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Distribution of P2Y₆ and P2Y₁₂ receptor: their colocalization with calbindin, calretinin and nitric oxide synthase in the guinea pig enteric nervous system

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Abstract The distribution of P2Y₆ and P2Y₁₂ receptor-immunoreactive (ir) neurons and fibers and their coexistence with calbindin, calretinin and nitric oxide synthase (NOS) has been investigated with single and double labeling immunostaining methods. The results showed that 30–36% of the ganglion cells in the myenteric plexus are strongly P2Y₆ receptor-ir neurons; they are distributed widely in the myenteric plexus of stomach, jejunum, ileum and colon, but not in the submucosal plexus, with a typical morphology of multipolar neurons with a long axon-like process. About 42–46% of ganglion cells in both the myenteric and submucosal plexuses show P2Y₁₂ receptor-ir. About 28–35% of P2Y₆ receptor-ir neurons were found to coexist with NOS and 41–47% of them coexist with calretinin, but there was no coexistence of P2Y₆ receptor-ir with calbindin. In contrast, all P2Y₁₂ receptor-ir neurons were immunopositive for calbindin, although occasionally P2Y₁₂ receptor-ir neurons without calbindin immunoreactivity were found, while none of the P2Y₁₂ receptor-ir neurons were found to coexist with calretinin or NOS in the gastrointestinal system of guinea pig. The P2Y₁₂ receptor-ir neurons coexpressing calbindin-ir in the small intestine are Dogiel type II/AH, intrinsic primary afferent neurons.

Keywords P2Y₆ receptor · P2Y₁₂ receptor · Immunocytochemistry · Enteric nervous system · Guinea pig

Introduction

The P2 family includes P2X receptors equivalent to intrinsic calcium-permeable cation channels and metabotropic P2Y receptors that are G-protein-coupled receptors (Ralevic and Burnstock 1998). Currently there are seven cloned P2X (P2X₁ to P2X₇) receptor subunits (Lazarowski et al. 1997; Khakh et al. 2001) and at least eight P2Y receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄) (Burnstock 2004). Adenosine 5'-triphosphate (ATP) and uridine 5'-triphosphate (UTP) may provide complex regulation in the enteric nervous system (ENS) via activation of P2 receptors at pre- or postsynaptic or postjunctional sites in the gut (Burnstock 2001a). The distribution patterns of P2 receptor-immunoreactivity (-ir) and colocalizations with other neurotransmitters or neuropeptides in the ENS would help researchers to further understand their physiological functions. The distribution of P2X receptor-ir and colocalization between P2X receptor-ir and other neurotransmitters/neuropeptides were reported intensively during recent years (Hu et al. 2001; Castelucci et al. 2002; Poole et al. 2002; Van Nassauw et al. 2002; Xiang and Burnstock 2004a, b). However, in comparison with P2X receptors, descriptions of the distribution and colocalization of P2Y receptors with other neurotransmitters/neuropeptides are few. The proteins of P2Y₁, P2Y₂ and P2Y₄ receptors in the submucosal and myenteric plexuses of rat distal colon and the gene products of P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₂ receptors in the submucous plexus were reported (Giaroni et al. 2002; Christofi et al. 2004; Cooke et al. 2004). There are no systematic studies on the distribution of P2Y₆ and P2Y₁₂ receptors in the gut. In this study we used polyclonal antibodies to P2Y₆ and P2Y₁₂ receptors to study their distributions of colocalizations with calbindin, calretinin

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and nitric oxide synthase (NOS) in the guinea pig gastrointestinal tract.

Materials and methods

Animals and tissue preparation

Breeding, maintenance and killing of the animals used in this study followed principles of good laboratory animal care and experimentation in compliance with Home Office (UK) regulations covering Schedule One Procedures and in accordance with the Animals (Scientific Procedures) Act, 1986, governing the use of animals. Protocols were approved by the local animal ethics committee. Five guinea pigs were used in this study. Animals were killed by asphyxiation with CO₂ and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4. Stomach (gastric corpus), jejunum, ileum, proximal and distal colon were dissected out. Immediately after the segments of the digestive tract were removed, the contents of the lumen were removed with saline. One end of the segment was knotted with a silk thread and a fixative was injected into the lumen to fill it and the open end was also knotted with a silk thread. The fixative-filled segment was then immersed in a fixative and refixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer pH 7.4 overnight. This was applied to all the segments of the intestine examined, including the stomach, from which the contents was removed via the duodenum which was then closed by a knotted silk thread and filled with a fixative from the oesophagus. The oesophagus was then closed such that the stomach resembled an irregular balloon. Once fixed, whole-mount preparations could be prepared from the flattened area of the stomach. The myenteric plexuses of whole-mount preparations of the stomach, jejunum, ileum, proximal and distal colon, and the submucosal plexuses of whole-mount preparations of jejunum, ileum, proximal and distal colon were prepared under a dissection microscope.

Immunocytochemistry

The immunocytochemical method was modified from our previous report (Xiang et al. 1998). Briefly the preparations were washed 3×5 min in 0.01 mol/l pH 7.2 phosphate-buffered saline (PBS), then incubated in 1.0% H₂O₂ for 30 min to block endogenous peroxidase. Preparations were pre-incubated in 10% normal horse serum (NHS), 0.2% Triton X-100 in PBS for 30 min, followed by an incubation with P2Y₆ or P2Y₁₂ receptor antibodies (Alomone Laboratories, Jerusalem, Israel), diluted 1:300 in an antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS) overnight at 4°C. Subsequently, preparations were incubated with biotinylated donkey-anti-rabbit IgG (Jackson ImmunoResearch, PA, USA) diluted 1:500 in an antibody dilution

solution for 1 h at 37°C and then with streptavidin-HRP (Sigma Chemical Co., Poole, UK) diluted 1:1000 in PBS for 1 h at 37°C. Finally, a nickel-intensified diaminobenzidine (DAB) reaction was used to visualize immunoreactivity. All incubations and reactions were separated by 3×10-min washes in PBS. The preparations were mounted, dehydrated, cleared and covered.

The following protocol was used for double-immunostaining of P2Y₆ and P2Y₁₂ receptors with calbindin, calretinin, NOS and PGP9.5 (PGP9.5 was used as a general neuronal marker). The preparations were washed 3×5 min in PBS, then pre-incubated in an antibody dilution solution for 30 min, followed by an incubation with P2Y₆ and P2Y₁₂ receptor antibodies diluted 1:300 and NOS antibody (sheep-anti-rat) diluted 1:1,000, or calbindin (mouse-anti-rat; SWANT, Bellinzona, Switzerland) diluted 1:5,000 or calretinin (mouse-anti-rat; SWANT) diluted 1:2,000 or PGP9.5 antibody (mouse-anti-rat) diluted 1:6,000 in an antibody dilution solution overnight at 4°C. Subsequently the preparations were incubated with Cy3 conjugated donkey-anti-rabbit IgG (Jackson) diluted 1:300 for P2Y₆, P2Y₁₂ antibodies and FITC conjugated donkey-anti-mouse or sheep IgG (Jackson) diluted 1:200 in an antibody dilution solution for calbindin, calretinin, NOS and PGP9.5 for 1 h at room temperature. All the incubations and reaction were separated by 3×10-min washes in PBS. The preparations were evaluated with fluorescence microscopy.

Controls

Control experiments were carried out with P2Y₆ and P2Y₁₂ receptor antibodies pre-absorbed with cognate peptide at a concentration of 25 µg/ml.

Photomicroscopy

Images of immunofluorescence labeling were taken with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). Filter sets included the following: for Cy3, excitation, 510–550 nm, emission, 590 nm for FITC, 470 nm excitation, 525 nm emission. Images were imported into a graphics package (Adobe Photoshop 5.0, San Jose, USA). The two-channel readings for green and red fluorescence were merged by using Adobe-Photoshop 5.0.

Quantitative analysis

Whole-mount preparations were also used to perform a quantitative analysis, as described previously (Van Nassauw et al. 2002). Briefly, the immunoreactive-positive neurons in the myenteric ganglia were counted per visual field (0.3 mm²) in whole-mount preparations. Ten randomly chosen fields in each whole mount preparation were studied, and the number of immunoreactive neurons was calculated.

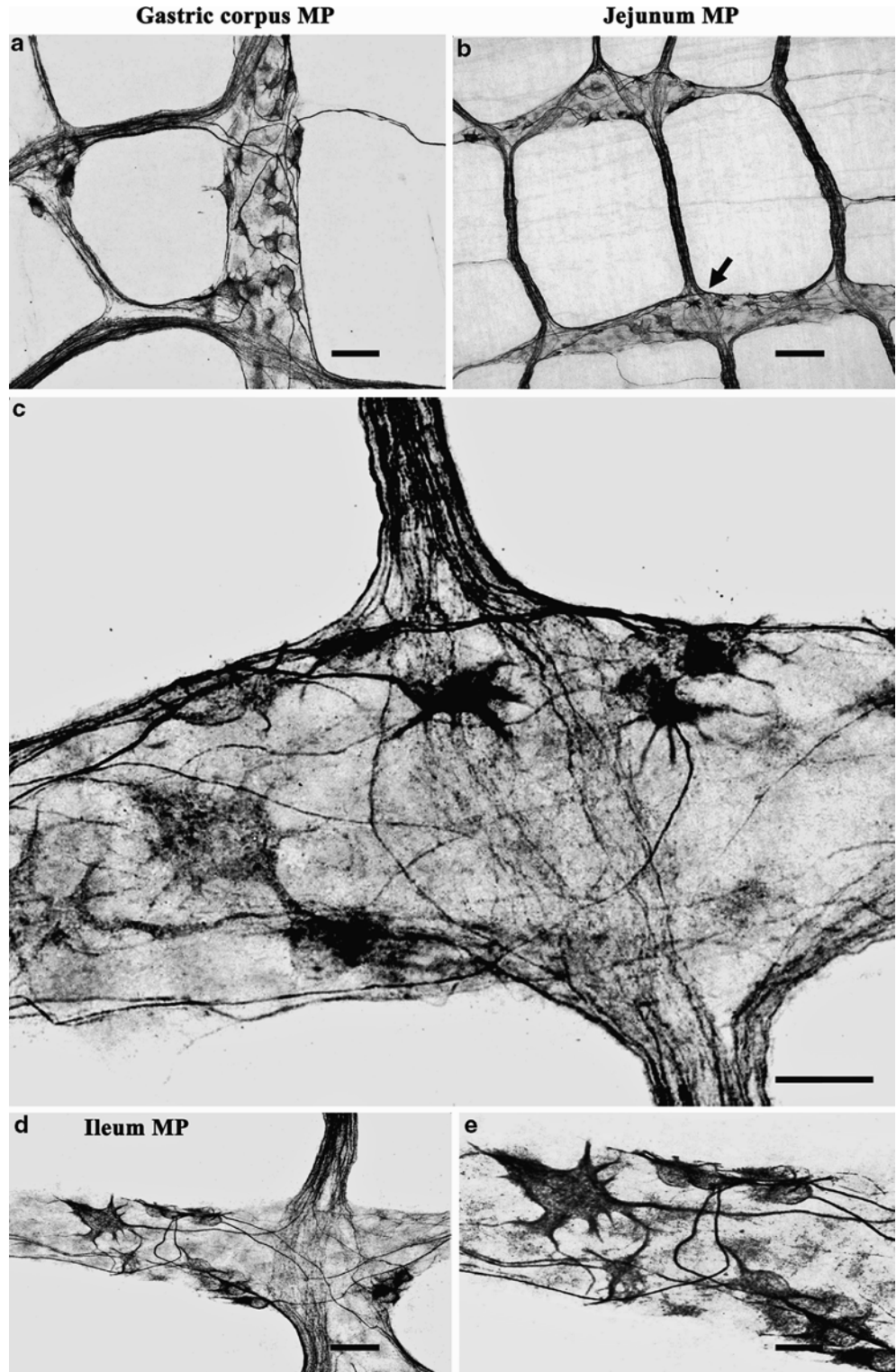
Results

Localization of P2Y₆ receptor immunoreactivity (-ir)

P2Y₆ receptor-ir was found in the myenteric plexus of gastric corpus, jejunum, ileum, proximal and distal co-

lon. Most of the P2Y₆ receptor-ir positive cells were shown to have a long axon-like process with strong positive staining. The axon-like processes projected out of the ganglia to join the nerve fiber bundles that connected the neighboring ganglia. These fiber bundles were strongly immunostained by the P2Y₆ receptor antibody.

Fig. 1 P2Y₆ receptor-ir (black) in stomach and small intestine of guinea pig. Note that most of the P2Y₆ receptor-ir positive cells have a long axon-like process with strong positive staining. The axon-like processes projected from the ganglia to join nerve fiber bundles that connected the neighboring ganglia. These fiber bundles were strongly immunostained by the P2Y₆ receptor antibody. **a** P2Y₆ receptor-ir in the myenteric plexus (MP) of gastric corpus. **b** P2Y₆ receptor-ir in the MP of jejunum. **c** High magnification of the area indicated by an arrow in **b**. **d** P2Y₆ receptor-ir in the MP of ileum. **e** High magnification of **d**. Note that both strong and weakly stained neurons are present. Scale bar = 80 μm in **a**, **b**; 25 μm in **c** and 35 μm in **d**, **e**



Most of the positive cells were multipolar with several short dendrites and a long axon. Immunostaining was observed in the cytoplasm of the cell body, but not the nucleus (Figs. 1, 2). In the submucous plexus no P2Y₆ receptor-ir neurons were demonstrated, although a few P2Y₆ receptor-ir fibers were present (Fig. 3e, f).

In the gastric corpus myenteric plexus, about 32% of ganglion cells were immunostained with the P2Y₆ receptor antiserum and most of the positive ganglion neurons were moderately stained (Fig. 1a). In the small intestine myenteric plexus of whole-mount preparations, P2Y₆ receptor-ir neurons were found in all ganglia and 34–36% of ganglion cells were positively immunostained for the P2Y₆ receptor in the jejunum and ileum, respectively. Both strong and weak stained ir-positive ganglion neurons were present (Fig. 1b, c, d, e). In the

myenteric plexus of proximal and distal colon, about 30–35% of ganglion neurons were positively stained by the P2Y₆ receptor antiserum. Positive ganglion neurons that were stained strongly or weakly were also present in the myenteric plexus of colon (Fig. 2a, b, c). Table 1 summarizes the quantitative analysis of P2Y₆ receptor-ir neurons in the myenteric plexus of stomach, jejunum, ileum, proximal and distal colon.

Colocalization of P2Y₆ receptor-ir with Calbindin, Calretinin and NOS

P2Y₆ receptor-ir was found to coexist with calretinin and NOS in the myenteric plexuses of gastric corpus, small intestine and distal colon (Figs. 3, 4a, b, c, d).

Fig. 2 P2Y₆ receptor-ir (black) in the guinea pig colon. Note that the morphology and distribution pattern of P2Y₆ receptor-ir neurons in the myenteric plexus of proximal and distal colon were similar with those in the stomach and small intestine. **a** P2Y₆ receptor-ir in the myenteric plexus (MP) of proximal colon. **b** P2Y₆ receptor-ir in the MP of distal colon. **c** Higher magnification of the area indicated by an arrow in b. Scale bar = 50 μm in a, 8 μm in b and 25 μm in c

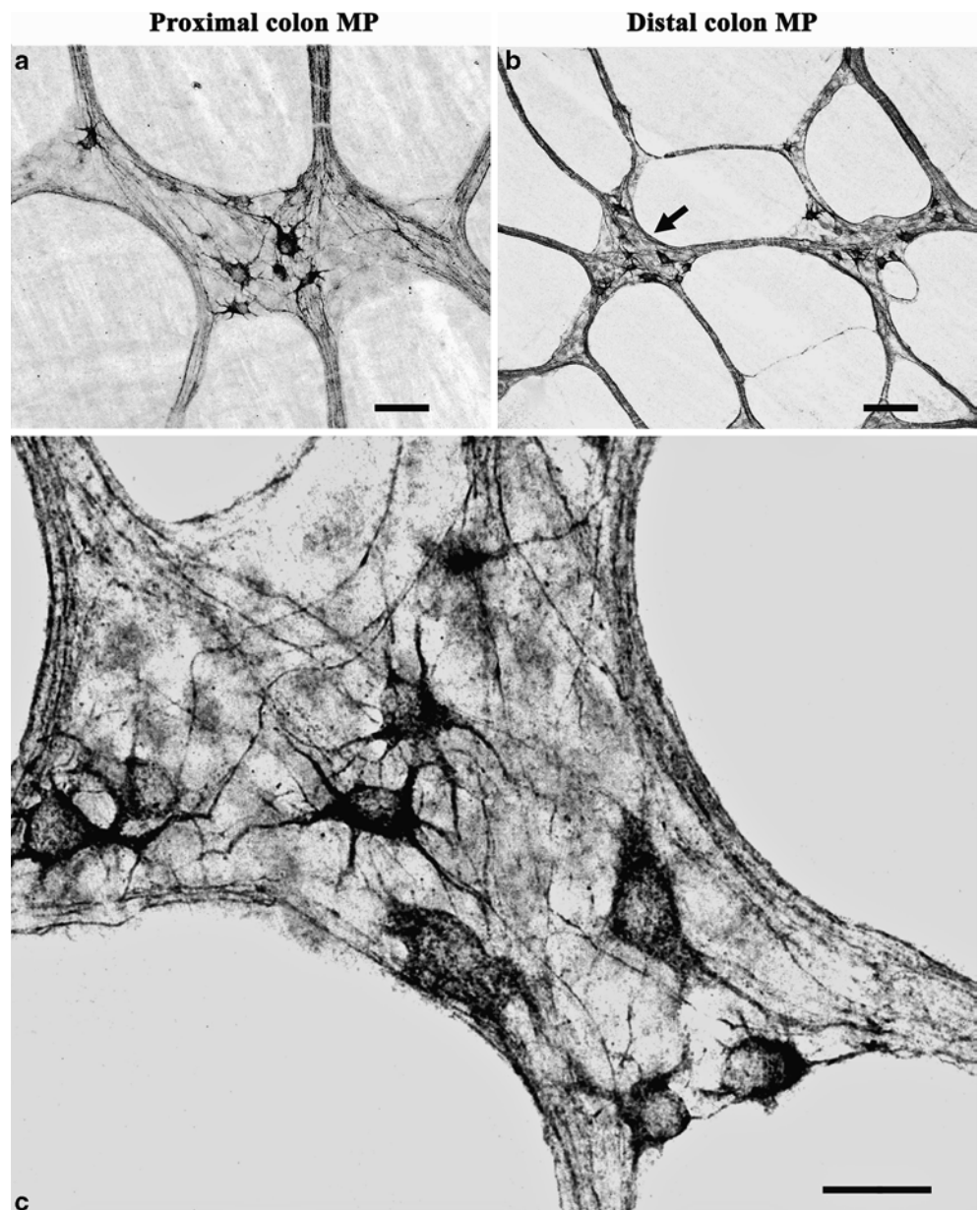
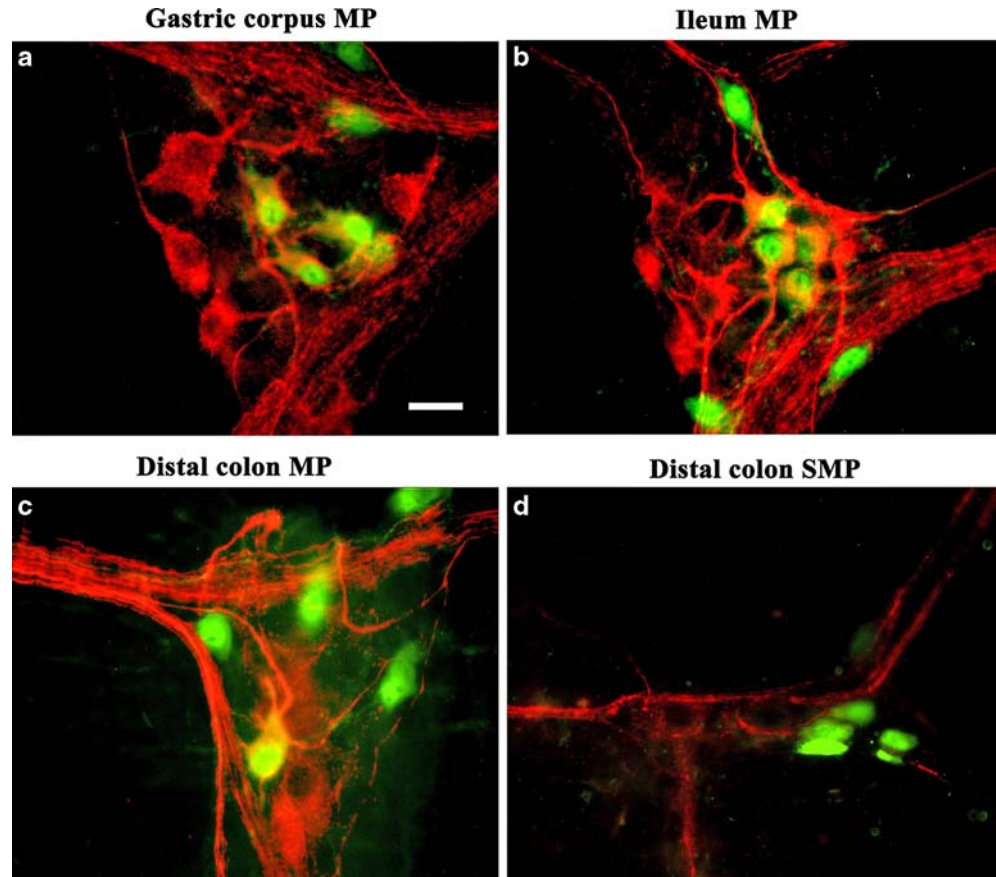


Fig. 3 P2Y₆ receptor-ir (red) coexists (yellow/orange) with calretinin (green) in a subpopulation of neurons in the guinea pig intestine. **a** In the myenteric plexus (MP) of gastric corpus. **b** In the MP of ileum. **c** In the MP of distal colon. **d** In the submucous plexus (SMP) of distal colon. No P2Y₆ receptor-ir neurons were found in the submucosal plexus along the whole length of the intestine. Scale bar in all figures = 50 μm



However, no P2Y₆ receptor-ir ganglion cells were found to be immunoreactive for calbindin (Fig. 4e, f, g).

The percentages of P2Y₆ receptor-ir neurons that were also immunoreactive for calretinin in the myenteric plexuses of gastric corpus, jejunum, ileum, proximal and distal colon were 45, 47, 44, 44 and 41%, respectively (Fig. 3, Table 2). The percentages of P2Y₆ receptor-ir neurons that were also immunoreactive for NOS in the myenteric plexuses of gastric corpus, jejunum, ileum, proximal and distal colon were 28, 29, 32, 30 and 35%, respectively (Fig. 4a, b, c, d, Table 3). In the submucosal plexus, no neurons double-labeled with P2Y₆ receptor-ir and calretinin or NOS were found as

Table 1 Number of P2Y₆ receptor-immunoreactive neurons in the myenteric plexus of guinea pig gastric corpus, jejunum, ileum, proximal and distal colon, expressed as the mean number of neurons ± SE mean, then expressed as a percentage (the mean number of P2Y₆ receptor-immunoreactive neurons was divided by the total number of PGP9.5-positive neurons in the same whole-mount preparations × 100%)

Regions	P2Y ₆ -ir	PGP9.5-ir	Percentage P2Y ₆ -ir
Stomach	176 ± 18	550 ± 23	32 ± 3
Jejunum	185 ± 21	544 ± 31	34 ± 4
Ileum	179 ± 23	497 ± 24	36 ± 5
Proximal colon	154 ± 15	513 ± 27	30 ± 3
Distal colon	178 ± 29	508 ± 34	35 ± 6

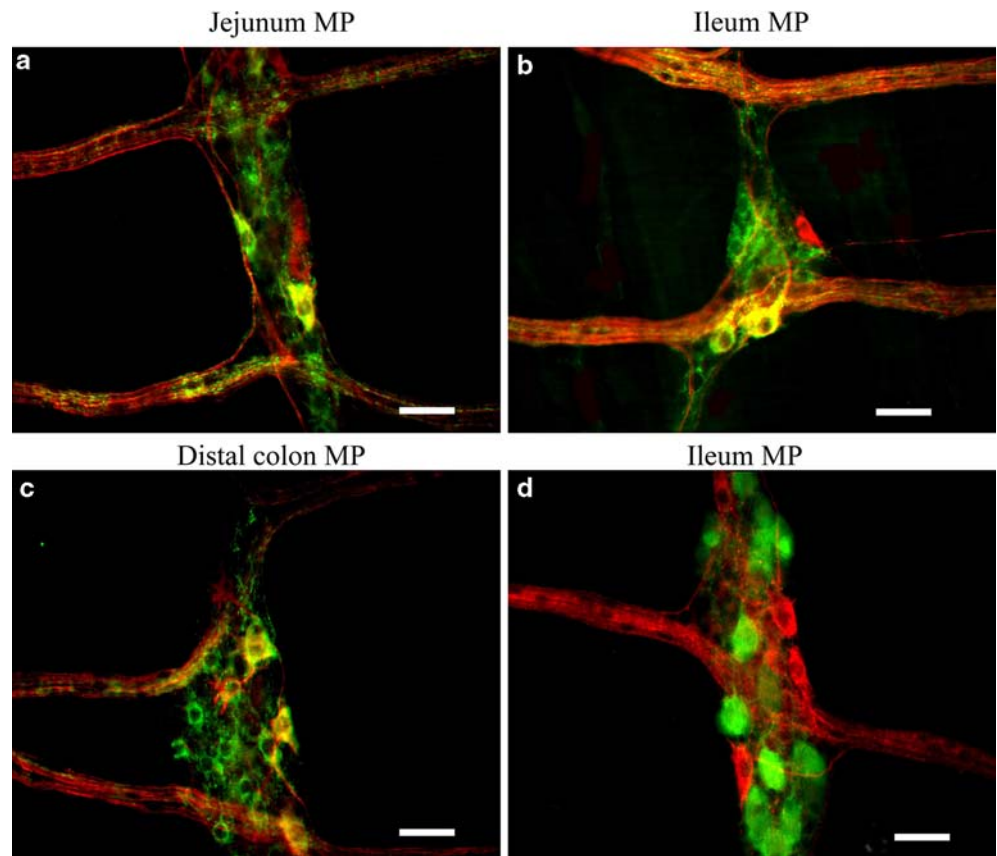
no P2Y₆ receptor-ir neurons were found, although a few nerve fibres appeared to show colocalization (Fig. 4g).

Localization of P2Y₁₂ receptor-immunoreactivity (-ir)

P2Y₁₂ receptor-ir was found in the myenteric plexuses of gastric corpus, jejunum, ileum, proximal and distal colon, and in submucosal plexuses of jejunum, ileum, proximal and distal colon. The P2Y₁₂ receptor-ir was localized in the cell bodies and most of the positive ganglion neurons were stained strongly. No positively-stained processes were demonstrated in either the myenteric or submucosal plexuses (Fig. 5a–f).

Table 4 shows the quantitative analysis of P2Y₁₂ receptor-ir neurons in both the myenteric and submucosal plexus. In the myenteric plexus of the gastric corpus, about 43% of ganglion cells were immunostained with the P2Y₁₂ receptor antiserum. In the small intestine myenteric plexus of whole-mount preparations, P2Y₁₂ receptor-ir neurons were found in all ganglia and about 42 and 46% of ganglion cells were positively-immunostained for the P2Y₁₂ receptor antibody in the jejunum and ileum, respectively. In the myenteric plexus of the proximal and distal colon, about 40 and 45% of ganglion neurons were positively immunostained by the

Fig. 4 P2Y₆ receptor-ir (red) colocalization (yellow/orange) with NOS (green), or calbindin (green). **a** P2Y₆ receptor-ir coexistence with NOS in the myenteric plexus (MP) of jejunum. **b** P2Y₆ receptor-ir coexistence with NOS in the MP of ileum. **c** P2Y₆ receptor-ir coexistence with NOS in the MP of distal colon. **d** Shows no coexistence between calbindin and P2Y₆ receptor-ir in nerve cell bodies (possibly some in nerve fibers) of MP of ileum. Scale bar in all figures = 50 μm



P2Y₁₂ receptor antiserum. P2Y₁₂ receptor-ir neurons were also found in the submucosal plexus of the jejunum, ileum, proximal and distal colon.

Colocalization of P2Y₁₂ receptor-ir with calbindin, calretinin and NOS

P2Y₁₂ receptor-ir was found to coexist with calbindin in the myenteric plexus of the gastric corpus and in both

myenteric and submucosal plexuses of small intestine and distal colon (Fig. 6). No P2Y₁₂ receptor-ir ganglion cells were found to be immunoreactive for calretinin and NOS in both myenteric and submucosal plexuses (Fig. 7a, b).

All the calbindin-ir neurons were also immunoreactive for P2Y₁₂ receptors, although occasionally a P2Y₁₂ receptor-ir ganglion cell was not immunoreactive for calbindin in both myenteric and submucosal plexuses (Fig. 6).

Table 2 Quantitative analysis of double labeling studies between P2Y₆ receptors and calretinin (CR) in the myenteric plexus (MP) of guinea pig gastric corpus, jejunum, ileum, proximal and distal colon. The first column shows the mean number of P2Y₆-ir neurons also labeled with CR ± SE mean, expressed as a percentage underneath. The second column shows the mean number of P2Y₆-ir neurons that were not immunopositive for CR ± SE mean,

expressed as a percentage underneath. The third column shows the mean number of CR-ir neurons also immunopositive for P2Y₆ receptors ± SE mean, expressed as a percentage underneath. The final column shows the mean number of CR-ir neurons that were not immunopositive for P2Y₆ receptors ± SE mean, expressed as a percentage underneath

Region	P2Y ₆ -ir +		CR-ir +	
	CR-ir +	CR-ir -	P2Y ₆ -ir +	P2Y ₆ -ir -
Stomach MP	76 ± 9 45 ± 5%	96 ± 8 55 ± 5%	76 ± 9 42 ± 5%	106 ± 18 58 ± 10%
Jejunum MP	84 ± 8 47 ± 4%	96 ± 12 53 ± 7%	84 ± 8 30 ± 35%	148 ± 16 70 ± 6%
Ileum MP	81 ± 12 44 ± 7%	102 ± 14 46 ± 8%	81 ± 12 34 ± 5%	157 ± 19 66 ± 8%
Proximal colon MP	68 ± 8 44 ± 5%	87 ± 11 56 ± 7%	68 ± 8 28 ± 3%	178 ± 15 78 ± 6%
Distal colon MP	71 ± 10 41 ± 6%	104 ± 16 59 ± 9%	71 ± 10 30 ± 4%	167 ± 14 70 ± 6%

Table 3 Quantitative analysis of double labeling studies between P2Y₆ receptors and NOS in the myenteric plexus (MP) of guinea pig gastric corpus, jejunum, ileum, proximal and distal colon. The first column shows the mean number of P2Y₆-ir neurons also labeled with NOS ± SE mean, expressed as a percentage underneath. The second column shows the mean number of P2Y₆-ir neurons that were not immunopositive for NOS ± SE mean,

expressed as a percentage underneath. The third column shows the mean number of NOS-ir neurons also immunopositive for P2Y₆ receptors ± SE mean, expressed as a percentage underneath. The final column shows the mean number of NOS-ir neurons that were not immunopositive for P2Y₆ receptors ± SE mean, expressed as a percentage underneath

Region	P2Y ₆ -ir +		NOS-ir +	
	NOS-ir +	NOS-ir -	P2Y ₆ -ir +	P2Y ₆ -ir -
Stomach MP	46 ± 8 28 ± 5%	121 ± 9 72 ± 6%	46 ± 8 23 ± 4%	157 ± 15 77 ± 7%
Jejunum MP	51 ± 9 29 ± 5%	126 ± 11 71 ± 6%	51 ± 9 23 ± 4%	168 ± 13 77 ± 6%
Ileum MP	61 ± 10 32 ± 5%	132 ± 16 68 ± 8%	61 ± 10 27 ± 4%	167 ± 17 66 ± 7%
Proximal colon MP	44 ± 6 30 ± 4%	103 ± 10 70 ± 7%	44 ± 6 21 ± 3%	162 ± 19 79 ± 9%
Distal colon MP	64 ± 12 35 ± 7%	118 ± 16 75 ± 9%	64 ± 12 27 ± 5%	169 ± 14 73 ± 6%

Discussion

In the present study, the distribution patterns and morphological characteristics of P2Y₆ and P2Y₁₂ receptor-ir neurons have been studied systematically in all the major regions of the gastrointestinal tract of the guinea pig. The results showed that P2Y₆ receptor-ir neurons are distributed widely in the myenteric plexus of stomach, jejunum, ileum and colon, but not in the submucosal plexus, while P2Y₁₂ receptor-ir neurons are distributed widely in both myenteric and submucosal plexuses.

In this study 28–35% of P2Y₆ receptor-ir neurons were found to coexist with NOS and 41–47% to coexist with calretinin, but none of them coexisted with calbindin in the gastrointestinal tract of the guinea pig. In contrast, all the P2Y₁₂ receptor-ir neurons were also immunoreactive for calbindin, while none of the P2Y₁₂ receptor-ir neurons were found to coexist with calretinin and NOS. These data suggest that P2Y₆ receptor-ir neuron and P2Y₁₂ receptor-ir neuron play different physiological roles. Calretinin-ir neurons were shown to be Dogiel type I in the guinea pig ileum (Costa et al. 1996). The majority of them were cholinergic neurons, which belong to excitatory motor neurons supplying longitudinal muscle or ascending interneurons (Costa et al. 1996). Taken together with our data this suggests that a subset of cholinergic neurons express the P2Y₆ receptor and would be regulated by UDP in the myenteric plexus of guinea pig gastrointestinal tract. A subset of NOS-ir neurons express P2Y₆ receptors and would also be regulated by UDP as the P2Y₆ receptor is potently activated by UDP (Nicholas et al. 1996).

P2Y₆ receptor-ir neurons were not found in the submucosal plexus of any segments of the guinea pig gut examined, although a few P2Y₆ receptor-ir nerve fibers were demonstrated. This result appears to be contradictory to a previous report that P2Y₆ receptor mRNA was found in the submucosa of rat distal colon by RT-

PCR (Christofi et al. 2004). However, the report that P2Y₆ receptors were expressed in mucosal epithelial cells, but not in the submucosa (Köttgen et al. 2003) might explain this apparent discrepancy, since it would not be easy to avoid mucosal cell contamination with RT-PCR analysis.

Previous studies have identified Dogiel type II neurons with cell bodies in the myenteric plexus of guinea pig ileum to be intrinsic primary afferent neurons. These neurons also have distinctive electrophysiological characteristics (AH neurons) and 82–84% are immunoreactive for calbindin. They are the only calbindin-ir neurons in the ileal plexus (Furness et al. 1988, 1990; Clerc et al. 1998; Quinson et al. 2001), although in other regions (stomach, proximal and distal colon) nerve cells with other shapes and functions have calbindin immunoreactivity (Furness et al. 1988; Messenger et al. 1994; Reiche et al. 1999). In the present work all the calbindin-ir neurons were also immunoreactive for P2Y₁₂ receptors. So the majority of P2Y₁₂ receptor-ir neurons are Dogiel type II/AH (the intrinsic primary afferent neurons). The fact that no P2Y₆ receptor-ir neurons were found to express calbindin in the small intestine in the present work implied that P2Y₆ receptor-ir neurons were not Dogiel type II/AH (the intrinsic primary afferent neurons). The percentage of neurons immunopositive for P2Y₁₂/calbindin in the ileum was found to be significantly higher (46%) than that reported by the group of Furness (20%; Furness et al. 1990). This may reflect the different methods used. Furness' group reported that calbindin-immunoreactive neurons comprised about 20% of myenteric neurons, which was less than previous estimates (30%), as the total population had been underestimated (Young et al. 1993; Furness et al. 1990). In another report from Furness' group, 38% of the neurons in guinea pig ileum were immunoreactive for calbindin (Pompolo and Furness 1988). Perhaps the total number of the neurons in this study was underestimated if PGP9.5 did not stain all the neurons (Phillips

Fig. 5 P2Y₁₂ receptor-ir (black) in the guinea pig intestine. **a** P2Y₁₂ receptor-ir in the myenteric plexus (MP) of gastric corpus. **b** P2Y₁₂ receptor-ir in the MP of ileum. **c** High magnification of the area indicated by an *arrow* in **b**. **d** P2Y₁₂ receptor-ir in the MP of distal colon. **e** P2Y₁₂ receptor-ir in submucous plexus (SMP) of ileum. **f** P2Y₁₂ receptor-ir in SMP of distal colon. Scale bar = 50 μ m in a, c, d, e, f and 160 μ m in b

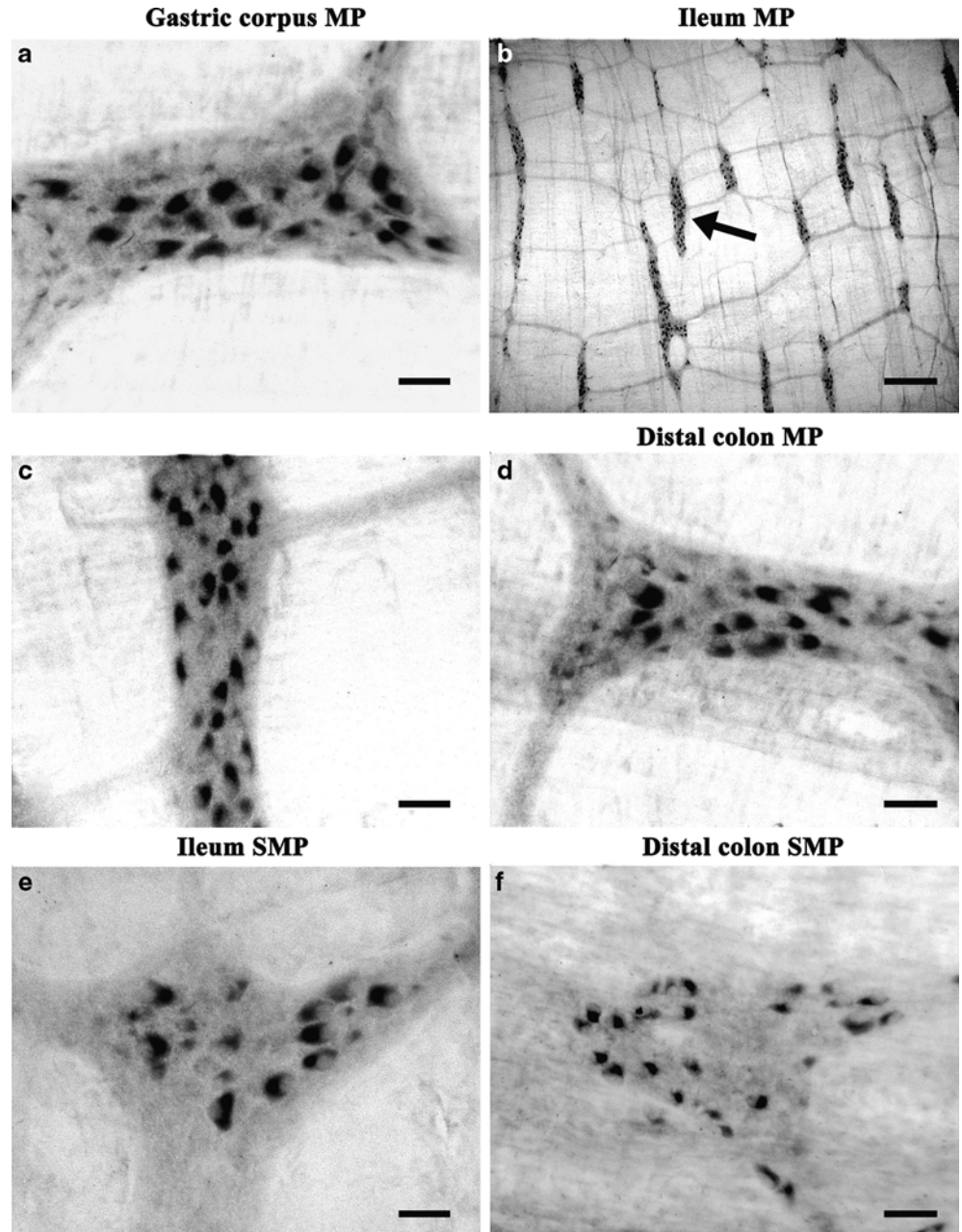


Table 4 Number of P2Y₁₂ receptor-immunoreactive neurons in the myenteric plexus (MP) and/or submucous plexus (SMP) of guinea pig gastric corpus, jejunum, ileum, proximal and distal colon, expressed as the mean number of neurons \pm SE mean, then

expressed as a percentage (the mean number of P2Y₁₂ receptor-immunoreactive neurons was divided by the total number of PGP9.5-positive neurons in the same whole-mount preparations \times 100%)

Regions	P2Y ₁₂ -ir	PGP9.5-ir	Percentage P2Y ₁₂ -ir
Stomach MP	195 \pm 16	453 \pm 26	43 \pm 4
Jejunum MP	181 \pm 23	431 \pm 31	42 \pm 5
Jejunum SMP	122 \pm 11	347 \pm 23	35 \pm 3
Ileum MP	196 \pm 23	426 \pm 36	46 \pm 5
Ileum SMP	116 \pm 15	351 \pm 25	33 \pm 4
Proximal colon MP	164 \pm 17	410 \pm 16	40 \pm 4
Proximal colon SMP	104 \pm 15	347 \pm 33	30 \pm 5
Distal Colon MP	178 \pm 19	396 \pm 21	45 \pm 5
Distal Colon SMP	118 \pm 12	337 \pm 16	35 \pm 4

Fig. 6 P2Y₁₂ receptor-ir (red) colocalization (yellow/orange) with calbindin (green). **a'** Calbindin in the myenteric plexus (MP) of gastric corpus. **a''**. P2Y₁₂ receptor-ir in the MP of gastric corpus. **a** The merged image from **a'** and **a''**. **b** P2Y₁₂ receptor-ir coexistence with calbindin in the MP of jejunum. **c** P2Y₁₂ receptor-ir coexistence with calbindin in the MP of distal colon. **d** P2Y₁₂ receptor-ir coexistence with calbindin in the submucous plexus (SMP) of distal colon. Scale bar in a to d = 20 μm, in **a'** and **a''** = 40 μm

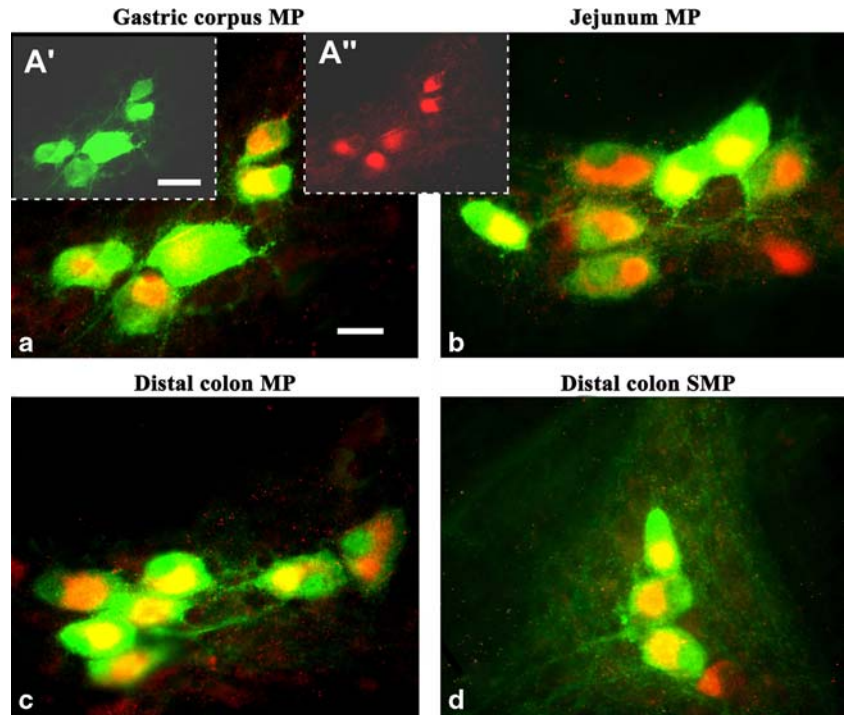
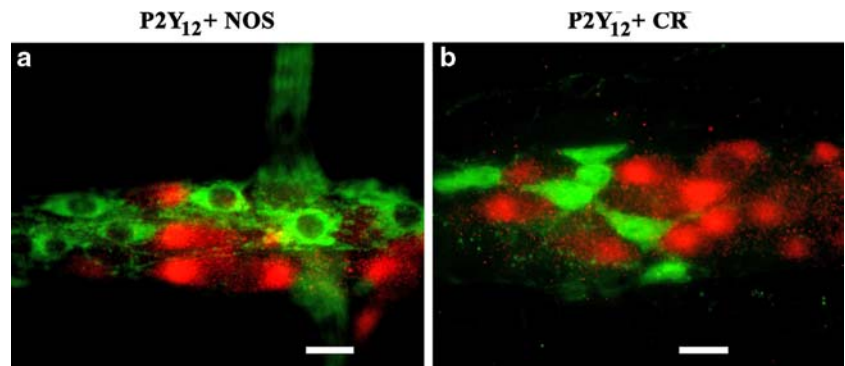


Fig. 7 P2Y₁₂ receptor-ir (red) colocalization with NOS (green) and calretinin (CR; green) in the jejunum myenteric plexus. **a** P2Y₁₂ receptor-ir colocalization with NOS. Note there is no coexistence between P2Y₁₂ receptors and NOS. **b** P2Y₁₂ receptor-ir colocalization with CR. Note there is no coexistence between P2Y₁₂ receptors and CR. All scale bars = 50 μm



et al. 2004). Another reason for the greater number of positive neurons for calbindin in this study may be the different antibodies used, which might have had a different (greater) affinity and dilution. In our hands, about 43, 42, 46, 40 and 45% of the total neurons were immunoreactive for calbindin in the myenteric plexus of stomach, jejunum, ileum, proximal colon and distal colon, respectively.

In the myenteric plexus almost all the P2Y₁₂ receptor-ir neurons were also immunoreactive for calbindin, but none of the P2Y₆ receptor-ir neurons were immunoreactive for calbindin, so clearly P2Y₆ receptor-ir neurons and P2Y₁₂ receptor-ir neurons were different cell types. The total number of P2Y₆ and P2Y₁₂ receptor-ir neurons account for 75, 76, 90, 70 and 80% of the total number of myenteric plexus neurons, as demonstrated by PGP9.5 staining in the myenteric plexus of stomach, jejunum, ileum, proximal colon and distal colon,

respectively, although recent data has showed PGP9.5 as a pan-neuronal marker which might underestimate the total neuron number (Phillips et al. 2004). In other words, the physiological functions of the majority of neurons in the myenteric plexus would be regulated by endogenous ADP (P2Y₁₂ receptor agonist) or UDP (P2Y₆ receptor agonist).

In summary, P2Y₆ receptor-ir neurons and fibers were found to be distributed widely in the myenteric, but not the submucous, plexus of the gastric corpus, jejunum, ileum, proximal and distal colon of the guinea pig. The typical morphology of the P2Y₆ receptor-ir neurons was multipolar with a long axon-like process with strong immunostaining. 30–36% of the ganglion cells were immunoreactive for P2Y₆ receptors in the myenteric plexus of the gastric corpus, jejunum, ileum, proximal and distal colon. A subset of calretinin-ir and NOS-ir neurons express the P2Y₆ receptor in the myenteric

plexus. P2Y₁₂ receptor-ir neurons were also distributed widely in both the myenteric and submucous plexuses of the gastric corpus, jejunum, ileum, proximal and distal colon of guinea pig. All the calbindin-ir neurons were also labeled by the P2Y₁₂ receptor antibody in both the myenteric and submucous plexuses, although occasionally P2Y₁₂ receptor-ir neurons without calbindin immunoreactivity were found. These P2Y₁₂ receptor-ir neurons with calbindin immunoreactivity in the small intestine are Dogiel type II/AH, intrinsic primary afferent neurons.

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