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1 **Localization of cannabinoid and cannabinoid related receptors in the cat gastrointestinal tract**

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8 **Abstract**

9 A growing body of literature indicates that activation of cannabinoid receptors may exert beneficial effects on
10 gastrointestinal inflammation and visceral hypersensitivity. The present study aimed to
11 immunohistochemically investigate the distribution of the canonical cannabinoid receptors CB1 (CB1R) and
12 CB2 (CB2R) and the putative cannabinoid receptors G protein-coupled receptor 55 (GPR55), nuclear
13 peroxisome proliferator-activated receptor alpha (PPAR α), transient receptor potential ankyrin 1 (TRPA1), and
14 serotonin receptor 5-HT1a (5-HT1aR) in tissue samples of the gastrointestinal tract of the cat. CB1R-
15 immunoreactivity (CB1R-IR) was observed in gastric epithelial cells, intestinal enteroendocrine cells (EECs)
16 and goblet cells, lamina propria mast cells (MCs), and enteric neurons. CB2R-IR was expressed by EECs,
17 enterocytes, and macrophages. GPR55-IR was expressed by EECs, macrophages, immunocytes, and MP
18 neurons. PPAR α -IR was expressed by immunocytes, smooth muscle cells, and enteroglial cells. TRPA1-IR
19 was expressed by enteric neurons and intestinal goblet cells. 5-HT1a receptor-IR was expressed by
20 gastrointestinal epithelial cells and gastric smooth muscle cells. Cannabinoid receptors showed a wide
21 distribution in the feline gastrointestinal tract layers. Although not yet confirmed/supported by functional
22 evidences, the present research might represent an anatomical substrate potentially useful to support, in feline
23 species, the therapeutic use of cannabinoids during gastrointestinal inflammatory diseases.

24 **Introduction**

25

26 Cannabinoid receptors regulate gastrointestinal tract (GIT) motility and secretion, sensation, emesis, satiety,
27 and inflammation (Hornby and Prouty 2004; Izzo 2004; Duncan et al. 2005a, 2008; Storr and Sharkey 2007;
28 Wright et al. 2008; Sharkey and Wiley 2016; Lee et al. 2016; Di Patrizio 2016).

29 Several evidences indicate that substances acting on GIT cannabinoid receptors may be beneficial for gut
30 discomfort and pain (Di Carlo and Izzo 2003; Hornby and Prouty 2004). The primary and most studied
31 cannabinoid receptors are two G protein-coupled receptors: cannabinoid receptors type 1 (CB1R) and type 2
32 (CB2R). CB1R is mostly expressed in the central and peripheral nervous system (Hu and Mackie 2015), while
33 CB2R is mainly expressed in immune cells (Di Marzo and Izzo 2006).

34 CB1R-immunoreactivity (IR) is also expressed by enteric neurons (Kulkarni-Narla and Brown 2000; Van
35 Sickle et al. 2001; Coutts et al. 2002; Duncan et al. 2005a; Galiazzo et al. 2018), enteroendocrine cells (EECs),
36 and lamina propria cells (Adami et al. 2002; Hornby and Prouty 2004; Galiazzo et al. 2018) of several
37 mammalian species.

38 CB2R may be expressed by GIT macrophages, plasma cells, mast cells, dendritic cells, lymphocytes, epithelial
39 cells, and enteric glial cells (Facci et al. 1995; Wright et al. 2005, 2008; Duncan et al. 2005b, 2008; Svensson
40 et al. 2010; Ke et al. 2016; Galiazzo et al. 2018). Several studies suggest that CB1R or CB2R might have a
41 protective role in inflammatory bowel disease (IBD), and support the possible value of targeting these
42 pathways with pharmacological agents, such as phytocannabinoids or synthetic cannabinoid agonists, for
43 therapeutic gain (Di Marzo and Izzo 2006; Duncan et al. 2008; Di Marzo and Piscitelli 2011; Di Patrizio 2016;
44 Gyires and Zádori 2016; Fabisiak and Fichna 2017).

45 Other receptors, such as G protein-coupled receptors 3 (GPR3), 6 (GPR6), 12 (GPR12), and 55 (GPR55), and
46 nuclear peroxisome proliferator-activated receptors alpha (PPAR α) and gamma (PPAR γ), are considered
47 cannabinoid-related receptors (Di Marzo et al. 2002; Brown et al. 2005; Lauchner et al. 2008; Izzo and Sharkey
48 2010; Lin et al. 2011; Petrosino and Di Marzo 2016; Tuduri et al. 2017; Laun and Song 2017; Laun et al. 2019).
49 In addition, phytocannabinoids may also act on transient receptors potential channels (TRP) vanilloid 1
50 (TRPV1) and ankyrin 1 (TRPA1), and serotonergic receptors such as 5-HT1a, 5-HT2a and 5-HT3 (Pertwee
51 2015).

52 Cannabidiol (CBD) is currently one of the most studied cannabinoids and its use is spreading throughout
53 human and veterinary medical practice. Notably, CBD also is a non-psychoactive compound with proved anti-
54 inflammatory, analgesic, anti-anxiety and anti-tumoral properties (Mechoulam et al. 2007; Morales et al.
55 2017). CBD seems to act preferentially on cannabinoid-related receptors, such as GPR3 (inverse agonist),
56 GPR6 (inverse agonist), GPR12 (inverse agonist), GPR55 (antagonist), TRPA1 (agonist), TRPV1 (agonist),
57 and serotonergic receptors 5-HT1a (agonist), 5-HT2a (partial agonist), and 5-HT3 (antagonist) (Iannotti et
58 al. 2014; Morales et al. 2017; Russo 2018; Laun et al. 2019). However, in the present study, due to their indirect
59 correlation with CBD, CB1R- and CB2R-immunolabelings were investigated. In fact, there are studies
60 reporting a weak CB1R antagonist effect of CBD (Thomas et al. 2007) and others indicating that CBD may
61 regulate the endocannabinoid system by inhibiting the uptake of the endogenous CB1R ligand anandamide
62 (AEA) or by inhibiting AEA enzymatic hydrolysis (entourage effect) (Ligresti et al. 2016).

63 Another non-psychoactive therapeutic endocannabinoid-like substance, palmitoylethanolamide (PEA), a lipid
64 mediator that is structurally related to the endocannabinoid anandamide, is used in human and veterinary
65 clinical practice for its neuroprotective, anti-neuroinflammatory, analgesic, and antipruritic properties (Re et
66 al. 2007; Gabrielsson et al. 2016; Petrosino and Di Marzo 2016; Cremon et al. 2017). Several investigators
67 have identified different mechanism of action for PEA (Iannotti et al. 2016; Petrosino and Di Marzo 2016),
68 which seems to have a direct (agonist) effect upon the cannabinoid receptors G-protein coupled receptor 55
69 (GPR55) (Ryberg et al. 2007) and PPAR α (Lo Verme et al. 2005a, b; Gabrielsson et al. 2016). PEA indirectly
70 activates CB1R and CB2R through an entourage effect, which increases AEA levels in tissues (Re et al. 2007;
71 Petrosino and Di Marzo 2016). PEA, which seems to also act favorably on visceral pain (Jaggat et al. 1998;
72 Farquhar-Smith et al. 2002; Gabrielsson et al. 2016), represents a promising natural approach for management
73 of hypersensitivity/pain derived from intestinal inflammation.

74 To explore the therapeutic potential of non-psychoactive phytocannabinoids in pathological GIT conditions, it
75 is important to investigate the cellular distribution of their receptors. Therefore, the present study aimed to
76 immunohistochemically characterize the cellular expression of six receptors (CB1R, CB2R, GPR55, PPAR α ,
77 TRPA1, and 5-HT1a receptor) on GIT tissue samples from cats.

78 The findings of the present study might be useful to support and strengthen the therapeutic use of non-
79 psychotropic cannabinoid agonists in feline gastritis and enteritis.

80 **Material and methods**

81 **Animals**

82 Gastrointestinal tissues were collected from five European cats (Table 1), that did not have a history of
83 gastrointestinal disorders and did not show gross alterations of the gastrointestinal wall. The animals died
84 spontaneously or were euthanized for human reasons due to different diseases, and their tissues were collected
85 following owners' permission.

86 According to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010
87 on the protection of animals used for scientific purposes, the Italian legislation (D. Lgs. n. 26/2014) does not
88 require any approval by competent authorities or ethics committees, because this research did not influence
89 any therapeutic decisions.

90 **Tissue collection**

91 GIT samples (pylorus, descending duodenum, ileum, and distal colon) were harvested within 1 h of the
92 animals' death and were longitudinally opened along the gastric small curvature (pylorus) and mesenteric
93 border (intestine). Tissues were then washed in phosphate-buffered saline (PBS), fixed and processed to obtain
94 cryosections (2.0 cm × 0.5 cm), which were later processed for immunohistochemistry, as described in
95 previous studies (Chiocchetti et al. 2015).

96 **Immunofluorescence**

97 Cryosections were hydrated in PBS and processed for immunostaining. To block non-specific bindings, the
98 sections were incubated in a solution containing 20% normal goat or donkey serum (Colorado Serum Co.,
99 Denver, CO, USA), 0.5% Triton X-100 (Sigma Aldrich, Milan, Italy, Europe), and bovine serum albumin (1%)
100 in PBS for 1 h at room temperature (RT). The cryosections were incubated in a humid chamber overnight at
101 RT with the antibodies directed against the cannabinoid and cannabinoid-related receptors (single
102 immunostaining) or a cocktail of primary antibodies (double immunostaining) (Table 2) diluted in 1.8% NaCl
103 in 0.01 M PBS containing 0.1% sodium azide. After washing the sections in PBS (3 × 10 min), they were
104 incubated for 1 h at RT in a humid chamber with the secondary antibodies (Table 3) diluted in PBS.
105 Cryosections were then washed in PBS (3 × 10 min) and mounted in buffered glycerol at pH 8.6 with 4,6-
106 diamidino-2-phenylindole—DAPI (Santa Cruz Biotechnology, CA, USA).

107 To identify the cellular types expressing cannabinoid receptors, we utilized specific antibodies. To identify
108 enteric neurons and glial cells (EGCs), we co-localized the antibodies anti-cannabinoid receptors with the anti-
109 HuC/HuD and anti-glial fibrillary acidic protein (GFAP) antibodies, respectively; to identify mast cells (MCs),
110 we utilized an antibody anti-tryptase (MCs protease). To identify macrophages, we utilized the antibody anti-
111 ionized calcium binding adapter molecule 1 (IBA1). A subclass of plasma cells was identified with an antibody
112 anti-IgA. To identify enteroendocrine cells (EECs), antibodies anti-chromogranin A (CGA), -serotonin (5-HT),
113 or -cholecystokinin (gastrin/cholecystokinin, GAS/CCK) were used.

114 **Specificity of the primary antibodies**

115 The specificity of anti-CB1R (Abcam, ab23703) (immunogen: c-terminal amino acids 461–472 of human
116 CB1R), -CB2R, and -PPAR α antibodies utilized in the present research has been tested on the cat tissues by
117 Western blot (Wb) analysis by Miragliotta et al. (2018).

118 We utilized the antibody anti-PPAR α (Novus Biol; NB600-636) (Galiazzo et al. 2018); the same antibody,
119 tested with Wb on feline tissues by Miragliotta et al. (2018), was until recently also available with another
120 code (Novus Biol; NBP1-03288). At present, Novus Biol. markets this antibody only with the code NB600-
121 636.

122 The specificity of the anti-GPR55 antibody, recently tested on mouse and dog tissues (Galiazzo et al. 2018),
123 was tested in the present study by Wb analysis (Fig. 1a).

124 The antibody anti-TRPA1 was raised using, as immunogen, a synthetic peptide (CEKQHELIKLIQKME)
125 corresponding to amino acids 1060–1075 of rat TRPA1. The alignment of the immunogen sequence with the
126 target protein in the cat is 100% (<https://blast.ncbi.nlm.nih.gov.ezproxy.unibo.it/Blast.cgi>); therefore, the
127 commercially available antibody anti-rat TRPA1 should also recognize the same receptor in the cat. However,
128 the specificity of the anti-TRPA1 antibody was tested in the present study by Wb analysis (Fig. 1b).

129 The antibody anti-5-HT $1a$ receptor (5-HT $1aR$) was raised using, as immunogen, a synthetic peptide
130 corresponding to amino acids 100–200 (conjugated to keyhole limpet haemocyanin) of rat 5-HT $1aR$. The
131 alignment of the immunogen with the target protein sequence in the cat is only 36% (<https://blast.ncbi.nlm.nih.gov.ezproxy.unibo.it/Blast.cgi>). The specificity of the anti-5-HT $1aR$ antibody was tested in the present
132 study by Wb analysis (Fig. 1c).
133

134 In addition, since the suppliers of the primary antibodies anti-TRPA1 and -5-HT $1aR$ employed in the present
135 study state them to rat-specific, rat ileum, and colon were used as control tissues for comparison purposes
136 (authorization no. 112/2018-PR of 12 February 2018). The supplier of the primary antibody anti-GPR55

137 (human GPR55) does not indicate cross-reactivity (based on sequence identity) with rat GPR55; however, we
138 tested the anti-GPR55 antibody also on rat ileum.

139 The details on the distribution of the study receptors in the rat were out of the scope of the present study and
140 were not evaluated.

141 The antibody mouse anti-human tryptase (Dako, M 7052—Clone AA1) was already employed on cat tissues
142 by Kleinschmidt et al. (2010). The goat anti-porcine specific IgA antibody (Novus Biol., NB100-1028) was
143 co-localized with the rabbit anti-human specific IgA antibody (Bethyl Lab., A80-103A). Since the two
144 antibodies perfectly co-localized in the same feline plasma cells (data not shown), in the present research we
145 utilized the goat anti-IgA antibody to identify immunocytes. The specificity of the goat anti-IBA1 antibody
146 (Novus Biol. NB100-1028; immunogen: peptide with sequence C-TGPPAKKAISELP corresponding to C-
147 Terminus), a marker for macrophages and CNS microglia (Pierezan et al. 2014), has not been tested on cat
148 tissues. Nevertheless, this antibody should recognize GIT macrophages in the feline GIT as well.

149 To avoid false positive staining, due to the presence of receptors for the Fc fragment of the secondary antibodies
150 on some immunocytes or inflammatory cells, we utilized two F(ab)2 fragment antibodies: goat F(ab)2 anti-
151 rabbit (FITC) (Abcam; ab98430) and goat F(ab)2 anti-mouse (TRITC) (Abcam; ab51379). We tested the
152 specificity of the other secondary antibodies employed in the present study (Table 2), as already described in
153 a previous work (Sadeghinezhad et al. 2013).

154 **Fluorescence microscopy**

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

160 **Western blot: specificity of the primary antibodies**

161 Intestinal tissue samples (feline duodenum and colon) were collected, frozen in liquid nitrogen, and stored
162 at -80°C until sample processing. Primary antibodies were tested in our laboratories according to standardized
163 protocols (Giancola et al. 2016; Galiazzo et al. 2018). Wb analysis of GPR55 (1:500) revealed a band of ~ 40
164 kDa (theoretical molecular weight of feline GPR55 is 40 kDa) (Fig. 1a). TRPA1 (1:500) revealed a major band
165 at ~ 100 kDa (theoretical molecular weight 100 kDa) (Fig. 1b) and 5-HT1aR (1:3000) presented a major band
166 at ~ 50 kDa (theoretical molecular weight 46 kDa) (Fig. 1c). Overall, Wb analysis confirmed the specificity of
167 the primary antibodies anti-GPR55, -TRPA1 and -5-HT1aR utilized in the present study.

168 **Results**

169 **CB1 receptor immunoreactivity**

170 CB1R-IR was observed in different cell types of the mucosa, such as gastric mucous cells (Fig. 2a–c) and
171 EECs, intestinal CGA (Fig. 2d–f) and CCK immunoreactive EECs, goblet cells (Fig. 2d–l), and lamina propria
172 tryptase immunoreactive MCs (Fig. 3a–c). Faint CB1R-IR was also displayed by smooth muscle cells of the
173 tunica muscularis. Specifically, in goblet cells, recognizable for their shape and the presence of compressed
174 nuclei confined in the deepest part of the cell, CB1R-IR was absent in the cytoplasm but was very bright at
175 membrane level. In the other cells types, the CB1R immunolabelling was diffused in the cytoplasm whereas
176 its localization on the cell membranes was less identifiable. Faint, granular cytoplasmic CB1R-IR was also
177 displayed by myenteric plexus (MP) (Fig. 3d–f) and submucosal plexus neurons (SMP) (data not shown) and
178 by smooth muscle cells of the tunica muscularis and muscularis mucosae.

179 **CB2 receptor immunoreactivity**

180 CB2R-IR was expressed by gastric and intestinal EECs, enterocytes, and lamina propria macrophages. Only a
181 few IgA immunoreactive plasma cells showed faint CB2R immunolabelling (data not shown). In the pylorus

182 and intestine, CB2R-IR was expressed by CGA (Fig. 4a–d) and 5-HT immunoreactive EECs (Fig. 4e–h).
183 Notably, epithelial cells of the small and large intestine showed bright immunolabelling; however, the
184 distribution of the epithelial CB2R-IR among the intestinal tracts (and animals) was rather unusual and not
185 constant. CB2R-IR in the colon was often evident also along the lateral portions of epithelial cells (Fig. 4i).
186 Goblet cells in the small intestine were CB2R negative, whereas those of the large intestine were
187 immunolabelled (Fig. 4i). Large intestinal EECs, in particular within the ileum and colon, showed impressive
188 granular CB2R-IR of the cytoplasm (Fig. 4j–l). IBA1 immunoreactive macrophages of the lamina propria
189 showed weak CB2R-IR (Fig. 4m–p).

190 CB2R-IR was also displayed by the smooth muscle cells of the muscular layers (data not shown). No CB2R-
191 IR was observed in neurons and glial cells of the enteric nervous system (ENS).

192 **GPR55 immunoreactivity**

193 GPR55-IR was expressed by epithelial cells, EECs, immunocytes, and enteric neurons. In particular, bright
194 and granular GPR55-IR was expressed by the cytoplasm of CGA immunoreactive EECs scattered along the
195 pylorus, small and large intestine (Fig. 5a–f); large intestinal EECs showed larger dimensions compared to the
196 small intestinal ones. Crypt epithelial cells in the large intestine displayed faint and granular GPR55-IR of the
197 cytoplasm (Fig. 5a–c). Immunocytes of lamina propria and Peyer’s patches nodules showed bright GPR55-IR
198 (Fig. 5g). Co-localization studies showed that no mucosal CD3-IR T lymphocytes or IgA-IR plasma cells
199 showed GPR55-IR (Fig. 5h, i). Notably, gastric MP (Fig. 5j–l) and intestinal MP and SMP neurons (data not
200 shown) showed moderate GPR55-IR. In the rat ileum and colon cryosections, no GPR55-IR was observed.

201 **PPAR α immunoreactivity**

202 PPAR α -IR was expressed by intestinal immunocytes (Fig. 6a, b), smooth muscle cells (Fig. 6c), and EGCs
203 (Fig. 6d–f).

204 **TRPA1 immunoreactivity**

205

206 TRPA1-IR was expressed by gastric MP neurons (Fig. 7a, b), and intestinal MP (Fig. 7c) and SMP neurons
207 (data not shown), whose phenotype was however not investigated. In enteric ganglia, it was also possible to
208 observe bundles of TRPA1-IR nerve fibers. In the small and large intestine, TRPA1-IR was also expressed by
209 goblet cells (Fig. 7d–f). Also in the rat ileum and colon, TRPA1-IR was expressed by goblet cells and enteric
210 neurons (Supplementary Fig. 1a–f).

211 **5-HT $1a$ receptor immunoreactivity**

212 5-HT $1a$ R-IR was expressed by the cell membrane (latero-basal) of gastrointestinal epithelial cells (Fig. 7g–i).
213 Bright 5-HT $1a$ R-IR was also observed around the nucleus of the epithelial cells. 5-HT $1a$ R-IR was observed in
214 gastric smooth muscle cells of the tunica muscularis, muscularis mucosae and submucosal blood vessels (data
215 not shown). In the rat ileum and colon, faint 5-HT $1a$ R-IR was expressed by epithelial cells and enteric neurons
216 (Supplementary Fig. 1g–l).

217 **Discussion**

218 **CB1 receptor**

219 Faint CB1R immunoreactive neurons were observed in the cat enteric neurons; this data is consistent with the
220 findings observed in many other species, such as rodents, ferrets, dogs, and humans (Kulkarni-Narla and
221 Brown 2000; Van Sickle et al. 2001; Coutts et al. 2002; Storr et al. 2004; Duncan et al. 2005a; Wright et al.
222 2005; Marquez et al. 2009; Galiazzo et al. 2018; Grill et al. 2019). In rodents, CB1R is mainly expressed by
223 cholinergic excitatory motor neurons. However, the phenotype of CB1R immunolabelled neurons was not
224 investigated in the present study.

225 CB1R-IR was displayed by different types of cat epithelial cells, such as pyloric mucous- secreting epithelial
226 cells, goblet cells, and EECs. This is in line with what was observed in human GIT mucosa, where CB1R has
227 been identified on gastric parietal cells, colonic epithelial cells, goblet cells, and EECs (Wright et al. 2005;
228 Pazos 2008; Marquez et al. 2009; Ligresti et al. 2016). The presence of CB1R in epithelial cells seems to be
229 crucial for their regeneration (Wright et al. 2005) and for the regulation of intestinal permeability (Karwad et
230 al. 2017). The expression of CB1R on mucous-secreting epithelial cells in the cat indicates that cannabinoids
231 might influence (reduce?) the GIT mucous secretion. In humans, CB1R was observed on parietal cells (Pazos
232 2008); in the cat (and dog; Galiazzo et al. 2018), we did not identify this receptor on parietal cells, whereas we
233 identified the receptor on gastric and intestinal EECs. It is known that under physiological conditions, the
234 activation of CB1R reduces gastric acidic secretion and regulates the release of enteroendocrine peptides, such
235 as CCK secreted by small intestinal EECs (Sykaras et al. 2012). The reduction of acid secretion by parietal
236 cells might be due to the effects of cannabinoids on the secretion of gastrin and histamine by gastric EECs;
237 these two hormones regulate, in fact, the acidic secretion of parietal cells (Adami et al. 2002; Schubert 2016).
238 The intestinal hormone CCK, acting on vagal terminals, induces a state of satiety (Owyang and Heldsinger
239 2011) in the cat as well (Bado et al. 1991); therefore, it is plausible to consider that cannabinoids may reduce
240 the secretion of CCK and increase the sense of hunger. In fact, Moss et al. (2012) showed that, in rodents,
241 intestinal EECs expressed the CB1R and that CB1R stimulation suppressed secretion of glucose-dependent
242 insulinotropic polypeptide (GIP), an enteroendocrine hormone secreted by a subclass of small intestinal EECs
243 (K cells), promoting storage of glucose and fat. For the sake of truth, CB1R agonist did not affect the secretion
244 of glucagon-like peptide-1 (GLP-1), thus the hypothesis that cannabinoids might reduce the secretion of CCK
245 remains to be elucidated.

246 Cat MCs expressed CB1R-IR. Historically, MCs play a key role in allergic reactions, being one of the major
247 sources of the mediator histamine (Walls et al. 2001). Usually, MCs are associated with pathological conditions
248 such as asthma (Wouters et al. 2016) or play a role in host–pathogen interactions, especially with parasites
249 (Marshall 2004). Up until the last two decades, MCs were recognized to be widely involved in a number of
250 non-allergic diseases including infection, inflammatory bowel disease (IBD), and food intolerance (Shea-
251 Donohue et al. 2010; Boeckxstaens 2015; Zhang et al. 2016; Wouters et al. 2016; Bednarska et al. 2017).
252 Studies demonstrated that CB1R and CB2R play a role in the regulation of the immune system in general, and
253 of MCs in particular. Growing evidence suggests that these receptors inhibit MCs activation and thus contribute
254 to the suppression of secretory responses (Samson et al. 2003; Harvima et al. 2014). In the GIT, MCs play
255 many different roles: they regulate vascular and epithelial permeability, ion secretion, angiogenesis, peristalsis,
256 fibrosis and tissue repair, innate and adaptive immunity, bacterial defense, chemotaxis and nociception. An
257 uncontrolled MCs activation can break gut homeostasis and contribute to the development of GIT diseases,
258 such as IBD (Bischoff and Krämer 2007). At the same time, MCs are fundamental for defending against
259 infections and for the regulation of innate and adaptive immune responses (Abraham and St John 2010).

260 **CB2 receptor**

261 It has to be remarked that CB2R immunoreactivity shows a wide distribution among the epithelial cells of the
262 small and large intestine. There are evidences supporting the role of CB2R in preserving gut mucosa integrity
263 (Harvey et al. 2013; Yang et al. 2014). A study demonstrated that CB2R activation inhibits tumor necrosis
264 factor-alpha (TNF-alpha)-induced secretion of interleukin-8 from the colonic epithelial cell line (Ihenetu et al.
265 2003).

266 The expression of CB2R-immunoreactivity on lamina propria cells was expected, due to the reported presence
267 of these receptors among different classes of immunocytes and inflammatory cells (Wright et al. 2008; Izzo
268 and Sharkey 2010; Gyires and Zádori 2016; Lee et al. 2016; Grill et al. 2019). We noted a great amount of
269 IBA1 immunoreactive macrophages co-expressing the CB2R-IR. The endocannabinoid system acts as a
270 regulator of immune homeostasis in the gut (Acharya et al. 2017). The CB2R of immune cell types, such as
271 macrophages, dendritic cells, and B cells, modulate their cytokine production and responsiveness (Ziring et al.
272 2006). Recently, Acharya et al. (2017) demonstrated that, in mice, the engagement of the CB2R augmented
273 the number and immune suppressive function of CX3CR1hi regulatory macrophages.

274 The expression of CB2R immunoreactivity by EECs suggests that endocannabinoids and cannabinoid agonists
275 acting on CB2R may exert a functional role in hormone production and secretion and may play a key role in
276 energy homeostasis. It has been shown that, in the pancreas, CB2R agonists may regulate pancreatic β -cell
277 function (Li et al. 2010; Gruden et al. 2016). At present, to the best of our knowledge, no information related
278 to the effects of CB2R agonists on enteroendocrine hormone production is available.

279 **GPR55**

280 The observation of GPR55-IR by cat enteric neurons was not a surprise; in fact, a growing body of evidence
281 indicates that this receptor is widely distributed in the ENS of humans and rodents (Lin et al. 2011; Ross 2011;
282 Li et al. 2013; Goyal et al. 2017). GPR55 agonists slow down gut transit and reduce colonic muscle contractility
283 during lipopolysaccharide (LPS)-induced motility disturbances in rodents. Since activation of GPR55 is not
284 associated with central sedation, the GPR55 may be considered as a future target for the treatment of colonic
285 motility disorders (Li et al. 2013). Since PEA shows affinity for GPR55 (Petrosino and Di Marzo 2016), it is
286 plausible to consider that PEA might influence GIT dysmotility during acute and chronic intestinal
287 inflammation. Unlike PEA, CBD acts as an antagonist of the GPR55 (Ligresti et al. 2016); however, Capasso
288 et al. (2008) showed that CBD may as well selectively inhibit inflammatory hypermotility in mice.

289 A large number of cat lamina propria and Peyer's patches immunocytes showed bright GPR55-IR. In other
290 species, GPR55 has already been identified in a large number of cell types, such as macrophages, plasma cells,
291 neutrophils, natural killer cells, monocytes, or lymphatic cells (T cells) (Balenga et al. 2011; Stancic et al.
292 2015; Chiurchiù et al. 2015; Taylor et al. 2015; Lanuti et al. 2015; Galiazzo et al. 2018; Grill et al. 2019). Data
293 related to the role played by GPR55 during intestinal inflammation is controversial; in fact, its proinflammatory
294 nature has also been hypothesized, since administration of GPR55 antagonists or inverse agonists seems to
295 reduce intestinal inflammation and decrease pro-inflammatory cytokines and leukocyte recruitment (Stancic
296 et al. 2015; Tuduri et al. 2017; Grill et al. 2019). The evidence of bright GPR55 immunolabelling of
297 gastrointestinal EECs, indicates that this receptor may play a role in the secretory function of these endocrine
298 cells. It has been shown that the activation of other specific G protein coupled receptors expressed on EECs
299 (e.g. GPR41, GPR43, GPR119 and TGR5) triggers the secretion of glucagon-like peptides (GLP-1 and GLP-
300 2) and PYY, gut peptides which are known to control energy homeostasis, glucose metabolism, gut barrier
301 function and metabolic inflammation (Cani et al. 2013). GPR55 plays an important role in the regulation of
302 mouse and human islet physiology (Liu et al. 2016), it is thus plausible that GPR55 on EECs may influence
303 gut hormone secretion.

304 The crypt cells of the colon also showed faint and granular GPR55-IR of the cytoplasm, which is in line with
305 the findings of Grill et al. (2019) in mice.

306 The rat tissues did not show any GPR55 immunolabeling. This finding may be due to the reduced homology
307 between the specific amino acid sequences of human and rat GPR55 (73.6%), whereas that of the cat is greater
308 (83%) (<https://blast.ncbi.nlm.nih.gov.proxy.unibo.it/Blast.cgi>). In addition, the supplier of the primary
309 antibody anti-GPR55 does not indicate cross-reactivity with rat GPR55. Thus, it is probably that the antibody
310 employed in the present study was not able to recognize the rat GPR55 epitope.

311 **PPAR α**

312 This receptor is a ligand-activated transcription factor belonging to the superfamily of nuclear hormone-
313 receptors. PPAR α may induce or repress transcription of a large number of different genes related to the
314 regulation of glucose, lipid, and cholesterol metabolism. PPAR α , as well as the other PPAR receptors (PPAR γ
315 and PPAR β/δ), can be activated by dietary fatty acids such as PEA (Petrosino and Di Marzo 2016) and PPAR α
316 agonists (Rigano et al. 2017). The activation of PPAR α within the GIT can lead to anti-nociceptive and anti-
317 inflammatory effects (Escher et al. 2001; Azuma et al. 2010; Petrosino and Di Marzo 2016). When activated
318 by PEA, PPAR α seems to biochemically react with the TRPV1 channel (i.e. the capsaicin receptor of
319 nociceptive neurons), and this interaction (entourage effect; Ambrosino et al. 2013) seems to desensitize the
320 TRPV1 receptor. Also, in the cat, as we observed in the dog (Galiazzo et al. 2018), PPAR α was located at the
321 level of EGCs (Liu et al. 2013; Sharkey 2015), which are functionally comparable to CNS astrocytes. EGCs

322 may release soluble factors acting as chemo attractants during inflammation; it has been reported that EGCs
323 activation may amplify intestinal inflammation, by releasing IL-6, monocyte chemotactic protein 1 and S100B
324 protein, which activate a biochemical cascade leading to the transcription of different cytokines and inducible
325 nitric oxide synthase protein (Cirillo et al. 2011; Ochoa-Cortes et al. 2016). Esposito et al. (2014) showed that
326 S100B protein recruits macrophages in the mucosa and therefore may amplify gut inflammation; in addition,
327 the same authors demonstrated that PEA mitigates, via a PPAR α manner, the course of intestinal inflammation
328 by reducing glial expression of S100 and toll like receptor 4.

329 The localization of PPAR α in the smooth muscle cells of LML suggests a role of this receptor on feline GIT
330 motility. We also observed a similar muscular pattern of PPAR α -IR in the dog (Galiazzo et al. 2018), and other
331 mammals such as pig and horse (personal observation of Dr. R. Chiocchetti). At present, we are not able to
332 speculate on the pathophysiological meaning of this different receptor distribution. A study of Azuma et al.
333 (2011) demonstrated that clofibrate, a PPAR α agonist, relaxes the LML of the mouse colon through a PPAR α -
334 independent mechanism.

335 **TRPA1**

336 TRPA1 is an ion channel that detects specific chemicals in food, and transduces mechanical, cold, and chemical
337 stimulation. Its presence in sensory neurons, also in cats (personal observation by Dr. R. Chiocchetti) is well
338 known, and evidence indicates that it is expressed by some enteric neurons (mainly inhibitory motoneurons;
339 Poole et al. 2011) and gastrointestinal enteroendocrine cells (EEC) (Cho et al. 2014). In the present study, we
340 observed enteric TRPA1-IR neurons. However, we did not characterize the phenotype of cat enteric TRPA1-
341 IR neurons. In addition, the immunoreactivity for this receptor was expressed by goblet cells, suggesting that
342 this receptor might directly influence the secretory function of these cells.

343 Our findings in cat are consistent with those obtained in rat.

344 **5-HT1a receptor**

345 The 5-HT1aR is expressed in a variety of CNS and enteric neurons (Kirchgessner et al. 1996). In the present
346 study, we did not observe a 5-HT1aR positivity in enteric neurons; however, the epithelial cells of the
347 gastrointestinal tract were brightly 5-HT1a immunolabelled. Serotonin plays a pivotal role in regulating gut
348 motility, visceral sensitivity, and fluid secretion via specific receptors. Among these receptors, it is probable
349 that, in the cat, 5-HT1a receptor may exert a role in cellular homeostasis and secretion. However, we did not
350 find similar data in the literature, with the exception of few studies carried out on transfected cultured kidney
351 epithelial cells (Langlois et al. 1996; Darmon et al. 1998).

352 Our findings in cat are partially consistent with those obtained in rat, in which 5-HT1aR immunoreactive
353 enteric neurons were also observed.

354 **Conclusion**

355 Our data show that, overall, “classic” and “new” cannabinoid receptors are widely distributed in the feline
356 digestive system, as we have recently demonstrated in the dog as well (Galiazzo et al. 2018). Due to their
357 localization, it seems plausible to consider that also in cats the endocannabinoid system is very important in
358 the control of intestinal functions such as the maintenance of epithelial barrier integrity, motor and sensory
359 activity, appetite, nausea, emesis, and the correct cellular microenvironment (Pertwee 2001; Uranga et al.
360 2018). These morphological findings, which should be supported by physiological or pharmacological
361 evidence, suggest that cannabinoid receptor agonists might have a therapeutic potential for controlling
362 gastrointestinal inflammatory conditions and visceral hypersensitivity in this species as well. The hypothesis
363 is supported by a great deal of evidence on the intestinal protective effects of the most studied naturally
364 occurring cannabinoid receptor ligands, CBD (Couch et al. 2017), and PEA (Borrelli et al. 2015).

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372 **Author information**

373 Agnese Stanzani and Giorgia Galiazzo contributed equally as co-first authors.

374 **Conflict of interest**

375 The authors declare that they have no conflict of interests.

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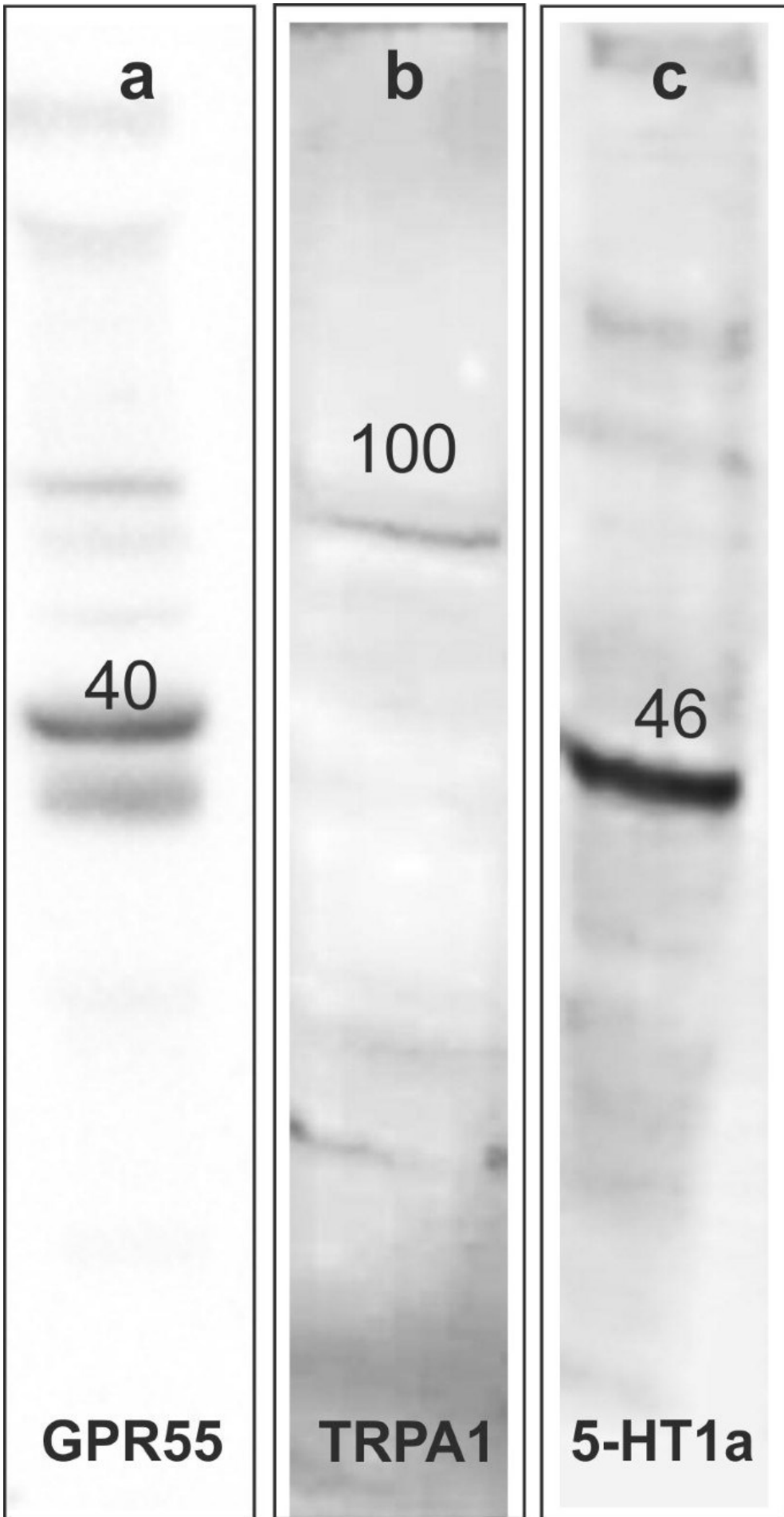
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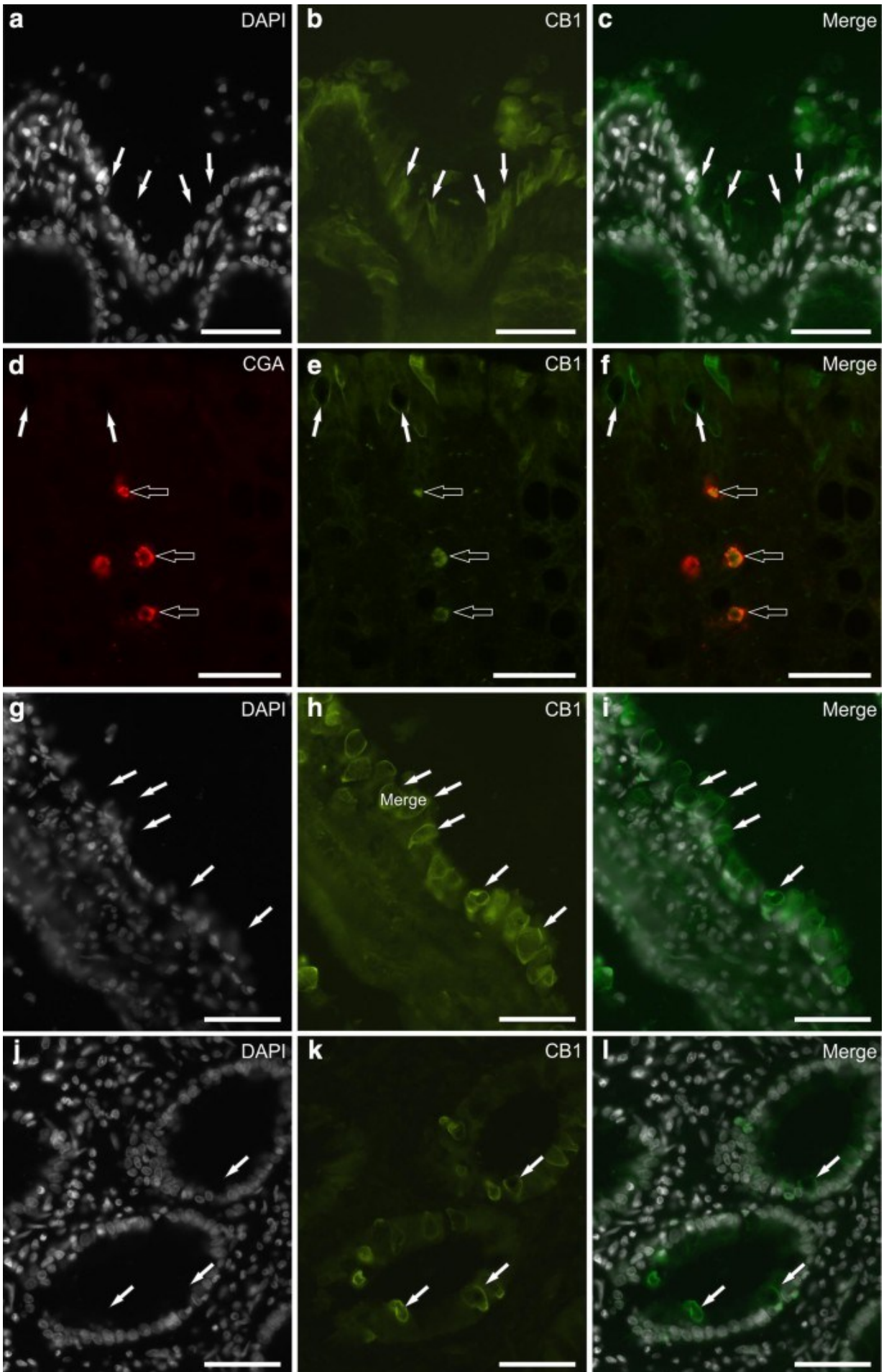
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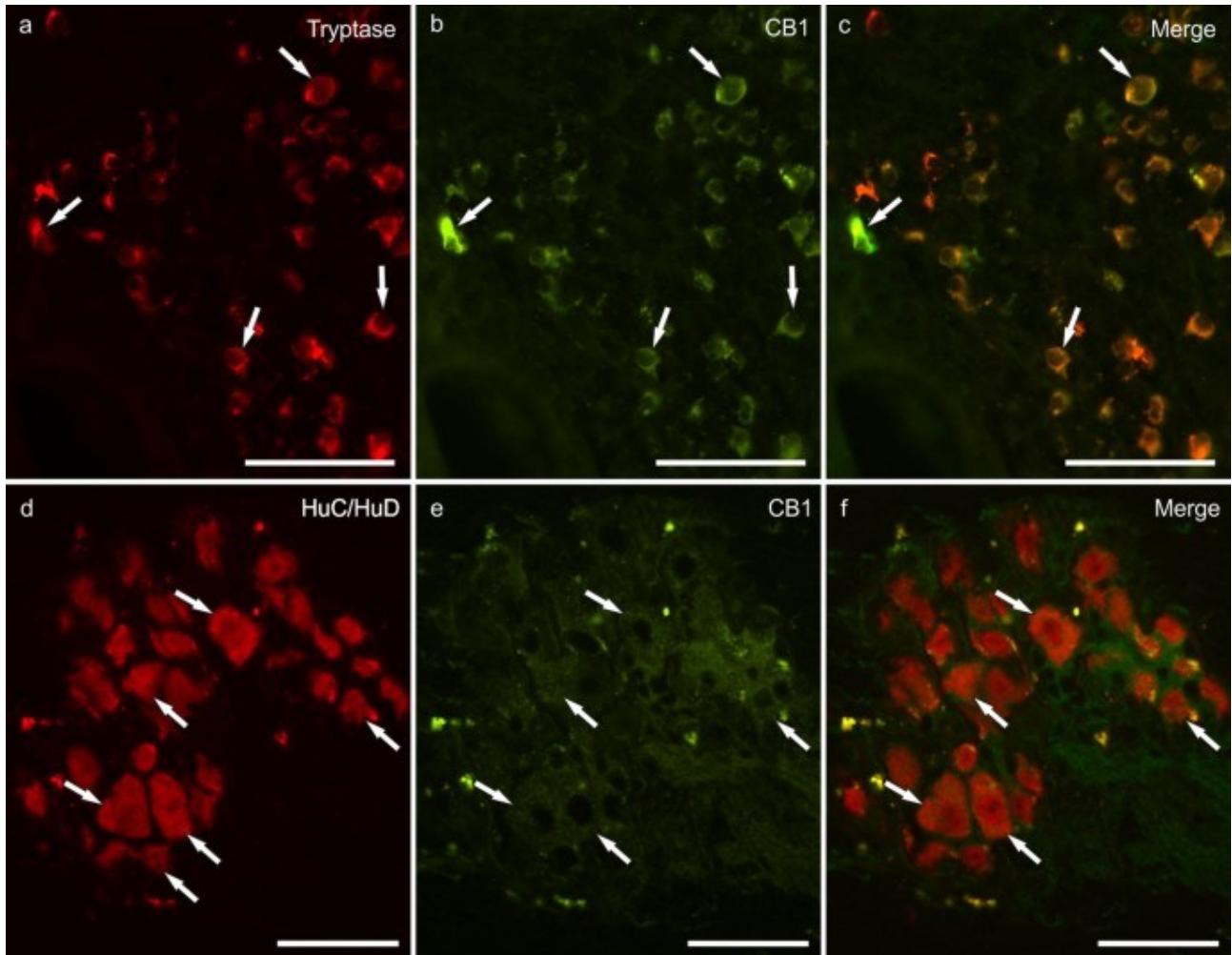
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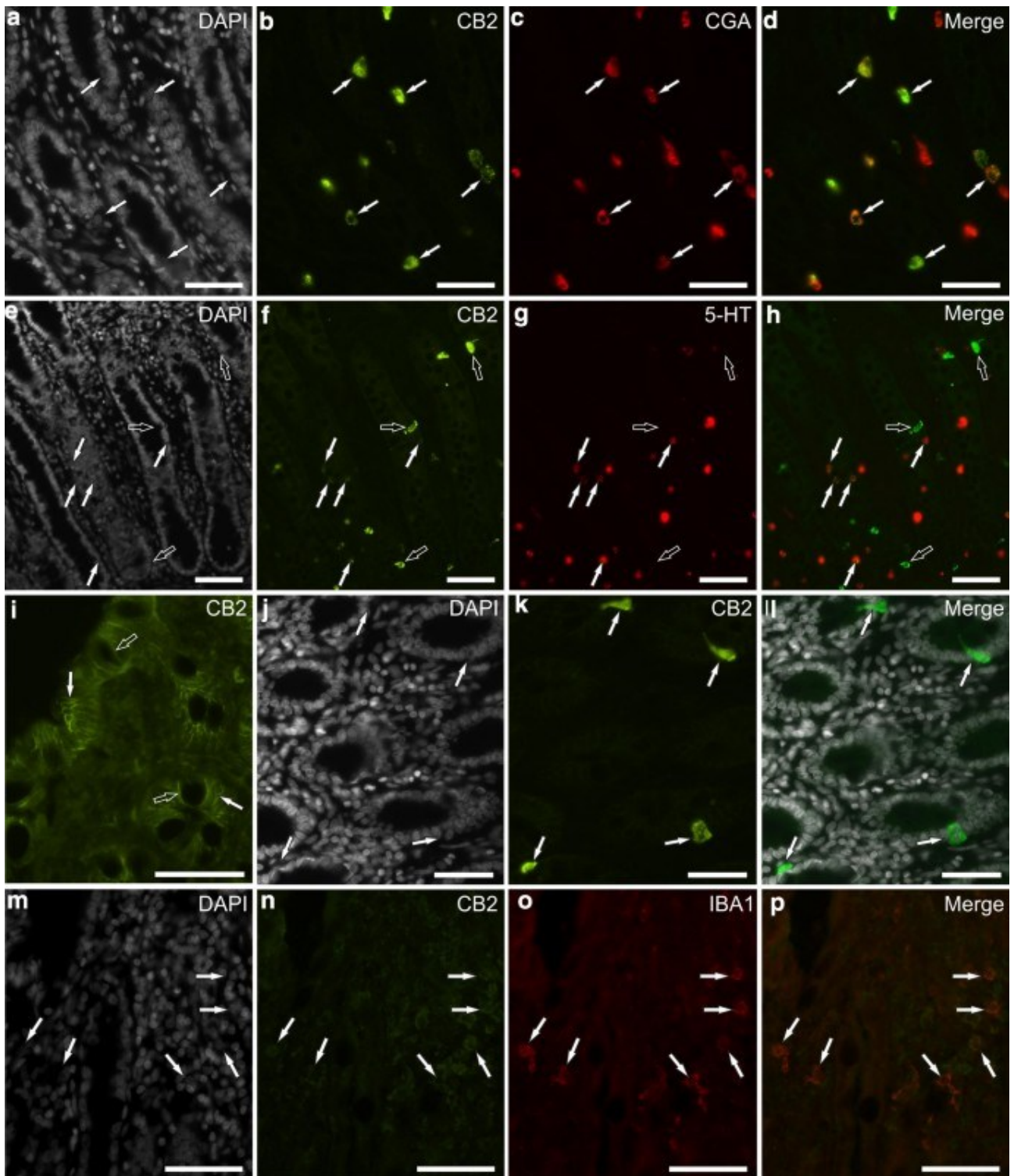
679 **FIG 1.** Western blot (Wb) analysis showing the specificity of the following primary antibodies utilized: a rabbit
680 anti-G protein-coupled receptor 55 (GPR55); b rabbit anti-transient receptor potential ankyrin 1 (TRPA1); c
681 rabbit anti-serotonin receptor 5-HT1a (5-HT1a); a the antibody anti-GPR55 showed a major band close to the
682 theoretical molecular weight (about 40 kDa). b The antibody anti-TRPA1 showed a major band close to the
683 theoretical molecular weight (100 kDa). c The antibody anti-5-HT1a receptor showed a major band close to
684 the theoretical molecular weight (46 kDa). The numbers on each line indicates the molecular weight. The
685 images of the immunoblots were slightly adjusted in brightness and contrast to match their backgrounds



687 FIG. 2. Photomicrograph showing cryosections of the cat gastrointestinal tract immunolabeled with the
 688 antibody anti-cannabinoid receptor 1 (CB1) (a–c). Arrows indicate some of the pyloric elongated mucosal
 689 cells, which show bright CB1 receptor immunoreactivity (d–f). Open arrows indicate three enteroendocrine
 690 cells of the colon identified with the antibody anti-chromogranin A (CGA) (c), co-expressing CB1 receptor
 691 immunoreactivity (e). White arrows indicate mucous goblet cells expressing CB1 receptor immunoreactivity.
 692 In f the merge image. (g–l) Arrows indicate some of the small intestine (g–i) and large intestine (j–l) mucous
 693 goblet cells expressing bright CB1 receptor immunoreactivity of the cell membrane. Scale bar: a–l, 50 μ m



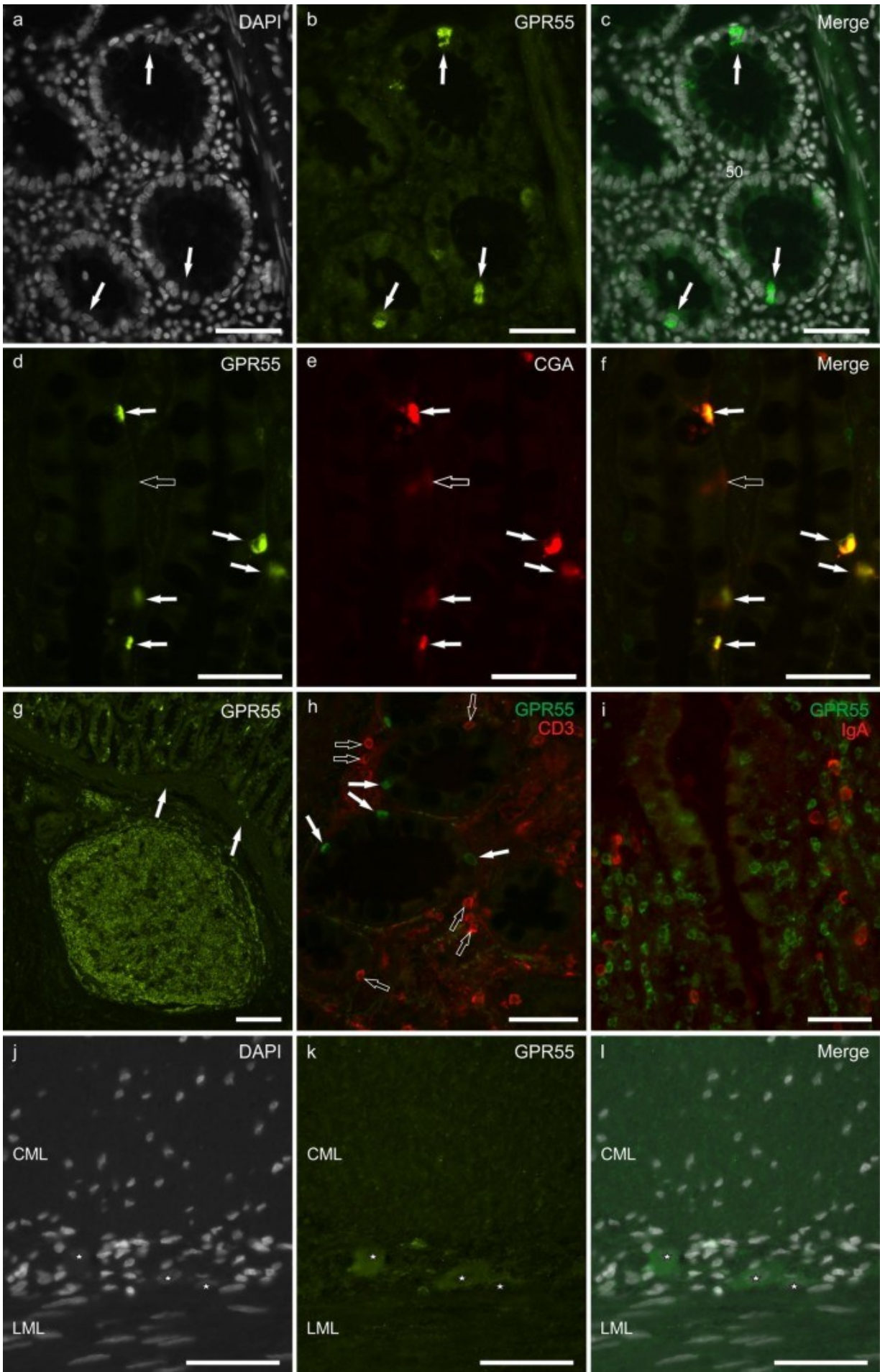
694
 695 FIG. 3. Photomicrograph showing cryosections of the cat gastrointestinal tract immunolabeled with the
 696 antibody anti-cannabinoid receptor 1 (CB1). Cellular nuclei were labelled with the nuclear stain DAPI. Arrows
 697 indicate four of the numerous lamina propria mast cells co-expressing tryptase (a) and bright CB1 receptor (b)
 698 immunoreactivity. In c the merge image. d–f HuC/HuD immunoreactive myenteric plexus neurons (arrows)
 699 showed weak-to-moderate granular cytoplasmic CB1 receptor immunoreactivity. In f the merge image. Scale
 700 bar: a–f, 50 μ m



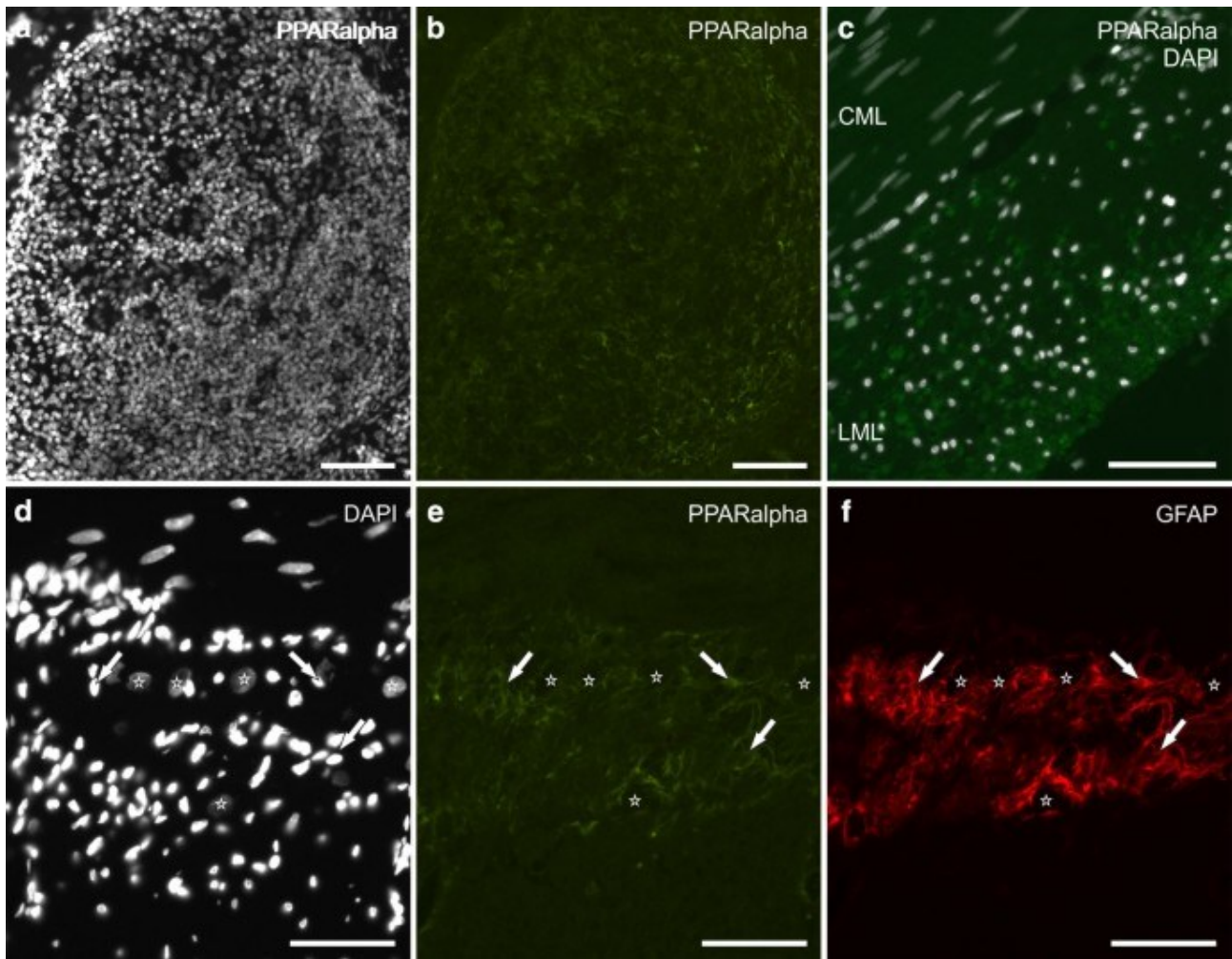
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702 FIG. 4. a–p Photomicrograph showing cryosections of cat intestinal tract immunolabelled with the antibody
 703 anti-cannabinoid receptor 2 (CB2). Cellular nuclei were labelled with the nuclear stain DAPI. a–d Arrows
 704 indicate some of the numerous enteroendocrine cells identified with the antibody anti-chromogranin A (CGA)
 705 (c) co-expressing bright CB2 receptor immunoreactivity (b). In d the merge image. e–h Arrows indicate some
 706 of the numerous enteroendocrine cells identified with the antibody anti-serotonin (5-HT) (g) co-expressing
 707 bright CB2 receptor immunoreactivity (f). Open arrows indicate CB2 receptor immunoreactive cells which
 708 were 5-HT negative. In h the merge image. i In the colon, CB2 receptor immunoreactivity was expressed by
 709 the cell membrane of crypts epithelial cells (white arrows) and goblet cells (open arrows). j–l Arrows indicate
 710 bright CB2 receptor immunolabelling (k) of the enteroendocrine cells of the colon. m–p The lamina propria

711 macrophages, recognized for their IBA1 immunoreactivity, co-expressed moderate CB2 immunolabelling
712 (arrows). In p the merge image. Scale bar: a-p, 50 μ m

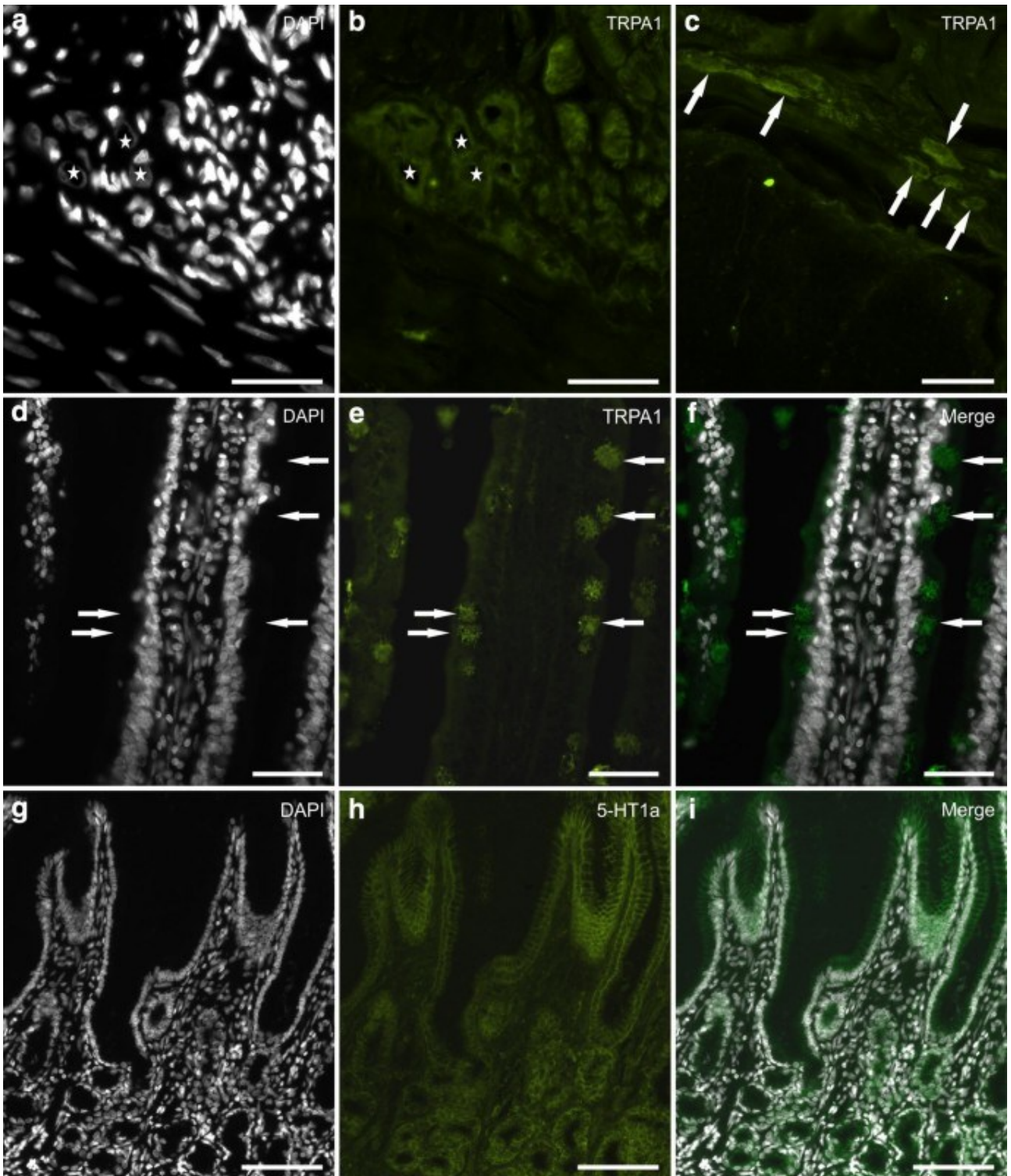


714 FIG. 5. Photomicrograph showing cryosections of cat small and large intestine immunolabelled with the
 715 antibody anti-GPR55. Cellular nuclei were labelled with the nuclear stain DAPI. a–c Arrows indicate bright
 716 GPR55 immunolabelling (b) of the enteroendocrine cells of the colon. d–e Arrows indicate enteroendocrine
 717 cells of the colon co-expressing GPR55 (d) and chromogranin A (CGA) (e) immunoreactivity; the open arrow
 718 indicates a CGA immunoreactive cell which was GPR55 negative. In f the merge image. g–h Intestinal
 719 lymphatic nodules in which a large number of immunocytes showed bright GPR55 immunoreactivity. The
 720 arrows indicate the muscularis mucosae. i Lamina propria IgA immunoreactive plasma cells (red color) did not
 721 co-express GPR55 immunoreactivity (green color). j–l Gastrointestinal subsets of myenteric plexus neurons
 722 expressed moderate GPR55 immunoreactivity. The white stars indicate three neuronal nuclei. CML circular
 723 muscle layer, LML longitudinal muscle layer. Scale bar: a–f, h–l, 50 μ m; g, 100 μ m



724

725 FIG. 6. Photomicrograph showing cryosections of cat intestine immunolabelled with the antibody anti-PPAR α
 726 (a–f). Cellular nuclei were labelled with the nuclear stain DAPI. a, b Intestinal lymph node in which a large
 727 percentage of immunocytes showed PPAR α immunoreactivity. c PPAR α immunoreactivity was observed also
 728 in the smooth muscle cells of the longitudinal muscle layer (LML). d–f Stars indicate the nucleus of some
 729 myenteric plexus neurons. Arrows indicate the nuclei of three GFAP immunoreactive glial cells (i) which co-
 730 expressed PPAR α immunoreactivity. CML circular muscle layer. Scale bar: a–f, 50 μ m



731

732 FIG. 7. Photomicrograph showing cryosections of cat gastrointestinal tract immunolabelled with the antibody
 733 anti-TRPA1 (a–f) and 5-HT1a receptor (g–i). Cellular nuclei were labelled with the nuclear stain DAPI. a, b
 734 Stars indicate three nuclei of the pyloric myenteric plexus neurons which showed TRPA1 immunoreactivity. c
 735 Arrows indicate duodenal myenteric plexus TRPA1 immunoreactive neurons. d–f Arrows indicate small
 736 intestinal goblet cells which showed bright TRPA1 immunoreactivity. g–i Pyloric mucosa in which mucous
 737 and glandular cells expressed 5-HT1a receptor immunoreactivity. Scale bar: a–i, 50 μ m

738

739

740 **Table 1 – Animals included in the study**

Animals	Age	Sex	Breed	Cause of death
#Ctrl1	13 yrs	M	Half-breed	Oral neoplasia
#Ctrl2	14 yrs	M	Half-breed	Iliac thrombosis
#Ctrl3	6 yrs	M	Half-breed	Head trauma (car accident)
#Ctrl4	4 yrs	M	Half-breed	Head trauma (car accident)
#Ctrl5	3 yrs	F	Half-breed	Urethral injury (car accident)

741

742

743 **Table 2 - Primary antibodies used in the study**

Primary antibody	Host	Code	Dilution	Source
CB1	Rabbit	Orb10430	1:200	Biorbyt
CB1	Rabbit	ab23703	1:100	abcam
CB2	Rabbit	ab45942	1:200	abcam
GAS/CCK	Mouse	CURE/CCD	1:1000	CURE/CCD
Chromogranin A	Mouse	MON9014	1:200	Monosan
Chymase	Goat	NBP2-27551	1:50	Novus Biol.
GFAP	Chicken	AB4674	1:800	abcam
GPR55	Rabbit	NB110-55498	1:200	Novus Biol.
IBA1	Goat	NB100-1028	1:80	Novus Biol.
IgA	Rabbit	A80-103A	1:1000	Bethyl Lab.
IgA	Goat	NB724	1:1000	Novus Biol.
PPAR α	Rabbit	NB600-636	1:200	Novus Biol.
Serotonin	Mouse	Ab16007; # 5HT-H209	1:500	abcam
5-HT1a receptor	Rabbit	ab85615	1:100	abcam
TRPA1	Rabbit	ab58844	1:100	abcam
Tryptase	Mouse	M 7052; #AA1	1:200	Dako

744

745 **Primary antibodies Suppliers:** abcam, Cambridge, UK; Bethyl Laboratories, Montgomery, TX,
746 USA; Biorbyt Ltd., Cambridge, UK; Dako Cytomation, Golstrup, Denmark; Monosan, Uden, The
747 Netherlands; Novus Biologicals, Littleton, CO, USA.

748

749

750 **Table 3 - Secondary antibodies used in the study**

Secondary antibody	Host	Code	Dilution	Source
Anti-mouse F(ab') ₂ fragment TRITC	Goat	Ab51379	1:50	abcam
Anti-rabbit F(ab') ₂ fragment FITC	Goat	98430	1:300	abcam
Anti-goat IgG 594	Donkey	AB150132	1:600	abcam
Anti-chicken TRITC	Donkey	703-025-155	1:200	Jackson
Anti-rat Alexa 594	Donkey	A-21209	1:50	Thermo Fisher

751 **Secondary antibodies Suppliers:** abcam, Cambridge, UK; Jackson Immuno Research
752 Laboratories, Inc. Baltimore Pike, PA, USA. Thermo Fisher Scientific, Waltham, MA USA.
753