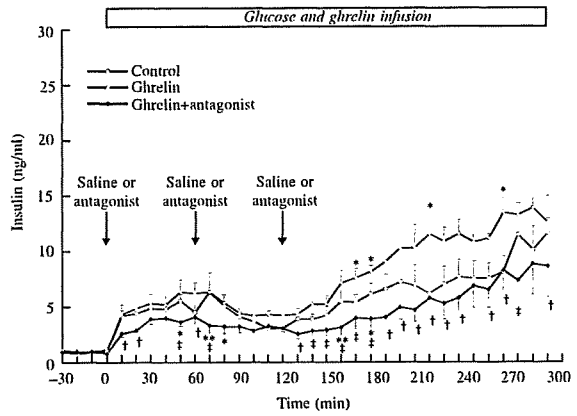

Figure 4 Average insulin plasma concentrations in meal-fed sheep continuously receiving saline (control) or ghrelin (ghrelin, 0·04 μg/kg body weight per min) during hyperglycemic clamp. Saline vehicle or D-lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) was administered every 60 min during the first half of hyperglycemic clamp. Values are means ±S.E.M. ($n=4$). *$P<0·05$ versus control, **$P<0·01$ versus control, †$P<0·05$ versus ghrelin, ‡$P<0·01$ versus ghrelin.

Changes in plasma insulin levels in the meal-fed state are presented in Fig. 4. Plasma insulin levels were significantly ($P<0·01$) increased by glucose infusion in all groups. There was a biphasic insulin increment in control and ghrelin-infused group. Ghrelin significantly ($P<0·05$) enhanced only the second phase of insulin secretion. The ghrelin antagonist significantly ($P<0·05$) depressed both the first- and second phase of insulin secretion.

Discussion

Effects of ghrelin on insulin secretion are bi-directional, since ghrelin exerts both stimulatory and inhibitory effects on insulin secretion. Ghrelin inhibits insulin secretion in fasted humans (Broglio *et al.* 2001) and mice (Reimer *et al.* 2003). By contrast, i.v. injection of ghrelin accelerates insulin secretion in free-feeding rats (Lee *et al.* 2002) and fed sheep (Takahashi *et al.* 2006). Ghrelin stimulates insulin secretion from isolated pancreatic islets of freely fed rats (Date *et al.* 2002). Taken together, these reports suggest that ghrelin differentially modulates insulin secretion, dependent on feeding states. The present study has clearly demonstrated that ghrelin inhibits and stimulates glucose-induced insulin secretion in fasting- and meal-fed state respectively.

Baseline of plasma ghrelin fluctuates between 0·5 and 2·0 ng/ml depending on the feeding states in sheep. The fluctuation of plasma ghrelin makes it difficult for us to determine the effects of ghrelin, since it is uncertain which level of plasma ghrelin is most effective on insulin secretion in different feeding states. In order to determine physiological effects of ghrelin as far as possible, we avoided administering ghrelin to the fasting animals in which plasma ghrelin levels had reached plateau (2·0 ng/ml), while we did not administer the ghrelin antagonist alone to the fed animals in which plasma ghrelin levels had reached nadir (0·5 ng/ml). Our previous study showed that ghrelin significantly enhanced glucose-stimulated insulin secretion at 1·0 ng/ml of plasma level in the meal-fed state (Takahashi *et al.* 2006). Therefore, the effect of the antagonist on insulin response at plasma ghrelin levels between 1·0 and 1·5 ng/ml during the HGC in the fasting states would be comparable with the effect of ghrelin administration in the meal-fed state.

In the meal-fed state, exogenous ghrelin enhanced glucose-induced insulin secretion as shown previously (Takahashi *et al.* 2006). The enhancement by ghrelin of glucose-stimulated insulin secretion was delayed in the present study when compared with the previous study. This may be related to weak insulin response to glucose in the animals newly applied to the HGC. Repeated doses of a ghrelin antagonist, D-Lys3-GHRP-6, counteracted the stimulatory effect of exogenous ghrelin on insulin secretion, suggesting that ghrelin stimulates insulin secretion via GHS-R1a in the meal-fed state. In addition, even lower insulin secretion by ghrelin plus antagonist compared with control suggests the possibility that basal ghrelin in the meal-fed state may also enhance insulin secretion.

In the fasting state, repeated doses of D-Lys3-GHRP-6 significantly enhanced glucose-induced insulin release, suggesting that endogenous ghrelin suppresses insulin secretion in the fasting state. The present result is supported by the finding that GHS-R antagonist enhances glucose-induced insulin release from perfused rat pancreas and increases plasma insulin levels in rats (Dezaki *et al.* 2006). In the present study, the insulin secretory response to the ghrelin antagonist emerged 60 min after the last dose of the drug. The hypoglycemic effect of the ghrelin antagonist emerges 2 days after the commencement of daily injections in diabetic mice where plasma ghrelin levels are high (Dong *et al.* 2006). In the fasting state, therefore, high levels of circulating ghrelin might compete with the ghrelin antagonist for GHS receptors, thus delaying the action of the drug.

Ghrelin stimulates GH secretion in sheep (Iqbal *et al.* 2006, Takahashi *et al.* 2006). In the present study, therefore, plasma GH concentrations were measured as an indicator for ghrelin receptor blockade. In the fed-state, D-Lys3-GHRP-6 attenuated ghrelin-induced GH secretion, indicating that the antagonist certainly blocked the ghrelin receptors. In the fasting state, however, the antagonist did not affect GH secretion, probably because GHS-R1a might not be involved in GH elevation after glucose infusion. Furthermore, ghrelin receptor mRNA expression is upregulated by fasting in the hypothalamus and pituitary (Kim *et al.* 2003). This may be related to the ineffectiveness of the ghrelin antagonist in GH suppression in the fasting state.

In conclusion, ghrelin inhibits and stimulates glucose-induced insulin secretion via GHS-R1a in fasting- and meal-fed state respectively. Further studies are required to identify

underlying mechanisms for alteration in the effects of ghrelin on insulin secretion in different feeding states.

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References

Adeghate E & Ponery AS 2002 Ghrelin stimulates insulin secretion from the pancreas of normal and diabetic rats. *Journal of Neuroendocrinology* **14** 555–560.

Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S *et al.* 2001 Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *Journal of Clinical Endocrinology and Metabolism* **86** 4753–4758.

Broglio F, Arvat E, Benso A, Gottero C, Muccioli G, Papotti M, van der Lely AJ, Deghenghi R & Chigo E 2001 Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. *Journal of Clinical Endocrinology and Metabolism* **86** 5083–5086.

Colombo M, Gregersen S, Xiao J & Hermansen K 2003 Effects of Ghrelin and other neuropeptides (CART, MCTH, Orexin A and B, and GLP-1) on the release of insulin from isolated rat islets. *Pancreas* **27** 161–166.

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

Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H *et al.* 2002 Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes* **51** 124–129.

Dezaki K, Hosoda H, Kakei M, Hashiguchi S, Watanabe M, Kangawa K & Yada T 2004 Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca^{2+} signaling in B-cells. *Diabetes* **53** 3142–3151.

Dezaki K, Sone H, Koizumi M, Nakata M, Kakei M, Nagai H, Hosoda H, Kangawa K & Yada T 2006 Blockade of pancreatic islet-derived ghrelin enhances insulin secretion to prevent high-fat diet-induced glucose intolerance. *Diabetes* **55** 3486–3493.

Dong J, Peeters TL, De Smet B, Moechars D, Delporte C, Vanden Berghe P, Coulie B, Tang M & Depoortere I 2006 Role of endogenous ghrelin in the hyperphagia of mice with streptozotocin-induced diabetes. *Endocrinology* **147** 2634–2642.

Fujino K, Inui A, Asakawa A, Kihara N, Fujimura M & Fujimiya M 2003 Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. *Journal of Physiology* **550** 227–240.

Iqbal J, Kurose Y, Canny B & Clarke IJ 2006 Effects of central infusion of ghrelin on food intake and plasma levels of growth hormone, luteinizing hormone, prolactin, and cortisol secretion in sheep. *Endocrinology* **147** 510–519.

Kim MS, Yoon CY, Park KH, Shin CS, Park KS, Kim SY, Cho BY & Lee HK 2003 Changes in ghrelin and ghrelin receptor expression according to feeding status. *Neuroreport* **14** 1317–1320.

Kojima M, Hosoda H, Date Y, Nakazato M, Matuo H & Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402** 656–660.

Lee HM, Wang G, Englander EW, Kojima M & Greeley GH, Jr 2002 Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. *Endocrinology* **143** 185–190.

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

Reimer MK, Pacini G & Ahren B 2003 Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology* **144** 916–921.

Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozone S, Hosoda H, Kangawa K & Matsukura S 2002 Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *Journal of Clinical Endocrinology and Metabolism* **87** 240–244.

Sugino T, Hasegawa Y, Kikkawa Y, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K & Terashima Y 2002 A transient ghrelin surge occurs just before feeding in scheduled meal-fed sheep. *Biochemical and Biophysical Research Communications* **295** 255–260.

Takahashi H, Kurose Y, Kobayashi S, Sugino T, Kojima M, Kangawa K, Hasegawa Y & Terashima Y 2006 Ghrelin enhances glucose-induced insulin secretion in scheduled meal-fed sheep. *Journal of Endocrinology* **189** 83–91.

Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A *et al.* 2000 Ghrelin strongly stimulates growth hormone release in humans. *Journal of Clinical Endocrinology and Metabolism* **85** 4908–4911.

Tschop M, Smiley DL & Heiman ML 2000 Ghrelin induces adiposity in rodents. *Nature* **407** 908–913.

Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA & Bloom SR 2001 Ghrelin enhances appetite and increases food intake in humans. *Journal of Clinical Endocrinology and Metabolism* **86** 5992–5995.

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Review

The role of PYY in feeding regulation

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Abstract

Peptide YY (PYY), a 36-amino-acid peptide, is secreted primarily from L-cells residing in the intestinal mucosa of the ileum and large intestine. PYY, which belongs to a family of peptides including neuropeptide Y (NPY) and pancreatic polypeptide, is released into the circulation as PYY_{1-36} and PYY_{3-36}; the latter is the major form of PYY in gut mucosal endocrine cells and throughout the circulation. Plasma PYY levels begin to rise within 15 min after starting to eat and plateau within ~90 min, remaining elevated for up to 6 h. Exogenous administration of PYY_{3-36} reduces energy intake and body weight in both humans and animals. Via Y2 receptors, the satiety signal mediated by PYY inhibits NPY neurons and activates pro-opiomelanocortin neurons within the hypothalamic arcuate nucleus. Peripheral PYY_{3-36} binds Y2 receptors on vagal afferent terminals to transmit the satiety signal to the brain. PYY_{3-36} may have therapeutic potential in human obesity.

Keywords: Anorectic peptide; Appetite control; PYY; Vagus nerve; Y2 receptor

Contents

1. Introduction

Obesity is closely linked to disability, disease, and death [1–3]. Public health officials and organizations have repeatedly issued warnings regarding the dangers of obesity, reporting it as a cause of significant years of life lost [4,5]. Food intake and energy homeostasis are tightly regulated by redundant neurohumoral factors in the central nervous system, especially within the hypothalamus [6–8]; the critical mechanisms underlying this regulation, however, have yet to be clarified. To develop effective treatments for obesity, it is necessary to understand the system by which energy homeostasis is regulated.

Peptide YY (PYY) is a gastrointestinal peptide that is critical in the regulation of food intake and energy homeostasis. In this review, we discuss the structural characteristics, tissue distribution, and physiological functions of PYY.

2. Structural characteristics and tissue distribution of PYY

PYY is a 36-amino-acid peptide originally isolated from porcine intestine in 1980 [9]. Tyrosine (Y) residues at both the N and C termini are the basis for its name. PYY belongs to a family of peptides that includes neuropeptide Y (NPY), a potent orexigenic peptide, and pancreatic polypeptide (PP), which is

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     1                                   36
PYY  YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY
NPY  --S--DN-----PA-DMA---SA----I--I------
PP   A-LE-VY--DN-TP-Q--Q-AAD--R---ML--P--
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Fig. 1. Amino-acid structures of human PYY, NPY, and PP. Shaded amino-acid abbreviations indicate the positions shared by all peptides. – indicates that the same amino acid as that of PYY exists at that position. A = alanine; D = aspartic acid; E = glutamic acid; G = glycine; H = histidine; I = isoleucine; K = lysine; L = leucine; M = methionine; N = asparagine; P = proline; Q = glutamine; R = arginine; S = serine; T = threonine; V = valine; Y = thyrosine.

secreted from pancreatic endocrine cells (PP cells). PYY has approximately 70% homology to NPY and PP (Fig. 1); the amino-acid structure of this peptide is also highly conserved between species [10,11]. All three peptides share a common tertiary structure, the PP-fold, which defines a U-shaped structure with an extended polyproline helix and α helix connected by β turn. PYY is secreted primarily from L-cells within the intestinal mucosa of the ileum and large intestine. The apical microvilli of these cells contact the intestinal lumen, allowing L-cells to sense nutrients and substances present within the lumen. The human PYY content is less than 10 pmol/g tissue in the proximal intestine, but increasing along the ileum to peak at 480 pmol/g tissue in the rectum [12]. PYY immunoreactivity can also be found in a subset of neurons within the central nervous system, including the hypothalamus, medulla, pons, and spinal cord [13]. PYY is released into the circulation as PYY_{1-36} and PYY_{3-36} [14,15]. PYY_{3-36}, the major form in both gut mucosal endocrine cells and the circulation, is created by cleavage of the N-terminal Tyr and Pro residues by dipeptidyl peptidase IV [15,16].

3. Receptors for PYY

Five Y receptors (Y1, Y2, Y4, Y5, and Y6) mediate the actions of PYY, NPY, and PP. Activation of these Y receptors, members of the G-protein-coupled receptor family, causes the induction of inhibitory responses, such as cAMP degradation [17–19]. The receptors subtypes differ in their distribution, function, and affinity for PYY, NPY, and PP. PYY_{1-36} binds all of the Y receptors, while PYY_{3-36} has a high affinity for the Y2 receptor and moderate affinity for Y1 and Y5 receptors [20]. Y2 receptors are distributed throughout the hypothalamus, primarily the arcuate nucleus, as well as the hippocampus, intestine, and neuronal cell bodies of the vagal nodose ganglion [21].

4. Secretion of PYY

PYY secretion is stimulated by neuronal and humoral factors as well as luminal nutrient content [14]. Plasma PYY levels begin to rise within 15 min of beginning to eat, reaching maximal levels within approximately 90 min and remaining elevated for as long as 6 h [14]. The peak plasma PYY concentration is dependent on the amount of calories ingested [22]. Plasma PYY levels are significantly lower in obese subjects in comparison to normal-weight subjects. Fasting PYY levels correlated negatively with body-mass index [23,24]. In humans, total plasma PYY levels after the intake of a high-protein meal are higher than that after the intake of high-fat or high-carbohydrate isocaloric meals [24]. High-protein diet induces greater increases in PYY levels than a normal-protein diet in mice [24]. In addition, long-term administration (16 weeks) of a high-protein diet enhances colonic and ileal PYY mRNA expression in comparison with a normal-protein diet in mice [24].

In addition to nutrients, bile acid, gastric acid, vasoactive intestinal polypeptide, and cholecystokinin stimulate PYY release [25–27]. In rats, intraduodenal liquid chyme induces the releases of PYY from the distal intestine before the food content reaches the ileum. This result suggests that PYY release occurs via a neural reflex, probably mediated by the vagus nerve [28–30].

5. PYY regulates feeding behavior

Preprandial decreases and postprandial increases in plasma PYY concentrations suggest that PYY is a satiety signal. A dose-dependent reduction in food intake following peripheral PYY_{3-36} administration occurred in both fasting and freely-feeding rodents [21,31–33]. In healthy volunteers, intravenous infusion of PYY_{3-36} caused a sustained decrease in appetite and food intake for more than 24 h [23]. Energy intake by obese subjects during a buffet lunch was reduced by 30% after intravenous infusion of PYY_{3-36} [23]. Chronic administration of PYY_{3-36} inhibited food intake and reduced body weight gain in mice, rabbits, and rhesus macaques [31,34,35]. In addition, daily food intake, body weight, and body fat increased in PYY knockout mice in comparison to wild-type mice [24]. These results suggest that PYY_{3-36} suppresses appetite and promotes weight loss.

Peripheral administration of PYY_{3-36} did not decrease food intake in Y2 receptor-deficient mice [31]. Deletion of the Y2 receptor from the hypothalamus resulted in a significant decrease in body weight and a significant increase in food intake in mice [36]. These results suggest that PYY_{3-36} inhibits food intake via the Y2 receptor. Peripheral administration of PYY_{3-36} decreased neural activity in the arcuate nucleus and inhibited hypothalamic NPY mRNA expression [31]. Direct injection of PYY_{3-36} into the arcuate nucleus suppressed the electrical activity of NPY-containing neurons [31]. Y2 receptor mRNA is expressed in greater than 80% of NPY-positive neurons in the arcuate nucleus [37]. Peripheral PYY_{3-36} administration activated approximately 20% of the neurons producing α-melanocyte stimulating hormone (α-MSH), an anorectic peptides generated from the α-MSH precursor pro-opiomelanocortin (POMC), within the arcuate nucleus [21,23]. PYY_{3-36} also suppresses NPY release and/or NPY neuron activation via presynaptic Y2 receptors. PYY_{3-36} also inhibits the electrical activity of NPY nerve terminals, activating neighboring POMC neurons. Recently, Koda et al. reported that the vagal afferent system is the major pathway conveying PYY_{3-36} satiety signals [21]. Peripheral administration of PYY_{3-36} to abdominally vagotomized rats did not alter food intake or activate neurons in the arcuate nucleus (Fig. 2A–B).

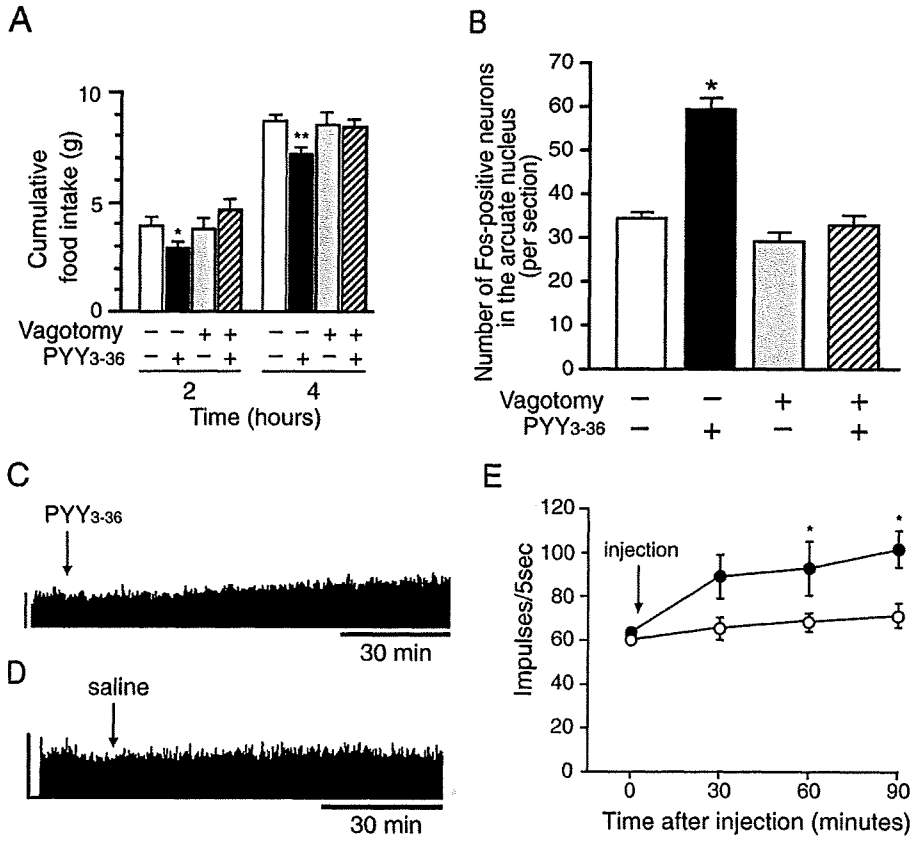

Fig. 2. Peripheral PYY_{3-36} transmits the satiety signal to arcuate nucleus via vagal nerve. (A) Cumulative food intake (means±SEM) of rats with bilateral subdiaphragmatic vagotomy or sham operation was measured after a single intraperitoneal administration of either PYY_{3-36} (3 nmol) or saline. $^{*}P<0.05$, $^{**}P<0.01$ vs. sham-operated rats given saline alone. (B) The numbers of Fos-positive, a marker of neuronal activation, neurons in the arcuate nuclei of rats after undergoing vagotomy or sham operation was determined after a single intrapenitoneal administration of PYY_{3-36} (5 nmol) or saline, respectively. Data are expressed as the means±SEM. $^{*}P<0.01$ vs. sham-operated rats given saline administration. (C) Representative data of gastric vagal afferent discharge in rats after a single intravenous administration of PYY_{3-36} (0.3 nmol) or saline (D). Vertical bar, 100 impulses per 5 s; horizontal bar, 30 min. (E) PYY_{3-36} stimulates gastric vagal afferent activity; no effect was observed after injection of saline. Data are expressed as the means±SEM. $^{*}P<0.01$ vs. value at 0 min. Open circles, saline; closed circles, PYY_{3-36}. (Redrawn from data in Ref. [21]).

Y2 receptors are synthesized within the rat nodose ganglion and transported to vagal afferent terminals by axonal transport. The firing frequency of gastric vagal afferent nerve was enhanced by intravenous administration of PYY_{3-36} (Fig. 2C–E). Bilateral midbrain transections rostral to the solitary tract nucleus also abolished PYY_{3-36}-induced reductions in feeding. These results indicate that peripheral PYY_{3-36} binds Y2 receptors at vagal afferent terminals to transmit satiety signals to the brain via vagal afferent pathways (Fig. 3).

In contrast to the peripheral administration of PYY, injection of PYY_{1-36} or PYY_{3-36} directly into the third, lateral, or forth cerebral ventricles of rodents potently stimulated food intake [38–40]. This orexigenic action of PYY is reduced in both Y1 receptor- and Y5 receptor-deficient mice [41]. Y1 and Y5 receptors thus play a critical role in the orexigenic action of centrally-administered PYY.

6. Other physiological functions of PYY_{3-36}

PYY demonstrated inhibition of jejunal and colonic motility and intestinal vasoconstriction. Intravenous infusion of PYY

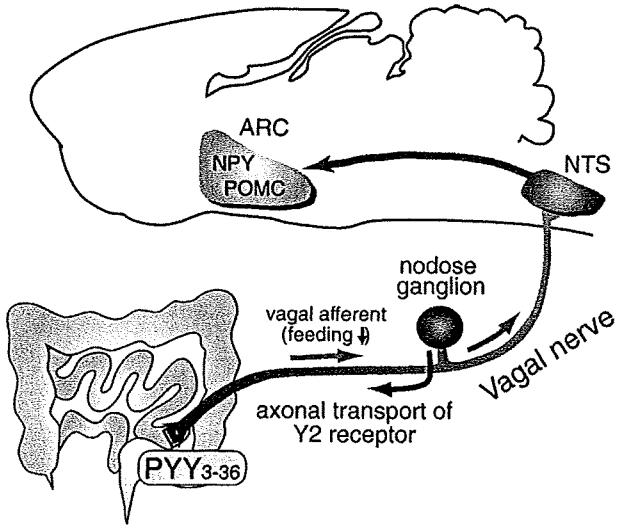

Fig. 3. Pathways of PYY_{3-36} action in the regulation of feeding. PYY_{3-36}'s signal for satiation to the brain is conveyed via gastric vagal afferent nerves. ARC; arcuate nucleus, NTS; nucleus of solitary tract, NPY; neuropeptide Y, POMC; pro-opiomelanocortin.

delayed both gastric emptying and mouth-to-cecum transit time in humans [42]. Intraluminal instillation of lipid into the terminal ileum markedly inhibits jejunal pressure wave activity. PYY is thought to mediate an "ileal brake mechanism", an inhibitory effect on upper gastrointestinal motor activity triggered by intraluminal fat in the terminal ileum [43,44]. PYY inhibits the gastric acid and pepsin secretion stimulated by pentagastrin, cholinergic agonists, vagal activity, and histamine in several species, including humans [12]. PYY also inhibits the cephalic phase of pancreatic exocrine secretion in humans and dogs [45,46]. These functions all support the role of PYY as a satiety signal.

In humans, intravenous PYY infusion also increased both systolic and diastolic blood pressures [47]. This result may be due to the vasoconstrictive effect of PYY on several vascular beds; in particular, PYY significantly reduces intestinal blood flow [48]. Therefore, the inhibitory effects of PYY on gastrointestinal secretion and motor activity may be mediated, at least in part, by effects on the splanchnic vasculature.

Intravenous PYY infusion in humans induced a 10% decrease in glomerular filtration rate, a 30% decrease in renin levels, and a 30% increase in sodium excretion [49]. These findings suggest that PYY also mediates postprandial naturesis.

7. Conclusions and perspectives

Peripheral PYY_{3-36} acts as a satiety signal to hypothalamic arcuate nucleus, likely via vagal afferent nerve after binding to Y2 receptors (Fig. 3). Exogenous administration of PYY_{3-36} reduces caloric intake and body weight in both lean and obese animals, even humans. PYY_{3-36} has been proposed as a potential therapeutic agent for human obesity. Clinical use of PYY is limited by its short-half life and the need for parenteral administration. A Phase I clinical study of PYY_{3-36} by the nasal drug delivery system has recently been completed [50]. While use of the PYY_{3-36} nasal spray offers major advantages over subcutaneous or intravenous injection for long-term therapy, large-scale clinical trials are needed to establish PYY's therapeutic potential. PYY_{3-36} is promising as a novel effective weapon in anti-obesity treatment.

References

[1] Hubert HB, Feinleib M, McNamara PM, Castelli WP. Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. Circulation 1983;67:968–77.

[2] Mokdad AH, Bowman BA, Ford ES, Vinicor F, Marks JS, Koplan JP. The continuing epidemics of obesity and diabetes in the United States. JAMA 2001;286:1195–200.

[3] Colditz GA, Willett WC, Rotnitzky A, Manson JE. Weight gain as a risk factor for clinical diabetes mellitus in women. Ann Intern Med 1995;122: 481–6.

[4] Fontaine KR, Redden DT, Wang C, Westfall AO, Allison DB. Years of life lost due to obesity. JAMA 2003;289:187–93.

[5] Peeters A, Barendregt JJ, Willekens F, Mackenbach JP, Al Mamun A, Bonneux L. NEDCOM, the Netherlands Epidemiology and Demography Compression of Morbidity Research Group. Obesity in adulthood and its consequences for life expectancy: a life-table analysis. Ann Intern Med 2003;138:24–32.

[6] Cummings DE, Schwartz MW. Genetics and pathophysiology of human obesity. Annu Rev Med 2003;54:453–71.

[7] Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG. Central nervous system control of food intake. Nature 2000;404:661–71.

[8] Spiegelman BM, Flier JS. Obesity and the regulation of energy balance. Cell 2001;104:531–43.

[9] Tatemoto K, Mutt V. Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. Nature 1980;285:417–8.

[10] Conlon JM. The origin and evolution of peptide YY (PYY) and pancreatic polypeptide (PP). Peptides 2002;23:269–78.

[11] Tatemoto K, Carlquist M, Mutt V. Neuropeptide Y — a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 1982;296:659–60.

[12] Adrian TE, Savage AP, Sagor GR, Allen JM, Bacarese-Hamilton AJ, Tatemoto K, Polak JM, Bloom SR. Effect of peptide YY on gastric, pancreatic, and biliary function in humans. Gastroenterology 1985;89: 494–9.

[13] Ekblad E, Sundler F. Distribution of pancreatic polypeptide and peptide YY. Peptides 2002;23:251–61.

[14] Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM, Bloom SR. Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology 1985;89:1070–7.

[15] Eberlein GA, Eysselein VE, Schaeffer M, Layer P, Grandt D, Goebell H, Niebel W, Davis M, Lee TD, Shively JE, et al. A new molecular form of PYY: structural characterization of human PYY(3–36) and PYY(1–36). Peptides 1989;10:797–803.

[16] Grandt D, Schimiczek M, Beglinger C, Layer P, Goebell H, Eysselein VE, Reeve Jr JR. Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1–36 and PYY 3–36. Regul Pept 1994;51:151–9.

[17] Naveilhan P, Neveu I, Arenas E, Ernfors P. Complementary and overlapping expression of Y1, Y2 and Y5 receptors in the developing and adult mouse nervous system. Neuroscience 1998;87:289–302.

[18] Parker RM, Herzog H. Regional distribution of Y-receptor subtype mRNAs in rat brain. Eur J Neurosci 1999;11:1431–48.

[19] Larhammar D. Structural diversity of receptors for neuropeptide, peptide YY and pancreatic polypeptide. Regul Pept 1996;65:165–74.

[20] Dumont Y, Fournier A, St-Pierre S, Quirion R. Characterization of neuropeptide Y binding sites in rat brain membrane preparations using [125I][Leu31,Pro34]peptide YY and [125I]peptide YY3–36 as selective Y1 and Y2 radioligands. J Pharmacol Exp Ther 1995;272:673–80.

[21] Koda S, Date Y, Murakami N, Shimbara T, Hanada T, Toshinai K, Niijima A, Furuya M, Inomata N, Osuye K, Nakazato M. The role of the vagal nerve in peripheral PYY3–36-induced feeding reduction in rats. Endocrinology 2005;146:2369–75.

[22] Konturek SJ, Konturek JW, Pawlik T, Brzozowski T. Brain–gut axis and its role in the control of food intake. J Physiol Pharmacol 2004;55: 137–54.

[23] Batterham RL, Cohen MA, Ellis SM, Le Roux CW, Withers DJ, Frost GS, Ghatei MA, Bloom SR. Inhibition of food intake in obese subjects by peptide YY3–36. N Engl J Med 2003;349:941–8.

[24] Batterham RL, Heffron H, Kapoor S, Chivers JE, Chandarana K, Herzog H, Le Roux CW, Thomas EL, Bell JD, Withers DJ. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. Cell Metab 2006;4:223–33.

[25] Ballantyne GH, Longo WE, Savoca PE, Adrian TE, Vukasin AP, Bilchik AJ, Sussman J, Modlin IM. Deoxycholate-stimulated release of peptide YY from the isolated perfused rabbit left colon. Am J Physiol 1989;257: G715–24.

[26] Kogire M, Izukura M, Gomez G, Uchida T, Greeley Jr GH, Thompson JC. Terbutaline, a beta 2-adrenoreceptor agonist, inhibits gastric acid secretion and stimulates release of peptide YY and gastric inhibitory polypeptide in dogs. Dig Dis Sci 1990;35:453–7.

[27] Ballantyne GH, Goldenring JR, Savoca PE, Kranz HK, Adrian TE, Bilchik AJ, Modlin IM. Cyclic AMP-mediated release of peptide YY (PYY) from the isolated perfused rabbit distal colon. Regul Pept 1993;47: 117–26.

[28] Fu-Cheng X, Anini Y, Chariot J, Castex N, Galmiche JP, Roze C. Mechanisms of peptide YY release induced by an intraduodenal meal in rats: neural regulation by proximal gut. Pflugers Arch 1997;433:571–9.
[29] Zhang T, Uchida T, Gomez G, Lluis F, Thompson JC, Geeley Jr GH. Neural regulation of peptide YY secretion. Regul Pept 1993;48:321–8.
[30] Rudnicki M, Rigel DF, McFadden DW. Vagal cooling blocks circulating neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) release. J Surg Res 1991;51:40–5.
[31] Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD, Bloom SR. Gut hormone PYY(3–36) physiologically inhibits food intake. Nature 2002;418:650–4.
[32] Challis BG, Pinnock SB, Coll AP, Carter RN, Dickson SL, O'Rahilly S. Acute effects of PYY3–36 on food intake and hypothalamic neuropeptide expression in the mouse. Biochem Biophys Res Commun 2003;311:915–9.
[33] Halatchev IG, Ellacott KL, Fan W, Cone RD. Peptide YY3–36 inhibits food intake in mice through a melanocortin-4 receptor-independent mechanism. Endocrinology 2004;145:2585–90.
[34] Sileno AP, Brandt GC, Spann BM, Quay SC. Lower mean weight after 14 days intravenous administration peptide YY3–36 (PYY3–36) in rabbits. Int J Obes 2006;30:68–72.
[35] Koegler FH, Enriori PJ, Billes SK, Takahashi DL, Martin MS, Clark RL, Evans AE, Grove KL, Cameron JL, Cowley MA. Peptide YY(3–36) inhibits morning, but not evening, food intake and decreases body weight in rhesus macaques. Diabetes 2005;54:3198–204.
[36] Sainsbury A, Schwarzer C, Couzens M, Fetissov S, Furtinger S, Jenkins A, Cox HM, Sperk G, Hokfelt T, Herzog H. Important role of hypothalamic Y2 receptors in body weight regulation revealed in conditional knockout mice. Proc Natl Acad Sci USA 2002;99:8938–43.
[37] Broberger C, Landry M, Wong H, Walsh JN, Hokfelt T. Subtypes Y1 and Y2 of the neuropeptide Y receptor are respectively expressed in pro-opiomelanocortin- and neuropeptide-Y-containing neurons of the rat hypothalamic arcuate nucleus. Neuroendocrinology 1997;66:393–408.
[38] Clark JT, Sahu A, Kalra PS, Balasubramaniam A, Kalra SP. Neuropeptide Y (NPY)-induced feeding behavior in female rats: comparison with human NPY ([Met17]NPY), NPY analog ([norLeu4]NPY) and peptide YY. Regul Pept 1987;17:31–9.
[39] Corp ES, McQuade J, Krasnicki S, Conze DB. Feeding after fourth ventricular administration of neuropeptide Y receptor agonists in rats. Peptides 2001;22:493–9.
[40] Stanley BG, Daniel DR, Chin AS, Leibowitz SF. Paraventricular nucleus injections of peptide YY and neuropeptide Y preferentially enhance carbohydrate ingestion. Peptides 1985;6:1205–11.
[41] Kanatani A, Mashiko S, Murai N, Sugimoto N, Ito J, Fukuroda T, Fukami T, Morin N, MacNeil DJ, Van der Ploeg LH, Saga Y, Nishimura S, Ihara M. Role of the Y1 receptor in the regulation of neuropeptide Y-mediated feeding: comparison of wild-type, Y1 receptor-deficient, and Y5 receptor-deficient mice. Endocrinology 2000;141:1011–6.
[42] Savage AP, Adrian TE, Carolan G, Chatterjee VK, Bloom SR. Effects of peptide YY (PYY) on mouth to caecum intestinal transit time and on the rate of gastric emptying in healthy volunteers. Gut 1987;28:166–70.
[43] Van Citters GW, Lin HC. The ileal brake: a fifteen-year progress report. Curr Gastroenterol Rep 1999;1:404–9.
[44] Spiller RC, Trotman IF, Higgins BE, Ghatei MA, Grimble GK, Lee YC, Bloom SR, Misiewicz JJ, Silk DB. The ileal brake-inhibition of jejunal motility after ileal fat perfusion in man. Gut 1984;25:365–74.
[45] Lluis F, Gomez G, Fujimura M, Greeley Jr GH, Thompson JC. Peptide YY inhibits nutrient-, hormonal-, and vagally-stimulated pancreatic exocrine secretion. Pancreas 1987;2:454–62.
[46] Konturek SJ, Bilski J, Pawlik W, Tasler J, Domschke W. Adrenergic pathway in the inhibition of pancreatic secretion by peptide YY in dogs. Gastroenterology 1988;94:266–73.
[47] Adrian TE, Sagor GR, Savage AP, Bacarese-Hamilton AJ, Hall GM, Bloom SR. Peptide YY kinetics and effects on blood pressure and circulating pancreatic and gastrointestinal hormones and metabolites in man. J Clin Endocrinol Metab 1986;63:803–7.
[48] Lundberg JM, Tatemoto K, Terenius L, Hellstrom PM, Mutt V, Hokfelt T, Hamberger B. Localization of peptide YY (PYY) in gastrointestinal endocrine cells and effects on intestinal blood flow and motility. Proc Natl Acad Sci USA 1982;79:4471–5.
[49] Playford RJ, Mehta S, Upton P, Rentch R, Moss S, Calam J, Bloom S, Payne N, Ghatei M, Edwards R, et al. Effect of peptide YY on human renal function. Am J Physiol 1995;268:F754–9.
[50] Johnson PH, Quay SC. Advances in nasal drug delivery through tight junction technology. Expert Opin Drug Deliv 2005;2:281–98.

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Neuronal interactions between neuropeptide W- and orexin- or melanin-concentrating hormone-containing neurons in the rat hypothalamus

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Abstract

Neuropeptide W (NPW) was recently discovered as the endogenous ligand for GPR7 and GPR8, which are orphan G protein-coupled receptors isolated from the porcine brain. These receptors are assumed to be involved in feeding regulation and/or energy homeostasis. Recent anatomical studies have revealed that high levels of GPR7 mRNA are distributed in the brain, including the hypothalamus and amygdala. However immunohistochemical studies on the distribution and localization of NPW have revealed differing results concerning whether or not NPW-containing cell bodies and their processes are present in the hypothalamus. Only a few immunohistochemical reports have been published concerning the presence of NPW-containing neurons in the brains of rodents, while there have been no anatomical studies of the co-localization of this neuropeptide with other transmitters. On this basis, we used a specific antiserum against NPW to determine immunohistochemically the presence of NPW-containing neurons in the rat hypothalamus. Many NPW-like immunoreactive cell bodies and their processes could be detected in the caudal region of the lateral hypothalamus but not in its anterior or middle regions. Given this positive identification of NPW-containing neurons in the lateral hypothalamus, we further studied the nature of interaction between NPW-containing neurons and neurons containing feeding regulating peptides such as orexim- and melanin-concentrating hormone (MCH). Very close interactions between NPW-containing nerve processes and orexin- and MCH-containing neuronal cell bodies and processes could be observed. These morphological findings strongly suggest that NPW is involved in the regulation of feeding and/or sleep/arousal behavior through orexin- and/or MCH-mediated neuronal pathways.

Keywords: Immunohistochemistry; Morphology; Rat; Hypothalamus; Neuropeptide

1. Introduction

GPR7 and GPR8 are two structurally-related G-protein-coupled orphan receptors (GPCR) that were originally isolated from the porcine brain. These receptors share significant homology with sequences encoding for transmembrane regions of the opioid and somatostatin-like receptor family genes from human genomic DNA [1]. While GPR7 is found both in humans and rodents, GPR8 is apparently expressed only in humans [2]. Both receptors are widely expressed in brain and peripheral tissues and organs. RT-PCR studies have shown that GPR7 mRNA is highly expressed in the brain and uterus of the rat [3]. In addition, *in situ* hybridization studies have revealed that GPR7 mRNA is present in the rat hypothalamus, including the arcuate (ARC), ventromedial (VMH), dorsomedial (DMH), paraventricular (PVN) and supraoptic (SO) nuclei [2,4]. These nuclei in the central nervous system (CNS) are well known to be involved in feeding regulation and energy homeostasis. However, a detailed study to compare the distributions of both GPR7 and GPR8 in the brain has not yet been done. GPR7 mRNA appears to be expressed in the hippocampus [2] but radio ligand binding assays have not demonstrated the presence of its protein [5].

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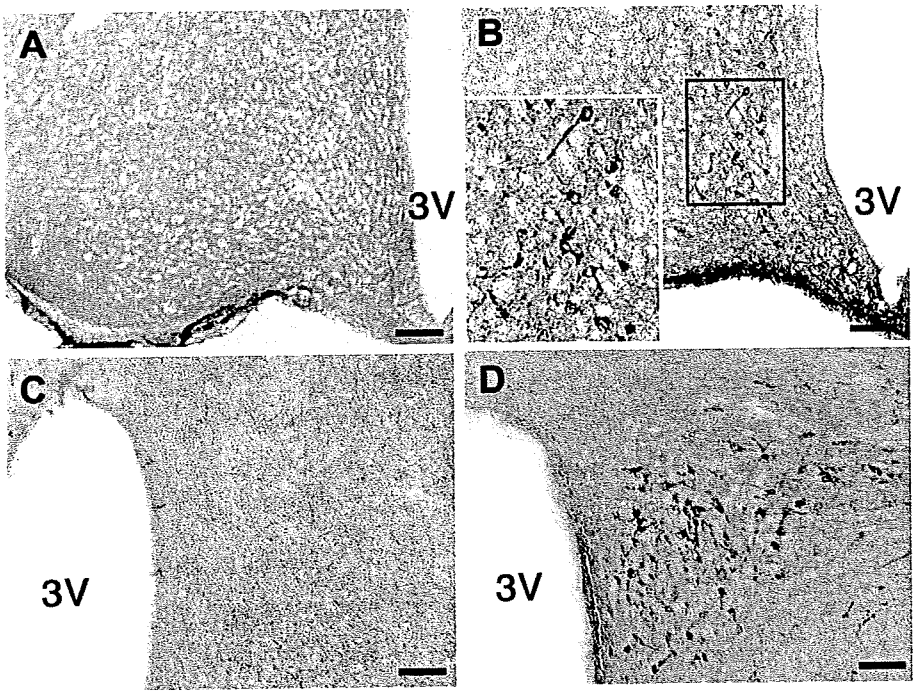

Fig. 1. Photomicrographs showing the effect of colchicine treatment on the detection of NPW-LI in the rat hypothalamus. NPW-LI cell bodies and fibers in brain sections from rats pre-treated with colchicine were observed in the ARC (A, B) and PVN (C, D). A, C; Control sections. B, D; Sections from colchicine-treated animal. Scale bar=100 μm (A, B), 50 μm (C, D). 3V: third ventricle.

NPW, a novel peptide consisting of 23- and 30-amino acid isoforms (NPW23 and NPW30), was isolated from the porcine hypothalamus as an endogenous ligand for GPR7 and GPR8 [6,7]. Both these peptides were reported to bind to GPR7 and GPR8 with similar affinity [6]. According to a recent report on the anatomical distribution of NPW, high levels of NPW mRNA were detected by *in situ* hybridization in the amygdala and hypothalamus [3]. Moreover, gene expression of NPW mRNA was found in the periaqueductal gray matter (PAG), ventral tegmental area (VTA), Edinger-Westphal (EW) nucleus, and dorsal raphe nucleus (DR), particularly in its dorsal part (DRD) [7,8]. Immunohistochemical study has shown that NPW-like immunoreactive (NPW-LI) cell bodies are distributed in the PVN (mainly in its parvocellular division), in SO, in accessory neurosecretory nuclei, dorsal and lateral hypothalamic areas (LH), perifornical nucleus, ARC and anterior and posterior pituitary gland [9]. In contrast, NPW-LI fibers are observed in the lateral septum, bed nucleus of the stria terminalis (BST), dorsomedial and posterior

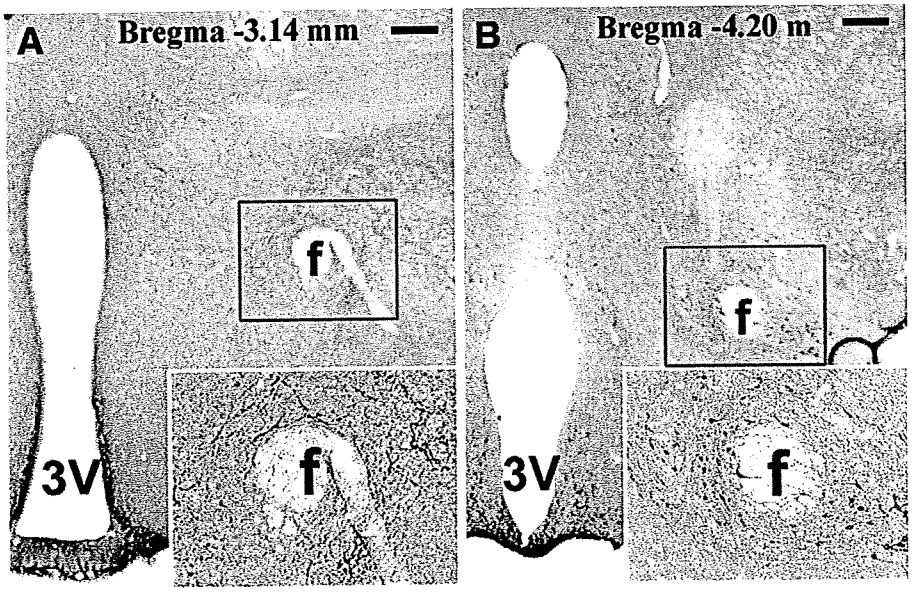

Fig. 2. Photomicrographs of brain sections from colchicine-treated rats showing NPW-LI in the LH. A: NPW-LI fibers in the anterior LH. B: NPW-LI cell bodies in the caudal LH. 3V: third ventricle. f: fornix. Scale bar=50 μm.

hypothalamus, central amygdaloid nucleus, CA1 field of the hippocampus, interpeduncular nucleus, inferior colliculus, lateral parabrachial nucleus, facial nucleus and hypoglossal nucleus [8]. Abundant NPW-LI was found in the central amygdaloid nucleus and the BST, these regions being implicated in the neural processing of fear and anxiety [8].

GPR7 knockout mice are known to be hyperphagic and become obese [10]. In addition, physiological studies have reported that intracerebroventricular (i.c.v.) infusion of NPW in rats initially provokes acute food intake [6]. However i.c.v. infusion of NPW in free-feeding suppresses feeding for an extended hours [11]. Another recent study has shown that administration of NPW increases c-Fos expression in the LH and PVN. These data suggest an appetite-regulating function of NPW in the hypothalamus and other brain regions.

In the LH, it is clearly shown that there exist several neuropeptides, including orexin [12] and melanin-concentrating hormone (MCH) [13], involved in feeding regulation. Since NPW-LI nerve processes are distributed in the LH [9], it could be postulated that the target of these NPW-containing neurons is orexin- and/or MCH-containing neurons, though this remains to be clarified. On this basis, we carried out double-staining immunohistochemistry experiments to explore the neural interactions between NPW-containing neurons and orexin and/or MCH-containing neurons in the rat brain.

2. Materials and methods

Male Wistar rats (Saitama Experimental Animal Center, Saitama, Japan; weight 250–300 g; $n=3$) were maintained on a 12/12 h light/dark cycle and supplied with standard laboratory chow and tap water *ad libitum*. All protocols used in these studies were reviewed and approved by the Institutional Animal Care and Use Committee of Showa University.

Rats were placed under deep Nembutal anesthesia (40 mg/kg, i.p.) and colchicine (200 g/25 μl saline) injected into the third ventricle of the brain. After 48 h, the rats were perfused through the ascending aorta with 50 ml of saline (37 °C), followed by 250–300 ml of 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH. 7.4) fixative for 20 min. The brains were enucleated, trimmed, and immersed in the same fixative for 12 h at 48 °C. After washing, the fixed brains were transferred to a solution containing 20% sucrose in 0.1 M PB for 2 days at 4 °C. For light microscopic study, the sections were single immunostained according to the following series of incubation steps: 5% normal horse serum for 20 min; mouse anti-NPW antiserum [11] (1/5000) overnight at room temperature; biotinylated anti-mouse IgG (DAKO, Carpinteria, CA, USA) for 1 h at room temperature; ABC complex for 45 min at room temperature (Vectastain elite ABC kit, Vector Laboratories, Burlingame, CA, USA) and visualized using a peroxidase substrate (DAB) kit (Vector Laboratories) and hydrogen peroxide (0.005%) in 0.05 M Tris buffer. Between each step, except for that between step 1 and 2, the sections were carefully rinsed with PB three times, each for 5 min. After dehydration, the sections were carefully rinsed and mounted on glass slides for examination using a light microscope (Olympus, Tokyo, Japan).

The frozen brains were cut into the coronal sections (thickness 7 m) on a cryostat (MICROM HM 500; MICROM, Heidelberg, Germany) and mounted on glass slides coated with 3% poly-lysine solution (Sigma, St. Louis, USA). The sections were

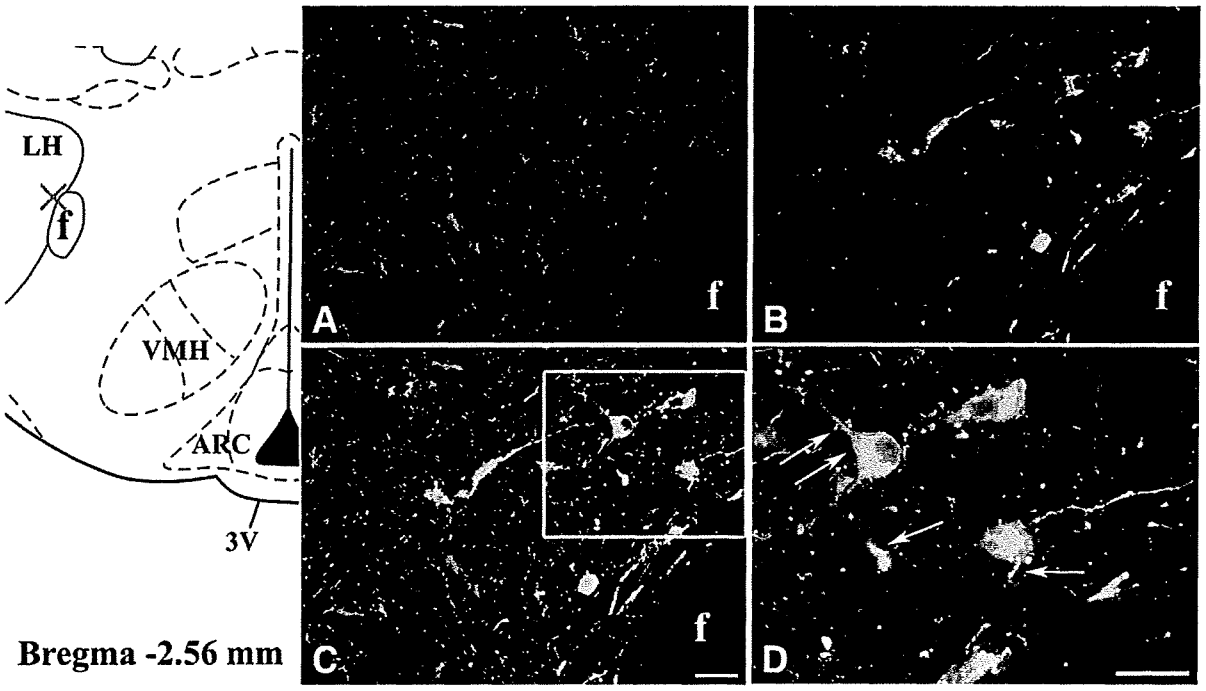

Fig. 3. Immunofluorescence photomicrographs of the LH after direct dual-labeling studies combining antiserum to NPW (A) with antiserum to orexin (B). Immunoreactivity for NPW (green, A) and orexin (red, B) are localized in the LH. The merged image (C) shows some NPW-positive terminals in direct contact with orexin-containing neurons (arrows). Magnified image of area in (C) is shown in (D). Scale bar=20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

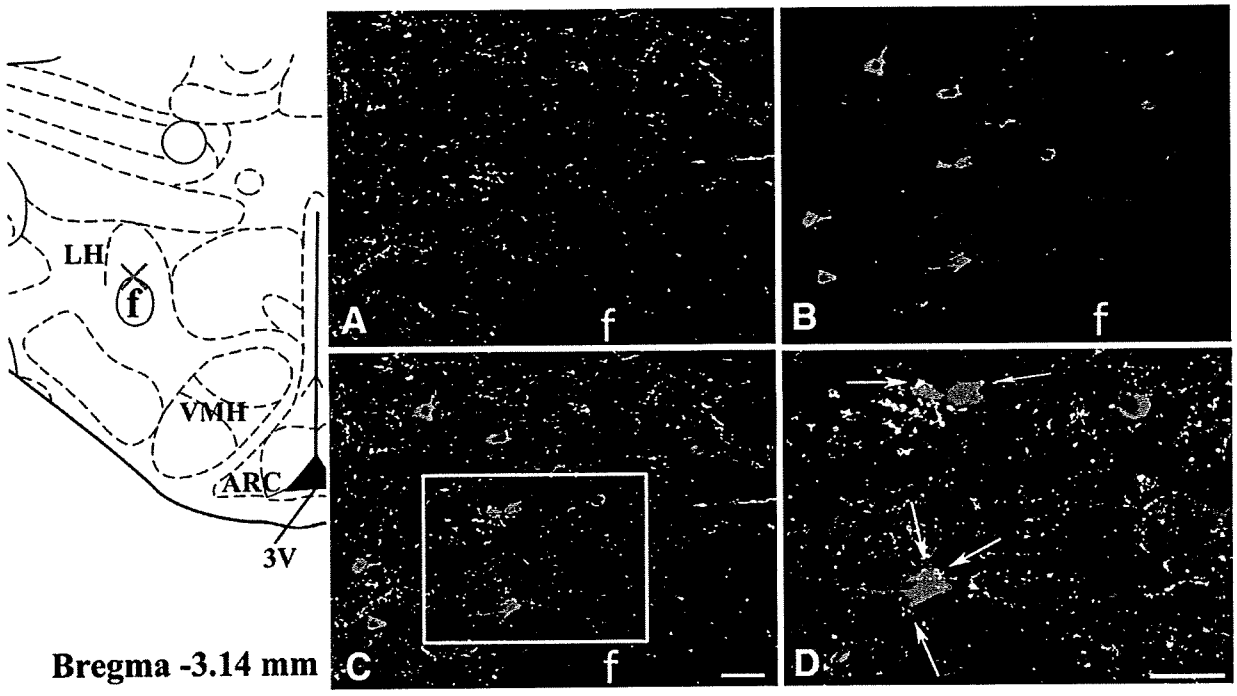

Fig. 4. Immunofluorescence photomicrographs of the LH after direct dual-labeling studies combining antiserum to NPW (A) with antiserum to MCH (B). Immunoreactivity for NPW (green, A) and MCH (red, B) are localized in the LH. The merged image (C) shows some NPW-positive terminals in direct contact with MCH-containing neurons (arrows). Magnified image of area in (C) is shown in (D). Scale bar=20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

washed in phosphate-buffered saline (PBS) for 15 min and incubated with 0.3% Triton X-100 in PBS for 5 days at 4 °C. The sections were subsequently rinsed three times for 5 min with PBS, blocked with 10% normal horse serum in PBS for 1 h at room temperature, and then incubated with rabbit anti-orexin antiserum (1/1000; CHEMICON International, USA) or sheep anti-MCH antiserum (1/10,000; CHEMICON international) for 2 days at 4 °C. After washing with PBS, sections were incubated with Alexa 488-labeled goat anti-mouse IgG antibody (1/400, Molecular Probes, Eugene, OR, USA) for 1.5 h at room temperature. For dual immunostaining, the coronal sections were first incubated with mouse anti-NPW antiserum [11] (1/10,000) for 2 days at 4 °C and then, after washing with PBS, incubated with Alexia 546-labeled anti-mouse antibody (1/400, Molecular Probes, Eugene, OR, USA) for 1.5 h at room temperature. Sections were examined with the aid of an Olympus AX-70 fluorescence microscope (Olympus, Tokyo, Japan).

Some sections to serve as controls were subjected to the same incubation process but were not exposed to anti-NPW antiserum. No specific immunofluorescence was observed in these control sections, signifying that any immunopositive reactions in the treated sections should be specific to the antibody used.

3. Results

We found that NPW-like immunoreactivity (NPW-LI) on tissue sections was dramatically enhanced when animals were pre-treated with colchicine compared to sections from animals not pre-treated with colchicine (Fig. 1). Identifiable NPW-LI cell bodies and processes were dramatically increased in number and density and were detected in the caudal part of the ARC (Fig. 1B), in both the magnocellular and parvocellular divisions of the PVN (Fig. 1D), and in the caudal part of the LH (Fig. 2A and B). Interestingly, a high density of NPW-LI fibers and their terminals were found around the fornix in the anterior part (Bregma −2.30~−3.80 mm) of the LH region (Fig. 2A) At this level, very few NPW-LI cell bodies-only were detected (Fig. 2A). However, many NPW-LI cell bodies were observed in the region where the mammillary recess of the 3rd ventricle appears (Fig. 2B). NPW-LI cell bodies in the LH were thus observed within a narrow area in the caudal hypothalamus. In addition, orexin- and MCH-containing neurons were found to be distributed abundantly in the LH (Figs. 3B and 4B).

We subsequently employed dual immunostaining to examine the relationship between neurons containing NPW and orexin and/or MCH in the LH given that the immunostaining experiments described above highlighted the abundant distribution of neurons containing these peptides in the same regions. Many NPW-LI nerve fibers were found to be in direct contact with orexin-containing neurons in the LH (Fig. 3C). Moreover, NPW-IL nerve fibers were also found to make direct contact both with MCH-containing neuronal cell bodies and with their processes in the LH (Fig. 4F).

4. Discussion

In this work, we found that the number and density of identifiable NPW-LI cell bodies and processes in brain slices could be

dramatically increased by pre-treating animals with colchicine. The NPW-LI cell bodies and processes could be detected in the caudal part of the ARC, the PVN and the LH. In addition, we provide here the first anatomical evidence of an NPW efferent neuron population in the hypothalamus, and show that these neurons interact with feeding-regulating neurons such as orexin- and MCH-containing neurons in the LH.

In rodents, NPW is considered an endogenous ligand for GPR7 [1–3,7,14], which is expressed in the feeding-regulating center in the hypothalamus. While the physiological role of NPW is yet to be fully elucidated, it has been reported that i.c.v. infusion of NPW increases food intake for 2 h in the light phase in rats [6]. On the other hand, in the dark phase, i.c.v. infusion of NPW reduces food intake for 48 h, up-regulates energy expenditure, and decreases body weight [11]. Baker et al. [15] reported that, compared to controls, rats that received an i.c.v. infusion of NPW drank significantly more water in the first two hours.

Interestingly, high levels of NPW mRNA are detected in the rat hippocampus, amygdala and hypothalamus [3]. On the other hand, NPW-LI neurons have been reported to be widely distributed in the PVN, dorsal hypothalamus, LH, ARC and the perifornical region of the rat brain [9]. In this study we observed NPW-LI cell bodies and their processes in the ARC and PVN as well as the LH. Further to this, it is clearly shown that feeding-regulating peptides such as orexin [12] and MCH [16] are present in the LH area. Recently, it was reported that i.c.v. injection of NPW activates c-Fos expression in the rat brain, predominantly in hypothalamic areas such as the LH, ARC, ventromedial and dorsomedial nuclei, as well the PVN [17]. Moreover, the authors of that study confirmed, using a double-labeling method, the upregulation of c-Fos immunoreactivity in orexin/hypocretin-containing neurons in the LH after i.c.v. injection of NPW. When NPW was injected into the PVN, a significant increase in food consumption was observed in the first 24 h. However, when high doses of NPW were injected into the LH, food intake in the first four hours was increased compared to control, but, in contrast to the effect following NPW injection into the PVN, had no effect at 24 h [17]. Based on these findings, and considering the morphological results of the present study showing the interactions of NPW-containing nerve processes with orexin- and MCH-containing neurons in the LH, we consider that NPW plays a crucial role in the regulation of food intake via orexin/MCH-mediated effects in the LH.

Contradictory data have been published concerning the distribution of NPW in the brains of rodents. One report described a sex-related difference, with the number of NPW-LI neurons in the male hypothalamus more than that in the female hypothalamus [9]. Different distribution patterns of NPW-LI neuronal cell bodies and fibers in brain have also been reported. Dun et al. [9] reported that NPW-LI cell bodies or fibers are observed in the PVN, SON, perifornical region, and median eminence. However, Kitamura et al. [8] found no evidence of NPW-LI cell bodies in some of these same regions [9], while we, in contrast, found NPW-LI cell bodies in many more places in the hypothalamus. The authors of those aforementioned studies [8,9] also used colchicine treatment, but we suggest that the dose and duration of the treatment we used were probably more effective. In spite of differences in distribution, there seems to be a consensus that NPW-LI processes are abundantly distributed in the LH [8,9,17]. In this study, we have demonstrated the presence of NPW-LI both in cell bodies and in nerve fibers in the LH. We observed in detail the distribution of NPW-LI neurons from the rostral to the caudal end of the LH and that that NPW-LI fibers only (i.e. not cell bodies) are present in the anterior region of LH. In contrast, NPW-LI cell bodies are present in abundance along a narrow strip in the caudal region of the LH.

As to the function of NPW in the hypothalamic PVN, Taylor et al. [18] reported that NPW-containing neurons in the parvocellular division activate the hypothalamo-pituitary-adrenal axis (HPA) through the release of corticotrophin-releasing hormone. In addition, Niimi et al. [19] reported that i.c.v. infusion of NPW stimulates significant c-Fos expression in the PVN, suggesting that NPW is related to stress-response signal transduction. To this extent, we have also detected NPW-LI cell bodies and their processes in the parvocellular division of the PVN (unpublished data).

The peptide-mediated regulation of food intake involves several complex neuronal networks in the LH [20]. Orexin-containing neurons are also known to interact with neurons containing various types of transmitter. In the LH, leptin receptor immunoreactivity was reported to be present in some MCH-containing neurons [21], while almost all orexin-containing neurons express leptin receptor immunoreactivity [22]. Orexin is recognised as a peptide that promotes food intake as well as to play a role in the control of sleep and wakefulness [12]. In addition, GPR7 is also expressed in the suprachiasmatic nucleus, an area involved in the control of circadian rhythms [2]. These results suggest that NPW may also be involved in the regulation of sleep/wakefulness and circadian rhythms.

In conclusion, we have demonstrated the projection of NPW-containing neurons to orexin- and MCH-containing neurons in the lateral hypothalamus and synaptic contacts made between these different peptide-containing neurons. Further studies at the ultrastructural level are required to demonstrate the interactions of NPW-containing neurons with other neurons in the LH and thereby clarify NPW's role in the regulation of feeding, sleep/wakefulness and circadian rhythms.

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References

[1] O'Dowd BF, Scheideler MA, Nguyen T, Cheng R, Rasmussen JS, Marchese A, et al. The cloning and chromosomal mapping of two novel human opioid-somatostatin-like receptor genes, GPR7 and GPR8, expressed in discrete areas of the brain. Genomics 1995;28:84–91.

[2] Lee DK, Nguyen T, Porter CA, Cheng R, George SR, O'Dowd BF. Two related G protein-coupled receptors: the distribution of GPR7 in rat brain

and the absence of GPR8 in rodents. Brain Res Mol Brain Res 1999;71:96–103.
[3] Fujii R, Yoshida H, Fukusumi S, Habata Y, Hosoya M, Kawamata Y, et al. Identification of a neuropeptide modified with bromine as an endogenous ligand for GPR7. J Biol Chem 2002;277:34010–6.
[4] Jackson VR, Lin SH, Wang Z, Nothacker HP, Civelli O. A study of the rat neuropeptide B/neuropeptide W system using in situ techniques. J Comp Neurol 2006;497:367–83.
[5] Singh G, Davenport AP. Neuropeptide B and W: neurotransmitters in an emerging G-protein-coupled receptor system. Br J Pharmacol 2006;148:1033–41.
[6] Shimomura Y, Harada M, Goto M, Sugo T, Matsumoto Y, Abe M, et al. Identification of neuropeptide W as the endogenous ligand for orphan G-protein-coupled receptors GPR7 and GPR8. J Biol Chem 2002;277: 35826–32.
[7] Tanaka H, Yoshida T, Miyamoto N, Motoike T, Kurosu H, Shibata K, et al. Characterization of a family of endogenous neuropeptide ligands for the G protein-coupled receptors GPR7 and GPR8. Proc Natl Acad Sci U S A 2003;100:6251–6.
[8] Kitamura Y, Tanaka H, Motoike T, Ishii M, Williams SC, Yanagisawa M, et al. Distribution of neuropeptide W immunoreactivity and mRNA in adult rat brain. Brain Res 2006;1093:123–34.
[9] Dun SL, Brailoiu GC, Yang J, Chang JK, Dun NJ. Neuropeptide W-immunoreactivity in the hypothalamus and pituitary of the rat. Neurosci Lett 2003;349:71–4.
[10] Ishii M, Fei H, Friedman JM. Targeted disruption of GPR7, the endogenous receptor for neuropeptides B and W, leads to metabolic defects and adult-onset obesity. Proc Natl Acad Sci U S A 2003;100: 10540–5.
[11] Mondal MS, Yamaguchi H, Date Y, Shimbara T, Toshinai K, Shimomura Y, et al. A role for neuropeptide W in the regulation of feeding behavior. Endocrinology 2003;144:4729–33.
[12] Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 1998;92:573–85.
[13] Bittencourt JC, Presse F, Arias C, Peto C, Vaughan J, Nahon JL, et al. The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. J Comp Neurol 1992;319: 218–45.
[14] Brezillon S, Lannoy V, Franssen JD, Le Poul E, Dupriez V, Lucchetti J, et al. Identification of natural ligands for the orphan G protein-coupled receptors GPR7 and GPR8. J Biol Chem 2003;278:776–83.
[15] Baker JR, Cardinal K, Bober C, Taylor MM, Samson WK. Neuropeptide W acts in brain to control prolactin, corticosterone, and growth hormone release. Endocrinology 2003;144:2816–21.
[16] Kawauchi H, Kawazoe I, Tsubokawa M, Kishida M, Baker BI. Characterization of melanin-concentrating hormone in chum salmon pituitaries. Nature 1983;305:321–3.
[17] Levine AS, Winsky-Sommerer R, Huitron-Resendiz S, Grace MK, de Lecea L. Injection of neuropeptide W into paraventricular nucleus of hypothalamus increases food intake. Am J Physiol Regul Integr Comp Physiol 2005;288:R1727–32.
[18] Taylor MM, Yuill EA, Baker JR, Ferri CC, Ferguson AV, Samson WK. Actions of neuropeptide W in paraventricular hypothalamus: implications for the control of stress hormone secretion. Am J Physiol Regul Integr Comp Physiol 2005;288:R270–5.
[19] Niimi M, Murao K. Neuropeptide W as a stress mediator in the hypothalamus. Endocrine 2005;27:51–4.
[20] Funahashi H, Takenoya F, Guan JL, Kageyama H, Yada T, Shioda S. Hypothalamic neuronal networks and feeding-related peptides involved in the regulation of feeding. Anat Sci Int 2003;78:123–38.
[21] Hakansson ML, Brown H, Ghilardi N, Skoda RC, Meister B. Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. J Neurosci 1998;18:559–72.
[22] Funahashi H, Hori T, Shimoda Y, Mizushima H, Ryushi T, Katoh S, et al. Morphological evidence for neural interactions between leptin and orexin in the hypothalamus. Regul Pept 2000;92:31–5.

Characterization of obestatin in rat and human stomach and plasma, and its lack of acute effect on feeding behavior in rodents

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Abstract

Obestatin is a 23-amino acid peptide, initially isolated from rat stomach as an endogenous ligand for the orphan G-protein-coupled receptor. Obestatin is derived from proteolytic cleavage of a 117-amino acid precursor, preproghrelin. Ghrelin increases food intake, body weight, and gastric emptying, whereas obestatin has the opposite effects. In this study, we characterized obestatin in both rat and human stomach, and investigated the peptide's effect on feeding behavior. Using reversed-phase high-performance liquid chromatography coupled with RIAs specific for rat and human obestatin, we detected a very small amount of obestatin, compared with ghrelin, in the gastric fundi. The ratios of obestatin to ghrelin are 0·0039 and 1·94% respectively in the rat and human gastric fundi. In humans, plasma obestatin accounted for 5·21% of the ghrelin concentration, whereas it was undetectable in rat plasma. Plasma ghrelin concentration decreased after a meal in normal subjects, whereas obestatin concentration did not change. When administered centrally or peripherally, obestatin did not suppress food intake in either free-feeding or fasted rodents. Administration of obestatin did not antagonize ghrelin-induced feeding. These findings indicate that obestatin is present at very low levels compared with ghrelin in both rat and human, and has no acute effect on feeding behavior.

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Introduction

Ghrelin, a 28-amino acid peptide with an *n*-octanoyl modification, was originally discovered in human and rat stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R; Kojima *et al.* 1999). Ghrelin is derived from amino residues 24–51 of its 117-amino acid precursor, preproghrelin. Ghrelin is predominantly synthesized in X/A-like cells of the gastric body and consists of two major molecular forms, acylated ghrelin (ghrelin) and des-*n*-octanoyl ghrelin (des-acyl ghrelin; Date *et al.* 2000, Hosoda *et al.* 2000). When administered either centrally or peripherally, ghrelin stimulates GH secretion and food intake (Kojima *et al.* 1999, Tschöp *et al.* 2000, Wren *et al.* 2000, Nakazato *et al.* 2001). In addition, ghrelin downregulates energy expenditure and conserves body fat; it also increases body weight gain, adipogenesis, and gastric emptying (Nakazato *et al.* 2001, Tschöp *et al.* 2001, Tack *et al.* 2005). Circulating ghrelin in humans and rodents increases before meals and decreases after meals, suggesting a possible role in meal initiation (Cummings *et al.* 2001).

A comprehensive evaluation of the amino acid sequences of preproghrelin in 11 mammalian species identified a novel conserved peptide, named obestatin (Zhang *et al.* 2005). Obestatin, a 23-amino acid peptide with C-terminal amidation, was isolated from the rat stomach as a possible endogenous ligand for an orphan G-protein-coupled receptor, GPR39, that belongs to the GHS-R family. GPR39 is expressed in various regions of brain and peripheral tissues in both rats and humans (McKee *et al.* 1997). Obestatin is derived from amino acid residues 76–98 of rat and human preproghrelin. Obestatin was initially reported to have actions opposite to ghrelin, such as reduction of food intake and body weight, and delaying gastric emptying (Zhang *et al.* 2005). In addition, obestatin was recently shown to promote pancreatic β-cell and human islet cell survival and stimulate the expression of main regulatory β-cell genes in the pancreas (Granata *et al.* 2008). Several subsequent studies of the actions of obestatin on gut motility and feeding behavior yielded conflicting findings, with some groups reporting positive findings (Bresciani *et al.* 2006, Carlini *et al.* 2007) and others reporting negative findings (Gourcerol *et al.* 2006, Bassil *et al.* 2007, Gourcerol & Taché 2007, de Smet *et al.* 2007). Furthermore, several groups were unable to reproduce the observation that obestatin binds to GPR39 and increases intracellular Ca^{2+} rise in GPR39-expressing cells (Lauwers *et al.* 2006, Chartrel *et al.* 2007, Holst *et al.* 2007). Taken together, these conflicting data indicate that further experiments are needed to define the role of obestatin in energy homeostasis.

In this study, we first developed two RIAs specific for rat and human obestatin, and characterized obestatin immunoreactive

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molecules in the rat and human stomach by using reversed-phase high-performance liquid chromatography (RP-HPLC). We also studied plasma obestatin response to a meal in normal subjects. Using the same experimental designs as used in the original publication, we examined the action of centrally or peripherally administered obestatin on feeding behavior in freely fed and fasted rodents. Finally, we investigated the antagonistic effect of obestatin on ghrelin activity by co-administration of both peptides. Here, we report that the ratio of obestatin to ghrelin in the rat and human stomach is extremely low (0·0038 and 1·94% respectively) and that obestatin does not affect acute feeding behavior.

Materials and Methods

Animals

Male Wistar rats (Charles River Japan, Inc., Shiga, Japan) weighing 300–350 g and male C57BL/6 mice (Charles River Japan, Inc.) weighing 30–35 g were maintained in individual cages under controlled temperature (21–23 °C) and light (light on 0800–2000 h) with *ad libitum* access to food and water. Animals were anesthetized by i.p. injections of sodium pentobarbital (Abbott Laboratories), and i.c.v. cannulae were implanted into the lateral cerebral ventricles of rats and mice as previously described (Nakazato *et al.* 2001). Proper placement of the cannula was verified at the end of the experiments by dye administration. Animals were sham injected before the study, and weighed and handled daily. Only animals that showed progressive weight gain after the surgery were used in subsequent experiments. All experiments were repeated two or three times. All procedures were approved by the University of Miyazaki Animal Care and Use Committee and were in accordance with the Japanese Physiological Society's guidelines for animal care.

Peptides

Rat and human obestatin, human cocaine and amphetamine-regulated transcript (CART), and rat ghrelin were provided from Peptide Institute, Inc. (Osaka, Japan).

Preparation of antisera

Rat and human obestatin were conjugated with bovine thyroglobulin by the glutaraldehyde method (Mondal *et al.* 1999). This method links the carrier molecule to the N-terminus of the peptide, thereby producing an antiserum recognizing the C-terminus portion of the peptide. These conjugated peptides were used as immunogens for the generation of polyclonal antibodies. Antibodies for rat ghrelin were raised as described elsewhere (Hosoda *et al.* 2000). Antibodies raised against the N- and C-terminal regions of rat ghrelin recognize the acylated and total ghrelin forms respectively.

RIA

Synthetic rat and human obestatin were radioiodinated by the lactoperoxidase method, and the ^{125}I-labeled peptides were purified by RP-HPLC. A diluted sample or standard peptide solution (100 µl) was incubated for 24 h with 100 µl antiserum diluent (final dilutions were 1/200 000 for anti-rat obestatin antiserum and 1/90 000 for anti-human obestatin antiserum). Following the addition of tracer solution (16 000 c.p.m./100 µl), mixtures were incubated for 24 h. The second antibody method was used to separate bound and free ligands. The samples were assayed in duplicate and all procedures were performed at 4 °C. The respective intra- and inter-assay coefficients of variation at 50% binding for rat obestatin were 2·8 and 3·2% respectively; whereas for human obestatin, they were 3·1 and 3·7% respectively. Rat obestatin (1 ng) and ^{125}I-rat obestatin (5000 c.p.m.) were added to the plasma sample extracts and then applied to SepPak C-18 cartridges (Waters, Milword, MA, USA); recoveries were 91·5±0·3% (S.E.M.) and 90·7±0·5% respectively. The recoveries of human obestatin (1 ng) and ^{125}I-human obestatin (5000 c.p.m.) were 90·2±0·5% (S.E.M.) and 89·5±0·2% respectively. Ghrelin RIA was performed as described elsewhere (Hosoda *et al.* 2000).

Quantification of obestatin and ghrelin in stomach and plasma

Stomach Rat gastric fundi were obtained from three *ad libitum* fed adult male Wistar rats. Following anesthesia with sodium pentobarbital, gastric fundi were immediately resected and boiled at 95–100 °C for 10 min in a tenfold volume of water to inactivate intrinsic proteases. Human gastric fundi were obtained from three patients who underwent surgery and gave prior written consent. The study was approved by the Ethics Committee for Human Research of University of Miyazaki. After the samples were cooled to 4 °C, CH_3COOH and HCl were added to respective final concentrations of 1 M and 20 mM. Stomach tissue was homogenized in a Polytron for 3 min, after which the homogenate was centrifuged at 28 000 *g* for 30 min at 4 °C. Stomach samples were applied to SepPak C-18 cartridges, and peptides were eluted with a 60% acetronitrile (CH_3CN) solution containing 0·1% trifluoroacetic acid (TFA). Portions of each sample, equivalent to 50 mg wet weight, were lyophilized and then subjected to obestatin and ghrelin RIAs. Other portions of eluates were subjected to RP-HPLC under the conditions described in the legend to Fig. 2. All the HPLC fractions were assayed by obestatin RIAs.

Plasma Rat truncal blood (n=3, 4 ml each) was obtained by decapitation after anesthesia with sodium pentobarbital. Samples were collected into cooled polypropylene tubes containing EDTA·2 NA (1 mg/ml blood) and immediately centrifuged. Human blood was collected from 16 normal healthy volunteers. The plasma samples were diluted 1:1 with

0·9% saline and applied to SepPak C-18 cartridges pre-equilibrated with 0·9% saline. The cartridges were then washed, first with 0·9% saline, and then with a 10% CH_3CN solution containing 0·1% TFA. Adsorbed peptides were eluted with a 60% CH_3CN solution containing 0·1% TFA. Some portions of the eluates were subjected to obestatin and ghrelin RIAs. Aliquots of rat plasma eluate were subjected to RP-HPLC and then RIA for rat obestatin.

Meal test

Healthy volunteers (eight men and eight women; mean age ± S.E.M., 22·2 ± 2·5 years; body mass index, 19·4–24·9, mean ± S.E.M., 21·5 ± 0·6) who had fasted overnight were given 450 kcal/450 ml liquid meal (RACOL; Otsuka Co., Ltd, Iwate, Japan; 62% carbohydrate, 20% fat, and 18% protein) over 3 min at 0900 h. Blood was collected at −5, 30, 60, 90, 120, and 180 min after taking the liquid meal. The Institutional Committee of Faculty of Medicine, University of Miyazaki approved the protocol, and all the subjects provided informed consent before participation. Plasma (3 ml) samples were acidified to prevent degradation of octanolyated ghrelin before application to a SepPak cartridge, and then the plasma concentrations of obestatin and ghrelin were measured by RIAs.

Feeding experiments

Experiments were performed 1 week after the implantation of i.c.v. cannulae. First, rat obestatin (0·3 or 3 nmol/5 µl saline), human obestatin (3 nmol/5 µl saline), or CART (1 nmol/5 µl saline) was administered i.c.v. to rats fed *ad libitum* 10 min before the beginning of the dark phase (1950 h), after which 1-, 2-, 4-, and 12-h food intakes were measured ($n=6$ per group). Secondly, rat obestatin (0·3 nmol/2 µl saline) or CART (0·3 nmol/2 µl saline) was administered i.c.v. to mice fed *ad libitum* at 1950 h, after which 1-, 3-, and 5-h food intakes were measured ($n=7$ per group). Thirdly, rats were fasted overnight for 8 h and then rat obestatin (1 nmol/5 µl saline) or CART (1 nmol/5 µl saline) was administered i.c.v. at 1000 h, after which 1- and 2-h food intakes were measured ($n=6$–8 per group). Fourthly, rat obestatin (125 or 1000 nmol/kg body weight/50 µl saline) was administered i.p. to mice fed *ad libitum* at 1950 h, after which 1-, 3-, and 5-h food intakes were measured ($n=5$ per group). Fifth, rat ghrelin (40 nmol/kg body weight/50 µl saline) or rat ghrelin (40 nmol/kg body weight) plus rat obestatin (125 nmol/kg body weight) were administered i.p. to mice fed *ad libitum* at 1000 h, after which 1- and 2-h food intakes were measured ($n=5$ per group). These feeding tests were performed using a crossover design experiments in which animals were randomized to receive either test substance with a washout period of 3 days between each administration.

Statistical analysis

All data are expressed as means ± S.E.M. Groups of data (means ± S.E.M.) were compared using ANOVA and *post hoc* Fisher's test. P values less than 0·05 are considered significant.

Results

Obestatin RIA

Figure 1 shows the amino acid sequences (upper panel) of rat and human obestatin and (lower panel) the standard RIA curves of rat and human obestatin. Half-maximum inhibition

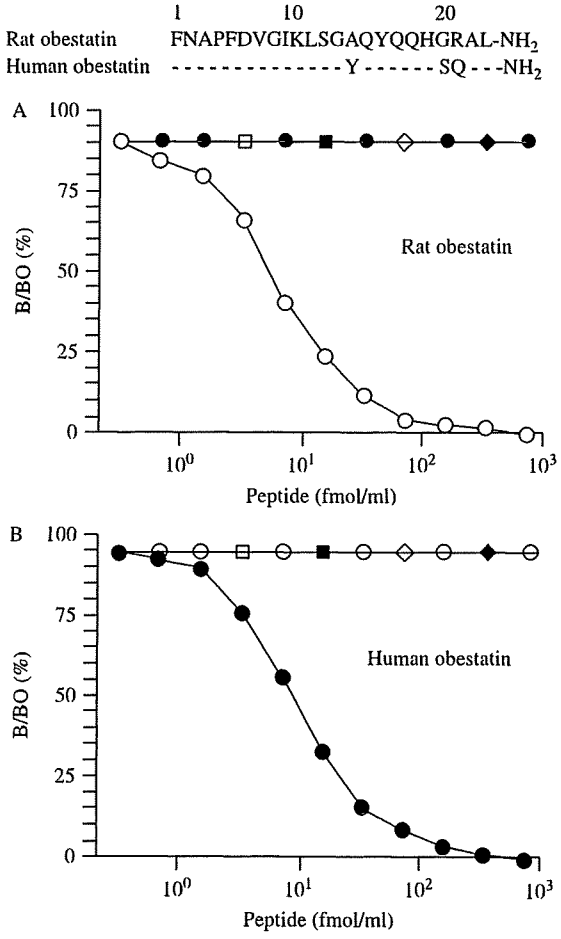
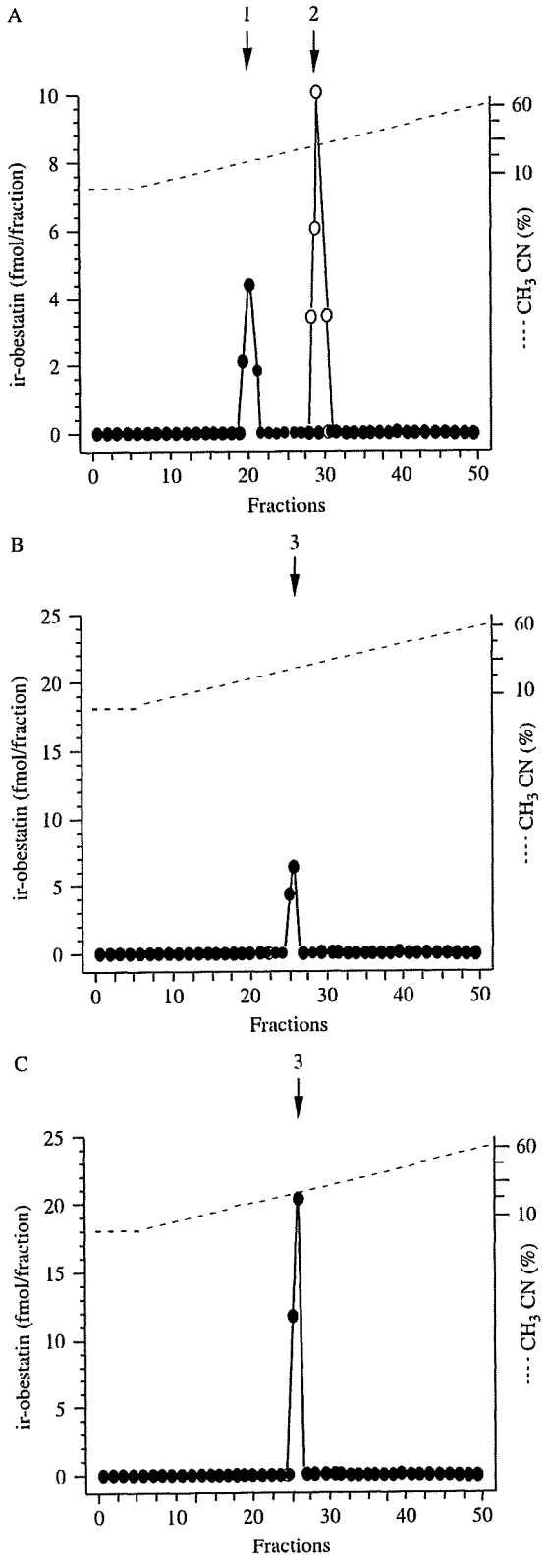


Figure 1 (Upper panel) Alignment of amino acid sequences of rat and human obestatin. Numbers indicate amino acid positions relative to the N-termini. (Lower panel) Standard RIA curves for rat and human obestatin. (A) Inhibition of ^{125}I-rat obestatin binding to anti-obestatin antiserum by serial dilution of rat obestatin (○), human obestatin (●), rat ghrelin (□), human gastrin (■), human gastrin-releasing peptide (◇), and human somatostatin (◆). (B) Inhibition of ^{125}I-human obestatin binding to anti-obestatin antiserum by serial dilution of human obestatin (●), rat obestatin (○), rat ghrelin (□), human gastrin (■), human gastrin-releasing peptide (◇), and human somatostatin (◆).



by rat obestatin was 3·2 fmol/ml, and the peptide was detectable at concentrations as low as 0·2 fmol/ml (Fig. 1A). Anti-rat obestatin antiserum recognizes the C-terminal region of rat obestatin, and had no cross-reactivity with human obestatin. Half-maximum inhibition by human obestatin on the standard RIA curve was 7·8 fmol/ml, and the peptide was also detectable at the low level of 0·2 fmol/ml (Fig. 1B). Anti-human obestatin antiserum recognizes the C-terminal region of human obestatin and had no cross-reactivity with rat obestatin. Neither anti-rat obestatin nor anti-human obestatin antiserum had any cross-reactivity with rat or human ghrelin, human gastrin, human gastrin-releasing peptide, or human somatostatin.

HPLC characterization of ir-obestatin and ghrelin in gastric fundus and plasma

RP-HPLC coupled with obestatin RIAs were used to characterize immunoreactive obestatin molecules in the rat and human gastric fundi and plasma. In the rat gastric fundus, one immunoreactive peak was eluted at the position of synthetic rat obestatin (Fig. 2A) and another immunoreactive peak was detected 4 min later. In both the human gastric fundus and plasma, only one immunoreactive peak was eluted at the position of synthetic human obestatin (Fig. 2B and C).

Obestatin and ghrelin contents in the gastric fundus and plasma

Obestatin contents in the rat and human gastric fundi accounted for 0·0039 and 1·94% respectively of their ghrelin contents (Table 1). Plasma obestatin concentration in humans accounted for 5·21% of plasma ghrelin concentration. Plasma obestatin was below the detectable value (less than 0·2 fmol) in rats.

Meal test

The plasma des-acyl ghrelin concentration of normal subjects significantly decreased after taking a test meal, reaching a nadir of 53% of the basal level 60 min after meal; obestatin concentration did not change after meal (Fig. 3).

Feeding behavior

A single i.c.v. administration of rat or human obestatin to rats did not suppress dark-phase food intake (Fig. 4A). Likewise, a single i.c.v. administration of rat obestatin to mice did not

Figure 2 Representative RP-HPLC profiles of obestatin immunoreactivities in (A) rat gastric fundus, (B) human gastric fundus, and (C) human plasma. Sample amounts of rat and human gastric fundi were 1·9 and 1·0 g wet tissue weight respectively. The sample volume of human plasma was 17 ml. Samples were loaded on a TSK ODS SIL 120A column (4·6×150 mm). RP-HPLC was done for 40 min at 1·0 ml/min with a linear gradient of CH_3CN (10–60%) in 0·1% TFA. Each fraction was 0·5 min. Arrows indicate the elution positions of 1) rat obestatin, 2) unknown immunoreactive peak, and 3) human obestatin.

Table 1 Obestatin and ghrelin amounts in rat and human gastric fundi and plasma. Values are mean±S.E.M. ($n=3$, human plasma $n=16$)

	Obestatin	Ghrelin	Ratio of obestatin to ghrelin (%)
Rat gastric fundus	0·18±0·03 fmol/mg	4633·5±440·1 fmol/mg	0·0039
Rat plasma	Not detected	344·4±40·6 fmol/ml	–
Human gastric fundus	0·17±0·02 fmol/mg	8·75±1·90 fmol/mg	1·94
Human plasma	6·9±0·28 fmol/ml	132·4±13·1 fmol/ml	5·21

suppress feeding (Fig. 4B), nor did an i.c.v. administration of rat obestatin suppress feeding in rats that had been fasted overnight (Fig. 4C). In these experiments, CART, which was used as a positive control, significantly suppressed food intake. Next, we studied the effect of peripheral administration of rat obestatin on feeding. A single i.p. administration of 125 or 1000 nmol/kg rat obestatin to mice did not suppress food intake (Fig. 4D). By contrast, i.p. administration of ghrelin significantly increased food intake in mice, but co-administration of obestatin did not antagonize ghrelin-induced feeding (Fig. 4E). All experiments were done in a crossover format, in which all animals received an injection of obestatin, CART or ghrelin on separate days.

Discussion

The use of evolutionary genomics screening approach led to the discovery of obestatin (Zhang *et al.* 2005). The authors identified the presence of obestatin in the rat stomach by peptide sequence analysis. They also reported that central or peripheral administration of obestatin to rats suppressed feeding. We first characterized obestatin immunoreactive molecules in the stomach of rats and humans by two RIAs specific for rat and human obestatin respectively. Both rat and human gastric fundi contain obestatin immunoreactive molecules, but their obestatin contents accounted for as little as 0·0038 and 1·94% of their ghrelin contents respectively. Zhang *et al.* (2005) also identified a C-terminal 13-amino acid fragment of obestatin eluted after obestatin itself in their HPLC analyses of rat stomach; consistent with this, our HPLC analysis of rat gastric fundus detected obestatin and another obestatin immunoreactive peak in fractions 27–31 eluted after obestatin. Rat and human obestatin differ by only three amino acids at positions 14, 20, and 21 (Fig. 1); however, the rat obestatin antiserum used in this study showed no cross-reactivity with human obestatin, suggesting that this antiserum recognized the C-terminal portion of rat obestatin. The obestatin immunoreactive peptide in fractions 27–31 may be a C-terminal 13-amino acid peptide fragment of obestatin or an N-terminally extended longer form of obestatin. Further sequence determination is needed to identify this unknown obestatin-related peptide.

Obestatin accounted for only 5·21% of ghrelin concentration in the human plasma, but was undetectable in the rat plasma. Obestatin contents in the gastric fundi of rats and humans are nearly equal (0·18±0·03 and 0·17±0·02 fmol/mg respectively). The ratio of obestatin to ghrelin in the gastric fundi of rats and humans is extremely low (0·0039% and 1·94% respectively). The presence of very low obestatin content in rat stomach may be due to rapid degradation of obestatin and/or a low processing rate of obestatin from preproghrelin. We therefore speculate may be due to this reason obestatin immunoreactivity was not detectable in the rat plasma. Bioactive peptides are cleaved from precursor proteins via limited cleavage and often undergo post-translational modifications indispensable for their biological activities. Processing of ghrelin after the initial removal of signal peptide involves a single cleavage, whereas the processing of obestatin requires cleavages at both the amino and carboxy termini (Garg 2007). Because the proposed proteolytic sites for cleavage of obestatin from proghrelin lack the more efficient dibasic residues, lysine and arginine, these cleavages might occur with reduced efficiency. *In vitro* digestion of proghrelin using several convertases fails to generate obestatin (Ozawa *et al.* 2007). These results suggest that obestatin may be a post-translational by-product of preproghrelin with no relevant physiological bioactivity.

Circulating human ghrelin is suppressed by food intake in humans (Cummings *et al.* 2001, Shiiya *et al.* 2002); we therefore compared the plasma obestatin with plasma ghrelin in response to meal in normal subjects. The plasma des-acyl ghrelin concentration significantly decreased after a meal, while the plasma obestatin concentration did not change. Human plasma obestatin concentration did not change even after intake of a 1550 kcal meal (Huda *et al.* 2008). Thus, obestatin secretion does not appear to be influenced by dietary nutrients.

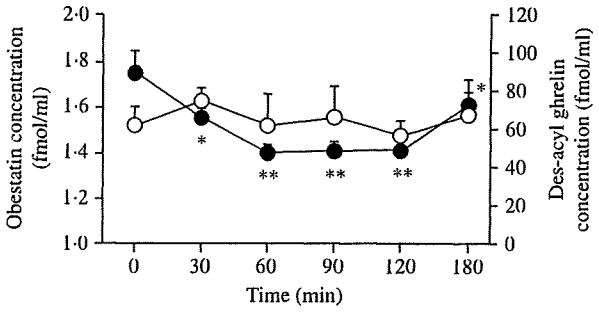


Figure 3 Human plasma obestatin (○) and des-acyl ghrelin (●) levels in a meal test in normal subjects. *$P<0·05$, **$P<0·001$ versus des-acyl ghrelin at 0 min; $n=16$.

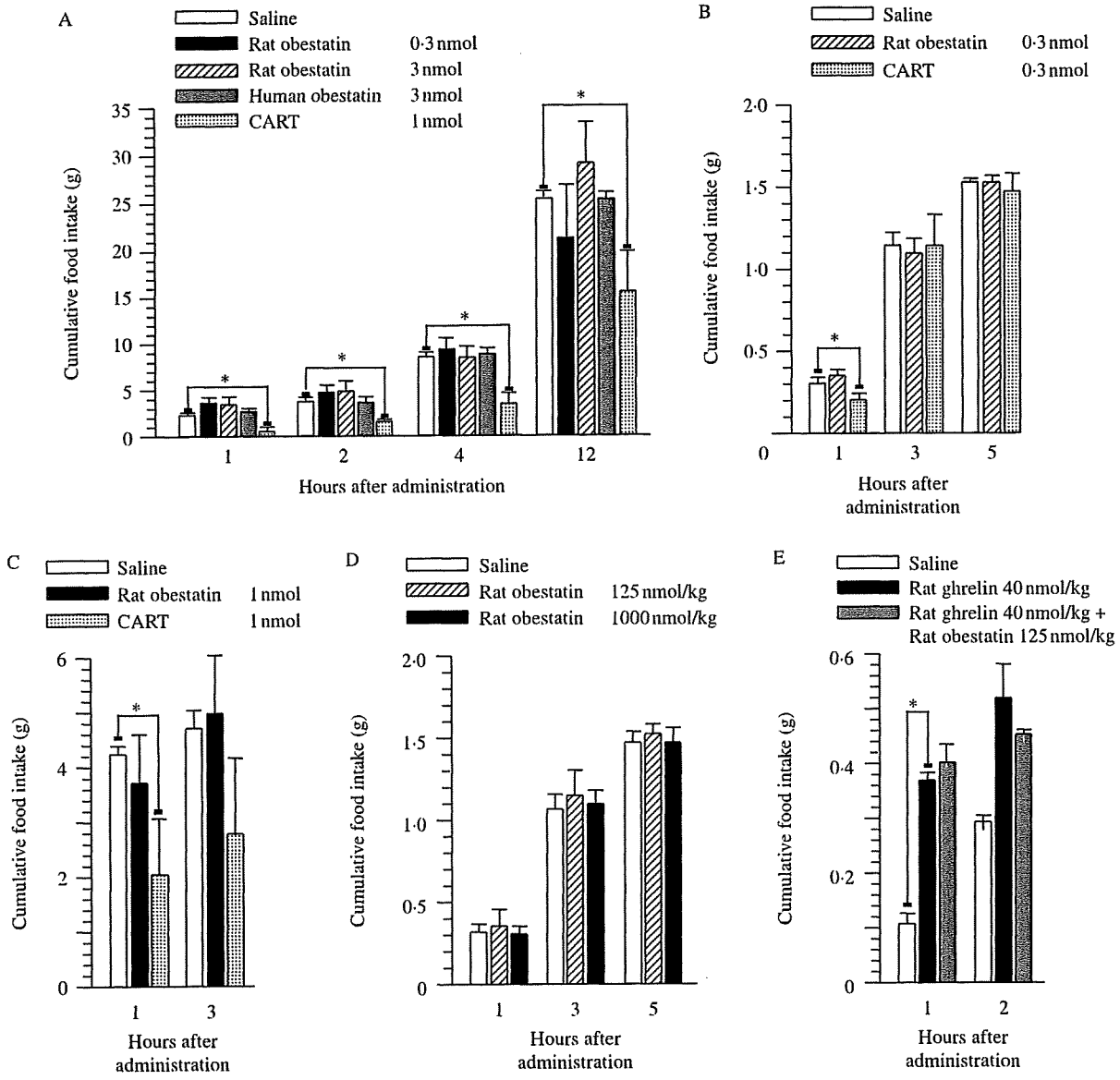


Figure 4 No effect on central or peripheral administration of obestatin on food intake. (A) Cumulative food intake in free-feeding rats in the dark phase ($n=6$ per group) following i.c.v. administration of saline, rat obestatin (0·3, 3·0 nmol), human obestatin (3·0 nmol), or CART (1 nmol). (B) Cumulative food intake in free-feeding mice in the dark phase ($n=7$ per group) following i.c.v. administration of saline, rat obestatin (0·3 nmol), or CART (0·3 nmol). (C) Cumulative food intake in fasted rats in the light phase ($n=6$–8 per group) was measured following i.c.v. administration of saline, rat obestatin (1 nmol), or CART (1 nmol). (D) Cumulative food intake in free-feeding mice in the dark phase ($n=5$ per group) following i.p. administration of rat obestatin (125, 1000 nmol/kg). (E) Cumulative food intake in free-feeding mice in the light phase ($n=5$ per group) following i.p. administration of rat ghrelin (40 nmol/kg) or administration of rat ghrelin (40 nmol/kg) plus rat obestatin (125 nmol/kg). *$P<0·01$ versus saline controls.

The present study showed that a central or peripheral administration of obestatin had no suppressive effects on food intake in either rats or mice fed *ad libitum*. The administration of obestatin at a very high dose (1000 nmol/kg, i.p.) did not exert any suppressive effects in mice, in contrast to the original report (Zhang *et al.* 2005). Similar negative findings using 1000 nmol/kg obestatin in mice were reported by other groups (Gourcerol *et al.* 2006, Holst *et al.* 2007, Nogueiras *et al.* 2007). The administration of obestatin to fasted rats failed to suppress food intake in this study, also consistent with recent reports (Gourcerol *et al.* 2006, de Smet *et al.* 2007). We used a satiety peptide, CART, as a positive control in all our feeding experiments. CART decreased food intake in rats and mice, thus confirming our findings regarding obestatin.

The functional antagonism of obestatin against ghrelin was assessed in this study by co-administration of obestatin and

ghrelin. In contrast to the original report (Zhang *et al.* 2005), the administration of obestatin did not antagonize ghrelin-induced feeding. Our findings show that obestatin does not function in feeding behavior as a satiety peptide. The hypothalamic arcuate nucleus is critical for feeding regulation, as it contains neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons, whose activation stimulates feeding, and pro-opiomelanocortin (POMC) and CART neurons, whose activation suppresses feeding (Schwartz *et al.* 2000). Ghrelin administration activated NPY/AgRP neurons, as demonstrated by enhanced *c-fos* expression on NPY and AGRP neurons and their mRNA expression (Nakazato *et al.* 2001). Chronic i.c.v. administration of obestatin for 7 days did not change mRNA expression of NPY, AGRP, POMC, or CART (Nogueiras *et al.* 2007). Taken together, these data suggest that obestatin does not exert any effects on the hypothalamic circuits that control energy balance.

Several groups, including ours, failed to reproduce obestatin's anorexigenic effect upon acute administration (Gourcerol *et al.* 2006, Bassil *et al.* 2007, Gourcerol & Taché 2007, de Smet *et al.* 2007). Differences in experimental conditions and species used in the original and the subsequent negative reports do not appear to be involved. The administration of obestatin by i.p., s.c., or i.c.v., was equally inefficient to influence food intake (Gourcerol & Taché 2007, Holst *et al.* 2007, Nogueiras *et al.* 2007). Negative findings were even obtained under testing conditions mimicking the initial report, and use of different doses, spontaneous feeding or fasting, and species (rat or mice; Gourcerol & Taché 2007, Holst *et al.* 2007, Nogueiras *et al.* 2007). By contrast, the administration of CART and ghrelin as control peptides in our experiments under similar conditions resulted in either suppression or stimulation of food intake respectively. Investigation of human and rat/mouse obestatin, which share 87% homology, under nocturnal fasting, 50% food deprived conditions, and in the darkness and light phases, all resulted in the same negative outcome (Gourcerol & Taché 2007, Holst *et al.* 2007, Nogueiras *et al.* 2007). These findings further confirm that obestatin does not hold its promise so far to regulate food intake in rodents.

In summary, the present study demonstrated that obestatin immunoreactive molecules are present in the rat and human gastric fundi at very low levels compared with ghrelin. In addition, neither central nor peripheral administration of obestatin suppressed food intake in freely fed or fasted rodents, and obestatin did not antagonize ghrelin-induced feeding. Thus, obestatin derived from preproghrelin may not act as a biological active peptide, as initially reported, to regulate acute feeding behavior in rodents.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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References

Bassil AK, Haglund Y, Brown J, Rudholm T, Hellström PM, Näslund E, Lee K & Sanger GJ 2007 Little or no ability of obestatin to interact with ghrelin or modify motility in the rat gastrointestinal tract. *British Journal of Pharmacology* **150** 58–64.

Bresciani E, Rapetti D, Donà F, Bulgarelli I, Tamiazzo L, Locatelli V & Torsello A 2006 Obestatin inhibits feeding but does not modulate GH and corticosterone secretion in the rat. *Journal of Endocrinological Investigation* **29** 16–18.

Carlini VP, Schiöth HB & Debarioglio SR 2007 Obestatin improves memory performance and causes anxiolytic effects in rats. *Biochemical and Biophysical Research Communications* **352** 907–912.

Chartrel N, Alvear-Perez R, Leprince J, Iturrioz X, Reaux-Le Goazigo A, Audinot V, Chomarat P, Coge F, Nosjean O, Rodriguez M *et al.* 2007 Comment on 'Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake'. *Science* **315** 766.

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

Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K & Nakazato M 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* **141** 4255–4261.

Garg A 2007 Commentary: the ongoing saga of obestatin: is it a hormone? *Journal of Clinical Endocrinology and Metabolism* **92** 3396–3398.

Gourcerol G & Taché Y 2007 Obestatin-a ghrelin-associated peptide that does not hold its promise to suppress food intake and motility. *Neurogastroenterology and Motility* **19** 161–165.

Gourcerol G, Million M, Adelson DW, Wang Y, Wang L, Rivier J, St-Pierre DH & Taché Y 2006 Lack of interaction between peripheral injection of CCK and obestatin in the regulation of gastric satiety signaling in rodents. *Peptides* **27** 2811–2819.

Granata R, Settanni F, Gallo D, Trovato L, Biancone L, Cantaluppi V, Nano R, Annunziata M, Campiglia P, Arnoletti E *et al.* 2008 Obestatin promotes survival of pancreatic β-cells and human islets and induces expression of genes involved in the regulation of β-cell mass and function. *Diabetes* **57** 967–979.

Holst B, Egerod KL, Schild E, Vickers SP, Cheetham S, Gerlach LO, Storjohann L, Stidsen CE, Jones R, Beck-Sickinger AG *et al.* 2007 GPR39 signaling is stimulated by zinc ions but not by obestatin. *Endocrinology* **148** 13–20.

Hosoda H, Kojima M, Matsuo H & Kangawa K 2000 Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochemical and Biophysical Research Communications* **279** 909–913.

Huda MS, Durham BH, Wong SP, Deepak D, Kerrigan D, McCulloch P, Ranganath L, Pinkney J & Wilding JP 2008 Plasma obestatin levels are lower in obese and post-gastrectomy subjects, but do not change in response to a meal. *International Journal of Obesity* **32** 129–135.

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