

Immunolocalization of the PP family and its receptors in the gastrointestinal tract of house musk shrew, *Suncus murinus*

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Abstract

OBJECTIVES: To investigate the immunolocalization of the pancreatic polypeptide (PP)-fold peptide family, important regulatory factors for food intake, in the gastrointestinal tract of *Suncus murinus*, and to discuss the relation with the obesity-resistance, visceral fat accumulation-resistance phenomenon in *Suncus murinus*.

METHODS: The gastrointestinal tract of adult *Suncus murinus*, except for the stomach and pylorus, was divided into five sections (S1, corresponding to the duodenum, S2, S3 and S4, corresponding to the jejunum and ileum, and S5, corresponding to the colon and rectum in other mammals), to investigate the PP family and their receptor-producing cells by means of immunohistochemistry.

RESULTS: NPY, PYY, Y1 and Y4-immunoreactive cells were distributed widely throughout the gastrointestinal tract, moreover, the PP family and their receptor-immunoreactive cells were predominantly distributed at the end of the gastrointestinal tract, the rectum.

CONCLUSION: In this study, we investigated the distribution of the PP family and their receptor-producing cells in the gastrointestinal tract of *Suncus murinus* in detail for the first time. It was presumed that the wide distribution of Y4 in the gastrointestinal tract may be related to (associated with) the phenomenon of natural obesity-resistance, visceral fat accumulation-resistance in aging *Suncus murinus*.

INTRODUCTION

The musk shrew, *Suncus murinus* (*S. murinus*), is an insectivore that retains many primitive characteristics of the lowest order of eutherians. Therefore, it has been noted that the shrew is suitable as a new experimental animal as an insectivore

(Oda & Kondo 1977; Yi *et al.* 2003 and 2010). Consequently, some morphological and functional phenotypes similar to those of primates may be recognized, and some may closely reflect features of human beings. Furthermore, our studies and those of other groups have demonstrated that *S. murinus* has a visceral system that is very simi-

lar to that of humans, and thus it is a useful model of human physiology and pathophysiology of the human gastrointestinal tract (GI) (Yi *et al.* 2003, 2004, 2005, 2006, and 2007).

Interestingly, our recent studies revealed an obesity-resistance phenomenon in *S. murinus*, whose body weight does not change during ageing from 2 to 12 months of age, and less visceral fat accumulated. In particular, mesenteric fat accumulation does not occur in this shrew (Yi *et al.* 2010). Furthermore, we have investigated the distribution of ghrelin-producing cells in the stomach and the effect of ghrelin administration in *S. murinus* does not change the rate of food intake, but resulted in an increase in body weight compared with the control group, the results are different from for other experimental animals, e.g. rats and mice (Li *et al.* 2010).

The pancreatic polypeptide (PP) family together with neuropeptide Y (NPY), one of the most abundant neuropeptides in the central and peripheral nervous systems, and peptide YY (PYY), mainly present in the intestine, constitute the PP-fold peptide family. The family comprises homologues of 36 amino acid-peptides. Five cloned receptors (Y1, Y2, Y4, Y5, and Y6) of the PP-fold family have been identified. This family is involved in the regulation of several gut functions, including absorption/secretion of water and electrolytes, gastric and pancreatic secretion, and gut motility (PYY), furthermore, they were recently shown to be strongly involved in the regulation of appetite (Ferrier *et al.* 2002). PP has been shown to be involved in the regulation of food intake, acting as a circulating factor (Batterham *et al.* 2003; Stanley *et al.* 2004; Huda *et al.* 2006). NPY has most notably been characterized as a potent orexigenic agent, and exogenous NPY administration causes robust increases in food intake and body weight (Morton & Schwartz 2001; Williams *et al.* 2001). It has been suggested that NPY may act as an integrating factor between signals for energy balance and neuroendocrine reproductive function (Smith & Grove 2002).

Gastrointestinal endocrine cells that are dispersed in the epithelium and gastric glands of the digestive tract synthesize various kinds of gastrointestinal hormones, and play an important role in physiological functions such as the overall regulation of digestive processes such as nutrient absorption, the secretion by intestinal and associated glands, gut motility and intestinal blood flow (Bell 1979). Kanamori *et al.* (1989) and Kitamura *et al.* (1990) have performed immunohistochemical studies on the distribution of endocrine cells, which contain gastrin, cholecystokinin, somatostatin, secretin, serotonin, glucagon, gastric inhibitory polypeptide, motilin, neurotensin and bovine pancreatic polypeptide, in the GI of *S. murinus*. So far, the localization of the PP family in the GI in *S. murinus* has not been reported.

As Y5 receptors are rarely observed in peripheral tissues, being predominantly expressed in the central nervous system (Cox 2007a), in the present study, the

PP family and their receptor (Y1 Y2 and Y4)-producing cells in the GI of *S. murinus* were identified, and their frequencies and immunolocalization were compared with previous reports on the occurrence and distribution of such cells in other mammals, including man.

MATERIALS AND METHODS

Animals

Ten male laboratory house musk shrews, *Suncus murinus* (4-weeks-old, weighing 49.86 ± 2.09 g), were obtained from a closed breeding colony in our laboratories. The mother colony, JIC: KAT-c, is maintained in the Central Institute for Experimental Animals, Nagoya, Japan. The animals were housed and handled in accordance with the *Guide for the Care and Use of Laboratory Animals* and the *Guide for the Care and Use of Experimental Animals* of the Canadian Council on Animal Care. Briefly, all shrews were kept individually after weaning (20 d after birth) in plastic cages equipped with a wooden nestbox containing paper strips in a conventionally conditioned animal room: $28 \pm 2^\circ\text{C}$, no humidity control, and 12: 12h light/dark cycle, lights on at 08:00, commercial feed pellets for trout (a specific and natural food of *S. murinus*, insectivores) (Oriental Yeast Co., Ltd., Bioindustry Division, Chiba, Japan) and water being supplied *ad libitum* (Yi *et al.* 2003, 2005 and 2007).

Tissue preparation

After animals had been completely narcotized by an intraperitoneal injection of a urethane solution (sodium ethylcarbamate, 900 mg/kg), the thoracic cavity was opened. Perfusion was performed with 4% paraformaldehyde (PFA) in 0.01 M PBS (pH 7.4) through the left cardiac ventricle, with the right cardiac atrium opened. Then the GI was further fixed with 4% PFA in 0.01 M PBS (pH 7.4) at 4°C overnight and subsequently dissected out. The stomach was separated from the esophagus and duodenum, and then either the stomach was opened along the greater curvature, emptied and completely bathed in fixative, or tissue samples were taken immediately after opening. The whole intestine was slit open longitudinally along the mesenteric side, pinned to a wooden board, and then immersed in fixative overnight. *S. murinus* has a unique GI lacking a cecum with a relatively short intestine (about 20 cm long), it is difficult to distinguish each intestinal segment, and there is no clear boundary between the small and large intestines (Kurohmaru *et al.* 1980; Kanamori *et al.* 1989; Kitamura *et al.* 1990). Therefore, after fixation, the whole intestinal tract was divided uniformly into five sections (i.e. it was cut into five equal portions) from the duodenum to the rectum (S1, corresponding to the duodenum, S2, S3 and S4, corresponding to the jejunum and ileum, and S5, corresponding to the colon and rectum in other mammals) (Figure 1). Then the specimens were dehydrated in a graded ethanol series

and embedded in paraffin. 5 µm-thick serial sections were cut from each paraffin block. The stomach was sectioned in a plane that passed through the greater and lesser curvatures. Representative sections of each tissue were stained with haematoxylin and eosin (HE) and subjected to immunohistochemistry for light microscopic examination.

Immunohistochemical procedures

Sets of seven consecutive sections were stained with HE, and immunostained using antisera to NPY, PYY, PP, Y1, Y2, and Y4. The immunohistochemical procedures were performed according to our previous papers (Yi *et al.* 2004; Li *et al.* 2010). Briefly, after rinsing the fixed tissue specimens in 0.01 M PBS (pH 7.4), endogenous peroxidase activity was inhibited by 30-min incubation in methanol containing 0.3% (v/v) hydrogen peroxide. After rinsing in PBS, the sections were blocked with Protein Block Serum-Free (ready-to-use, X0909, DAKO, Denmark) for 1 h at room temperature

(RT), and then incubated with the primary antibodies overnight at 4 °C in a humidified chamber, followed by the secondary antibodies for 1 h at RT. Subsequently, the avidin-biotin-complex technique (ABCComplex/HRP, K 0355, DAKO, Denmark) was performed by incubating the sections with the ABC complexes for 1 h at RT, and then they were treated for 1 min with 3-3'-diaminobenzidine and 0.005% H₂O₂, as chromogens. The sections were counterstained with Harris' hematoxylin for 50 s, dehydrated in a graded ethanol series and xylene, and then coverslipped with Entellanu (Merck, Germany).

The primary antibodies are summarized in Table 1. The secondary antibodies were biotin-conjugated goat anti-rabbit IgG (DAKO) and rabbit anti-goat IgG (Santa Cruz Biotechnology), respectively.

Furthermore, the control experiments involved the following: (1) omission of the primary antiserum; (2) substitution of the primary antibody with 0.05 M Tris-BSA buffer; and (3) an adsorption control: substitution

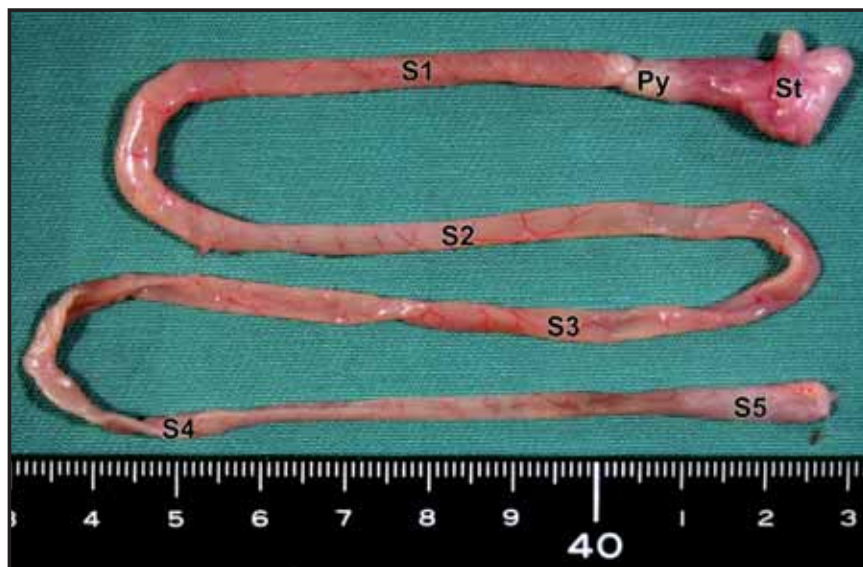


Fig. 1. Photograph of the gastrointestinal tract of *S. murinus*, which was divided uniformly into five segments (S1, corresponding to the duodenum, S2, S3, and S4, corresponding to the jejunum and ileum, and S5, corresponding to the colon and rectum in other mammals). The stomach (St) and pylorus (Py) were ignored.

Tab. 1. The first and secondary antibodies used in this study.

Antigen	Host	Dilution	Source & Code No.
human NPY	rabbit polyclonal	1:100	Santa Cruz Bio., sc-28943
human PYY	rabbit polyclonal	1:100	Progen Bio., 11431
human PP	rabbit polyclonal	1:50	Zymed Lab., 40380313
human NPY1-R	goat polyclonal	1:100	Santa Cruz Bio., sc-21992
human NPY2-R	rabbit polyclonal	1:100	Santa Cruz Bio., sc-28950
human NPY4-R	goat polyclonal	1:50	Santa Cruz Bio., sc-23842
rabbit anti-goat IgG		1:200	Santa Cruz Bio., sc-2774
goat anti-rabbit IgG		1:100	DAKO, Z 0421

of the primary antibody with antiserum pre-adsorbed with the various hormones at a concentration 10^{-6} M. These experiments were carried out on sections at the same time as the treatment with the primary antibody.

Enumeration of immunoreactive cells and statistical analysis

The numbers of immunoreactive epithelial cells with clearly recognizable nuclei were determined in randomly selected sections from every tissue block of each animal, after staining with each antiserum. The distribution and relative frequency of immunoreactive cells are summarized in Table 2 and Figure 4. The immunoreactive cells were graded subjectively into five groups according to their relative frequencies as confirmed under a light microscope, (-), absent; (+/-), rare and not found in every section; (+), few; (++) , moderate in number; and (+++) , numerous, respectively.

RESULTS

The following terminology has been used throughout: NPY, PYY, PP, Y1, Y2, and Y4-producing/immunoreactive cells are called NPY, PYY, PP, Y1, Y2, and Y4 cells, respectively.

Immunolabelling did not occur in all control experiments. Pre-adsorption of antisera with the hormones completely abolished any staining.

Morphology of the gastric mucosa and intestine

Our findings are consistent with what was found in another recent study on the morphology and histology of the stomach of *S. murinus*. Morphologically, the stomach of *S. murinus* has a single cavity, i.e., there is no forestomach, and consists of cardia, fundus, greater curvature, lesser curvature, and pylorus regions. The general appearance of the gastric mucosa of *S. murinus* is similar to the human gastric mucosa, and differs from those of some other widely used experimental animals such as hamsters, rats, and mice (Kanamori *et al.* 1989; Li *et al.* 2010).

Typical villous structures were recognized in S1–S4, but these villous structures tended to gradually disappear towards the anal region. Brunner’s glands were observed in small regions near the oral end of S1. As judged from this lack of villi, much of the end of S5, approximately 1.5 cm length to the anus, most likely corresponds to the large intestine and rectum of other animals (Figure 1).

Distribution of immunoreactive cells

NPY cells were detected throughout the GI and they showed the highest frequency in the rectum. In the pylorus, NPY cells were mainly distributed in the submucosa (Figure 2A), while in the intestinal tract, NPY was distributed in the neurons in both the submucous and myenteric plexuses (Figures 2B and C), and also detected in the rectal epithelium.

PYY cells, although few in number, were randomly scattered, and varied in number throughout the GI, but were most abundant in the rectum. PYY cells were detected in the cryptic areas of the intestinal villi and rectal epithelium (Figures 2D and E).

PP cells were restricted to the end of the intestine, i.e., the rectum, and mainly distributed and located close to the basolateral domain, i.e., cryptic areas, of the rectal epithelium, often being observed in the vicinity of PYY cells (Figure 2F).

Although few in number, Y1 cells were distributed randomly in varicosities located close to the basolateral domain of the crypt epithelia in all parts of the GI. However, in the rectum, Y1 cells were the most abundant in both the submucous and myenteric neurons (Figure 3A).

Y2 cells were only detected in the rectum, mainly being found in both the submucous and myenteric neurons (Figure 3B).

Y4 cells were abundantly detected throughout the GI. In the stomach, numerous Y4 cells were distributed in the epithelium, moreover, most of the Y4 cells were detected among the pyloric glands cells (Figures 3D and G). In the intestinal tract portion, Y4 cells increased gradually from being few in number in the

Tab. 2. Distribution and relative frequencies of the PP family and their receptor-producing cells in the gastrointestinal tract of *S. murinus*.

	St	Py	S1	S2	S3	S4	S5
NPY	±	+	+	+	+	+	+++
PYY	±	+	+	+	+	+	++ ~ +++
PP	-	-	-	-	-	-	+ ~ ++
Y1	±	+	+	+	±	±	+++
Y2	-	-	-	-	-	-	++
Y4	++	+++	+	+	+ ~ ++	++ ~ +++	+++

-, not detected; ±, rare and not detected in every animal; +, few but detected in every animal; ++, moderate; +++, numerous. NPY, neuropeptide Y; PP, pancreatic polypeptide; PYY, peptide YY; St, stomach; Py, pylorus; Y1, Y2 and Y4, receptors of the PP family; S1~S5, the intestinal tract from the duodenum to the rectum divided uniformly into five sections.

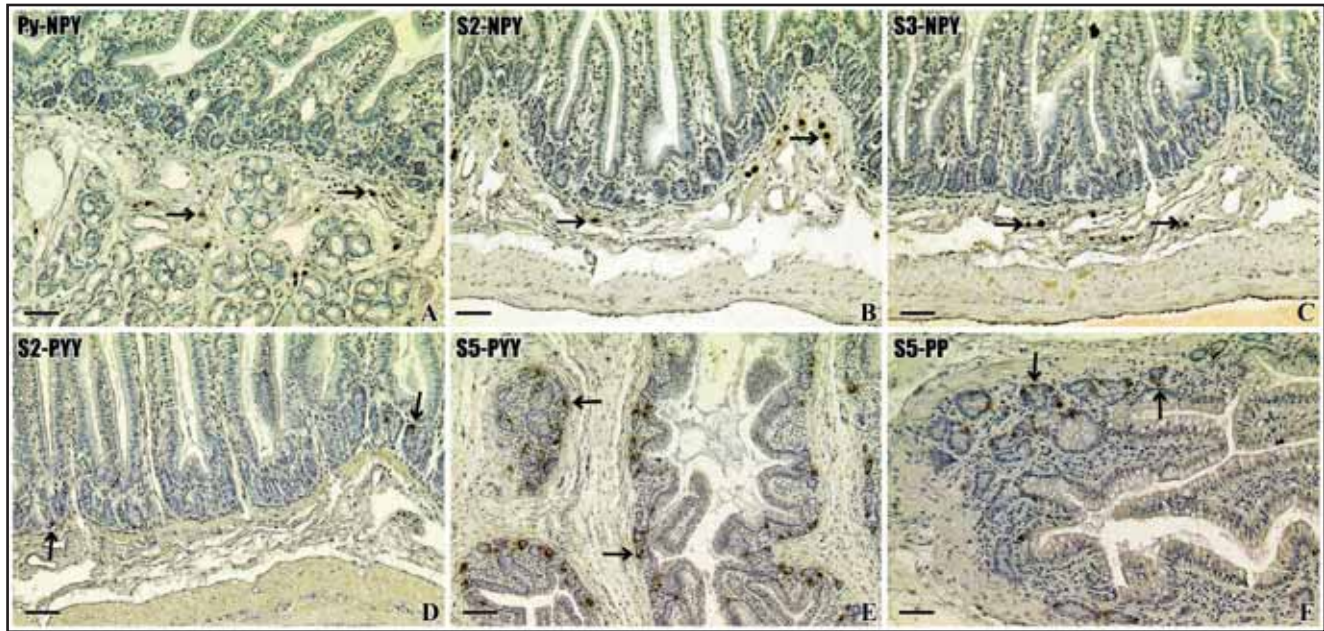


Fig. 2. Immunolocalization of NPY in the pylorus (Py) (A), S2 (B), and S3 (C); PYY in S2 (D) and S5 (E); PP in S5 (F). Arrows indicate immunoreactive cells. Scale bars: E = 100 μ m; others = 50 μ m.

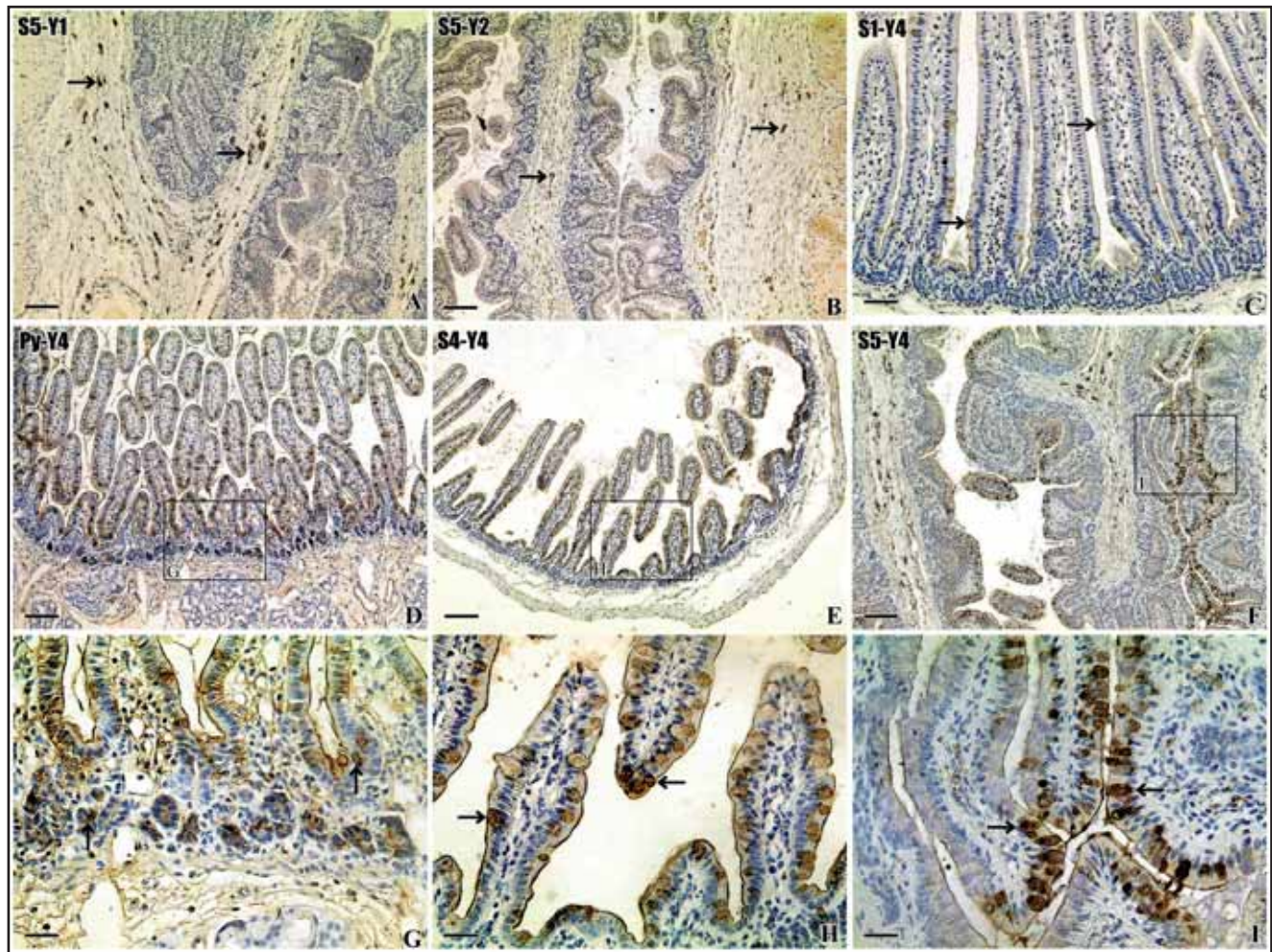


Fig. 3. Immunolocalization of Y1 in S5 (A); Y2 in S5 (B); Y4 in S1 (C), the pylorus (D), S4 (E), and S5 (F). (G), (H), and (I): higher magnifications of the areas indicated in (D), (E), and (F), respectively. Arrows indicate immunoreactive cells. Scale bars: A, B, D, E, and F = 100 μ m, C = 50 μ m, G, H, and I = 25 μ m.

duodenum, moderate in number in the distal intestinal tract and numerous in the rectum (Table 2). Y4 cells were apparently co-localized with goblet cells and on the basal lamina of intestinal villi throughout the whole GI (Figures 3C, E, and H). In the rectum, Y4 cells were also detected either in the rectal epithelium, or both the submucous and myenteric neurons (Figures 3F and I).

In summary, the topographical distribution and relative frequency of PP family cells in the GI are summarized in Table 2 and Figure 4. The results demonstrated that the total number of Y4 cells was much larger than those of other cells, while the PP cells were not detected in other intestinal regions except for the rectum. Without exception, for all of the PP family members, the largest numbers were observed in the rectum.

DISCUSSION

In the present study, the immunolocalization of the PP family and their receptors in the GI of *S. murinus* was focused on for the first time. The results showed NPY, PYY, Y1, and Y4 cells were distributed widely throughout the GI, moreover, the PP family and their receptors were predominantly distributed at the end of the GI, the rectum. In general mammals, PYY cells are almost completely absent in the stomach, relatively few in number in the duodenum and jejunum, dramatically increased in number in the ileum and colon, and at very high levels in the rectum (El-Salhy *et al.* 1983a and b; Ali-Rachedi *et al.* 1984; Agungpriyono *et al.* 1994; Baltazar *et al.* 1998). The total population of endocrine cells in the lower gut has been reported to be higher distally (rectum) in other species as well, including man (Cristina *et al.* 1978; Kitamura *et al.* 1985; Calingasan *et al.* 1984). The importance of the colon and rectum as

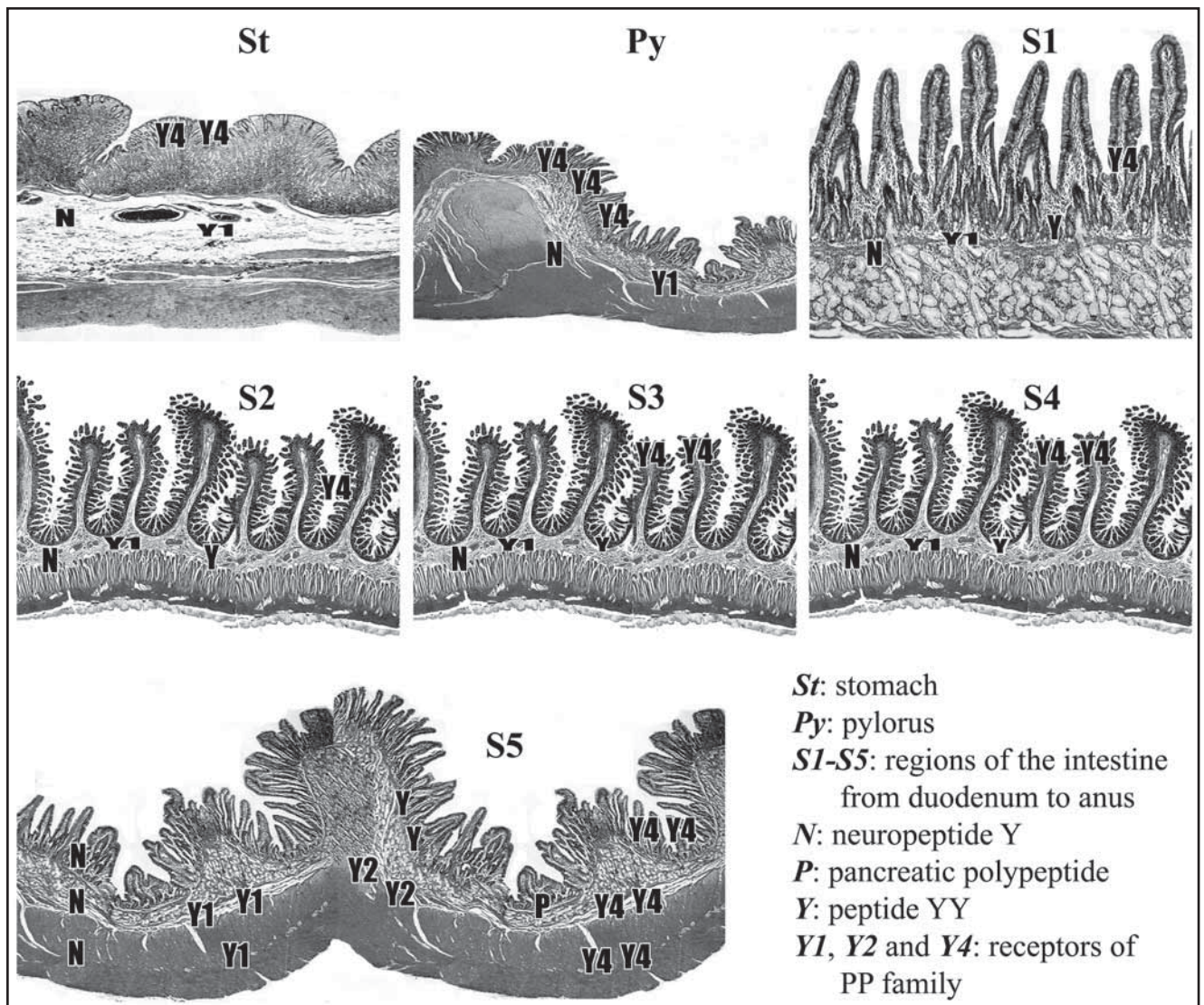


Fig. 4. Schema summarizing the topographical distribution and relative frequencies of the PP family and their receptor –producing cells in the GI.

endocrine organs has been considered (Lluis & Thompson 1988). The lower gut, especially the rectum, as an endocrine organ in *S. murinus* may also be important from the aspect of the number and variety of the PP family and their receptor, -producing cells. It is possible that they may be involved in feed-back control of secretory and motile functions.

PYY has been reported to be present in the myenteric plexus and endocrine pancreas of many species, but not in man (Ekblad & Sundler 2002). These reports are consistent with the present immunohistochemical results. In *S. murinus*, PYY cells were observed in all portions of the intestine, but were few in number in the stomach. In addition, PYY cells were detected in the cryptic areas of the intestinal villi and rectal epithelium in *S. murinus*. In contrast to PYY, NPY was also widely distributed throughout the GI except for the stomach, and distributed mainly in the neurons of the submucous plexus of the small intestine, and of both the submucous and myenteric plexuses of the rectum. Moreover, the overall quantity of PYY cells was far less than that of NPY cells in the GI.

PP is primarily expressed in the endocrine cells of the pancreas, particularly those in the duodenal portion, which formed ~10% of endocrine cell population, and PP cells are also found elsewhere in small numbers, i.e., in the stomachs of adult opossum, cat and dog (Larsson *et al.* 1976; Sundler *et al.* 1984; Ekblad & Sundler 2002). Low numbers of PP cells have been reported to occur in the human colon and rectum (Cristina *et al.* 1978; Sjölund *et al.* 1983). In *Suncus*, the PP cells are distributed predominantly in the right lobe of the pancreas, which corresponds to the ventral pancreas embryologically, occupying approximately 57.4% of the islets (Yi *et al.* 2003 and 2004). PP cells are also present in the GI, being restricted to the rectum, and located close to the basolateral domain, i.e., cryptic areas, of the rectal epithelium. These PP cells in the rectum were distinct from PYY cells, which is consistent with findings in other animals (El-Salhy *et al.* 1983b).

Generally, Y2 receptors mediate NPY and PYY absorptive effects indirectly via enteric submucous neurons in the human and mouse colon. Y1 receptors mediate absorption predominantly (but not exclusively) via epithelial mechanisms in the mouse descending colon. Y4 and Y1 receptors mediate epithelial actions, while neuronal Y2 and Y1 receptors mediate absorptive effects in the isolated human colon (Cox 2007b). PYY binds with high affinity to all Y receptors, whereas PP binds with greatest affinity to Y4 receptors, which are also called PP1 receptors (Ferrier *et al.* 2002; Stanley *et al.* 2004).

Few immunohistochemical studies have been performed to date, but RT-PCR and Northern blot analyses have consistently revealed Y4 mRNA in the rat proximal colon (Feletou *et al.* 1998; Ferrier *et al.* 2000 and 2002), and distal colon (Ferrier *et al.* 2002), and within both muscle layers of the rat jejunum (Goumain

et al. 1998); though not in the human ileum, colon or rectum smooth muscle (Ferrier *et al.* 2002). Y4 receptor mRNA is present (as evidenced by RT-PCR) in epithelia along the small intestine crypt-villus axis (in the rat), the apparently highest level of expression being in the colonic epithelium (Goumain *et al.* 1998). Furthermore, Campbell *et al.* (2003) reported Y4 receptor immunoreactivity apparently localized in goblet cells and on the basal lamina of intestinal villi in the rat. In the present study, it was shown that Y4 cells were apparently co-localized with goblet cells and on the basal lamina of intestinal villi throughout the whole GI in *S. murinus*. In addition, in the rectum, Y4 cells were also detected in either the rectal epithelium or both the submucous and myenteric neurons in *S. murinus*.

PP is produced in response to food intake, but the time-course is different to that reported for PYY (Ekblad & Sundler 2002; Stanley *et al.* 2004; Huda *et al.* 2006). PYY is not co-localized with PP in these intestinal endocrine cells (El-Salhy *et al.* 1983a). In our recent study (Yi *et al.* 2010), we found the phenomenon (characteristic) of natural obesity-resistance, visceral fat accumulation-resistance in *S. murinus*. It could be presumed that the wide GI distribution of Y4 is related to the phenomenon of natural obesity-resistance, visceral fat accumulation-resistance in aging *S. murinus*. Further physiological studies are required to elucidate the ways in which the distribution of PP family and their receptors, especially Y4 cells, may be involved in the regulatory characteristics of gut motility, absorption of lipid, and energy metabolism in this animal.

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