**Name of journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO: 224**

**Columns: ORIGINAL ARTICLES**

**Predominant mucosal expression of 5-HT4(+h) receptor splice variants in pig stomach and colon**

Priem EKV *et al.* Pig and 5-HT4 receptor distribution

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**Supported by** the Fund for Scientific Research Flanders

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**Received:** August 17, 2012 **Revised:** December 3, 2012

**Accepted:** December 15, 2012

**Published online:**

**Abstract**

**AIM**: To investigate cellular 5-HT4(-h/+h) receptor distribution, with attention to the epithelial layer, by laser microdissection and PCR in porcine gastrointestinal tissues.

**METHODS**: A stepwise approach was used to evaluate RNA quality and to study cell-specific 5-HT4 receptor mRNA expression in porcine gastric fundus and colon descendens. After freezing, staining, laser microdissection and pressure catapulting (LMPC), RNA quality was evaluated by the experion automated electrophoresis system. 5-HT4 receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was examined by endpoint RT-PCR in mucosal and muscle-myenteric plexus (MMP) tissue fractions, in mucosal and MMP parts of hematoxylin&eosin (H&E) stained tissue sections and in microdissected patches of the epithelial and circular smooth muscle cell layer in these sections. In 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 H&E staining, the epithelial and circular smooth muscle cell layer of pig colon descendens and the epithelial cell layer of gastric fundus were morphologically identified and isolated by LMPC. Enterochromaffin (EC) cells of pig gastric fundus were successfully stained by IHC and isolated by LMPC. Freezing, H&E and IHC staining, and LMPC had no influence on RNA quality. 5-HT4 receptor and GAPDH mRNA expression was detected in mucosa and MMP tissue fractions, and in mucosal and MMP parts of H&E stained tissue sections of pig colon descendens and gastric fundus. In the mucosa tissue fractions of both gastrointestinal regions, the expression of h-exon containing receptor (5-HT4(+h) receptor) mRNA was significantly higher (*P* < 0.01) compared to 5-HT4(-h) receptor expression, and a similar trend was obtained in the mucosal part of H&E 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-HT4 receptor and GAPDH mRNA expression. No 5-HT4 receptor mRNA expression was detected in gastric LMPC-isolated EC cells from IHC stained tissues, while the cells were positive for GAPDH.

**CONCLUSION:** Porcine gastrointestinal mucosa predominantly expresses 5-HT4(+h) receptor splice variants, suggesting their contribution to 5-HT4 receptor-mediated mucosal effects of 5-HT.

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

Priem EKV, De Maeyer JH, Vandewoestyne M, Deforce D, Lefebvre RA. Predominant mucosal expression of 5-HT4(+h) receptor splice variants in pig stomach and colon. *World J Gastroenterol* 2012; 18

**Available from:** URL: http://www.wjgnet.com/1007-9327/14/0000.asp

**DOI:** http://dx.doi.org/10.3748/wjg.14.0000

**INTRODUCTION**

The 5-HT4 receptor is a G-protein coupled receptor (GPCR) which activates the adenylyl cyclase/cyclic adenosine monophosphate/protein kinase A pathway in response to serotonin (5-HT). The 5-HT4 receptor is expressed on excitatory motor neurons in the gut, facilitating acetylcholine release hereby stimulating gastrointestinal (GI) motility[1-3]. This presynaptic facilitation is thought to be the principal mechanism for the prokinetic action of 5-HT4 receptor agonists, explaining their therapeutic use in GI dysmotility related disorders such as chronic constipation, gastroparesis and gastroesophageal reflux disease[4]. The selective 5-HT4 receptor agonist prucalopride is now used in patients with chronic laxative-resistant constipation; it indeed facilitates acetylcholine release from cholinergic neurons towards human colonic circular[5] as well as longitudinal[6] smooth muscle. The non-selective 5-HT4 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]. Also prucalopride accelerates gastric emptying in man[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-HT4 receptors on GI cholinergic neurons, as the presence of facilitatory 5-HT4 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-HT4 receptor in colon and stomach have been proposed. In the human colon, 5-HT4 receptors were reported to be present on circular smooth muscle cells, inducing relaxation[13]. A functional study by Borman and Burleigh in 1996[14] reported that 5-HT-induced secretion in human sigmoid colon is mediated *via* 5-HT2A receptors but that in the ascending colon a combination of 5-HT2A and 5-HT4 receptors appears to be involved. Still a recent study showed the presence of mRNA of several 5-HT4 receptor splice variants in the mucosal layer of human sigmoid colon[15]; 5-HT4 receptor mRNA was also reported in pig colonic mucosa[16]. In rat colon, it has been suggested that 5-HT-induced mucosal ion transport and Cl- secretion is mediated by 5-HT4 receptors[17- 20]. Immunohistochemical and functional assays showed the presence of 5-HT4 receptors in mouse colonic epithelial cells, including enterochromaffin cells and goblet cells, inducing mucosal 5-HT release and Cl- secretion[20]. The presence of 5-HT4 receptor transcripts detected by RT-PCR has also been reported in the mucosa of human[21, 22] and pig[16] stomach, 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-HT4 receptor, is needed in human enteric neuronal subpopulations, mast cells and epithelial cells leading to a better understanding of function and activity of 5-HT4 receptors in the GI wall, which may offer new therapeutic perspectives[23]. To date, the majority of information on 5-HT4 receptor distribution is based on functional studies[12] or on 5-HT4 receptor expression studies using homogenates of tissues[15, 16, 21, 22]. However, homogenates of tissues limit the potential of expression studies, since 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 laser microdissected enteric ganglia of the human intestine, 5-HT3A receptor mRNA expression was described[25] and in microdissected human colonic mucosal epithelium, transcripts encoding for 5-HT3A, 5-HT3C, 5-HT3D and 5-HT3E subunits were detected[26]. In different species, 5-HT4 receptors show splice variation in the intracellular C-terminal starting after the common amino acid structure L358. In man, 9 splice variants have been described (Figure 1A). In pig, at least another 9 different splice variants, not described in man, have been reported (Figure 1A), as well as unique splicing variation with variants composed of duplicated exons[16]. Splice variants in the extracellular loops of G-protein coupled receptors are rare[27] but the 5-HT4 receptor can have an extra insertion of 14 amino acids in the second extracellular loop, encoded by the h-exon (Figure 1A). In human, this H variant has been described in combination with the b-terminal exon (5-HT4(hb))[28]. When comparing the pharmacology of this 5-HT4(hb) splice variant, transiently expressed in cells being CV-1 (simian) in origin, and carrying the SV40 genetic material (COS)-7 cells, with that of the 5-HT4(b) and 5-HT4(a) splice variant, it showed a smaller fraction of receptors coupled to G-protein and the 5-HT4 receptor antagonist GR113808 behaved as a partial agonist[28]. In the human gastrointestinal tract, the h exon could be amplified in combination with the b exon only from the lower esophageal sphincter but h exon carrying 5-HT4 transcripts were also obtained in other parts of the gastrointestinal tract, suggesting that the h-exon might be expressed in combination with other C-terminal exons[28]. In pig, De Maeyer *et al*[16] (2008) indeed showed that the 5-HT4(h) splice variant also exists in combination with other C-terminal exons than 5-HT4(b), namely 5-HT4(ha), 5-HT4(hm) and 5-HT4(hr); h-exon containing 5-HT4 transcripts were also found along the porcine gastrointestinal 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-HT4 receptor (with and without the h exon) distribution at the cellular level in laser microdissected porcine GI tissues with special attention for 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, Belgium. All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

The pigs were anaesthetized with an intramuscular injection of 5 mL Zoletil 100 (containing 50 mg/mL tiletamine and 50 mg/mL zolazepam; Virbac Belgium SA, 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% CO2/95% O2) phosphate buffered saline (PBS) at pH 7.4 (Life Technologies Europe, 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 gastrointestinal 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 a RNase-free vial (Life Technologies Europe, Belgium), rapidly frozen in liquid N2 and stored at -80°C. After frozen tissue homogenization and before RNA extraction, MMP samples were treated with proteinase K (Qiagen, 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, Belgium). RNA from mucosa and MMP fractions was extracted using the RNeasy Mini Kit (Qiagen, Belgium) according to manufacturer’s guidelines and RNA samples were stored at -80°C.

**Freezing tissues for section preparation and laser microdissection:** Whole tissues, containing the mucosal as well as the smooth muscle layer, were cut into full-thickness small pieces with a sterile scalpel, placed in tissue embedding medium PELCO CryO-Z-T (Pelco International, United States), rapidly frozen in liquid N2 containing cold isopentane and stored at -80°C. The frozen tissue samples were cut into 8 µm-thick sections using a cryostat (Leica CM 1950; Leica Microsystems, Belgium) with disposable RNase-free knifes. Sections at 8 µM thickness are considered to represent a monolayer of cells[29, 30]. The sections were placed on chilled (-20°C) nuclease free polyethylene naphthalate (PEN)-covered membrane slides (Carl Zeiss, Germany) and immediately stored at -80°C until staining procedure. The membrane slides used for immunohistochemistry were extra coated with poly-L-Lysine (Sigma, Belgium), which was diluted with 0.1% diethylpyrocarbonate (DEPC)-treated water. All materials (pincets, brushes,) were treated with RNase ZAP (Sigma, Belgium) and glassware and pincets were heated for 6 h at 200°C, to remove all exogenous RNases.

To morphologically distinguish the different layers of the tissue sections for laser microdissection, the frozen tissue sections were stained with hematoxylin and eosin (Sigma, Belgium) in RNase-free conditions. Hematoxylin-eosin (H&E) 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°C; aqueous solutions were pre-cooled at 4°C. All solutions were diluted with 0.1% DEPC-treated water, kept in 50 mL RNase-free conical tubes (Life Technologies Europe, Belgium) and kept on ice during the staining procedure.

**Immunohistochemistry:** To distinguish and isolate enterochromaffin (EC) cells with the laser microdissection and pressure catapulting technique (LMPC), visualization with cell-specific antibodies of these cells is needed. To extract integer 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 15 s rinsed with cold (4°C) PBS (pH 7.4; Life Technologies Europe, Belgium) and then 5 min fixed in ice-cold (-20°C) aceton. Aceton was removed by a cold PBS rinse (15 s) and slides were incubated for 30 min at 4°C with blocking buffer (0.25% Triton X-100, 1% bovine serum albumin, 10% goat serum) supplemented with 1 M NaCl. Then, sections were shortly rinsed with cold PBS and incubated overnight at 4°C with the rat anti-serotonin primary antibody MAB352 (Milipore, Belgium), used as a marker for enterochromaffin cells. MAB352 was diluted 1: 200 in PBS supplemented with 1 M NaCl. Unbound primary antibody was removed by rinsing 3 times with cold PBS supplemented with 1 M NaCl. Sections were then incubated with chicken anti-rat secondary antibody Alexa Fluor 488 (Life Technologies, Belgium) diluted 1: 100 in PBS with 1 M NaCl for 2 h at 4°C. Unbound secondary antibody was removed by rinsing 3 times with cold PBS with 1 M 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 prior to laser microdissection.

***Laser microdissection and pressure catapulting***

LMPC was performed using the laser microdissection system from PALM Technologies (Carl Zeiss, Germany) containing a PALM Microbeam, RoboStage and a PALM RoboMover [PALM RoboSoftware version 4]. Under direct microscopic visualization, LMPC permits procurement of histologically or immunohistologically defined tissue and cell samples (Figure 2). Approximately 15 large patches of cells from the epithelium or circular smooth muscle layer in H&E 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, Belgium). 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 still preserved after 2 h. The samples were homogenized by vortexing, spinned down and then placed at -80°C for later use. 7 EC cell collections were pooled to 1 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, Belgium) according to the manufacturer’s instructions.

***Endpoint RT-PCR***

Quantification of RNA was determined using a Nanodrop ND-1000 spectrophotometer (Isogen Life Science, Belgium) and the quality of RNA extracted from tissue fractions and tissue sections was assessed by using the Experion automated electrophoresis system (BioRad, 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 transfer of sample RNA to cDNA by reverse transcriptase (RT) was carried out according to manufacturer’s instructions using SuperScript III Reverse Transcriptase SuperMix (Life Technologies Europe, Belgium) containing random hexamers and oligo(dT)20. The obtained cDNA was stored at -20°C before PCR. cDNA amplification reactions were carried out using the AccuPrime *Pfx* SuperMix (Life Technologies Europe, Belgium). The template cDNA of mucosa and MMP tissue fractions for amplification was diluted 1:10. Expression of the 5-HT4 receptor within the samples was analysed using 5-HT4 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°C followed by 36 cycles with annealing temperature of 54°C. Because of the low RNA output of LMPC samples, PCR reactions to amplify the cDNA for both 5-HT4 receptor and GAPDH, were performed using 2 rounds of PCR [15], according to the following protocol: a first reaction with 5 min at 95°C followed by 36 cycles at 54°C annealing temperature was performed, 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°C followed by 36 cycles) with the same primers but with a higher annealing temperature of 56°C to increase specificity. RT and endpoint PCR reactions were run on a C1000 Thermal Cycler (BioRad, Belgium). PCR products were separated by 2% agarose gel electrophoresis and visualised by ethidium bromide staining. The primers (Eurogentec, Belgium) used were previously published by De Maeyer *et al*[16] and are listed as follows:

5-HT4R forward primer (5’-ACAGGAACAAGATGACCCCT-3’); 5-HT4R reverse primer (5’-AGGAGGAACGGGATGTAGAA-3’); GAPDH forward 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-HT4 receptor and GAPDH RNA was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Data presented are means ± standard error of the mean for *n* animals. 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 first to efficiently and selectively isolate the desired cells and second to obtain 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 enzymes. First, to select the desired cells from the heterogeneous GI tissues, a good visualization of the tissue layers and cells under direct microscopy is necessary. This requires preserved morphology of the tissue, as fractures and air bubbles within the specimen will hamper the view. Tissues for section preparation and LMPC were therefore frozen in liquid N2 containing isopentane. After H&E 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 28 S and 18 S and pre-18 S ribosomal peaks to a set of degradation standards using the Experion automated electrophoresis system, the RNA quality indicator (RQI) returning 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), H&E staining (Figure 4B), LMPC (Figure 4C) or after IHC staining (Figure 4D). However, electropherograms of RNA collected by LMPC could not systematically be analysed due to a too low amount of RNA collected. In Figure 4C, RNA quality from large microdissected patches of either epithelial or smooth muscle cells is shown, indicating that RNA was still mainly intact but the small ribosomal RNA peaks (18 S/28 S) indicate a low amount of RNA.

***Expression of the 5-HT4 receptor in porcine tissue fractions***

Tissue samples from pig colon descendens and gastric fundus were dissected into the mucosal-submucosal (mucosa) fraction and the muscular-myenteric plexus (MMP) fraction before freezing in liquid N2. After RNA extraction and endpoint PCR analysis of these fractions, 5-HT4 receptor expression was detected in mucosa as well as MMP fractions of colon descendens and gastric fundus (Figure 5). All tissue samples were positive for the GAPDH housekeeping gene, confirming the integrity of the samples and completed PCR reactions. In all samples 5-HT4 receptors containing the h-exon (277 bp; 5-HT4(+h) receptor) as well as 5-HT4 receptors without h-exon (235 bp; 5-HT4(-h) receptor) were present (Figure 5). However, a third extra band, corresponding to a fragment of more than 300 bp was also observed. Therefore, we isolated this unknown PCR band (QIAquick Gel extraction kit, Qiagen, Belgium) and determined the DNA sequence of this band (ABI3130XL sequencer, Life Technologies, Belgium). After sequence analysis, we aligned the unknown sequence with the 5-HT4(+h) and 5-HT4(-h) receptor sequence and observed that the unknown band contained the same sequence and length as the 5-HT4(+h) receptor, but chromatogram details suggested the presence of additional nucleotides in the tail of the sequence, possibly due to formation of a heteroduplex with a 5-HT4(+h) strand and a 5-HT4(-h) strand, or formation of a triplex with other PCR fragments, resulting in a different electrophoresis separation.

Mucosa fractions of colon descendens and gastric fundus contained relatively more h-exon containing 5-HT4 receptor. Semi-quantification by expressing the intensity of the bands towards the housekeeping gene GAPDH and statistical analysis (Figure 6), confirmed the significantly (*P* < 0.01) more pronounced expression of 5-HT4(+h) receptor within the mucosa fractions (the ratio versus GAPDH was in colon descendens: 0.55 ± 0.10; gastric fundus: 0.69 ± 0.13; *N*=4) compared to the expression of the 5-HT4(-h) receptor (colon descendens: 0.12 ± 0.03; gastric fundus: 0.21 ± 0.10; *N* =4). Within the MMP fraction of colon descendens and gastric fundus there was no difference in expression of 5-HT4(+h) receptor (colon descendens: 0.18 ± 0.06; and gastric fundus: 0.18 ± 0.05; *N* =4) and 5-HT4(-h) receptor (colon descendens: 0.26 ± 0.12; gastric fundus: 0.17 ± 0.09; *N* =4).

***Expression of the 5-HT4 receptor in porcine H&E stained tissue sections***

Whole tissue sections, and the mucosal or MMP part of tissue sections of colon descendens and gastric fundus were scraped off a membrane slide and 5-HT4 receptor expression was analyzed.

**Colon descendens:**5-HT4 receptor and GAPDH expression was detected in the whole tissue sections, and in the mucosal and MMP parts of tissue sections of colon descendens (Figure 7A). After semi-quantification, the values for 5-HT4 receptor expression were within whole tissue sections (5-HT4(+h)R: 1.21 ± 0.66 and 5-HT4(-h)R: 0.63 ± 0.45; *n* =3), within the mucosal part of tissue sections (5-HT4(+h)R: 0.86 ± 0.39, 5-HT4(-h)R: 0.28 ± 0.14; *n* =3) and within the MMP part of tissue sections (5-HT4(+h)R: 0.47 ± 0.05, 5-HT4(-h)R: 0.24 ± 0.11; *n* =3). The tendency for more pronounced expression of the h-exon containing splice variant did not reach significance.

**Gastric fundus:** 5-HT4 receptor and GAPDH expression was also detected in the whole tissue sections, and mucosal and MMP parts of tissue sections of gastric fundus (Figure 7B). After semi-quantification, expression values were 0.51 and 0.19 for 5-HT4(+h)R and 0.23 and 0.11 for 5-HT4(-h)R in whole tissue sections, and 1.13 and 0.24 for 5-HT4(+h)R and 0.31 and 0.05 for 5-HT4(-h)R in the mucosal part of tissue sections. In the 2 samples with the MMP part of tissue sections, the 5-HT4(-h) receptor was found in both (0.24 and 0.45) while the 5-HT4(+h) receptor was only found in one of the samples (0.53) (Figure 7B lane 3, showing the result with only the 5-HT4(-h) receptor detected).

***Expression of the 5-HT4 receptor in LMPC-isolated cell populations from H&E stained porcine tissue sections***

After H&E 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. Due to low RNA yield, only after a second round of PCR, with the same 5-HT4 receptor specific-primers, 5-HT4 receptor expression was detected in large microdissected patches of epithelial cells (Figure 8A and 8B) or smooth muscle cells (Figure 8A). Aspecific amplification occasionally occurred because of the high number of cycles (Figure 8A). In *colon descendens* (Figure 8A) 5-HT4(+h) receptor was more expressed compared to 5-HT4(-h) receptor within the LMPC-isolated epithelial cells (5-HT4(+h)R: 1.03 and 0.85; 5-HT4(-h)R: 0.24 and 0.11), while the opposite occurred for the smooth muscle cells (5-HT4(+h)R: 1.14 and 0.91; 5-HT4(-h)R: 5.07 and 4.03). In gastric fundus epithelial cells (Figure 8B) the expression appeared similar for the 5-HT4(+h)R (0.45 and 0.20) and 5-HT4(-h)R (0.67 and 0.24).

***Expression of the 5-HT4 receptor in LMPC-isolated EC cells from IHC stained porcine tissue sections***

After IHC staining, EC cells (MAB352; Fig 8C), were isolated by LMPC from gastric fundus tissue sections. After a second round of PCR with the same 5-HT4 receptor specific-primers, 5-HT4 receptor expression was not detected in LMPC-isolated EC cells although cells were positive for the GAPDH housekeeping 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-HT4 receptor and GAPDH expression was detected.

**DISCUSSION**

The aim of this study was to investigate the 5-HT4 receptor distribution in pig GI tract by confining gene expression analysis to site-specific regions of interest, with special attention 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 H&E 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 H&E 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, hereby avoiding contamination, and the preserved cellular integrity[32]. The main obstacles, when using LMPC to analyse 5-HT4 receptor mRNA expression in different cell types, are first to recognize the cells of interest and second to obtain RNA of good quality. To recognize the cells of interest while preserving RNA integrity, a H&E protocol and a cell-specific IHC protocol was developed in RNase-free conditions. Developing a suitable IHC staining procedure was more complex in contrast to the H&E staining, because a standardized 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[33]. Our attempts to develop a fast IHC protocol resulted in diminished visualization due to 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 addition of 1 M NaCl in 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, and hence, prevents the release of intracellular RNases[34]. Our results confirm the preservation of RNA after H&E 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 MAB352 antibody against 5-HT (Figure 3). Penkova *et al*[35] indeed showed that MAB352 antibody is selective for EC cells in the human gastric mucosa as verified by electron microscopy.

In this study, 5-HT4 receptor expression was detected in both mucosa and MMP tissue fractions (Figure 5 and 6) and mucosal and MMP parts of H&E 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]; expression of both a variant with (5HT4(+h) receptor, 277 bp) and one without the h-exon (5HT4(-h) receptor, 235 bp) was observed. This is possible by analysis of the 5-HT4 receptor mRNA expression profile by using forward and reverse primers in exon 4 and 5, designed to amplify part of the common receptor region encoded by exon 4 and 5, exons that flank exon h (Figure 1B). The study was not designed to discriminate the C-terminal tail associated with the h-exon; the 5HT4(+h) receptor detected might indeed be associated with several C-terminal splice variants as 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-HT4(+h) receptor was significantly higher compared to the 5-HT4(-h) receptor, and a similar trend was obtained in the mucosal part of H&E stained tissue sections. While evaluation of 5-HT4 receptor RNA expression in human GI full-thickness tissue samples, showed similar levels in the stomach compared to 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 pig gastric fundus mucosa however, the expression of the 5-HT4 receptor was similar as in the mucosa of the colon descendens with clearcut preponderance of the h-exon containing receptor. The predominant mucosal location of h-exon containing 5-HT4 receptor splice variants might correspond to the preferential involvement of this type of 5-HT4 receptor splice variants in mucosal effects of 5-HT4 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-HT4 receptors has been suggested at least in some regions in some species such as enterochromaffin cells, smooth muscle cells of the muscularis mucosae and submucosal intrinsic neurons in the mucosal part; 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-HT4 receptors, cell layers or particular cell types were isolated by LMPC. In colon descendens, patches of the epithelial cell layer obtained by LMPC showed expression of 5-HT4 receptors, predominantly the 5HT4(+h) receptor. Possible cell types involved might be enterochromaffin cells and goblet cells, which were recently shown to express 5-HT4 receptors in mouse intestine[21]. In mouse, application of 5-HT4 receptor agonists led to mucosal 5-HT release and mucus secretion in a tetrodotoxin-insensitive way indicating direct activation of stimulatory 5-HT4 receptors on the enterochromaffin cells and goblet cells respectively[21]; in porcine and human small intestine however, analysis of 5-HT release suggested the presence of inhibitory 5-HT4 receptors on the enterochromaffin cells[42]. As relaxant 5-HT4 receptors have been proposed on circular smooth muscle in human colon on the basis of functional data[13], patches of cells were also obtained from the circular muscle layer of the pig colon descendens indeed revealing 5-HT4 receptor expression. Still, some authors were not able to confirm the presence of relaxant 5-HT4 receptors in human colonic circular muscle strips[43] and we did not obtain evidence for muscular 5-HT4 receptors in pig colonic circular muscle strips [12]. We can thus not exclude that the 5-HT4 receptor expression observed in LMPC-isolated cell patches from the pig colonic circular muscle layer represents the 5-HT4 receptors on intercalated interstitial cells of Cajal. Also in pig gastric fundus, LMPC-isolated cell patches of the epithelial cell layer showed 5-HT4 receptor expression. As the human gastric mucosa was shown to contain a considerable number of enterochromaffin cells scattered within the lining epithelium[35], we stained enterochromaffin cells in porcine gastric mucosa immunohistochemically and isolated them by LMPC. Although the 5-HT4 receptor was still detected in the full mucosal part of these IHC stained sections, 5-HT4 receptor expression was not detected in the isolated enterochromaffin cells. As the enterochromaffin cells showed expression of GAPDH, this might be related to the small amount of 5-HT4 receptor RNA even when pooling 500 LMPC-isolated cells. In gastric fundus, LMPC was not applied to obtain cell patches from the muscle layer, as there are no functional data suggesting 5-HT4 receptors to be present on muscle cells in the stomach.

In conclusion, This study using endpoint RT-PCR confirms the presence of 5-HT4 receptors in the mucosal and in the MMP part of porcine gastric fundus and colon descendens, and shows that the mucosa predominantly expresses h-exon containing 5-HT4 receptors. The mucosal h-exon containing 5-HT4 receptors might form additional sites of action for 5-HT4 receptor agonists. 5-HT4 receptors were detected in LMPC-isolated epithelial cell patches in gastric fundus and colon descendens, and circular muscle cell patches in the colon descendens. No 5-HT4 receptor expression was detected in gastric LMPC-isolated enterochromaffin cells stained by immunohistochemistry, the expression of 5-HT4 receptors in individual cell types might be too low to pick them up by LMPC and endpoint PCR.

**ACKNOWLEDGEMENTS**

The authors thank Mrs. An Neesen, Sandra Soetaert and Trees Lepez for their technical help. The study was financially supported by grant G.0061.08 from the Fund for Scientific Research Flanders.

**COMMENTS**

***Background***

5-HT4 receptors are distributed in the gastrointestinal tract. They are expressed on excitatory motor neurons facilitating the stimulatory effect of these neurons on gastrointestinal motility; this explains the therapeutic use of 5-HT4 receptor agonists in conditions with impaired gastrointestinal motility such as constipation. Several reports indicate that 5-HT4 receptors are also expressed in the gastrointestinal mucosa. 5-HT4 receptors are G-protein coupled receptors with their amino acid chain passing 7 times through the cellular membrane. The intracellular tail shows splice variation with different lengths of the intracellular amino acid sequence. Quite exceptional for G-protein coupled receptors, 5-HT4 receptors can also have an extra insertion of 14 amino acids in the second extracellular loop, encoded by the h-exon (42 base pairs). Distribution studies of 5-HT4 receptors in the gastrointestinal 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, since important cell-specific transcript information is lost because of the cellheterogeneity of tissues such as gastrointestinal 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, hereby avoiding contamination, and the preserved cellular integrity. The main concerns when using LMPC to analyse 5-HT4 receptor mRNA expression of a specific cell type is first to efficiently and selectively isolate the right cells and second to obtain RNA of good quality. To address these issues and to optimize a LMPC experimental design for gastrointestinal tissue which is highly heterogeneous, rich in endogenous RNase and enzymes, a systematic approach was needed to evaluate the impact on RNA integrity of different critical steps. Therefore RNA yield and quality was determined with the Experion automated electrophoresis system after tissue collection, hematoxylin and eosin (H&E) 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 in contrast to the H&E 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 attempts to develop a fast IHC protocol resulted in diminished visualization due to the short antibody labeling time. Therefore, the authors used an overnight antibody incubation for our IHC staining protocol whereby RNA integrity could be maintained by addition of 1Mol/L NaCl in all aqueous solutions, resulting in superior protection of RNA. It is not yet clear why a saline solution preserves RNA, although the authors can speculate that normal saline protects the integrity of cell membranes, and hence, prevents the release of intracellular RNases.

***Applications***

This study using endpoint RT-PCR and LMPC confirms the presence of 5-HT4 receptors in the mucosal and in the MMP part of porcine gastric fundus and colon descendens, and shows that the mucosa predominantly expresses h-exon containing 5-HT4 receptors. The mucosal h-exon containing 5-HT4 receptors might be preferentially involved in the mucosal response to 5-HT4 receptor activation and might form potential drug targets for 5-HT4(+h) receptor selective agonists..

***Terminology***

5-HT4 receptors: The 5-HT4 receptor is a G-protein coupled receptor which activates the adenylyl cyclase/cyclic adenosine monophosphate/protein kinase A pathway in response to serotonin (5-hydroxytryptamine; 5-HT). The 5-HT4 receptor is expressed on excitatory motor neurons in the gut, facilitating acetylcholine release hereby stimulating gastrointestinal motility; Laser microdissection and pressure catapulting (LMPC): Under direct microscopic visualization, LMPC permits procurement of histologically or immunohistologically defined tissue and cell samples. LMPC system uses a focused pulsed nitrogen UV-A laser beam (wavelength 355 nm) of which the 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. Then, the beam is 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 in the cap of the vial.

***Peer review***

This manuscript describes the expression of the +h 5-HT4 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 HTR4 variant (+ exon h) at 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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**P-Reviewe**r Hori M, Tache Y **S-Editor** Huang XZ  **L-Editor E-Editor**

**Figure 1 Schematic representation** A:The porcine and human 5-HT4 receptor splice variants;B:cDNA of the porcine 5-HT4(hb) receptor based on gene browsing (transcript ID: [ENSSSCT00000015770](http://www.ensembl.org/Sus_scrofa/Transcript/Summary?db=core;g=ENSSSCG00000014428;r=2:156451710-156556790;t=ENSSSCT00000015770) on http://www.ensembl.org/Sus\_scrofa/Gene). The 5-HT4 receptor variants have identical sequences up to Leu358, or Leu372 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-HT4 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.

**Figure 2 Photomicrographs of hematoxylin&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

**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.

**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 H&E stained tissue sections of colon descendens; C: Large patches of epithelial cells and smooth muscle cells obtained by LMPC from (H&E stained tissue sections and from the whole H&E 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; H&E: Hematoxylin&eosin; IHC: Immunohistochemically; RQI: RNA quality indicator

**Figure 5 Invert color image of endpoint PCR analysis of the 5-HT4 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-HT4(+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 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.

**Figure 6 Expression of 5-HT4(+h) and 5-HT4(-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 expression. The line indicates the mean of *N* = 4 for each region-fraction. b*P* < 0.01 versus values for 5-HT4(-h) receptors in colon descendens or gastric fundus. MMP: Muscle-myenteric plexus.

**Figure 7 Invert color image of endpoint PCR analysis**. 5-HT4 receptor and glyceraldehyde-3-phosphate dehydrogenase expression is shown in a Hematoxylin&eosin (H&E) stained whole tissue section, and in the mucosal as well as the muscle-myenteric plexus part of a H&E stained tissue section of colon descendens (A) and gastric fundus (B). The size of the expected PCR products is indicated.

**Figure 8 Invert color representation of the double round of endpoint PCR analysis.** A,B: 5-HT4 receptor and glyceraldehyde-3-phosphate dehydrogenase expression is shown in large patches of epithelium cells and smooth muscle cells obtained by laser microdissection and pressure catapultin (LMPC) from Hematoxylin&eosin (H&E) 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 immunohistochemicall (IHC) stained tissue sections of gastric fundus; C: For comparison, the result obtained in the mucosal part of the H&E or IHC stained tissue section of gastric fundus is also shown in B and C. The size of the expected PCR products and presence of aspecific amplification are indicated (\*).