	19	3371.22	ghrelin-(1-28)(C10:1)	17.50
	20	3371.22	ghrelin-(1-28)(C10:1)	2.50
F	21	3371.13	ghrelin-(1-28)(C10:1)	3.75
	22	3372.77	ghrelin-(1-28)(C10:0)	10.00
G	23	3356.37	ghrelin-(1-28)(C10:0)	1.25
H	24	3412.68	ghrelin-(1-28)(C13:1)	0.63
I	25	3413.35	ghrelin-(1-28)(C13:0)	1.50

region (UTR) and 3′-UTR (153 and 136 bp, respectively), but the length of the coding region was 354 bp (DDBJ/EMBL/GenBank accession no. AB089201) or 351 bp (DDBJ/EMBL/GenBank accession no. AB089202). The deduced amino acid sequences of the coding regions of the two cDNAs indicated that prepro-ghrelin and prepro-des-Gln^{14}-ghrelin are composed of 117 and 116 amino acids, respectively (Fig. 2). The amino acid sequence of feline prepro-ghrelin was 79% and 82% identical to that of rat and human, respectively (Fig. 3A). From this cDNA analysis, the unidentified third amino acid was determined to be serine, as seen in other species except bullfrog, in which the residue is threonine.

3.3. Structural determination

To determine the molecular weights of the feline ghrelins, isolated peptides were analyzed by MALDI-TOF mass spectrometry. Table 1 shows the actual measured molecular masses of the isolated peptides, the deduced molecular forms, and the isolated yields. From the molecular masses in addition to the analyses of peptide and cDNA sequences, four types of

prepro-ghrelin

```
   1 GGCAGAGAGAAAGGGAGAGATCGAGAATCCCAAGCAGCATGGAGCTTGAT  50
     GCAGGGATCGAACTCATGNGACTGTGAGATCATGACCTGAGCTGAAACCA 100
  51 AGAATCAGATGCTTAACTGACTTCCACCAGGAATCCCAGGCCCACCTGAC 150
 101 ACCATGCCCTCCCCGGGGACCGTGTGCAGCCTGCTGCTCTTCAGCATGCT 200
       M  P  S  P  G  T  V  C  S  L  L  L  F  S  M  L (16)
 151 GTGGGCAGACTTGGCCATGGCAGGCTCCAGCTTCCTGAGCCCCGAACACC 250
       W  A  D  L  A  M  A  G  S  S  F  L  S  P  E  H  (32)
 201 AGAAAGTACAGCAGAGAAAGGAATCCAAGAAGCCACCAGCCAAACTGCAG 300
 251 Q  K  V  Q  Q[14] R  K  E  S  K  K  P  P  A  K  L  Q  (49)
     CCCCGAGCTCTGGAAGGCTTGATCCACCCAGAAGACACAAGTCAAGTGGA 350
 301 P  R  A  L  E  G  L  I  H  P  E  D  T  S  Q  V  E  (66)
     AGGGGCAGAGGATGAACTAGAAATCCGGTTCAACGCCCCTTTTGATGTTG 400
 351   G  A  E  D  E  L  E  I  R  F  N  A  P  F  D  V   (82)
 401 GAATCAAGCTGTCAGGGGCTCAGTACCACCAGCATGGCCAGGCGCTGGGG 450
     G  I  K  L  S  G  A  Q  Y  H  Q  H  G  Q  A  L  G  (99)
 451 AAGTTTCTTCAGGACGTCCTTTGGGAAGAGGCCGATGAGGTCCTGGCAGA 500
       K  F  L  Q  D  V  L  W  E  E  A  D  E  V  L  A  D (116)
 501 TGAGTGATCATCCACTAGAACGACCCACTTGCCTTCCTCCCAACCTGACA 550
       E  *                                              (117)
 551 GCGCCCACCTGGCTTTTAAACTGTTTCTGCAACAACATCCAGTTCTGAGT 600
 601 GGTACTAGCTTAAGAAGTGTATAAACATTCATGCTGTATGCCG         643
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prepro-des-Gln[14]-ghrelin

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   1 GGCAGAGAGAAAGGGAGAGATCGAGAATCCCAAGCAGCATGGAGCTTGAT  50
     GCAGGGATCGAACTCATGNGACTGTGAGATCATGACCTGAGCTGAAACCA 100
  51 AGAATCAGATGCTTAACTGACTTCCACCAGGAATCCCAGGCCCACCTGAC 150
 101 ACCATGCCCTCCCCGGGGACCGTGTGCAGCCTGCTGCTCTTCAGCATGCT 200
       M  P  S  P  G  T  V  C  S  L  L  L  F  S  M  L (16)
 151 GTGGGCAGACTTGGCCATGGCAGGCTCCAGCTTCCTGAGCCCCGAACACC 250
       W  A  D  L  A  M  A  G  S  S  F  L  S  P  E  H  (32)
 201 AGAAAGTACAGAGAAAGGAATCCAAGAAGCCACCAGCCAAACTGCAGCCC 300
 251 Q  K  V  Q  R  K  E  S  K  K  P  P  A  K  L  Q  P  (49)
     CGAGCTCTGGAAGGCTTGATCCACCCAGAAGACACAAGTCAAGTGGAAGG 350
 301 R  A  L  E  G  L  I  H  P  E  D  T  S  Q  V  E  G  (66)
     GGCAGAGGATGAACTAGAAATCCGGTTCAACGCCCCTTTTGATGTTGGAA 400
 351   A  E  D  E  L  E  I  R  F  N  A  P  F  D  V  G   (82)
 401 TCAAGCTGTCAGGGGCTCAGTACCACCAGCATGGCCAGGCGCTGGGGAAG 450
     I  K  L  S  G  A  Q  Y  H  Q  H  G  Q  A  L  G  K  (99)
 451 TTTCTTCAGGACGTCCTTTGGGAAGAGGCCGATGAGGTCCTGGCAGATGA 500
       F  L  Q  D  V  L  W  E  E  A  D  E  V  L  A  D  E (116)
 501 GTGATCATCCACTAGAACGACCCACTTGCCTTCCTCCCAACCTGACAGCG 550
       *
 551 CCCACCTGGCTTTTAAACTGTTTCTGCAACAACATCCAGTTCTGAGTGGT 600
 601 ACTAGCTTAAGAAGTGTATAAACATTCATGCTGTATGCCG           640
```

Fig. 2. Nucleotide sequence and deduced amino acid sequence of feline ghrelin cDNA. The feline ghrelin cDNA was 643 bp (prepro-ghrelin) and 640 bp (prepro-des-Gln[14]-ghrelin) in length. The mature sequence of feline ghrelin is *underlined*. The nucleotide sequence of the feline ghrelin precursor has been deposited in the DDBJ/EMBL/GenBank databases with the accession nos. AB089201 (prepro-ghrelin) and AB089202 (prepro-des-Gln[14]-ghrelin).

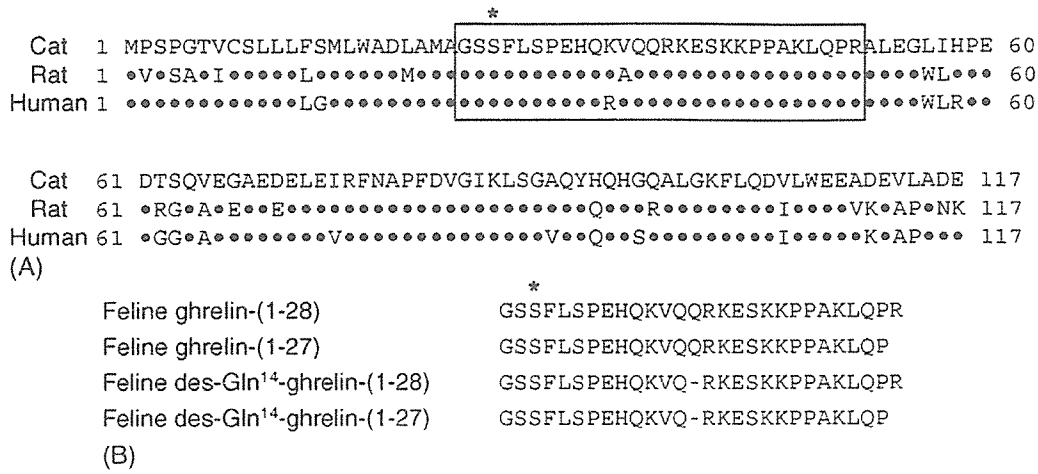

Fig. 3. Structure of feline ghrelin. (A) Amino acid sequences of feline, rat, and human prepro-ghrelin. Mature ghrelin is boxed. The dots indicate identical amino acids with cat. (B) Sequence comparison of feline ghrelin. The asterisk indicates Ser modified by fatty acid.

amino acid sequence were predicted, as shown in Fig. 3B. We concluded that all the isolated peptides were feline ghrelin and its isoforms. The major feline ghrelin, with isolated yield of approximately 105 pmol, was a peptide isolated from group D, peak 13. The expected peptide sequence was ghrelin-(1-28) with saturated octanoic acid (C8:0) (Table 2).

3.4. GH release in response to ghrelin injection

Ghrelin caused significant GH release at doses of 0.5 and 2.5 μg/kg ($P < 0.05$ versus saline) and the response was monophasic (Fig. 4A). The highest dose ghrelin injection caused a peak GH value of 123.9 ng/ml after 20 min, and this value was 18 times higher than the basal level. Plasma GH levels did not increase after injection of ghrelin at 0.05 μg/kg. The GH level returned to its basal value by 80 min after administration of all doses.

3.5. Ghrelin response to feeding

Plasma active ghrelin levels were significantly increased by almost 2.5-fold after fasting for 15 h (before fasting 10.3 ± 4.13 versus after fasting 28.3 ± 4.55 fmol/ml; $P < 0.05$;

Table 2
The molar yield of purified feline ghrelin and ghrelin-derived molecules

	(1-28)	(1-27)	des-Gln^{14}-(1-28)	des-Gln^{14}-(1-27)
C8:1	18.75			
C8:0	107.50	20.00	8.00	8.75
C10:2	21.25	5.63		
C10:1	38.75	10.63		
C10:0	11.25	3.13		
C13:1	0.63			
C13:0	1.50			

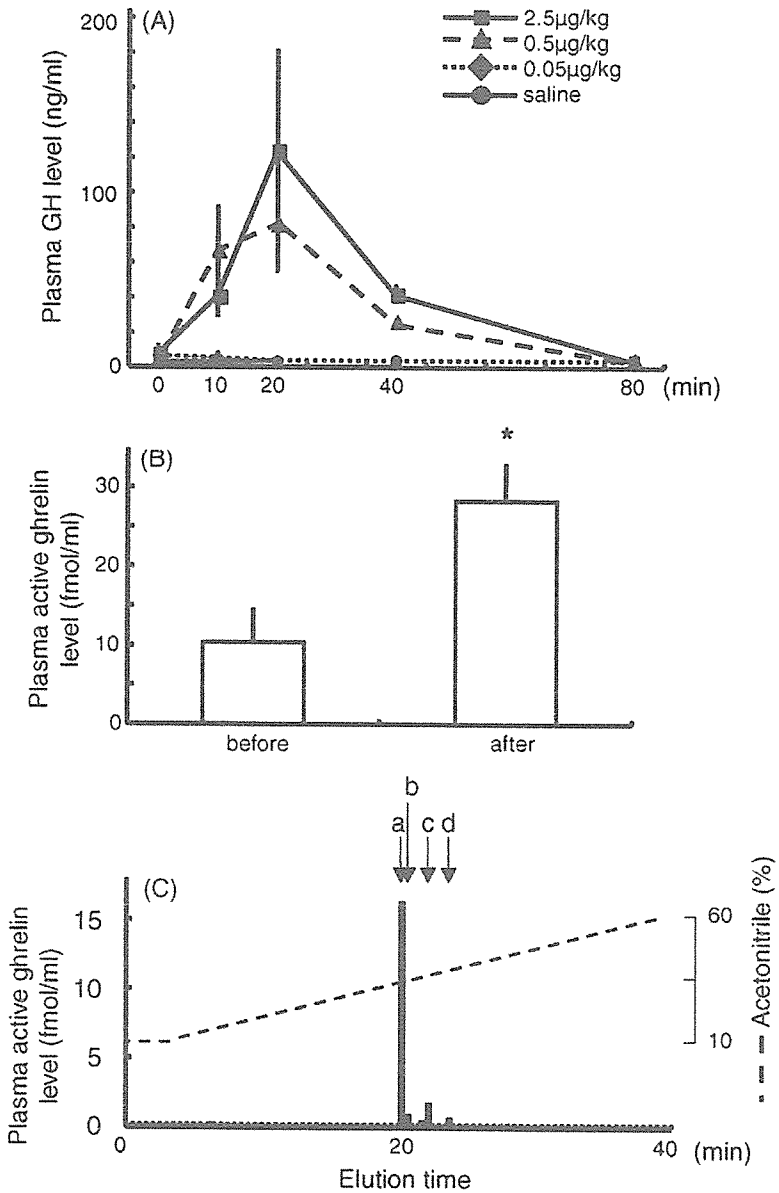

Fig. 4. Biological activity of ghrelin in cats. (A) Time courses of plasma growth hormone concentrations after intravenous injections of synthetic rat ghrelin into cats. Each symbol and vertical line on the line graph represents the mean ± S.E.M. of data from five cats. (B) Effect of fasting for 15 h on plasma active ghrelin levels in cats. Data are means ± S.E.M. from eight cats. $^{*}P < 0.05$. (C) RP-HPLC of feline plasma monitored by active ghrelin ELISA kit. *Solid bars* indicate active fractions. A portion of each fraction (250 μl of plasma equivalent) was subjected to ELISA for ghrelin. Arrows indicate the expected molecular forms of ghrelin from retention time. a: ghrelin-(1-28)(C8:0), b: ghrelin-(1-28)(C10:2), c: ghrelin-(1-28)(C10:1), and d: ghrelin-(1-28)(C10:0).

Fig. 4B). The principle of the active ghrelin ELISA kit is a two-site sandwich enzyme-linked immunosorbent assay. Each antibody was raised against a N-terminal fragment (positions 1–10) and a C-terminal fragment (positions 13–28) of human ghrelin. The ELISA kit is possible for measuring human besides, rat and mouse ghrelin-(1-28)(C8:0). Amino acid sequences of both N- and C-terminal fragments for feline ghrelin are exactly the same

sequence with human, rat and mouse. Therefore, it was believed that this assay was validated for feline ghrelin. To clarify whether the ELISA kit can measure other types of ghrelin in addition to feline ghrelin-(1-28)(C8:0), we used it to measure plasma ghrelin fractions separated by RP-HPLC (Fig. 4C). The retention time of the main fraction detected as ghrelin almost agreed with that of feline ghrelin-(1-28)(C8:0). Although the ELISA kit could detect other fractions (other types of feline ghrelin), it remains unclear whether the measured concentration is exactly correct or whether the ELISA can measure all types of feline ghrelin.

4. Discussion

In the present study, we report the purification and characterization of feline ghrelin and other minor ghrelin-derived molecules from cat stomach. In addition, we show that injected ghrelin can alter GH levels in cats and plasma levels of endogenous ghrelin change on fasting. The major active form of feline ghrelin is a 28-amino acid peptide with an *n*-octanoyl modification at Ser^3. The major form of acyl modification of feline ghrelin was *n*-octanoic acid, as in all the known mammalian and non-mammalian ghrelins except rainbow trout ghrelin [1,3–6]. We also identified from its molecular weight and peptide sequence a decanoylated form that comprised approximately 35% of the isolated feline ghrelin. This finding is similar to that in human [3], chicken [5] and bullfrog [6], in which the decanoylated form represents 23%, 50% and 33% of the total ghrelin population, respectively. Recently, it has been reported that rat and human decanoylated ghrelin shows a potency similar to that of the octanoylated form in CHO-GHSR62 cells [20]. Interestingly, the ratio of saturated to unsaturated octanoylated and decanoylated ghrelins was observed to be 2:1 in cat in contrast to 11:1 in human [3]. In addition, we report the first observations of ghrelin-(1-28)(C8:1), ghrelin-(1-28)(C10:2) and ghrelin-(1-27)(C10:2) from a mammalian species, and of ghrelin-(1-28)(C13:0) and ghrelin-(1-28)(C13:1) from any species. Most of the ghrelin isolated from rainbow trout is modified by unsaturated *n*-decanoic acid [4]. The mechanisms governing the acylation of ghrelin are still unknown, but feeding conditions or food composition may influence the type and extent of acyl modification of ghrelin.

Peptide sequencing, the isolation of two cDNAs and the determination of molecular masses led to the identification of four ghrelin isoforms in cat. It is likely that the ghrelin-(1-27) isolated from human is produced through alternative C-terminal processing of the same precursor as for human ghrelin-(1-28) [3]. Although rat ghrelin-(1-27) is present only at a very low level in rat stomach, cDNA analysis has demonstrated two types of ghrelin precursor in that organ, a 117-amino acid precursor (prepro-ghrelin) and a 116-amino acid precursor (prepro-des-Gln^{14}-ghrelin) [2]. Des-Gln^{14}-ghrelin-(1-28), a splice variant of ghrelin, is the second endogenous ligand for the GHS-R. However, the des-Gln^{14}-ghrelin-(1-28) peptide was not identified in human stomach [3]. In this study, we observed both feline ghrelin-(1-27) and des-Gln^{14}-ghrelin-(1-28) as well as des-Gln^{14}-ghrelin-(1-27). Therefore, it is likely that feline ghrelin is composed of more isoforms than are rat or human ghrelins.

Injection of synthetic rat ghrelin at 2.5 μg/kg into cats caused GH secretion to rise to a level 18 times higher than the basal level by 20 min after injection. The time course and dose–response relationship of ghrelin for GH secretion were closely similar to those

previously reported in humans [21]. There is only one amino acid difference between rat and feline ghrelin. Our study demonstrates that synthetic rat ghrelin is efficacious in stimulation of GH secretion in cats.

We observed that plasma active ghrelin levels increased after fasting. In the commercial ELISA kit, 'active ghrelin' means human ghrelin-(1-28)(C8:0) [22]. On the basis of comparison of the structures of feline and human ghrelin, the kit seems to be suitable for measurement of feline ghrelin-(1-28)(C8:0), although it remains unknown whether other minor ghrelin-derived molecules are recognized by this procedure. Then, we used the ELISA kit to detect ghrelin after separation of feline plasma by RP-HPLC. By this method, we could detect minor ghrelin-derived molecules in feline plasma, although it was unclear whether the measured concentrations were exactly correct or whether all types of feline ghrelin can be measured with the ELISA kit. These minor ghrelin-derived molecules were able to increase calcium levels in CHO-GHSR62 cells and therefore might be able to stimulate feeding behavior in cats. Human studies on the effect of fasting on plasma ghrelin levels have produced conflicting results [23–25], and it is possible that these discrepancies are related to the existence of minor ghrelins. Future studies should investigate the effects of both the major and minor forms of ghrelins on feeding behavior.

In summary, four isoforms of ghrelin with multiple types of acylation were isolated from feline stomach. All feline ghrelin and its multiple isoforms were biologically active in an assay using CHO-GHSR62 cells expressing rat GHS-R. In cats, synthetic rat ghrelin stimulated the release of GH and plasma active ghrelin levels increased after fasting. Further analysis will be required to determine the physiological significance of the various different forms of feline ghrelin. Furthermore, there are many similarities in pathophysiology between humans and cats. Therefore, we anticipate that studies of the physiological functions of ghrelin in cats, including effects on GH release and feeding behavior, will help us to understand the role of ghrelin in human pathophysiology.

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Peripheral ghrelin transmits orexigenic signals through the noradrenergic pathway from the hindbrain to the hypothalamus

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Summary

Ghrelin, a gastrointestinal peptide, stimulates feeding when administered peripherally. Blockade of the vagal afferent pathway abolishes ghrelin-induced feeding, indicating that the vagal afferent pathway may be a route conveying orexigenic ghrelin signals to the brain. Here, we demonstrate that peripheral ghrelin signaling, which travels to the nucleus tractus solitarius (NTS) at least in part via the vagus nerve, increases noradrenaline (NA) in the arcuate nucleus of the hypothalamus, thereby stimulating feeding at least partially through α-$_1$ and β-$_2$ noradrenergic receptors. In addition, bilateral midbrain transections rostral to the NTS, or toxin-induced loss of neurons in the hindbrain that express dopamine β hydroxylase (an NA synthetic enzyme), abolished ghrelin-induced feeding. These findings provide new evidence that the noradrenergic system is necessary in the central control of feeding behavior by peripherally administered ghrelin.

Introduction

Ghrelin, a newly discovered member of the family of gut-brain peptides, functions in feeding control and growth hormone (GH) secretion by binding to the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999; Nakazato et al., 2001; Tschöp et al., 2000; Wren et al., 2000). This peptide, which is produced primarily by endocrine cells of the stomach, is released into the circulation (Date et al., 2000; Dornonville de la Cour et al., 2001). Ghrelin is also produced by neurons of the hypothalamus, where it serves as part of the neural networks (Cowley et al., 2003). GHS-R is extensively distributed throughout the brain, including the hypothalamus and brainstem where are essential for energy homeostasis. Given the GHS-R expression pattern, ghrelin, when given centrally, peripherally, or both, may increase food intake directly via effects on neurons present in the hypothalamus or brainstem. We recently demonstrated, however, that blockade of the gastric vagal afferent pathway abolished peripheral ghrelin-induced feeding (Date et al., 2002). A similar study demonstrated that intraperitoneal injection of ghrelin into vagotomized mice did not stimulate food intake (Asakawa et al., 2001). These findings suggest that the gastric vagal afferent pathway as well as the humoral pathway may have some significant part in conveying ghrelin-mediated orexigenic signals to the brain.

Several gastrointestinal hormones, including ghrelin, cholecystokinin (CCK), peptide YY, and glucagon-like peptide 1, transmit signals of starvation and satiety to the brain at least in part via the vagal afferent system (Date et al., 2002; Smith et al., 1981; Koda et al., 2005; Abbott et al., 2005). Feeding-related information, travels directly to the nucleus tractus solitarius (NTS), where it can be converted to additional signals that transmit a feeling of hunger or fullness to the hypothalamus. In the present study, we focused on the importance of the neural pathways from the NTS to the hypothalamus in transmitting peripheral ghrelin signals.

To investigate the neural pathways involved in the transmission of ghrelin orexigenic signals from the NTS to the hypothalamus, we examined the effects of bilateral midbrain transections on ghrelin-induced feeding. The NTS contains the A2 noradrenergic cell group, which projects to regions of the hypothalamus that include the arcuate nucleus (ARC) (Sawchenko and Swanson, 1981). Therefore, we examined the role of the central noradrenaline (NA) system in peripheral ghrelin feeding stimulation. Using real-time PCRs, we quantified the expression of dopamine β hydroxylase (DBH), an enzyme necessary to convert dopamine into NA, within the NTS. We also measured overflow NA within or near the ARC after intravenous administration of ghrelin using in vivo microdialysis. We studied the effects of adrenergic antagonists and the elimination of NA innervation within the ARC on ghrelin-induced food intake. Using immunohistochemical techniques, we demonstrated that the NPY neurons activated following intravenous administration of ghrelin are innervated by DBH-containing fibers.

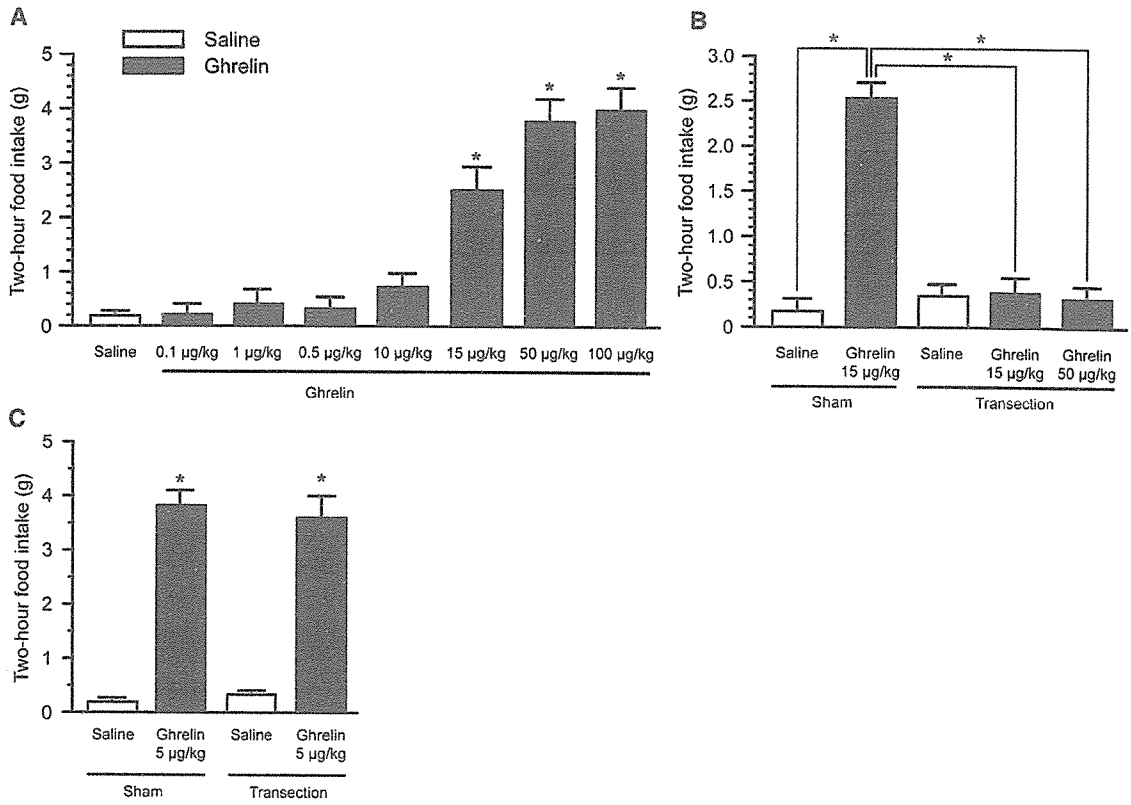

Figure 1. Effect of bilateral midbrain transections on ghrelin-induced feeding behavior

A) Two hour food intake (mean ± SEM) of sham-treated rats after a single intravenous administration of ghrelin (0.1–100 µg/kg). *$p < 0.0001$ versus saline.
B) Food intake of rats with bilateral midbrain transections after a single intravenous administration of ghrelin (15 and 50 µg/kg). *$p < 0.0001$.
C) Food intake of rats with bilateral midbrain transections after single intracerebroventricular administration of ghrelin (5 µg/kg). *$p < 0.0001$.
Error bars represent the SEM.

Results and Discussion

Midbrain transections and peripheral ghrelin-induced feeding

To investigate if intravenous administration of ghrelin stimulates feeding via the ascending efferent fibers of the NTS, we examined ghrelin-induced food intake in rats with bilateral midbrain transections (Crawley et al. 1984). Before this experiment, we confirmed that there were no significant differences in body weight or food intake between control and actual transected groups up to eight days after the surgery (see Supplemental Results and Figure S1 in the Supplemental Data available with this article online). There were also no significant differences in the feeding response after fasting for 12 hr, energy expenditure, locomotor activity, body fat, or food preference between the two groups seven days after surgery (Supplemental Results and Figure S2). Therefore, we performed feeding experiments using rats seven days after the surgery. The lowest effective dose of intravenously (i.v.) administered ghrelin for rats subjected to sham surgery (sham-treated rats) was 15 µg/kg; this value was used as the standard dose in the subsequent experiments (Figure 1A). Intravenous administration of ghrelin (≥ 15 µg/kg) significantly increased food intake (10:00–12:00 hr) in sham-treated rats, whereas ghrelin-induced feeding was absent in midbrain transected rats (Figure 1B) (n = 10 per group). Because bilateral midbrain transections may nonspecifically suppress feeding in response to ghrelin, we tested the orexigenic effect of centrally administered ghrelin in the midbrain transected rats. Intracerebroventricular administration of ghrelin similarly increased food intake in the transected and control groups (Figure 1C) (n = 7 per group). This finding demonstrates that bilateral midbrain transections specifically blocked peripherally administered ghrelin-induced feeding, but did not affect centrally administered ghrelin-induced feeding. Centrally and peripherally administered ghrelin may therefore stimulate feeding by distinct mechanisms. Midbrain transections severing the ascending efferent fibers of the NTS block feeding reduction of CCK that transmits satiety signals to the brain via the afferent limb of the vagus nerve (Crawley et al., 1984). In contrast, Grill and Smith showed that CCK-induced feeding reduction is still observed in chronic decerebrate rats (Grill and Smith, 1988). We described some differences in the surgery between midbrain transection and chronic decerebration in Supplementary Methods (Grill and Norgren, 1978) (Supplemental Experimental Procedures).

We have already shown the possibility that peripheral ghrelin signals for starvation are transmitted to the neuropeptide Y

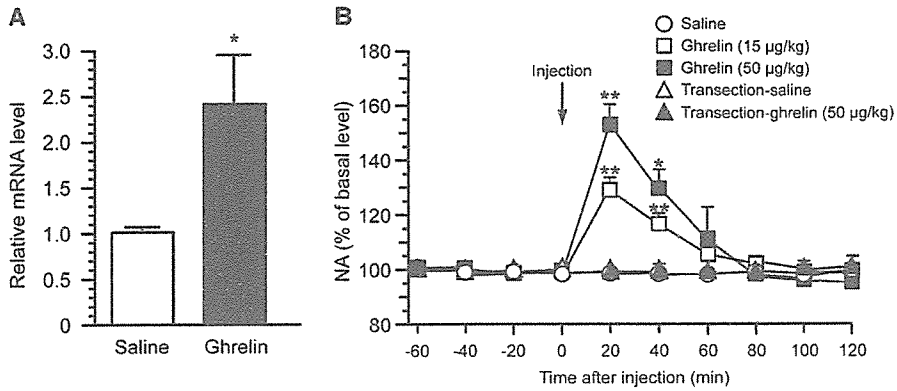

Figure 2. Ghrelin stimulates feeding via the NA system

A) DBH mRNA levels in rats receiving either ghrelin (15 μg/kg, i.v.) or saline. *$p < 0.03$ versus saline.

B) Effect of intravenous ghrelin on NA levels within the ARC in sham-treated and midbrain-transected rats. NA levels are represented as percentages of the mean concentration of NA in four consecutive dialysate samples taken before ghrelin injection. *$p < 0.01$, **$p < 0.0001$ versus sham saline.

Error bars represent the SEM.

neurons of the ARC at least partially via the vagal afferent pathway (Date et al., 2002). The possibility remains, however, that i.v. administered ghrelin may bind directly to receptors present on neurons in the ARC, as the ARC, situated at the base of the hypothalamus, is incompletely isolated from the general circulation by the blood-brain barrier (Banks and Kastin, 1985; Merchenthaler, 1991). The present study shows that ghrelin-induced feeding was abrogated in transected rats. This result indicates that neural pathways ascending from the NTS may play an important role in the transmission of ghrelin orexigenic signals to the hypothalamus. Conveyed to the NTS, these signals could be relayed to the hypothalamus through other transmitters produced by neurons located in the NTS.

NA system and peripheral ghrelin-induced feeding

Although afferent projections from the NTS to the hypothalamus are not exclusively noradrenergic, the noradrenergic pathway is the major constituent. We here showed that DBH mRNA levels increased significantly in the NTS after ghrelin (15 μg/kg) administration (Figure 2A). Considering that the NTS is the termination area of the vagal afferent fibers that receive vicerosensory information from the gastrointestinal tract, it seems reasonable to expect that peripheral ghrelin induces Fos expression in the NTS. We were not, however, able to detect any increase in the number of Fos-expressing neurons in the NTS (Date et al., 2005). This finding is consistent with previous data from other groups (Wang et al., 2002; Rüter et al., 2003). These results may depend on the fact that peripherally administered ghrelin decreases the firing rate of gastric vagal afferent fibers by binding to its receptor present in the vagal afferent terminals (Asakawa et al., 2001; Date et al., 2002). Thus, inhibitory signals caused by peripherally administered ghrelin may affect DBH expression in the NTS.

Peripherally administered ghrelin and synthetic GHSs primarily activate neurons located in the ARC (Hewson and Dickson, 2000). Most peripheral ghrelin-induced Fos-positive neurons in the ARC express NPY (Wang et al., 2002; Date et al., 2002). NPY and agouti-related protein (AgRP), which are colocalized in neurons of the ARC, have been implicated in the stimulation of feeding behavior. Pharmacological examinations indicated that centrally administered NPY Y-1 receptor antagonists block the orexigenic effect of ghrelin injected peripherally (Asakawa et al., 2001). Furthermore, Chen et al. demonstrated that peripherally administered ghrelin does not induce food intake in $NPY^{-/-}$, $AgRP^{-/-}$ double-knockout mice (Chen et al., 2004). These findings suggest that the ARC plays a crucial role in regulating peripheral ghrelin signals. In order to examine whether peripherally administered ghrelin affects the release of NA in the ARC, which is not only a noradrenergic terminal area but also a target site of peripheral ghrelin signals, we monitored overflow NA within or near the ARC using a microdialysis system. Overflow NA is thought to include both newly released NA and NA that was not subject to reuptake. Examination of overflow NA within or near the ARC after intravenous administration of 15 and 30 μg/kg ghrelin to sham-treated rats ($n = 7$ per group) revealed significantly increased NA concentrations within and near the ARC, reaching 129.7 ± 4.7% and 152.8 ± 7.5% of the control levels, respectively (Figure 2B). Ghrelin administration, however, did not induce NA release in transected rats. Hindbrain noradrenergic neurons innervating the hypothalamus are implicated in mediation of the feeding response to glucose deprivation (Ritter et al., 2001), suggesting that the NA system in the brain contributes significantly to feeding regulation and/or energy homeostasis. The present study demonstrates that ghrelin, an orexigenic signal produced in the periphery, increases DBH mRNA levels in the NTS and increases NA levels within the ARC. These results suggest that noradrenergic inputs, projecting from the hindbrain to the ARC, are critical for the feeding behavior induced by peripheral ghrelin. This study, however, has yet to elucidate whether peripheral ghrelin signals transmitted via the vagal afferent pathway affect the NA system in the ARC or whether ghrelin bound to the receptor present in the area postrema or NTS stimulates it. To clarify this issue, further examinations to evaluate NA overflow in the ARC of vagotomized animals are needed.

CCK, an anorectic peptide produced by the gastrointestinal tract, increases the firing rate of the vagal afferent fibers, and thereby transmits satiety information to the NTS. Recently, Sutton et al. showed that the CCK-induced reduction in feeding is modulated by a melanocortinergic pathway through extracellular signal-regulated kinase signaling in the NTS (Sutton

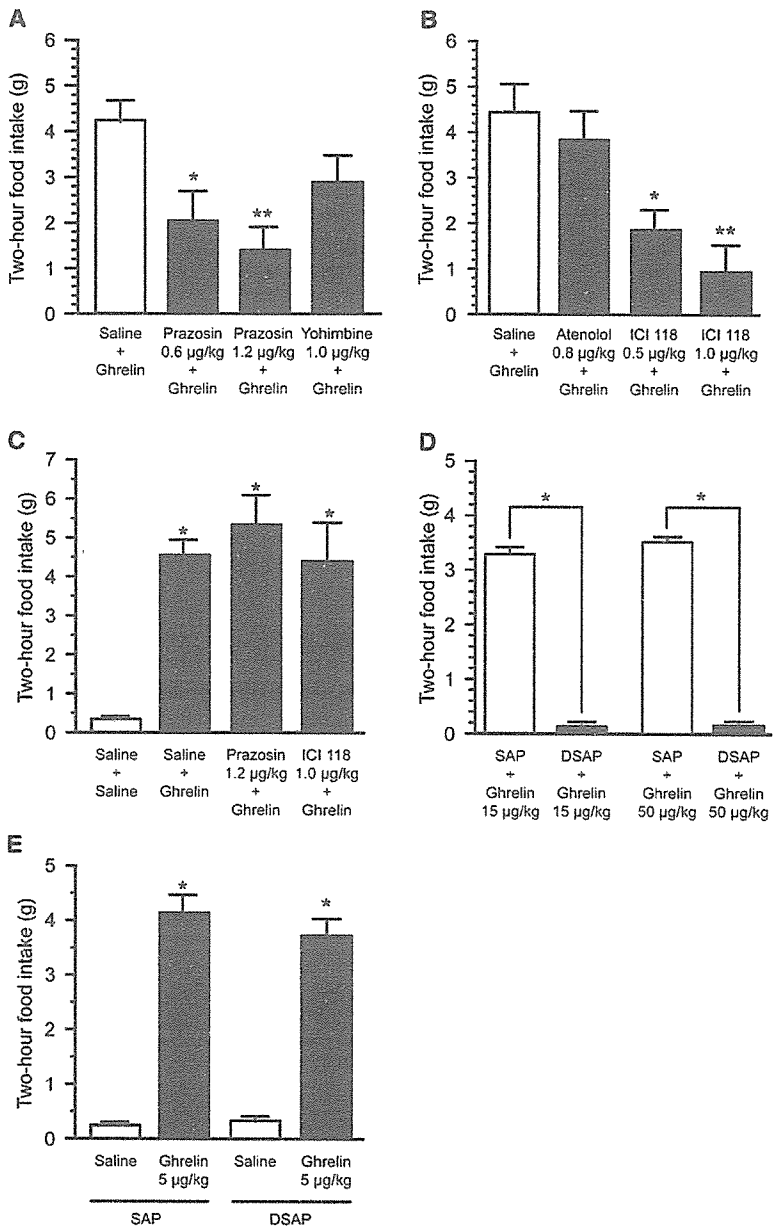

Figure 3. The effects of either pretreatment with adrenoceptor antagonists or disruption of DBH-containing neurons on ghrelin-induced feeding

A) Effect of i.c.v.-administered α_1 or α_2 antagonists on feeding induced by ghrelin (15 µg/kg). *$p < 0.05$, **$p < 0.005$ versus rats given saline plus ghrelin.

B) Effect of i.c.v.-administered β_1 or β_2 antagonists on feeding induced by ghrelin (15 µg/kg). *$p < 0.005$, **$p < 0.001$ versus rats given saline plus ghrelin.

C) Food intake of rats treated with an α_1 or a β_2 antagonist after a single intracerebroventricular administration of ghrelin (5 µg/kg). *$p < 0.0005$ versus saline.

D) Effect of DSAP treatment on ghrelin-induced feeding. *$p < 0.0001$.

E) Food intake of DSAP-treated rats after a single intracerebroventricular administration of ghrelin (5 µg/kg). *$p < 0.0001$.

Error bars represent the SEM.

et al., 2005). There is also a report that fourth ventricular administration of the MC4-melanocortin receptor antagonist SHU9119 completely blocked the peripherally administered CCK-induced decrease in feeding (Fan et al., 2004). These findings indicated that the NTS is crucial for the integration of peripheral ascending signals with descending signals from the hypothalamus that relate to feeding. The precise molecular mechanisms that underlie the effect of peripheral ghrelin signals on NTS function remain to be elucidated. To fully understand the noradrenergic pathway from the NTS to the hypothalamus, further investigations into the identities of the intracellular signaling systems in the NTS that are mobilized by peripheral ghrelin, and the signals from the forebrain that modulate peripheral ghrelin signaling in the NTS are required.

NA can utilize at least four distinct receptor subtypes: α_1, α_2, β_1, and β_2 (O'Dowd et al., 1989). We examined which of these receptors was involved in ghrelin-induced feeding by treatment with an antagonist for each adrenoceptor. Ghrelin-induced feeding was attenuated in rats pretreated with either the specific α_1 antagonist prazosin or the specific β_2 antagonist ICI 118, but not the α_2 antagonist yohimbine or the β_1 antagonist atenolol (Figures 3A and 3B) (n = 7 per group). After injection of these adrenergic antagonists intracerebroventricularly (i.c.v.), rats were observed for behavioral signs of nausea (elongation of the body, gaping, raising the tail, and lowering the belly to the floor), ataxia, sedation, and anxiety (locomotion within the cage and avoidance of the front of the cage). The rats did not exhibit any of these signs during the testing period. We also tested

the orexigenic effect of centrally administered ghrelin in rats treated with prazosin or ICI 118, as these antagonists may nonspecifically suppress feeding in response to ghrelin. Centrally administered ghrelin increased feeding similarly in the prazosin- and ICI 118-treated groups and the control group (Figure 3C). This result suggests that although NA antagonists specifically suppressed feeding induced by peripherally administered ghrelin, centrally administered ghrelin induces feeding by a mechanism that is independent of the noradrenergic system. Considering that NA excites approximately 50% of the neurons in the ARC, probably due to a direct postsynaptic response through α_1- or β-adrenoceptors (Kang et al., 2000), peripherally administered ghrelin may activate NPY/AgRP neurons in the ARC through the NA system. A recent study also suggested the possibility that the GABAergic system is involved in ghrelin-induced feeding. Cowley et al. showed that ghrelin induced depolarization of ARC NPY neurons and hyperpolarization of ARC proopiomelanocortin (POMC) neurons using hypothalamic slices (Cowley et al., 2003). Given that NPY/AgRP neurons expressing are GABAergic, central ghrelin may induce the release of GABA from NPY axonal terminals and thereby modulate the activity of postsynaptic POMC neurons.

NA exerts a variety of responses that depend on the type of neurons and the expression of different adrenoceptor subtypes (Nicoll et al., 1990). Infusion of exogenous NA can cause either increases or decreases in food intake (references in Wellman, 2000), which may depend on the site of application or changes in the numbers of adrenoceptors according to the circadian cycle. For example, NA injection into the hypothalamic paraventricular nucleus (PVN) increases feeding through PVN α_2-adrenoceptors, whereas it decreases feeding through PVN α_1-adrennoceptors (Goldman et al., 1985; Wellman et al., 1993). The circadian pattern in the number of α_2-adrenoceptors within the PVN exhibits a sharp increase in α_2-adrenoceptors at the onset of the dark phase, a time when feeding is greatly enhanced. Taken together, it may be difficult to determine whether microinjection of an NA agonist or antagonist into the hypothalamic nuclei results in a physiologically significant effect. Therefore, in the present study, we focused on the role of endogenous NA induced by peripherally administered ghrelin in the control of food intake. We demonstrated that α_1- and β_2-receptor antagonists attenuated feeding induced by ghrelin. This result indicates that α_1- and/or β_2-adrenoceptors in the ARC play an important role in peripheral ghrelin-induced feeding.

To eliminate NA innervation of the ARC, we used DSAP, a monoclonal antibody specific for DBH, the enzyme that converts dopamine into NA, conjugated to saporin (SAP) (Fraley and Ritter, 2003). DSAP, an immunotoxin that allows an antibody against the NA synthetic enzyme DBH to selectively deliver the saporin toxin, can successfully destroy hindbrain neurons that contain DBH (Rinaman, 2003). Bilateral DSAP injections into the ARC induced an approximately 70% reduction in DBH-positive neurons in the NTS in comparison to the number of DBH neurons present in rats treated with an SAP control solution (data not shown). DSAP injections also completely disrupted peripherally administered ghrelin-induced feeding (Figure 3D) (n = 7 per group). We also tested the orexigenic effect of centrally administered ghrelin in the DSAP-treated rats. Centrally administered ghrelin increased feeding similarly in the DSAP-treated group and the control group (Figure 3E) (n = 7 per group). This finding suggests that the noradrenergic system in the ARC is not involved in centrally administered ghrelin-induced feeding.

There are several catecholaminergic neuronal cell groups in the hindbrain. DBH-positive neurons projecting to the hypothalamus are found within the A2 cell group located in the caudal medial and commissural NTS and the A1/C1 cell group located in the ventrolateral medulla (VML). Most NA neurons within the A2 group directly project to the hypothalamus, central nucleus of the amygdala, and bed nucleus of the stria terminalis, whereas the A2 NA neurons also project to these forebrain areas in part via the A1/C1 group. As viscerosensory signals from the gastrointestinal tract are carried to the caudal medial and commissural NTS via the vagal afferent pathway, NA neurons in A2 may be an integral component of the brainstem circuits that mediate ghrelin-induced feeding. Given the projection from the A2 group to the A1/C1 group, these integrative circuits would include a role for NA neurons in the VML. Our findings suggest that NA neurons in the hindbrain are necessary to convey ghrelin-related orexigenic signals to the hypothalamus.

Innervation of NPY neurons by DBH-containing fibers

To examine the effect of peripheral ghrelin signals ascending from the NTS on neurons in the ARC, we investigated DBH innervation and ghrelin-induced Fos expression using unilateral midbrain-transected rats as described previously (Ericsson et al., 1994; Sawchenko, 1988). We compared DBH innervation and Fos expression in the ARC ipsilateral and contralateral to the lesion. Midbrain transections significantly decreased the DBH-imunoreactive innervation ipsilateral to the lesion (Figures 4A and 4B). This finding is consistent with the fact that the ascending catecholamine input to the hypothalamus is largely unilateral. In lesioned rats, peripherally administered ghrelin resulted in a significant increase in Fos expression in the ARC that was contralateral to the lesion (ipsilateral side, 24.3 ± 1.8 neurons; contralateral side, 50.6 ± 1.9 neurons; $p < 0.001$) (Figures 4C and 4D). When saline was injected i.v. to lesioned rats, Fos expression did not differ significantly on the two sides of the brain (ipsilateral side, 11.6 ± 1.3 neurons; contralateral side, 11.9 ± 1.0 neurons; $p > 0.1$) (data not shown). These results suggest that the midbrain transections that were effective in reducing DBH-positive innervation blocked the response of neurons in the ARC to peripherally administered ghrelin.

Electron microscope immunohistochemistry demonstrated that NPY-immunoreactive perikaryon and dendritic process often received synapses from DBH-containing axon terminals (Figures 4E–4G). Approximately 40%–50% of hypothalamic NPY neuron innervation arises from catecholaminergic neurons in the hindbrain (Everitt and Hokfelt, 1989). NPY, a potent orexigenic peptide, is thought to be the final mediator of ghrelin feeding signals. To examine the anatomical linkage of NPY neurons, which are activated by ghrelin, with DBH-immunoreactive fibers, we performed immunohistochemistry. Intravenous ghrelin injection significantly increased Fos expression in 53% of the NPY neurons in the ARC (Figure 4H), in accordance with previous studies (Date et al., 2002). Triple labeling immunofluorescence demonstrated that 54% of these NPY neurons in the ARC induced to express Fos by ghrelin treatment were innervated by DBH-immunoreactive fibers (Figure 4I). These results suggest that ghrelin signals activate NPY neurons via the noradrenergic pathway ascending from the NTS to the ARC, resulting in increased feeding.

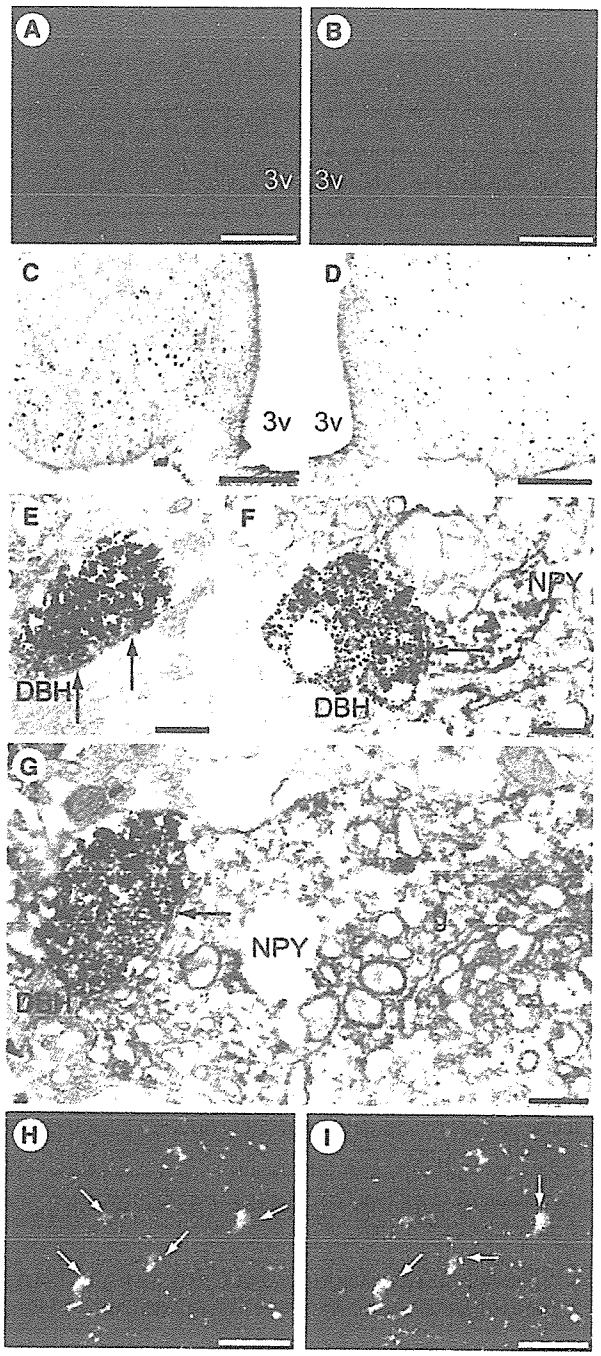

Figure 4. The effect of unilateral midbrain transections on ghrelin-induced Fos expression and activation of NPY neurons by ghrelin via the catecholaminergic pathways

A) DBH-immunoreactive fibers project to the ARC contralateral to the lesion.
B) DBH-immunoreactive innervation ipsilateral to the lesion decreases as compared to that on the contralateral side.
C) Peripherally administered ghrelin (15 μg/kg) induces Fos protein expression contralateral to the lesion.
D) Ghrelin-induced Fos expression ipsilateral to the lesion decreases as compared to that on the contralateral side.
E) DBH-immunoreactive axon terminal making synapses with immunonegative dendritic process (arrow, synapse).
F) DBH-immunoreactive axon terminal making synapses with NPY-immunoreactive dendritic process (arrow, synapse).
G) DBH-immunoreactive axon terminal making synapses with NPY-immunoreactive perikaryon (arrow, synapse).
H) Intravenous administration of ghrelin (15 μg/kg) upregulates Fos expression in NPY neurons of the ARC (arrows) (blue, Fos; green, NPY).
I) Fifty-four percent of ghrelin-activated NPY neurons receive projections from DBH-immunoreactive fibers (arrows) (blue, Fos; green, NPY; red, DBH). g; Golgi apparatus; 3v, third ventricle. The scale bar represents, respectively, 100 μm (**A and B**), 200 μm (**C and D**), 400 nm (**E–G**), and 50 μm (**H and I**).

The present study focused on the hypothesis that the neural pathway from the brainstem to the ARC plays a crucial role in transmitting peripheral ghrelin signals and peripheral ghrelin regulates feeding at least partially via NA-mediated neuronal transmission. Although the central circuits for feeding may have been altered in response to bilateral midbrain transections or DSAP treatment, the results shown here are consistent with the hypothesis. The hypothesis, if correct, is a counterpoint to the most widely accepted model for neuroendocrine energy balance regulation. We have shown previously that peripheral ghrelin-induced feeding is absent in either vagotomized or capsaicin-treated rats. We showed here that ghrelin-induced feeding is also canceled in midbrain transected rats. Thus, it may seem that peripheral ghrelin signals for starvation are transmitted to the hypothalamus only via the vagal afferent pathway and neural pathways from the NTS. However, we have to consider the possibility that vagotomy and/or midbrain transections affect several peripheral substances as well as central circuits relative to feeding. In addition, the present study has yet to address the direct relationship between peripheral ghrelin signals via the vagal afferent pathway and the NA system in the ARC. Taken together, it may be difficult to assert that peripheral ghrelin signals are transmitted only via the neural pathways. We, therefore, think that the humoral pathway and the neural pathway are important routes to convey peripheral energy balance information to the brain. Very recently, we found that peripherally administered leptin decreased 2 hr and 4 hr food intake in vagotomized, midbrain transected, and sham-operated rats, and the leptin-induced reduction in feeding was less pronounced in vagotomized and transected rats than in the sham-operated rats (unpublished data). These findings suggest that the vagal afferent pathway and/or the ascending efferent pathway from the brainstem to the hypothalamus are necessary elements for the effectual action of leptin on feeding and energy homeostasis. Feeding is regulated by a complicated interaction of many orexigenic and anorectic signals; sophisticated interactions between humoral pathways and neural pathways may be necessary to maintain energy homeostasis. We have shown that the central noradrenergic system is a candidate to mediate peripheral ghrelin signals. Although the pathways linking peripheral ghrelin to NA transmission are likely to be more complicated given the remarkable number of signals that provide input to the NTS and ARC, we believe that this study provides an important clue to understanding the feedback loops linking the brain and peripheral tissues in the control of feeding and energy homeostasis.

Experimental procedures

Experimental animals

We maintained male Wistar rats (Charles River Japan, Inc.), weighing 255.9 ± 2.0 g, under controlled temperature and light conditions (0800–2000 hr light).

For feeding and microdialysis experiments, an intravenous cannula was implanted into the right jugular vein of each rat under anesthesia. We performed unilateral or bilateral midbrain transections 5 days after implantation, as described details in the Supplemental Experimental Procedures. To confirm that the transection surgeries were successful, the brains were immunostained using an anti-DBH antiserum diluted 1:1000 (Chemicon International, Inc.) by the avidin-biotin complex method (Date et al., 1999) after the feeding tests were completed (Figure S3A). To facilitate the penetration of a microdialysis probe, a guide cannula (500 μm outside diameter; AG-12, Eicom) was stereotaxically implanted 1.0 mm above the ARC (0.2 mm lateral to the midline, 2.4 mm caudal to the bregma, and 9.0 mm ventral to the dura), fixed to the skull with acrylic dental cement, and sealed with a dummy cannula (350 μm external diameter; AD-12, Eicom). To inject adrenergic receptor antagonists into the rats, we implanted intracerebroventricular cannulae into the lateral cerebral ventricle. To block noradrenergic innervation of the ARC, we microinjected either a SAP-conjugated DBH-specific mouse monoclonal antibodies (DSAP; Advanced Targeting Systems; 42 ng/0.2 μl in phosphate buffer [pH 7.4], n = 6) or SAP-conjugated normal mouse IgG (SAP control solution) (Advanced Targeting Systems; 8.82 ng/0.2 μl, n = 6) bilaterally into the ARC (Ritter et al., 2001). Only animals exhibiting progressive weight gain after these surgeries were used in subsequent experiments. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Food intake

First, rat ghrelin (Peptide Institute, Inc.) at 0.1–100 μg/kg (100 μl), or saline alone (100 μl) was administered i.v. at 1000 hr to ad libitum-fed rats that had undergone a sham operation (n = 7 per group). Second, rat ghrelin (15 or 50 μg/kg [100 μl]) was administered i.v. to rats that had undergone bilateral midbrain transection. Third, rat ghrelin (15 or 50 μg/kg [100 μl]) was administered i.v. to rats that had been treated with either DSAP or SAP control solution. Fourth, ghrelin (5 μg/kg [10 μl]) was injected i.c.v. at 1000 hr into rats that had undergone bilateral midbrain transections or sham operations, or into rats that had been treated with either DSAP or SAP control solution. The dose of centrally administered ghrelin (5 μg/kg) is often used as a standard while investigating the effect of i.c.v.-administered ghrelin on food intake under various conditions (Nakazato et al., 2001; Kamegai et al., 2000; Toshinai et al., 2003). Thus, this dosage is recognized as the most appropriate in constantly inducing food intake when administered i.c.v. Therefore, this dosage was also selected as a standard to evaluate i.c.v. administered ghrelin induced feeding. After ghrelin injection, rats were immediately returned to their cages. Two hour food intake was then measured.

Quantitative RT-PCR

Two hours after intravenous administration of ghrelin (15 μg/kg) or saline to rats, total RNA was extracted from the NTS using TRIZOL Reagent (Invitrogen Corp.). Quantitative RT-PCR for DBH was conducted with a LightCycler system (Roche Diagnostics) using a LightCycler-Fast Start DNA Master SYBR Green I kit (Roche) and the following primer set for rat DBH: 5′-CTAGGGCCCTGGGCGCCAAGGCATT-3′ and 5′-GCCAGAGGAGTCGCG CCGGCCTT-3′. Known amounts of DBH cDNA were used to obtain a standard curve. Rat rRNA levels were also measured as an internal control.

Microdialysis

One week after midbrain transection, the rats were lightly anesthetized with isoflurane, and the dummy cannula was replaced with a microdialysis probe. The tip of the microdialysis probe, covered with hollow fibers (1.0 mm in length, 220 μm external diameter, regenerated cellulose membrane with a molecular weight cutoff of 48 kDa; Eicom), was set to extend 1 mm beyond the guide cannula to reach the ARC. Microdialysis was performed under free-moving conditions. A microinfusion pump was used to continually perfuse the probe with modified physiological Ringer's solution (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$ [pH 6.5]) at a constant flow rate of 1 μl/min. To measure NA, chromatographic analysis of dialysates was carried out by HPLC with electrochemical detection as described previously (Ishizuka et al., 2000). The perfusate from the ARC was automatically injected into the HPLC every 20 min. After a 3 hr stabilization period, baseline NA levels were assessed in four consecutive dialysate samples. At the end of each experiment, rats were sacrificed with an overdose of pentobarbital sodium; the brains were then fixed in 10% neutral buffered formalin. Placement of the microdialysis probe was verified histologically in 40-μm cresyl violet-stained coronal sections (Figure S3B).

Effect of adrenoceptor blockers on ghrelin-induced feeding

At 0930 hr, rats were i.c.v. administered either vehicle alone (saline, n = 6) or one of the specified adrenergic receptor antagonists: prazosin (selective α_1 antagonist: 0.6 or 1.2 μg/kg, n = 6 each) (Sigma Chemical Co.), yohimbine (selective α_2 antagonist: 1.0 μg/kg, n = 6) (Sigma), atenolol (selective β_1 antagonist: 0.8 μg/kg, n = 10) (Sigma), or ICI 118 (selective β_2 antagonist: 0.5 or 1.0 μg/kg, n = 6 each) (Sigma). Thirty minutes after adrenergic receptor antagonist injection, ghrelin (50 μg/kg) was administered intraperitoneally to rats; 2 hr food intake was measured. We also tested the orexigenic effect of centrally administered ghrelin in rats that had been injected with prazosin or ICI 118. Thirty minutes after prazosin (1.2 μg/kg, n = 6) or ICI 118 (1.0 μg/kg, n = 6) injection, ghrelin (5 μg/kg) was administered i.c.v. to rats; 2 hr food intake was measured. The rats fasted between the two injections.

Immunohistochemistry

Ghrelin (15 μg/kg) or saline was injected i.v. into rats 90 min before transcardial perfusion with fixative containing 4% paraformaldehyde (n = 5 per group). The brains of animals were then cut into 20-μm thick sections. The sections were first incubated with anti-c-Fos antiserum (1:500, Santa Cruz Biotechnology), and then with Alexa Flour 350-conjugated donkey anti-goat IgG (Molecular Probes, Inc.). Next, samples were incubated with anti-NPY antiserum (1:500, ImmunoStar, Inc.), then with Alexa Flour 488-conjugated chicken anti-rabbit IgG (Molecular Probes, Inc.). Finally, the samples were incubated with anti-DBH antiserum (1:1,000, Chemicon International, Inc.), then with Alexa Flour 568-conjugated goat anti-mouse IgG (Molecular Probes, Inc.). Samples were then observed under a BH2-RFC microscope (Olympus Corp.). We counted the number of Fos-immunoreactive cells in the bilateral ARCs (bregma: −2.30 to −3.30 from Paxinos and Watson's rat brain atlas). Sections from unilaterally transected rats were also incubated with anti-DBH antiserum, and then with Alexa Flour 568-conjugated goat anti-mouse IgG (Molecular Probes, Inc.). A significant (>60%) depletion of DBH-immunoreactive fibers was determined by semi-quantitative comparison of the strength of the DBH-positive innervation of the ARC ipsilateral and contralateral to the lesion by two independent observers (Sawchenko, 1988). Sections from unilaterally transected rats were incubated with anti-c-Fos antiserum (Santa Cruz Biotechnology), and then stained by the avidin-biotin complex method (Date et al., 1999). The number of Fos-immunoreactive cells was compared in the ARC ipsilateral and contralateral to the letion. Fos-expressing cells of the ARC in a 0.7-mm right triangle (0.245 mm^2) were counted in every fifth section (ten tissue sections per rat) using a cell-counting program written for NIH Image (v1.62; NIH).

Electron microscope immunohistochemistry

Three Wistar rats were perfused as described above. The brain was cut into 30–40 μm thick sections using an Oxford vibratome (Oxford Instruments). Electron microscope immunohistochemistry was performed using anti-NPY antiserum and anti-DBH antiserum as described previously (Toshinai et al., 2003).

Statistical analysis

We analyzed groups of data (means ± SEM) using analysis of variance (ANOVA) and post hoc Fisher tests. p values less than 0.05 were considered to be significant (two-tailed tests).

Supplemental data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, Supplemental References, and three figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/4/323/DC1/.

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Peripheral Interaction of Ghrelin with Cholecystokinin on Feeding Regulation

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Ghrelin and cholecystokinin (CCK) are gastrointestinal hormones regulating feeding. Both transmitted via the vagal afferent, ghrelin elicits starvation signals, whereas CCK induces satiety signals. We investigated the interaction between ghrelin and CCK functioning in short-term regulation of feeding in Otsuka Long-Evans Tokushima fatty (OLETF) rats, which have a disrupted CCK type A receptor (CCK-AR), and their lean littermates, Long-Evans Tokushima Otsuka (LETO) rats. Intravenous administration of ghrelin increased 2-h food intake in both OLETF and LETO rats. Because OLETF rats are CCK insensitive, iv-administered CCK decreased 2-h food intake in LETO, but not in OLETF, rats. Although preadministration of CCK to LETO rats blocked food intake induced by ghrelin, CCK preadministration to OLETF rats did not affect ghrelin-induced food intake. Conversely, preadministration of ghrelin to LETO rats blocked feeding reductions induced by CCK. In electrophysiological studies, once gastric vagal afferent discharges were altered by ghrelin or CCK administration, they could not be additionally affected by serial administrations of either CCK or ghrelin, respectively. The induction of Fos expression in the hypothalamic arcuate nucleus by ghrelin was also attenuated by CCK preadministration. Using immunohistochemistry, we also demonstrated the colocalization of GH secretagogue receptor (GHS-R), the cellular receptor for ghrelin, with CCK-AR in vagal afferent neurons. These results indicate that the vagus nerve plays a crucial role in determining peripheral energy balance. The efficiency of ghrelin and CCK signal transduction may depend on the balance of their respective plasma concentration and/or on interactions between GHS-R and CCK-AR. (*Endocrinology* 146: 3518–3525, 2005)

IN ADULT ANIMALS and humans, body weight usually remains within a relatively narrow range, despite large day-to-day changes in the amount of food consumed. Even when the restriction of food intake or excessive overfeeding induces changes in body adiposity, both body weight and adiposity in humans and animals return to baseline levels after the resumption of regular feeding (1, 2, 3). Multiple peripheral signals (*e.g.* nutrients, nutrient metabolites, or hormones) regulate short-term and long-term food intake and energy balance through diverse but interacting pathways (4). Signals affecting short-term food uptake have significantly different mechanisms than the long-term regulators of energy homeostasis activated in proportion to both body adipose stores and the food consumed over prolonged periods.

Using an intracellular calcium assay of stable cell lines expressing rat GH secretagogue receptor (GHS-R), we recently discovered in rat stomach a novel endogenous ligand for the GHS-R (5) named ghrelin. Ghrelin, produced primarily in endocrine cells of the stomach, is released into circulation (5–7). Whereas multiple gastrointestinal hormones have been implicated in feeding regulation (8–12), ghrelin stimulates appetite, food intake, and GH secretion when administered to humans and rodents (5, 13–17). In humans, the circulating ghrelin levels increase before and decrease after every meal (18–22), suggesting that ghrelin functions as a meal initiator. The effect of ghrelin on feeding is rapid and short-lived, implying that ghrelin functions in short-term regulation of feeding. The inverse correlation between ghrelin levels and body mass index, as well as ghrelin-mediated promotion of adipogenesis, suggests that ghrelin may also participate in long-term regulation of body weight (14, 19, 23–27).

Most gastrointestinal hormones regulating feeding, with the exception of ghrelin, inhibit food intake (28, 29). Cholecystokinin (CCK) decreases meal size in rats and humans when administered peripherally (30–32). This peptide, released from the proximal small intestine, functions as a postprandial satiety signal (33–36). The anorectic effect of CCK is also rapid and short-lived; long-term peripheral administration of CCK does not reduce overall food intake or induce maintained weight loss (37). These results suggest that CCK plays an essential role in the short-term regulation of feeding.

Although ghrelin has an opposite effect on feeding as CCK, this peptide exhibits characteristics similar to CCK on the short-term regulation of feeding. Both ghrelin and CCK, after release from the gastrointestinal tract, transmit starva-

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Abbreviations: CCK, Cholecystokinin; CCK-AR, CCK type A receptor; GHS-R, GH secretagogue receptor; LETO, Long-Evans Tokushima Otsuka; NPY, neuropeptide Y; NTS, the nucleus of the solitary tract; OLETF, Otsuka Long-Evans Tokushima fatty; PBN, parabranchial nucleus.

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tion and satiety signals to the brain through receptors, GHS-R and CCK type A receptor (CCK-AR), respectively, located in the vagal capsaicin-sensitive afferents (38–42). Thus, vagal afferent fibers represent a major target of these peripheral feeding regulators, ghrelin and CCK.

In this study, we examined the functional relationship between ghrelin and CCK in the short-term regulation of food intake using CCK-AR-deficient Otsuka Long-Evans Tokushima fatty (OLETF) rats and their lean littermates, Long-Evans Tokushima Otsuka (LETO) rats. We also investigated the colocalization of GHS-R with CCK-AR in rat vagal afferents. Because iv administration of ghrelin induces Fos expression in the hypothalamic arcuate nucleus of rats through gastric vagal afferents, we examined the induction of Fos expression in the arcuate nucleus by iv administration of ghrelin after CCK treatment. The electrical discharge of gastric vagal afferents is attenuated by ghrelin and stimulated by CCK (38, 42–48). In this study, we evaluated changes in vagal afferent firing induced by iv treatment of ghrelin and CCK after CCK and ghrelin administration, respectively.

Materials and Methods

Animals

Ten-week-old OLETF and lean littermate LETO rats (body weight: OLETF, 403.2 ± 6.0 g; LETO, 392.8 ± 2.6 g; $P > 0.1$; n = 20), obtained from Otsuka Pharmaceutical (Tokushima, Japan), were used in the experiments for feeding. Male Wistar rats (body weight: 361.6 ± 1.3 g; n = 20) (Charles River Japan, Inc., Shiga, Japan) were used for immunohistochemistry, Fos expression, and electrophysiological studies. Rats were housed individually in plastic cages at constant room temperature in a 12-h light (0800–2000)/12-h dark cycle. Animals were given standard laboratory chow and water *ad libitum*. Intravenous cannulas were implanted into the right jugular vein under anesthesia after an ip injection of sodium pentobarbital (80 mg/kg body weight) (Abbott Laboratories, Chicago, IL). Rats were sham-injected before the study and weighed and handled daily. We also injected heparin daily (1 U/100 μl 0.9% saline) into the cannulas of the animals to prevent coagulation. Only animals exhibiting progressive weight gain after surgery were used in subsequent experiments. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Preparation of anti-GHS-R serum

The [Cys0]-rat GHS-R [342–364] peptide was synthesized using the Fmoc solid-phase method on a peptide synthesizer (433A; Applied Biosystems, Foster City, CA), then purified by reverse phase-HPLC. The synthesized peptide (10 mg) was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (6 mg) (mcKLH; Pierce, Rockford, IL) in conjugation buffer (Pierce). The conjugate was emulsified with an equal volume of Freund's complete adjuvant and was used to immunize New Zealand white rabbits by intracutaneous and sc injection. Animals were boosted every 2 wk and bled 7 d after each injection. The specificity of the antisera was confirmed by immunoreactivity of GHS-R-expressing (CHO-GHSR62 cells), but not of control cells.

Feeding experiments

Experiments were performed 1 wk after iv cannulation. First, CCK (Peptide Institute, Inc., Osaka, Japan) was dissolved in 0.9% saline, and this solution (10 pmol-5 nmol/100 μl) was administered iv at 1000 h to LETO rats after fasting for an 8-h period to determine the lowest effective dose of CCK on feeding (n = 10 per group). Second, a solution of rat ghrelin (Peptide Institute) dissolved in 0.9% saline (1.5 nmol/100 μl or 3 nmol/100 μl) was administered iv at 1000 h to OLETF and LETO rats fed *ad libitum* (n = 10 per group). After injection, rats were immediately returned to their cages, then 2-h food intake was measured. Third, a solution of CCK dissolved in 0.9% saline (1 nmol/100 μl) was administered iv at 1000 h to OLETF and LETO rats after fasting for an 8-h period (n = 10 per group). After returning rats to their cages immediately after injection, 2-h total food intake was measured. Fourth, after fasting for an 8-h period, OLETF and LETO rats were treated with CCK (1 nmol/100 μl). They were not given any food until the next injections. Animals were subsequently given ghrelin (3 nmol/100 μl) or saline (100 μl) 30 min after CCK injection (n = 10 per group). After ghrelin or saline injection, 2-h food intake was measured. Fifth, after an 8-h fasting period, LETO rats were first treated with ghrelin (3 nmol/100 μl), then subsequently given CCK (1 nmol/100 μl) or saline (100 μl) 30 min after ghrelin injection (n = 10 per group). The rats were fasted between ghrelin and CCK or saline injections. After this second injection, 2-h food intake was measured. These feeding studies were performed in a crossover design. Rats were allowed at least 4 d without injections between experimental days.

Immunohistochemical double-staining

Three Wistar rats, weighing 300–350 g, were perfused transcardially with 0.1 M phosphate buffer (pH 7.4), then with 4% paraformaldehyde in a 0.1 M phosphate buffer. The nodose ganglia were sectioned into 12 μm-thick slices at −20 C using a cryostat. Sections were stored at −80 C. Primary neurons were also obtained from the nodose ganglia of five Wistar rats, ranging from 5–6 wk of age. These neurons were submitted to collagenase dispersion as described (49, 50), then seeded and cultured for 4 d in polyethylenimine-coated Lab-Tek chamber slides (Electron Microscopy Sciences, Hatfield, PA) in complete DMEM (25 mM glucose) containing 5% newborn calf serum, 5% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 30 ng/ml nerve growth factor 2.5S (Sigma Chemical Co., St. Louis, MO), and 2 mM L-glutamine at 37 C in 5% CO_2. The medium was replaced every 2 d. Slides were washed in 0.01 M PBS (pH 7.4), then fixed in 10% formaldehyde. The slides of both the primary culture and sectioned nodose ganglia were incubated overnight at 4 C in rabbit anti-GHS-R antiserum (dilution 1/1000). Antibody staining was detected using Alexa Fluor 594-conjugated chicken antirabbit IgG (Molecular Probes, Inc., Eugene, OR). Samples were subsequently incubated with anti-CCK-AR antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; dilution 1/100), then with Alexa Fluor 488-conjugated donkey antigoat IgG (Molecular Probes, Inc.). Slides were observed by fluorescence microscopy (BH2-RFC; Olympus, Tokyo, Japan). The number of neurons expressing GHS-R or CCK-AR immunoreactivity in the nodose ganglion was quantified by counting two randomly selected visual fields in two sections from each of the three rats.

Fos expression and image analysis

The lowest effective dose of ghrelin or CCK on feeding was used for Fos expression studies. CCK (1 nmol/100 μl) or saline was injected iv into three male Wistar rats weighing 341.6 ± 1.3 g. Ghrelin (1.5 nmol/100 μl) was then injected into these rats 30 min after CCK or saline injection. Ninety minutes after ghrelin or saline injection, rats were perfused transcardially with fixative containing 4% paraformaldehyde. The brain was sectioned into 40-μm-thick samples. Immunohistochemistry of Fos was performed as described (51). Quantitation of Fos-immunoreactive cells in the nucleus of the solitary tract (NTS), parabranchial nucleus (PBN), and hypothalamic arcuate nucleus (bregma: −11.30 to −14.60 for NTS, −9.16 to −10.04 for PBN, −2.30 to −3.30 for the arcuate nucleus from Paxinos and Watson's rats brain atlas) was performed bilaterally. Fos-expressing cells of the arcuate nucleus for a 0.7-mm right triangle (0.245 mm^2) were counted in every fifth section (200 μm frequency) (five tissue sections per rats) using a cell counting program written for NIH Image (version 1.62; National Institutes of Health, Bethesda, MD).

Electrophysiological study

Multiunit neural discharge in gastric vagal afferent fibers was recorded extracellularly. Male Wistar rats, fasted for an 8-h period, were anesthetized by an ip injection of urethan (1 g/kg) (Sigma). The electrophysiological study was performed under anesthetization throughout. The rat trachea was intubated, and the electrocardiogram was recorded. Body temperature was maintained at 37 C. Standard methods of extracellular recording from vagal nerve filaments were used, as described in detail elsewhere (52). After laparotomy, a small catheter (Intramedic PE-10; Clay Adams, Parsippany, NJ) was inserted into the

inferior vena cava. After gastric branches of the vagus nerve were visualized, we placed filaments isolated from the peripheral cut end of the ventral branch for recording of afferent nerve activity on a pair of silver wire electrodes. Silver wire electrodes, connected through an alternating current-coupled differential amplifier (DAP-10E; Dia Medical Systems, Co., Tokyo, Japan) to an oscilloscope and magnetic tape recorder, were used for display and storage of the neural activity. A window discriminator (DSE-325A) converted spikes to constant amplitude pulses for analysis of spike frequency with a rate meter that reset at 5-sec intervals. Output from the rate meter was recorded on a chart paper (8K20 recorder; NEC-SAN E1 Co., Tokyo, Japan). Ghrelin (0.03 pmol–1.5 nmol/100 μl) or CCK (0.01 pmol–1 nmol/100 μl) was administered iv to rats through a catheter inserted into the inferior vena cava (n = 10 per group). After administration, nerve discharges from the multiunit afferents were recorded for 60 min and analyzed. In addition, CCK (1 nmol/100 μl) or ghrelin (1.5 nmol/100 μl), considered as a standard dose for feeding (38), was administered iv to rats through a catheter inserted into the inferior vena cava (n = 5 per group) before recording the multiunit afferent nerve discharges for 30 min. After the subsequent iv administration of either ghrelin (1.5 nmol/100 μl) or CCK (1 nmol/100 μl) to these rats, multiunit afferent nerve discharges were recorded for 30 min and analyzed.

Statistical analysis

Groups of data (mean ± SEM) were compared using ANOVA and *post hoc* Fisher's test. $P < 0.05$ was considered to be significant.

Results

Effects of ghrelin and CCK on feeding in OLETF or LETO rats

We first tested various doses of CCK ranging from 10 pmol to 5 nmol in a food intake experiment using LETO rats fasted for an 8-h period (Fig. 1A). The lowest effective dose of CCK administered iv was 1 nmol, which also applied to feeding examination using Wistar rats (data not shown). Therefore, we used 1 nmol as a standard dose of CCK in a subsequent series of experiments. The lowest effective dose of ghrelin on feeding in Wistar rats was 1.5 nmol (38); however, a single iv administration of 1.5 nmol ghrelin to OLETF and LETO rats did not induce feeding (LETO: saline, 0.35 ± 0.19 g; 1.5 nmol ghrelin, 0.28 ± 0.14 g; $P > 0.7$, n = 10; OLETF: saline, 0.36 ± 0.19 g; 1.5 nmol ghrelin, 0.33 ± 0.21 g; $P > 0.9$, n = 10). Because a single iv administration of 3.0 nmol ghrelin significantly increased food intake in both OLETF and LETO rats (Fig. 1B), we used this dose as a standard dose of ghrelin in feeding experiments. Although a single iv administration of CCK significantly decreased food intake in LETO rats, it did not affect food intake in CCK-AR-deficient OLETF rats (Fig. 1C). When CCK was administered iv to LETO rats 30 min before ghrelin administration, we could not observe a ghrelin-induced increase in food intake (Fig. 2A). Administration of CCK iv to OLETF rats 30 min before ghrelin treatment, however, induced similar increases in food intake as those seen in rats without pretreatment (Fig. 2A). Conversely, when ghrelin was administered iv to LETO rats 30 min before CCK treatment, the CCK-induced feeding reduction could not be observed (Fig. 2B).

Immunohistochemistry

GHS-R- and CCK-AR-immunoreactive neurons were found throughout the nodose ganglion (Fig. 3A). Approximately 70% of GHS-R-immunoreactive neurons in the nodose ganglion also expressed CCK-AR (Fig. 3, B–D). Double-staining studies also demonstrated the colocalization of GHS-R with CCK-AR in cultured nodose ganglion neurons (Fig. 3, E–G). GHS-R immunoreactivity was observed in GHS-R-expressing CHO cells (CHO-GHSR62 cells), but not in control CHO cells (data not shown). No GHS-R-specific immunoreactivity could be detected in the nodose ganglion using either normal rabbit serum or antiserum absorbed with excess synthetic GHS-R [342–364] (Fig. 3H).

Fos expression

A single iv administration of ghrelin induced Fos protein in the arcuate nucleus of the hypothalamus of rats (Fig. 4A). Fos-positive neurons were mainly distributed from the anterior to the middle region of the arcuate nucleus. Ghrelin-induced Fos expression in the NTS and PBN was not found (data not shown). When CCK was administered to rats iv 30 min before ghrelin treatment, the number of Fos-expressing neurons was significantly decreased compared with that induced by ghrelin alone (Fig. 4, B and E) and was not different from that of control rats (Fig. 4, C and E).

Electrophysiological study

Intravenous administration of ghrelin to rats significantly suppressed gastric vagal afferent activity (Fig. 5A), whereas iv administration of CCK significantly enhanced the afferent activity (Fig. 5B). The lowest effective doses of ghrelin and CCK were 0.3 pmol and 0.1 pmol, respectively (Fig. 5, A and

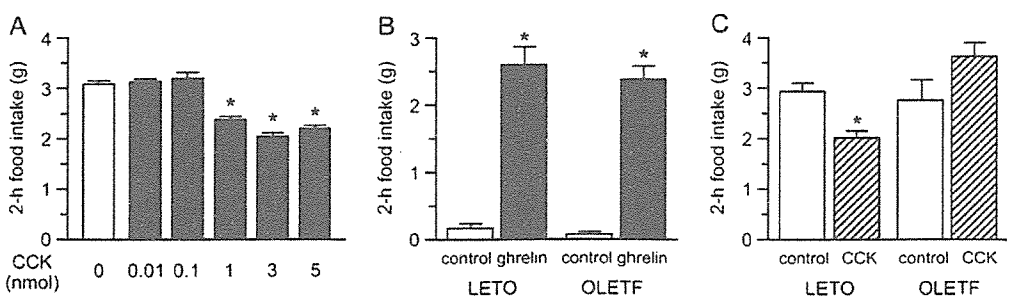

FIG. 1. The effect of iv administration of ghrelin or CCK on food intake in LETO and OLETF rats. A, Two-hour food intake (mean ± SEM) of 8-h fasting rats after a single iv administration of CCK (0.01–5 nmol). *, $P < 0.0005$ *vs.* control. B, After a single iv administration of ghrelin (3 nmol) or saline to LETO and OLETF rats, 2-h food intake from 1000–1200 h was measured. *, $P < 0.0001$ *vs.* control. C, After a single iv administration of CCK (1 nmol) or saline to LETO and OLETF rats after an 8-h fasting period, 2-h food intake from 1000–1200 h was measured. Control rats were administered saline iv. *, $P < 0.001$ *vs.* control.

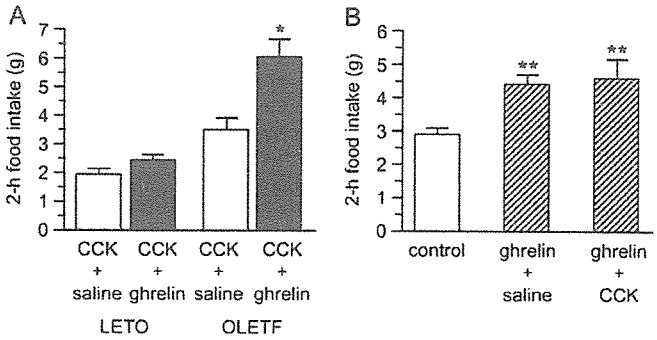

FIG. 2. The interaction between ghrelin and CCK on food intake in LETO and OLETF rats. A, After a single iv administration of ghrelin or saline after CCK treatment of LETO and OLETF rats after an 8-h fasting period, 2-h food intake from 1000–1200 h was measured. *, $P < 0.01$ *vs.* OLETF rats administered saline after CCK treatment. B, After a single iv administration of CCK or saline after ghrelin or saline treatment of LETO rats after an 8-h fasting period, 2-h food intake was measured from 1000–1200 h. Control rats were administered saline iv. **, $P < 0.001$ *vs.* control.

B). To investigate the interaction between ghrelin and CCK in electrophysiological studies, we used 1.5 nmol ghrelin and 1 nmol CCK, considered as their respective standard doses for feeding. When ghrelin was administered to rats after CCK treatment, gastric vagal afferent activity was not suppressed (Fig. 5C). Conversely, when CCK was administered to rats after ghrelin treatment, the CCK-mediated enhancement of afferent activity could not be observed (Fig. 5D).

Discussion

Signals produced within gastrointestinal tract affect feeding patterns (53). Distension of the stomach inhibits feeding, while nutrients in the small intestine induce the release of several gastrointestinal hormones, including CCK, an intestinal anorectic peptide (8, 9). The vagus nerve plays an important role in regulating feeding behavior by transmitting chemosensory and mechanosensory information from the viscera (54). Both neural and humoral signals for satiety and starvation generated in the gastrointestinal tract can be conveyed to the brain via the vagal afferent nerve and the blood circulation.

In the present study, we investigated peripheral interaction of ghrelin, an orexigenic gut peptide, with CCK on feeding regulation. Ghrelin, discovered in the stomach, stimulates food intake and GH secretion after iv administration (5, 16, 38, 55). Because the existence of an orexigenic peptide-based system in the periphery has yet to be discovered, ghrelin is thought to be the first peptide acting in the periphery as a starvation signal. Plasma ghrelin levels are up-regulated under conditions of negative energy balance including starvation, whereas they are down-regulated under conditions of positive energy balance (19, 56–59). Glucose load and food intake lead to a rapid fall in plasma ghrelin concentration, indicating that endogenous ghrelin serves as an indicator of short-term energy balance (19). Very recently, Cummings *et al.* (22) showed the preprandial increase of ghrelin levels among humans without time- or food-related cues and the overlap between these levels and hunger scores. These findings indicate ghrelin would be a candidate for peripheral meal initiator. In contrast, CCK, the most well-studied gastrointestinal peptide functioning in feeding, transmits a satiety signal to the NTS via vagal afferents (41, 43, 60). CCK decreases food intake when peripherally administered to rats (30). In humans, postprandial CCK levels are increased about five times higher than fasting levels (35, 61, 62). Thus, CCK has been thought to be a meal terminator.

Ghrelin is produced not only in the stomach but also in the hypothalamus (5, 63). Centrally administered ghrelin also stimulates both food intake and GH secretion (5, 13, 15, 64–66), and the ghrelin receptor is expressed in neuropeptide Y (NPY)- and GHRH-producing neurons in the hypothalamic arcuate nucleus, where it is incompletely isolated from the general circulation by the blood-brain barrier (67).

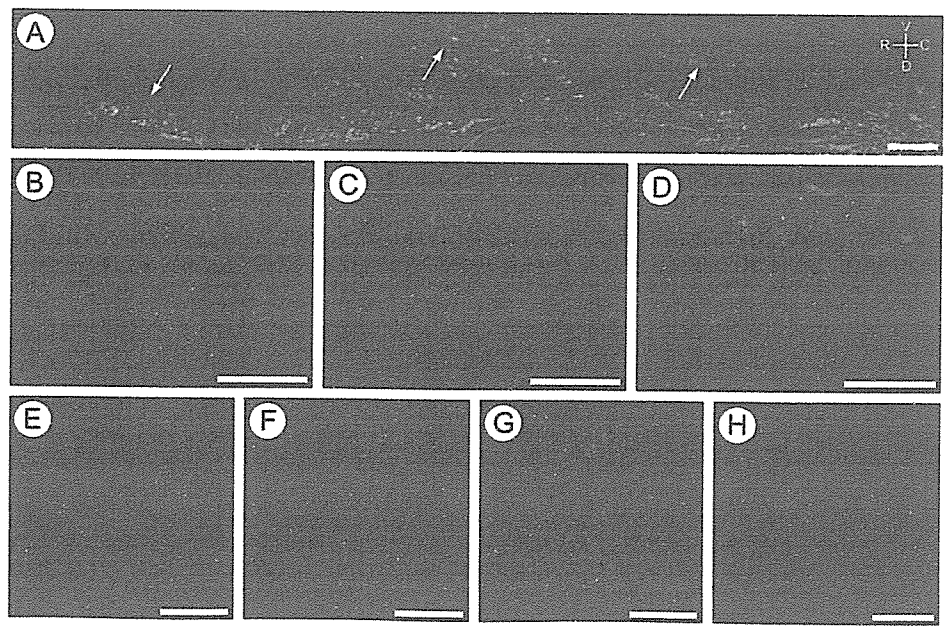

FIG. 3. Colocalization of GHS-R with CCK-AR in neurons of the nodose ganglion. A, GHS-R-immunoreactive neurons (*arrows*) are distributed throughout the nodose ganglion. Antisera for GHS-R [342–364] (A, B, D, E, and G) and CCK-AR (C, D, F, and G) were used to assess the (D) immunofluorescence double staining of GHS-R and CCK-AR in the nodose ganglion or (G) immunofluorescence double staining of GHS-R and CCK-AR in primary cultured neurons of the nodose ganglion. H, No GHS-R-immunoreactivity was observed with antiserum absorbed with excessive synthetic GHS-R [342–364]. R, Rostral; C, caudal; D, dorsal; V, ventral. *Scale bar*, 200 μm (A), 100 μm (B–H)