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Localization of the 5-hydroxytryptamine 4 receptor in equine enteric neurons and extrinsic sensory fibers

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1 Localization of the 5-hydroxytryptamine 4 receptor in equine 2 enteric neurons and extrinsic sensory fibers

3
4 F. Giancola^{1,2}, A. M. Rambaldi¹, F. Bianco^{1,2}, S. Iusco¹, N. Romagnoli¹, C. Tagliavia¹, C. Bombardi¹, P.
5 Clavenzani¹, R. De Giorgio², R. Chiocchetti¹

6
7 ¹Department of Veterinary Medical Sciences
8 (UNI EN ISO 9001:2008), University of
9 Bologna, Bologna, Italy

10 ²Department of Medical and Surgical
11 Sciences, University of Bologna, Bologna, Italy

12 **Correspondence:**

13 Prof. Roberto Chiocchetti, DVM, PhD,
14 Department of Veterinary Medical Sciences,
15 University of Bologna, Bologna, Italy.
16 Email: roberto.chiocchetti@unibo.it

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18
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23

24 **Abstract**

25 **Background**

26 Serotonin plays a pivotal role in regulating gut motility, visceral sensitivity, and fluid secretion via
27 specific receptors. Among these receptors, 5-HT₄ exerts a prominent control on gut motor function.
28 Although the prokinetic effect exerted by 5-HT₄ agonists is well known, the cellular sites of 5-HT₄
29 expression remain poorly understood in large mammals, e.g., horses. In this study, we evaluated the
30 distribution of 5-HT₄ in the horse intestine and in foals with enteric aganglionosis, reminiscent of
31 human Hirschsprung's disease.

32 **Methods**

33 The intestine and spinal ganglia were obtained from three healthy horses and two foals with hereditary
34 ileocolonic aganglionosis. Tissues were processed for immunohistochemistry using a specific
35 antibody to 5-HT₄ and a variety of neuronal markers. Myenteric and submucosal plexus 5-HT₄-
36 immunoreactive (IR) neurons were quantified as relative percentage (mean±SD) to the total number
37 of neurons counted. Furthermore, the density of 5-HT₄-IR nerve fibers was evaluated in the mucosa
38 and *tunica muscularis*.

39 **Key Results**

40 The 5-HT₄ immunoreactivity was localized to large percentages of myenteric neurons ranging from
41 28±9% (descending colon) to 63±19% (ileum), and submucosal neurons ranging from 54±6%
42 (ileum) to 68±14% (duodenum). The 5-HT₄-immunoreactivity was co-expressed by some substance
43 P-IR (SP-IR) spinal ganglion neurons and extrinsic sensory fibers of aganglionic foals.

44 **Conclusions** **&** **Inferences**
45 The presence of 5-HT₄ in many enteric and extrinsic sensory neurons and nerve fibers provides solid
46 morphological evidence of the cellular sites of 5-HT₄ expression in horses. The evidence of SP-IR
47 sensory neurons positive for 5-HT₄ suggests its role in visceral sensitivity.

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

54 **1 Introduction**

55 Serotonin, or 5-hydroxytryptamine (5-HT), is a crucial transmitter controlling different functions in
56 the central (CNS) and peripheral nervous system, including the enteric nervous system (ENS).1 About
57 95% of serotonin synthesis occurs in enterochromaffin cells (ECs) of the gut mucosal layer,1, 2 and a
58 further minor component is synthesized in neurons of the myenteric plexus (MP).3, 4 The importance
59 of serotonin in gastrointestinal (GI) physiology and pathophysiology is due to its dual action as a
60 mucosal messenger and neurotransmitter.5, 6 Mechanical and chemical stimuli in the gut lumen are
61 able to induce ECs to release serotonin in the *lamina propria*,2 which acts on specific receptors
62 expressed by mucosal projections of extrinsic primary afferent neurons; thereby, leading to sensory
63 transmission of nausea, discomfort, and pain to the CNS.1, 7 Serotonin has a direct effect on the
64 intramural innervation; in fact, ENS submucosal intrinsic primary afferent neurons (IPANs) and MP
65 neurons respond to serotonin signaling initiating peristaltic and secretory reflexes.1, 3 Serotonin
66 produced by MP neurons3, 4 is involved in GI motility patterns by regulating fast and slow
67 neurotransmission.1, 8

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

70 Among the serotonin receptor types, 5-HT₄ exerts a crucial role in controlling gut motility.10, 11 The
71 activation of 5-HT₄ is known to evoke the release of acetylcholine (and other messengers, e.g.,
72 substance P [SP]) from motor neurons of the MP resulting in a prokinetic effect in the gut.
73 Furthermore, 5-HT₄ stimulation contributes to enteric fluid secretion and probably plays a role in
74 visceral sensitivity. 5-HT₄ agonists have been used in the clinical arena to counteract motor function
75 abnormalities observable in functional bowel disorders because of the prominent prokinetic effect.
76 From this, different 5-HT₄ agonists have been developed and used in humans as prokinetic agents for
77 the treatment of chronic constipation.12 Among them, a selective 5-HT₄ full agonist, prucalopride,8,
78 13 stimulates intestinal propulsion, mucosal secretion,14 and exhibits enteric neuroprotective
79 properties.15-18

80 Pharmacological, electrophysiological, and molecular studies showed the presence of 5-HT₄ in
81 different intestinal cell types of rodents and humans.8, 13, 15, 16, 19-21 Concerning the
82 characterization of 5-HT₄, few studies have performed an immunohistochemical localization of the
83 cellular expression of this receptor and has mainly been carried out on mice.16, 22

84 The present study has been undertaken to establish the cellular sites of 5-HT₄ expression in horses as
85 a paradigm of a large mammalian species. Indeed, 5-HT₄ seems to play a fundamental role in GI
86 physiology and seems to be a promising pharmacological target for equine GI disorders.²³⁻³³ In
87 addition, we investigated 5-HT₄ expression in visceral extrinsic sensory nerves by analyzing spinal
88 ganglion neurons from healthy horses and intestinal tissues from foals with lethal white foal syndrome
89 (LWFS), i.e., ileocolonic aganglionosis, reminiscent of Hirschsprung's disease in humans.³⁴

90 **2 Materials and Methods**

91 **2.1 Animals and tissues collection**

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

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

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

104 Whole-thickness pieces of pelvic flexure were immediately frozen in liquid nitrogen and stored at
105 -80°C for testing anti-5-HT₄ antiserum specificity by Western blot analysis.

106 **2.2 Immunofluorescence**

107 Cryosections were hydrated in phosphate-buffered saline (PBS) and processed for immunostaining.
108 To block non-specific bindings, the sections were incubated in a solution containing 20% normal goat
109 or donkey serum (Colorado Serum Co., Denver, CO, USA) and 0.5% Triton X-100 (Sigma Aldrich,
110 Milan, Italy, Europe) in PBS for 1 hour at room temperature (RT). The cryosections were incubated
111 overnight in a humid chamber at RT with primary antibodies (Table 1) diluted in 1.8% NaCl in
112 0.01 M PBS containing 0.1% sodium azide. Enteric neurons were identified with the anti-human
113 neuronal protein (HuC/HuD) antiserum.

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

118 **2.3 Specificity of the antibodies**

119 The polyclonal rabbit anti-5-HT₄ antibody utilized in the present research is predicted to work on
120 horse tissues as specified in the datasheet provided by the manufacturer (rabbit anti-5HT₄, AB60359;
121 Abcam, Cambridge, UK, Europe). To confirm its specificity, we tested this antibody by WB analysis.
122 The specificity of the rabbit anti-choline acetyltransferase (ChAT), neuronal nitric oxide synthase

123 (nNOS), and SP antibodies has been previously tested on horse tissues.³⁵ In the present research, we
124 also utilized the goat anti-ChAT antibody (AB144P; Millipore, Darmstadt, Germany, Europe), which
125 was co-localized with the validated antibody rabbit anti-ChAT³⁵ in neurons and nerve fibers by
126 double staining (Fig. S1).

127 The specificity of the mouse anti-vasoactive intestinal polypeptide (VIP) antibody³⁶ has not been
128 tested on horse tissues. Nevertheless, in the present research, we co-localized this antiserum with the
129 rabbit anti-VIP antibody (Sc-20727; Santa Cruz Biotechnology). The two antibodies were co-
130 localized in neurons and fibers (Fig. S2).

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

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

134 **2.4 Western blotting**

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

138 Western blot analysis was performed according to a validated protocol¹⁸ and incubating membranes
139 with the primary antibody (Table 1) and related peroxidase-conjugated secondary antibody (Table 2).

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

144 For 5-HT₄ antibody, a unique band of ~45 kDa (theoretical molecular weight ~44 kDa)
145 (<http://www.uniprot.org/>) was present in extracts from the descending colon and in the positive
146 control; and no band was detected in the negative control (Figure 1). Western blot analysis confirmed
147 the specificity of the primary antibody utilized in the present study.

148 **2.5 Analysis of the sections**

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

154 **2.6 Quantification of 5-HT₄ receptor localization**

155 The proportions of neurons that were HuC/HuD-immunoreactive (IR) and that co-expressed 5-HT₄-
156 IR were quantified in each double-stained cryosections. At least 200 HuC/HuD-IR neurons were
157 counted in the MP and submucosal plexus (SMP) of each investigated gut segment/animal (n=3).
158 Data were expressed as relative percentage (mean \pm SD).

159 The quantitative analysis for the density of 5-HT₄-IR nerve fibers was performed in all intestinal
160 tracts considered. For each layer (*tunica mucosa*, circular muscle layer [CML] and longitudinal

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

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

171 **3 Results**

172 **3.1 5-HT₄ immunoreactivity in enteric neurons and nerve fibers**

173 **3.1.1 Neurons**

174 Large percentages of HuC/HuD-IR neurons showed 5-HT₄-immunoreactivity (5-HT₄-IR) in the MP
175 and SMP of all the GI segments under investigation (Figure 2). 5-HT₄-IR showed different degrees
176 of brightness, varying from weak to strong. The pattern of immunoreactivity was preferentially
177 located into the cytoplasm rather than along the plasma membrane. In the myenteric neuropil, nerve
178 fibers and varicosities embracing neurons showed a bright 5-HT₄-IR (Figure 2A-F).

179 A total number of 5226 HuC/HuD-IR neurons were counted in all the intestinal tracts considered of
180 healthy adult horses. In the MP, we counted 2595 HuC/HuD-IR neurons and 43% of these cells
181 showed 5-HT₄-IR. In the SMP, we counted 2531 HuC/HuD-IR neurons and 63% of these cells
182 showed 5-HT₄-IR. Considering MP and SMP together, the small and large intestine shared the same
183 percentage of 5-HT₄-IR neurons (54%). The MP of the small and large intestine showed the same
184 percentage (43%) of 5-HT₄-IR neurons, whereas the SMP of the small and large intestine harbored
185 larger percentages of 5-HT₄-IR neurons (62% and 66%, respectively). In more detail, in the small
186 intestine MP, the greatest percentage of immunoreactive neurons was observed in the ileum
187 (63±19%) followed by duodenum (44±25%) and jejunum (35±20%). In the large intestine MP, pelvic
188 flexure 5-HT₄-IR neurons largely outnumbered (47±13%) the density of those observed in the
189 descending colon (28±9%) (Figure 2A-F). Submucosal neurons, which showed in general brighter 5-
190 HT₄-IR than myenteric ones, were observed in the inner and outer SMP layers (Figure 2G-L). In the
191 duodenal submucosa, the 5-HT₄-IR neurons were closely related to Brunner's glands (Figure 2J-L).
192 Nevertheless, no 5-HT₄-IR nerve fibers were observed within the glands. The percentages of 5-HT₄-
193 IR SMP neurons were similar in the pelvic flexure, duodenum, and jejunum (69±13%, 68±14%, and
194 67±3%, respectively), and slightly decreased in the descending colon (60±23%) and ileum (54±6%).
195 Figure 4A graphically represents the data related to the distribution of the 5-HT₄-IR neurons.

196 **3.1.2 Nerve fibers**

197 Bright 5-HT₄-IR nerve fibers were widely distributed in all layers of the small and large intestine.

198 The mucosal layer was widely innervated by 5-HT₄-IR nerve fibers, especially in the duodenum. The
199 5-HT₄-IR nerve fibers were distributed in the *muscularis mucosae*,³⁸ around intestinal glands, and in
200 the *lamina propria* (Figure 3A and D). In the mucosa of small intestine, the 5-HT₄-IR nerve fibers
201 were more visible on the upper half of the villi, whereas in the large intestine these positive fibers
202 were more prominent in the basal portion of the glands.

203 In the submucosa, a delicate/thin network of the 5-HT₄-IR fibers and varicosities encircled SMP
204 neurons (Figure 3E), while only a few immunoreactive fibers were visible around blood vessels.

205 In the *tunica muscularis*, the greatest density of the 5-HT₄-IR nerve fibers was observed in the CML
206 (Figure 3F) of the duodenum and descending colon. In the LML, the 5-HT₄-IR nerve fibers were well
207 represented in the descending colon and ileum and were scantily represented in the other intestinal
208 tracts. Figure 4B graphically represents the data related to the distribution of the 5-HT₄-IR nerve
209 fibers.

210 **3.2 5-HT₄ immunoreactivity in extrinsic innervation**

211 **3.2.1 Intestinal extrinsic sensory fibers**

212 In horses, most of the SP-IR innervation derives from enteric neurons supplying the CML
213 (Figure 3G).³⁴ Double immunohistochemistry carried out on adult horse tissues showed that 5-HT₄-
214 and SP-IR fibers widely co-localized (Figure S3). Notably, also in LWFS tissues, supplied only by
215 extrinsic nerves, SP-IR processes co-expressed 5-HT₄-IR (Figure 3H and I).

216 **3.2.2 Spinal ganglion neurons**

217 A weak to moderate 5-HT₄-IR was expressed by small- and medium-sized spinal ganglion neurons.
218 Double immunohistochemistry showed that the 5-HT₄-IR neurons co-expressed SP-IR (Figure 3J-
219 N).

220 **3.3 Neurochemical coding of 5-HT₄-IR enteric neurons**

221 In the MP, 5-HT₄-IR was expressed by excitatory neurons, detected for their immunoreactivity to
222 ChAT (Figure 5A and B) and SP (Figure 5C and D) and by inhibitory neurons, immunoreactive to
223 nNOS (Figure 5E and F). In the SMP, 5-HT₄-IR was co-expressed by large neurons co-expressing
224 SP-IR and by small putative secretomotor neurons, showing VIP-IR (Figure 5G and H). Notably,
225 subsets of myenteric and submucosal neurons showed triple co-localization 5-HT₄/SP/VIP-IRs
226 (Figure S4).

227 **3.4 5-HT immunoreactivity**

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

231 **3.5 Extra-neuronal 5-HT₄-IR distribution**

232 Extra-neuronal sites of the small and large intestine of 5-HT₄-IR included smooth muscle cells of the
233 *tunica muscularis*, a subset of ECs (Figure 6A-C), and endothelial cells of small vessels (data not
234 shown). In contrast, 5-HT₄-IR was not detectable in enterocytes and interstitial cells of Cajal.

235 **4 Discussion**

236 The present study provides solid evidence of 5-HT₄-IR in the MP and SMP neurons of horse intestine.
237 Despite some functional investigations carried out on horse small and large intestine indicated the
238 presence of 5-HT₄ in the ENS of this animal model,²³⁻³³ the only immunohistochemical study

239 carried out on horse ENS failed to detect 5-HT₄ in enteric neurons, but confirmed the presence of this
240 serotonergic receptor on muscular layers.³¹ Furthermore, a recent study by Delesalle et al.³⁹
241 questioned the presence of 5-HT₄ on cholinergic neurons of horse small intestine. In this line, our
242 results represent strong morphological support indicating that 5-HT₄ is expressed in MP and SMP
243 neuronal cell bodies and nerve fibers of horse GI tracts. The presence of large percentages of MP and
244 SMP 5-HT₄-IR neurons suggests that 5-HT₄ agonists may influence both intestinal propulsion and
245 secretion. In fact, the expression of 5-HT₄-IR by SMP SP-IR neuronal cell bodies (likely IPANs⁴⁰)
246 and related nerve fibers supports the role of this receptor in intramural reflex activities. Once activated
247 by 5-HT, the 5-HT₄ expressed by IPANs begins peristalsis through the recruitment of MP excitatory
248 ChAT-/SP-IR⁴¹ and inhibitory nNOS-IR⁴² motoneurons. Our findings are consistent with Cellek
249 et al.¹³ who showed the effect of 5-HT₄ agonists on both excitatory and inhibitory enteric neurons of
250 the human colon.

251 Concerning the secretory role, it is known that the active mucosal secretion can be triggered by a
252 serotonin-dependent activation of neurogenic responses mediated by 5-HT₄, 5-HT₃, and 5-HT_{1p}
253 receptors expressed by intrinsic submucosal neurons. This neurogenic mechanism leads secretomotor
254 neurons to release acetylcholine and VIP, inducing Cl⁻ and bicarbonate secretion by epithelial
255 cells.^{10, 43} Our findings that show the co-expression of 5-HT₄-IR in SMP VIP-, and SP-IR neurons,
256 suggest the involvement of 5-HT₄ in secretory and vasodilatory mechanisms in horses. This confirms
257 and expands previous work by Burns and Cummings³⁸ and Moore et al.⁴⁴

258 In veterinary medicine, selective 5-HT₄ agonists have been not yet used as prokinetic agents to treat
259 dysmotility disorders of the horse. The only exception is represented by the 5-HT₄ agonist mosapride
260 which effectively attenuated the decline of small intestinal motility in an experimental model of
261 postoperative ileus in the healthy horse. As a matter of fact, in veterinary clinical practice, the use of
262 5-HT₄ is still extremely limited to personal experience and, similarly to the routine practice in
263 humans, metoclopramide, whose effects rely upon a combination of receptor interaction (i.e.,
264 dopamine receptor antagonism, 5-HT₃ antagonism and 5-HT₄ agonism), is the most commonly
265 used.⁴⁵ While there is no evidence of side effects elicited by selective 5-HT₄ agonists,
266 metoclopramide evokes extrapyramidal manifestations in a similar fashion to what may happen in
267 humans chronically treated with this drug.

268 The expression of 5-HT₄-IR by horse spinal ganglion neurons does not necessarily indicate that these
269 neurons contribute to the intestinal innervation; nevertheless, the presence of 5-HT₄-IR on extrinsic
270 sensory fibers observed in aganglionic tissues of LWFS foals suggests that drugs acting on 5-HT₄
271 might influence the extrinsic visceral sensory pathway, as shown in humans.⁴⁶ This is the first
272 morphological evidence of 5-HT₄-IR in the horse spinal ganglia. Splice variants of this receptor were
273 already observed in the rat spinal ganglia.²² As 5-HT₄-IR was co-expressed by extrinsic SP-IR
274 sensory fibers of aganglionic LWFS foals, it is plausible that this receptor might play a role in
275 nociception.^{47, 48} In support of this notion, 5-HT₄ mRNA has been shown to be expressed by
276 nociceptive neurons of rat spinal ganglia.^{49, 50} 5-HT₄-IR was also co-expressed by SP-negative
277 spinal ganglion neurons, suggesting that this receptor might be involved in other aspects of the
278 sensory function, such as mechanosensitivity. In fact, tegaserod, a 5-HT₄ partial agonist, has been
279 shown to have an inhibitory effect on intramural mechanoreceptors of a cat rectum.⁵¹ Nevertheless, to
280 identify and confirm a role of 5-HT₄ in the visceral sensitivity of horses, functional and
281 pharmacological investigations are needed.

282 Gut vasodilation is regulated by intrinsic reflex circuitry involving SMP neurons via the activation of
283 5-HT₃ and 5-HT₄ receptors.⁵⁰ In the present study, endothelial cells of small vessels expressed 5-
284 HT₄-IR. This finding is consistent with studies indicating that 5-HT₄ mRNA (together with 5-HT₁, 5-
285 HT₂, and 5-HT₇ mRNA) is expressed by endothelial and vascular smooth muscle cells.^{52, 53}

286 Furthermore, it is known that endothelial 5-HT₄ regulates angiogenesis and that 5-HT₄ agonist
287 mosapride can inhibit proliferation and migration of endothelial cells in the human umbilical vein.⁵⁴

288 Growing evidence indicates that 5-HT₄ stimulation enhances development, survival, and
289 neurogenesis of enteric neurons^{18, 55, 56} and 5-HT₄ agonists facilitate neurogenesis from
290 transplanted stem cells in intestinal anastomosis.⁵⁷ Thus, 5-HT₄ agonists may have a
291 neuroregenerative potential that can be useful to treat horses subjected to colic surgery.

292 In conclusion, our results provide a consistent demonstration that 5-HT₄-IR is widely expressed by
293 different subsets of enteric neurons involved in secretory and motor reflexes. The morphological data
294 support previous pharmacological evidence and bear implication in the treatment of equine intestinal
295 dysmotility and constipation. Furthermore, the presence of 5-HT₄ on extrinsic nerve pathways widens
296 the role of serotonergic receptors in visceral perception mechanisms.

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305 **Disclosure**

306 The authors have no competing interests.

307 **Author Contribution**

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

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- 449

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

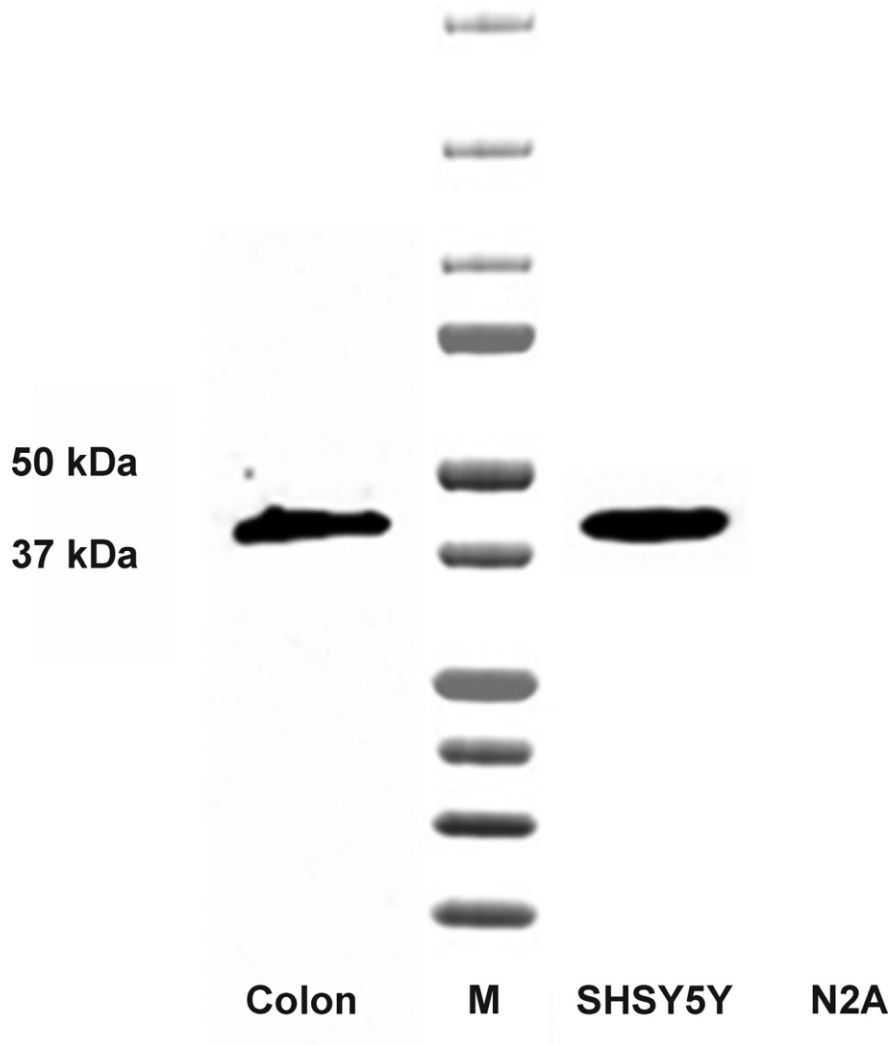
Antibody	Immunogen	Source, cat. no., species	RRID	Dilution
HuC/HuD	Human HuC/HuD neuronal protein	Life Technologies, A21271; Mouse monoclonal; Clone 16A11	AB_221448	IHC 1:200; WB 1:200
ChAT	Peptide fragment of purified porcine ChAT (GLFSSYRLPGHTQDTLVAQKSS) aminoacids:168-189	Generous gift of M. Schemann, 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 ₄ R	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/DDR C; 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

451 Suppliers: Abcam, Cambridge, UK, Europe; CURE/DDRC, DDD, University of California Los
 452 Angeles, Los Angeles, CA, USA; Fitzgerald Industries Int., Inc. Concord, MA, USA; Life
 453 Technologies, Carlsbad, CA, USA; Merck Millipore, Merck KGaA, Darmstadt, Germany,
 454 Europe; Santa Cruz Biotechnology, Santa Cruz, CA, USA.
 455 5-HT₄R, 5-hydroxytryptamine receptor 4; ChAT, choline acetyltransferase; HuC/HuD, human
 456 neuronal protein; IHC, Immunohistochemistry; nNOS, neuronal nitric oxide synthase; RRID,
 457 Research Resource Identifiers; SP, Substance P; VIP, vasoactive intestinal polypeptide; WB,
 458 Western blot.

459 **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

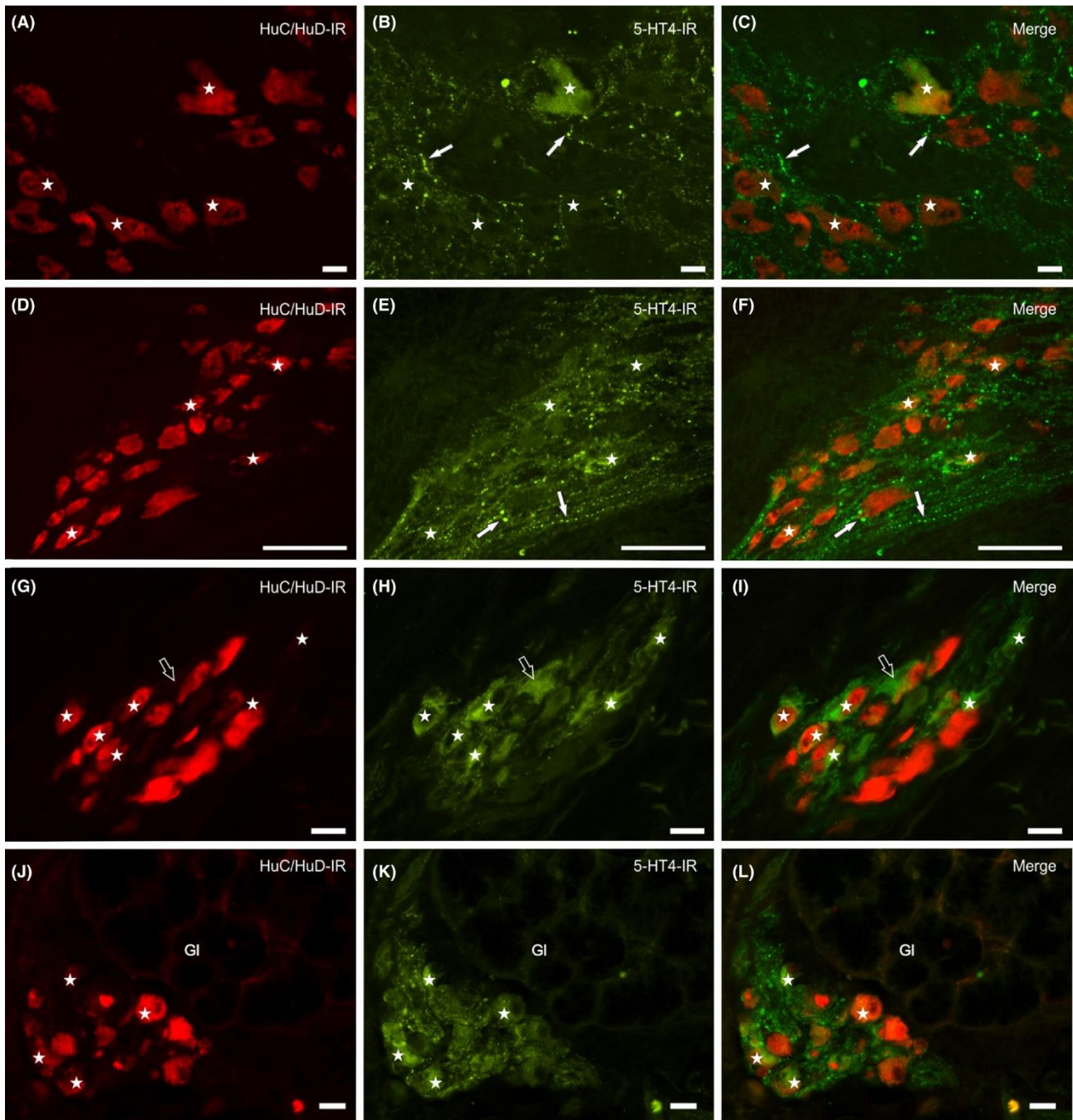
460 Suppliers: Abcam, Cambridge, United Kingdom, Europe; Biotium Inc., Hayward, California,
 461 USA; Jackson ImmunoResearch Laboratories, Inc, Pennsylvania, USA; Life Technologies,
 462 Carlsbad, Ca, USA; Merck Millipore, Darmstadt, Germany, Europe; Sigma Aldrich, Milan, Italy,
 463 Europe; Vector Laboratories,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



468

469 **Figure 1**

470 Validation of 5-HT₄ antibody in horse tissue by Western blot. Total protein lysate from horse colon
471 (lane 1), marker of molecular weight (M) (lane 2), SH-SY5Y human cell line as positive control (lane
472 3), murine Neuro2A (N2A) cell line as negative control (lane 4). A specific band of ~45 kDa was
473 detected in horse tissue (lane 1) and in the positive control (lane 3). No bands were detected in the
474 negative control (lane 4)



475

476

Figure 2

477

Micrographs showing HuC/HuD- and 5-HT₄-immunoreactivity (IR) in tangential cryosections of

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myenteric and submucosal plexus of horse ileum (A-C), pelvic flexure (D-F), and duodenum (J-L).

479

(A-F) Stars indicate some HuC/HuD-IR myenteric plexus neurons of the ileum (A-C) and pelvic

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flexure (D-F) which showed 5-HT₄-IR. Arrows indicate nerve fiber varicosities showing strong 5-

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HT₄-IR. (G-L) Stars indicate some ileal and duodenal submucosal plexus HuC/HuD-IR neurons co-

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expressing 5-HT₄-IR. (G-I) The open arrow indicates one 5-HT₄-IR ileal submucosal neuron showing

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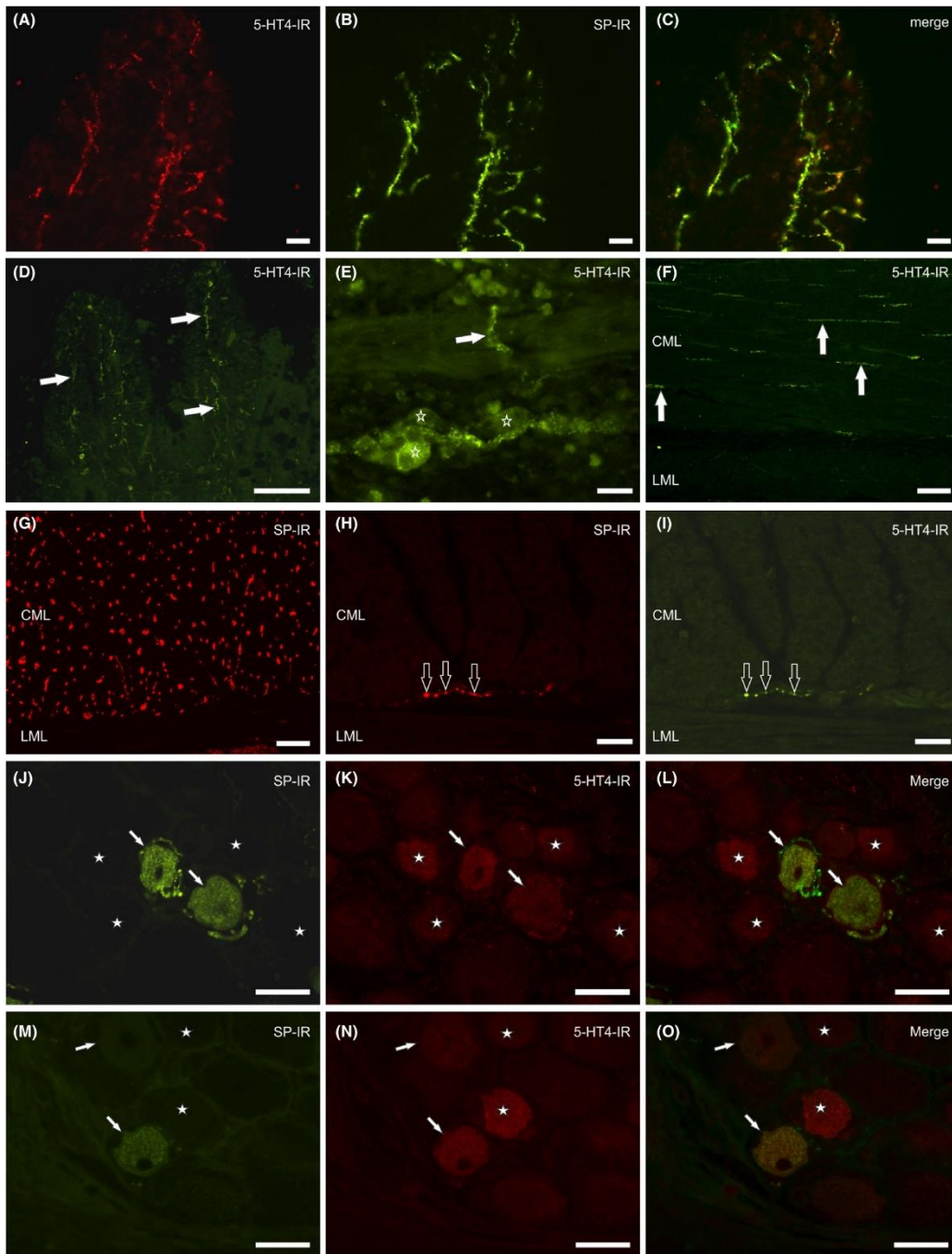
very faint HuC/HuD-IR. HuC/HuD-IR showed several degrees of immunoreactivity, from strong

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nuclear and cytoplasmic immunoreactivity to very weak (or almost undetectable). Bars = A-C; G-L:

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20 μm; D-F: 100 μm



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Figure 3

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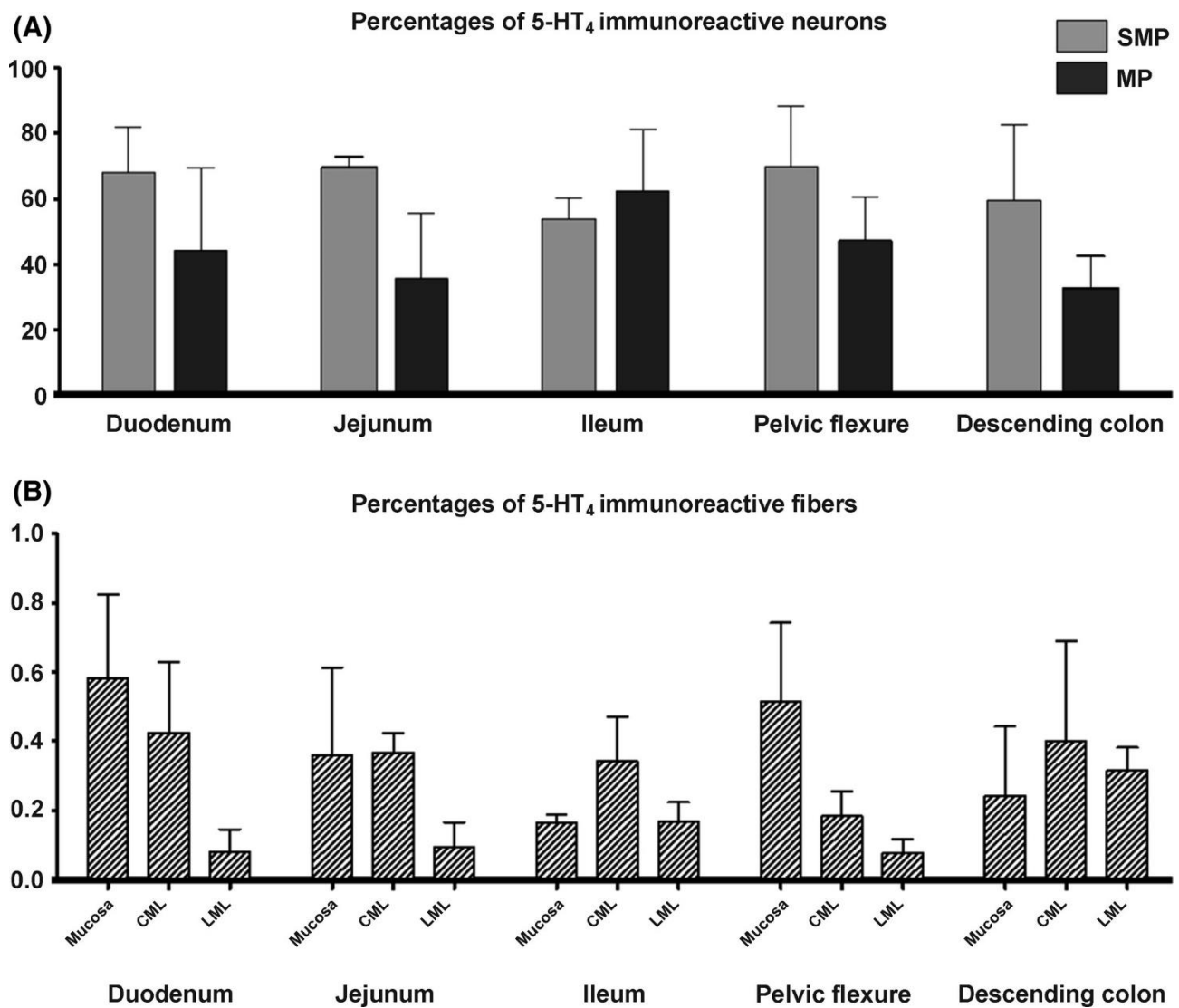
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(A-F) Micrographs showing 5-HT₄-immunoreactive nerve fibers (arrows) in the *lamina propria* (A-C, D), *muscularis mucosae* (E) and circular (CML) and longitudinal muscle layer (LML) (transverse cryosections) (F) of the horse ileum; stars (E) indicate some 5-HT₄-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-HT₄-IR. (J-O) Cryosections of horse spinal ganglion neurons; the arrows indicate SP-IR sensory neurons which co-expressed 5-HT₄-IR; stars indicate other 5-HT₄-IR neurons which were SP-negative. Bars = A-C, E, H and I: 20 μm; D, F, G, J-L: 100 μm



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Figure 4

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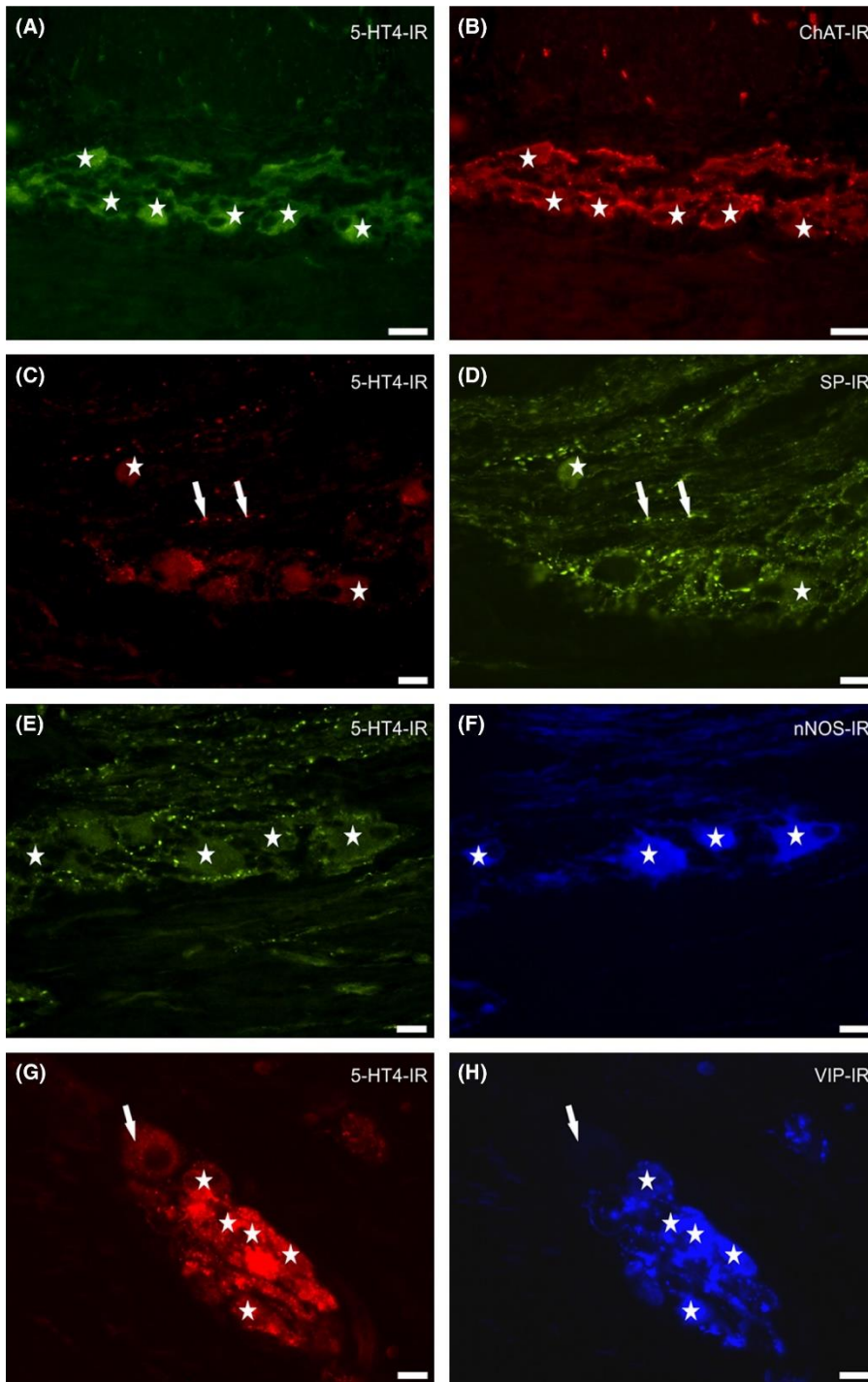
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(A) Graphical representation of the percentages of 5-HT₄-immunoreactive (-IR) neurons in the horse small and large intestine. Black bars indicate the percentages of 5-HT₄-IR neurons in the myenteric plexus of duodenum (161/452 cells), jejunum (147/497 cells), ileum (348/571 cells), pelvic flexure (297/606 cells), and descending colon (165/469 cells). Gray bars indicate the percentages of 5-HT₄-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-HT₄-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



510

511

Figure 5

512

Micrographs showing the co-localization of 5-HT₄-immunoreactivity (IR) with choline

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acetyltransferase (ChAT), substance P (SP), neuronal nitric oxide synthase (nNOS), and vasoactive

514

intestinal polypeptide (VIP) in the myenteric (A-F) and submucosal plexus (G-H) of the horse ileum.

515

(A and B) Stars indicate some myenteric plexus 5-HT₄-IR neurons co-expressing ChAT-IR. (C and

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D) Stars and arrows indicate myenteric plexus neurons and nerve fibers showing co-localization

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between 5-HT₄- and SP-IR. (E and F) Stars indicate 5-HT₄-IR myenteric plexus neurons co-

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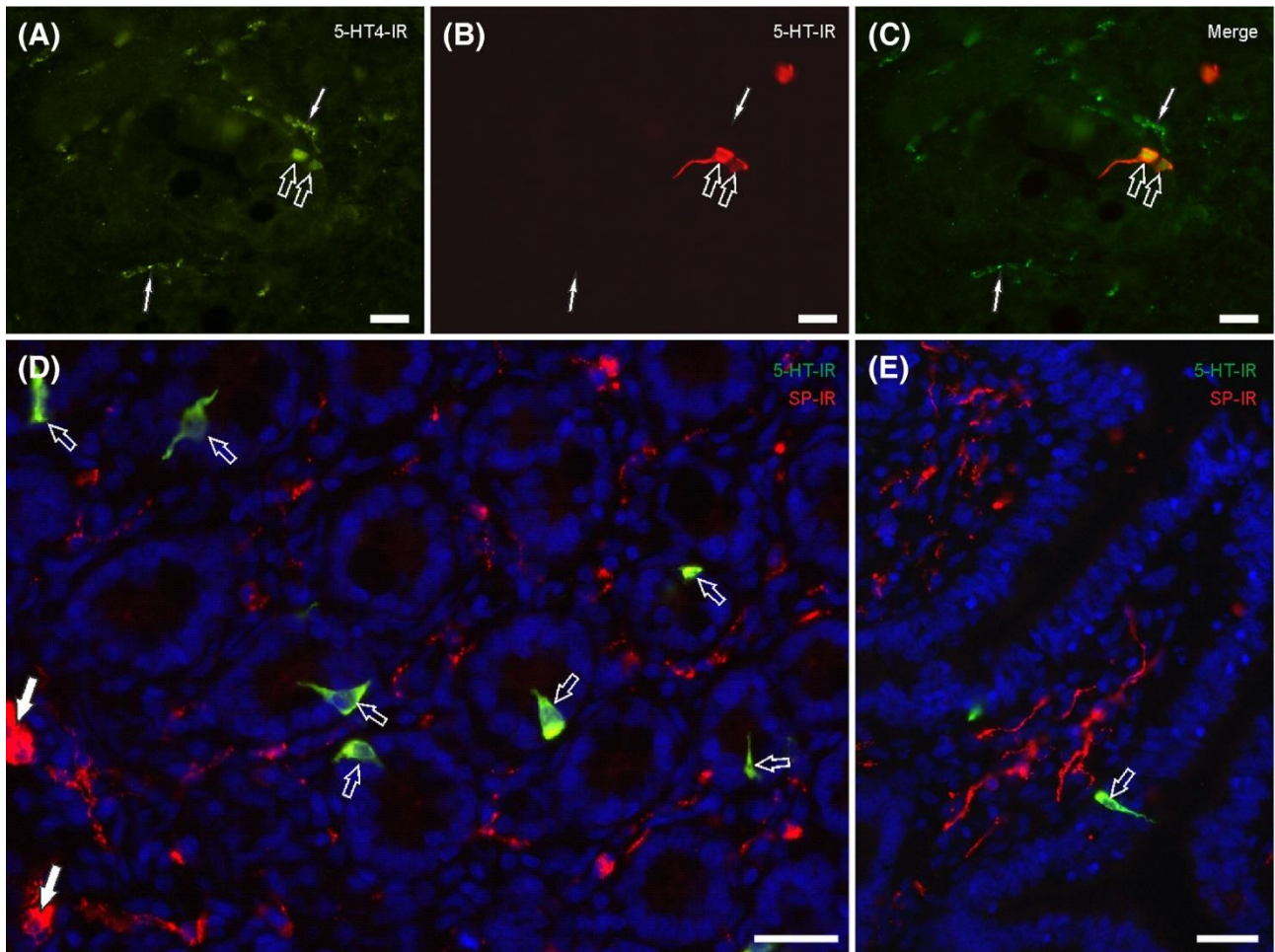
expressing nNOS-IR. (G and H) Stars indicate some submucosal plexus neurons expressing strong

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5-HT₄ and VIP-IR. The arrow indicates a 5-HT₄-IR neuron, which was VIP-negative. Bar = A-H:

520

20 μm



521

522 **Figure 6**

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

529