Gastrointestinal hormones (anorexigenic peptide YY and orexigenic ghrelin) influence neural tube development

Hideki Yuzuriha,* Akio Inui,[†,1] Akihiro Asakawa,* Naohiko Ueno,* Masato Kasuga,* Michael M. Meguid,[‡] Jun-ichi Miyazaki,[§] Maiko Ninomiya,[‖] Herbert Herzog,[¶] and Mineko Fujimiya[‖]

*Division of Diabetes, Digestive and Kidney Diseases, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan; [†]Department of Behavioral Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan; [‡]Department of Neuroscience and Surgery, SUNY Upstate Medical University, Syracuse, New York, USA; [§]Department of Nutrition and Physiological Chemistry, Osaka University Graduate School of Medicine, Osaka, Japan; [‖]Department of Anatomy, Shiga University of Medical Science, Otsu, Japan; and [¶]Neurobiology Program, Garvan Institute of Medical Research, Sydney, Australia

ABSTRACT **Gastrointestinal (GI) hormones play an important role in GI secretion, motility, and eating behaviors. It was recently suggested that GI hormones may have a trophic role in GI tract. Here we demonstrate that two principal GI hormones, anorexigenic peptide YY (PYY) and orexigenic ghrelin, affect neural tube development. Chronic administration into the pregnant mice or transgenic overexpression of PYY led to a neural tube defect (NTD) in the embryos that was blocked by ghrelin. PYY Y1 receptor antagonist prevented the occurrence of NTD induced not only by PYY but also by vitamin A, a well-known teratogen in humans and animals. Y1 receptor deficiency also engendered NTDs, indicating the need to maintain normal Y1 receptor signaling. The present study is the first linking GI hormones to the leading cause of infant mortality and provides a novel insight for neurogenesis in which materno-fetal communication through GI hormones appears to be important.—Yuzuriha, H., Inui, A., Asakawa, A., Ueno, N., Kasuga, M., Meguid, M. M., Miyazaki, J-i., Ninomiya, M., Herzog, H., Fujimiya, M. Gastrointestinal hormones (anorexigenic peptide YY and orexigenic ghrelin) influence neural tube development. *FASEB J.* 21, 2108–2112 (2007)**

Key words: neural tube defect · transgenic mice · gut hormone

Peptide YY (PYY) is a 36 amino acid gastrointestinal hormone that is structurally related to neuropeptide Y (NPY) in the brain and pancreatic polypeptide (PP) in the pancreas (1, 2). PYY is synthesized and released from endocrine L cells from the distal gut in response to oral nutrient ingestion (2). PYY has been characterized primarily for its postprandial inhibitory effects on the gastrointestinal tract, including inhibition of gastric emptying and appetite (2–4). The appetite-reducing effects have been shown in both healthy volunteers and obese subjects (5). The biological effects of PYY are mediated by four cloned Y receptors known as Y1, Y2, Y4, and Y5 (6). PYY displays high affinities for the Y1, Y2, and Y5 receptors, carboxyl-terminal fragments for the Y2 receptor, and PP for the Y4 receptor.

Ghrelin is an endogenous ligand of a growth hormone (GH) secretagogue receptor that was discovered in the stomach (7, 8). Ghrelin is a potent stimulator of feeding as well as GH secretion, and integrates the control of food intake with digestive processes and growth (7, 9–11). The peptide shows a competitive interaction with PYY and improves anorexia and cachexia in patients with cancer and other diseases after intravenous administration (9, 12).

To examine the trophic role of gastrointestinal (GI) hormones in tissues other than the GI tract, we developed mice overexpressing PYY with use of the mouse PYY cDNA and the cytomegalovirus immediate-early enhancer chicken β-actin hybrid promoter (pCAGGS) (13, 14). Since overexpression of PYY resulted in abnormalities in forebrain formation known as neural tube defects (NTDs) in humans (15), we administered PYY, its analogs, and ghrelin into normal pregnant mice or Y receptor knockout mice to see whether this occurs in the embryos. Furthermore, we examined the interactions between PYY and nutritional components involved in NTDs such as vitamin A to see whether GI hormones are involved in the naturally occurring human pathology.

MATERIALS AND METHODS

Plasmid construction and production of transgenic mice

Plasmid pCAGGS-PYY was constructed by inserting a mouse PYY cDNA into the unique *Eco*RI site between the CAG

[1] Correspondence: Department of Behavioral and Internal Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, 8–35-1 Sakuragaoka, Kagoshima 890-8520, Japan. E-mail: inui@m.kufm.kagoshima-u.ac.jp

doi: 10.1096/fj.06-7621com

0892-6638/07/0021-2108 © FASEB

promoter and 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector (16). Mouse PYY cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from RNA isolated from mouse intestine. The DNA fragment was excised from its plasmid by digestion with *Sal*I and *Bam*HI, then purified and microinjected into the pronuclei of fertilized eggs obtained from BDF1 or C57BL/6 female mice, as reported previously (14). Transgenic mice were usually identified by PCR and Southern blot analyses. All experiments were approved by the Kobe University animal care committee.

PYY administration into normal mice

PYY, a COOH-terminal fragment $NPY_{13–36}$, and PP were administered i.p. to pregnant BDF1 mice at a dose of 3 nmol/mouse twice a day for 11 days beginning on the first postcoital day. Ghrelin and Y receptor antagonists such as BIBO3304(Y1) and L152804(Y5:9-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-en-1-yl)-3,3-dimethyl-2,3,4,9-tetrahydro-1H-xanthen-1-one) were also administered i.p. at the dose of 3 nmol/mice and 15 nmol/mice, respectively, twice a day for 11 days. Daily injections were performed at 7 AM and 7 PM. All the mice had received an i.p. injection with pregnant mare's serum (5.0 IU) and human chorionic gonadotropin (5.0 IU) 3 and 1 days before the experiment, respectively (17). Mouse PYY and mouse $NPY_{13–36}$ were obtained from Peninsula Labs Inc. (Belmont, CA, USA), mouse ghrelin from Peptide Institute Inc. (Osaka, Japan), and mouse PP was custom-synthesized by Sawady Technology Co. Ltd. (Tokyo, Japan). BIBO3304 was supplied by Boehringer-Ingelheim Pharma GmbH (Rheinland Palatinate, Germany) and L152804 was from Banyu (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan). Each drug was diluted in 100 μl physiological saline immediately before the i.p. injection.

PYY administration into Y1 receptor knockout mice

Germline deletion of Y1 receptor gene was achieved as described previously (18). All mice generated were maintained on mixed C57BL/6–129/SvJ backgrounds. PYY was administrated i.p. to pregnant Y1 receptor knockout mice at a dose of 3 nmol/mouse twice a day for 11 days beginning on the first postcoital day.

Real-time RT-PCR

The mice were killed by cervical dislocation 30 min after the final (fourth) administration of PYY. The stomach was removed immediately, frozen on dry ice, and stored at −80°C until preparation of real-time RT-PCR. RNA was isolated using the RNeasy Mini Kit (Qiagen Inc., Tokyo, Japan). Quantification of mRNA levels was performed with SYBR-green chemistry (Qiagen Inc., Tokyo, Japan) using a one-step RT-PCR reaction on an ABI PRISM 7700 Sequence Detection System purchased from Applied Biosystems Japan, Ltd. (Tokyo, Japan). The reaction was performed under standard conditions recommended by the manufacturer. We used the mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene as an internal control. All expression data were normalized to a G3PDH expression level from the same individual sample. The following primers were used in real-time RT-PCR: G3PDH forward, ATGGTGAAGGTCGGTGTGAA; and reverse, GAGTGGAGTCATACTGGAAC. Ghrelin forward, AGCATGCTCTGGATGGACATG; and reverse, GCAGTTTAGCTGGTGGCTTCTT. Leptin forward, CTGTGGCTTTGGTCCTATCT; and reverse, TGATAGACTGCCAGAGTCTG.

Dietary manipulation

Dietary manipulation was made using vitamin A or folic acid, which induces or prevents NTDs in lower animals and humans (19, 20). In addition to a normal diet, vitamin A or folic acid was administered to normal pregnant mice with a liquid diet containing vitamin A (16 mg/100 g, Elental) or with water containing folic acid (63 mg/100 g) throughout the experiments. The mice were found to have taken effective doses of vitamin A (18 mg (884 IU)/kg/day) or folic acid (68 mg/kg/day).

Brain histological analysis and immunohistochemistry for PYY

Pregnant mice were anesthetized with an i.p. injection of sodium pentobarbital (100 mg/kg; Nembutal; Abbott Laboratories, Chicago, IL, USA). Embryos were removed from the uterus and immersed in a fixative containing 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid (21). Cryostat sagittal or frontal sections of embryos were treated in the free-floating state with hematoxylin-eosin or antirat PYY used at a dilution of 1:10,000 (22).

Food intake and measurement of plasma PYY and acylated ghrelin

Food intake and plasma levels of PYY and ghrelin were examined in mice that received PYY or saline injection in free-feeding and pair-fed groups. Blood samples were obtained 5 h after final injection of PYY or saline on embryonic day 11. Blood samples were immediately transferred to chilled poly-propylene tubes containing Na2EDTA and aprotinin, and centrifuged at 4°C. For ghrelin assay, plasma samples were acidified with hydrogen chloride for a final concentration of 0.1N. Plasma PYY levels were measured with an enzyme immunoassay kit (YK081, Yanaihara Institute, Shizuoka, Japan), and acylated ghrelin levels were measured with an active ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Tokyo, Japan).

Statistical analysis

The incidence of NTDs was expressed as (number of affected fetuses/number of total examined fetuses) ×100 on embryonic day 12 or 15. Data on gene expression were expressed as a percentage of physiological saline-injected controls. ANOVA, followed by a Bonferroni's *t* test to assess differences among the groups. *P* values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

PYY-overexpressed mice develop neural tube defect

Overexpression of PYY appeared to result in midgestational lethality. To ascertain why PYY transgenic embryos died *in utero*, the embryos were histologically examined. Examination of hematoxylin and eosin-stained cryostat sections revealed that all of the major organs were normal except for the brain (**Fig. 1*A–D***). Transgenic embryos displayed exencephaly, an abnormality of forebrain formation that strikingly resembles a corresponding human syndrome caused by NTDs, the leading cause of infant mortality (15). Thus, PYY may

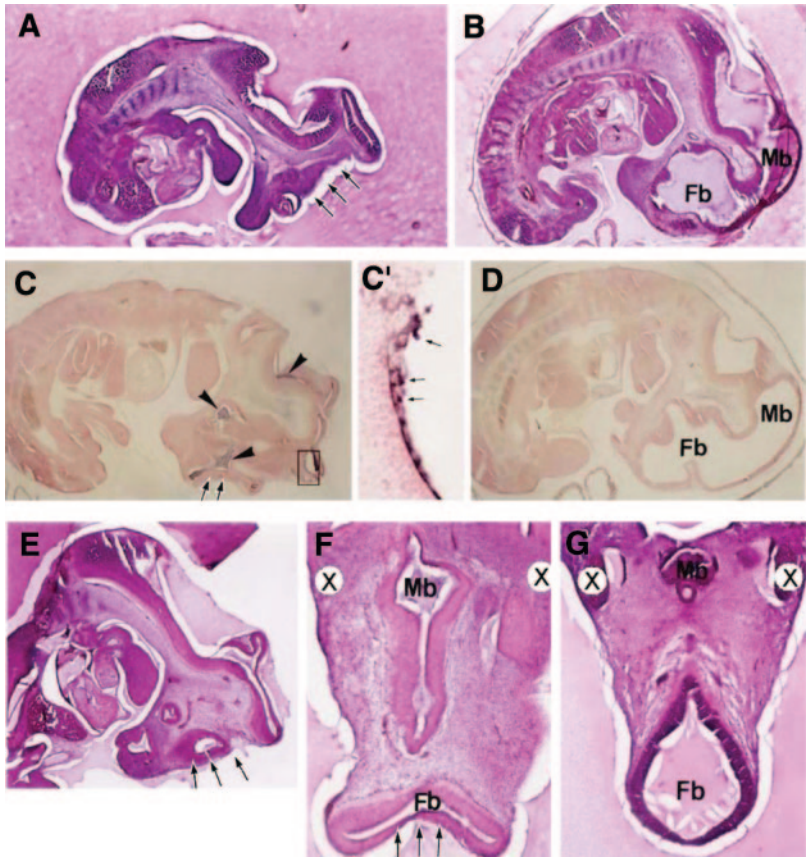

Figure 1. *A–D)* Histological comparison between PYY-overexpressing (*A, C*) and wild-type (*B, D*) embryos at ED12 in BDF1 background. Hematoxylin-eosin staining (*A, B*) and PYY immunohistochemisty (*C, D*) of the sagittal sections from the embryos are shown. Details of the procedure were reported previously (12). Forebrain formation is severely disrupted in the PYY-overexpressing embryos (arrows in panels *A*, and *C*) compared with the wild-type controls (*B, D*). PYY immunoreactivity is seen in the ventricle in transgenic embryos (arrowheads in panel *C*), in a higher magnification a positive reaction is seen in the ependymal cells (*C'* arrows). *E–G)* ED12 embryos of BDF1 mice obtained from maternal PYY administration (3 nmol administered intraperitoneally twice a day for 11 days during pregnancy) (*E, F*) and from control saline injection (*G*). Hematoxylin-eosin staining of the sagittal sections (*E*) and frontal sections (*F, G*) at the level of the optic vesicles (X) is shown. Disrupted forebrain formation is seen in PYY-treated embryos (arrows in panel *E*), which is equivalent to that seen in the PYY-overexpressing embryos (*A*). In the frontal sections, the forebrain is flattened in the PYY-treated embryos (arrows in panel *F*) compared with the saline injected controls (*G*). Fb, forebrain; Mb, midbrain.

be a part of the gene regulatory and metabolic pathways for NTDs (19). Anencephaly and spina bifida are the two most common forms of NTDs, occurring in 1 in 1000 pregnancies in the U.S. and an estimated 300,000 or more newborns worldwide each year (15). Overexpression of PYY was confirmed immunohistochemically in tissues such as the ependymal cells of the cerebral ventricle in transgenic mice, but not in the controls (Fig. 1*C, C'*).

Maternal PYY administration produces neural tube defect in fetuses

To examine the PYY effect in a more physiological situation, PYY was administered to pregnant mice at a dosage that reduces food intake in this species (4, 23). Chronic PYY administration into maternal peritoneum was able to recapture the phenotype in the embryos (Fig. 1*E–G*). Thus, either derived maternally or from embryos, PYY does cause NTDs. This malformation occurred in 65% of the fetuses on embryonic day 12 of pregnant mice treated with PYY (**Table 1**). In mouse embryos, the neural tube forms during days 8–10 of gestation (19). PYY immunoreactivity is already apparent in the pancreatic islet on embryonic day 9.5 (24), and Y1 receptor mRNA or protein also appear at around the same time (25, 26). PYY can evoke this effect through an inhibition of ghrelin, since stomach ghrelin was down-regulated by PYY (not shown) while preventing the PYY-induced NTDs when maternally administered (Table 1). Since ghrelin is undetectable or marginally expressed in the stomach of the developing fetus or in the placenta at earlier stages of pregnancy (11, 27 and unpublished data), maternal ghrelin could have a protective role in the development of NTDs. When analyzed later on embryonic day 15, >80% of embryos treated with PYY revealed the abnormality.

Food intake and GI hormone levels in PYY-treated mice

To examine whether the development of NTDs is derived secondarily from the anorexia induced by PYY, we mea-

TABLE 1. *Incidence of neural tube defects (NTDs) in normal mice*[a]

Treatment	Incidence of NTDs (%)[b]	
Control (saline)	5/33	(15.1)
PYY	28/46	(60.9)***
PP	6/22	(27.2)
$NPY_{13–36}$	5/20	(25.0)
PYY + Y1 antagonist	0/14	(0)†††
PYY + Y5 antagonist	12/28	(42.8)
PYY + folic acid	6/14	(42.9)
PYY + ghrelin	4/15	(26.7)†
Vitamin A + saline	7/11	(63.6)**
Vitamin A + Y1 antagonist	3/13	(23.0)$^{\#}$

[a] Peptides and drugs were administered into the peritoneum, and vitamin A and folic acid were administered with a liquid diet and water, respectively. [b] Incidence of NTDs is analyzed on embryonic day 12 and includes mild forms. (Number of affected fetuses/number of total examined fetuses)×100. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* saline controls; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ *vs.* PYY-treated group; $^{\#}P < 0.05$ *vs.* vitamin A-treated controls.

sured food intake and body weight of PYY-treated animals (n=4). In contrast to normal mice (23), PYY failed to decrease food intake [4.73±0.08 g/day (PYY) *vs.* 4.27±0.26 g/day (saline) and body weight increase [8.0±1.2 g/11 days (PYY) *vs.* 8.5 g±1.1 g/11 days (control)] in pregnant mice that exhibited an ~1.5-fold increase in food intake compared with nonpregnant mice. The increased appetite in pregnancy is known to be associated with increased activity of orexigenic signals such as neuropeptide Y in the hypothalamus (28, 29). To examine whether the teratogenic effect of PYY has physiological relevance, we measured blood PYY and acylated ghrelin in PYY-treated animals (n=4). PYY levels tended to increase in PYY-treated mice [1.91±0.15 ng/ml (PYY) *vs.* 1.39±0.22 ng/ml (saline), 1.46 ± 0.19 ng/ml (pair-fed)] and acylated ghrelin levels tended to decrease [44.7±6.41fmol/ml (PYY) *vs.* 49.7±19.6 fmol/ml (saline), 56.0 ± 13.5fmol/ml (pair-fed)] 5 h after the final PYY administration. Although a considerable amount of blood is needed to measure PYY and acylated ghrelin hampers the exact evaluation of hormonal changes in mice, the increase in PYY levels appeared to be limited in length despite chronic administration. Normally, the blood-brain barrier prevents exposure of neurological tissues to circulating GI hormones except through the circumventricular organs. However, the blood-brain barrier is likely to be leaky in the fetuses, which may leave the developing brain susceptible to the changes of circulating GI hormones in the mother, as observed after PYY or ghrelin (30) administration. Future studies should address whether more subtle changes of circulating GI hormones are associated with the NTDs observed in control animals.

PYY receptor involved in neural tube defect

To examine the receptor mechanism involved, we administered PP (a Y4 agonist) and $NPY_{13–36}$ (a Y2 agonist), as well as the Y1 (BIBO3304) and Y5 (L152804) receptor antagonists (10, 23) to pregnant mice (Table 1). We found that neither PP nor $NPY_{13–36}$ produced statistically significant effects on NTDs. However, the Y1, but not the Y5, receptor antagonist eliminated the teratogenic activity of PYY when coadministered, suggesting a Y1 receptor-mediated event. To further clarify the Y receptor involved, we repeated experiments in Y1 receptor knockout mice (**Table 2**). Y1 knockout mice already had significantly higher incidence of NTDs compared with normals (Tables 1 and 2), but PYY treatment failed to further increase the rate of NTDs in these mice. The finding that both the lack and the overstimulation of the Y1 receptor can cause an increased incidence of NTDs suggests that the Y1 receptor may be a physiological regulator in neural tube closure.

TABLE 2. *Incidence of neural tube defects (NTDs) in Y1 receptor knockout (KO) mice*

Treatment	Incidence of NTDs (%)[a]
Y1 KO + saline	25/41(61.0)
Y1 KO + PYY	9/16(56.3)

[a] Incidence of NTDs is analyzed on embryonic day 12 and includes mild forms. (Number of affected fetuses/number of total examined fetuses)×100.

Y1 receptor is involved in nutritionally induced NTDs

Previous studies demonstrated a link between nutrition and the occurrence of NTDs (15). Retinoids (vitamin A and its derivatives) are known to play important roles in maintaining various tissues in the adult vertebrate and to be essential for diverse embryological processes. Experiments in mice showed that retinoids can be teratogenic, and this has been confirmed in humans (15, 20). On the other hand, folate deficiency and disturbed folate metabolism have been implicated in the etiology of NTDs (15), and supplementation of folic acid partly reduced the incidence of NTDs in both animals and humans (15, 31). Therefore, a final group of experiments was performed to clarify the potential link between these micronutrients and PYY-induced NTDs. We found that the Y1 antagonist significantly inhibited hypervitaminosis A-induced teratogenesis in the mouse model, whereas folic acid failed to prevent the teratogenic effect of PYY (Table 1). There are few effective preventions/treatments of folic acid-resistant NTDs that consist of at least 30% of the cases (15). Since PYY-induced NTDs are folate resistant but ghrelin sensitive, maternal GI hormones may play a role in the development of intractable NTDs.

Here we reported that GI hormones may have a physiological role for neural tube closure. Overexpression of PYY by transgenic technique, chronic administration of PYY into normal pregnant mice, or deletion of PYY Y1 receptor produced NTDs in the embryos, which is a common congenital malformation in humans and the leading cause of infant mortality. The teratogenic effect of PYY is exerted at the biologically active dose and involves a specific mechanism mediated by the Y1 receptor. The findings that the Y1 antagonist significantly reduced the incidence of NTDs induced by vitamin A, a well-known teratogen in humans (15, 21), suggest a fundamental role of the Y1 receptor in neural tube closure. In contrast to PYY, ghrelin protected against NTDs induced by PYY when maternally administered. Ghrelin may thus have a specific role in neural tube closure in addition to its effect on fetal growth (30). The present study demonstrates a novel developmental role for GI hormones in the brain and provides an excellent candidate from mouse NTD models to be investigated in human NTDs. Further investigation of GI hormones might lead to an understanding of molecular mechanisms of the materno-fetal communication by which fetal development and neurogenesis can be regulated. FJ

This work was partly supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (A.I. and M.F.).

REFERENCES

1. Tatemoto, K., and Mutt, V. (1980) Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. *Nature* **285,** 417–418
2. Taylor, I. L. (1989) Pancreatic polypeptide family: pancreatic polypeptide, neuropeptide Y and peptide YY. In *Handbook of Physiology,* Vol. 2 (Rauner, B. B., Makhlouf, G. M., and Schultz, S. D., eds) pp. 475–543, American Physiological Society, Bethesda, MD
3. Adrian, T. E., Savage, A. P., Sagor, G. R., Allen, J. M., Bacarese-Hamilton, A. J., Tatemoto, K., Polak, J. M., and Bloom, S. R. (1985) Effect of peptide YY on gastric, pancreatic, and biliary function in humans. *Gastroenterology* **89,** 494–499
4. Batterham, R. L., Cowley, M. A., Small, C. J., Herzog, H., Cohen, M. A., Dakin, C. L., Wren, A. M., Brynes, A. E., Low, M. J., Ghatei, M. A., Cone, R. D., and Bloom, S. R. (2002) Gut hormone PYY(3–36) physiologically inhibits food intake. *Nature* **418,** 650–654
5. Batterham, R. L., Cohen, M. A., Ellis, S. M., Le Roux, C. W., Withers, D. J., Frost, G. S., Ghatei, M. A., and Bloom, S. R. (2003) Inhibition of food intake in obese subjects by peptide YY3–36. *N. Engl. J. Med.* **349,** 941–948
6. Michel, M. C., Beck-Sickinger, A., Cox, H., Doods, H. N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., and Westfall T XVI. (1998) International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol. Rev.* **50,** 143–150
7. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402,** 656–660
8. Tomasetto, C., Karam, S. M., Ribieras, S., Masson, R., Lefebvre, O., Staub, A., Alexander, G., Chenard, M. P., and Rio, M. C. (2000) Identification and characterization of a novel gastric peptide hormone: the motilin-related peptide. *Gastroenterology* **119,** 395–405
9. Inui, A. (2001) Ghrelin: an orexigenic and somatotrophic signal from the stomach. *Nat. Rev. Neurosci.* **2,** 551–560
10. Asakawa, A., Inui, A., Kaga, T., Yuzuriha, H., Nagata, T., Ueno, N., Makino, S., Fujimiya, M., Niijima, A., Fujino, M. A. (2001) Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* **120,** 337–345
11. Gualillo, O., Caminos, J., Blanco, M., Garcia-Caballero, T., Kojima, M., Kangawa, K., Dieguez, C., and Casanueva, F. (2001) Ghrelin, a novel placental-derived hormone. *Endocrinology* **142,** 788–794
12. Neary, N. M., Small, C. J., Wren, A. M., Lee, J. L., Druce, M. R., Palmieri, C., Frost, G. S., Ghatei, M. A., Coombes, R. C., and Bloom, S. R. (2004) Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. *J. Clin. Endocrinol. Metab.* **89,** 2832–2836
13. Asakawa, A., Inui, A., Kaga, T., Katsuura, G., Fujimiya, M., Fujino, M. A., and Kasuga, M. (2003) Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. *Gut* **52,** 947–952
14. Ueno, N., Inui, A., Iwamoto, M., Kaga, T., Asakawa, A., Okita, M., Fujimiya, M., Nakajima, Y., Ohmoto, Y., Ohnaka, M., *et al.* (1999) Decreased food intake and body weight in pancreatic polypeptide-overexpressing mice. *Gastroenterology* **117,** 1427–1432
15. Botto, L. D., Moore, C. A., Khoury, M. J., and Erickson, J. D. (1999) Neural-tube defects. *N. Engl. J. Med.* **341,** 1509–1519
16. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108,** 193–200
17. Hogan, B., Baddington, R., Costantini, F., and Lacy, E. (1994) Manipulating the mouse embryo. *A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Sainsbury, A., Schwarzer, C., Couzens, M., Fetissov, S., Furtinger, S., Jenkins, A., Cox, H. M., Sperk, G., Hokfelt, T., and Herzog, H. (2002) Important role of hypothalamic Y2 receptors in body weight regulation revealed in conditional knockout mice. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 8938–8943
19. Harris, M. J., and Juriloff, D. M. (1999) Mini-review: toward understanding mechanisms of genetic neural tube defects in mice. *Teratology* **60,** 292–305
20. Rothman, K. J., Moore, L. L., Singer, M. R., Nguyen, U. S., Mannino, S., and Milunsky, A. (1995) Teratogenicity of high vitamin A intake. *N. Engl. J. Med.* **333,** 1369–1373
21. Fujimiya, M., Okumiya, K., Yamane, T., and Maeda, T. (1997) Distribution of serotonin-immunoreactive nerve cells and fibers in the rat gastrointestinal tract. *Histochem. Cell Biol.* **107,** 105–114
22. Fujimiya, M., Miyazaki, M., Fujimura, M., and Kimura, H. (1995) Effects of carbachol on the release of peptide YY from isolated vascularly and luminally perfused rat ileum. *Peptides* **16,** 939–944
23. Asakawa, A., Inui, A., Yuzuriha, H., Ueno, N., Katsuura, G., Fujimiya, M., Fujino, M. A., Niijima, A., Meguid, M. M. (2003) Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. *Gastroenterology* **124,** 1325–1336
24. Upchurch, B. H., Aponte, G. W., and Leiter, A. B. (1994) Expression of peptide YY in all islet cell types in the developing mouse pancreas suggests a common peptide YY-producing progenitor. *Development* **120,** 245–252
25. Leroux, P. (2002) Localization and characterization of NPY/PYY receptors in rat frontoparietal cortex during development. *J. Comp. Neurol.* **442,** 35–47
26. Jazin, E. E., Zhang, X., Soderstrom, S., Williams, R., Hokfelt, T., Ebendal, T., and Larhammar, D. (1993) Expression of peptide YY and mRNA for the NPY/PYY receptor of the Y1 subtype in dorsal root ganglia during rat embryogenesis. *Brain Res. Dev. Brain Res.* **76,** 105–113
27. Rindi, G., Necchi, V., Savio, A., Torsello, A., Zoli, M., Locatelli, V., Raimondo, F., Cocchi, D., and Solcia, E. (2002) Characterisation of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues. *Histochem. Cell Biol.* **117,** 511–519
28. Inui A. (2000) Transgenic approach to the study of body weight regulation. *Pharmacol. Rev.* **52,** 35–61
29. Kalra, S. P., Dube, M. G., Pu, S., Xu, B., Horvath, T. L., and Kalra, P. S. (1999) Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocrine Rev.* **20,** 68–100
30. Nakahara, K., Nakagawa, M., Baba, Y., Sato, M., Toshinai, K., Date, Y., Nakazato, M., Kojima, M., Miyazato, M., Kaiya, H., *et al.* (2006) Maternal ghrelin plays an important role in rat fetal development during pregnancy. *Endocrinology* **147,** 1333–1342
31. Zhao, Q., Behringer, R. R., and de Crombrugghe, B. (1996) Prenatal folic acid treatment suppresses acrania and meroanencephaly in mice mutant for the Cart1 homeobox gene. *Nat. Genet.* **13,** 275–283

Received for publication November 5, 2006.
Accepted for publication February 1, 2007.