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REGULAR ARTICLE

Piezo proteins: incidence and abundance in the enteric nervous system. Is there a link with mechanosensitivity?

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Abstract

Piezo channels play fundamental roles in many physiological processes. Their presence and functional role in the enteric nervous system is still not known. We hypothesize that they play a role in mechanotransduction in enteric neurons. Our aims are to quantify the presence of both Piezo1 and 2 in enteric neurons throughout the gastrointestinal tract using immunohistochemistry and analyze their function(s) using neuroimaging techniques and pharmacological investigations. In order to perform a systematic and comparative study, we performed our experiments in gastrointestinal tissue from guinea pigs, mice and humans. Piezo1 (20–70%) is expressed by both enteric neuronal cell bodies and fibers in the myenteric and submucosal plexi of all the species investigated. Generally, Piezo1 expressing somata are more numerous in the submucosal plexus (50–80%) than in the myenteric plexus (15–35%) apart from the stomach where Piezo1 is expressed in up to 60% of cell bodies. Myenteric Piezo1 neurons mainly (60–100%) but not exclusively, also express nitric oxide synthase, a minority express choline acetyltransferase. In the submucosal plexus, Piezo1 neurons co-express vasoactive intestinal peptide (40–90%). Conversely, expression of Piezo2 is extremely rare in the somata of enteric neurons and is present in few neurites. In functional experiments, 38–76% of the mechanosensitive neurons expressed Piezo1 channels. Statistical analysis showed a positive significant correlation between mechanosensitive and Piezo1 positive neurons. However, pharmacological experiments using an activator and an inhibitor of Piezo channels did not demonstrate changes in mechanotransduction. A major role of Piezo1 in the mechanosensitivity of enteric neurons can be excluded.

Keywords Enteric nervous system · Gastrointestinal · Mechanosensitivity · Piezo channels · Immunohistochemistry

Introduction

We recently identified multifunctional mechanosensitive enteric neurons (MEN) in various regions of the gastrointestinal tract of guinea pig, mice and human (Mazzuoli and Schemann 2009, 2012; Kugler et al. 2015; Mazzuoli-Weber and Schemann 2015b). These neurons have cell bodies located in the gastrointestinal wall and contribute to a neuronal network largely independent from the central nervous system: the enteric nervous system (ENS). The ENS can indeed control all intestinal functions autonomously; however those can be modulated by extrinsic sympathetic and parasympathetic nerve fibers (Furness 2012). The ENS consists mainly of two networks of ganglia, one residing just underneath the mucosa known as the submucosal plexus and one located between the two smooth muscle layers of the gastrointestinal tract known as the myenteric plexus.

Potential candidates for the putative channels responsible for MEN mechanotransduction include the Piezo proteins. The Piezo1 (Fam38A) and Piezo2 (Fam38B) multipass transmembrane proteins have been identified in the last decade as mechano-activated cation channels in different eukaryotic cells (Coste et al. 2010, 2012; Gottlieb and Sachs 2012). Mutations in human Piezo proteins have been associated with a variety of disorders including hereditary xerocytosis and

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several syndromes with aberrant muscular contracture as a prominent feature (Bagriantsev et al. 2014).

Expression of Piezo1 has been demonstrated in the bladder, colon, kidney, lung, skin, endothelial cells, erythrocytes and periodontal ligament cells (Coste et al. 2010; Jin et al. 2015). Piezo2 is highly expressed in the bladder, colon, lung, Merkel cells and in sensory trigeminal and in dorsal root ganglia (DRG) neurons (Coste et al. 2010; Ikeda and Gu 2014; Woo et al. 2014).

The sequences and structures of Piezo proteins are conserved among various species and their presence seems to be critical for homeostasis (Coste et al. 2012; Bagriantsev et al. 2014). Piezo proteins have been described to have rather distinct biological functions. They are activated by mechanical stimuli but also modulated by a voltage-gating mechanism (Moroni et al. 2018). Piezo1 was identified as an important component in the blood vessel response to shear stress via downstream signaling pathways involving endothelial nitric oxide synthase (eNOS) (Li et al. 2014). Piezo1 channels act as an endothelial sensor of blood flow promoting cell organization and alignment in the flow direction (Li et al. 2014). The lack of Piezo1 specifically in endothelial cells leads to abnormal vascular and lymphatic system development (Li et al. 2014; Lukacs et al. 2015). Piezo1 was also described to have a general role in in epithelial cell adhesion by maintaining integrin activation (McHugh et al. 2010). In the epithelial cells of the gastrointestinal tract, Piezo1 channels are sensors for cell crowding and migration (Eisenhoffer et al. 2012; Yang et al. 2014). In the urinary tract, Piezo1 acts as a stretch-activated cationic channel in renal tubular epithelial cells and bladder urothelial cells (Peyronnet et al. 2013; Miyamoto et al. 2014; Martins et al. 2016). Piezo1 is responsible for sensing shear stress and changes in cell volume in red blood and vascular endothelial cells (Peyronnet et al. 2013; Faucherre et al. 2014; Li et al. 2014; Miyamoto et al. 2014; Ranade et al. 2014; Martins et al. 2016; Wu et al. 2017). Piezo1 was shown to participate in integrin activation and knockdown of Piezo1 in lung epithelial cells decreased cell adherence and promoted cell migration (McHugh et al. 2010).

Unlike the various rather distinct roles of Piezo1, Piezo2 seems to play an important role in light touch, pain and visceral sensation. Knockout studies of Piezo2 revealed that the functionality of this protein is connected to the majority of mechanically activated currents in DRG (Bagriantsev et al. 2014) and Merkel cells (Woo et al. 2014). Co-localization of Piezo2 with small-diameter unmyelinated nociceptors in DRG cells suggested a role in noxious mechanosensitivity (Bagriantsev et al. 2014). This was also confirmed by the observation that Piezo2 is expressed in DRGs with transient receptor potential vanilloid 1 (TRPV1) channels (Coste et al. 2010). Recent work suggested a potential role for Piezo2 in the mediation of visceral sensation (Yang et al. 2016). In control rats, Piezo2 knockdown resulted in a decreased visceromotor response to innocuous but not to noxious stimuli (Yang et al. 2016). Moreover, Piezo2 is the major mechanotransducer of mammalian proprioceptors (Woo et al. 2015). In the gastrointestinal tract, Piezo2 channels are important for enterochromaffin cells mechanosensitivity (Wang et al. 2017). Recently, it has been described that Piezo2 or Piezo1 channels expressed in DRGs were inhibited upon activation of TRPV1 by capsaicin (Borbiro et al. 2015).

Data on the expression of Piezo proteins in the gastrointestinal tract were recently reviewed (Alcaino et al. 2017). However, presence and expression of Piezo proteins in the ENS have not yet been described fully in the literature. The focus of our work is to investigate the expression and function of Piezo channels within the myenteric and submucosal plexus of the ENS. We want to describe the co-localization of Piezo proteins with different neuronal markers. Finally, we wish to investigate whether Piezo channels are involved in the mechanotransduction process of MEN.

To this end, we used immunohistochemical techniques to determine the expression of Piezo channels in the ENS, as well as neuroimaging experiments with specific pharmacological tools to determine the role of Piezo channels in mechanotransduction. Commercial antibodies against Piezo1 and 2 are now available but have not been tested on enteric neurons. Since we had no information of the possible role of Piezo proteins in enteric neurons, we investigated the co-expression of Piezo1 and 2 channels with a wide range of markers for enteric neuronal populations For the pharmacological experiments, Piezo1-dependent currents were reversibly inhibited by the tarantula venom peptide GsMTx4, which has been shown to inhibit Piezo1 activity selectively in transfected HEK293 cells (Bae et al. 2011). Whether GsMTx4 is also effective against Piezo2 is unclear; in DRG neurons, GsMTx4 fails to suppress rapidly desensitizing mechano-activated currents thought to be mediated by Piezo2 (Drew et al. 2007). We tested these toxin evaluating MEN responses before and after drugs application. Very recently, Yoda 1, a synthetic small molecule that appears to act as a Piezo1 activator was identified (Syeda et al. 2015). We tested neuronal responses following application of this compound.

Material and methods

All procedures and techniques have been previously described in detail (Michel et al. 2011; Mazzuoli and Schemann 2012; Kugler et al. 2015; Mazzuoli-Weber and Schemann 2015b).

Ethics statement

All animal work with guinea pig was conducted according to the German guidelines for animal care and welfare (Deutsches

Tierschutzgesetz) and approved by the Bavarian state ethics committee (Regierung Oberbayern, which serves as the Institutional Care and Use Committee for the Technische Universität München) according to §4 and §11 Deutsches Tierschutzgesetz under reference number 32-568-2.

Procedures for the work with human samples were approved by the ethical committee of the Technische Universitaet Muenchen (1746/07; informed consent was obtained from all patients).

Tissue samples

Male "Dunkin Hartley" guinea pigs (Harlan GmbH, Borchen, Germany) (330 ± 50 g) and male mice C57BI/6 (Charles River, Sulzfeld, Germany) that were 16 weeks old upon arrival were used. All animals were killed by cervical dislocation followed by exsanguination. The whole gastrointestinal tract was removed and dissected in Krebs solution containing (in mM): 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$ and 11 glucose aerated with carbogen (95% O_2, 5% CO_2; pH = 7.40). Myenteric plexus preparations were obtained from the stomach (seven guinea pigs) and submucosal and myenteric plexus preparation from duodenum (three guinea pigs each), ileum (six and seven guinea pigs, respectively), proximal (three guinea pigs each) and distal colons (seven guinea pigs each). These preparations were used for immunohistochemistry. Tissue from 13 guinea pigs and two mice were used for electrophysiology experiments (*n* numbers are also indicated in the results sections). Preparations used for electrophysiology (5 × 10 mm) were pinned onto a silicone ring placed in a recording chamber continuously perfused with 37 °C carbogen-bubbled Krebs solution with a rate of perfusion of 10 ml/min.

Human samples of large (three) and small intestines (three) were obtained from six patients (two male, four female, mean age 64 years) undergoing surgery at the medical clinics of Freising and Rechts der Isar in Munich (Germany). Samples were taken from macroscopically unaffected areas. After surgery, tissue samples were placed in ice-cold-oxygenated sterile Krebs solution and immediately transferred to our laboratory. Tissues were dissected in ice-cold-oxygenated sterile Krebs solution to obtain whole-mount submucosal and myenteric plexus preparations. Human tissue samples were only used for immunohistochemistry.

Primary culture of myenteric neurons

Primary myenteric neuron cultures from guinea pig small intestine ($n = 4$) and primary cultures of DRG neurons ($n = 3$) were obtained following a previously published method (Buhner et al. 2014; Kugler et al. 2015, 2018). The cultures were grown for at least 7 days before experiments or fixation for immunohistochemistry.

For recordings, the dishes were placed in custom-made holders on an inverted microscope (Zeiss Axio Observer.A1; Munich, Germany). During the experiment, the culture dishes were continuously superfused with 37 °C HEPES-modified Krebs solution (pH = 7.4) containing (in mM): 1 $MgCl_2$, 2 $CaCl_2$, 150 NaCl, 5 KCl, 10 Glucose and 10 HEPES.

Immunohistochemistry

Tissue specimens or culture dishes were fixed overnight at room temperature in a solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 mol/l phosphate buffer and then washed (3 × 10 min) in phosphate buffer. The preparations were first incubated in phosphate-buffered saline (PBS)/NaN3 (0.1%)/horse serum (HS, 4%) for 1 h at room temperature followed by 48 h and 12 h incubation with the primary and secondary antibody, respectively. Primary antibodies used are listed in Table 1; secondary antibodies are listed in Table 2.

Human anti-Hu antibodies, also referred to as type 1 antineuronal nuclear antibodies or ANNA-1, were used as pan neuronal markers (Li et al. 2016). For each region, a considered minimum number of 30 ganglia from 3 different animals was used.

For the Piezo1 antibody, double and triple staining for the co-localization study was performed.

Specimens were washed in PBS, mounted on poly-l-lysine-coated slides and cover slipped with a solution of PBS (pH 7.0)/NaN3 (0.1) containing 65% glycerol. The preparations were examined with an epifluorescence microscope (Olympus) equipped with appropriate filter blocks. Pictures were acquired with a video camera connected to a computer and controlled by Scion image software (Scion Corp., Frederick, MD, USA). Frame integration and contrast enhancement were employed for image processing. Besides specifications provided by the suppliers, specificity of primary antibodies were previously published for rabbit anti-nitric oxide synthase (NOS) (Kummer et al. 1992) and goat anti-choline acetyltransferase (ChAT) (Li and Furness 1998; Pfannkuche et al. 1998). Human anti-Hu specificity was shown by our group (Li et al. 2016); moreover, the staining was blocked by the anti-Hu antigen.

Specificity of the Piezo immunohystochemical labeling was assessed with the following control experiments: substitution of the primary antibodies with PBS/NaN_3/HS and incubation of the primary antibody (Piezo1) pre-absorbed with the homologous peptide (1:100; NBP1–78446PEP; Novus Biological) overnight (Fig. 1a, b).

In addition, further appropriate controls for the double-labeling technique were performed to ensure the primary antisera do not cross react. Tissue from the same sample was used for sequential staining for each antibody. Results of these experiments were compared with experiments during which staining with multiple antibodies occurred simultaneously.

Table 1 List of primary antibodies used

Antibody	Source	Dilution
Rabbit anti-PIEZO1 (NBP1-78446)	Novus Biological, Cambridge, UK	1:20000/2000
Rabbit anti-PIEZO2 (NBP1-78624)	Novus Biological, Cambridge, UK	1:2000
Human: Mouse Anti-HU Biotin (A-21272)	Thermo Fisher Scientific	1:50
Guinea pig/Mouse: Human anti-Hu	(Prof. M. Schemann) (Li et al. 2016)	1:10000
Goat anti-choline acetyltransferase (ChAT; AB144P)	Chemicon, Limburg, Germany	1:100
Guinea pig: Sheep anti-nitric oxide synthase (NOS; AB1529)	Chemicon, Limburg, Germany	1:1000
Guinea pig: Rabbit anti-vasoactive intestinal peptide (VIP; RIN-7161)	Peninsula laboratories international, Inc., USA	1:5000
Mouse/Human: Mouse Anti- vasoactive intestinal peptide (VIP; MaVIP)	East Acres Biological, Southbridge, MA, USA	1:6000/1:2000
Mouse/Human: Rabbit Anti- nitric oxide synthase (210 501R025)	Alexis, Enzo Life Sciences GmbH, Lörrach Germany	1:5000/1:2000
Goat anti-VR1(P19) (SC-12503)	Santa Cruz, Heidelberg, Germany	1:500/1:1000
Sheep anti-TH (AB1542)	Chemicon, Limburg, Germany	1:5000
Mouse anti-calbindin (300)	SWant, Marly, Switzerland	1:200
Rabbit anti-CGRP (CA-08-220)	Genosys, Cambridgeshire, UK	1:6000

Secondary antibodies were also tested to ensure they recognize the appropriate antigen-antibody complexes. For this experiment, we replaced the primary antibody with the same amount of normal serum from the same species. With no primary antibody to bind the secondary antibody, no labeling was observed.

Three tissues (ileum myenteric plexus) from two guinea pigs were treated for 24 h with 100 μM colchicine and then stained (Fig. 1c–f).

Ultra-fast neuroimaging technique

An ultra-fast neuroimaging technique coupled with a voltage-sensitive dye was used (Neunlist et al. 1999; Mazzuoli and Schemann 2012). Single ganglia were loaded with fluorescent voltage-sensitive dye Di-8-ANEPPS (1-(3-sulfanatopropyl)-4-[beta[2-(di-n-octylamino)-6-naphthyl]vinyl] pyridinium betaine) (Invitrogen, Karlsruhe, Germany) by local application through a microejection pipette loaded with 20 μM Di-8-ANEPPS dissolved in DMSO and pluronic F-127 containing Krebs solution. Cultured myenteric neurons were stained by incubating 10 μM Di-8-ANEPPS for 10 min at room temperature in the dark. The recording chamber was mounted onto an inverted epifluorescence Olympus IX 71 microscope (Olympus, Hamburg, Germany) equipped with a 75-W xenon arc lamp (Optosource, Cairn Research Ltd., Faversham, UK). The light emitted by a green LED (LE T S2W, Osram, Munich, Germany) excites Di-8-ANEPPS through a modified Cy3 fluorescence filter. This setup allowed us to measure relative changes in the fluorescence ($\Delta F/F$), which is linearly related to changes in the membrane potential (Neunlist et al. 1999). Changes in fluorescence intensity were detected by a CCD camera (80 × 80 pixels; RedShirt Imaging, Decatur, USA) at a sampling rate of 1 kHz. The recordings were taken with a × 40 objective lens resulting in a spatial resolution of ~ 30 μm^2/pixel for intact tissue and with a × 100 objective lens (4.68 μm^2/pixel) for cultured neurons. The fluorescent images were acquired and processed by the Neuroplex 10.1.2 software (RedShirt Imaging).

To evoke fast EPSPs via electrical stimulation of interganglionic fiber tracts, a Teflon-coated platinum electrode (diameter 25 μm) connected to a constant-voltage stimulator was used (600 μs pulse duration with amplitudes ranging from 1 to 8 V that were supra-threshold).

For the mechanical stimulation paradigm, we used intraganglionic injections of small volumes of Krebs solution

Table 2 List of secondary antibodies used

Antibody	Source	Dilution
Donkey anti-rabbit Cy2 (711–225-152)	Dianova, Hamburg, Germany	1:200
Donkey anti-rabbit Cy3 (711–165-152)	Dianova	1:500
Donkey anti-goat Cy5 (705–175-147)	Dianova	1:500
Donkey anti-goat Cy3 (705–165-147)	Dianova	1:500
Donkey anti-human AMCA (709–155-149)	Dianova	1:50
Streptavidin Cy3 (016–160-084)	Dianova	1:500
Donkey anti-mouse Cy3 (715–165-151)	Dianova	1:500
Donkey anti-sheep Cy2 (713–225-147)	Dianova	1:200

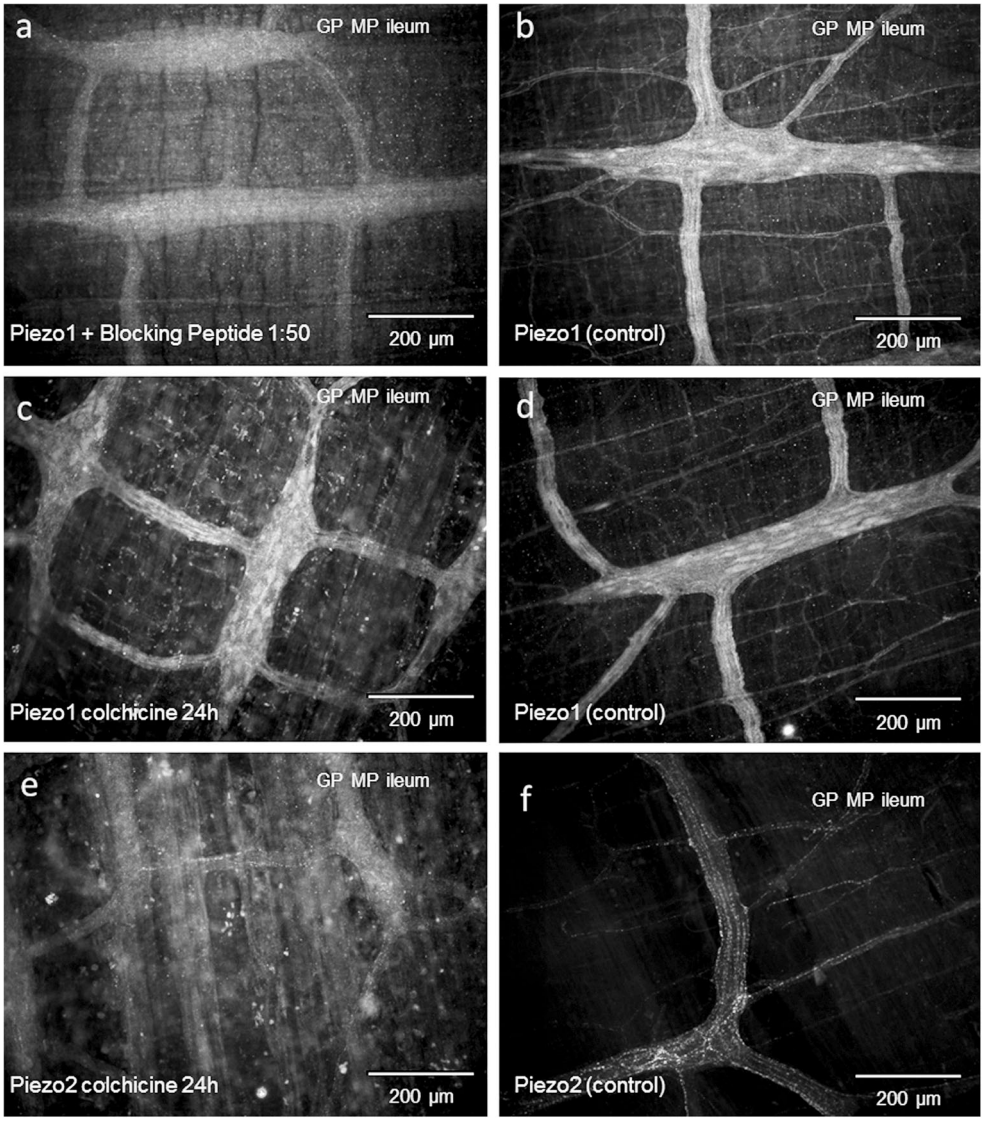

Fig. 1 **a** Control to test the specificity of Piezo1 antibody immunohistochemical staining. The primary antibody (NBP1–78446, Novus Biological, Cambridge, UK) was pre-absorbed overnight with the homologous peptide (1:100; NBP1–78446PEP; Novus Biological). The tissue sample, in this case guinea pig ileum myenteric plexus, only shows background staining; **b** control staining. **c** Myenteric plexus from guinea pig ileum stained with the Piezo1 antibody after 24 h incubation with 100 μM colchicine. In the colchicine treated tissues the cell bodies staining intensity was stronger compared with the control; **d** control staining. **e** Guinea pig myenteric plexus labeled with Piezo2 antibody (NBP1–78624, Novus Biological) after 24 h incubation with 100 μM colchicine. Here, the colchicine treatment did not change staining intensity; **f** control staining

(already applied successfully in the guinea pig ileum and stomach and in the mouse small and large intestines) (Mazzuoli and Schemann 2009, 2012; Mazzuoli-Weber and Schemann 2015b). Cultured neurons were probed with an 8 μm diameter carbon fiber used as von Frey hair (Kugler et al. 2015). These kinds of mechanical stimuli allow the identification of normal stress/compression-sensitive MEN, which mainly show a rapid adaptation behavior (Kugler et al. 2015). To characterize the adaptation pattern of MEN, we adopted an adaptation index (AI, described already in Kugler et al. 2015, Mazzuoli-Weber and Schemann 2015b and Kugler et al. 2018) comparing the firing frequency between the first 500 ms and the one of the remaining recording period. With the AI, we could differentiate between neurons with a rapid adaptation pattern vs. neurons with a slow-adaptation response pattern. Respectively, these were named rapidly and slowly adapting mechanosensitive enteric neurons (RAMEN and SAMEN) (Mazzuoli-Weber and Schemann 2015a).

Pharmacology

In order to perform pharmacological studies, the following substances were used: GsMTx4 (L) (Dr. Sachs, Suny at Buffalo Physiology, USA) 5 μM and Yoda 1 (Tocris Bioscience) 20 μM.

GsMTx4 was added to the Krebs solution inside the recording chamber and incubated for 25 min. During this time, the solution into the chamber was bubbled with carbogen. Before, during incubation and after 30 min washout of each drug, we performed mechanical stimulations. For statistical analysis, we only considered neurons that were still responding after washout.

Yoda 1 was applied directly onto the enteric ganglia with a local perfusion system.

Data analysis and statistics

For the tissue that underwent immunohistochemistry, we counted the number of neurons per ganglia (Hu-antibody

positive cell bodies) and this was taken as 100%. Then, we counted how many of those neurons were co-labeled with the Piezo1 or 2 antibodies. For the co-localization study, we counted the number of neurons that were labeled by the Piezo 1 antibody and were also labeled by ChAT, NOS, or VIP antibodies. Here, Piezo1 expressing neurons were taken as 100%.

For the neuroimaging experiments, we counted the number of labeled neurons in each ganglion and we analyzed the number of mechanosensitive neurons per ganglion and the frequency of their action potential discharge.

For signal and image analysis, we used Neuroplex 9.9.6 (RedShirt Imaging), Igor Pro 6.03 (Wavemetrics Inc., Lake Oswego, OR, USA) and Image J 1.43u (Wayne Rasband, National Institute of Health, USA) software. The statistical analyses and graphics were performed with Sigmaplot 12.0 (Systat Software Inc., Erkrath, Germany) and GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). All data are presented as mean ± standard deviation or, when not normally distributed, as median values together with the 25 and 75% quartiles given in brackets. Data were tested for normal distribution using the Shapiro-Wilk test. In case of normally distributed data, a Students *t* test was performed for unpaired experiments. Data from pharmacology experiments, which were obtained in a paired experimental design were analyzed using a paired Students *t* test. For data that were not normally distributed (action potential frequency before and after treatment), Wilcoxon rank sum tests were performed. Contingency was tested with chi-square test of independence. For all analyses, the difference between data groups was defined significant for *P* values below 0.05.

Results

Presence of Piezo1 and Piezo2 in the ENS: immunohistochemistry

In the guinea pig gastrointestinal tract, Piezo1 was expressed both in somata and in nerve fibers. In particular, in the myenteric plexus of the stomach 62 ± 14% of the neurons

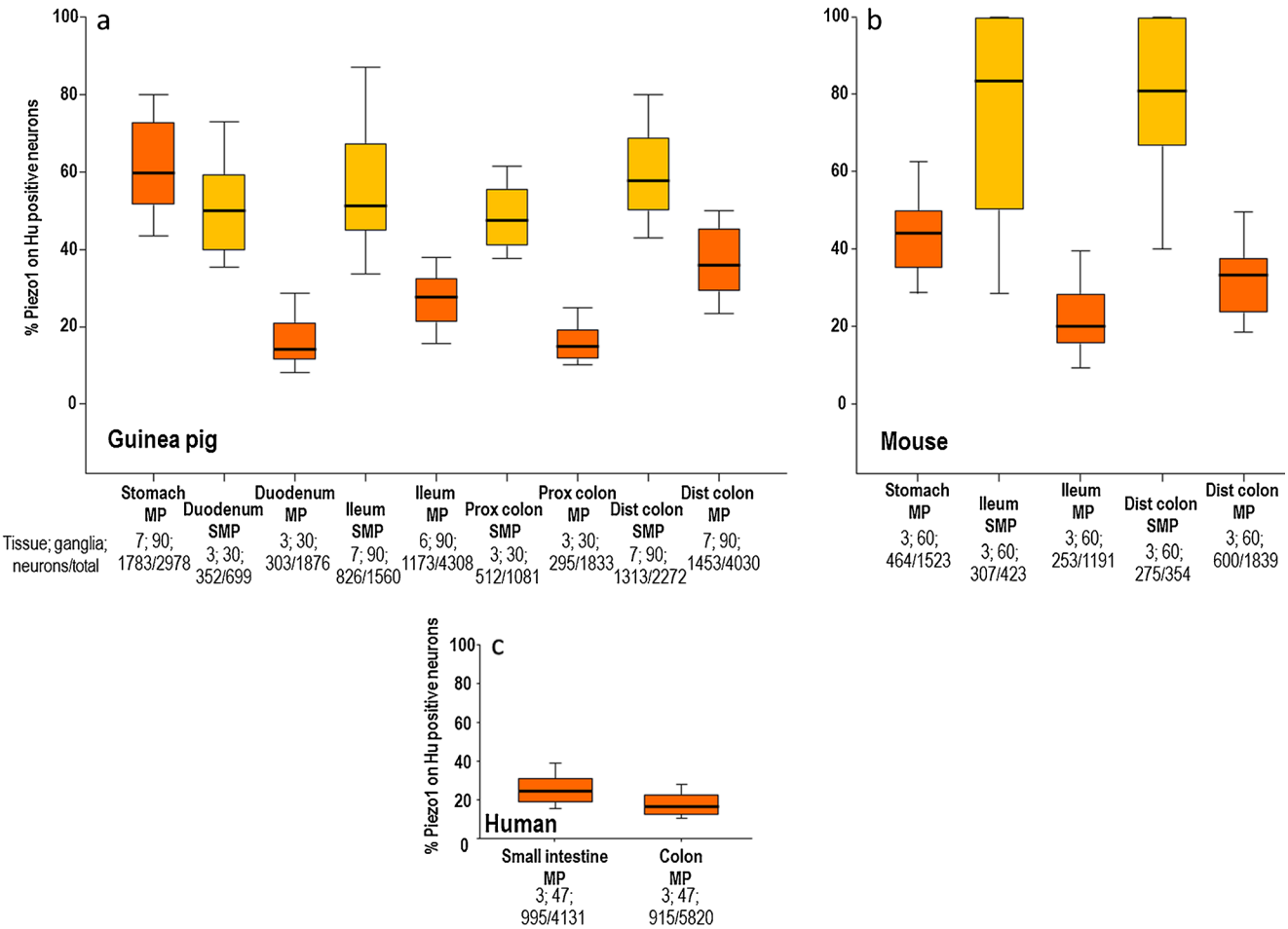

Fig. 2 Presence of Piezo1 expressing neurons in all studied regions of the **a** guinea pig, **b** mouse and **c** human gastrointestinal tract. Piezo1 antibody-stained neurons are expressed as percentage of anti-Hu positive neurons (100%). Both myenteric plexus (MP, orange box plots) and submucosal plexus (SMP, yellow box plots) neurons were counted. The number of tissue (animals), ganglia and neurons are reported

per ganglion expressed Piezo1; in the duodenum 16 ± 8%, in the ileum 27 ± 8%, in the proximal colon 17 ± 8% and in the distal colon 37 ± 11%. In the submucous plexus of the duodenum, 52 ± 16% of the neurons expressed Piezo1; in the ileum 57 ± 20%, in the proximal colon 48 ± 9% and in the distal colon 60 ± 16% (Figs. 2a and 3a–d). In the colchicine-treated tissues, there was a higher intensity of staining in the somata compared with untreated samples (Fig. 1c, d).

In the mouse gastrointestinal tract, Piezo1 was also expressed both in somata and in nerve fibers. In the myenteric plexus of the stomach, 44 ± 15% of the neurons per ganglion expressed Piezo1; in the ileum 23 ± 10% and in the colon 33 ± 11%. In the submucous plexus of the ileum 76 ± 29% and in the colon 77 ± 26% of the neurons expressed Piezo 1 (Figs. 2b and 3f, h).

In the human small and large intestines, Piezo1 was expressed on nerve fibers in both submucous and myenteric

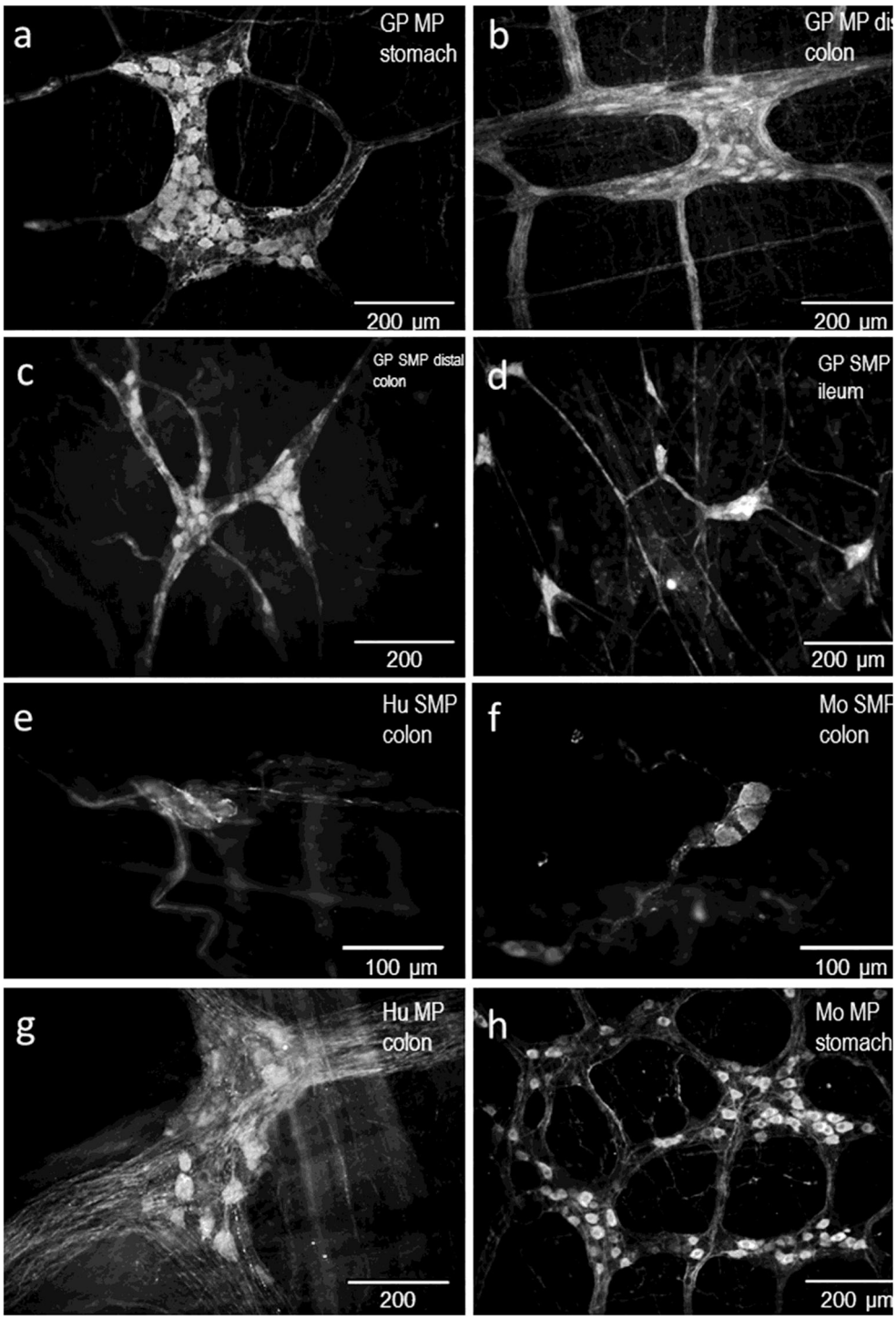

Fig. 3 Guinea pig Piezo1 expressing neurons in the MP of the **a** stomach, **b** distal colon and in the **c** SMP of the distal colon and **d** ileum. Staining for Piezo1 antibody in the human (Hu) colon **e** SMP and **g** MP. In **f** and **h** Piezo1 antibody staining was performed in the mouse (Mo) SMP colon and MP stomach, respectively

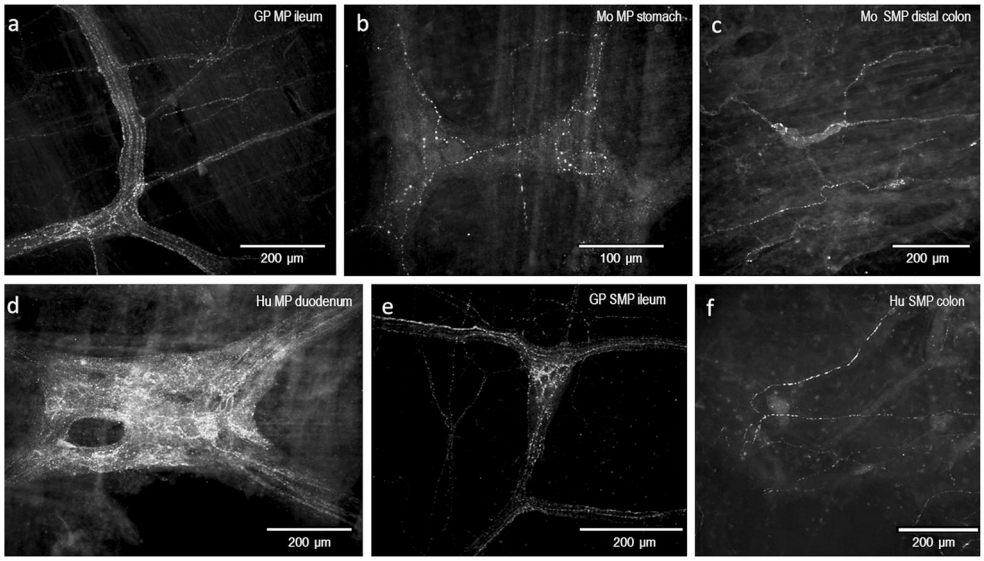

Fig. 4 Example of Piezo2 antibody staining in different regions and species. This antibody was staining mostly neuronal fibers

plexus but it was almost only present in the somata of the myenteric plexus. In particular, in the small intestine 26 ± 8% and in the colon 18 ± 6% of the neurons expressed Piezo1 (Figs. 2c and 3e, g).

Piezo2 was instead overall mainly expressed in nerve fibers and very rarely in neuronal cell bodies (Fig. 4). In particular, Piezo2 was expressed in nerve fibers running together with the blood vessels and within the muscle. The colchicine treatment did not change the staining intensity (Fig. 1e, f).

In cultured myenteric neurons of the guinea pig ileum, Piezo1 was expressed on both the somata and neurites in 55 ± 4% of the neurons (3/10/129) (Fig. 5a–c). Piezo2 was rarely present in sparse neurites.

In DRG-cultured neurons, both Piezo1 (91 ± 2%) and Piezo2 (89 ± 4%) were expressed on both the somata and neurites of the vast majority of the cells (Fig. 5d–f).

Co-expression studies

In the guinea pig myenteric plexus of all investigated regions, Piezo1-expressing neurons also mainly expressed NOS (Figs. 6a and 7a–c), while the minority co-expressed ChAT (Figs. 6a and 7d–f). Only in the stomach was the difference less pronounced. Co-localization was present on both the somata and neurites (Fig. 7).

In all observed regions of the guinea pig submucosal plexus, Piezo1-expressing neurons mostly expressed VIP and to a lesser extent ChAT (Fig. 7g–i).

Piezo1-expressing neuronal fibers were not present in nerves expressing TRPV1. Similarly, no co-expression was observed with markers for TH, Calb, or CGRP.

In the mouse gastrointestinal tract, similar observations were made (Figs. 6b and 8). Two differences were noted: (1) in the stomach myenteric plexus, the variance between

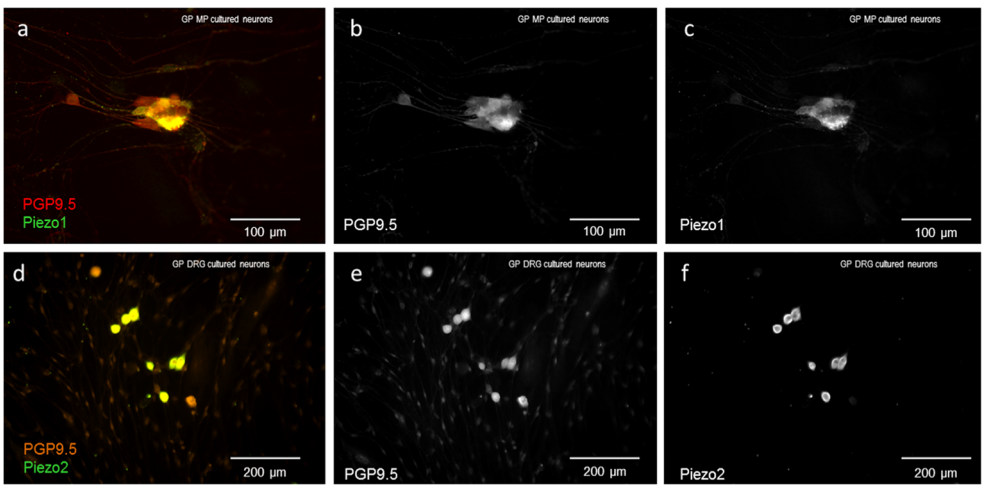

Fig. 5 Piezo antibody staining of cultured neurons. **a** Merged picture of myenteric cultured neurons stained in red for the pan-neuronal marker PGP 9.5 antibody and green for Piezo1 antibody. Neurons positive for both antibodies appear yellow. **b**, **c** Black and white pictures showing single staining. **d** Merged picture of DRG cultured neurons stained in dark orange for PGP 9.5 antibody and in green for Piezo2 antibody. A high percentage of DRG neurons expressed Piezo2. **e**, **f** Single labelling is shown

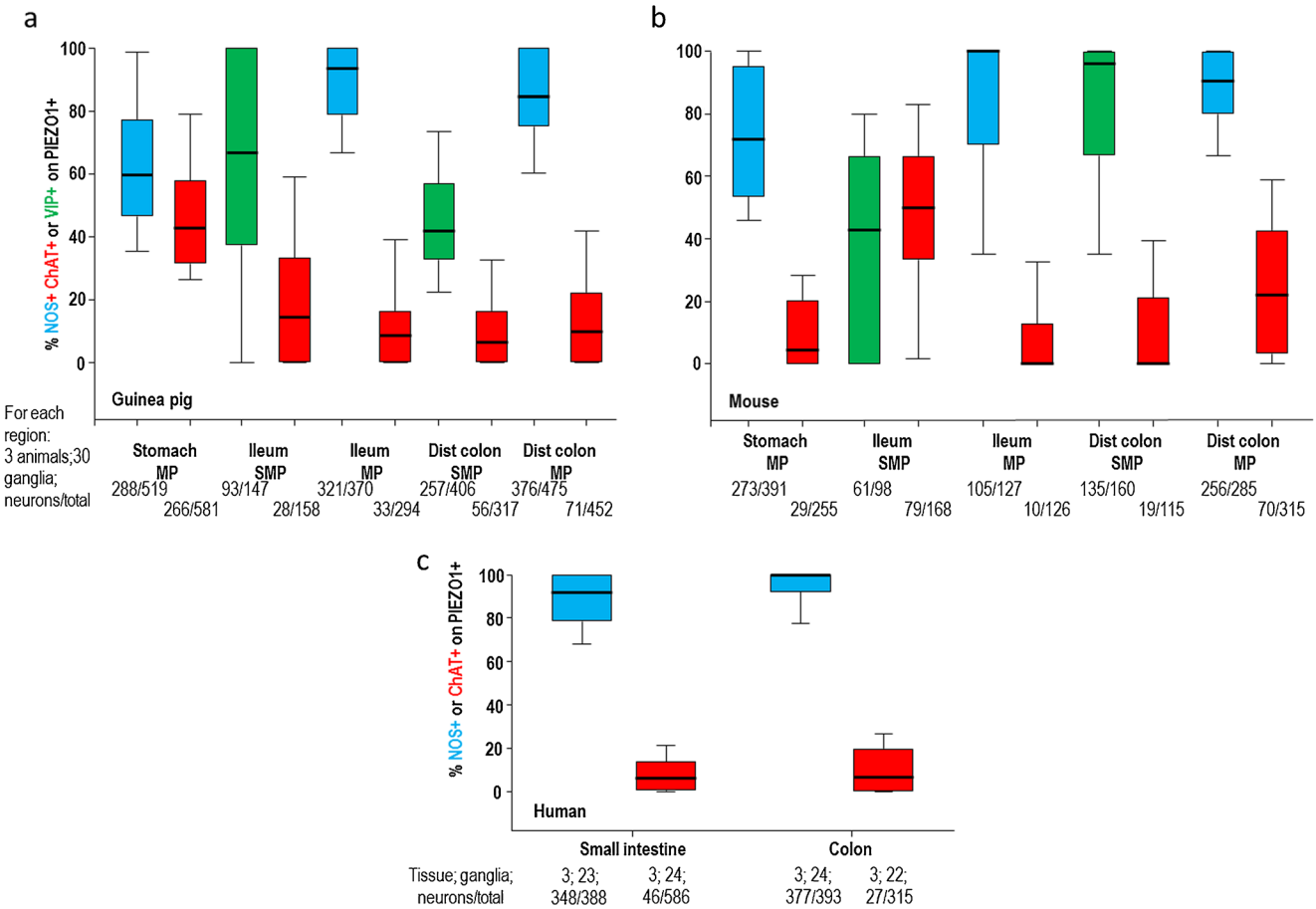

Fig. 6 Piezo1 antibody co-localization study in **a** guinea pig and mouse **b** MP of the stomach and in the SMP and MP of the ileum and distal colon. Neurons co-expressing Piezo1 and NOS, Piezo1 and ChAT and Piezo1 and VIP are represented by the blue, red and green box and whisker plots, respectively. It is consistent that the majority of Piezo1 expressing neurons co-express NOS and VIP. **c** Piezo1 co-localization study in human MP small and large intestine. Neurons co-expressing Piezo1 and NOS and Piezo1 and ChAT are represented by the blue and red box and whisker plots, respectively

neurons expressing Piezo1 and also NOS- and ChAT-immunoreactivity was more pronounced and (2) in the submucous plexus of the ileum, neurons expressing both Piezo1 and ChAT were more numerous than neurons expressing Piezo1 and VIP (Figs. 6b and 8).

In human small intestine and colon myenteric plexus, the vast majority of Piezo1-expressing neurons co-expressed NOS (Figs. 6c and 9).

Neuronal fibers in guinea pig and mouse labeled by the Piezo 2 antibody were not co-stained with antibodies raised against TRPV1, TH, Calb, or CGRP.

Mechanosensitivity and pharmacological studies

Eight ganglia from four guinea pigs were perfused with GMsTx4. There was no significant change in the proportion of MEN before (21 ± 5%) and after (18 ± 4%) the treatment. The action potential frequency also remained the same after the perfusion: 1.1 (0.6/1.7) Hz.

Yoda 1 was applied onto eight ganglia from three guinea pigs. No neuronal activity was observed in response to this application.

Immunostaining on normal stress/compression-sensitive MEN

In eight ganglia from the ileum of three guinea pigs, 50 MEN were identified. Of these, 19 (38%) expressed Piezo1 (example in Fig. 10a). Of these neurons, 15 were rapidly adapting MEN (RAMEN), four were slowly adapting MEN (SAMEN). A chi-square test of independence showed a significant relationship between the two populations ($p = 0.0015$).

In eight ganglia from the stomach of three guinea pigs we identified 47 MEN. Of these, 28 (60%) expressed Piezo1. Twenty-two of these neurons were RAMEN, six SAMEN.

In the mouse distal colon (two animals, 14 ganglia), we identified 52 MEN. Of these, 26 (50%) expressed Piezo1. Twenty were RAMEN and six SAMEN.

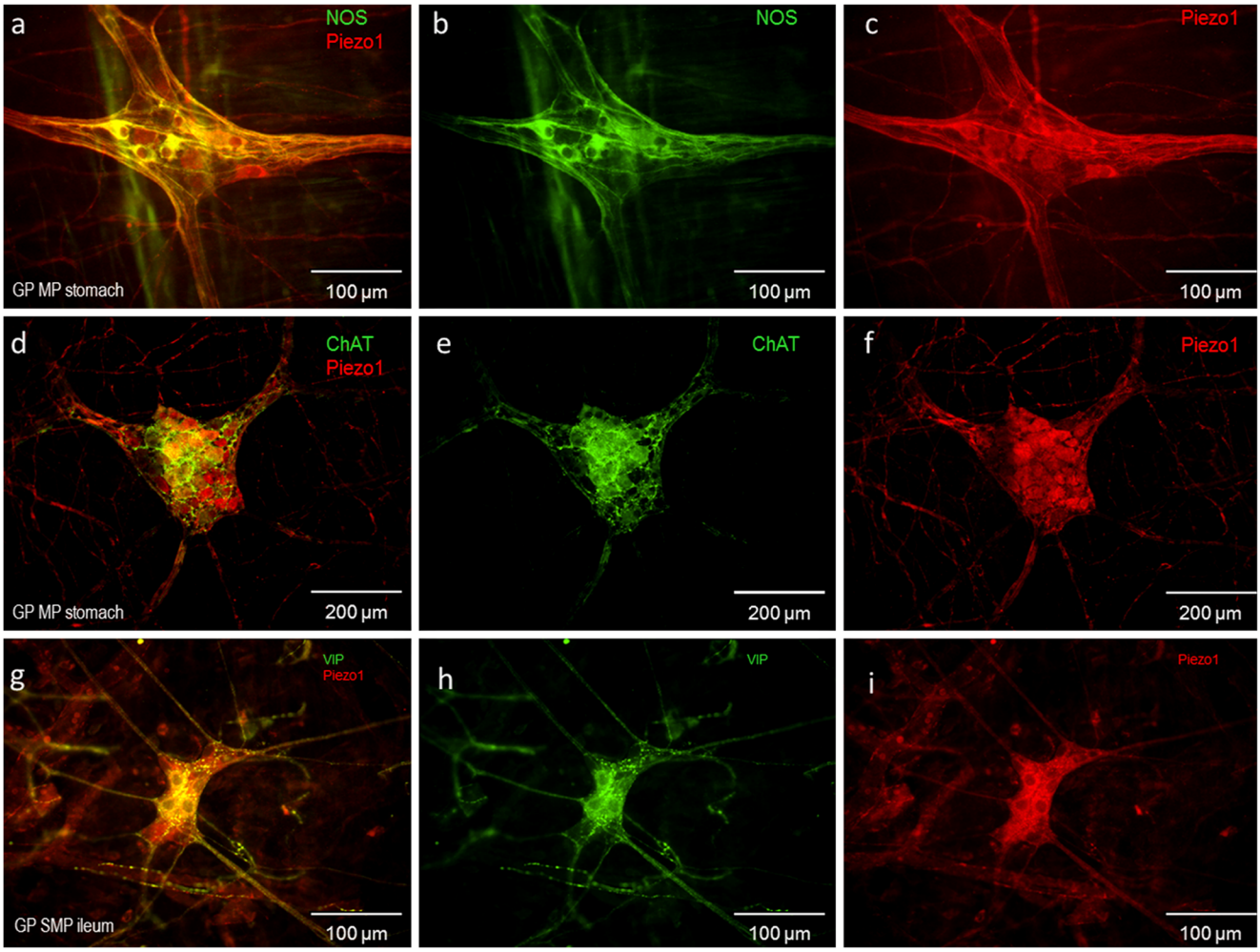

Fig. 7 Piezo1 antibody co-localization study in guinea pig stomach and ileum MP. **a** Merged picture example of co-staining between Piezo1 (red) and NOS (green) antibodies. **b**, **c** The single labeling is shown. **d** Staining for Piezo1 (red) and ChAT (green) antibodies are shown. **e**, **f** Single channel labeling is shown. **g** Shows staining for Piezo1 (red) and VIP (green) antibodies. **h**, **i** Single channel labeling is shown

In four neuronal clusters of primary cultured myenteric neurons from two guinea pigs, 21 MEN were characterized. Of them, 16 (76%) expressed Piezo1. Three RAMEN and 13 SAMEN (Fig. 10b, c).

Discussion

A recently published review of Piezo proteins in the digestive tract described the presence and suggested the putative role of these channels in the different compartments of the gastrointestinal wall (Alcaino et al. 2017). For instance, Piezo2 is involved in the mechanosensitivity of enterochromaffin cells (Wang et al. 2017). Along the gastrointestinal tract, neurons of the ENS autonomously regulate digestive functions directly within the gut wall. Recently, we described a subpopulation of these neurons with mechanosensitive properties and called them MEN (Mazzuoli-Weber and Schemann 2015a). In an attempt to discover which channel regulated mechanotransduction processes in MEN, we realized there was a dearth of information on the role of these channels in enteric neurons. Thus, the aim of our study was to investigate the presence of Piezo proteins in the ENS and to reveal if these channels could play a role in enteric neuronal mechanotransduction. For this purpose, we used immunohistochemistry, electrophysiology and pharmacology experiments comparing different gastrointestinal regions and species.

Our main findings are: (1) Piezo1 but not Piezo2 is expressed by enteric neurons in all investigated regions and species; (2) Piezo1 antibody staining in neurons co-localizes mainly but not exclusively, with the NOS expressing cells in the myenteric plexus and with VIP expressing cells in the submucosal plexus; (3) It is unlikely that Piezo1 plays a major role in the mechanosensitivity of enteric neurons.

Our work describes the presence of Piezo channels in the neurons of the ENS. While Piezo1 is expressed by 20–70% of

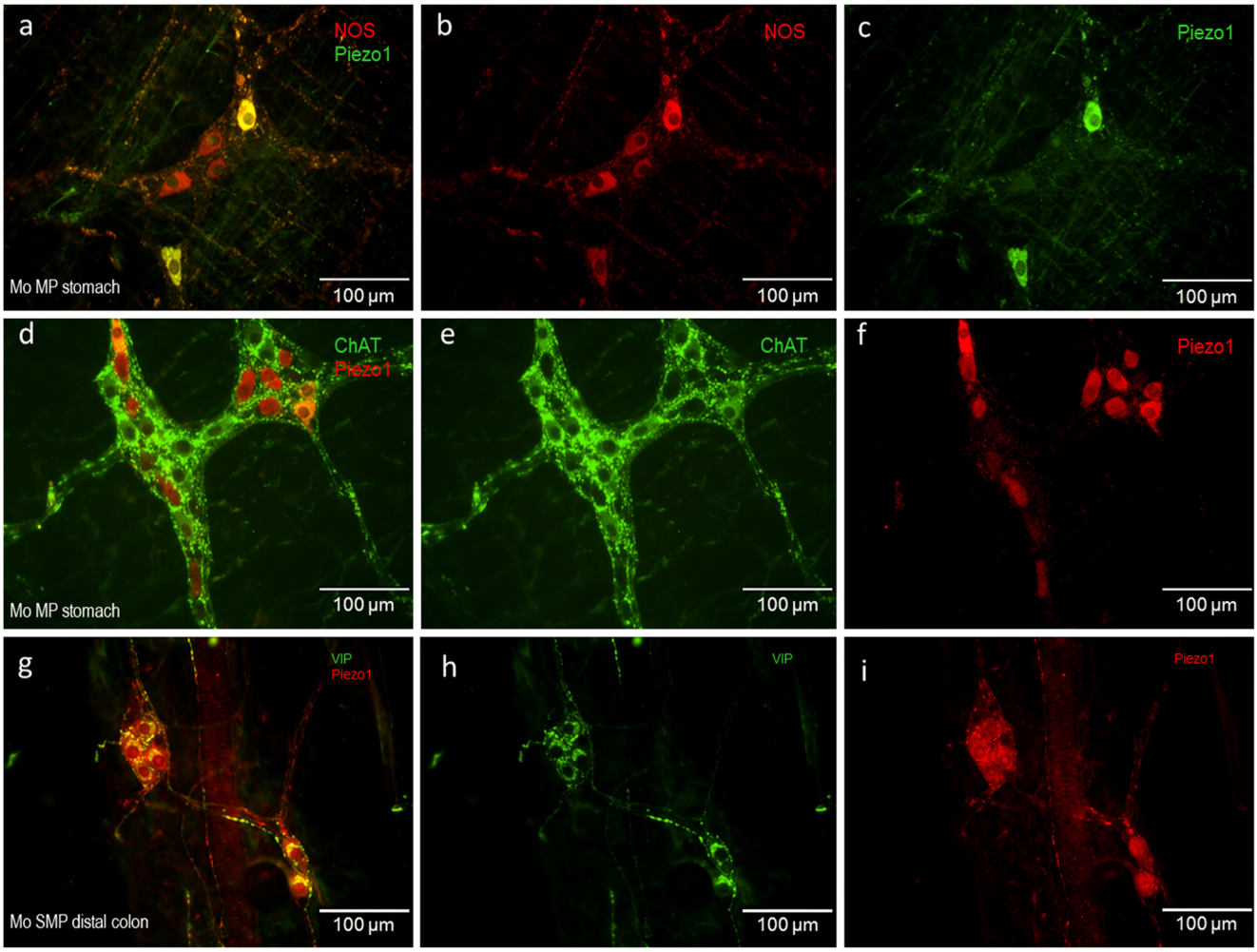

Fig. 8 Piezo1 antibody co-localization study in mouse stomach MP and distal colon SMP. In **a** merged picture example of co-staining between Piezo1 (green) and NOS (red) antibodies. **b**, **c** Present the single-channel staining. In **d** VIP (green) and Piezo1 (red) antibodies staining and in **g** ChAT (green) and Piezo1 (red) antibodies staining are shown. **e**, **f**, **h**, and **i** show the single channel labeling

the enteric neurons in all species and regions studied, Piezo2 seems to be extremely rare in enteric neurons somata and only expressed in a few neuronal projections. Generally, Piezo1 positive somata were more numerous in the submucosal plexus (50–80%) than in the myenteric plexus (15–35%). The only exception was in the guinea pig stomach myenteric plexus, where we observed a relatively high (up to 60%) Piezo1 expression. In primary cultured myenteric neurons, Piezo1 expression was higher than in the intact tissue of the same region. This could be explained by the role of Piezo1 in integrin activation (McHugh et al. 2010) leading the positive Piezo1 enteric neurons to attach more strongly to the cell culture dish surface.

In the myenteric plexus, enteric neurons tended to co-express Piezo1 and NOS (60–100%). Nitrergic neurons are involved in the peristaltic reflex and mediate aboral relaxation. Thus, we can conclude that the myenteric Piezo1-NOS neurons could be inhibitory motor neurons. If we suppose that Piezo1 neurons play a role in mechanosensitivity, they could functionally be involved in the intrinsically mediated nerve-dependent, nitric oxide-mediated muscle relaxation during gastric volume accommodation (Paton and Vane 1963; Desai et al. 1991; Hennig et al. 1997). These data are supported by our finding in the gastric corpus: (1) in this region 60% of the MEN were labeled by the Piezo1 antibody; (2) we recently demonstrated that around twice as many gastric MEN have a NOS antibody immunoreactive phenotype compared with the small intestine (Mazzuoli-Weber and Schemann 2015b).

In the submucosal plexus of all regions, the Piezo1 expressing neurons were co-localized with VIP antibody labelling (40–90%). These neurons are likely to be non-cholinergic secretomotor/vasodilator neurons, which could also be involved in mechanosensitivity, since distention of submucosal preparations in the guinea pig intestine evoked nerve-mediated secretion (Weber et al. 2001; and personal observations).

Since in all systems investigated prior to this study, especially sensory systems, Piezo2 played a role in mechanosensitivity,

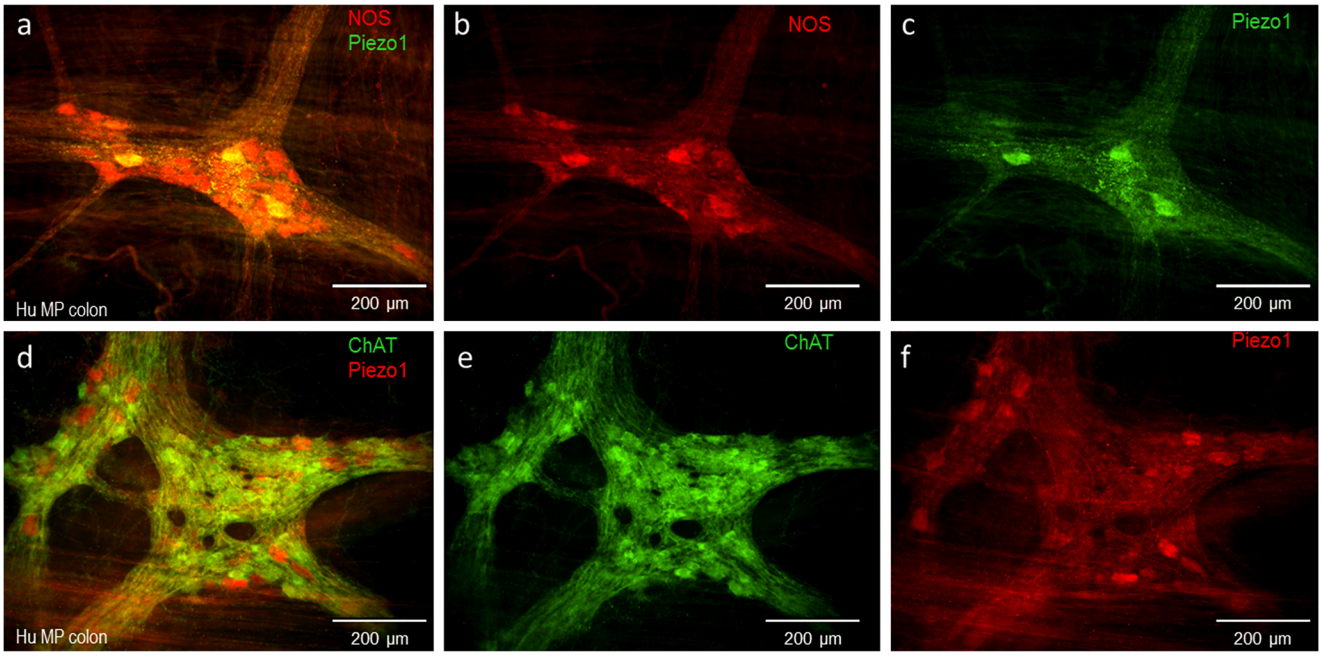

Fig. 9 Piezo1 antibody co-localization study in human colon MP. **a** Immunohistochemical staining of Piezo1 (green) and NOS (red) antibodies and **d** Piezo1 (red) and ChAT (green) antibodies. In **b**, **c**, **e** and **f** single channel staining is shown

we expected to have similar results in our experimental series. However, this was not the case. Piezo2 was very rarely expressed in cell bodies but was observed in some neuronal processes. These were likely to be extrinsic afferent nerve fibers for the following reason: we did not observe Piezo2 antibody staining in primary-cultured enteric neurons. It has been previously described that Piezo2 is expressed in DRG neurons, which play a role in visceral sensation (Yang et al. 2016).

Conversely, the prominent proportion of Piezo1 expressing neurons made us consider a role for this channel in MEN mechanotransduction. Perhaps surprisingly, in pharmacological experiments with GsMTx4 or Yoda 1, we did not find any evidence to support our hypothesis. GsMTx4 did not affect

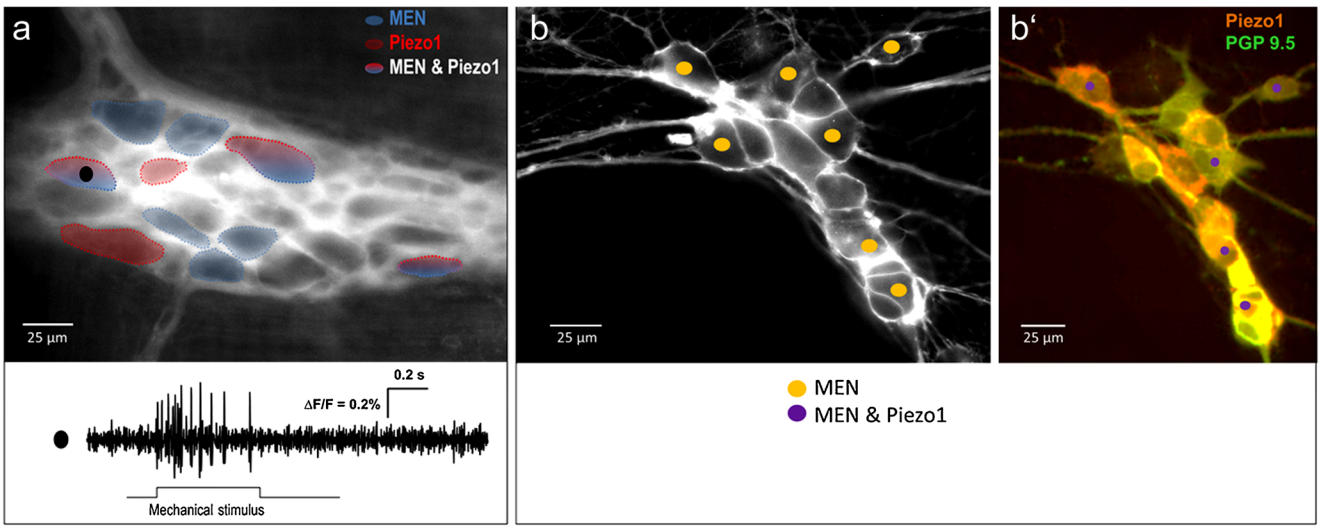

Fig. 10 Immunostaining on normal stress/compression-sensitive mechanosensitive enteric neurons (MEN). **a** Myenteric ganglion loaded with the voltage-sensitive dye Di-8-ANEPPS. The dye incorporates into the cell membrane revealing the outlines of the neurons in white. MEN are marked in blue; Piezo1 neurons are marked in red and MEN expressing Piezo1 are marked with both blue and red. In the lower part of the picture an example trace of a neuron responding with action potentials to mechanical stimulus is shown. The corresponding neuron is marked with a black dot. **b** Primary cultured enteric neurons are labeled with the voltage-sensitive dye Di-8-ANEPPS. The neurons showing mechanosensitive properties are marked with orange dots. **b'** Picture of the same cell cluster taken after immunohistochemical staining for PGP9.5 (green) antibody and Piezo1 (dark orange) antibody. Here, the neurons that expressed Piezo1 and were mechanosensitive are marked with purple dots

mechanosensitive responses, suggesting that Piezo1 is not involved in mechanosensitive responses in enteric neurons. It is possible that neuronal activation is compensated for and therefore not observed in this study, due to the fact that this toxin has many off-target effects and its general mechanism of action via the lipids of the plasma membrane (Suchyna et al. 2009; Redaelli et al. 2010). One hypothesis could be that the toxin is not as effective in guinea pig enteric neurons as in the original study where transfected HEK cells were used (Bae et al. 2011). This could be supported by the fact that GsMTx4 failed to block mechanically activated currents in sensory (DRG) neurons (Drew et al. 2007). The authors of this publication concluded that ubiquitous stretch-activated ion channels do not mediate mechanically activated currents in sensory neurons (Drew et al. 2007). Another possible explanation of the negative results of pharmacological experiments is that with our mechanical stimulation protocol we may activate in addition other mechanosensitive channels that could mask the contribution of Piezo1. Functional experiments, where we identified MEN and tested them for the presence of Piezo1, provided results partially confirming our hypothesis. In the guinea pig ileum, 38% of the identified MEN expressed Piezo1, in the stomach they were up to 60%. A similar picture could be observed in the primary enteric neuronal culture (76% of the MEN expressed Piezo1) as well as in the mouse colon (50%). Of course, these data could reflect regional differences. The statistical analysis performed showed a significant relationship between the two populations (Piezo1 and MEN); however, this seems not consistent enough to definitely prove a prominent role for Piezo1 on enteric neuronal mechanotransduction. The fact that not all MEN are stained by the Piezo1 antibody confirmed the findings of experiments with various blockers of mechanosensitive channels (data only partially published see Mazzuoli-Weber and Schemann 2015b), which point to the involvement of non-Piezo channels/mechanisms mediating enteric neuronal mechanosensation.

In conclusion, this work is the first comprehensive study on Piezo proteins and ENS. With it, we revealed the presence of Piezo1 channels in enteric neurons and provided some preliminary data on their possible functional role within the ENS.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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