Research Article

Distribution and morphology of P2X3-immunoreactive subserosal afferent nerve endings in the rat gastric antrum

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Abstract

The present study investigated the morphological characteristics of subserosal afferent nerve endings with immunoreactivity for the P2X3 purinoceptor (P2X3) in the rat stomach by immunohistochemistry of whole-mount preparations using confocal scanning laser microscopy. P2X3 immunoreactivity was observed in subserosal nerve endings proximal and lateral to the gastric sling muscles in the distal antrum of the lesser curvature. Parent axons ramified into several lamellar processes to form net-like complex structures that extended two-dimensionally in every direction on the surface of the longitudinal smooth muscle layer. The axon terminals in the periphery of P2X3-immunoreactive net-like structures were flat and looped or leaf-like in shape. Some net-like lamellar structures and their axon terminals with P2X3 immunoreactivity were also immunoreactive for P2X2. P2X3-immunoreactive nerve fibers forming net-like terminal structures were closely surrounded by S100B-immunoreactive terminal Schwann cells, whereas axon terminals twined around these cells and extended club-, knob-, or thread-like protrusions in the surrounding tissues. Furthermore, a retrograde tracing method using fast blue dye indicated that most of these nerve endings originated from the nodose ganglia of the vagus nerve. These results suggest that P2X3-immunoreactive subserosal nerve endings have morphological characteristics of mechanoreceptors and contribute to sensation of a mechanical deformation of the distal antral wall associated with antral peristalsis.

Keywords: Stomach, P2X purinoceptor, Afferent nerve endings, Mechanoreceptor,

PRID: AB_2157930, PRID: AB_476701, PRID: AB_2299063, PRID AB_477499

1 INTRODUCTION

The unilocular stomach of mammals is part of the upper gastrointestinal tract and its functional role can be divided into two motor activities: acting as a flexible reservoir and gastric peristalsis. The fundus and corpus serve as a flexible reservoir to accommodate a large amount of ingesta, whereas the antrum functions as a peristaltic pump to triturate, mix, and then propel gastric contents into the duodenum. The gastric wall is innervated by vagal afferents that project from the nodose ganglion (NG). A previous electrophysiological study revealed that gastric muscle afferents in the rat vagus nerve are unmyelinated C-fibers sensitive to mechanical stimuli associated with gastric distension and peristaltic contraction (Ozaki, Sengupta, & Gebhart, 1999). The mechanosensory signals from the gastric muscle afferents in the vagus nerve are transmitted to the nucleus of the solitary tract via the NG, and trigger vago-vagal reflexes such as gastric accommodation, activation of antral peristalsis, and enterogastric inhibition (Andrews, Grundy, & Lawes, 1980; Cannon & Lieb, 1913; Sengupta & Gebhart, 1994).

Vagal afferent nerve endings distributed in the gastric smooth muscle layers have been morphologically classified into two subtypes by anterograde tracing into the rat NG; intraganglionic laminar endings (IGLEs) associated with myenteric ganglia, and intramuscular arrays (IMAs) within the muscle layers (Berthoud & Powley, 1992). IGLEs arborized laminar terminal branches covering myenteric ganglion neurons between smooth muscle layers, and were electrophysiologically characterized as tension-sensitive mechanoreceptors with highly focal receptive fields (Zagorodnyuk,

Chen, & Brookes, 2001), whereas IMAs formed rectilinear terminal branches that run parallel to smooth muscle fibers and therefore are suggested to be stretch (or length) mechanoreceptors (Phillips & Powley, 2000; Powley & Phillips, 2011; Powley et al., 2008). Furthermore, anterograde tracing studies revealed vagal afferents composing IMAs within the longitudinal muscle layer to form the honeycombed network structures of lamellar neurites, called “antral web endings”, in subserosal tissue (SS) of the rat antral lesser curvature (Powley et al., 2012, 2013; Powley, Hudson, McAdams, Baronowsky, & Phillips, 2016).

Extracellular adenosine 5’-triphosphate (ATP) is one of the important excitatory transmitters/modulators in the peripheral nervous system (Burnstock, 2000; Nakatsuka & Gu, 2006). In the sensory system, ATP binds to ionotropic P2X purinoceptors to activate sensory nerves (Burnstock, 2000). Functionally, ATP plays a role in mechanosensory transduction via P2X3 homomeric and/or P2X2/P2X3 heteromeric purinoceptors in various organs including the urinary bladder, ureter, esophagus, and stomach (review see, Burnstock, 2009). Some P2X2 and/or P2X3 purinoceptors-expressing mechanosensitive nerve endings have been shown to be distributed in connective tissue associated with stretchable organs, such as carotid baroreceptor endings in the adventitial layer of the carotid sinus wall (Yokoyama, Settai, Nakamuta, & Yamamoto, 2019) and laminar nerve endings in the visceral pleura (Pintelon, Brouns, De Proost, Van Meir, Timmermans, & Adriaensen, 2007). A previous immunohistochemical study revealed that immunoreactivity for P2X2 purinoceptor was detected in IGLEs associated with myenteric ganglia in the mouse gastrointestinal tract including stomach (Castelucci, Robbins, & Furness, 2003). Furthermore, P2X3

purinoceptors are expressed in IGLEs and IMAs in the rat gastric smooth muscle layers (Xiang & Burnstock, 2004). However, it is unknown whether P2X purinoceptors-expressing nerve endings are distributed in SS in the rat stomach.

In several mechanoreceptors, such as Ruffini endings, lanceolate endings of the sinus hair, and carotid baroreceptors, axon terminals were ensheathed by S100- or S100B-immunoreactive terminal Schwann cells (Takahashi-Iwanaga, 2000; Takahashi-Iwanaga, Maeda, & Abe, 1997; Yokoyama, Settai, Nakamuta, & Yamamoto, 2019), and axonal spines or finger-like protrusions of the terminals extended through slits of these cells to the surrounding connective tissue for detecting mechanical stimuli (Maeda, Ochi, Nakakura-Ohshima, Youn, & Wakisaka, 1999; Nakakura-Ohshima, Maeda, Ohshima, Noda, & Takano, 1995; Takahashi-Iwanaga, 2000). Thus, the detailed morphological characteristics of subserosal afferent nerve terminals and the spatial relationship between nerve terminals and terminal Schwann cells are needed to analyze how the terminals detect mechanical stimuli in the rat stomach.

In the present study, we examined the distribution and morphology of P2X3-immunoreactive afferent nerve endings in the rat gastric subserosa using immunohistochemistry with confocal laser scanning microscopy. Since immunohistochemistry with whole-mount preparations has been widely used in morphological analyses of sensory nerve endings (Pintelon, Brouns, De Proost, Van Meir, Timmermans, & Adriaensen, 2007; Song et al., 2012; Suzuki, Ebara, Koike, Tonomura, & Kumamoto, 2012; Takahashi, Nakamura, & Yamamoto, 2016; Yamamoto & Nakamuta, 2018; Yokoyama, Settai, Nakamuta, & Yamamoto, 2019), we employed this method to demonstrate the morphological characteristics of nerve endings in the

gastric subserosa. We also attempted to elucidate the interrelationships between P2X3-immuhnoreactive nerve endings, visceral smooth muscle cells, and terminal Schwann cells. Furthermore, the origin of P2X3-immunoreactive nerve endings were examined by use of a combination of labeling with retrograde tract tracing using fast blue (FB) and immunohistochemistry.

2 MATERIALS AND METHODS

2.1 Animal procedure

All animal experiments in the present study were approved by and conducted under the authority of the Iwate Medical University Institutional Animal Care and Use Committee (accession number #30-026). Male Wistar rats (8-10 weeks old; totally n = 45) were purchased from Japan SLC, Inc. (Slc: Wistar, Japan SLC, Hamamatsu, Japan).

The procedures for preparing whole-mount preparations of the serosa with the smooth muscle layer of the stomach were conducted as previously reported with minor modifications (Powley et al., 2012, 2013). Thirty-three rats were transcardially perfused with Ringer's solution (200 ml) under deep anesthesia by an intraperitoneal pentobarbital injection (50 mg/kg), and the entire stomach was removed along with the thoracic esophagus and duodenal bulb. The stomach was trimmed by cutting at the gastroesophageal junction (lower esophageal sphincter region) and duodenal bulb, opened with fine scissors along the greater curvature from the pylorus to the cardiac notch (Figure 1a). Tissues were pinned on a silicone board, and then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 12-18 h. After washing with phosphate-buffered saline (PBS; pH 7.4), the gastric mucosa and submucosal tissue were removed using fine scissors under a binocular dissecting microscope in order to obtain whole-mount preparations of the serosa with the smooth muscle layer of the stomach. Whole-mount preparations were divided by cutting into three parts: the dorsal and ventral sides of the gastric fundus/corpus, and the gastric

antrum (Figure 1b). Regarding cryostat sections, five rats were anesthetized by pentobarbital and transcardially perfused with Ringer's solution (200 ml) followed by the same fixative (200 ml). The stomachs were dissected out and further fixed at 4 °C for 12-18 h. Tissues were soaked in PBS containing 30% sucrose and frozen at -80 °C with compound medium (Tissue-Tek O.C.T. compound, Sakura Finetech, Tokyo, Japan).

2.2 Immunohistochemistry

Whole-mount preparations of the stomach were stained by indirect immunofluorescence for P2X3. Preparations were incubated with non-immune donkey serum (1:50 dilution, S30, Merck Millipore, Billerica, MA) diluted with 0.01 M PBS containing 0.5% Triton X-100 (PBS-T, pH 7.4) at room temperature for 120 min. After incubation with normal donkey serum, preperations were incubated with rabbit polyclonal anti-P2X3 (Neuromics, Edina, MN) at 4 °C for at least 5 days. Preparations were then incubated with an Alexa 488-labeled donkey antibody against rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at 4 °C for 24 h after rinsing with PBS-T. To penetrate antibodies, PBS-T was used as a diluent. Preparations were then incubated with 4',6-diamidino-2-phenylindole (DAPI) solution (1 μg/ml; Dojindo, Kumamoto, Japan) for nuclear staining. Preparations were mounted on glass slides coated with chrome alum-gelatin and coverslipped with aqueous mounting medium (Fluoromount; Diagnostic Biosystems, Pleasanton, CA). Regarding double immunofluorescence, whole-mount preparations were incubated with a mixture of the antibody for P2X3 and

that for the P2X2 purinoceptor (P2X2; Neuromics), Alpha-smooth muscle actin (ASMA; Sigma-Aldrich, Saint Louis, MO), or S100B (Sigma-Aldrich). Antibodies for ASMA and S100B were used for the visualization of smooth muscle cells and terminal Schwann cells, respectively. Preperations were then incubated with a mixture of Alexa 488-labeled anti-rabbit IgG and Cy3-labeled donkey antibody against mouse IgG or guinea pig IgG (Jackson ImmunoResearch) at 4 °C for 24 h. The details of the primary antibodies used in the present study were described in Section 2.5 and tabulated in Table 1.

Regarding cryostat sections, stomachs were serially sectioned at 50 μm using a cryostat (CM 1900; Leica, Wetzlar, Germany) and collected in PBS. After incubation with normal donkey serum at room temperature for 30 min, free-floating sections were then incubated with a mixture of the antibody for P2X3 and that for ASMA at 4 °C for at least 2 days. Sections were subsequently incubated with a mixture of Alexa 488-labeled anti-rabbit IgG and Cy3-labeled anti-mouse IgG at room temperature for 2 h. Finally, free-floating sections were incubated with DAPI solution for nuclear staining, and mounted on glass slides coated with chrome alum-gelatin and coverslipped with aqueous mounting medium.

2.3 Observations

Preparations were examined with a confocal scanning laser microscope (C1; Nikon, Tokyo, Japan). Projection images were made from z-stacks of confocal images (3-48 series at 0.1-1 μm intervals) using computer software (NIS-elements; Nikon). Other

images were reconstructed in a three-dimensional view from intact confocal images of z-stack series or binary images converted from the original. All digital images were analyzed with the use of Photoshop CC (Adobe Systems, San José, CA) in addition to NIS-Elements.

2.4 Retrograde labeling

Seven rats were anesthetized by intraperitoneal injection of medetomidine hydrochloride (0.15 mg/kg), midazolam (2 mg/kg), and butorphanol tartrate (2.5 mg/kg), and a midline incision was made through the linea alba to expose the stomach. A retrograde tracer (0.4 μl of 2.5% FB in 10% dimethyl sulfoxide (FB; Polysciences, Warrington, PA) was injected into SS in the ventral aspect of the distal antrum at a distance of approximately 1 mm lateral to the sling muscle at three points using a glass micropipette (inner diameter of approximately 50 μm at the tip) connected to a Hamilton micro-syringe (Hamilton Syringe Company, Anaheim, CA). The injection site of FB was selected the area at which P2X3-immunoreactive nerve endings were observed. After surviving for 7 days, animals were fixed by transcardial perfusion with 4% paraformaldehyde as described above, and the jugular ganglia (JG), NG, and dorsal root ganglia (DRG) at Th8-Th11 were bilaterally dissected. The stomach was also collected to confirm dye injection sites. Twenty-micrometer-thick serial cryostat sections were stained by indirect immunofluorescence for P2X3. Epifluorescence microscopy (BX50; Olympus, Tokyo, Japan) was used to count FB-labeled neurons in all sections. Some sections containing FB-labeled neurons were photographed using a

confocal scanning laser microscope.

2.5 Antibody characterization

2.5.1 P2X3

A rabbit polyclonal anti-P2X3 antibody (RA10109; Neuromics, PRID AB_2157930) was raised against the synthetic peptide (VEKQSTDGAYSIGH) corresponding to amino acid residues 383-397 of the C-terminal region of rat P2X3. An immunoblotting analysis of this antibody labeled a band near 57-kDa in a rat DRG lysate (Xu & Huang, 2002), a 64-kDa band in a mouse DRG lysate (Cho & Chaban, 2012), and multiple glycosylated forms of P2X3 at 250- and 75-kDa (Hemmings-Mieszczak, Dorn, Natt, Hall, & Wishart, 2003). Immunohistochemical analyses of this antibody labeled ramified sensory nerve endings in the rat tracheal mucosa (Yamamoto & Nakamuta, 2018) and sensory nerve endings associated with type I cells in the rat carotid body (Yokoyama, Yamamoto, Hirakawa, Kato, & Saino, 2020). This antibody has also been used for immunohistochemistry to label ganglion neurons in the rat DRG (Huang, Greenwood, Thorne, & Housley, 2005; Vulchanova et al., 1997) and the rat trigeminal ganglion (Ichikawa & Sugimoto, 2004). In the present study, we performed a preabsorption test of the anti-P2X3 antibody for an immunohistochemical control. The antibody for P2X3 (1:1,000 dilution) was incubated with a synthetic polypeptide for P2X3 (2 μg/μl) at 4°C for 24 h. Thereafter, whole-mount preparations were incubated with the preabsorbed P2X3 antibody and an anti-P2X2 antibody, and processed for

double immunofluorescence as described above.

2.5.2 ASMA

A mouse monoclonal anti-ASMA antibody (clone 1A4; A2547; Sigma-Aldrich, PRID AB_476701) was produced using a mouse-derived hybridoma (Skalli et al., 1986). An immunoblotting analysis labeled a single band of 42-43 kDa in human gingival fibroblast cultures (Çomut, Shortkroff, Zhang, & Spector, 2000) and ovine fetal kidneys (Yang, Woolf, Quinn, & Winyard, 2001). This antibody has also been used for immunohistochemical analyses of smooth muscle cells in the rat bronchial artery (Jones, Jacobson, & Steudel, 1999) and the rat carotid sinus (Yokoyama, Settai, Nakamuta, & Yamamoto, 2019).

2.5.3 P2X2

A guinea-pig polyclonal anti-P2X2 antibody (GP14106; Neuromics, PRID AB_2299063) was raised against the synthetic peptide (DSTSTDPKGLAQL) corresponding to amino acid residues 460-472 of the C-terminal region of rat P2X2. An immunoblotting analysis showed a 62-kDa band in HEK cells transfected with a plasmid encoding the P2X2-FLAG protein or mouse brain tissue (Chaumont et al., 2008). Immunohistochemical analyses of this antibody labeled ramified intraepithelial endings in the laryngeal and tracheal mucosa (Takahashi, Nakamura, & Yamamoto, 2016; Yamamoto & Nakamuta, 2018), and sensory nerve endings associated with type I

cells in the rat carotid body (Yokoyama, Yamamoto, Hirakawa, Kato, & Saino, 2020). This antibody has also been used for immunohistochemistry to label cerebellar Purkinje cells and granule cells in the dentate gyrus of the mouse brain (Chaumont et al., 2008).

2.5.4 S100B

A mouse monoclonal anti-S100B antibody (clone SH-B1; S2532; Sigma-Aldrich, PRID AB_477499) recognized a single band of 11 kDa by immunoblotting in the rat peripheral nerves (Liao, Wang, Huang, & Tseng, 2013). This antibody has also been used for immunohistochemical analyses to label astrocytes of the rat brain (Henriksson & Tjälve, 2000) and terminal Schwann cells associated with baroreceptor endings in the rat carotid sinus (Yokoyama, Settai, Nakamuta, & Yamamoto, 2019).

3 RESULTS

3.1 Morphology of P2X3-immunoreactive subserosal nerve endings

In whole-mount preparations, P2X3-immunoreactive nerve endings were observed in SS of the gastric antrum, but not in the dorsal or ventral sides of the gastric fundus and corpus (Figure 2). P2X3-immunoreactive subserosal nerve endings consisted of web-like complex structures and peripheral variform axon terminals. The P2X3-immunoreactive parent axon of the nerve endings was relatively thin in diameter (approximately 1-2 μm), and was ramified repeatedly to form honeycombed- or net-like complicated structures with lamelliform processes (Figure 2a). In the periphery of the net-like terminal structures, the lamellar processes terminated with pleomorphic, looped, or flattened axon terminals. A high magnification image showed that axon terminals of the nerve endings contained punctate P2X3-immunoreactive products (Figure 2b). The area at which the P2X3-immunoreactive nerve endings occupied was ranged from 0.002 to 0.45 mm^2, and the average was 0.074 mm^2 (55 nerve endings from 21 preparations). The distribution of P2X3-immunoreactive nerve endings was confined to a limited area of the gastric distal antrum in the lesser curvature at a distance of approximately 1 mm lateral to both sides of sling muscles (Figure 2c). The range of area at which the nerve endings distributed was 4.1-4.5 mm in the major axis and 3.3-3.7 mm in the minor axis. In our experiment, no P2X3-immunoreactive products were observed in any regions of P2X2-immunoreactive subserosal nerve endings of the gastric antrum when a preabsorbed antibody was used (2 μg/μl; The Supporting Information Figure S1a-c). In

free-floating cryostat sections of the antral lesser curvature, P2X3-immunoreactive nerve endings were located in SS, but not in longitudinal muscle (LM) and circular muscle (CM) layers, that were characterized by several layers of visceral smooth muscle cells immunoreactive for ASMA (Figure 2d). As observed in whole-mount preparations, P2X3-immunoreactive nerve fibers arborized net-like terminal structures in SS, but did not enter ASMA-immunoreactive smooth muscle cell layers.

In some cases, large P2X3-immunoreactive nerve endings distributing 0.45 mm^2 emerged from several parent axons (Figure 3). Some P2X3-immunoreactive parent axons entered SS from the longitudinal smooth muscle layer, and formed lamelliform processes of the net-like terminal structures without branching, whereas others ramified into several axonal branches and formed processes. The lamelliform processes derived from each parent axons appeared to tightly intertwine to form the net-like terminal structures.

Occasionally, P2X3-immunoreactive net-like terminal structures were formed by beaded thin nerve fibers with numerous small oval or polygonal bulges (Figure 4a). Higher magnifications showed filiform and pleomorphic protrusions with P2X3 immunoreactivity of the beaded thin fibers (Figure 4b). In other cases, P2X3-immunoreactive thin parent axons arborized to form tree-like terminal structures with flat leaf-like axon terminals (Figure 4c). At a higher magnification, pleomorphic or lamelliform protrusions arose from the flat leaf-like terminals (Figure 4d).

In whole-mount preparations stained by double immunofluorescence for P2X3 and ASMA, P2X3-immunoreactive lamelliform nerve fibers composing net-like terminal structures extended in every direction, regardless of the axis of the longitudinal

smooth muscle layer with ASMA immunoreactivity (Figure 5a). Three-dimensional reconstruction views revealed that P2X3-immunoreactive net-like complex structures distributed two-dimensionally in SS on the surface of the ASMA-immunoreactive longitudinal smooth muscle layer (Figure 5b). A projection view and 3D reconstructed figure showed that branched net-like processes projected club-like and knob-like protrusions, and terminated with flat leaf-like axon terminals (Figure 5c, d). DAPI-labeled oval cell nuclei were observed in gaps between net-like processes immunoreactive for P2X3.

In whole-mount preparations stained with double immunofluorescence for P2X3 and P2X2, weak P2X2 immunoreactivity was observed in P2X3-immunoreactive net-like terminal structures and their pleomorphic or looped axon terminals (Figure 6a-c). Punctate P2X2 immunoreactivity was weak or not observed in P2X3-immunoreactive lamelliform processes of the net-like structures (Figure 6d-f). P2X3-immunoreactive net-like structures surrounded several DAPI-labeled oval cell nuclei. Higher magnifications of the net-like structures showed that flattened processes contained numerous P2X3-immunoreactive puncta, and some immunoreactive punctate products were co-localized with P2X2-immunoreacive puncta (Figure 6g-i). P2X3-immunoreactive flattened processes with some punctate products with P2X2 immunoreactivity surrounded oval cell nuclei.

In double immunofluorescence for P2X3 and S100B, P2X3-immunoreactive beaded nerve fibers of net-like terminal structures partially surrounded terminal Schwann cells with S100B immunoreactivity (Figure 7a-c). S100B-immunoreactive terminal Schwann cells consisted of a perinuclear region with an oval nucleus and

elongated cytoplasmic processes. Some oval cell nuclei of putative fibroblast without S100B immunoreactivity were also observed around P2X3-immunoreactive net-like terminal structures. In the peripheral regions of web-like network structures, P2X3-immunoreactive axon terminals surrounded terminal Schwann cells immunoreactive for S100B (Figure 7d-f). Higher magnification views showed that hederiform terminals immunoreactive for P2X3 twined around the perinuclear cytoplasm and slender cytoplasmic processes of S100B-immunoreactive terminal Schwann cells (Figure 7g-i). Small pleomorphic and thread-like protrusions immunoreactive for P2X3 arose from the hederiform terminals.

3.2 Retrograde tracer study

After the injection of FB retrograde tracer into SS in the ventral aspect of the antral lesser curvature corresponding with the distribution of P2X3-immunoreactive afferent nerve endings, FB-labeled neurons were observed bilaterally in the NG and DRG at the levels of Th8-Th11, but not in the JG (Table 2; Figure 8). In sections of the antral lesser curvature including the FB injected site, most of the injected dye was observed within SS, and only a small amount had diffused to the longitudinal muscle layer (Figure not shown). Some FB-labeled neurons in the NG were also immunoreactive for P2X3 (Figure 8a-c). FB-labeled neurons with P2X3 immunoreactivity were small and medium in size (14-39 μm in diameter, 52 neurons from 18 tissue sections). FB-labeled neurons immunoreactive for P2X3 were not observed in the DRG at Th8-Th11 (Figure 8d-f). Based on experiments on seven rats, a total of 1,291 and 172 neurons were labeled with

FB in the left and right sides of the NG, respectively. Of these FB-labeled neurons, 490/1,291 (38.0%) and 62/172 (36.0%) cells were also immunoreactive for P2X3 (Figure 8g). In the DRG at Th8-Th11, a total of 432 and 787 neurons were labeled with FB in the left and right sides of these ganglia, respectively. However, a total of 1,219 FB-labeled neurons in both sides of the DRG at Th8-Th11 were not immunoreactive for P2X3.

4 DISCUSSION

The present study demonstrated that P2X3-immunoreactive nerve endings were distributed in the SS of the distal antrum in the lesser curvature, particularly in the lateral region of the gastric sling muscles, the putative oblique muscles that span between the gastroesophageal junction and the region of the antropyloric junction in the rat stomach (Powley et al., 2013). Furthermore, P2X3-immunoreactive nerve endings shown in Figures 2, 3, 5, and 6, formed honeycombed- or net-like terminal structures with ramified lamelliform processes, and terminated with pleomorphic axon terminals in the edge of net-like structures. The restricted distribution and characteristic morphology of these P2X3-immunoreactive nerve endings were consistent with those of antral web endings, which have been detected by anterograde tracing into the rat NG (Powley et al., 2012, 2013). Thus, some P2X3-immunoreactive subserosal nerve endings observed in the present study should be the antral web endings as previously reported. However, other P2X3-immunoreactive nerve endings appeared to be morphologically distinct from the antral web endings: net-like terminal structures formed by beaded thin fibers (Figures 4a, b, and 7) and tree-like terminal structures with flat leaf-like axon terminals (Figure 4c, d). These P2X3-immunoreactive subserosal nerve endings may be the morphological subtype of the antral web endings.

P2X3-immunoreactive lamellar processes of subserosal nerve endings extended in every direction on the surface of longitudinal smooth muscle layers, unlike muscle spindle afferents and vagal afferent IMAs that are arranged in axes with intrafusal muscle fibers and gastric smooth muscle layers, respectively (Powley et al., 2013;

Schoultz & Swett, 1974). Furthermore, two-dimensionally extended net-like terminal structures formed by multiple parent axons of P2X3-immunoreactive nerve endings have been shown to resemble mechanosensory endings such as the laminar endings in the laryngeal mucosa (Soda & Yamamoto, 2012), and sensory endings in airway smooth muscle (Brouns et al., 2006) and visceral pleura (Pintelon, Brouns, De Proost, Van Meir, Timmermans, & Adriaensen, 2007). Since antral web endings were formed by a subset (41%) of vagal afferents forming IMAs in the longitudinal muscle layers, as shown by anterograde tracing into the rat NG (Powley et al., 2012; Powley, Hudson, McAdams, Baronowsky, & Phillips, 2016), P2X3-immunoreactive subserosal nerve endings may be a subtype of vagal mechanoreceptors in the distal antrum of the lesser curvature. Video spatiotemporal mapping techniques using *ex vivo* preparations of the rat stomach have revealed that intraluminal infusion of saline induced-antral peristalsis dynamically changes the area strain rate of the distal antrum in the lesser curvature (Lentle, Reynolds, Hulls, & Chambers, 2016). Thus, the restricted distribution of P2X3-immunoreactive nerve endings may be suitable for receiving a deformation of the distal antral wall in every direction, which is produced by contraction and distension during antral peristalsis. Since antral peristalsis is responsible for the trituration, mixing, and emptying of gastric contents, P2X3-immunoreactive subserosal nerve endings may be activated under these digestive processes. The total number of IGLEs and the volume of muscle sheets innervated by IMAs in the gastric antrum were smaller than those of the fundus and corpus, as shown by anterograde tracing into the rat NG (Berthoud, Patterson, Neumann, & Neuhuber, 1997; Powley, Hudson, McAdams, Baronowsky, & Phillips, 2016). These findings suggest that large net-like structures of P2X3-

immunoreactive subserosal nerve endings compensate for the mechanoreceptive field during antral peristalsis. The most prominent characteristics of P2X3-immunoreactive subserosal nerve endings are the net-like terminal structures with pleomorphic, looped, or flat leaf-like axon terminals. P2X3 immunoreactivity expressed in the net-like terminal structures and variform terminals suggest that subserosal afferent endings are activated by ATP. Therefore, the mesh-like planar structures with variform axon terminals of P2X3-immunoreactive nerve endings may enlarge the receptive area in SS covering the distal antral wall in the lesser curvature, and multiple parent axons may play a role in the summation of sensory impulses from lamelliform processes forming net-like structures. In the mechanoreceptors of tracheal smooth muscles, sensory nerve endings were located in smooth muscle bundles in order to respond to the stretch of smooth muscle produced by tracheal pressure changes (Baluk & Gabella, 1991; Brouns et al., 2006). However, P2X3-immunoreactive nerve endings were located in SS, but did not make contact with ASMA-immunoreactive smooth muscle cells in the gastric antrum of the lesser curvature. Since sensory nerve endings were surrounded by connective tissue fibers in SS, P2X3-immunoreactive nerve endings may be indirectly activated by the stretching of smooth muscle layers via connective tissue fibers.

Variform axon terminals of P2X3-immunoreactive subserosal nerve endings extended protrusions in various shapes, such as filiform, club-, or knob-like protrusions. Electron microscopic studies of other mechanoreceptors, such as lanceolate endings and periodontal Ruffini endings, have revealed that axon terminals protrude finger- or thread-like projections into the surrounding collagen mesh in order to respond to mechanical stimuli (Nakakura-Ohshima, Maeda, Ohshima, Noda, & Takano, 1995;

Takahashi-Iwanaga, 2000). These findings suggest that protrusions arising from axon terminals are important morphological features for detecting mechanical stimuli in mechanoreceptors. Since P2X3-immunoreactive terminals extended around putative terminal Schwann cell nuclei and terminated as pleomorphic and thread-like protrusions in the surrounding tissues, these protrusions may also be suitable for receiving mechanosensory signals in the gastric antrum of the lesser curvature.

P2X2 immunoreactivity has been observed in the axon terminals of various P2X3-immunoreactive sensory endings: ramified intraepithelial endings in the laryngeal and tracheal mucosa (Takahashi, Nakamura, & Yamamoto, 2016; Yamamoto & Nakamuta, 2018), ramified endings in lung neuroepithelial bodies (Brouns et al., 2006), IGLEs in the gastrointestinal tract (Wang & Neuhuber, 2003; Xiang & Burnstock, 2004), arterial baroreceptors (Song et al., 2012; Yokoyama, Settai, Nakamuta, & Yamamoto, 2019), and nerve endings in the taste buds (Ishida et al., 2009). However, P2X2-immunoreactivity was weak or not observed in P2X3-immunoreactive subserosal nerve endings. Biochemical analyses revealed that P2X3 molecules in sensory neurons formed homometric and heterometric channels with P2X2 (Dunn, Zhong, & Burnstock, 2001). Weak or the lack of P2X2 immunoreactivity indicates that the P2X3 homomeric and P2X2/P2X3 heteromeric channels are both expressed in P2X3-immunoreactive nerve endings of the antral lesser curvature.

Previous immunohistochemical studies have revealed that terminal Schwann cells tightly surround axon terminals of mechanoreceptors, such as Ruffini endings in the periodontal ligaments (Maeda, Ochi, Nakakura-Ohshima, Youn, & Wakisaka, 1999; Takahashi-Iwanaga, Maeda, & Abe, 1997), laminar endings in the laryngeal mucosa

(Soda & Yamamoto, 2012), and arterial baroreceptor endings in the aortic arch and carotid sinus (Krauhs, 1979; Yokoyama, Settai, Nakamuta, & Yamamoto, 2019). In P2X3-immunoreactive subserosal nerve endings, axon terminals surrounded these cells and projected pleomorphic and thread-like protrusions to the surrounding tissues. Therefore, P2X3-immunoreactive axon terminals may mainly receive mechanosensory signals via protrusions in various shapes. However, P2X3-immunoreactive net-like structures and axon terminals made contact with terminal Schwann cells, suggesting ATP-mediated transmission between them. Terminal Schwann cells associated with lanceolate endings have been involved in glia-neuronal interactions via ATP, although the exact function of these cells is still unknown (Takahashi-Iwanaga & Habara, 2004). It is speculated that P2X3-immunoreactive subserosal nerve endings are activated by ATP released from terminal Schwann cells during deformation of the antral lesser curvature.

Previous retrograde tracing studies have revealed that primary afferents innervating the rat stomach derived from bilateral DRG at Th4-L1, with accumulation at Th8-Th11 (Neuhuber & Niederle, 1979; Ozaki & Gebhart, 2001; Su, Bishop, Power, Hamada, & Polak, 1987). The results of retrograde tracing with FB suggest that P2X3-immunoreactive subserosal nerve endings of the antral lesser curvature are derived from the NG, but not from the DRG at Th8-Th11, and the results was consistent with previous observations of antral web endings obtained from rats that received injections of an anterograde tracer into the NG (Powley et al., 2012, 2013). Since the ventral aspect of the antral lesser curvature was mainly innervated by the ventral (anterior) gastric branch derived from the left NG, as shown by anterograde tracing (Wang,

Young, & Kao, 2012), the larger number of FB-labeled neurons immunoreactive for P2X3 in the left NG than the right NG may be attributed to the laterality of vagal afferent innervation in the rat gastric antrum. On the other hand, FB-labeled NG neurons without P2X3 immunoreactivity appeared to express another chemical coding, for example, $GABA_A$ receptors, cholecystokinin A receptor, and neuronal nitric synthase, as reported by retrograde tracing combined with immunohistochemistry in NG neurons innervating the stomach (Page et al., 2009; Patterson, Zheng, & Berthoud, 2002; Smid, Young, Cooper, & Blackshaw, 2001).

In the present study, we demonstrated that P2X2-/P2X3-immunoreactive nerve endings had two-dimensionally-extended net-like terminal structures with pleomorphic flattened axon terminals in SS of the antral lesser curvature, and originated from NG neurons. The morphology of these endings may be suitable for receiving the mechanical deformation of the distal antral wall associated with antral peristalsis during gastric mixing and emptying. Furthermore, the net-like structures associated with axon terminals may interact with terminal Schwann cells via ATP for the regulation of mechanoreceptive function. Further studies on the electrophysiological and pharmacological properties of nerve endings and terminal Schwann cells are needed in order to clarify the precise functions of ATP in P2X2-/P2X3-immmunoreactive subserosal nerve endings in the rat antral lesser curvature.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for its integrity and the accuracy of the data analysis. Study concept and design: TY. Acquisition of data: MH, TY. Analysis and interpretation of data: MH, TY, YY, TS. Drafting of the article: MH. Critical revision of the manuscript for important intellectual content: TY.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Andrews, P. L., Grundy, D., & Lawes, I. N. (1980). The role of the vagus and splanchnic nerves in the regulation of intragastric pressure in the ferret. *The Journal of Physiology*, 307, 401-411.

Baluk, P., & Gabella, G. (1991). Afferent nerve endings in the tracheal muscle of guinea-pigs and rats. *Anatomy and Embryology*, 183, 81-87.

Berthoud, H. R., Patterson, L. M., Neumann, F., & Neuhuber, W. L. (1997). Distribution and structure of vagal afferent intraganglionic laminar endings (IGLEs) in the rat gastrointestinal tract. *Anatomy and Embryology*, 195, 183-191.

Berthoud, H. R., & Powley, T. L. (1992). Vagal afferent innervation of the rat fundic stomach: morphological characterization of the gastric tension receptor. *The Journal of Comparative Neurology*, 319, 261-276.

Brouns, I., Pintelon, I., De Proost, I., Alewaters, R., Timmermans, J. P., & Adriaensen, D. (2006). Neurochemical characterisation of sensory receptors in airway smooth muscle: comparison with pulmonary neuroepithelial bodies. *Histochemistry Cell Biology*, 125, 351-367.

Burnstock, G. (2000). P2X receptors in sensory neurons. *British Journal of Anaesthesia*, 84, 476-488.

Burnstock, G. (2009). Purines and sensory nerves. *Handbook of Experimental Pharmacology*, 194, 333-392.

Cannon, W. B., & Lieb, C. W. (1913). The receptive relaxation of the stomach. *American Journal of Physiology*, 29, 267-273.

Castelucci, P., Robbins, H. L., & Furness, J. B. (2003). $P2X_2$ purine receptor immunoreactivity of intraganglionic laminar endings in the mouse gastrointestinal tract. *Cell and Tissue Research, 312*, 167-174.

Chaumont, S., Compan, V., Toulme, E., Richler, E., Housley, G. D., Rassendren, F., & Khakh, B. S. (2008). Regulation of P2X2 receptors by the neuronal calcium sensor VILIP1. *Science Signaling*, 1, ra8.

Cho, T., & Chaban, V. V. (2012). Interaction between P2X3 and oestrogen receptor (ER)α/ERβ in ATP-mediated calcium signalling in mice sensory neurones. *Journal of Neuroendocrinology*, 24, 789-797.

Çomut, A. A., Shortkroff, S., Zhang, X., & Spector, M. (2000). Association of fibroblast orientation around titanium in vitro with expression of a muscle actin. *Biomaterials*, 21, 1887-1896.

Dunn, P. M., Zhong, Y., & Burnstock, G. (2001). P2X receptors in peripheral neurons. *Progress in Neurobiology*, 65, 107-134.

Hemmings-Mieszczak, M., Dorn, G., Natt, F. J., Hall, J., & Wishart, W. L. (2003). Independent combinatorial effect of antisense oligonucleotides and RNAi-mediated specific inhibition of the recombinant rat P2X3 receptor. *Nucleic Acids Research*, 31, 2117-2126.

Henriksson, J., & Tjälve, H. (2000). Manganese taken up into the CNS via the olfactory pathway in rats affects astrocytes. *Toxicological Sciences : an Official Journal of the Society of Toxicology*, 55, 392-398.

Huang, L. C., Greenwood, D., Thorne, P. R., & Housley, G. D. (2005). Developmental regulation of neuron-specific P2X3 receptor expression in the rat cochlea. *The Journal of Comparative Neurology*, 484, 133-143.

Ichikawa, H., & Sugimoto, T. (2004). The co-expression of P2X3 receptor with VR1 and VRL-1 in the rat trigeminal ganglion. *Brain Research*, 998, 130-135.

Ishida, Y., Ugawa, S., Ueda, T., Yamada, T., Shibata, Y., Hondoh, A., Inoue, K., Yu, Y., & Shimada, S. (2009). P2X2- and P2X3-positive fibers in fungiform papillae originate from the chorda tympani but not the trigeminal nerve in rats and mice. *The Journal of Comparative Neurology*, 514, 131-144.

Jones, R., Jacobson, M., & Steudel, W. (1999). α-smooth-muscle actin and microvascular precursor smooth-muscle cells in pulmonary hypertension. *American Journal of Respiratory Cell and Molecular Biology*, 20, 582-594.

Krauhs, J. M. (1979). Structure of rat aortic baroreceptors and their relationship to connective tissue. *Journal of Neurocytology*, 8, 401-414.

Lang, H., Li, M., Kilpatrick, L. A., Zhu, J., Samuvel, D. J., Krug, E. L., & Goddard, J. C. (2011). Sox2 up-regulation and glial cell proliferation following degeneration of spiral ganglion neurons in the adult mouse inner ear. *Journal of the Association for Research in Otolaryngology*, 12, 151-171.

Lentle, R., Reynolds, G. W., Hulls, C. M., & Chambers, J. P. (2016). Advanced spatiotemporal mapping methods give new insights into the coordination of contractile activity in the stomach of the rat. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 311, G1064-G1075.

Liao, W. C., Wang, Y. J., Huang, M. C., & Tseng, G. F. (2013). Methylcobalamin facilitates collateral sprouting of donor axons and innervation of recipient muscle in end-to-side neurorrhaphy in rats. *PLoS One*, 8, e76302.

Maeda, T., Ochi, K., Nakakura-Ohshima, K., Youn, S. H., & Wakisaka, S. (1999). The Ruffini ending as the primary mechanoreceptor in the periodontal ligament: its morphology, cytochemical features, regeneration, and development. *Critical Reviews in Oral Biology and Medicine : an Official Publication of the American Association of Oral Biologists*, 10, 307-327.

Nakakura-Ohshima, K., Maeda, T., Ohshima, H., Noda, T., & Takano, Y. (1995). Postnatal development of periodontal ruffini endings in rat incisors: an immunoelectron microscopic study using protein gene product 9.5 (PGP 9.5) antibody. *The Journal of Comparative Neurology*, 362, 551-564.

Nakatsuka, T., & Gu, J. G. (2006). P2X purinoceptors and sensory transmission. *Pflügers Archiv - European journal of physiology*, 452, 598-607.

Neuhuber, W., & Niederle, B. (1979). Spinal ganglion cells innervating the stomach of the rat as demonstrated by somatopetal transport of horseradish peroxidase (HRP). *Anatomy and Embryology,* 155, 355-362.

Ozaki, N., & Gebhart, G. F. (2001). Characterization of mechanosensitive splanchnic nerve afferent fibers innervating the rat stomach. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 281, G1449-G1459.

Ozaki, N., Sengupta, J. N., & Gebhart, G. F. (1999). Mechanosensitive properties of gastric vagal afferent fibers in the rat. *Journal of Neurophysiology*, 82, 2210-2220.

Page, A. J., O'Donnell, T. A., Cooper, N. J., Young, R. L., & Blackshaw, L. A. (2009). Nitric oxide as an endogenous peripheral modulator of visceral sensory neuronal function. *The Journal of Neuroscience* 29, 7246-7255.

Patterson, L. M., Zheng, H., & Berthoud, H. R. (2002). Vagal afferents innervating the gastrointestinal tract and CCKA-receptor immunoreactivity. *The Anatomical Record*, 266, 10-20.

Phillips, R. J., & Powley, T. L. (2000). Tension and stretch receptors in gastrointestinal smooth muscle: re-evaluating vagal mechanoreceptor electrophysiology. *Brain Research*, 34, 1-26.

Pintelon, I., Brouns, I., De Proost, I., Van Meir, F., Timmermans, J. P., & Adriaensen, D. (2007). Sensory receptors in the visceral pleura: neurochemical coding and live staining in whole mounts. *American Journal of Respiratory Cell and Molecular Biology*, 36, 541-551.

Powley, T. L., Baronowsky, E. A., Gilbert, J. M., Hudson, C. N., Martin, F. N., Mason, J. K., McAdams, J. L., & Phillips, R. J. (2013). Vagal afferent innervation of the lower esophageal sphincter. *Autonomic Neuroscience : Basic & Clinical,* 177, 129-142.

Powley, T. L., Gilbert, J. M., Baronowsky, E. A., Billingsley, C. N., Martin, F. N., & Phillips, R. J. (2012). Vagal sensory innervation of the gastric sling muscle and antral wall: implications for gastro-esophageal reflux disease? *Neurogastroenterology and Motility*, 24, e526-e537.

Powley, T. L., Hudson, C. N., McAdams, J. L., Baronowsky, E. A., & Phillips, R. J. (2016). Vagal Intramuscular Arrays: The specialized mechanoreceptor arbors that

innervate the smooth muscle layers of the stomach examined in the rat. *The Journal of Comparative Neurology*, 524, 713-737.

Powley, T. L., & Phillips, R. J. (2011). Vagal intramuscular array afferents form complexes with interstitial cells of Cajal in gastrointestinal smooth muscle: analogues of muscle spindle organs? *Neuroscience*, 186, 188-200.

Powley, T. L., Wang, X. Y., Fox, E. A., Phillips, R. J., Liu, L. W., & Huizinga, J. D. (2008). Ultrastructural evidence for communication between intramuscular vagal mechanoreceptors and interstitial cells of Cajal in the rat fundus. *Neurogastroenterology and Motility*, 20, 69-79.

Schoultz, T. W., & Swett, J. E. (1974). Ultrastructural organization of the sensory fibers innervating the Golgi tendon organ. *The Anatomical Record*, 179, 147-162.

Sengupta, J. N., & Gebhart, G. F. (1994). Characterization of mechanosensitive pelvic nerve afferent fibers innervating the colon of the rat. *Journal of Neurophysiology,* 71, 2046-2060.

Skalli, O., Ropraz, P., Trzeciak, A., Benzonana, G., Gillessen, D., & Gabbiani, G. (1986). A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *The Journal of Cell Biology*, 103, 2787-2796.

Smid, S. D., Young, R. L., Cooper, N. J., & Blackshaw, L. A. (2001). $GABA_BR$ expressed on vagal afferent neurones inhibit gastric mechanosensitivity in ferret proximal stomach. *American Journal of physiology. Gastrointestinal and Liver Physiology*, 281, G1494-G1501.

Soda, Y., & Yamamoto, Y. (2012). Morphology and chemical characteristics of subepithelial laminar nerve endings in the rat epiglottic mucosa. *Histochemistry and Cell Biology*, 138, 25-39.

Song, X., Gao, X., Guo, D., Yu, Q., Guo, W., He, C., Burnstock, G., & Xiang, Z. (2012). Expression of P2X2 and P2X3 receptors in the rat carotid sinus, aortic arch, vena cava, and heart, as well as petrosal and nodose ganglia. *Purinergic Signalling*, 8, 15-22.

Su, H. C., Bishop, A. E., Power, R. F., Hamada, Y., & Polak, J.M. (1987). Dual intrinsic and extrinsic origins of CGRP- and NPY-immunoreactive nerves of rat gut and pancreas. *The Journal of Neuroscience*, 7, 2674-2687.

Suzuki, M., Ebara, S., Koike, T., Tonomura, S., & Kumamoto, K. (2012). How many hair follicles are innervated by one afferent axon? A confocal microscopic analysis of palisade endings in the auricular skin of thy1-YFP transgenic mouse. *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences*, 88, 583-595.

Takahashi-Iwanaga, H. (2000). Three-dimensional microanatomy of longitudinal lanceolate endings in rat vibrissae. *The Journal of Comparative Neurology*, 426, 259-269.

Takahashi-Iwanaga, H., & Habara, Y. (2004). Oscillatory calcium responses mediated by P2Y2 purinergic receptors in terminal Schwann cells of longitudinal lanceolate endings isolated from rat vibrissae. *The Journal of Comparative Neurology*, 475, 416-425.

Takahashi-Iwanaga, H., Maeda, T., & Abe, K. (1997). Scanning and transmission electron microscopy of Ruffini endings in the periodontal ligament of rat incisors. *The Journal of Comparative Neurology*, 389, 177-184.

Takahashi, N., Nakamuta, N., & Yamamoto, Y. (2016). Morphology of P2X3-immunoreactive nerve endings in the rat laryngeal mucosa. *Histochemistry and Cell Biology*, 145, 131-146.

Vulchanova, L., Riedl, M. S., Shuster, S. J., Buell, G., Surprenant, A., North, R. A., & Elde, R. (1997). Immunohistochemical study of the P2X2 and P2X3 receptor subunits in rat and monkey sensory neurons and their central terminals. *Neuropharmacology*, 36, 1229-1242.

Wang, F. B., Young, Y. K., & Kao, C. K. (2012). Abdominal vagal afferent pathways and their distributions of intraganglionic laminar endings in the rat duodenum. *The Journal of Comparative Neurology*, 520, 1098-1113.

Wang, Z. J., & Neuhuber, W. L. (2003). Intraganglionic laminar endings in the rat esophagus contain purinergic P2X2 and P2X3 receptor immunoreactivity. *Anatomy and Embryology*, 207, 363-371.

Xiang, Z., & Burnstock, G. (2004). Development of nerves expressing P2X3 receptors in the myenteric plexus of rat stomach. *Histochemistry and Cell Biology*, 122, 111-119.

Xu, G. Y., & Huang, L. Y. (2002). Peripheral inflammation sensitizes P2X receptor-mediated responses in rat dorsal root ganglion neurons. *The Journal of Neuroscience*, 22, 93-102.

Yamamoto, Y., & Nakamuta, N. (2018). Morphology of P2X3-immunoreactive nerve endings in the rat tracheal mucosa. *The Journal of Comparative Neurology*, 526, 550-566.

Yang, S. P., Woolf, A. S., Quinn, F., & Winyard, P. J. (2001). Deregulation of renal transforming growth factor-beta1 after experimental short-term ureteric obstruction in fetal sheep. *The American Journal of Pathology*, 159, 109-117.

Yokoyama, T., Saino, T., Nakamuta, N., Kusakabe, T., & Yamamoto, Y. (2016). Three-dimensional architectures of P2X2-/P2X3-immunoreactive afferent nerve terminals in the rat carotid body as revealed by confocal laser scanning microscopy. *Histochemistry and Cell Biology*, 146, 479-488.

Yokoyama, T., Settai, K., Nakamuta, N., & Yamamoto, Y. (2019). Distribution and morphology of baroreceptors in the rat carotid sinus as revealed by immunohistochemistry for P2X3 purinoceptors. *Histochemistry and Cell Biology,* 151, 161-173.

Yokoyama, T., Yamamoto, Y., Hirakawa, M., Kato, K., & Saino, T. (2020). Vesicular nucleotide transporter-immunoreactive type I cells associated with P2X3-immunoreactive nerve endings in the rat carotid body. *The Journal of Comparative Neurology*, 528, 1486-1501.

Zagorodnyuk, V. P., Chen, B. N., & Brookes, S. J. (2001). Intraganglionic laminar endings are mechano-transduction sites of vagal tension receptors in the guinea-pig stomach. *The Journal of Physiology*, 534, 255-268.

FIGURE LEGENDS

FIGURE 1 Schematic drawing of a whole-mount preparation of the smooth muscle layer with serosa of the stomach. Cut lines are indicated by red-colored dashed lines. (a) A ventral view of the stomach. The stomach is separated from the gastroesophageal junction and the duodenal bulb, and is opened along the greater curvature from the pylorus to the cardiac notch. (b) A serosal side of the stomach, opened on the greater curvature. A whole-mount preparation is cut into three parts: dorsal and ventral sides of the gastric fundus/corpus, and gastric antrum

FIGURE 2 Morphology and distribution of P2X3-immunoreactive subserosal nerve endings in the rat gastric antrum of the lesser curvature. The projection images are made from 11 images at 1 μm intervals in (a) and 16 images at 0.4 μm intervals in (d), respectively. (a) P2X3-immunoreactive subserosal nerve endings in a whole-mount preparation of the antral lesser curvature. A parent axon (*arrow*) diverges into several lamelliform processes to form web-like structures with pleomorphic (*arrowheads*) and looped (*small arrowhead*) axon terminals. *A small arrow* indicates the branching points of the parent axon. (b) A higher magnification of the *small arrowhead* in panel (a) shows the P2X3-immunoreactive puncta in the looped axon terminals. (c) Distribution of P2X3-immunoreactive nerve endings in a whole-mount preparation. P2X3-immunoreactive subserosal nerve endings (filled circles) are densely distributed lateral to the gastric sling muscle bundles in the distal antrum of the lesser curvature. (d) A free-floating cryostat section of the antral lesser curvature immunolabeled for P2X3 and

ASMA. P2X3-immunoreactive nerve endings are distributed in subserosal tissue (SS), but do not make contact with ASMA-immunoreactive smooth muscle cells in the longitudinal muscle (LM) and circular muscle (CM) layers. *Small arrowheads* indicate elongated nuclei of mesothelial cells in the visceral peritoneum. In (d), nuclei are labeled by DAPI (*blue*)

FIGURE 3 Axonal ramification of P2X3-immunoreactive subserosal nerve endings in a whole-mount preparation of the gastric antrum. (a, b) Projection view of confocal images (a) from 10 images at 0.3 μm intervals and trace drawings (b) of a P2X3-immunoreactive nerve ending. Two P2X3-immunoreactive parent axons (*arrows*) terminate in large net-like complicated structures without branching, whereas the other (*arrowhead*) ramifies into several axonal branches and terminates. *Asterisks* indicate the branching points of the parent axon. (b) A P2X3-immunoreactive nerve ending and the parent axons are indicated by black- and red-colored lines, respectively

FIGURE 4 Variations in P2X3-immunoreactive subserosal nerve endings in whole-mount preparations. Projection images are made from 10 images in (a-c) and 7 images in (d) at 1 μm intervals, respectively. (a, b) P2X3-immunoreactive web-like structures composed of beaded thin nerve fibers. (a) *Arrows* and *asterisk* indicate parent axons and the branching point of the web-like network structures, respectively. (b) Higher magnification views of the rectangles in panel (a) show that hederiform nerve fibers with numerous small bulges project pleomorphic and thread-like protrusions. (c, d) Tree-like terminal structures with P2X3-immunoreactive flat leaf-like terminals. (c)

Arrows indicate thin parent axons of the ending. (d) Higher magnification views of *an arrowhead* in panel (c) show that flat leaf-like terminals project pleomorphic protrusions (*arrows*)

FIGURE 5 (a, b) Double immunofluorescence for P2X3 and ASMA in a whole-mount preparation. A projection image (a) and three-dimensional reconstruction view (b) are made from 48 and 12 images at 0.2 μm intervals, respectively. P2X3-immunoreactive nerve fibers of net-like structures extend two-dimensionally in every direction on the longitudinal muscle layer with ASMA immunoreactivity. (c, d) A projection view (c) and three-dimensional reconstructed view from a binary image of z-stacks (d) of the rectangles in panel (a) show the net-like terminal region of the P2X3-immunoreactive nerve endings. Net-like processes project flattened axon terminals (*large arrow*), club-like (*small arrows*), and knob-like (*arrowheads*) protrusions

FIGURE 6 Double immunofluorescence for P2X3 and P2X2 in a whole-mount preparation. Confocal projection images are made from 3 images at 1 μm intervals. (a-c) P2X3-immunoreactive nerve endings are also weakly immunoreactive for P2X2. (d-f) Higher magnification views of the rectangles in panel (a-c) show that flat processes of P2X3- and P2X2-immunoreactive net-like terminal structures surround cell nuclei (*arrowheads*). (g-i) Higher magnification views of the rectangles in panel (d-f). P2X3-immunoreactive flat processes surrounding cell nuclei (*arrowhead*) also contain some P2X2-immunoreactive puncta (*arrows*). In (f) and (i), nuclei are labeled by DAPI (*blue*)

FIGURE 7 Double immunofluorescence for P2X3 and S100B in a whole-mount preparation. Confocal projection images are made from 4 images at 1 μm intervals in (a-c) and 16 images at 0.1 μm intervals in (d-f), respectively. (a-c) P2X3-immunoreactive beaded nerve fibers of net-like terminal structures partially surround the perinuclear cytoplasm (*arrowheads*) and slender cytoplasmic processes *(arrows)* of Schwann cells immunoreactive for S100B. *Asterisks* indicate oval cell nuclei of putative fibroblasts immunonegative for S100B. (d-f) P2X3-immunoreactive hederiform and flat leaf-like axon terminals surround some S100B-immunoreactive Schwann cells (*arrowheads*). (g-i) Higher magnification views of the rectangles in panel (d-f). P2X3-immunoreactive terminals wrap around S100B-immunoreactive cell bodies as a hederiform structure with pleomorphic (*arrows*) and thread-like protrusions (*arrowheads*). Nuclei are labeled by DAPI (*blue*)

FIGURE 8 Fast blue (FB)-labeled neurons in the nodose ganglion (NG) and dorsal root ganglion (DRG) after its injection into subserosal tissue in the antral lesser curvature. (a-c) FB-labeled neurons in the left NG are immunoreactive for P2X3 (*arrows*), whereas the other neuron is not (*arrowheads*). (d-f) The FB-labeled neuron in the left DRG at the level of Th8 is not immunoreactive for P2X3 (*arrowhead*). (g) Total number of FB-labeled neurons in left and right sides of NG and DRG at the levels of Th8-Th11 from seven experiments. Closed and open columns show P2X3-immunoreactive and P2X3-immunonegative neurons, respectively