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ORIGINAL ARTICLE

Predominant mucosal expression of 5-HT$_{4(+h)}$ receptor splice variants in pig stomach and colon

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Abstract

AIM: To investigate cellular 5-HT$_{4(-h/+h)}$ receptor distribution, particularly in the epithelial layer, by laser microdissection and polymerase chain reaction (PCR) in porcine gastrointestinal (GI) tissues.

METHODS: A stepwise approach was used to evaluate RNA quality and to study cell-specific 5-HT$_4$ receptor mRNA expression in the porcine gastric fundus and colon descendens. After freezing, staining and laser microdissection and pressure catapulting (LMPC), RNA quality was evaluated by the Experion automated electrophoresis system. 5-HT$_4$ receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressions were examined by endpoint reverse transcription (RT)-PCR in mucosal and muscle-myenteric plexus (MMP) tissue fractions, in mucosal and MMP parts of hematoxylin and eosin (HE) stained tissue sections and in microdissected patches of the epithelial and circular smooth muscle cell layer in these sections. Pig gastric fundus tissue sections were also stained immunohistochemically (IHC) for enterochromaffin cells (EC cells; MAB352); these cells were isolated by LMPC and examined by endpoint RT-PCR.

RESULTS: After HE staining, the epithelial and circular smooth muscle cell layer of pig colon descendens and the epithelial cell layer of gastric fundus were identified morphologically and isolated by LMPC. EC cells of pig gastric fundus were successfully stained by IHC and isolated by LMPC. Freezing, HE and IHC staining, and LMPC had no influence on RNA quality. 5-HT$_4$ receptor and GAPDH mRNA expressions were detected in mucosa and MMP tissue fractions, and in mucosal and MMP parts of HE stained tissue sections of pig colon descendens and gastric fundus. In the mucosa tissue fractions of both GI regions, the expression of h-exon containing receptor [5-HT$_{4(+h)}$ receptor] mRNA was significantly higher ($P < 0.01$) compared to 5-HT$_{4(-h)}$ receptor expression, and a similar trend was obtained in the mucosal part of HE stained tissue sections. Large microdissected patches of the epithelial and circular smooth muscle cell layer of pig colon descendens and of the epithelial cell layer of pig gastric fundus, also showed 5-HT$_4$ receptor and GAPDH mRNA expression. No 5-HT$_4$ receptor mRNA expression was detected in gastric LMPC-isolated EC cells from IHC stained tissues, which cells were positive for GAPDH.

CONCLUSION: Porcine GI mucosa predominantly expresses 5-HT$_{4(+h)}$ receptor splice variants, suggesting their contribution to the 5-HT$_4$ receptor-mediated mucosal effects of 5-HT.

Key words: 5-hydroxytryptamine 4 receptors; Pig; Gastric fundus; Colon descendens; Epithelium; Smooth muscle; Laser microdissection and pressure catapulting

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INTRODUCTION

The 5-HT$_4$ receptor is a G-protein coupled receptor (GPCR) that activates the adenylyl cyclase/cyclic adenosine monophosphate/protein kinase A pathway in response to serotonin (5-HT). The 5-HT$_4$ receptor is expressed on excitatory motor neurons in the gut, facilitating acetylcholine release, which stimulates gastrointestinal (GI) motility[1-3]. This presynaptic facilitation is thought to be the principal mechanism for the prokinetic action of 5-HT$_4$ receptor agonists, explaining their therapeutic use in GI dysmotility-related disorders, such as chronic constipation, gastroparesis and gastroesophageal reflux disease[4]. The selective 5-HT$_4$ receptor agonist prucalopride is now used in patients with chronic laxative-resistant constipation; indeed, it facilitates acetylcholine release from cholinergic neurons towards human colonic circular[5], as well as longitudinal[6], smooth muscle. The non-selective 5-HT$_4$ receptor agonist cisapride was, until it was withdrawn because of non-specific cardiac side effects, used for increasing gastric emptying in patients with gastroparesis[7]. In addition, prucalopride accelerates gastric emptying in humans[8], corresponding with its facilitating effect on acetylcholine release from cholinergic nerves towards human gastric circular muscle[9]. Our group has previously shown that the pig is a good model for human 5-HT$_4$ receptors on GI cholinergic neurons; the presence of facilitatory 5-HT$_4$ receptors on cholinergic neurons innervating pig gastric circular[10] and longitudinal[11] muscle and colonic circular muscle[12] was illustrated in functional assays.

However, apart from cholinergic neurons, other locations for the 5-HT$_4$ receptor in the colon and stomach have been proposed. In the human colon, 5-HT$_4$ receptors were reported to be present on circular smooth muscle cells, inducing relaxation[13]. A functional study by Borman *et al*[14] in 1996 reported that 5-HT-induced secretion in the human sigmoid colon is mediated *via* 5-HT$_{2A}$ receptors; however, in the ascending colon, a combination of 5-HT$_{2A}$ and 5-HT$_4$ receptors appears to be involved. Nevertheless, a recent study showed the presence of mRNA of several 5-HT$_4$ receptor splice variants in the mucosal layer of the human sigmoid colon[15]; 5-HT$_4$ receptor mRNA was also reported in the pig colonic mucosa[16]. In the rat colon, it has been suggested that 5-HT-induced mucosal ion transport and Cl^- secretion is mediated by 5-HT$_4$ receptors[17-20]. Immunohistochemical and functional assays showed the presence of 5-HT$_4$ receptors in mouse colonic epithelial cells, including enterochromaffin (EC) cells and goblet cells, inducing mucosal 5-HT release and Cl^- secretion[20]. The presence of 5-HT$_4$ receptor transcripts, detected by reverse transcription polymerase chain reaction (RT-PCR), has also been reported in the gastric mucosa of humans[21,22] and pigs[16], but cellular distribution within the epithelial layer has not yet been investigated.

More detailed information on the expression and localization of GPCRs, with special attention to the 5-HT$_4$ receptor, is needed in human enteric neuronal subpopulations, mast cells and epithelial cells, to provide a better understanding of function and activity of 5-HT$_4$ receptors in the GI wall, which may offer new therapeutic perspectives[23]. To date, the majority of information on 5-HT$_4$ receptor distribution is based on functional studies[12] or on 5-HT$_4$ receptor expression studies using homogenates of tissues[15,16,21,22]. However, homogenates of tissues limit the potential of expression studies: important cell-specific transcript information is lost because of the heterogeneity of tissues, such as GI tissues. Techniques have been developed to enable collection of particular cells from mixed populations, which generally involve either fluorescence activated cell sorting (FACS) purification of dissociated cells or laser-assisted microdissection. In contrast to FACS, microdissection can be applied to most tissues[24] and laser microdissection has already been used in previously reported gene expression studies to investigate site-specific gene expression. In the laser microdissected enteric ganglia of the human intestine, 5-HT$_{3A}$ receptor mRNA expression was described[25] and in microdissected human colonic mucosal epithelium, transcripts encoding 5-HT$_{3A}$, 5-HT$_{3C}$, 5-HT$_{3D}$ and 5-HT$_{3E}$ subunits were detected[26]. In different species, 5-HT$_4$ receptors show splice variation in the intracellular C-terminus starting after the common amino acid structure L358. In humans, nine splice variants have been described (Figure 1A). In pig, at least another nine different splice variants, not described in humans, have been reported (Figure 1A), as well as unique splice variation, with variants composed of duplicated exons[16]. Splice variants in the extracellular loops of GPCRs are rare[27]; however, the 5-HT$_4$ receptor can have an extra insertion of 14 amino acids in the second extracellular loop, encoded by the h-exon (Figure 1A). In humans, this H variant has been described in combination with the b-terminal exon [5-HT$_{4(hb)}$][28]. When comparing the pharmacology of the 5-HT$_{4(hb)}$ splice variant, when transiently expressed in cells being CV-1 (simian) in origin, and carrying the SV40 genetic material (COS)-7 cells, with that of the 5-HT$_{4(b)}$ and 5-HT$_{4(a)}$ splice variant, it showed a smaller fraction of receptors coupled to G-proteins and the 5-HT$_4$ receptor antagonist GR113808 behaved as a partial agonist[28]. In the human GI tract, the h exon could be amplified in combination with the b exon only from the lower esophageal sphincter; however, h exon-carrying 5-HT$_4$ transcripts were also obtained from other parts of the GI tract, suggesting that the h-exon might be expressed in combination with other C-terminal exons[28]. In pigs, De Maeyer *et al*[16] showed that the 5-HT$_{4(h)}$ splice variant also exists in combination with C-terminal

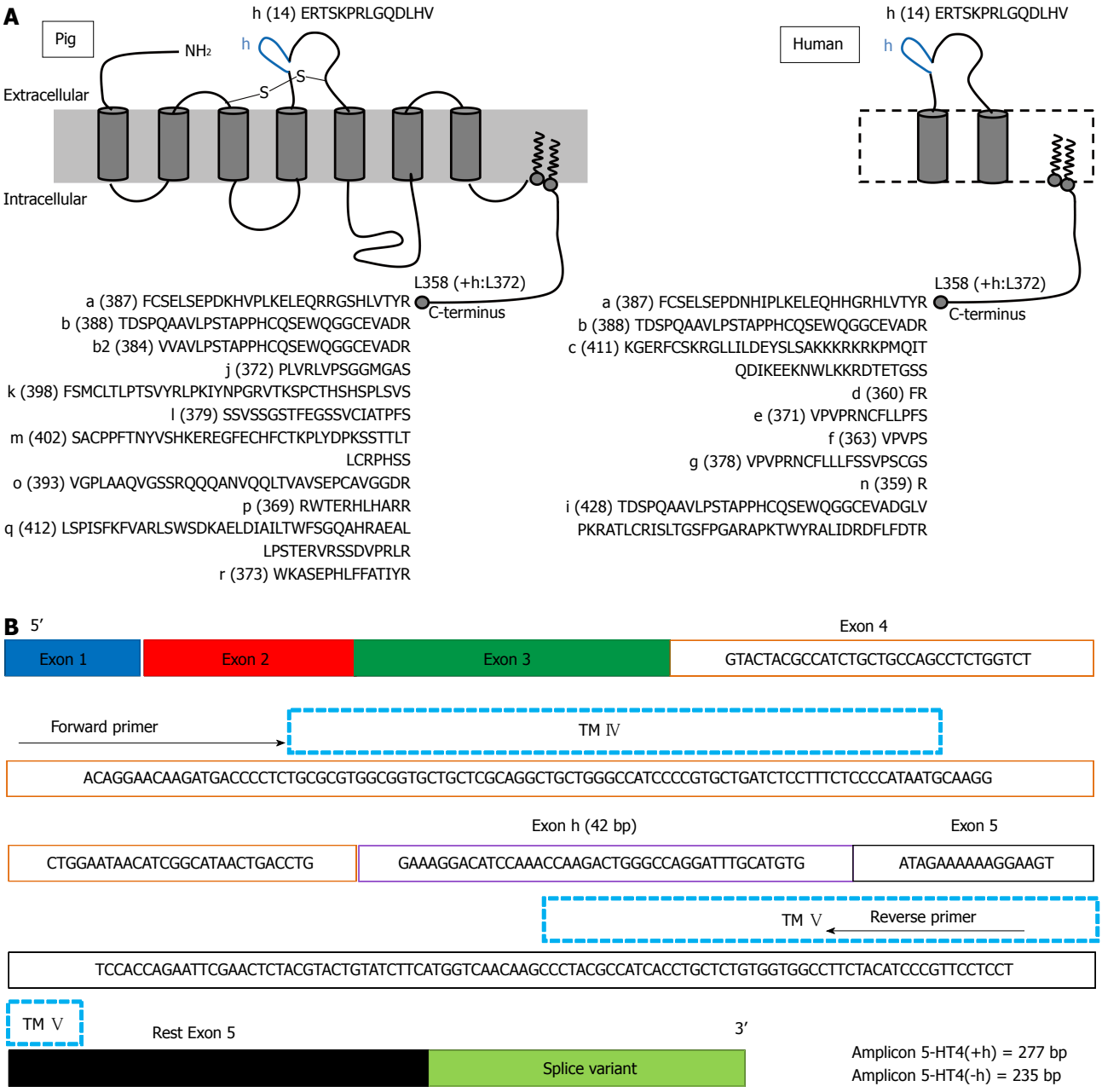

Figure 1 Schematic representation. A: The porcine and human 5-HT$_4$ receptor splice variants; B: cDNA of the porcine 5-HT$_{4(hb)}$ receptor based on gene browsing (transcript ID: ENSSSCT00000015770 on http://www.ensembl.org/Sus_scrofa/Gene). The 5-HT$_4$ receptor variants have identical sequences up to Leu^{358}, or Leu^{372} when exon h is included, and differ by the length and composition of their C-terminal domain. The presence of the h sequence of 14 amino acids in the second extracellular loop depends on the all or none inclusion of exon h between exon 4 and 5. The positions of the boundaries in between exons, the transmembrane (TM) domains Ⅳ and Ⅴ and the positions of primers used in this study are indicated. The primers will detect all 5-HT$_4$ receptor splice variants. Amplification will result in a 277 bp amplicon, when containing the 42 bp h-exon or a 235 bp amplicon, not containing the h-exon.

exons other than 5-HT$_{4(b)}$, namely 5-HT$_{4(ha)}$, 5-HT$_{4(hm)}$ and 5-HT$_{4(hr)}$. H-exon containing 5-HT$_4$ transcripts were also found along the porcine GI tract, with predominant expression in the mucosal layer. Therefore, the aim of the present study was to develop and validate an experimental protocol for the assessment of 5-HT$_4$ receptor distribution (with and without the h exon) at the cellular level in laser microdissected porcine GI tissues, paying special attention to the mucosal layer of pig colon descendens and gastric fundus.

MATERIALS AND METHODS

Tissue preparation and tissue processing

Young male pigs (10-12 wk, 15-25 kg-breed Line 36) were obtained from Rattlerow Seghers, Lokeren Belgium. The Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University approved all the experimental procedures.

The pigs were anaesthetized with an intramuscular injection of 5 mL Zoletil 100 (containing 50 mg/mL ti-

letamine and 50 mg/mL zolazepam; Virbac Belgium SA, Heverlee, Belgium). After exsanguination, the stomach and the colon descendens, prelevated 10 cm above the anus to the transverse colon, were removed and thoroughly washed in ice cold aerated (5% CO_2/95% O_2) phosphate buffered saline (PBS) at pH 7.4 (Life Technologies Europe, Ghent, Belgium). The gastric fundus was cut open along the lesser curvature and small pieces of tissue were cut in the direction of the circular muscle layer from the ventral side. The colon descendens was opened along the mesenteric border, fat tissue was removed and tissues were cut in the direction of the circular muscle layer.

Freezing tissue fractions for direct RNA processing: The GI tissues were divided by blunt dissection into a mucosal-submucosal (mucosa) fraction and a muscular-myenteric plexus (MMP) fraction. The fractions were cut into small pieces, put in an RNase-free vial (Life Technologies Europe), rapidly frozen in liquid N_2 and stored at -80 ℃. After frozen tissue homogenization and before RNA extraction, MMP samples were treated with proteinase K (Qiagen, Antwerp, Belgium) to increase the total RNA output. Proteinase K removes proteins such as the contractile proteins, connective tissue and collagen, which define a fibrous tissue such as the smooth muscle layer (Rneasy fibrous tissue handbook, Qiagen). RNA from mucosa and MMP fractions was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer's guidelines and RNA samples were stored at -80 ℃.

Freezing tissues for section preparation and laser microdissection: Whole tissues, containing the mucosal and the smooth muscle layers were cut into full-thickness small pieces with a sterile scalpel, placed in tissue embedding medium PELCO CryO-Z-T (Pelco International, CA, United States), rapidly frozen in liquid N_2 containing cold isopentane and stored at -80 ℃. The frozen tissue samples were cut into 8 μm-thick sections using a cryostat (Leica CM 1950; Leica Microsystems, Diegem, Belgium) with disposable RNase-free knives. Sections of 8 μm thickness are considered to represent a monolayer of cells[29,30]. The sections were placed on chilled (-20 ℃) nuclease free polyethylene naphthalate-covered membrane slides (Carl Zeiss, Oberkochen, Germany) and immediately stored at -80 ℃ until the staining procedure. The membrane slides used for immunohistochemistry were extra coated with poly-L-Lysine (Sigma, Bornem, Belgium), which was diluted with 0.1% diethylpyrocarbonate (DEPC)-treated water. All materials (pincers, brushes) were treated with RNase ZAP (Sigma) and glassware and pincers were heated for 6 h at 200 ℃, to remove all exogenous RNases.

To distinguish morphologically the different layers of the tissue sections for laser microdissection, the frozen tissue sections were stained with hematoxylin and eosin (Sigma) in RNase-free conditions. Hematoxylin and eosin (HE) staining started with fixing the slides in 70% ethanol for 1 min, followed by dipping the slides for 15 s in DEPC-treated water to remove PELCO CryO-Z-T embedding medium. Hematoxylin staining was carried out by placing the slides for 1 min in the hematoxylin solution (0.1%), followed by dipping the slides for 15 s in DEPC-treated water and 15 s in 70% ethanol. Slides were then placed for 1 min in eosin solution (0.25%), followed by dehydrating the slides for 15 s in the following order: DEPC-treated water, 70% ethanol, 100% ethanol. The staining procedure was finished with a 3 min xylene treatment and the slides were air dried for 10 min at room temperature, before scraping off the whole tissue section, or the mucosal and MMP part of the tissue section separately, or applying laser microdissection. Staining solutions based on ethanol and xylene were pre-cooled at -20 ℃; aqueous solutions were pre-cooled at 4 ℃. All solutions were diluted with 0.1% DEPC-treated water, kept in 50 mL RNase-free conical tubes (Life Technologies Europe) and kept on ice during the staining procedure.

Immunohistochemistry: To distinguish and isolate EC cells using the laser microdissection and pressure catapulting (LMPC) technique, visualization with cell-specific antibodies of these cells is required. To extract intact RNA of the cell samples, an immunohistochemically (IHC) protocol under RNase-free conditions was developed according to the staining procedure reported by Brown *et al*[24]. Cryosections were rinsed for 15 s with cold (4 ℃) PBS (pH 7.4; Life Technologies Europe) and then fixed for 5 min in ice-cold (-20 ℃) acetone. Acetone was removed by a cold PBS rinse (15 s) and slides were incubated for 30 min at 4 ℃ with blocking buffer (0.25% Triton X-100, 1% bovine serum albumin, 10% goat serum) supplemented with 1 mol/L NaCl. Then, sections were briefly rinsed with cold PBS and incubated overnight at 4 ℃ with the rat anti-serotonin primary antibody MAB352 (Milipore, Overijse, Belgium), used as a marker for EC cells. MAB352 was diluted 1:200 in PBS supplemented with 1 mol/L NaCl. Unbound primary antibody was removed by rinsing three times with cold PBS supplemented with 1 mol/L NaCl. Sections were then incubated with chicken anti-rat secondary antibody Alexa Fluor 488 (Life Technologies Europe) diluted 1:100 in PBS with 1 mol/L NaCl for 2 h at 4 ℃. Unbound secondary antibody was removed by rinsing three times with cold PBS with 1 mol/L NaCl and excess NaCl was removed by a PBS rinse (5 s). Sections were dehydrated in 70% and then 100% ethanol (3 min each) and air dried for 10 min at room temperature before laser microdissection.

Laser microdissection and pressure catapulting

LMPC was performed using the laser microdissection system from PALM Technologies (Carl Zeiss) containing a PALM Microbeam, RoboStage and a PALM RoboMover (PALM RoboSoftware version 4). Under direct microscopic visualization, LMPC permits the procurement of histologically or immunohistologically defined

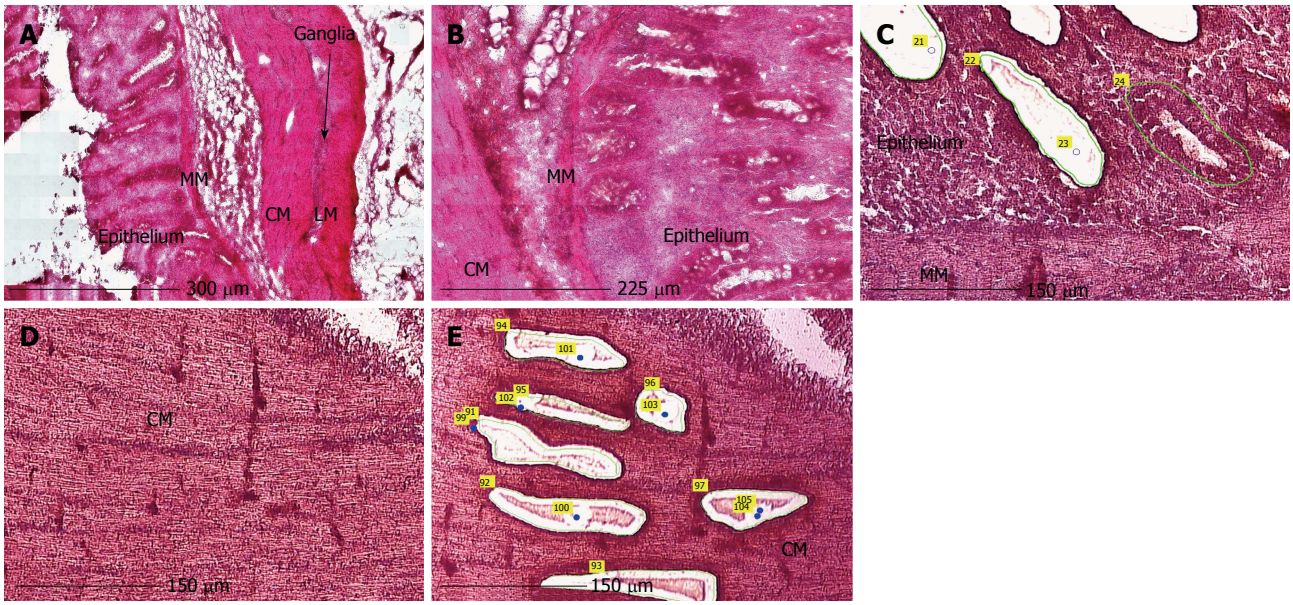

Figure 2 Photomicrographs of hematoxylin and eosin stained tissue sections of colon descendens: epithelium, muscularis mucosae, circular muscle layer, longitudinal muscle layer and ganglion. A: Overview of all layers in colon descendens; B: Detail of the epithelium; C: Epithelium with large patches microdissected by LMPC; D: Details of CM; E: CM with large patches of smooth muscle cells microdissected by LMPC. MM: Muscularis mucosae; CM: Circular muscle layer; LM: Longitudinal muscle layer; LMPC: Laser microdissection and pressure catapulting.

tissue and cell samples (Figure 2). Approximately 15 large patches of cells from the epithelium or circular smooth muscle layer in HE stained sections, or 70 EC cells in IHC stained sections were laser-dissected and pressure-catapulted in 50 μL RLT lysis buffer (RNeasy kit, Qiagen). The cell collecting time was limited to 2 h per slide and after 2 h of cell sampling, the remaining tissue on the membrane slide was scraped off and RNA was extracted to determine if RNA integrity was preserved after 2 h. The samples were homogenized by vortexing, centrifuged and then placed at -80 ℃ for later use. Seven EC cell collections were pooled into one sample with a final volume of 350 μL, resulting in a collection of approximately 500 cells per sample. Total RNA from the cell samples was extracted using the RNeasy Micro kit (Qiagen), according to the manufacturer's instructions.

Endpoint RT-PCR

Quantification of RNA was determined using a Nanodrop ND-1000 spectrophotometer (Isogen Life Science, Temse, Belgium) and the quality of RNA extracted from tissue fractions and tissue sections was assessed using the Experion automated electrophoresis system (BioRad, Nazareth Eke, Belgium).

cDNA of tissue fractions was prepared from 1 μg total RNA, whereas cDNA of whole tissue sections, parts of tissue sections and LMPC samples was prepared from the maximal input of total RNA as possible, as the amount of total RNA was less than 1 μg. The production of cDNA from sample RNA by RT was carried out according to manufacturer's instructions, using SuperScript Ⅲ Reverse Transcriptase SuperMix (Life Technologies Europe) containing random hexamers and oligo (dT)$_{20}$. The obtained cDNA was stored at -20 ℃ before PCR. cDNA amplification reactions were carried out using the AccuPrime Pfx SuperMix (Life Technologies Europe). The template cDNA of mucosa and MMP tissue fractions for amplification was diluted 1:10. Expression of the 5-HT$_4$ receptor within the samples was analysed using 5-HT$_4$ receptor-specific primers spanning exon-intron-exon junctions: an exon 4-specific forward primer and an exon 5-specific reverse primer (Figure 1B). These primers will detect alternative splicing of the h-exon because the h-exon is located between exon 4 and exon 5. The quality of cDNA produced was assessed by amplifying cDNA for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To amplify cDNA of tissue fractions and tissue sections, PCR reactions were performed using the following protocol: 5 min at 95 ℃, followed by 36 cycles with annealing temperature of 54 ℃. The LMPC samples had a low RNA output; therefore, PCR reactions to amplify the cDNA for both 5-HT$_4$ receptor and GAPDH, were performed using two rounds of PCR[15], according to the following protocol: a first reaction of 5 min at 95 ℃ followed by 36 cycles at 54 ℃ annealing temperature, followed by a second reaction using 1.5 μL of the product of the first reaction as a template for a second round of PCR (5 min at 95 ℃ followed by 36 cycles) with the same primers, but with a higher annealing temperature of 56 ℃ to increase specificity. RT and endpoint PCR reactions were processed on a C1000 Thermal Cycler (BioRad). PCR products were separated by 2% agarose gel electrophoresis and visualised by ethidium bromide staining. The primers (Eurogentec, Seraing, Belgium) used were published previously by De Maeyer *et al*[16]: 5-HT$_4$R forward primer (5'-ACAGGAACAAGATGACCCCT-3'); 5-HT$_4$R reverse primer (5'-AGGAGGAACGGGATGTAGAA-3'); GAPDH forward

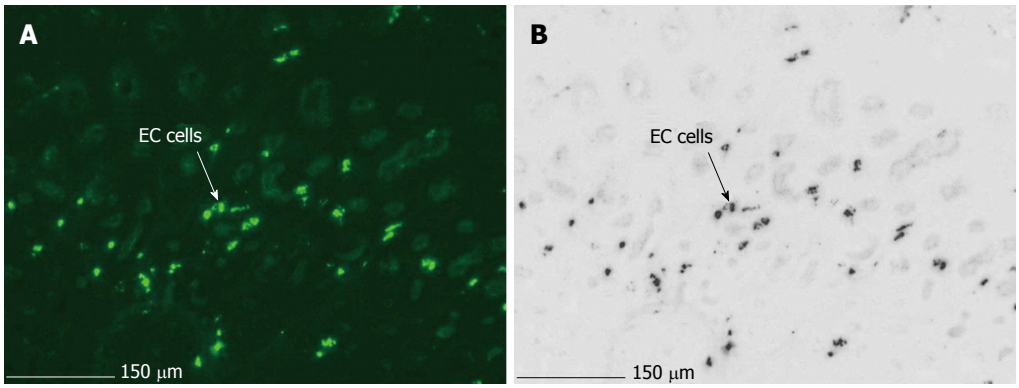

Figure 3 Photomicrographs of immunohistologically stained 8 μm sections of pig gastric fundus showing. A: MAB352 (1:200) immunofluorescent enterochromaffin cells (EC) in the epithelium (× 20); B: Invert color visualization of MAB352 (1:200) immunofluorescent EC cells.

primer (5'-ACCACAGTCCATGCCATCAC-3'); GAPDH reverse primer (5'-TCCACCCTGTTGCTGTA-3').

Statistical analysis

Semi-quantification of PCR products was determined by the intensity of PCR bands on the agarose gels using Image J 1.45 software. Band intensity was expressed as relative absorbance units, and the background of the image was determined and subtracted from the gel image. The ratio between the 5-HT$_4$ receptor and GAPDH RNA was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Data presented are mean ± SE of the mean for animals (n). Statistical analyses were performed using Graphpad Prism software v.5.01 (United States). Differences in intensity were determined by an unpaired t test; $P < 0.05$ was considered statistically significant.

RESULTS

Evaluating cell-specific visualization and RNA integrity

The main difficulties, when using LMPC to analyse gene expression of a specific cell type, are efficient and selective isolation of the desired cells, and obtaining RNA of good quality. Therefore, optimization of the LMPC experimental design was needed for the pig GI tissues, which are highly heterogeneous and rich in endogenous RNase and other enzymes. First, to select the desired cells from the heterogeneous GI tissues, a good visualization of the tissue layers and cells under direct microscopy was necessary. This requires that the morphology of the tissue be preserved, because fractures and air bubbles within the specimen will hamper the view. Tissues for section preparation and LMPC were therefore frozen in liquid N_2 containing isopentane. After HE staining, the different layers of colon descendens (Figure 2) and gastric fundus (not shown) could be identified based on their morphological characteristics. After cell-specific IHC staining, MAB352-immunoreactive EC cells (Figure 3) were present in the crypts, villi and epithelial lining of the mucous membrane of the gastric fundus and were isolated by LMPC.

To evaluate the impact of the different protocol steps on RNA integrity, a systematic approach was followed by evaluating RNA yield and quality after each protocol step. RNA quality was assessed by comparing 28S and 18S and pre-18S ribosomal peaks to a set of degradation standards using the Experion automated electrophoresis system, where the RNA quality indicator (RQI) returns a number between 10 (intact RNA) and 1 (highly degraded RNA)[31]. The analysis showed that RNA quality was not affected after tissue fraction collection (Figure 4A), HE staining (Figure 4B), LMPC (Figure 4C) or after IHC staining (Figure 4D). However, electropherograms of RNA collected by LMPC could not be analysed systematically because the amount of RNA collected was too low. In Figure 4C, RNA quality from large microdissected patches of either epithelial or smooth muscle cells is shown, indicating that RNA was remained mostly intact, but the small ribosomal RNA peaks (18S/28S) indicate a low amount of RNA.

Expression of the 5-HT$_4$ receptor in porcine tissue fractions

Tissue samples from pig colon descendens and gastric fundus were dissected into the mucosa fraction and the MMP fraction before freezing in liquid N_2. After RNA extraction and endpoint PCR analysis of these fractions, 5-HT$_4$ receptor expression was detected in mucosa as well as MMP fractions of the colon descendens and gastric fundus (Figure 5). All tissue samples were positive for GAPDH, confirming the integrity of the samples and completed PCR reactions. In all samples, 5-HT$_4$ receptors containing the h-exon [277 bp; 5-HT$_{4(+h)}$ receptor] as well as 5-HT$_4$ receptors without the h-exon [235 bp; 5-HT$_{4(-h)}$ receptor] were present (Figure 5). However, a third band, corresponding to a fragment of more than 300 bp was also observed. Therefore, we isolated this unknown PCR band using a QIA quick Gel extraction kit (Qiagen) and determined its DNA sequence with an ABI3130XL sequencer (Life Technologies Europe). After sequence analysis, we aligned the unknown sequence with the 5-HT$_{4(+h)}$ and 5-HT$_{4(-h)}$ receptor sequences and observed that the unknown band contained the same sequence and

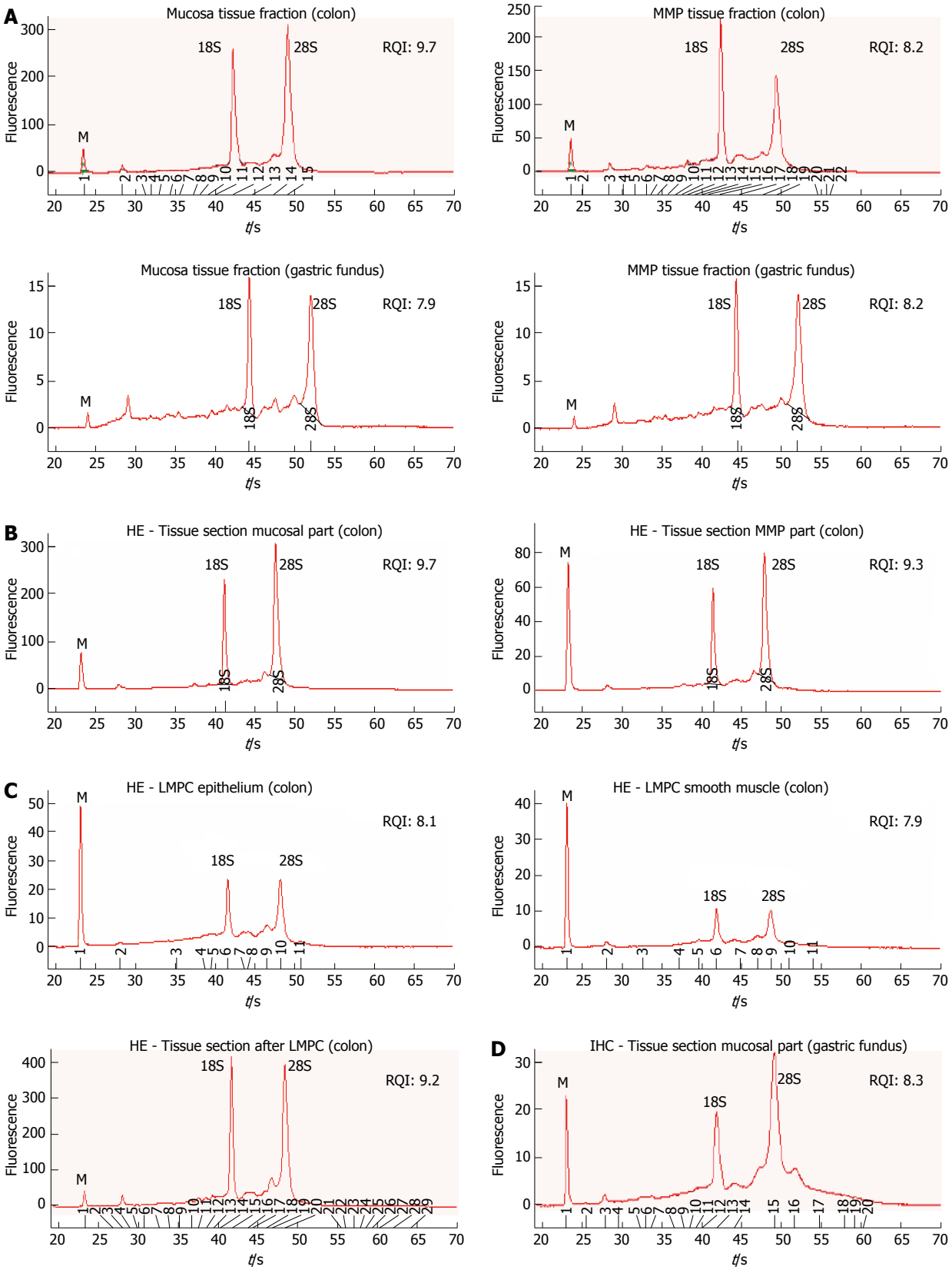

Figure 4 Representative experion electropherograms of collected RNA. A: Mucosa and MMP tissue fractions of colon descendens and gastric fundus; B: The mucosal part and the MMP part of HE stained tissue sections of colon descendens; C: Large patches of epithelial cells and smooth muscle cells obtained by LMPC from HE stained tissue sections and the whole HE stained tissue section scraped off after LMPC in colon descendens; D: The mucosal part of an IHC stained tissue section of gastric fundus. Electropherograms show fluorescence (ordinate) *vs* time (abscissa) with RQI values. Positions of 18S and 28S ribosomal RNA and marker (M) peaks are indicated. MMP: Muscle-myenteric plexus; HE: Hematoxylin and eosin; IHC: Immunohistochemically; RQI: RNA quality indicator.

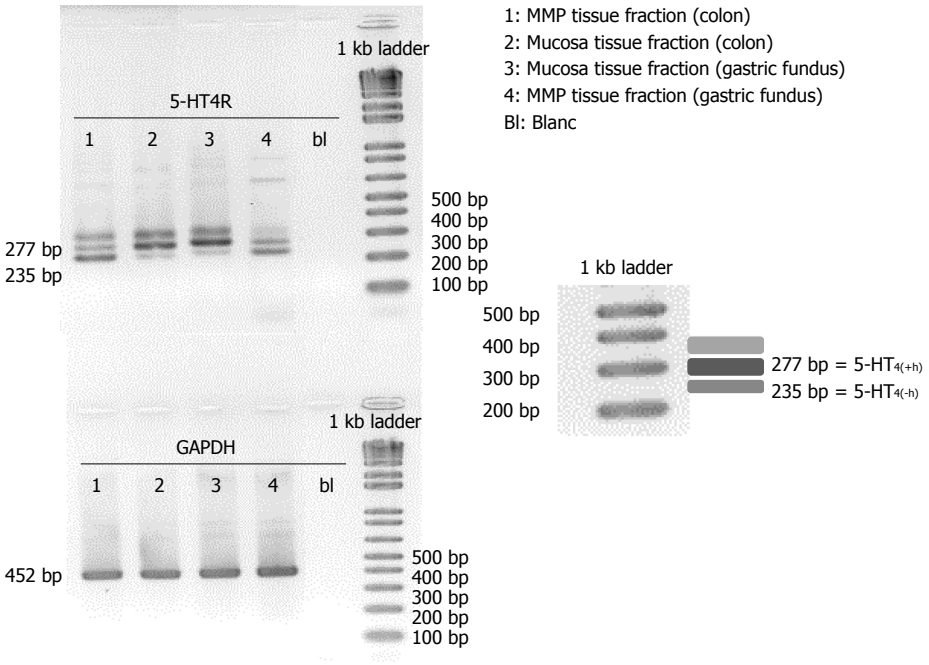

Figure 5 Invert color image of endpoint polymerase chain reaction analysis of the 5-HT$_4$ receptor and of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene expressed in mucosa and muscle-myenteric plexus tissue fractions of pig colon descendens and gastric fundus. Dominant expression of 5-HT$_{4(+h)}$ receptor in the mucosa of the colon descendens and gastric fundus is observed. Part of the ladder is increased to indicate the size of the expected polymerase chain reaction (PCR) products. A third unknown upper band is shown above the 277 bp band, due to dimerization of the PCR product with other PCR fragments after the PCR reaction. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MMP: Muscle-myenteric plexus.

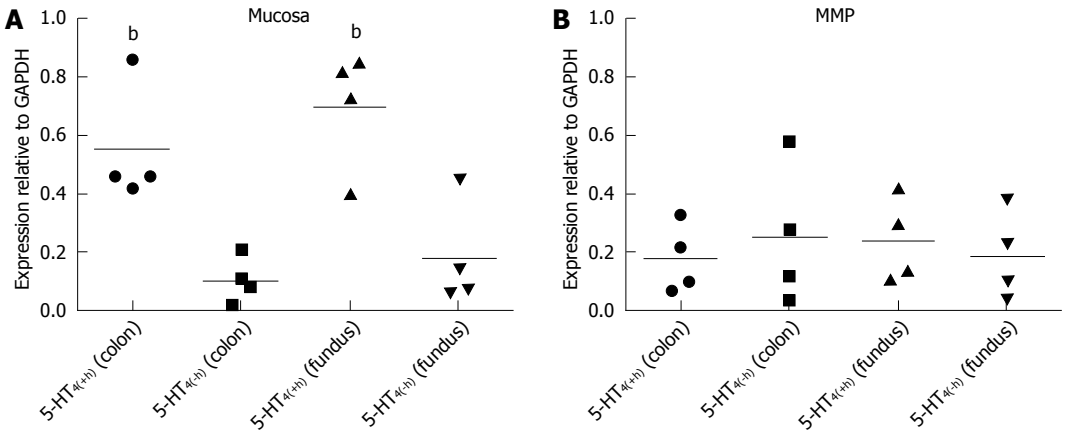

Figure 6 Expression of 5-HT$_{4(+h)}$ and 5-HT$_{4(-h)}$ receptors in the mucosa (A) and muscle-myenteric plexus (B) fractions of colon descendens and gastric fundus. Data are given as ratio relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The line indicates the mean of $n = 4$ for each region-fraction. $^bP < 0.01$ *vs* values for 5-HT$_{4(-h)}$ receptors in colon descendens or gastric fundus. MMP: Muscle-myenteric plexus.

length as the 5-HT$_{4(+h)}$ receptor, but chromatogram details suggested the presence of additional nucleotides in the tail of the sequence, possibly caused by the formation of a heteroduplex with a 5-HT$_{4(+h)}$ strand and a 5-HT$_{4(-h)}$ strand, or formation of a triplex with other PCR fragments, resulting in a different electrophoretic separation.

Mucosa fractions of the colon descendens and gastric fundus contained relatively more h-exon containing 5-HT$_4$ receptors. Semi-quantification by expressing the intensity of the bands compared with the intensity of GAPDH and statistical analysis (Figure 6), confirmed the significantly ($P < 0.01$) more pronounced expression of 5-HT$_{4(+h)}$ receptor within the mucosa fractions (the ratio *vs* GAPDH was 0.55 ± 0.10 in the colon descendens and 0.69 ± 0.13 in the gastric fundus; $n = 4$) compared to the expression of the 5-HT$_{4(-h)}$ receptor (colon descendens: 0.12 ± 0.03; gastric fundus: 0.21 ± 0.10; $n = 4$). Within the MMP fraction of the colon descendens and gastric fundus there was no difference in expression of the 5-HT$_{4(+h)}$ receptor (colon descendens: 0.18 ± 0.06; and gastric fundus: 0.18 ± 0.05; $n = 4$) and 5-HT$_{4(-h)}$ receptor (colon descendens: 0.26 ± 0.12; gastric fundus: 0.17 ± 0.09; $n = 4$).

Expression of the 5-HT$_4$ receptor in porcine HE stained tissue sections

Whole tissue sections, and the mucosal or MMP part of

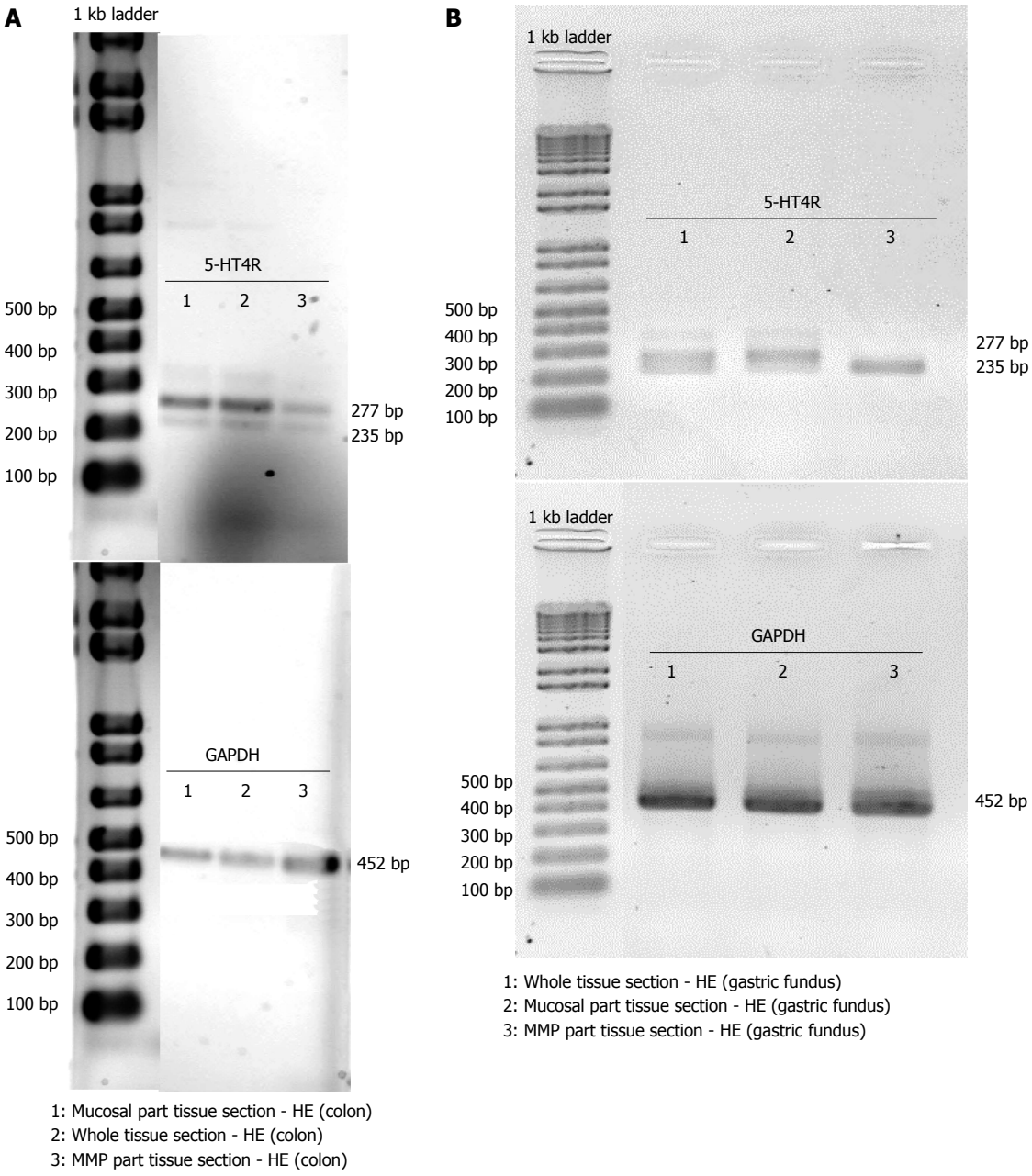

Figure 7 Invert color image of endpoint polymerase chain reaction analysis. 5-HT$_4$ receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown in a hematoxylin and eosin (HE) stained whole tissue section, and in the mucosal as well as the muscle-myenteric plexus (MMP) part of a HE stained tissue section of colon descendens (A) and gastric fundus (B). The size of the expected polymerase chain reaction products is indicated.

tissue sections of the colon descendens and gastric fundus were scraped off a membrane slide and 5-HT$_4$ receptor expression was analyzed.

Colon descendens: 5-HT$_4$ receptor and GAPDH expressions were detected in the whole tissue sections, and in the mucosal and MMP parts of tissue sections of the colon descendens (Figure 7A). After semi-quantification, the values for 5-HT$_4$ receptor expression were 5-HT$_{4(+h)}$R 1.21 ± 0.66 and 5-HT$_{4(-h)}$R 0.63 ± 0.45 within whole tissue sections (n = 3); 5-HT$_{4(+h)}$R 0.86 ± 0.39, 5-HT$_{4(-h)}$R 0.28 ± 0.14 within the mucosal part of tissue sections (n = 3); and 5-HT$_{4(+h)}$R 0.47 ± 0.05, 5-HT$_{4(-h)}$R 0.24 ± 0.11 within the MMP part of tissue sections (n = 3). The tendency for more pronounced expression of the h-exon containing splice variant did not reach significance.

Gastric fundus: 5-HT$_4$ receptor and GAPDH expression was also detected in the whole tissue sections, and mucosal and MMP parts of tissue sections of the gastric fundus (Figure 7B). After semi-quantification, expression values were 0.51 and 0.19 for 5-HT$_{4(+h)}$R, 0.23 and 0.11 for 5-HT$_{4(-h)}$R in whole tissue sections, 1.13 and 0.24 for 5-HT$_{4(+h)}$R and 0.31 and 0.05 for 5-HT$_{4(-h)}$R in the mucosal part of tissue sections. In the two samples with the MMP part of tissue sections, the 5-HT$_{4(-h)}$ receptor was found in both (0.24 and 0.45), while the 5-HT$_{4(+h)}$ receptor was only found in one of the samples (0.53) (Figure 7B

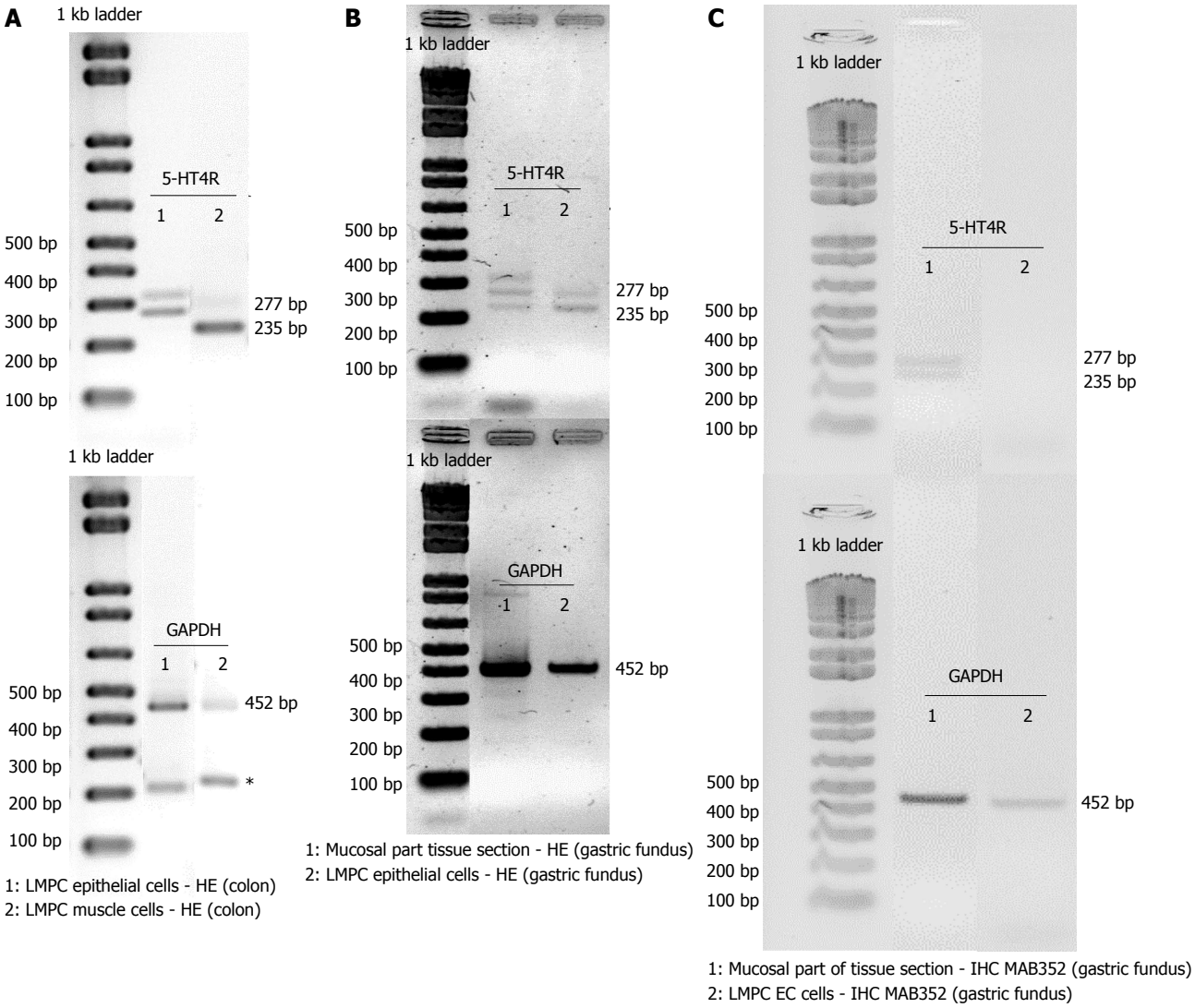

Figure 8 Invert color representation of the double round of endpoint polymerase chain reaction analysis. A, B: 5-HT$_4$ receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown in large patches of epithelium cells and smooth muscle cells obtained by laser microdissection and pressure catapulting (LMPC) from hematoxylin and eosin (HE) stained tissue sections of colon descendens (A) and gastric fundus (B, only epithelial cells were obtained), and in enterochromaffin cells obtained by LMPC from MAB352 immunohistochemical (IHC) stained tissue sections of gastric fundus (C). For comparison, the result obtained in the mucosal part of the HE or IHC stained tissue section of gastric fundus is also shown in B and C. The size of the expected polymerase chain reaction products and presence of non-specific amplification are indicated (asterisk).

lane 3, showing the result with only the 5-HT$_{4(-h)}$ receptor detected).

Expression of the 5-HT$_4$ receptor in LMPC-isolated cell populations from HE stained porcine tissue sections

After HE staining, large microdissected patches of cells were taken from the epithelium and the circular smooth muscle layer of the colon descendens and from the epithelium of the gastric fundus. Low RNA yields meant that only after a second round of PCR, with the same 5-HT$_4$ receptor specific-primers, was 5-HT$_4$ receptor expression detected in large microdissected patches of epithelial cells (Figure 8A and B) or smooth muscle cells (Figure 8A). Non-specific amplification was occasionally observed because of the high number of cycles (Figure 8A). In the colon descendens (Figure 8A), higher expression of the 5-HT$_{4(+h)}$ receptor was observed compared with the 5-HT$_{4(-h)}$ receptor within the LMPC-isolated epithelial cells [5-HT$_{4(+h)}$R 1.03 and 0.85, 5-HT$_{4(-h)}$R 0.24 and 0.11], while the opposite occurred for the smooth muscle cells [5-HT$_{4(+h)}$R 1.14 and 0.91, 5-HT$_{4(-h)}$R 5.07 and 4.03]. In the gastric fundus epithelial cells (Figure 8B), the expression appeared similar for the 5-HT$_{4(+h)}$R (0.45 and 0.20) and 5-HT$_{4(-h)}$R (0.67 and 0.24).

Expression of the 5-HT$_4$ receptor in LMPC-isolated EC cells from IHC stained porcine tissue sections

After IHC staining, EC cells (MAB352; Figure 8C), were isolated by LMPC from gastric fundus tissue sections. After a second round of PCR with the same 5-HT$_4$ receptor specific-primers, no 5-HT$_4$ receptor expression was detected in LMPC-isolated EC cells, although the cells were positive for the *GAPDH* gene, confirming the integrity of the samples and completed PCR reactions at

least for GAPDH. Additionally, in the mucosal part (Figure 8C) of IHC stained tissue sections, scraped off after LMPC of EC cells, 5-HT$_4$ receptor and GAPDH expressions were detected.

DISCUSSION

The aim of this study was to investigate the 5-HT$_4$ receptor distribution in the pig GI tract by confining gene expression analysis to site-specific regions of interest, with special attention being paid to the mucosal layer of the pig colon descendens and gastric fundus by isolating epithelial cells using the LMPC technique. A stepwise approach was used by first studying mucosal and MMP tissue fractions, then tissue sections where different cell layers were discerned morphologically by HE staining, and finally tissue sections stained for a particular cell type by IHC. The impact of the freezing method and staining method on the RNA quality was evaluated in mucosa and MMP tissue fractions (Figure 4A), and mucosal and MMP parts of HE stained tissue sections (Figure 4B) of the pig colon descendens and gastric fundus. The major advantages of LMPC are the isolation of biological material without direct user contact, thereby avoiding contamination, and the preservation of cellular integrity[32]. The main obstacles, when using LMPC to analyse 5-HT$_4$ receptor mRNA expression in different cell types, are firstly to recognize the cells of interest and secondly to obtain RNA of good quality. To recognize the cells of interest while preserving RNA integrity, an HE protocol and a cell-specific IHC protocol were developed in RNase-free conditions. Developing a suitable IHC staining procedure was more complex compared with the HE staining, because a standardized IHC procedure requires a long overnight antibody incubation to obtain good antibody labeling; however, long incubation in aqueous buffers activates endogenous RNases, resulting in RNA degradation[33]. Our attempts to develop a fast IHC protocol resulted in diminished visualization because of the short antibody labeling time. Therefore, our IHC staining protocol was based on the report published by Brown *et al*[24], where overnight antibody incubation and RNA integrity could be maintained with the addition of 1 mol/L NaCl to all aqueous solutions, resulting in superior protection of RNA. It is not yet clear why a saline solution preserves RNA, although we can speculate that normal saline protects the integrity of cell membranes, which may prevent the release of intracellular RNases[34]. Our results confirm the preservation of RNA after HE staining (Figure 4B) as well as after overnight IHC staining (Figure 4D). EC cells in the mucosal layer of the pig gastric fundus were visualized by IHC staining with the MAB352 antibody against 5-HT (Figure 3). Indeed, Penkova *et al*[35] showed that the MAB352 antibody is selective for EC cells in the human gastric mucosa, as verified by electron microscopy.

In this study, 5-HT$_4$ receptor expression was detected in both mucosa and MMP tissue fractions (Figures 5 and 6), and in mucosal and MMP parts of HE stained tissue sections (Figure 7) of the colon descendens, as well as of the gastric fundus, confirming previously reported data by De Maeyer *et al*[16]. Expressions of both a variant with the h-exon [5HT$_{4(+h)}$ receptor, 277 bp] and one without the h-exon [5HT$_{4(-h)}$ receptor, 235 bp] were observed. This was made possible by analysis of the 5-HT$_4$ receptor mRNA expression profile using forward and reverse primers based on exon 4 and 5, which were designed to amplify part of the common receptor region encoded by exon 4 and 5, which flank exon h (Figure 1B). The study was not designed to discriminate the C-terminal tail associated with the h-exon; the 5HT$_{4(+h)}$ receptor detected might indeed be associated with several C-terminal splice variants, because the h-exon has already been associated with the a, m and r C-terminal in pigs[16]. Semi-quantitative analysis revealed that in the mucosa tissue fractions of colon descendens and gastric fundus, the expression level of the 5-HT$_{4(+h)}$ receptor was significantly higher compared with the 5-HT$_{4(-h)}$ receptor, and a similar trend was obtained in the mucosal part of HE stained tissue sections. While evaluation of 5-HT$_4$ receptor RNA expression in human GI full-thickness tissue samples showed similar levels in the stomach compared with more distal levels[36], expression in human gastric mucosal specimens was much less pronounced than in mucosal specimens of more distal regions of the GI tract[21,22]. In the pig gastric fundus mucosa, however, the expression of the 5-HT$_4$ receptor was similar to that in the mucosa of the colon descendens, with a clear-cut preponderance of the h-exon-containing receptor. The predominant mucosal location of h-exon containing 5-HT$_4$ receptor splice variants might correspond to the preferential involvement of this type of 5-HT$_4$ receptor splice variant in the mucosal effects of 5-HT$_4$ receptor activation, such as goblet cell degranulation, chloride secretion and control of 5-HT release[21].

Both the mucosal and the MMP part of the GI tract contain several cell types on which the presence of 5-HT$_4$ receptors has been suggested, at least in some regions in some species, such as EC cells, smooth muscle cells of the muscularis mucosae and submucosal intrinsic neurons in the mucosal part; and myenteric cholinergic neurons, smooth muscle cells and interstitial cells of Cajal in the MMP part[37-41]. To obtain more information on the cell-specific distribution of the 5-HT$_4$ receptors, cell layers or particular cell types were isolated by LMPC. In the colon descendens, patches of the epithelial cell layer obtained by LMPC showed expression of 5-HT$_4$ receptors, predominantly the 5HT$_{4(+h)}$ receptor. Possible cell types involved might be EC cells and goblet cells, which were recently shown to express 5-HT$_4$ receptors in the mouse intestine[21]. In mouse, application of 5-HT$_4$ receptor agonists led to mucosal 5-HT release and mucus secretion in a tetrodotoxin-insensitive manner, indicating direct activation of stimulatory 5-HT$_4$ receptors on the EC cells and goblet cells[21]. In the porcine and human small intestine however, analysis of 5-HT release suggested the presence of inhibitory 5-HT$_4$ receptors on the EC cells[42]. Relaxant 5-HT$_4$ receptors have been proposed on circular smooth muscle in the human colon on the basis

of functional data[13]; therefore, patches of cells were also obtained from the circular muscle layer of the pig colon descendens, which revealed 5-HT$_4$ receptor expression. However, some authors were not able to confirm the presence of relaxant 5-HT$_4$ receptors in human colonic circular muscle strips[43]; we were also unable to obtain evidence for muscular 5-HT$_4$ receptors in pig colonic circular muscle strips[12]. Thus, we can not exclude the possibility that the 5-HT$_4$ receptor expression observed in LMPC-isolated cell patches from the pig colonic circular muscle layer represents the 5-HT$_4$ receptors on intercalated interstitial cells of Cajal. In addition, in the pig gastric fundus, LMPC-isolated cell patches of the epithelial cell layer showed 5-HT$_4$ receptor expression. The human gastric mucosa was shown to contain a considerable number of EC cells scattered within the lining epithelium[35]; therefore, we stained EC cells in porcine gastric mucosa immunohistochemically and isolated them by LMPC. Although the 5-HT$_4$ receptor was still detected in the full mucosal part of these IHC stained sections, 5-HT$_4$ receptor expression was not detected in the isolated EC cells. The EC cells showed expression of GAPDH; therefore, this might be related to the small amount of 5-HT$_4$ receptor RNA obtained, even when pooling 500 LMPC-isolated cells. In the gastric fundus, LMPC was not used to obtain cell patches from the muscle layer, as there are no functional data suggesting 5-HT$_4$ receptors to be present on muscle cells in the stomach.

In conclusion, this study, using endpoint RT-PCR, confirmed the presence of 5-HT$_4$ receptors in the mucosa and in the MMP part of porcine gastric fundus and colon descendens, and showed that the mucosa predominantly expresses h-exon-containing 5-HT$_4$ receptors. The mucosal h-exon-containing 5-HT$_4$ receptors might form additional sites of action for 5-HT$_4$ receptor agonists. 5-HT$_4$ receptors were detected in LMPC-isolated epithelial cell patches in the gastric fundus and colon descendens, and in circular muscle cell patches in the colon descendens. No 5-HT$_4$ receptor expression was detected in gastric LMPC-isolated EC cells stained by immunohistochemistry; the expression of 5-HT$_4$ receptors in individual cell types might be too low to detect by LMPC and endpoint PCR.

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COMMENTS

Background

5-HT$_4$ receptors are distributed throughout the gastrointestinal (GI) tract. They are expressed on excitatory motor neurons, promoting the stimulatory effect of these neurons on GI motility. This explains the therapeutic use of 5-HT$_4$ receptor agonists in conditions with impaired GI motility, such as constipation. Several reports indicate that 5-HT$_4$ receptors are also expressed in the GI mucosa. 5-HT$_4$ receptors are G-protein coupled receptors containing seven transmembrane domains. The intracellular tail shows splice variation, with different lengths of the intracellular amino acid sequence. Exceptionally among G-protein coupled receptors, 5-HT$_4$ receptors can also have an extra insertion of 14 amino acids in the second extracellular loop, encoded by the h-exon (42 base pairs). Previous distribution studies of 5-HT$_4$ receptors in the GI tract did not consider the all or none presence of the h-sequence.

Research frontiers

Using homogenates of tissues limits the potential to study cell-specific expression: important cell-specific transcript information is lost because of cell heterogeneity of tissues such as GI tissues. Techniques have been developed to enable collection of particular cells from mixed populations, which generally involve either fluorescence activated cell sorting (FACS) purification of dissociated cells or laser-assisted microdissection. In contrast to FACS, microdissection can be applied to most tissues.

Innovations and breakthroughs

The major advantages of laser microdissection and pressure catapulting (LMPC) are the isolation of biological material without direct user contact, thereby avoiding contamination, and the preservation of cellular integrity. The main concerns when using LMPC to analyse 5-HT$_4$ receptor mRNA expression of a specific cell type is the efficient and selective isolation of the right cells, and obtaining RNA of good quality. To address these issues, and to optimize a LMPC experimental design for GI tissue that is highly heterogeneous, rich in endogenous RNase and enzymes, a systematic approach was required to evaluate the impact on RNA integrity of different critical steps. Therefore, RNA yield and quality were determined using the Experion automated electrophoresis system after tissue collection, hematoxylin and eosin (HE) staining or immunohistochemistry (IHC) staining, and LMPC by analysing RNA extracted from mucosal and muscular myenteric plexus (MMP) tissue fractions, and from scraped off tissue sections after staining and after LMPC. Developing a suitable IHC staining procedure was more complex compared with the HE staining, because a standard IHC procedure requires a long overnight antibody incubation to obtain good antibody labeling, but long incubation in aqueous buffers activates endogenous RNases, resulting in RNA degradation. The authors attempted to develop a fast IHC protocol, which resulted in diminished visualization because the short antibody labeling time. Therefore, the authors used an overnight antibody incubation for the IHC staining protocol whereby RNA integrity was maintained by the addition of 1 mol/L NaCl to all aqueous solutions, resulting in superior protection of RNA. It is not yet clear why a saline solution preserves RNA, although the authors speculate that normal saline protects the integrity of cell membranes, preventing the release of intracellular RNases.

Applications

This study, using endpoint reverse transcription-polymerase chain reaction (PCR) and LMPC, confirms the presence of 5-HT$_4$ receptors in the mucosal and MMP parts of the porcine gastric fundus and colon descendens, and shows that the mucosa predominantly expresses h-exon containing 5-HT$_4$ receptors. The mucosal h-exon-containing 5-HT$_4$ receptors might be preferentially involved in the mucosal response to 5-HT$_4$ receptor activation and might form potential drug targets for 5-HT$_{4(+h)}$ receptor-selective agonists.

Terminology

5-HT$_4$ receptors: The 5-HT$_4$ receptor is a G-protein coupled receptor that activates the adenylyl cyclase/cyclic adenosine monophosphate/protein kinase A pathway in response to serotonin (5-hydroxytryptamine; 5-HT). The 5-HT$_4$ receptor is expressed on excitatory motor neurons in the gut, facilitating acetylcholine release, which stimulates GI motility; LMPC: Under direct microscopic visualization, LMPC permits sampling of histologically or immunohistologically defined tissue and cell samples. The LMPC system uses a focused pulsed nitrogen UV-A laser beam (wavelength 355 nm) whose source is positioned below the material and the high energy generated beam is focused through a microscope ocular lens onto the biological material on the slide.The RoboMover stage is used to move the sample through the laser beam path to allow the user to control the size and shape of the area to be cut. The beam is then defocused and this energy is used to catapult the membrane and corresponding biological material from the slide. When the laser beam strikes the material, it is blasted off the glass surface and catapulted into the cap of the vial.

Peer review

This manuscript describes the expression of the +h 5-HT$_4$ receptor variant in porcine stomach and colon. The significance of this work is the use of LMPC and end-point PCR to allow the positive identification of the specific HTR$_4$ variant (+ exon h) in discernible functional cell types and tissue regions relevant to serotoninergic responses. This study also discusses several technique modifications and improvements and artifacts in detail, which might be helpful for wider application of similar interests.

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