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ORIGINAL PAPER

Expression of $P2X_6$ receptors in the enteric nervous system of the rat gastrointestinal tract

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Abstract Expression of $P2X_4$ and $P2X_6$ receptor subunits in the gastrointestinal tract of the rat was studied with double-labeling fluorescence immunohistochemistry. The results showed that $P2X_6$ receptors were expressed widely in the submucosal and myenteric plexuses. In the myenteric plexus, $P2X_6$ receptors were expressed mainly in large size neurons which resembled Dogiel type II neurons. These $P2X_6$ receptor-immunoreactive (ir) neurons also expressed calbindin 28K, calretinin and neuronal nuclei (NeuN), proteins that are markers of intrinsic sensory neurons. In the submucosal plexus, all the calbindin 28K, calretinin and NeuN-ir cells were immunoreactive for $P2X_6$ receptors. $P2X_6$ receptors do not form homomultimers, but rather heteromultimers with either $P2X_2$ or $P2X_4$ receptors. $P2X_4$ receptors were not expressed in neurons, but were expressed in macrophages of the rat gastrointestinal tract. These data indicate that $P2X_6$ receptors are mainly expressed on intrinsic sensory neurons and that ATP, via $P2X_6$ receptors probably in heteromeric combination with $P2X_2$ receptors, may be involved in regulating the physiological functions of these neurons.

Keywords $P2X_6$ receptor · Enteric neuron · Gastrointestinal tract · Rat

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Introduction

ATP is a primitive signaling molecule that has been retained as a co-transmitter in every nerve type in both the peripheral and central nervous systems, although the relative role of ATP varies considerably in different species and pathological conditions (Burnstock 2007a). ATP acts on two types of P2 purinoceptors on effector tissues. P2X and P2Y purinoceptors are ligand-gated cation channels and G-protein-linked receptors, respectively (Ralevic and Burnstock 1998). ATP acting at P2X receptors may function as an excitatory neurotransmitter in central and sympathetic neurons and enteric ganglia (Dunn et al. 2001; Burnstock 2007a). Seven P2X receptor subunits have been cloned and each subunit has unique pharmacological and electrophysiological properties and it is established that three subunits form the cation channel either as homo- or hetero-multimers. The $P2X_6$ receptor does not form a homomultimer, but only a heteromultimer receptor with either $P2X_2$ or $P2X_4$ receptor subunits (North 2002). Morphological and functional data show that ATP, via P2X receptors, regulates the functions of the gastrointestinal tract (see Burnstock 2008). Pharmacological studies have shown that 90% of

cultured myenteric neurons of the guinea-pig small intestine respond to ATP via P2X purinoceptors, the properties of which are similar to those of cloned $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors (Zhou and Galligan 1996; Barajas-Lopez et al. 1996). Immunohistochemical studies have shown that $P2X_2$ (mouse, rat and guinea-pig), $P2X_3$ (mouse, rat and guinea-pig) and $P2X_5$ (mouse) receptors are expressed in neurons of the submucosal and myenteric ganglia in the gastrointestinal tract (Castelucci et al. 2002, 2003; Van Nassauw et al. 2002; Poole et al. 2002; Xiang and Burnstock 2004a, b; Ruan and Burnstock 2005). In a report from Van Crombruggen et al. (2007), $P2X_4$ receptor staining was found only in macrophages in the rat distal colon and $P2X_6$ receptor staining only in enteric glial cells. $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors are expressed widely in the central nervous system (Burnstock 2007a; Guo et al. 2008). Thus, it is possible that $P2X_4$ and $P2X_6$ receptors may be expressed in neurons of the submucosal and myenteric ganglia of the gastrointestinal tract. In this study, we used immunostaining methods to systematically study the expression patterns of $P2X_4$ and $P2X_6$ receptors on neurons of the rat gastrointestinal tract. Here, we report that the $P2X_6$ receptor is widely expressed in the submucosal and myenteric ganglia of the enteric nervous system of the rat, where it may form a heteromultimer receptor with $P2X_2$ receptors, rather than $P2X_4$ receptors, which are expressed on macrophages.

Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Second Military Medical University. Six adult rats (200–300 g) were used. The rats were anesthetized with sodium pentobarbitone and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/l phosphate buffer pH 7.4. The digestive tracts were removed and washed with phosphate buffered saline (PBS). One end of the segment was knotted with a silk thread and 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 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4, for 4–6 h. The mucosa was discarded and the submucosal and myenteric plexuses of the gastric corpus, jejunum, ileum, proximal and distal colon of the rats were used as whole-mount preparations.

Immunohistochemistry

The development and specificity of the $P2X_4$ and $P2X_6$ receptor polyclonal antisera have been previously reported (Xiang et al. 1998; Oglesby et al. 1999). The following protocol was used for double immunostaining of $P2X_6$ receptors with calbindin D-28K, NeuN (neuronal nuclei marker), calretinin, GFAP (enteric glial cell marker) and PGP9.5 (neuronal maker), and $P2X_4$ with ED1 (a marker of activated macrophages). The preparations were washed 3×5 min in PBS, and then preincubated in antiserum solution 1(10% normal bovine serum, 0.2% Triton X-100, 0.4% sodium azide in 0.01 mol/l PBS pH 7.2) for 30 min, followed by incubation with different combinations of $P2X_6$ antibody diluted 1:800, $P2X_4$ antibody diluted 1:400, calbindin antibody (mouse anti-rat; SWANT) diluted 1:500, calretinin antibody (mouse anti-rat; SWANT) diluted 1:500, NeuN antibody (mouse anti-rat; Chemicon) diluted 1:400, ED1(mouse anti-rat; chemicon) antibody diluted 1:400, GFAP antibody (mouse anti-rat; Biomeda) diluted 1:400, PGP9.5 antibody (mouse anti-rat; Neuromics) diluted 1:200 in antiserum solution 2 (1% normal bovine serum, 0.2% Triton X-100, 0.4% sodium azide in 0.01 mol/l PBS pH 7.2), at room temperature. Subsequently, the preparations were incubated with Cy3-conjugated donkey anti-rabbit IgG diluted 1:400 for $P2X_6$ and $P2X_4$ antibodies and FITC-conjugated donkey anti-mouse IgG diluted 1:200 for calbindin, calretinin, NeuN, GFAP, PGP9.5 and ED1 antibodies in antiserum solution 2 for 2 h at room temperature. Both Cy3-conjugated donkey anti-rabbit IgG and FITC-conjugated donkey anti-mouse IgG are specialized for multiple immunostaining from Jackson Immunoresearch Lab. All the incubations and reactions were separated by 3×10 min washes in PBS.

Control experiments were carried out with $P2X_4$ or $P2X_6$ antibody preabsorbed with $P2X_4$ or $P2X_6$ peptides. No staining was observed in those preparations incubated with antiserum solutions preabsorbed with $P2X_4$ or $P2X_6$ peptides. A further negative control of omitting the primary antibody was also carried out. No staining was also observed in those preparations.

In order to further confirm the specificity of the $P2X_4$ and $P2X_6$ receptor antibodies, western blot analysis was carried out. Rats were deeply anesthetized by sodium pentobarbital (60 mg/kg) and killed by decapitation. The proximal colon was rapidly removed, washed with ice-cold PBS and lysed with 20 mM Tris–HCl buffer, pH 8.0, containing 1% NP-40, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase and phosphatase inhibitors (Sigma). Protein concentration was determined by the bovine serum albumin (BCA) protein assay method using BCA as standard (BCA protein assay kit from Beyotime). Protein samples (100 μg) from the colon were loaded per lane, separated by SDS-PAGE (10% polyacrylamide gels) and then were electrotransferred onto nitrocellulose membranes. The membranes were blocked with 10% nonfat dry milk in Tris-buffered saline for 1 h and incubated overnight

at 4°C with $P2X_4$ or $P2X_6$ antibody (Roche Palo Alto, CA, USA) diluted 1:1,000 in 2% BSA in PBS. The membranes were then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:5,000 in 2% BSA in PBS for 1 h at room temperature. The color development was performed with 400 µg/ml nitro-blue tetrazolium, 200 µg/ml 5-bromo-4-chloro-3-indolyl phosphate and 100 mg/ml levamisole in TSM2 (0.1 mol/l Tris–HCl2 buffer, pH 9.5, 0.1 mol/l NaCl and 0.05 mol/l $MgCl_2$) in the dark. Bands were scanned using a densitometer (GS-700; Bio-Rad Laboratories).

Western blotting, performed on tissue extracts derived from the rat proximal colon (Fig. 1), assessed the specificity of the polyclonal $P2X_4$ and $P2X_6$ receptor antibody. An immunoreactive band was detected at about 43–44 kDa that corresponded to the molecular weight of the $P2X_4$ and $P2X_6$ receptor (Fig. 1, lanes 1 and 3). Preadsorption of the antiserum with the peptide antigen resulted in the absence of the band (Fig. 1, lanes 2 and 4), indicating that the antibodies detected the appropriate antigen sequence.

Quantitative analysis

Some of the preparations were counterstained with NeuN antiserum or PGP9.5 in order to assess the number of neurons in the ganglia (Figs. 4, 7, 9). For fluorescence immunocytochemistry, whole-mount preparations were used to perform a quantitative analysis as previously described (Van Nassauw et al. 2002). Briefly, positively stained neuron bodies in the submucosal and myenteric ganglia were counted per visual field (area of 0.62 mm^2). Ten randomly chosen fields in each whole-mount preparation and three preparations of each rat were analyzed. At least four rats were used for each marker. The percentage of neurons immunoreactive for a particular marker that were also positive for a different marker was calculated and expressed as mean ± standard error of the mean.

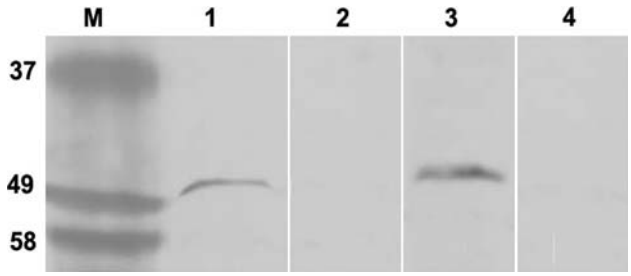

Fig. 1 Western blotting from colon extracts. *M* molecular weight marker. *Lanes 1* and *3* $P2X_4$ and $P2X_6$ receptor immunoreactive bands are located at about 43 kDa, respectively. *Lanes 2* and *4*, preabsorption of the $P2X_4$ and P2X6 receptor antisera with its peptide antigen, respectively, which resulted in the absence of the band

Photomicroscopy

Images were taken with the Nikon digital camera DXM1200 (Nikon, Japan) attached to a Nikon Eclipse E600 microscope (Nikon). Images were imported into a graphics package (Adobe Photoshop).

Results

$P2X_6$ receptor immunoreactivity occurred in nerve cell bodies in all regions that were examined in the myenteric plexus of the stomach, ileum, proximal and distal colon, and in the submucosal plexus of the jejunum, ileum, proximal and distal colon. Immunoreactivity occurred in the perikarya of the nerve cells, but not in the processes in either the submucosal or myenteric plexuses. In the myenteric plexus, the majority of positive cells were large (42 ± 6 µm long diameter), although a few small positive cells (18 ± 5 µm in diameter) were also found in some ganglia. The large positive cells had negative eccentric nuclei. In the gastric corpus myenteric plexus, about 10% of ganglion cells were immunostained with the $P2X_6$ receptor antiserum and most of the positive ganglion neurons were moderately immunostained (Figs. 2a, 3a, 4a). In the small intestine myenteric plexus of whole-mount preparations, $P2X_6$ receptor-immunoreactive (ir) neurons were found in all ganglia and about 23 and 27% of ganglion cells were positively immunostained for the $P2X_6$ receptor in the jejunum and ileum, respectively. Both large and small positive ganglion neurons were present, but the majority of them were large size cells (Figs. 2d, g, 3d, g, 4d, g). In the myenteric plexus of the distal colon, about 25% of ganglion neurons were positively stained by the $P2X_6$ receptor antiserum. Large and small positive ganglion neurons were also present in the myenteric plexus of the colon (Figs. 2j, m, 3j, m, 4j, m).

Double-labeling studies were conducted to identify the major classes of neurons with $P2X_6$ receptor immunoreactivity in the myenteric plexus of the gastric corpus, jejunum, ileum proximal and distal colon, and the submucosal plexus of the jejunum, ileum, and proximal and distal colon. In the myenteric plexus of all segments we examined, almost all the $P2X_6$ receptor-ir neurons were also immunoreactive for calbindin 28K and calretinin, although occasionally a few $P2X_6$ receptor-ir neurons, usually with small diameter cell bodies, were not found to be immunoreactive for calbindin 28K or calretinin (Figs. 2, 3). There are two different types of neurons in the myenteric plexus of all segments of the gastrointestinal tract. In the $NeuN_{NC}$ neurons, NeuN localizes in both the nucleus and cytoplasm, and in the $NeuN_N$ neurons, NeuN localizes only in the nucleus (Fig. 4). Almost all of the $P2X_6$ receptor-ir neurons

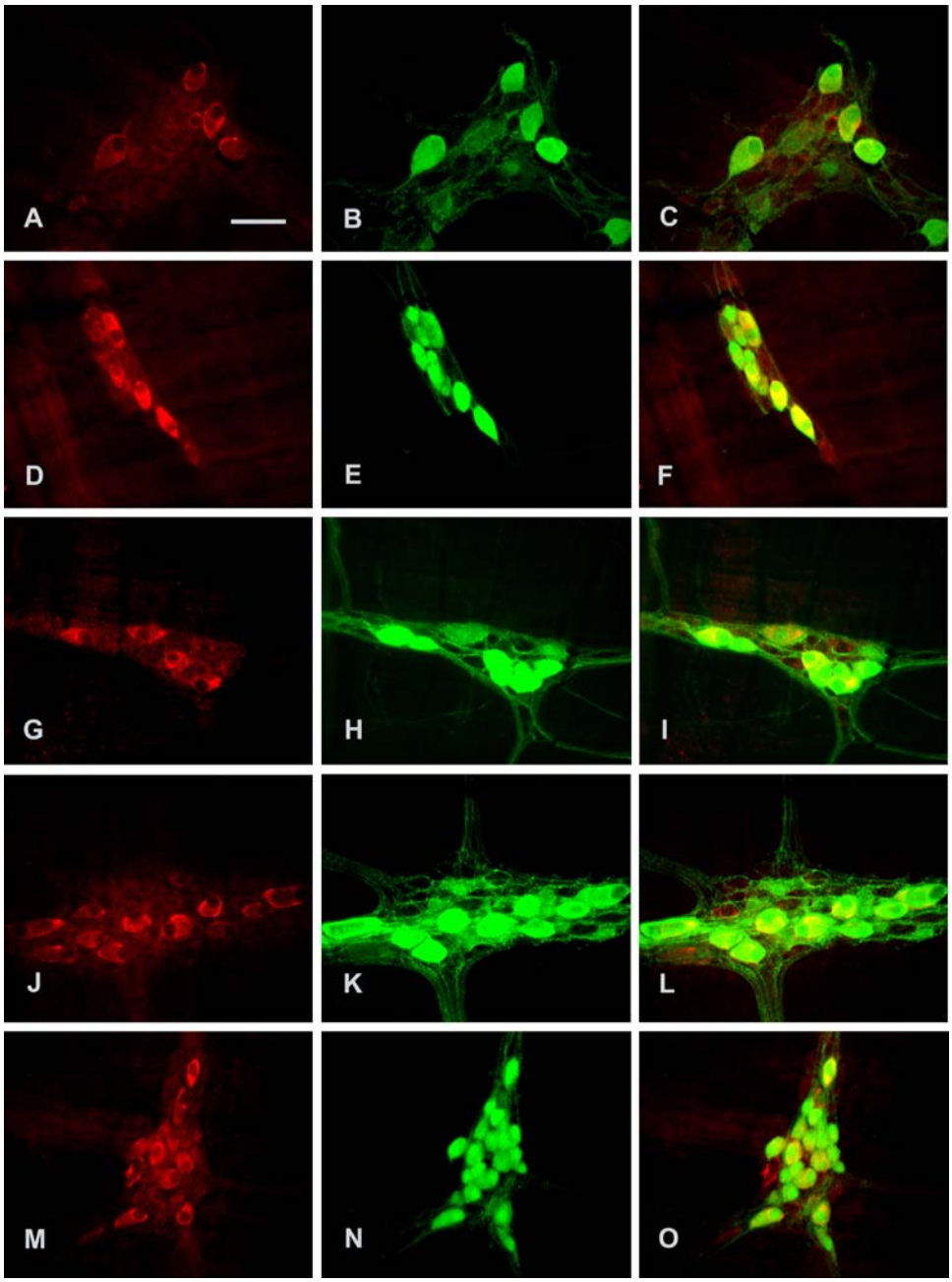

Fig. 2 Colocalization of $P2X_6$ receptor-ir and calbindin-ir in the myenteric plexus of the rat gastrointestinal tract. **a**, **d**, **g**, **j** and **m** $P2X_6$ receptor-ir cells (*red*) in the myenteric plexus of gastric corpus, jejunum, ileum, proximal and distal colon, respectively. **b**, **e**, **h**, **k** and **n** Calbindin 28K-ir cells (*green*) in the myenteric plexus of gastric corpus, jejunum, ileum, proximal and distal colon from the same fields as **a**, **d**, **g**, **j** and **m**, respectively. **c**, **f**, **i**, **l** and **o** The merged images from **a** and **b**, **d** and **e**, **g** and **h**, **j** and **k**, **m** and **o**, respectively. Note that all the large size cells express both $P2X_6$ receptor-ir and calbindin 28K-ir (*scale bars* 60 μm)

overlapped with the $NeuN_{NC}$ neurons expressing NeuN (Fig. 4). In the submucosal plexus of the jejunum, ileum and distal colon, about 52–60% of $P2X_6$ receptor-ir neurons were immunoreactive for calbindin 28K (Fig. 5), about 78–85% of $P2X_6$ receptor-ir neurons for calretinin (Fig. 6), and almost all the $P2X_6$ receptor-ir neurons were labeled with NeuN (Fig. 7). Table 1 summarizes the percentages of $P2X_6$ receptor-ir neurons that were immunoreactive for calbindin 28K, calretinin and NeuN in the submucosal plexus of the jejunum, ileum and distal colon.

$P2X_4$ receptor immunoreactivity did not occur in nerve cells in any regions that were examined in the myenteric plexus and submucosal plexus preparations of the rat gastrointestinal tract, but did occur in macrophage-like cells (Fig. 8a). Double immunostaining showed that these $P2X_4$ receptor-ir cells were also immunoreactive for ED1 (a marker of activated macrophage) (Fig. 8b, c).

In order to further confirm the type of cells expressing $P2X_6$ receptors in the enteric plexuses, a GFAP antibody was used. Double immunostaining clearly demonstrated that $P2X_6$ receptor-ir cells did not express GFAP-ir, which is a marker of the enteric glial cell (Fig. 9). As the cells with $P2X_6$ receptor-ir also expressed NeuN and PGP9.5, but did not express GFAP, all of these cells can be identified as neurons.

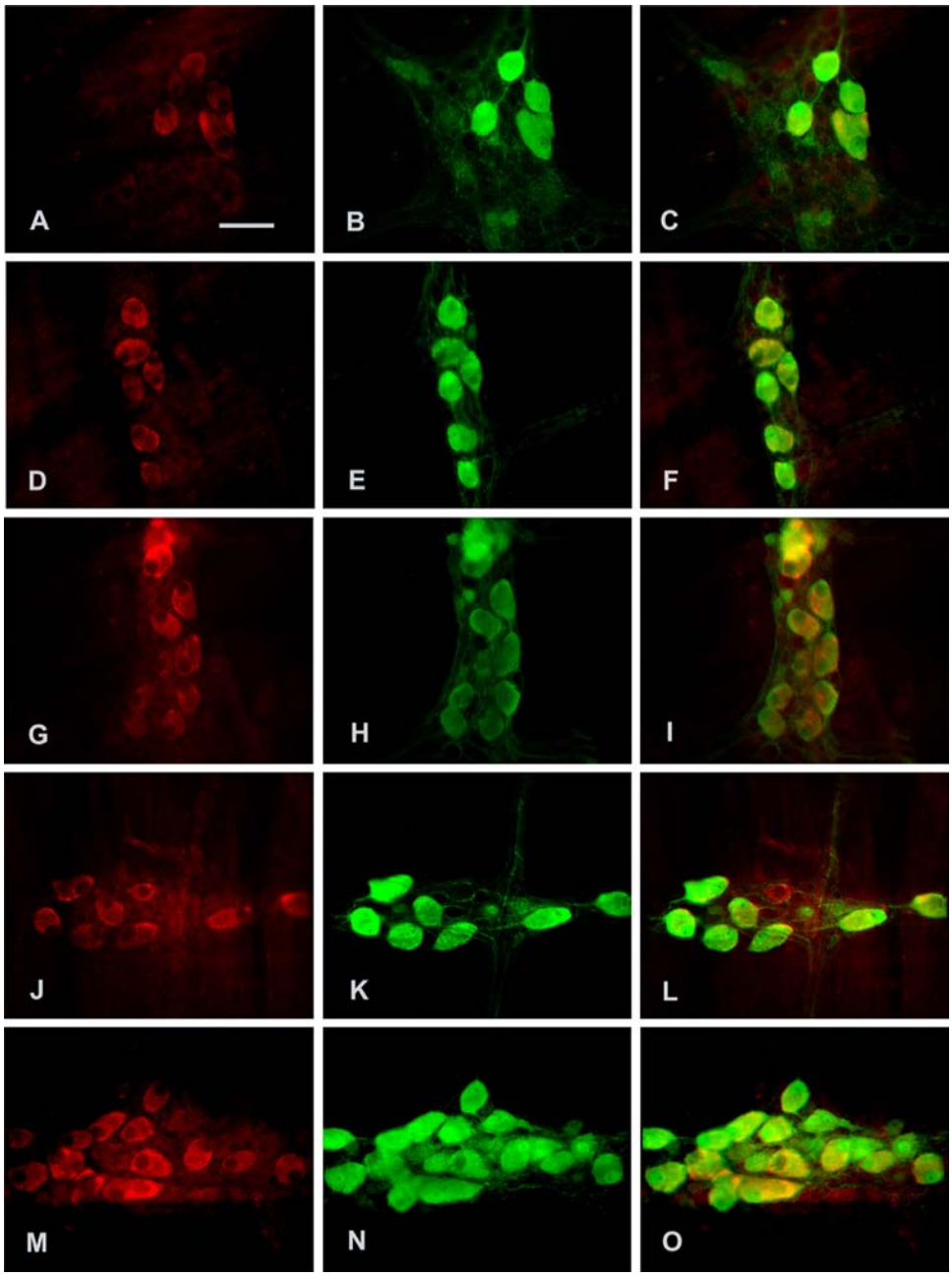

Fig. 3 Colocalization of $P2X_6$ receptor-ir and calretinin-ir in the myenteric plexus of the rat gastrointestinal tract. **a**, **d**, **g**, **j** and **m** $P2X_6$ receptor-ir cells (*red*) in the myenteric plexus of gastric corpus, jejunum, ileum, proximal and distal colon, respectively. **b**, **e**, **h**, **k** and **n** Calretinin-ir cells (*green*) in the myenteric plexus of gastric corpus, jejunum, ileum, proximal and distal colon from the same fields as **a**, **d**, **g**, **j** and **m**, respectively. **c**, **f**, **i**, **l** and **o** The merged images from **a** and **b**, **d** and **e**, **g** and **h**, **j** and **k**, **m** and **o**, respectively. Note that all the large size cells express both $P2X_6$ receptor-ir and calretinin-ir (*scale bars* 60 μm)

Discussion

In the present study, the distribution patterns and morphological characteristics of $P2X_6$ receptor-ir neurons have been studied systematically in all the major regions of the rat gastrointestinal tract. The results show that $P2X_6$ receptor-ir neurons are distributed widely in the myenteric plexus of stomach, jejunum, ileum and colon, and the submucosal plexus of jejunum, ileum and colon.

Several previous studies have shown that there are intrinsic sensory neurons in the intestine (Langley and Magnus 1905; Crema et al. 1970; Furness et al. 1995). They have been identified in the small intestine of the guinea-pig as Dogiel type II neurons (Kirchgessner et al. 1992; Kunze et al. 1995; Bertrand et al. 1997). Dogiel type II neurons with similar electrophysiological properties, projections and chemistries have been found in the large intestine of the guinea-pig and in the rat small intestine, which suggests that these neurons are also intrinsic sensory neurons in other regions and species (Mann et al. 1997; Lomax et al. 1999; Neunlist et al. 1999). Calbindin 28K is believed to be expressed in intrinsic sensory neurons of submucosal and myenteric plexuses of the guinea-pig (Furness 2000). Intrinsic sensory neurons, with Dogiel type II morphology, in the myenteric plexus of the rat ileum were also reported to express calbindin and calretinin.

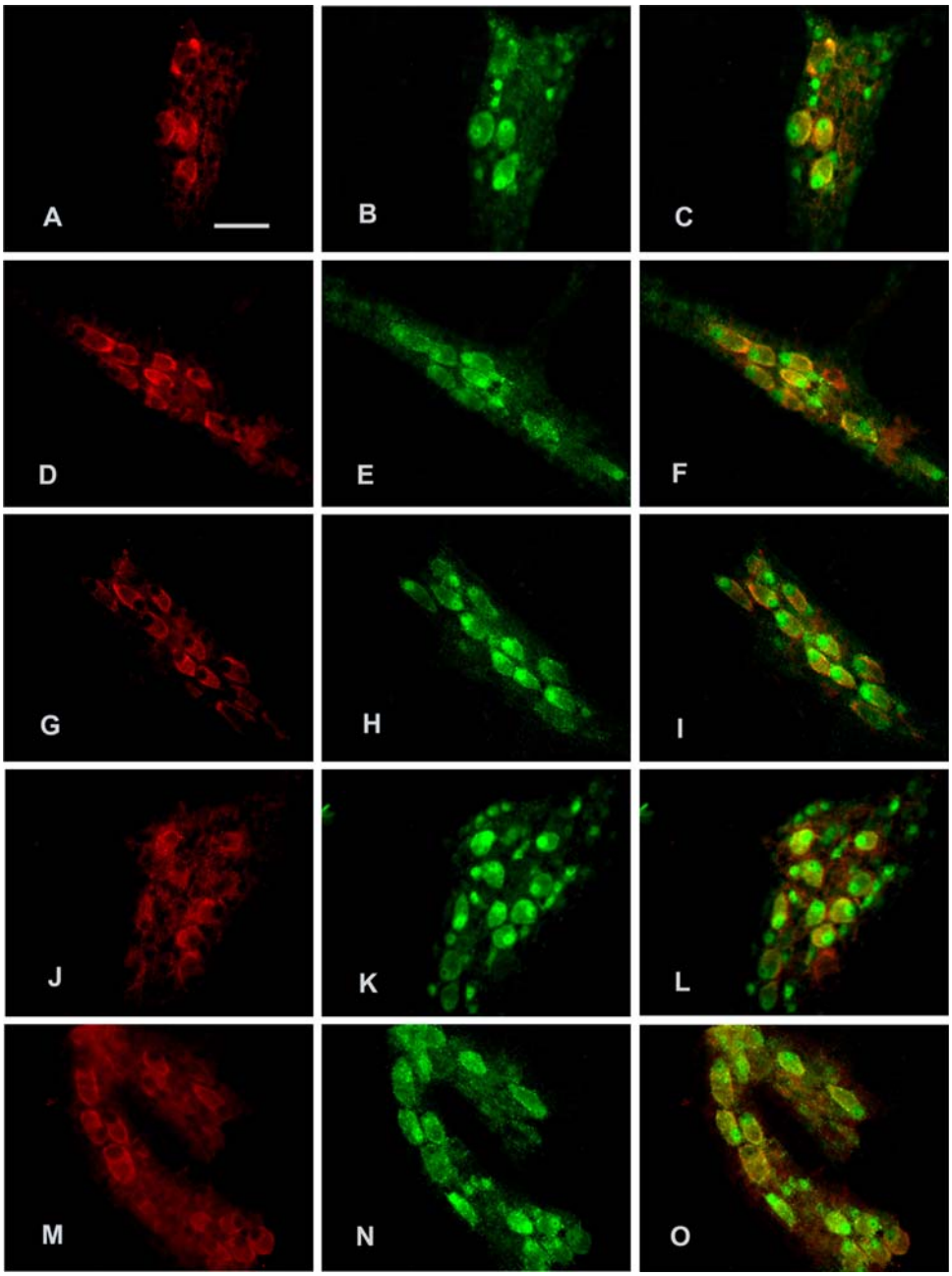

Fig. 4 Colocalization of $P2X_6$ receptor-ir and NeuN-ir in the myenteric plexus of the rat gastrointestinal tract. **a**, **d**, **g**, **j** and **m** $P2X_6$ receptor-ir cells (*red*) in the myenteric plexus of gastric corpus, jejunum, ileum, proximal and distal colon, respectively. **b**, **e**, **h**, **k** and **n** NeuN-ir cells (*green*) in the myenteric plexus of gastric corpus, jejunum, ileum, proximal and distal colon from the same fields as **a**, **d**, **g**, **j** and **m**, respectively. **c**, **f**, **i**, **l** and **o** The merged images from **a** and **b**, **d** and **e**, **g** and **h**, **j** and **k**, **m** and **o**, respectively. Note that all the large size cells express both $P2X_6$ receptor-ir and NeuN-ir (*scale bars* 60 μm)

Thus, calbindin and calretinin are also markers of intrinsic sensory neurons in the myenteric plexus of the rat ileum (Mann et al. 1999). In this study, we show that in the myenteric plexus the majority of $P2X_6$ receptor-positive cells were of the Dogiel II neuron shape, i.e., large size, and long diameter of about 42 ± 6 μm. The double immunostaining study showed that almost all the $P2X_6$ receptor-ir neurons were found to coexist with calbindin 28K and calretinin, although occasionally a few small $P2X_6$ receptor-ir neurons were not found to be immunoreactive for calbindin 28K and calretinin in some myenteric ganglia. It could be deduced that $P2X_6$ receptors in the myenteric plexus of the rat are expressed in intrinsic sensory neurons. In the submucosal plexus, 52–60% of neurons expressing $P2X_6$ receptors were also immunoreactive for calbindin, and 78–85% of these neurons for calretinin. Thus, a group of neurons expressing $P2X_6$ receptors also express both calbindin and calretinin.

In the myenteric plexus and submucosal plexus of the guinea-pig, calbindin is exclusively expressed on intrinsic sensory neurons and calretinin is exclusively expressed on cholinergic secretomotor and vasodilator neurons, but not on intrinsic sensory neurons (Furness 2000). In contrast, both calbindin and calretinin appear to be markers for intrinsic sensory neurons in mouse gastrointestinal tract (Sang and Young 1996, 1998), showing that there is a

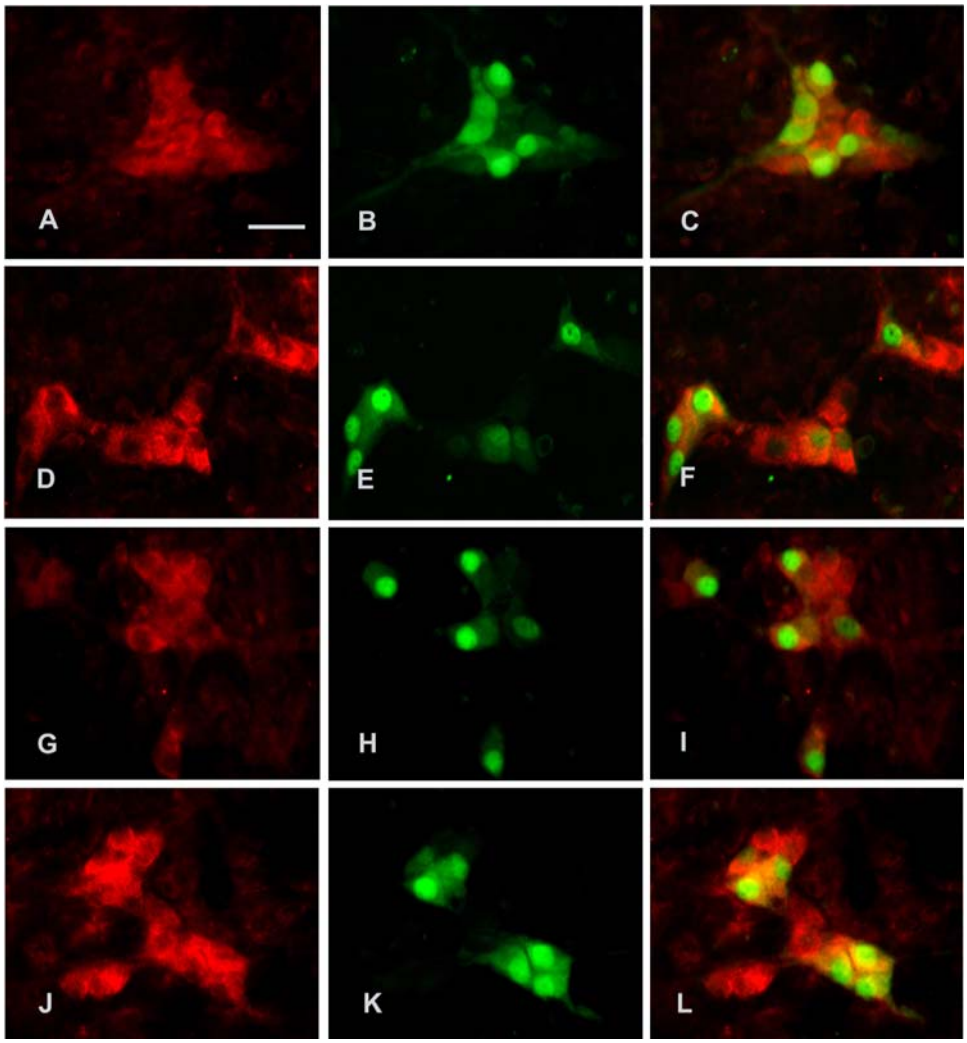

Fig. 5 Colocalization of $P2X_6$ receptor-ir and calbindin 28K-ir in the submucosal plexus of the rat gastrointestinal tract. **a**, **d**, **g** and **j** $P2X_6$ receptor-ir cells (*red*) in the submucosal plexus of jejunum, ileum, proximal and distal colon, respectively. **b**, **e**, **h** and **k** Calbindin-28K-ir cells (*green*) in the submucosal plexus of jejunum, ileum, proximal and distal colon from the same fields as **a**, **d**, **g**, **j** and **m**, respectively. **c**, **f**, **i** and **l** The merged images from **a** and **b**, **d** and **e**, **g** and **h**, **j** and **k**, respectively. Note that all the calbindin 28K-ir cells are also immunoreactive for $P2X_6$ receptors (*scale bars* 60 μm)

species difference in the expression patterns of these two calcium binding proteins in enteric neurons. The neuronal nuclei (NeuN) antibody was initially described as a result of attempts to identify novel proteins involved in the regulation of neuronal phenotype. The appearance of NeuN is coincident with neuronal differentiation and the neuron's exit from the cell cycle (Mullen et al. 1992). It has been recently shown that nerve cell bodies with the shape of Dogiel type II neurons in both myenteric and submucous ganglia in the guinea-pig ileum express NeuN immunoreactivity. It was suggested that NeuN is a selective marker for intrinsic sensory neurons (Costa et al. 2001; Poole et al. 2002). It is reported that NeuN labels the nuclei of almost all the enteric neurons (Chiocchetti et al. 2003), but that cytoplasmic expression of NeuN appears to be restricted to Dogiel type II neurons (Chiocchetti et al. 2003; Van Nassauw et al. 2005).

In the present study, two types of neurons expressing NeuN immunoreactivity are identified. The $NeuN_{NC}$ neurons expressing NeuN immunoreactivity in both the cytoplasm and nuclei were also immunoreactive for $P2X_6$ receptors in all segments of the rat gastrointestinal tract. To our knowledge, there are no reports about NeuN immunoreactivity in intrinsic sensory neurons of rat myenteric neurons. Thus, this study has demonstrated for the first time intrinsic sensory neurons of rat myenteric neurons that express NeuN. We have also shown that the pattern of expression of calbindin and calretinin appears to be comparable to that seen in the mouse, rather than the guinea-pig, both being markers for intrinsic sensory neurons. Furthermore, our study demonstrates that $P2X_6$ receptors are selectively expressed on intrinsic sensory neurons of the rat myenteric plexus. In the submucosal plexus, almost all the neurons expressing $P2X_6$ receptors are also immunoreactive for NeuN and vice versa. Only the $NeuN_{NC}$-ir neurons remain to be identified in the submucosal plexus of the rat intestinal tract. Further experiments need to be carried out in order to determine whether all the $NeuN_{NC}$ neurons in

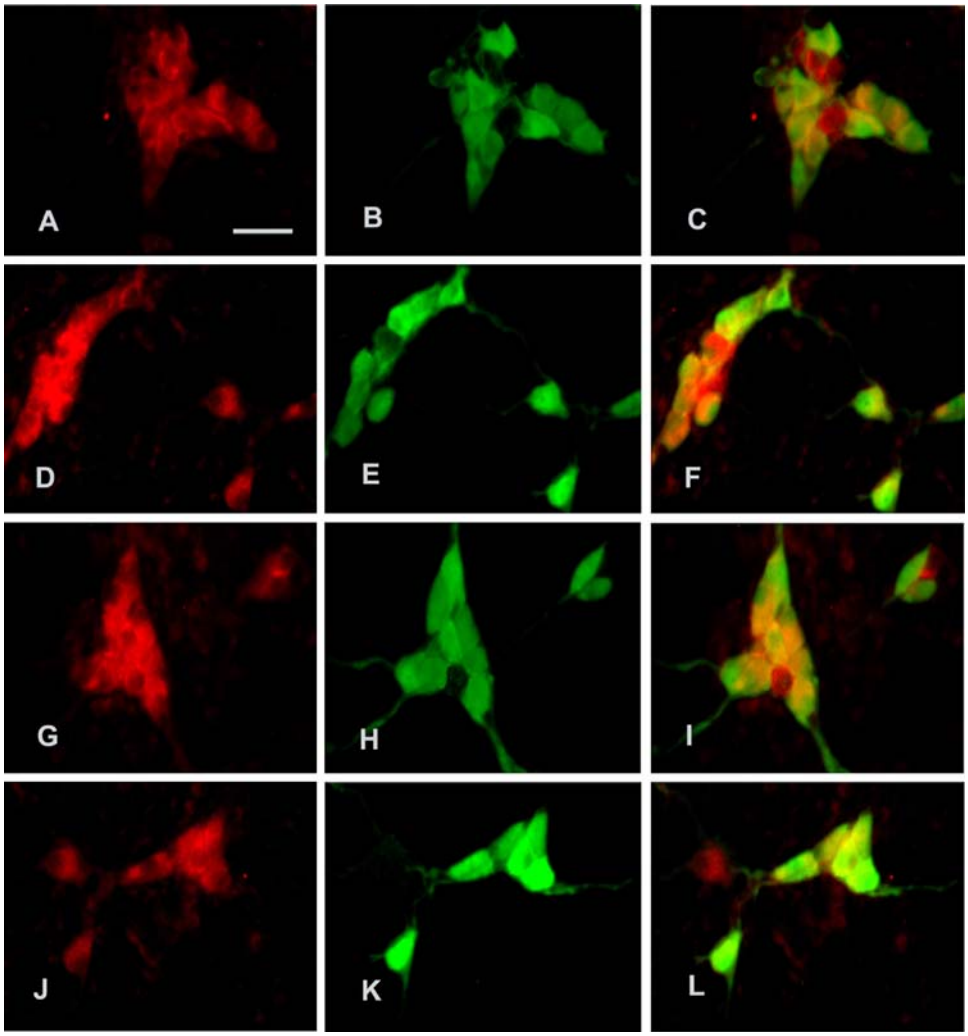

Fig. 6 Colocalization of $P2X_6$ receptor-ir and calretinin-ir in the submucosal plexus of the rat gastrointestinal tract. **a**, **d**, **g** and **j** $P2X_6$ receptor-ir cells (*red*) in the submucosal plexus of jejunum, ileum, proximal and distal colon, respectively. **b**, **e**, **h** and **k** Calretinin-ir cells (*green*) in the submucosal plexus of jejunum, ileum, proximal and distal colon from the same fields as **a**, **d**, **g**, **j** and **m**, respectively. **c**, **f**, **i** and **l** The merged images from **a** and **b**, **d** and **e**, **g** and **h**, **j** and **k**, respectively. Note that all the calretinin-ir cells are also immunoreactive for $P2X_6$ receptors

the submucsoal plexus of the rat intestinal tract are intrinsic sensory neurons.

Seven P2X receptor subunits have been cloned (Burnstock 2007b). There has been some ambiguity in the interpretation of electrophysiological results regarding which P2X receptor subunit(s) are present in myenteric ganglion neurons. Zhou and Galligan (1996) suggested that the electrophysiological properties of myenteric neurons in the small intestine indicated that 10% of them expressed $P2X_1$ or $P2X_3$ receptors and that most of them expressed $P2X_2$ or $P2X_5$ receptors. The report of Lepard et al. (1997) showed that the $P2X_2$ receptor was the main purinoceptor subunit, but Barajas-Lopez et al. (1996) suggested that $P2X_4$ and $P2X_6$, perhaps as heteromultimers, were dominantly expressed on myenteric neurons. Molecular cloning of P2X receptor subunits indicates that a subunit is composed of a 379- to 472-amino acid protein, in which the N and C terminals are in the cytoplasm connected by two-transmembrane-spanning segments and a large extracellular loop (Ralevic and Burnstock 1998; North 2002). It is speculated that a receptor is assembled with multiple subunits (Ralevic and Burnstock 1998; Khakh et al. 2001). P2X receptor subunits are able to form homo- and heteromeric multimers as the functional receptor channels. Homomeric $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_7$ channels and heteromeric $P2X_{1/2}$, $P2X_{1/5}$, $P2X_{1/4}$, $P2X_{2/3}$, $P2X_{2/6}$, $P2X_{4/6}$ and $P2X_{4/7}$ receptor channels have been characterized following heterologous expression (Khakh et al. 2001; Burnstock 2007b; Guo et al. 2007). Our previous morphological data showed that $P2X_2$ and $P2X_3$ receptors were widely expressed in neurons of the submucosal and myenteric ganglia in the rat gastrointestinal tract (Xiang and Burnstock 2004a, b). Some $P2X_2$ receptor-ir neurons in the rat myenteric ganglia are of the Dogiel type II shape, with large cell bodies, that were immunoreactive for both calbindin and calretinin (Xiang and Burnstock 2004b). Thus, $P2X_2$ and $P2X_6$ receptor subunits expressed on P2X receptor-containing neurons could assemble into homomeric $P2X_2$ channels or heteromeric

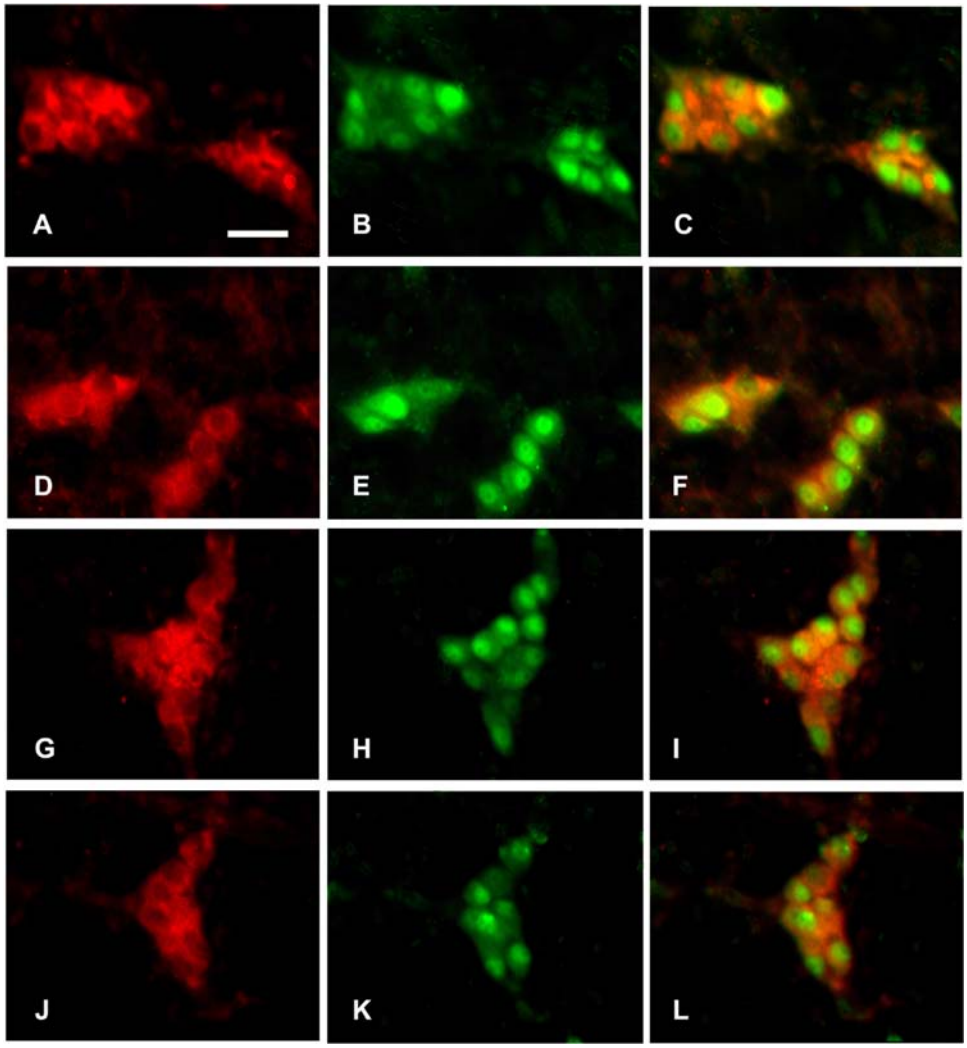

Fig. 7 Colocalization of $P2X_6$ receptor-ir and NeuN-ir in the submucosal plexus of the rat gastrointestinal tract. **a**, **d**, **g** and **j** $P2X_6$ receptor-ir cells (*red*) in the submucosal plexus of jejunum, ileum, proximal and distal colon, respectively. **b**, **e**, **h** and **k** NeuN-ir cells (*green*) in the submucosal plexus of jejunum, ileum, proximal and distal colon from the same fields as **a**, **d**, **g** and **j**, respectively. **c**, **f**, **i** and **l** The merged images from **a** and **b**, **d** and **e**, **g** and **h**, **j** and **k**, respectively. Note that almost all the NeuN-ir cells are also immunoreactive for $P2X_6$ receptors and vice versa (*scale bars* 60 μm)

Table 1 Quantitative analysis of double-labeling studies between $P2X_6$ receptors and calbindin (CB), $P2X_6$ receptors and calretinin (CR) and $P2X_6$ receptors and NeuN in the submucosal plexus of rat jejunum, ileum and distal colon

Region	$P2X_6$-ir+ CB-ir+	$P2X_6$-ir+ CB-ir–	$P2Y_6$-ir+ CR-ir+	$P2Y_6$-ir+ CR-ir–	$P2Y_6$-ir+ NeuN+	$P2Y_6$-ir+ NeuN-
Jejunum	80 ± 8	73 ± 12	123 ± 9	35 ± 8	146 ± 14	8 ± 3
	52 ± 5%	48 ± 8%	42 ± 6%	58 ± 5%	95 ± 9%	5 ± 2%
Ileum	94 ± 8	79 ± 10	143 ± 15	32 ± 11	168 ± 16	5 ± 2
	54 ± 5%	46 ± 6%	81 ± 9%	19 ± 6%	97 ± 9%	3 ± 1%
Distal colon	82 ± 8	54 ± 9	155 ± 9	27 ± 6	141 ± 12	6 ± 2
	60 ± 6%	40 ± 7%	85 ± 5%	15 ± 3%	96 ± 8%	4 ± 1%

The first column shows the mean number of $P2X_6$-ir neurons also labeled with CB ± SEM, expressed as a percentage underneath. The second column shows the mean number of $P2X_6$-ir neurons that were not immunopositive for CB ± SEM, expressed as a percentage underneath. The third column shows the mean number of $P2X_6$ receptors-ir neurons also immunopositive for CR ± SEM, expressed as a percentage underneath. The fourth column shows the mean number of $P2X_6$ receptors-ir neurons that were not immunopositive for CR ± SEM, expressed as a percentage underneath. The fifth column shows the mean number of $P2X_6$ receptors-ir neurons also immunopositive for NeuN ± SEM, expressed as a percentage underneath. The sixth column shows the mean number of $P2X_6$ receptors-ir neurons that were not immunopositive for NeuN ± SEM, expressed as a percentage underneath

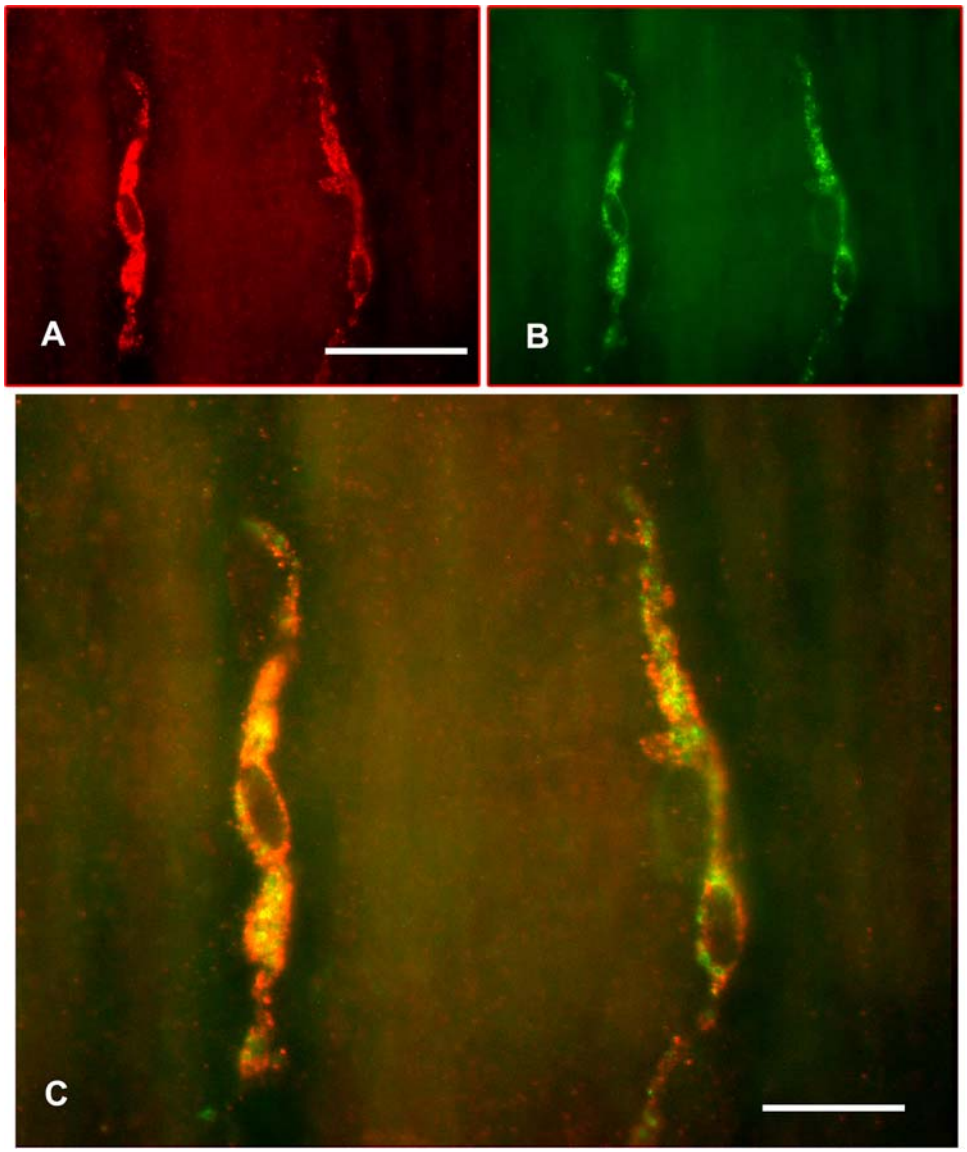

Fig. 8 $P2X_4$ receptor immunoreactivity in the myenteric plexus of rat ileum and co-localization with ED1 (a marker of activated macrophages). **a** $P2X_4$ receptor-ir cells (*red*). **b** ED1-ir cells (*green*) from the same field as **a**. **c** Merged image from **a** and **b**, note that all the $P2X_4$ receptor-ir cells are also immunoreactive for ED1. No neuron-like cells were found to be immunoreactive for $P2X_4$ receptors (*scale bar* **a**, **b** 60 μm, **c** 30 μm)

$P2X_{2/6}$ in enteric neurons of the rat gastrointestinal tract. Further studies using specific antagonists are required for unequivocal support of these possible functional combinations of P2X receptor multimers.

In this report we found that $P2X_4$ receptor subunits were expressed only in macrophages, which is consistent with previous reports, and that $P2X_6$ receptor subunits were expressed only in neurons of the enteric plexus, which differs from a previous report (Van Crombruggen et al. 2007). The specificity of the $P2X_6$ receptor antibody used in this study had been confirmed by previous reports (Oglesby et al. 1999; Loesch and Burnstock 2001; Glass et al. 2002), and further by preabsorption controls and Western blot experiments reported in this study. In the previous report by Van Crombruggen et al. (2007), the antibodies were obtained from different commercial suppliers and this may account for the differences in the expression patterns found between their study and ours.

In conclusion, the present study demonstrates that $P2X_6$ receptor subunits are distributed widely in the rat gastrointestinal tract. In the myenteric plexus the $P2X_6$ receptor localizes mainly in the intrinsic primary afferent neurons, as these $P2X_6$ receptor-ir cells are also immunoreactive for calbindin 28K, calretinin and NeuN, and their morphology resembles that of Dogiel type II neurons. In the submucosal plexus, all the calbindin 28K, calretinin and NeuN-ir cells are immunoreactive for $P2X_6$ receptors, although some of the $P2X_6$ receptor-ir cells are not immunoreactive for calbindin 28K and calretinin. The $P2X_4$ receptor was not expressed by neurons of the rat gastrointestinal tract, but was expressed by macrophages. These data indicate that $P2X_6$ receptors are mainly expressed on intrinsic sensory

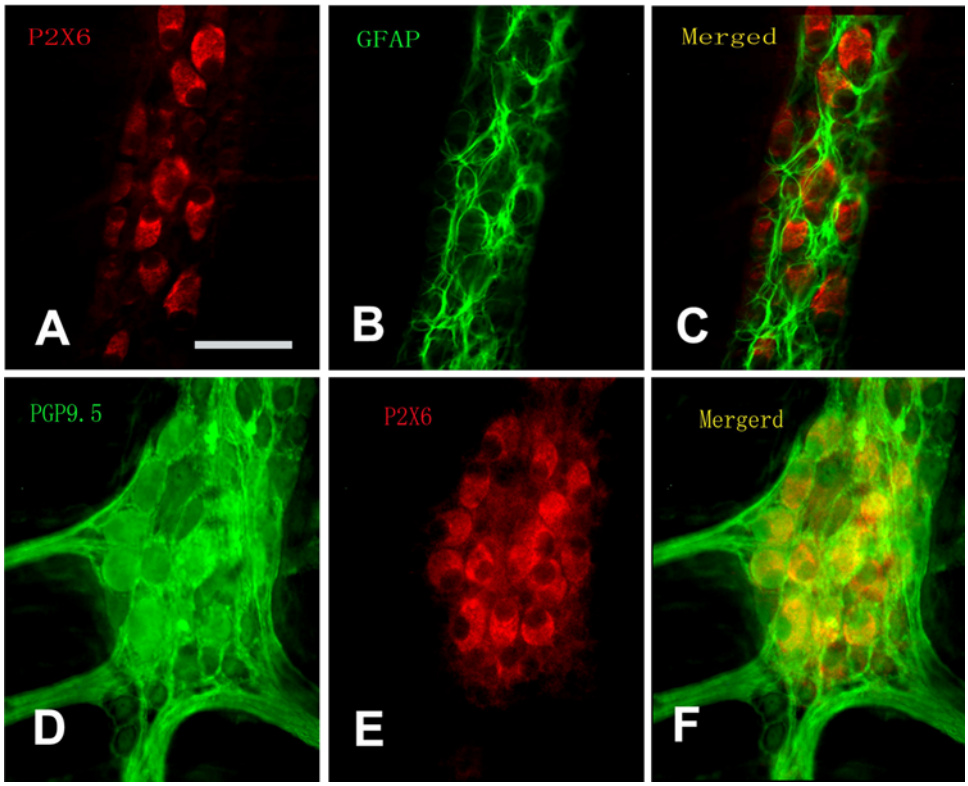

Fig. 9 Double immunostaining of $P2X_6$ receptors with GFAP or PGP9.5. **a** $P2X_6$ receptor-ir cells in the myenteric plexus of rat ileum. **b** GFAP-ir cells in the same field as **a**. **c** The merged image from **a** and **b**; note that no GFAP-ir and $P2X_6$ receptor-ir was found to coexist in the same cells. **d** PGP9.5-ir cells in the myenteric plexus of the proximal colon. **e** $P2X_6$ receptor-ir cells in the myenteric plexus of the proximal colon from the same field as **d**. **f** The merged image from **d** and **e**, note that all the $P2X_6$ receptor-ir cells also express PGP9.5-ir (*scale bars* **a**–**f** 60 μm)

neurons and that ATP, via $P2X_6$ receptors probably in heteromeric combination with $P2X_2$ receptors, may be involved in regulating the physiological functions of these neurons.

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