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Abstract

Background

Serotonin plays a pivotal role in regulating gut motility, visceral sensitivity, and fluid secretion via specific receptors. Among these receptors, 5-HT_4 exerts a prominent control on gut motor function. Although the prokinetic effect exerted by 5-HT_4 agonists is well known, the cellular sites of 5-HT_4 expression remain poorly understood in large mammals, e.g., horses. In this study, we evaluated the distribution of 5-HT_4 in the horse intestine and in foals with enteric aganglionosis, reminiscent of human Hirschsprung's disease.

Methods

The intestine and spinal ganglia were obtained from three healthy horses and two foals with hereditary ileocolonic aganglionosis. Tissues were processed for immunohistochemistry using a specific antibody to 5-HT_4 and a variety of neuronal markers. Myenteric and submucosal plexus 5-HT_4-immunoreactive (IR) neurons were quantified as relative percentage (mean±SD) to the total number of neurons counted. Furthermore, the density of 5-HT_4-IR nerve fibers was evaluated in the mucosa and *tunica muscularis*.

Key Results

The 5-HT$_4$ immunoreactivity was localized to large percentages of myenteric neurons ranging from 28±9% (descending colon) to 63±19% (ileum), and submucosal neurons ranging from 54±6% (ileum) to 68±14% (duodenum). The 5-HT$_4$-immunoreactivity was co-expressed by some substance P-IR (SP-IR) spinal ganglion neurons and extrinsic sensory fibers of aganglionic foals.

Conclusions & Inferences

The presence of 5-HT$_4$ in many enteric and extrinsic sensory neurons and nerve fibers provides solid morphological evidence of the cellular sites of 5-HT$_4$ expression in horses. The evidence of SP-IR sensory neurons positive for 5-HT$_4$ suggests its role in visceral sensitivity.

Abbreviations: ChAT, choline acetyltransferase; CML, circular muscle layer; CNS, central nervous system; ECs, enterochromaffin cells; ENS, enteric nervous system; GI, gastrointestinal; HuC/HuD, human neuronal protein; IPANs, intrinsic primary afferent neurons; IR, immunoreactive; LML, longitudinal muscle layer; LWFS, lethal white foal syndrome; MP, myenteric plexus; nNOS, neuronal nitric oxide synthase; RT, room temperature; SMP, submucosal plexus; SP, substance P; VIP, vasoactive intestinal polypeptide; WB, Western blot.

1 Introduction

Serotonin, or 5-hydroxytryptamine (5-HT), is a crucial transmitter controlling different functions in the central (CNS) and peripheral nervous system, including the enteric nervous system (ENS).1 About 95% of serotonin synthesis occurs in enterochromaffin cells (ECs) of the gut mucosal layer1, 2 and a further minor component is synthesized in neurons of the myenteric plexus (MP).3, 4 The importance of serotonin in gastrointestinal (GI) physiology and pathophysiology is due to its dual action as a mucosal messenger and neurotransmitter.5, 6 Mechanical and chemical stimuli in the gut lumen are able to induce ECs to release serotonin in the *lamina propria*,2 which acts on specific receptors expressed by mucosal projections of extrinsic primary afferent neurons; thereby, leading to sensory transmission of nausea, discomfort, and pain to the CNS.1, 7 Serotonin has a direct effect on the intramural innervation; in fact, ENS submucosal intrinsic primary afferent neurons (IPANs) and MP neurons respond to serotonin signaling initiating peristaltic and secretory reflexes.1, 3 Serotonin produced by MP neurons3, 4 is involved in GI motility patterns by regulating fast and slow neurotransmission.1, 8

The variety of effects mediated by serotonin in the body is determined by the activation of different pathways, depending on the type of serotonin receptors involved.9

Among the serotonin receptor types, 5-HT$_4$ exerts a crucial role in controlling gut motility.10, 11 The activation of 5-HT$_4$ is known to evoke the release of acetylcholine (and other messengers, e.g., substance P [SP]) from motor neurons of the MP resulting in a prokinetic effect in the gut. Furthermore, 5-HT$_4$ stimulation contributes to enteric fluid secretion and probably plays a role in visceral sensitivity. 5-HT$_4$ agonists have been used in the clinical arena to counteract motor function abnormalities observable in functional bowel disorders because of the prominent prokinetic effect. From this, different 5-HT$_4$ agonists have been developed and used in humans as prokinetic agents for the treatment of chronic constipation.12 Among them, a selective 5-HT$_4$ full agonist, prucalopride,8, 13 stimulates intestinal propulsion, mucosal secretion,14 and exhibits enteric neuroprotective properties.15-18

Pharmacological, electrophysiological, and molecular studies showed the presence of 5-HT$_4$ in different intestinal cell types of rodents and humans.8, 13, 15, 16, 19-21 Concerning the characterization of 5-HT$_4$, few studies have performed an immunohistochemical localization of the cellular expression of this receptor and has mainly been carried out on mice.16, 22

The present study has been undertaken to establish the cellular sites of 5-HT_4 expression in horses as a paradigm of a large mammalian species. Indeed, 5-HT_4 seems to play a fundamental role in GI physiology and seems to be a promising pharmacological target for equine GI disorders.[23-33] In addition, we investigated 5-HT_4 expression in visceral extrinsic sensory nerves by analyzing spinal ganglion neurons from healthy horses and intestinal tissues from foals with lethal white foal syndrome (LWFS), i.e., ileocolonic aganglionosis, reminiscent of Hirschsprung's disease in humans.[34]

2 Materials and Methods

2.1 Animals and tissues collection

Intestinal tissue samples were collected from three horses of different breeds, aged 18 months, and slaughtered at a public slaughterhouse. None had a history of GI disorders. Lumbar spinal ganglia were collected from the half-carcasses. Furthermore, the ileum and pelvic flexure of two new-born American paint male foals affected with LWFS were utilized.[34] According to Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes, the Italian legislation (D. Lgs. n. 26/2014) does not require any approval by the competent Authorities or by ethics committees.

The blood samples of selected horses were analyzed and hemato-biochemical parameters confirmed the general healthy state of the subjects.

Cryosections were obtained as described previously[34, 35] from small and large intestine (descending duodenum, jejunum, ileum, pelvic flexure, and descending colon) and spinal ganglia of three adult horses and from ileum and pelvic flexure of two LWFS foals.

Whole-thickness pieces of pelvic flexure were immediately frozen in liquid nitrogen and stored at −80°C for testing anti-5-HT_4 antiserum specificity by Western blot analysis.

2.2 Immunofluorescence

Cryosections were hydrated in phosphate-buffered saline (PBS) and processed for immunostaining. To block non-specific bindings, the sections were incubated in a solution containing 20% normal goat or donkey serum (Colorado Serum Co., Denver, CO, USA) and 0.5% Triton X-100 (Sigma Aldrich, Milan, Italy, Europe) in PBS for 1 hour at room temperature (RT). The cryosections were incubated overnight in a humid chamber at RT with primary antibodies (Table 1) diluted in 1.8% NaCl in 0.01 M PBS containing 0.1% sodium azide. Enteric neurons were identified with the anti-human neuronal protein (HuC/HuD) antiserum.

After washing in PBS (3×10 minutes), the sections were incubated for 1 hour at RT in a humid chamber with the secondary antibodies (Table 2) diluted in PBS. Cryosections were then washed in PBS (3×10 minutes) and mounted in buffered glycerol at pH 8.6 with 4′,6-diamidino-2-phenylindole – DAPI- (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.3 Specificity of the antibodies

The polyclonal rabbit anti-5-HT_4 antibody utilized in the present research is predicted to work on horse tissues as specified in the datasheet provided by the manufacturer (rabbit anti-5HT_4, AB60359; Abcam, Cambridge, UK, Europe). To confirm its specificity, we tested this antibody by WB analysis. The specificity of the rabbit anti-choline acetyltransferase (ChAT), neuronal nitric oxide synthase

(nNOS), and SP antibodies has been previously tested on horse tissues.[35] In the present research, we also utilized the goat anti-ChAT antibody (AB144P; Millipore, Darmstadt, Germany, Europe), which was co-localized with the validated antibody rabbit anti-ChAT[35] in neurons and nerve fibers by double staining (Fig. S1).

The specificity of the mouse anti-vasoactive intestinal polypeptide (VIP) antibody[36] has not been tested on horse tissues. Nevertheless, in the present research, we co-localized this antiserum with the rabbit anti-VIP antibody (Sc-20727; Santa Cruz Biotechnology). The two antibodies were co-localized in neurons and fibers (Fig. S2).

The serotonin molecule is identical in all species and the specificity of the antiserum should be similar in mammals and fish.[37]

The specificity of the secondary antibodies has been tested as described in a previous work.[35]

2.4 Western blotting

Colonic tissue samples were collected, frozen in liquid nitrogen, and stored at −80°C. Tissue was thawed and homogenized. Total protein content from human (SH-SY5Y) and murine (Neuro 2A) neuroblastoma cell lines were included as positive and negative controls, respectively.[18]

Western blot analysis was performed according to a validated protocol[18] and incubating membranes with the primary antibody (Table 1) and related peroxidase-conjugated secondary antibody (Table 2).

Immunoreactive bands were visualized using chemiluminescent substrate (Pierce ECL Western Blotting Substrate; Thermo Scientific, Milan, Italy, Europe). The intensity of luminescent signal was acquired on a C-DiGit Chemiluminescent Western Blot Scanner using Image Studio Digits Software Ver 3.1 (LI-COR Biotechnology, Cambridge, UK, Europe).

For 5-HT_4 antibody, a unique band of ~45 kDa (theoretical molecular weight ~44 kDa) (http://www.uniprot.org/) was present in extracts from the descending colon and in the positive control; and no band was detected in the negative control (Figure 1). Western blot analysis confirmed the specificity of the primary antibody utilized in the present study.

2.5 Analysis of the sections

Preparations were examined on a Nikon Eclipse Ni microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands, Europe) equipped with the appropriate filter cubes. The images were recorded with a DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV). Slight contrast and brightness adjustments were made using Corel Photo Paint, whereas the figure panels were prepared using Corel Draw (Mountain View, Ottawa, ON, Canada).

2.6 Quantification of 5-HT4 receptor localization

The proportions of neurons that were HuC/HuD-immunoreactive (IR) and that co-expressed 5-HT_4-IR were quantified in each double-stained cryosections. At least 200 HuC/HuD-IR neurons were counted in the MP and submucosal plexus (SMP) of each investigated gut segment/animal (n=3). Data were expressed as relative percentage (mean±SD).

The quantitative analysis for the density of 5-HT_4-IR nerve fibers was performed in all intestinal tracts considered. For each layer (*tunica mucosa*, circular muscle layer [CML] and longitudinal

muscle layer [LML]), three randomly selected high power fields (40×, longitudinal sections) were acquired at the same exposure time. Images were converted into an 8-bit file and were analyzed using ImageJ software (http://imagej.nih.gov/ij/). Threshold values were determined empirically by selecting a setting, which gave the most accurate binary image. The same threshold was used for all images. The resulting numbers of pixels corresponding to the percentage of immunoreactive area on the total area were measured. All graphical representations were prepared using commercial software (GraphPad Prism version 5.00 for Windows; GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as mean±SD.

Statistical analysis has not been performed because of the small number of animals examined in the present study (n=3).

3 Results

3.1 5-HT_4 immunoreactivity in enteric neurons and nerve fibers

3.1.1 Neurons

Large percentages of HuC/HuD-IR neurons showed 5-HT_4-immunoreactivity (5-HT_4-IR) in the MP and SMP of all the GI segments under investigation (Figure 2). 5-HT_4-IR showed different degrees of brightness, varying from weak to strong. The pattern of immunoreactivity was preferentially located into the cytoplasm rather than along the plasma membrane. In the myenteric neuropil, nerve fibers and varicosities embracing neurons showed a bright 5-HT_4-IR (Figure 2A-F).

A total number of 5226 HuC/HuD-IR neurons were counted in all the intestinal tracts considered of healthy adult horses. In the MP, we counted 2595 HuC/HuD-IR neurons and 43% of these cells showed 5-HT_4-IR. In the SMP, we counted 2531 HuC/HuD-IR neurons and 63% of these cells showed 5-HT_4-IR. Considering MP and SMP together, the small and large intestine shared the same percentage of 5-HT_4-IR neurons (54%). The MP of the small and large intestine showed the same percentage (43%) of 5-HT_4-IR neurons, whereas the SMP of the small and large intestine harbored larger percentages of 5-HT_4-IR neurons (62% and 66%, respectively). In more detail, in the small intestine MP, the greatest percentage of immunoreactive neurons was observed in the ileum (63±19%) followed by duodenum (44±25%) and jejunum (35±20%). In the large intestine MP, pelvic flexure 5-HT_4-IR neurons largely outnumbered (47±13%) the density of those observed in the descending colon (28±9%) (Figure 2A-F). Submucosal neurons, which showed in general brighter 5-HT_4-IR than myenteric ones, were observed in the inner and outer SMP layers (Figure 2G-L). In the duodenal submucosa, the 5-HT_4-IR neurons were closely related to Brunner's glands (Figure 2J-L). Nevertheless, no 5-HT_4-IR nerve fibers were observed within the glands. The percentages of 5-HT_4-IR SMP neurons were similar in the pelvic flexure, duodenum, and jejunum (69±13%, 68±14%, and 67±3%, respectively), and slightly decreased in the descending colon (60±23%) and ileum (54±6%). Figure 4A graphically represents the data related to the distribution of the 5-HT_4R-IR neurons.

3.1.2 Nerve fibers

Bright 5-HT_4-IR nerve fibers were widely distributed in all layers of the small and large intestine.

The mucosal layer was widely innervated by 5-HT_4-IR nerve fibers, especially in the duodenum. The 5-HT_4-IR nerve fibers were distributed in the *muscularis mucosae*,38 around intestinal glands, and in the *lamina propria* (Figure 3A and D). In the mucosa of small intestine, the 5-HT_4-IR nerve fibers were more visible on the upper half of the villi, whereas in the large intestine these positive fibers were more prominent in the basal portion of the glands.

In the submucosa, a delicate/thin network of the 5-HT_4-IR fibers and varicosities encircled SMP neurons (Figure 3E), while only a few immunoreactive fibers were visible around blood vessels.

In the *tunica muscularis*, the greatest density of the 5-HT_4-IR nerve fibers was observed in the CML (Figure 3F) of the duodenum and descending colon. In the LML, the 5-HT_4-IR nerve fibers were well represented in the descending colon and ileum and were scantly represented in the other intestinal tracts. Figure 4B graphically represents the data related to the distribution of the 5-HT_4-IR nerve fibers.

3.2 5-HT_4 immunoreactivity in extrinsic innervation

3.2.1 Intestinal extrinsic sensory fibers

In horses, most of the SP-IR innervation derives from enteric neurons supplying the CML (Figure 3G).34 Double immunohistochemistry carried out on adult horse tissues showed that 5-HT_4- and SP-IR fibers widely co-localized (Figure S3). Notably, also in LWFS tissues, supplied only by extrinsic nerves, SP-IR processes co-expressed 5-HT_4-IR (Figure 3H and I).

3.2.2 Spinal ganglion neurons

A weak to moderate 5-HT_4-IR was expressed by small- and medium-sized spinal ganglion neurons. Double immunohistochemistry showed that the 5-HT_4R-IR neurons co-expressed SP-IR (Figure 3J-N).

3.3 Neurochemical coding of 5-HT_4R-IR enteric neurons

In the MP, 5-HT_4-IR was expressed by excitatory neurons, detected for their immunoreactivity to ChAT (Figure 5A and B) and SP (Figure 5C and D) and by inhibitory neurons, immunoreactive to nNOS (Figure 5E and F). In the SMP, 5-HT_4-IR was co-expressed by large neurons co-expressing SP-IR and by small putative secretomotor neurons, showing VIP-IR (Figure 5G and H). Notably, subsets of myenteric and submucosal neurons showed triple co-localization 5-HT_4/SP/VIP-IRs (Figure S4).

3.4 5-HT immunoreactivity

As expected, 5-HT-IR was strongly expressed by EC cells (Figure 6A-C). In the mucosa, 5-HT positive EC cells were mainly detected in the basal portion of the *lamina propria*, in some proximity to SP-IR sensory nerve fibers (Figure 6D and E).

3.5 Extra-neuronal 5-HT_4-IR distribution

Extra-neuronal sites of the small and large intestine of 5-HT_4-IR included smooth muscle cells of the *tunica muscularis*, a subset of ECs (Figure 6A-C), and endothelial cells of small vessels (data not shown). In contrast, 5-HT_4-IR was not detectable in enterocytes and interstitial cells of Cajal.

4 Discussion

The present study provides solid evidence of 5-HT_4-IR in the MP and SMP neurons of horse intestine. Despite some functional investigations carried out on horse small and large intestine indicated the presence of 5-HT_4 in the ENS of this animal model,23-33 the only immunohistochemical study

carried out on horse ENS failed to detect 5-HT_4 in enteric neurons, but confirmed the presence of this serotonergic receptor on muscular layers.[31] Furthermore, a recent study by Delesalle et al.[39] questioned the presence of 5-HT_4 on cholinergic neurons of horse small intestine. In this line, our results represent strong morphological support indicating that 5-HT_4 is expressed in MP and SMP neuronal cell bodies and nerve fibers of horse GI tracts. The presence of large percentages of MP and SMP 5-HT_4-IR neurons suggests that 5-HT_4 agonists may influence both intestinal propulsion and secretion. In fact, the expression of 5-HT_4-IR by SMP SP-IR neuronal cell bodies (likely IPANs[40]) and related nerve fibers supports the role of this receptor in intramural reflex activities. Once activated by 5-HT, the 5-HT_4 expressed by IPANs begins peristalsis through the recruitment of MP excitatory ChAT-/SP-IR[41] and inhibitory nNOS-IR[42] motoneurons. Our findings are consistent with Cellek et al.[13] who showed the effect of 5-HT_4 agonists on both excitatory and inhibitory enteric neurons of the human colon.

Concerning the secretory role, it is known that the active mucosal secretion can be triggered by a serotonin-dependent activation of neurogenic responses mediated by 5-HT_4, 5-HT_3, and 5-HT_{1p} receptors expressed by intrinsic submucosal neurons. This neurogenic mechanism leads secretomotor neurons to release acetylcholine and VIP, inducing Cl^- and bicarbonate secretion by epithelial cells.[10, 43] Our findings that show the co-expression of 5-HT_4-IR in SMP VIP-, and SP-IR neurons, suggest the involvement of 5-HT_4 in secretory and vasodilatory mechanisms in horses. This confirms and expands previous work by Burns and Cummings[38] and Moore et al.[44]

In veterinary medicine, selective 5-HT_4 agonists have been not yet used as prokinetic agents to treat dysmotility disorders of the horse. The only exception is represented by the 5-HT_4 agonist mosapride which effectively attenuated the decline of small intestinal motility in an experimental model of postoperative ileus in the healthy horse. As a matter of fact, in veterinary clinical practice, the use of 5-HT_4 is still extremely limited to personal experience and, similarly to the routine practice in humans, metoclopramide, whose effects rely upon a combination of receptor interaction (i.e., dopamine receptor antagonism, 5-HT_3 antagonism and 5-HT_4 agonism), is the most commonly used.[45] While there is no evidence of side effects elicited by selective 5-HT_4 agonists, metoclopramide evokes extrapyramidal manifestations in a similar fashion to what may happen in humans chronically treated with this drug.

The expression of 5-HT_4-IR by horse spinal ganglion neurons does not necessarily indicate that these neurons contribute to the intestinal innervation; nevertheless, the presence of 5-HT_4-IR on extrinsic sensory fibers observed in aganglionotic tissues of LWFS foals suggests that drugs acting on 5-HT_4 might influence the extrinsic visceral sensory pathway, as shown in humans.[46] This is the first morphological evidence of 5-HT_4-IR in the horse spinal ganglia. Splice variants of this receptor were already observed in the rat spinal ganglia.[22] As 5-HT_4-IR was co-expressed by extrinsic SP-IR sensory fibers of aganglionotic LWFS foals, it is plausible that this receptor might play a role in nociception.[47, 48] In support of this notion, 5-HT_4 mRNA has been shown to be expressed by nociceptive neurons of rat spinal ganglia.[49, 50] 5-HT_4-IR was also co-expressed by SP-negative spinal ganglion neurons, suggesting that this receptor might be involved in other aspects of the sensory function, such as mechanosensitivity. In fact, tegaserod, a 5-HT_4 partial agonist, has been shown to have an inhibitory effect on intramural mechanoceptors of a cat rectum.[51] Nevertheless, to identify and confirm a role of 5-HT_4 in the visceral sensitivity of horses, functional and pharmacological investigations are needed.

Gut vasodilation is regulated by intrinsic reflex circuitry involving SMP neurons via the activation of 5-HT_3 and 5-HT_4 receptors.[50] In the present study, endothelial cells of small vessels expressed 5-HT_4-IR. This finding is consistent with studies indicating that 5-HT_4 mRNA (together with 5-HT_1, 5-HT_2, and 5-HT_7 mRNA) is expressed by endothelial and vascular smooth muscle cells.[52, 53]

Furthermore, it is known that endothelial 5-HT_4 regulates angiogenesis and that 5-HT_4 agonist mosapride can inhibit proliferation and migration of endothelial cells in the human umbilical vein.[54]

Growing evidence indicates that 5-HT_4 stimulation enhances development, survival, and neurogenesis of enteric neurons[18], [55], [56] and 5-HT_4 agonists facilitate neurogenesis from transplanted stem cells in intestinal anastomosis.[57] Thus, 5-HT_4 agonists may have a neuroregenerative potential that can be useful to treat horses subjected to colic surgery.

In conclusion, our results provide a consistent demonstration that 5-HT_4-IR is widely expressed by different subsets of enteric neurons involved in secretory and motor reflexes. The morphological data support previous pharmacological evidence and bear implication in the treatment of equine intestinal dysmotility and constipation. Furthermore, the presence of 5-HT_4 on extrinsic nerve pathways widens the role of serotoninergic receptors in visceral perception mechanisms.

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Disclosure

The authors have no competing interests.

Author Contribution

RC, FG, RDG, and PC co-designed (conception, planning, and initiation) the study; SI and NR recruited animals and defined the clinical features; SI, FG, AR, FB, CT, and CB performed the experiments; RC supervised the experiments; RC, FG, and RDG analyzed the data and wrote the manuscript. All authors contributed to the interpretation of the data and critically reviewed the manuscript. All authors approved the final version of the manuscript.

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450 **TABLE 1** Details of primary antibodies used in the present study

Antibody	Immunogen	Source, cat. no., species	RRID	Dilution
HuC/HuD	Human HuC/HuD neuronal protein	Life Technologies, A21271; Mouse monoclonal; Clone 16A11	AB_221448	IHC 1:200; WB 1:200
ChAT	Peptide fragment of purified porcine ChAT (GLFSSYRLPGHTQDTLVAQKSS) aminoacids:168-189	Generous gift of M. Schemann, Technische Universität München; Rabbit polyclonal; Clone P3YEB	AB_2314176	IHC 1:250
ChAT	Human placental enzyme	Millipore, AB144P; Goat polyclonal	AB_90661	IHC 1:25
5-HT_4R	Synthetic peptide: LMAILGNLLVMVAVCWDRQLRKIKTNYFIVSLAFADLLVS, corresponding to amino acids 31-70 of Human $5HT_4$ Receptor	Abcam, AB60359; Rabbit polyclonal	AB_2122438	IHC 1:200; WB 1:200
nNOS	Amino acids 2-300 of human NOS1	Santa Cruz, (A-11) sc-5302; Mouse monoclonal	AB_626757	IHC 1:50
Substance P	Substance P-BSA conjugate	Fitzgerald, 10-S15A; Rat monoclonal; Clone MO9205, Batch 115	AB_1288870	IHC 1:400
VIP55		CURE/DDRC; Mouse monoclonal; Clone #55		IHC 1:2500
VIP	Amino acids 1-95 of human VIP	Santa Cruz, Sc-20727; Rabbit polyclonal	AB_2304501	IHC 1:50

Suppliers: Abcam, Cambridge, UK, Europe; CURE/DDRC, DDD, University of California Los Angeles, Los Angeles, CA, USA; Fitzgerald Industries Int., Inc. Concord, MA, USA; Life Technologies, Carlsbad, CA, USA; Merck Millipore, Merck KGaA, Darmstadt, Germany, Europe; Santa Cruz Biotechnology, Santa Cruz, CA, USA.
5-HT_4R, 5-hydroxytryptamine receptor 4; ChAT, choline acetyltransferase; HuC/HuD, human neuronal protein; IHC, Immunohistochemistry; nNOS, neuronal nitric oxide synthase; RRID, Research Resource Identifiers; SP, Substance P; VIP, vasoactive intestinal polypeptide; WB, Western blot.

Table 2. Details of secondary antisera used in the present study

Secondary antibody	Host species	Source	Code	Dilution
Antimouse IgG Alexa 594	Goat	Life Technologies	A11005	IHC 1:200
Antimouse IgG biotinylated[a]	Goat	Vector Laboratories	BA-9200	IHC 1:200
Antirabbit IgG FITC	Goat	Merck Millipore	401314	IHC 1:200
Antirabbit IgG HRP	Goat	Sigma Aldrich	A0545	WB 1:35000
Antirabbit IgG Alexa 594	Donkey	Abcam	AB150132	IHC 1:600
Antirat IgG Alexa 594	Donkey	Life Technologies	A21209	IHC 1:50
Antirat IgG Alexa 488	Goat	Biotium	20023	IHC 1:100
Antigoat IgG TRITC	Donkey	Jackson	705-295-003	IHC 1:100

Suppliers: Abcam, Cambridge, United Kingdom, Europe; Biotium Inc., Hayward, California, USA; Jackson ImmunoResearch Laboratories, Inc, Pennsylvania, USA; Life Technologies, Carlsbad, Ca, USA; Merck Millipore, Darmstadt, Germany, Europe; Sigma Aldrich, Milan, Italy, Europe; Vector Laboratories, Burlingame, California, USA.
FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IHC: Immunohistochemistry; TRITC, Tetramethylrhodamine; WB, Western blot.
[a] Used with AMCA (Aminomethylcoumarin) streptavidine (1:100; SA-5008; Vector Laboratories).

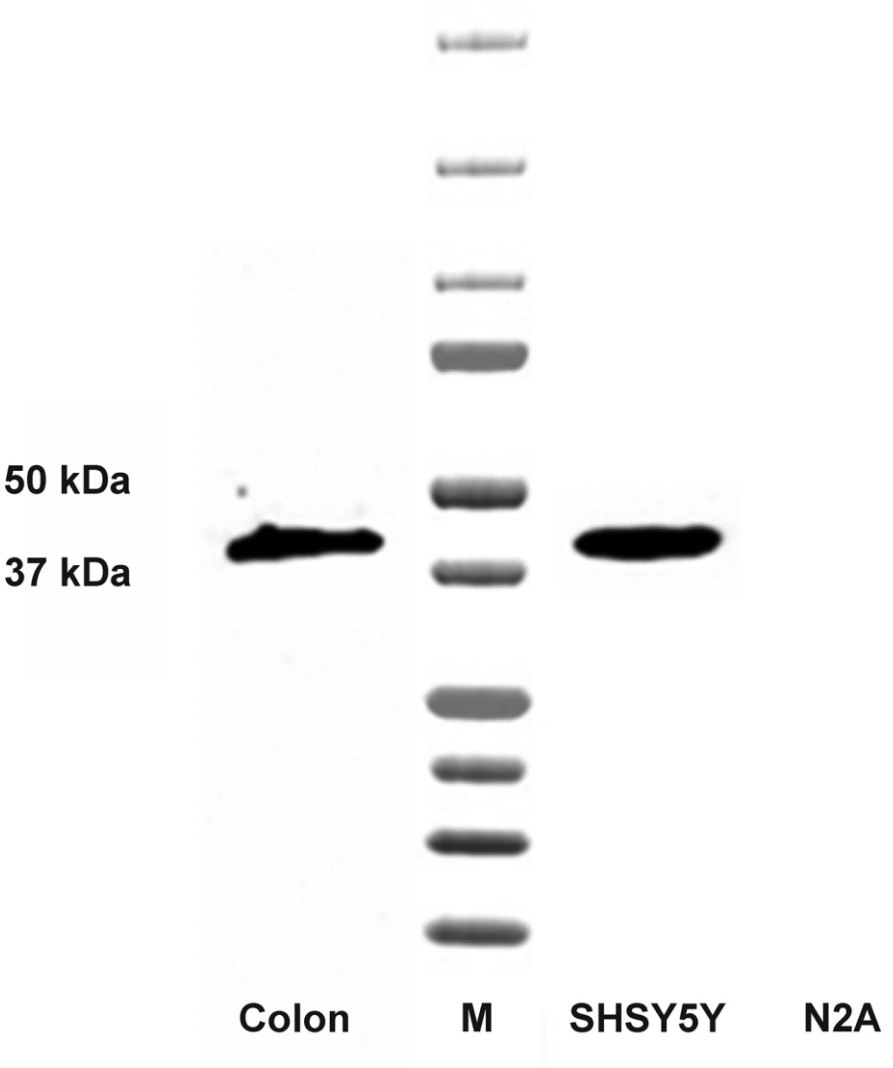

Figure 1

Validation of 5-HT_4 antibody in horse tissue by Western blot. Total protein lysate form horse colon (lane 1), marker of molecular weight (M) (lane 2), SH-SY5Y human cell line as positive control (lane 3), murine Neuro2A (N2A) cell line as negative control (lane 4). A specific band of ~45 kDa was detected in horse tissue (lane 1) and in the positive control (lane 3). No bands were detected in the negative control (lane 4)

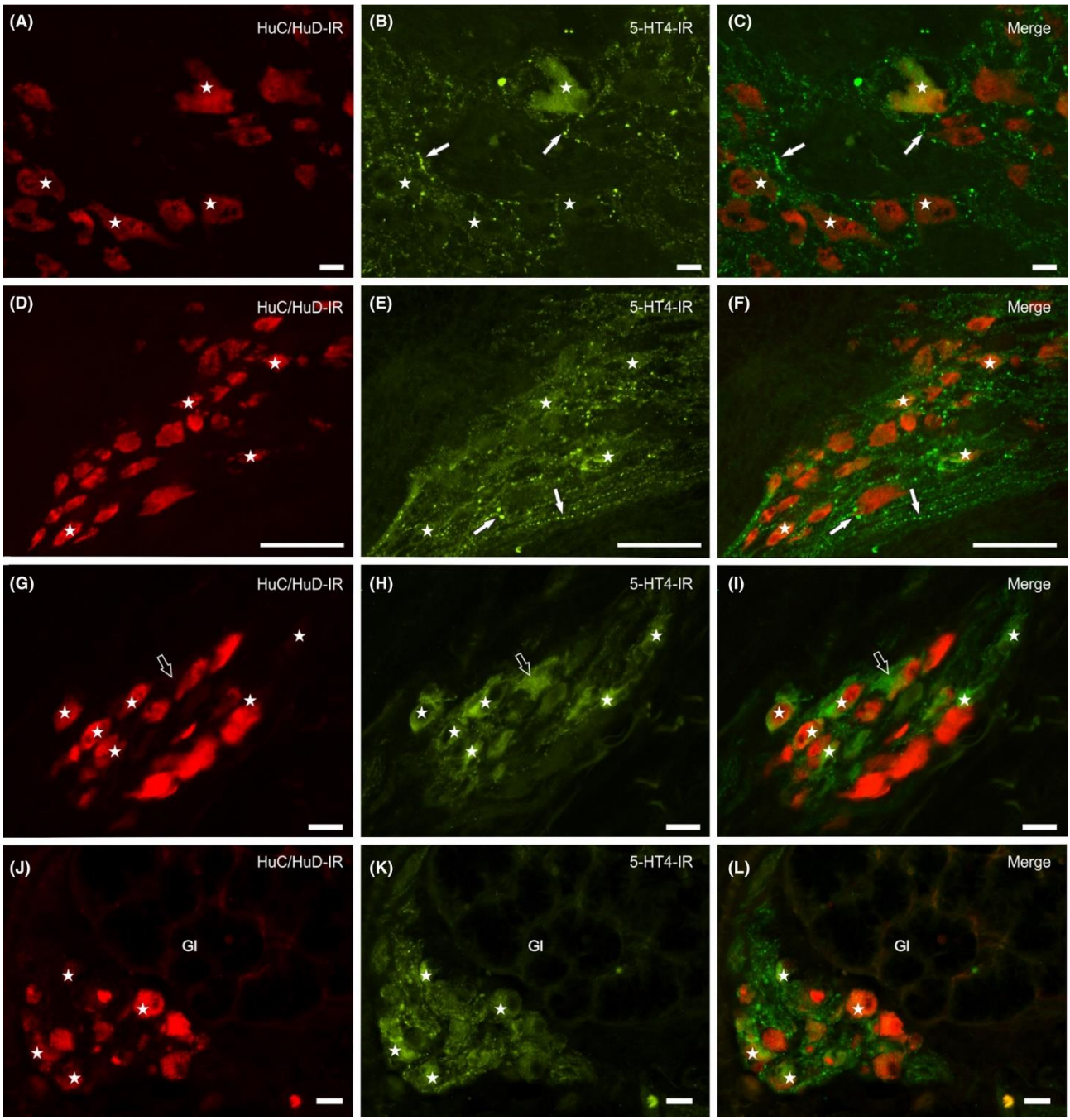

Figure 2

Micrographs showing HuC/HuD- and 5-HT_4-immunoreactivity (IR) in tangential cryosections of myenteric and submucosal plexus of horse ileum (A-C), pelvic flexure (D-F), and duodenum (J-L). (A-F) Stars indicate some HuC/HuD-IR myenteric plexus neurons of the ileum (A-C) and pelvic flexure (D-F) which showed 5-HT_4-IR. Arrows indicate nerve fiber varicosities showing strong 5-HT_4-IR. (G-L) Stars indicate some ileal and duodenal submucosal plexus HuC/HuD-IR neurons co-expressing 5-HT_4-IR. (G-I) The open arrow indicates one 5-HT_4-IR ileal submucosal neuron showing very faint HuC/HuD-IR. HuC/HuD-IR showed several degrees of immunoreactivity, from strong nuclear and cytoplasmic immunoreactivity to very weak (or almost undetectable). Bars = A-C; G-L: 20 μm; D-F: 100 μm

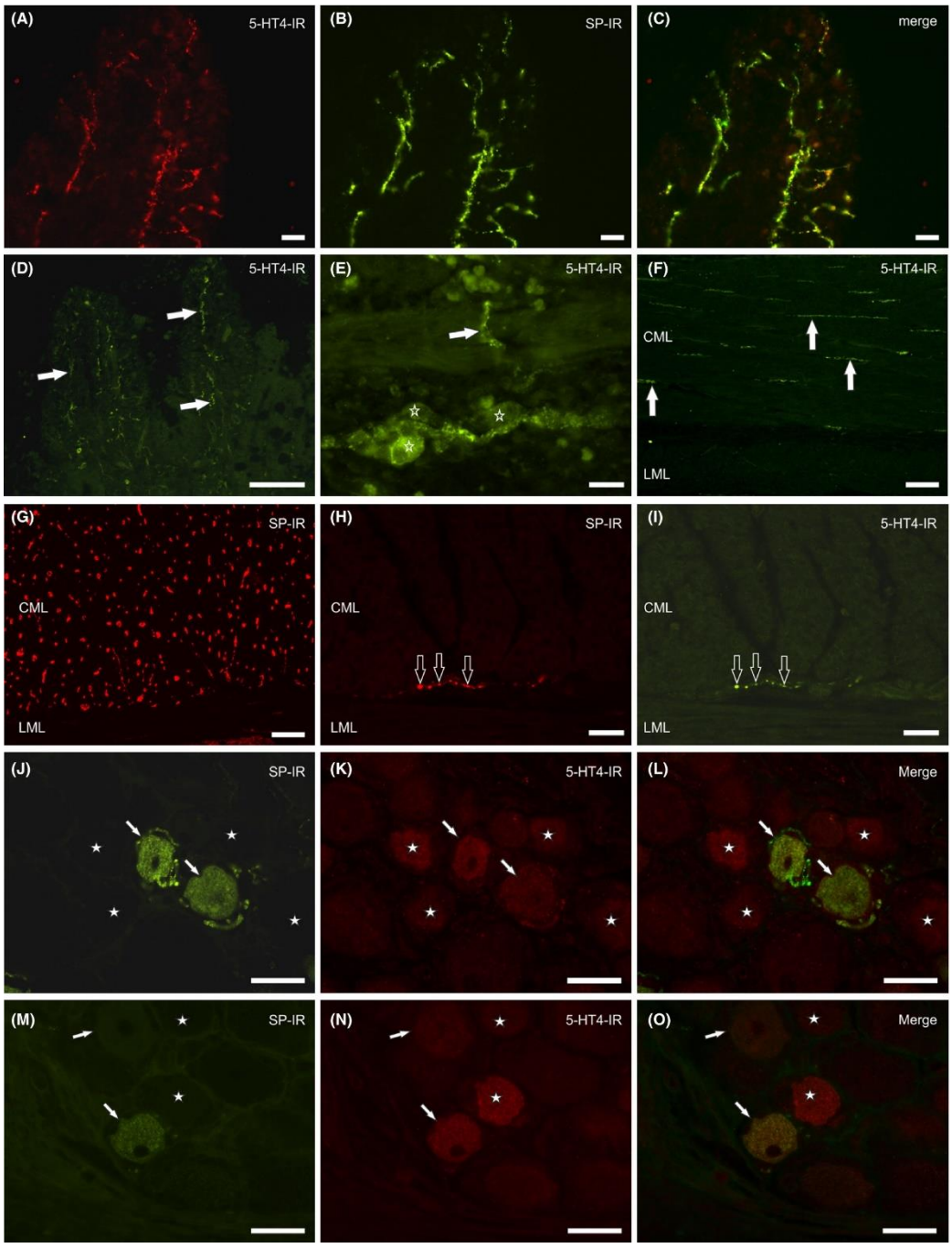

Figure 3

(A-F) Micrographs showing 5-HT_4-immunoreactive nerve fibers (arrows) in the *lamina propria* (A-C, D), *muscularis mucosae* (E) and circular (CML) and longitudinal muscle layer (LML) (transverse cryosections) (F) of the horse ileum; stars (E) indicate some 5-HT_4-IR submucosal neurons very close to the *muscularis mucosae*. (G) Longitudinal ileal cryosections showing the dense CML innervation supplied by substance P (SP) nerve fibers. (H and I) Longitudinal cryosections of the ileum of a lethal white foal syndrome animals, characterized by the absence of enteric neurons. The arrows indicate SP-IR sensory fibers of extrinsic origin running between CML and LML and co-expressing strong 5-HT_4-IR. (J-O) Cryosections of horse spinal ganglion neurons; the arrows indicate SP-IR sensory neurons which co-expressed 5-HT_4-IR; stars indicate other 5-HT_4-IR neurons which were SP-negative. Bars = A-C, E, H and I: 20 μm; D, F, G, J-L: 100 μm

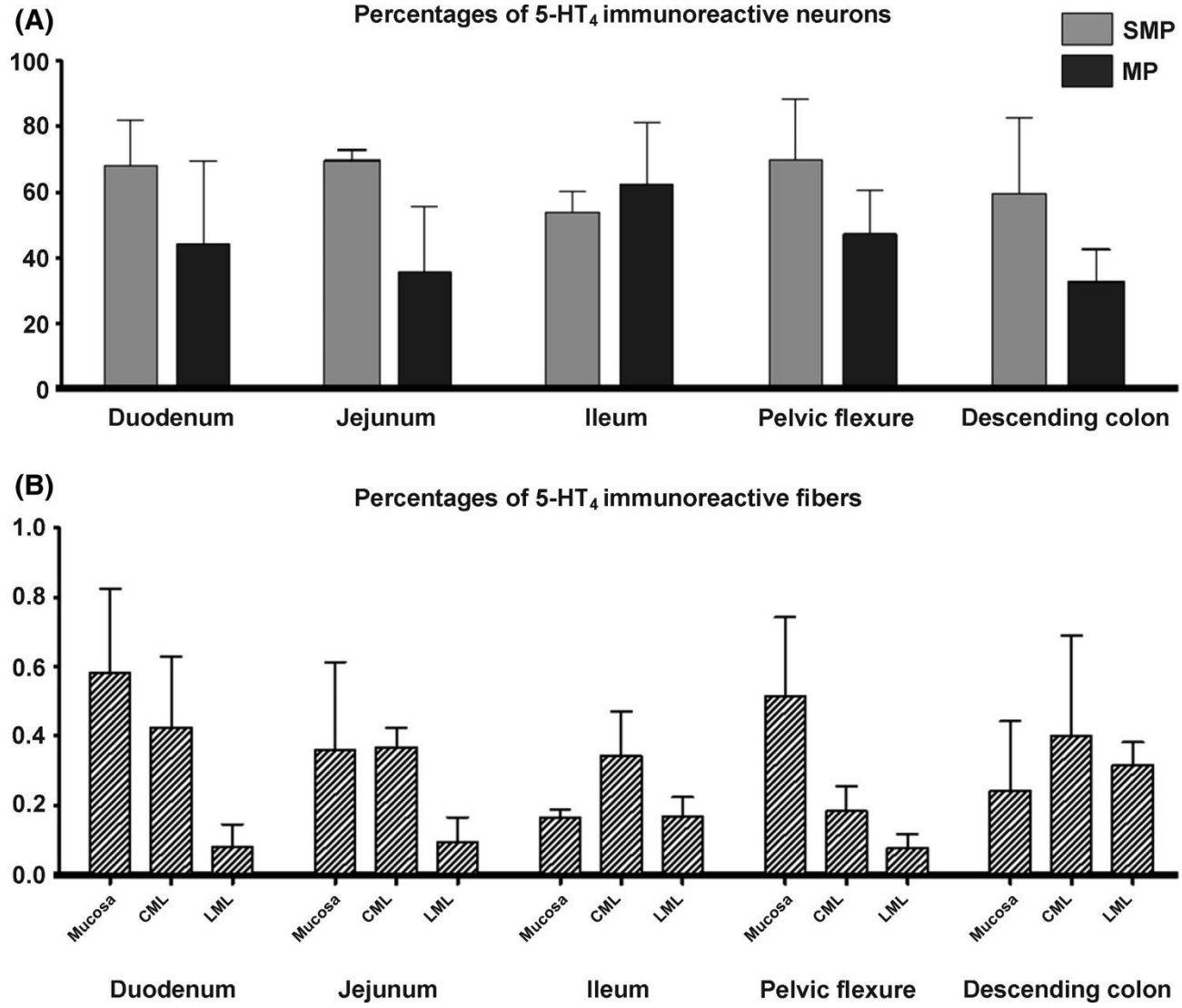

Figure 4

(A) Graphical representation of the percentages of 5-HT$_4$-immunoreactive (-IR) neurons in the horse small and large intestine. Black bars indicate the percentages of 5-HT4-IR neurons in the myenteric plexus of duodenum (161/452 cells), jejunum (147/497 cells), ileum (348/571 cells), pelvic flexure (297/606 cells), and descending colon (165/469 cells). Gray bars indicate the percentages of 5-HT$_4$-IR neurons in the submucosal plexus (SMP) of duodenum (394/586 cells), jejunum (262/404 cells), ileum (324/599 cells), pelvic flexure (377/540 cells), and descending colon (248/402 cells). Data are represented as mean±SD. (B) Graphical representation of the density of 5-HT$_4$-immunoreactive nerve fibers in the *tunica mucosa* (mucosa), circular muscle layer (CML) and longitudinal muscle layer and (LML) of duodenum, jejunum, ileum, pelvic flexure, and descending colon. Data are represented as mean±SD

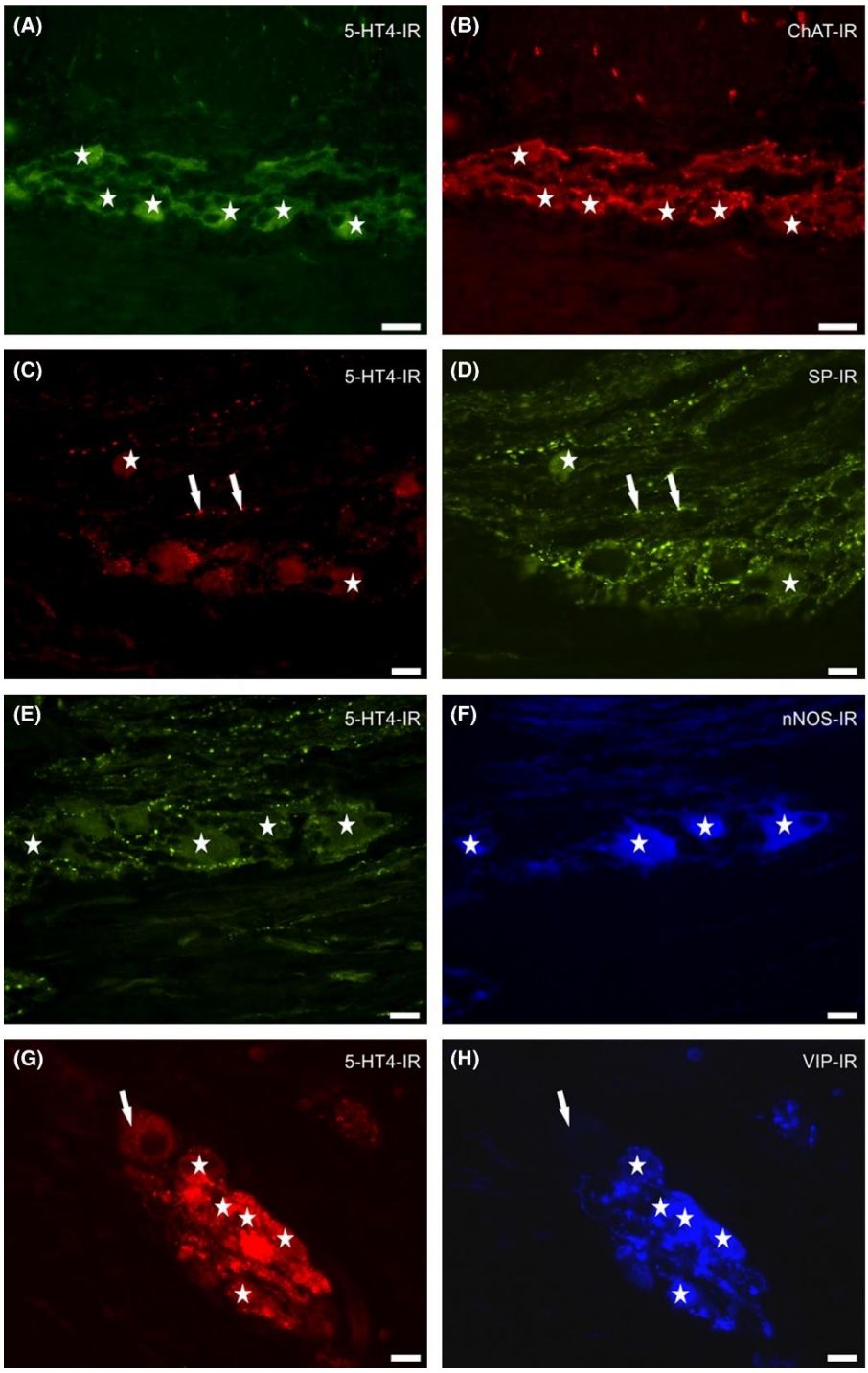

Figure 5

Micrographs showing the co-localization of 5-HT_4-immunoreactivity (IR) with choline acetyltransferase (ChAT), substance P (SP), neuronal nitric oxide synthase (nNOS), and vasoactive intestinal polypeptide (VIP) in the myenteric (A-F) and submucosal plexus (G-H) of the horse ileum. (A and B) Stars indicate some myenteric plexus 5-HT_4-IR neurons co-expressing ChAT-IR. (C and D) Stars and arrows indicate myenteric plexus neurons and nerve fibers showing co-localization between 5-HT_4- and SP-IR. (E and F) Stars indicate 5-HT_4-IR myenteric plexus neurons co-expressing nNOS-IR. (G and H) Stars indicate some submucosal plexus neurons expressing strong 5-HT_4 and VIP-IR. The arrow indicates a 5-HT_4-IR neuron, which was VIP-negative. Bar = A-H: 20 µm

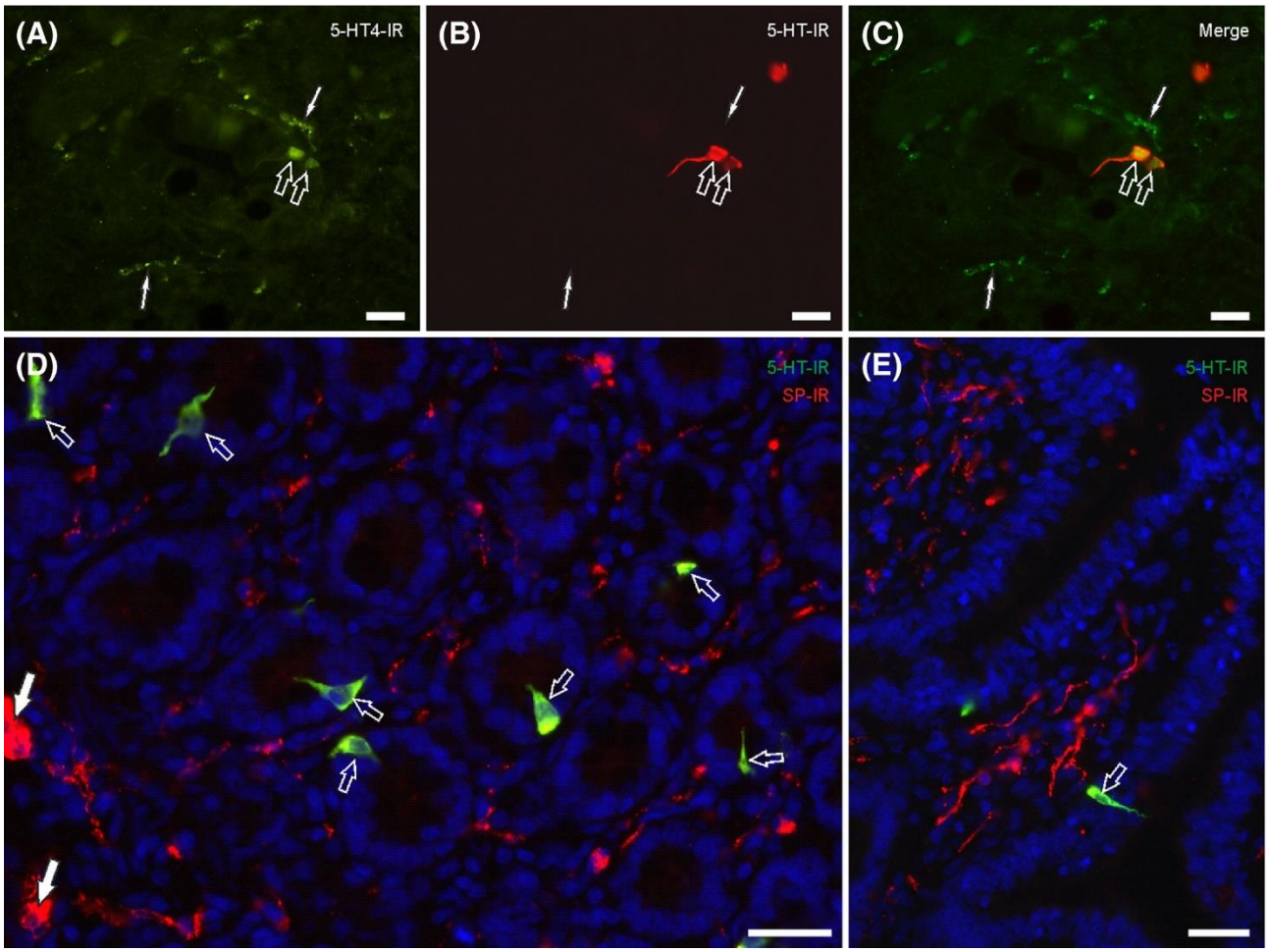

Figure 6

Micrographs showing serotonin (5-HT) and 5-HT_4-immunoreactivity (IR) in the mucosa of the horse ileum. (A-C) Arrows indicate 5-HT_4-IR expressed by nerve fibers surrounding a crypt in which two enterochromaffin cells (EC_S) (open arrows) co-expressed 5-HT4- and 5-HT-IR. (D and E) Bright 5-HT-IR EC (open arrows) lining the crypts (D) and villi (E) closely located to a dense network of Substance P (SP) immunoreactive sensory nerve fibers (red color). Arrows (D) indicate two SP-IR submucosal neurons. Bar = A-E: 20 µm