November 15, 2012

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 224-Review.docx).

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

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

**ESPS Manuscript NO: 224**

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

1. Revision has been made according to the suggestions of the reviewer
2. Comments of referee “**00001781”**:
* *Need to better define h-exon structure and function as study rationale.*

*Pig HTR4 is less familiar to most readers than its human and rodent counterparts despite the authors have published an extensive work in Physiol Genomics. How is the cryptic exon h (42 bp) within exon 4-5 important needs to be addressed in the introduction. Comparison to its human HTR4 isoforms in terms of GI tissue distribution/physiology and 5-HT drug sensitivity would make the aim of the study better received.*

*homo\_sapiens:5 AAGGAAAGGAGTCTAAACMAAGGCCTSGGCCAGGATTTTCATGYRGTATGTCAGAACAAGTATTCCTTAGGATTCTGAAAGAAATGCCG*

 *E R S L N K G L G Q D F H V*

*sus\_scrofa:2 CAGGAAAGGACATCCAAACCAAGACTGGGCCAGGATTTGCATGTGGTATGTGAGAACACGAGTTCCTTGGGCTTCTGAAGGAAATGCTT*

 *E R T S K P R L G Q D L H V*

*Fig 2A does depict other C-term isoforms but the ECL-2 sequence corresponding to h-exon (14 a.a.) is not included or should be added to Fig 2B.*

Information on the h-exon in man and pig is now given in the introduction (page 4). First the situation in man is described where the h-exon was so far only associated with the b variant of the C-terminal. The pharmacology of this hb variant has been compared with that of the a and b variant by Bender et al. (J. Neurochemistry 2000, 74, 478-489) and the result is summarized in the introduction. The hb variant was present in the human lower esophageal sphincter, but the PCR product of all h-exon carrying 5-HT4 transcripts was present in the other parts of the gastrointestinal tract so that Bender suggested possible association with other C-terminal sequences than b. In pig, the h-exon has indeed already been associated with a, m and r C-terminal sequences and h-exon containing 5-HT4 receptors were also present along the gastrointestinal tract (De Maeyer et al., Physiol. Genomics, 2008, 34, 22-33).
In this new part in the introduction there is referred to figure 1, which is the old figure 2, where the amino acid sequence of the h-exon is now added for both the human and porcine h-exon; the original figure 1 has now become figure 2.

* *Need to better distinguish 5-HT stained cell types.* *MAB352 staining is primarily to identify 5-HT immunopositive cell types that would include mucosal EC cells, MMP neurons and/or blood-derived platelets. EC cells needs other endocrine marker such as chromogranin A (ChgA) as defined in Fig 3. Likewise, it would be desirable to include ChgA or TPH1 PCR in Fig 8C to demonstrate that there are indeed EC cells in pooled LMPC since GAPDH is a general house-keeping gene regardless its overamplification produced artifact band in Fig 8A.*

The referee suggests that MAB352 stained 5-HT immunopositive cells in gastric fundus might not only include EC cells but also MMP neurons and blood-derived platelets, and suggests to use another endocrine marker such as chromogranin A. Although also chromogranin A can stain other enteric neuroendocrine cells than EC cells (Portela-Gomes et al., J. Histochem. Cytochem., 1997, 45, 815-822, we took up the suggestion to use chromogranin A. We immunostained cryosections of the stomach with chromogranin A (Chromogranin A mouse anti human, AbD Serotec, U.K.) yielding chromogranin A-stained cells in the mucosa (see right panel of the understanding figure).



**Figure**

Photomicrographs of immunohistologically stained 8 µm sections of pig gastric fundus showing A) MAB352 (1:200) immunofluorescent enterochromaffin cells (EC) (10X) and B) Chromogranin A (1:25) immunofluorescent EC cells (10X) in the epithelium.

However, when these cells were isolated by LPMC and PCR was performed for 5-HT4 receptors or GAPDH, even the PCR for GADPH remained negative, and the same applied for the mucosal part of the chromogranin A IHC stained tissue sections, scraped off after LMPC (n=2). This is in contrast to our results with MAB352 stained tissues (figure 8C in manuscript). We have no explanation for this negative result, as we used the same optimized procedure for IHC with chromogranin A, as we had developed for MAB352 to obtain optimal RNA integrity (see discussion page 12).

Still we believe that the great majority of MAB352 stained cells in our study represent EC cells. The MAB352 antibody indeed stains serotonin (5-HT) containing cells. Penkova et al. (Folia Med., 2010, 52, 31-37) used MAB352 antibody to stain EC cells in human gastric mucosa and verified these stained cells as EC cells by electron microscopy. They did not report any other 5-HT positive cells in the human gastric mucosa next to the EC cells. Additionally, Meyer and Brinck (Digestion, 1999, 60, 63-68) reported that the staining of enterochromaffin cells by serotonin or chromogranin A in the mucosa of the human stomach, small and large intestine was restricted to cells of the epithelial layer. This result is further confirmed by van Lelyveld et al. (Neurogastrenterol. Motil., 2007, 19, 342-348) by comparing the number of chromogranin A-immunoreactive cells and 5-HT-immunoreactive cells in the mucosa of the duodenum and stomach. They did not report any significant difference in staining between chromogranin A and 5-HT.

As for the other 5-HT containing cell types such as blood platelets and MMP neurons, mentioned by the referee, none of the previous referred authors showed any presence of blood platelets stained by 5-HT antibodies in the mucosa of the stomach. MMP neurons in the gastrointestinal (GI) tract can indeed be stained by 5-HT antibodies. Throughout the human and pig small and large intestine, serotonin-immunoreactive nerves are mainly detected in the myenteric plexus and, to a smaller extent, in the submucous plexus, while hardly any nerve fibers are seen in the mucosa (Crowe et al., Gastroenterology 1992;102, 461–467; Gershon and Tack, 2009; Kurian et al., Histochemistry, 1983, 78, 523–529; Tobe et al., Gastroenterol. 1966, 46, 34-37; Timmermans et al., Comp. Biochem. Physiol., 1997, 118, 331-340; Timmermans et al., Anatom Record, 2001, 262, 71-78 ). However, only few reports are available on the expression of 5-HT in the enteric nervous system in the human or pig stomach. Anlauf et al. (J. Comp. Neurol., 2003, 459, 90-111) did a thorough investigation to the chemical coding of the human GI nervous system, including the stomach. They report only a weak staining of the myenteric neurons by 5-HT antibodies in the stomach, small and large intestine and did not found any 5-HT-immunoreactivity in the submucosal neurons. Yet, for rat stomach Fujimiya et al. (Histochem. Cell Biol., 1997, 107, 105-114) and Yu et al. (Am. J. Gastrointest. Liver Physiol., 2001, 280, G1099-G1105) did report 5-HT immunoreactivity in submucosal fibers. However, in our study we also did not see any 5-HT immunoreactivity upon staining with the MAB352 antibody in the submucosa of the pig stomach. Isolation of MMP neurons can be excluded as we isolated only cells of the crypts, villi and the epithelial lining of the mucous membrane by LMPC.

More information about MAB352 staining and EC cell isolation is now given in the materials en methods section (page 6, line 26), in the results section (page 9, lines 12-14) and discussion (page 12, lines 11-12).

* *Which C-terminal variants are in combination with up-regulated h-exon? Authors discussed the possibility of h-exon may coexpress with a, b, m, and r exons. Is any particular isoform dominant or all about the same in the GI mucosa at increased level?*

The C-terminal sequences already associated with the h-exon in man and pig are now clearly described in the introduction. The actual study was not designed to discriminate which C-terminal sequences are associated with the h-exon as the primers used (forward in exon 4 and reverse in exon 5) amplify the common region in exon 4 and 5, flanking the h-exon, yielding PCR products with or without the h-exon but not including the C-terminal tail. This is now clearly mentioned in the discussion (page 12, lines 28-31).

1. Comments of referee “**00028211”**:
* *This manuscript investigated 5-HT4R distribution including splicing variants in porcine gastrointestinal tissue. Main conclusion is that h-exon containing 5-HT4(+h)R expressed higher than h-exon deleted 5-HT4(-h)R in mucosal region in both colon and gastric fundus. They used microdisssection and pressure catapulting (LMPC) and extracted RNA quality was evaluated by the Experion automated electrophoresis system. Obtained results are clear and quantitative, and the following discussion is logical and reasonable.*

No adaptations required.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology.*

Sincerely yours,

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