SHORT ARTICLE

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

Yukari Date,[1,8,*] Takuya Shimbara,[1,8] Shuichi Koda,[1,3] Koji Toshinai,[1] Takanori Ida,[4] Noboru Murakami,[4] Mikiya Miyazato,[5] Koichi Kokame,[6] Yuta Ishizuka,[2] Yasushi Ishida,[2] Haruaki Kageyama,[7] Seiji Shioda,[7] Kenji Kangawa,[5] and Masamitsu Nakazato[1]

[1] Third Department of Internal Medicine
[2] Department of Psychiatry
Miyazaki Medical College, University of Miyazaki, Miyazaki 889-1692, Japan
[3] Biomedical Research Laboratories, Daiichi Asubio Pharma Co., Ltd., Osaka 681-8513, Japan
[4] Department of Veterinary Physiology, Faculty of Agriculture, Miyazaki University, Miyazaki 889-2192, Japan
[5] Department of Biochemistry
[6] Department of Vascular Physiology
National Cardiovascular Center Research Institute, Osaka 565-8565, Japan
[7] Department of Anatomy, Showa University School of Medicine, Tokyo142-8555, Japan
[8] These authors contributed equally to this work.
*Correspondence: dateyuka@med.miyazaki-u.ac.jp

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.

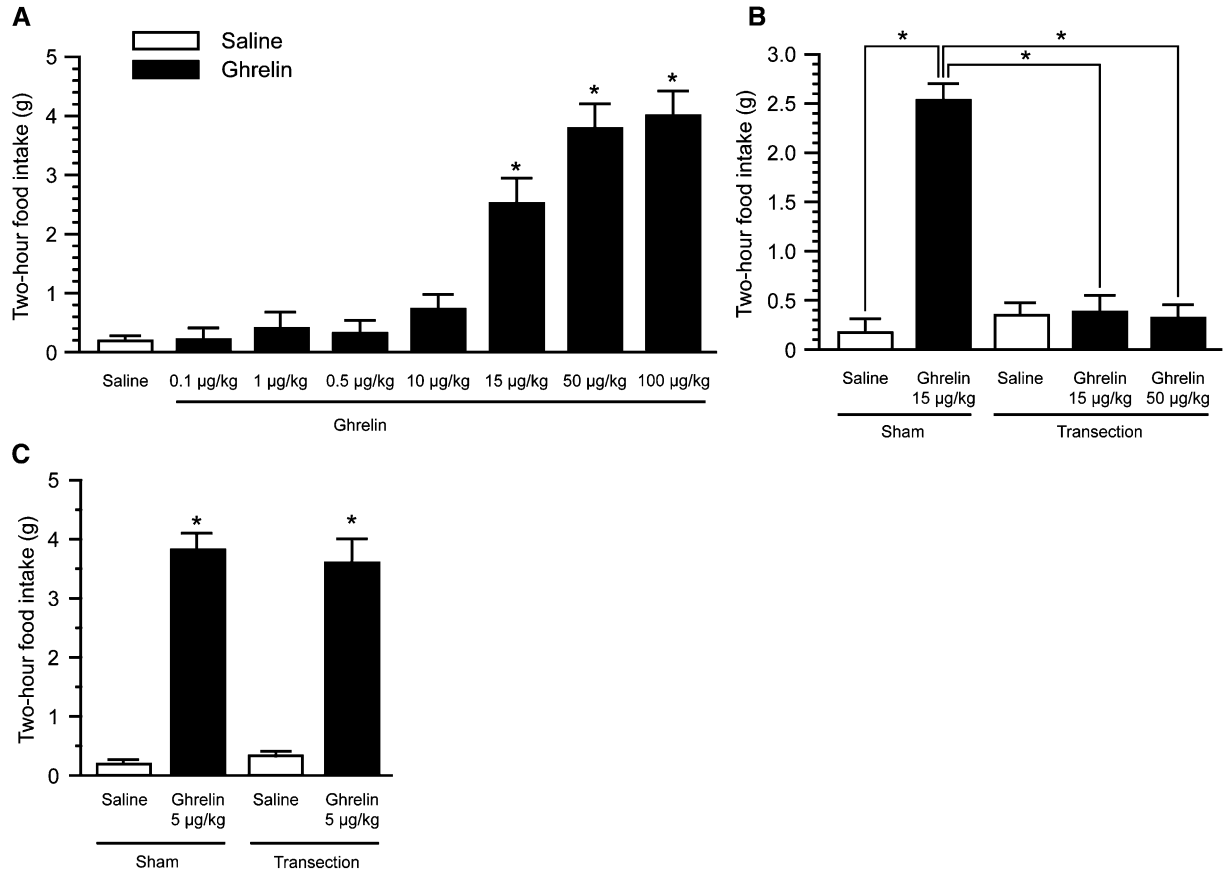

Figure 1. Effect of bilateral midbrain transections on ghrelin-induced feeding behavior

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

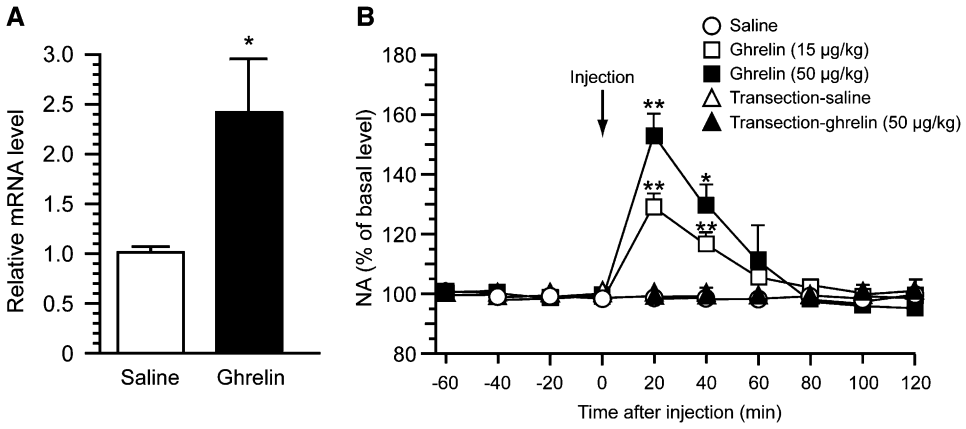

Figure 2. Ghrelin stimulates feeding via the NA system

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

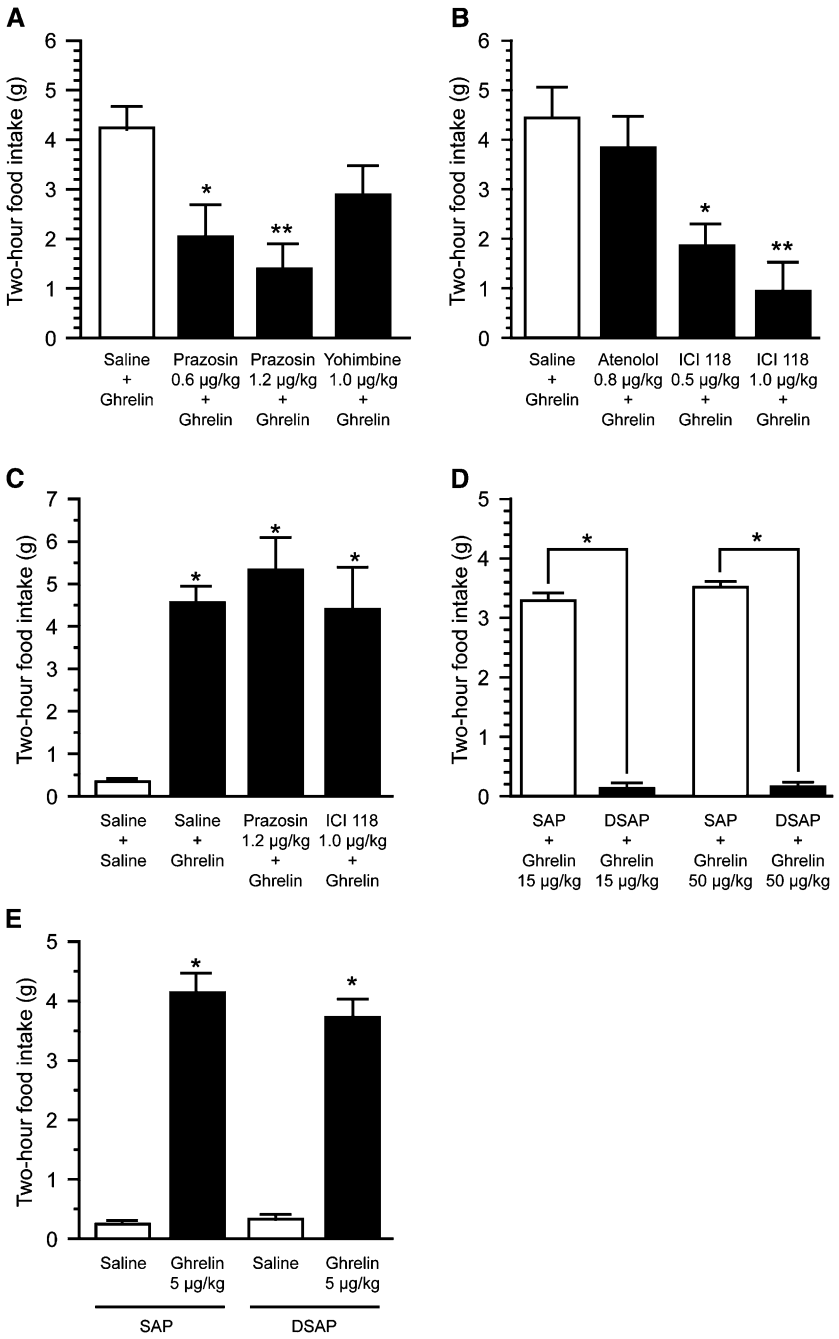

Figure 3. The effects of either pretreatment with adrenoceptor antagonists or disruption of DBH-containing neurons on ghrelin-induced feeding

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.

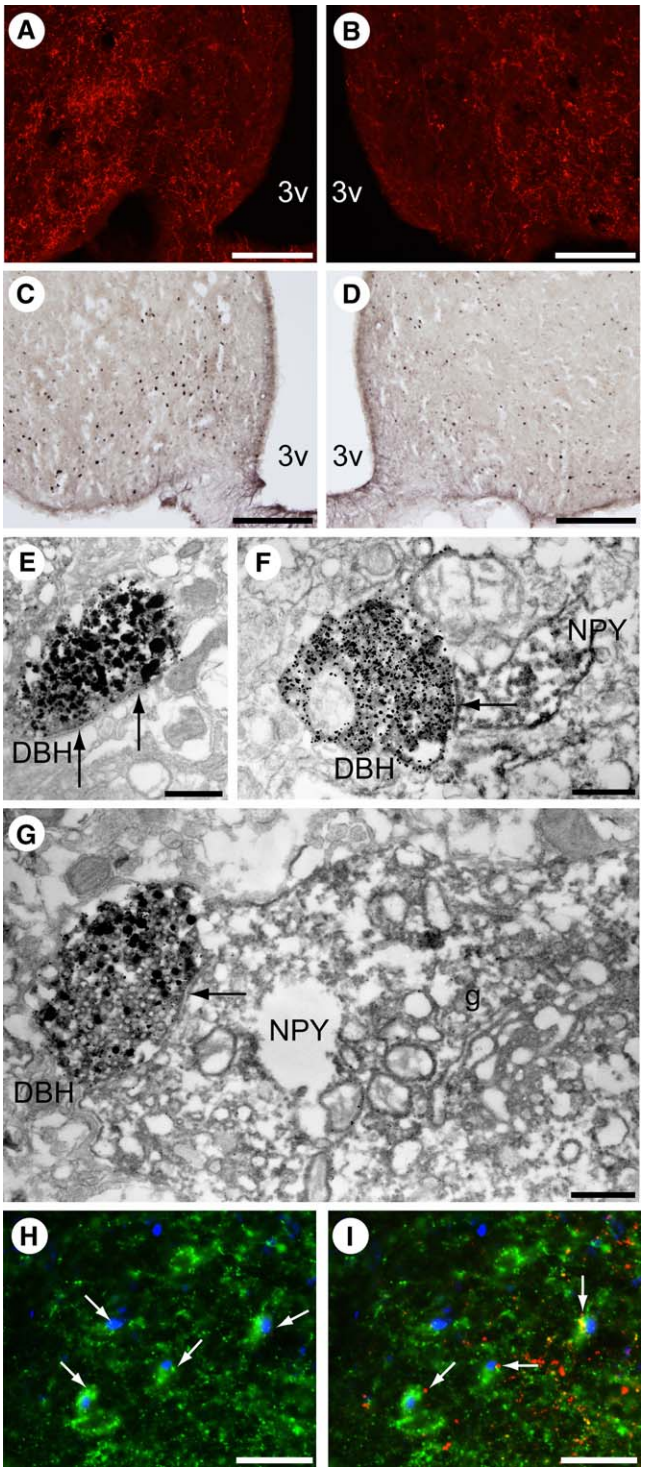

Figure 4. The effect of unilateral midbrain transections on ghrelin-induced Fos expression and activation of NPY neurons by ghrelin via the catecholaminergic pathways

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/.

Acknowledgments

We thank Rie Matsuura, Tomoko Tsuruta, and Yuko Nobe for their assistance. This study was supported in part by the 21st Century Center of Excellence Program (COE), the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO),

and by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Ministry of Health, Labor, and Welfare of Japan.

Received: October 15, 2005
Revised: July 19, 2006
Accepted: September 13, 2006
Published online: October 3, 2006

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