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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

Material and methods

Animals

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

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

Tissue collection

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

Immunofluorescence

Cryosections were hydrated in 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), 0.5% Triton X-100 (Sigma Aldrich, Milan, Italy, Europe), and bovine serum albumin (1%) in PBS for 1 h at room temperature (RT). The cryosections were incubated in a humid chamber overnight at RT with the antibodies directed against the cannabinoid and cannabinoid-related receptors (single immunostaining) or a cocktail of primary antibodies (double immunostaining) (Table 2) diluted in 1.8% NaCl in 0.01 M PBS containing 0.1% sodium azide. After washing the sections in PBS (3 × 10 min), they were incubated for 1 h at RT in a humid chamber with the secondary antibodies (Table 3) diluted in PBS. Cryosections were then washed in PBS (3 × 10 min) and mounted in buffered glycerol at pH 8.6 with 4,6-diamidino-2-phenylindole—DAPI (Santa Cruz Biotechnology, CA, USA).

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

Specificity of the primary antibodies

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

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

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

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

The antibody anti-5-HT1a receptor (5-HT1aR) was raised using, as immunogen, a synthetic peptide corresponding to amino acids 100–200 (conjugated to keyhole limpet haemocyanin) of rat 5-HT1aR. The 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-HT1aR antibody was tested in the present study by Wb analysis (Fig. 1c).

In addition, since the suppliers of the primary antibodies anti-TRPA1 and -5-HT1aR employed in the present study state them to rat-specific, rat ileum, and colon were used as control tissues for comparison purposes (authorization no. 112/2018-PR of 12 February 2018). The supplier of the primary antibody anti-GPR55

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

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

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

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

Fluorescence microscopy

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

Western blot: specificity of the primary antibodies

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

Results

CB1 receptor immunoreactivity

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

CB2 receptor immunoreactivity

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

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

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

GPR55 immunoreactivity

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

PPARα immunoreactivity

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

TRPA1 immunoreactivity

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

5-HT1a receptor immunoreactivity

5-HT1aR-IR was expressed by the cell membrane (latero-basal) of gastrointestinal epithelial cells (Fig. 7g–i). Bright 5-HT1aR-IR was also observed around the nucleus of the epithelial cells. 5-HT1aR-IR was observed in gastric smooth muscle cells of the tunica muscularis, muscularis mucosae and submucosal blood vessels (data not shown). In the rat ileum and colon, faint 5-HT1aR-IR was expressed by epithelial cells and enteric neurons (Supplementary Fig. 1g–l).

Discussion

CB1 receptor

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

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

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

CB2 receptor

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

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

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

GPR55

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

A large number of cat lamina propria and Peyer's patches immunocytes showed bright GRP55-IR. In other species, GPR55 has already been identified in a large number of cell types, such as macrophages, plasma cells, neutrophils, natural killer cells, monocytes, or lymphatic cells (T cells) (Balenga et al. 2011; Stancic et al. 2015; Chiurchiù et al. 2015; Taylor et al. 2015; Lanuti et al. 2015; Galiazzo et al. 2018; Grill et al. 2019). Data related to the role played by GPR55 during intestinal inflammation is controversial; in fact, its proinflammatory nature has also been hypothesized, since administration of GPR55 antagonists or inverse agonists seems to reduce intestinal inflammation and decrease pro-inflammatory cytokines and leukocyte recruitment (Stancic et al. 2015; Tuduri et al. 2017; Grill et al. 2019). The evidence of bright GPR55 immunolabelling of gastrointestinal EECs, indicates that this receptor may play a role in the secretory function of these endocrine cells. It has been shown that the activation of other specific G protein coupled receptors expressed on EECs (e.g. GPR41, GPR43, GPR119 and TGR5) triggers the secretion of glucagon-like peptides (GLP-1 and GLP-2) and PYY, gut peptides which are known to control energy homeostasis, glucose metabolism, gut barrier function and metabolic inflammation (Cani et al. 2013). GPR55 plays an important role in the regulation of mouse and human islet physiology (Liu et al. 2016), it is thus plausible that GPR55 on EECs may influence gut hormone secretion.

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

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

PPARα

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

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

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

TRPA1

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

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

5-HT1a receptor

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

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

Conclusion

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

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Conflict of interest

The authors declare that they have no conflict of interests.

References

Abraham, S.N., St John, A.L., 2010. Mast cell-orchestrated immunity to pathogens. Nat Rev Immunol 10. https://doi.org/10.1038/nri2782

Acharya, N., Penukonda, S., Shcheglova, T., Hagymasi, A.T., Basu, S., Srivastava, P.K., 2017. Endocannabinoid system acts as a regulator of immune homeostasis in the gut. Proc Natl Acad Sci USA 114. https://doi.org/10.1073/pnas.1612177114

Adami, M., Frati, P., Bertini, S., Kulkarni-Narla, A., Brown, D.R., Caro, G., Coruzzi, G., Soldani, G., 2002. Gastric antisecretory role and immunohistochemical localization of cannabinoid receptors in the rat stomach. Br J Pharmacol 135. https://doi.org/10.1038/sj.bjp.0704625

Ambrosino, P., Soldovieri, M.V., Russo, C., Taglialatela, M., 2013. Activation and desensitization of TRPV1 channels in sensory neurons by the PPARα agonist palmitoylethanolamide. Br J Pharmacol 168. https://doi.org/10.1111/bph.12029

Azuma, Y.T., Nishiyama, K., Matsuo, Y., Kuwamura, M., Morioka, A., Nakajima, H., Takeuchi, T., 2010. PPARα contributes to colonic protection in mice with DSS-induced colitis. Int Immunopharmacol 10. https://doi.org/10.1016/j.intimp.2010.07.007

Azuma, Y.T., Nishiyama, K., Morioka, A., Nakajima, H., Takeuchi, T., 2011. Clofibrate relaxes the longitudinal smooth muscle of the mouse distal colon through calcium-mediated desensitisation of contractile machinery. Pharmacology 88. https://doi.org/10.1159/000329418

Bado, A., Durieux, C., Moizo, L., Roques, B.P., Lewin, M.J., 1991. Cholecystokinin-A receptor mediation of food intake in cats. Am J Physiol 260. https://doi.org/10.1152/ajpregu.1991.260.4.R693

Balenga, N.A., Aflaki, E., Kargl, J., Platzer, W., Schröder, R., Blattermann, S., Kostenis, E., Brown, A.J., Heinemann, A., Waldhoer, M., 2011. GPR55 regulates cannabinoid 2 receptor-mediated responses in human neutrophils. Cell Res 21. https://doi.org/10.1038/cr.2011.60

Bednarska, O., Walter, S.A., Casado-Bedmar, M., Ström, M., Salvo-Romero, E., Vicario, M., Mayer, E.A., Keita, Å.V., 2017. Vasoactive intestinal polypeptide and mast cells regulate increased passage of colonic bacteria in patients with irritable bowel syndrome. Gastroenterology 153. https://doi.org/10.1053/j.gastro.2017.06.051

Bischoff, S.C., Krämer, S., 2007. Human mast cells, bacteria, and intestinal immunity. Immunol Rev 217. https://doi.org/10.1111/j.1600-065X.2007.00523.x

Boeckxstaens, G., 2015. Mast cells and inflammatory bowel disease. Curr Opin Pharmacol 25. https://doi.org/10.1016/j.coph.2015.11.005

Borrelli, F., Romano, B., Petrosino, S., Pagano, E., Capasso, R., Coppola, D., Battista, G., Orlando, P., Marzo, V., Izzo, A.A., 2015. Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal anti-inflammatory agent. Br J Pharmacol 172. https://doi.org/10.1111/bph.12907

Brown AJ, Ueno S, Suen K, Dowell SJ, Wise A (2005) Molecular identification of GPR55 as a third G protein-coupled receptor responsive to cannabinoid ligands. In: Brian T (ed). Symposium on the Cannabinoids. International Cannabinoid Research Society, Burlington, VT, pp 24–27, n.d.

Cani, P.D., Everard, A., Duparc, T., 2013. Gut microbiota, enteroendocrine functions and metabolism. Curr Opin Pharmacol 13. https://doi.org/10.1016/j.coph.2013.09.008

Capasso, R., Borrelli, F., Aviello, G., Romano, B., Scalisi, C., Capasso, F., Izzo, A.A., 2008. Cannabidiol, extracted from Cannabissativa, selectively inhibits inflammatory hypermotility in mice. Br J Pharmacol 154. https://doi.org/10.1038/bjp.2008.177

Carlo, G., Izzo, A.A., 2003. Cannabinoids for gastrointestinal diseases: potential therapeutic applications. Expert Opin Investig Drugs 12. https://doi.org/10.1517/13543784.12.1.39

Chiocchetti, R., Giancola, F., Mazzoni, M., Sorteni, C., Romagnoli, N., Pietra, M., 2015. Excitatory and inhibitory enteric innervation of horse lower esophageal sphincter. Histochem Cell Biol 143. https://doi.org/10.1007/s00418-014-1306-y

Chiurchiù, V., Lanuti, M., Bardi, M., Battistini, L., Maccarrone, M., 2015. The differential characterization of GPR55 receptor in human peripheral blood reveals a distinctive expression in monocytes and NK cells and a proinflammatory role in these innate cells. Int Immunol 27. https://doi.org/10.1093/intimm/dxu097

Cho, H.J., Callaghan, B., Bron, R., Bravo, D.M., Furness, J.B., 2014. Identification of enteroendocrine cells that express TRPA1 channels in the mouse intestine. Cell Tissue Res 356. https://doi.org/10.1007/s00441-013-1780-x

Cirillo, C., Sarnelli, G., Turco, F., Mango, A., Grosso, M., Aprea, G., Masone, S., Cuomo, R., 2011. Proinflammatory stimuli activates human-derived enteroglial cells and induces autocrine nitric oxide production. Neurogastroenterol Motil 23. https://doi.org/10.1111/j.1365-2982.2011.01748.x

Couch, D.G., Tasker, C., Theophilidou, E., Lund, J.N., O'Sullivan, S.E., 2017. Cannabidiol and palmitoylethanolamide are anti-inflammatory in the acutely inflamed human colon. Clin Sci 131. https://doi.org/10.1042/CS20171288

Coutts, A.A., Irving, A.J., Mackie, K., Pertwee, R.G., Anavi-Goffer, S., 2002. Localisation of cannabinoid CB(1) receptor immunoreactivity in the guinea pig and rat myenteric plexus. J Comp Neurol 448. https://doi.org/10.1002/cne.10270

Cremon, C., Stanghellini, V., Barbaro, M.R., Cogliandro, R.F., Bellacosa, L., Santos, J., Vicario, M., Pigrau, M., Alonso Cotoner, C., Lobo, B., Azpiroz, F., Bruley des Varannes, S., Neunlist, M., DeFilippis, D., Iuvone, T., Petrosino, S., Marzo, V., Barbara, G., 2017. Randomised clinical trial: the analgesic properties of dietary supplementation with palmitoylethanolamide and polydatin in irritable bowel syndrome. Aliment Pharmacol Ther 45. https://doi.org/10.1111/apt.14014

Darmon, M., Langlois, X., Suffisseau, L., Fattaccini, C.M., Hamon, M., 1998. Differential membrane targeting and pharmacological characterization of chimeras of rat serotonin 5-HT1A and 5-HT1B receptors expressed in epithelial LLC-PK1 cells. J Neurochem 71. https://doi.org/10.1046/j.1471-4159.1998.71062294.x

Duncan, M., Davison, J.S., Sharkey, K.A., 2005. Review article: endocannabinoids and their receptors in the enteric nervous system. Aliment Pharmacol Ther 22. https://doi.org/10.1111/j.1365-2036.2005.02648.x

Duncan M, Ho W, Shariat N, Pittman QJ, Mackie K, Patel KD, Sharkey KA (2005b) Distribution of the CB2 receptor in enteric nerves of the rat ileum. In: Symposium on the Cannabinoids, Burlington, VT, International Cannabinoid Research Society, p 159, n.d.

Duncan, M., Mouihate, A., Mackie, K., Keenan, C.M., Buckley, N.E., Davison, J.S., Patel, K.D., Pittman, Q.J., Sharkey, K.A., 2008. Cannabinoid CB2 receptors in the enteric nervous system modulate gastrointestinal contractility in lipopolysaccharide-treated rats. Am J Physiol Gastrointest Liver Physiol 295. https://doi.org/10.1152/ajpgi.90285.2008

Escher, P., Braissant, O., Basu-Modak, S., Michalik, L., Wahli, W., Desvergne, B., 2001. Rat PPARs: quantitative analysis in adult rat tissues and regulation in fasting and refeeding. Endocrinology 142. https://doi.org/10.1210/endo.142.10.8458

Esposito, G., Capoccia, E., Turco, F., Palumbo, I., Lu, J., Steardo, A., Cuomo, R., Sarnelli, G., Steardo, L., 2014. Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR-α activation. Gut 63. https://doi.org/10.1136/gutjnl-2013-305005

Fabisiak, A., Fichna, J., 2017. Cannabinoids as gastrointestinal anti-inflammatory drugs. Neurogastroenterol Motil 29. https://doi.org/10.1111/nmo.13038

Facci, L., Toso, R., Romanello, S., Buriani, A., Skaper, S.D., Leon, A., 1995. Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. Proc Natl Acad Sci USA 92. https://doi.org/10.1073/pnas.92.8.3376

Farquhar-Smith, W., Jaggar, S., Rice, A., 2002. Attenuation of nerve growth factor-induced visceral hyperalgesia via cannabinoid CB1 and CB2-like receptors. Pain 97. https://doi.org/10.1016/s0304-3959(01)00419-5

Gabrielsson, L., Mattsson, S., Fowler, C.J., 2016. Palmitoylethanolamide for the treatment of pain: pharmacokinetics, safety and efficacy. Br J Clin Pharmacol 82. https://doi.org/10.1111/bcp.13020

Galiazzo, G., Giancola, F., Stanzani, A., Fracassi, F., Bernardini, C., Forni, M., Pietra, M., Chiocchetti, R., 2018. Localization of cannabinoid receptors CB1, CB2, GPR55 and PPARα in the canine gastrointestinal tract. Histochem Cell Biol 150. https://doi.org/10.1007/s00418-018-1684-7

Giancola, F., Fracassi, F., Gallucci, A., Sadeghinezhad, J., Polidoro, G., Zini, E., Asti, M., Chiocchetti, R., 2016. Quantification of nitrergic neurons in the myenteric plexus of gastric antrum and ileum of healthy and diabetic dogs. Auton Neurosci 197. https://doi.org/10.1016/j.autneu.2016.04.004

Goyal, H., Singla, U., Gupta, U., May, E., 2017. Role of cannabis in digestive disorders. Eur J Gastroenterol Hepatol 29. https://doi.org/10.1097/MEG.0000000000000779

Grill, M., Hasenoehrl, C., Kienzl, M., Kargl, J., Schicho, R., 2019. Cellular localization and regulation of receptors and enzymes of the endocannabinoid system in intestinal and systemic inflammation. Histochem Cell Biol 151. https://doi.org/10.1007/s00418-018-1719-0

Gruden, G., Barutta, F., Kunos, G., Pacher, P., 2016. Role of the endocannabinoid system in diabetes and diabetic complications. Br J Pharmacol 173. https://doi.org/10.1111/bph.13226

Gyires, K., Zádori, Z.S., 2016. Role of cannabinoids in gastrointestinal mucosal defense and inflammation. Curr Neuropharmacol 14. https://doi.org/10.2174/1570159x14666160303110150

Harvey, B.S., Nicotra, L.L., Vu, M., Smid, S.D., 2013. Cannabinoid CB2 receptor activation attenuates cytokine-evoked mucosal damage in a human colonic explant model without changing epithelial permeability. Cytokine 63. https://doi.org/10.1016/j.cyto.2013.04.032

Harvima, I.T., Levi-Schaffer, F., Draber, P., Friedman, S., Polakovicova, I., Gibbs, B.F., Blank, U., Nilsson, G., Maurer, M., 2014. Molecular targets on mast cells and basophils for novel therapies. J Allergy Clin Immunol 134. https://doi.org/10.1016/j.jaci.2014.03.007

Hornby, P.J., Prouty, S.M., 2004. Involvement of cannabinoid receptors in gut motility and visceral perception. Br J Pharmacol 141. https://doi.org/10.1038/sj.bjp.0705783

494 Hu, S.S., Mackie, K., 2015. Distribution of the endocannabinoid system in the central nervous system. Handb
495 Exp Pharmacol 231. https://doi.org/10.1007/978-3-319-20825-1_3

496 Iannotti, F.A., Hill, C.L., Leo, A., Alhusaini, A., Soubrane, C., Mazzarella, E., Russo, E., Whalley, B.J., Marzo,
497 V., Stephens, G.J., 2014. Nonpsychotropic plant cannabinoids, cannabidivarin (CBDV) and cannabidiol
498 (CBD), activate and desensitize transient receptor potential vanilloid 1 (TRPV1) channels in vitro: potential
499 for the treatment of neuronal hyperexcitability. ACS Chem Neurosci 5. https://doi.org/10.1021/cn5000524

500 Iannotti, F.A., Marzo, V., Petrosino, S., 2016. Endocannabinoids and endocannabinoid-related mediators:
501 targets, metabolism and role in neurological disorders. Prog Lipid Res 62.
502 https://doi.org/10.1016/j.plipres.2016.02.002

503 Ihenetu, K., Molleman, A., Parsons, M.E., Whelan, C.J., 2003. Inhibition of interleukin-8 release in the human
504 colonic epithelial cell line HT-29 by cannabinoids. Eur J Pharmacol 458. https://doi.org/10.1016/S0014-
505 2999(02)02698-5

506 Izzo, A.A., 2004. Cannabinoids and intestinal motility: welcome to CB2 receptors. Br J Pharmacol 142.
507 https://doi.org/10.1038/sj.bjp.0705890

508 Izzo, A.A., Sharkey, K.A., 2010. Cannabinoids and the gut: new developments and emerging concepts.
509 Pharmacol Ther 126. https://doi.org/10.1016/j.pharmthera.2009.12.005

510 Jaggar, S.I., Sellaturay, S., Rice, A., 1998. The endogenous cannabinoid anandamide, but not the CB2 ligand
511 palmitoylethanolamide, prevents the viscero-visceral hyperreflexia associated with inflammation of the rat
512 urinary bladder. Neurosci Lett 253. https://doi.org/10.1016/S0304-3940(98)00621-1

513 Karwad, M.A., Couch, D.G., Theophilidou, E., Sarmad, S., Barrett, D.A., Larvin, M., Wright, K.L., Lund, J.N.,
514 O'Sullivan, S.E., 2017. The role of CB1 in intestinal permeability and inflammation. FASEB J 31.
515 https://doi.org/10.1096/fj.201601346R

516 Ke, P., Shao, B.Z., Xu, Z.Q., Wei, W., Han, B.Z., Chen, X.W., Su, D.F., Liu, C., 2016. Activation of cannabinoid
517 receptor 2 ameliorates DSS-induced colitis through inhibiting NLRP3 inflammasome in macrophages. PLoS
518 ONE 11. https://doi.org/10.1371/journal.pone.0155076

519 Kirchgessner, A.L., Liu, M.T., Raymond, J.R., Gershon, M.D., 1996. Identification of cells that express 5-
520 hydroxytryptamine1A receptors in the nervous systems of the bowel and pancreas. J Comp Neurol 364.
521 https://doi.org/10.1002/(SICI)1096-9861(19960115)364:3%3c439::AID-CNE5%3e3.0.CO;2-5

522 Kleinschmidt, S., Harder, J., Nolte, I., Marsilio, S., Hewicker-Trautwein, M., 2010. Phenotypical
523 characterization, distribution and quantification of different mast cell subtypes in transmural biopsies from the
524 gastrointestinal tract of cats with inflammatory bowel disease. Vet Immunol Immunopathol 137.
525 https://doi.org/10.1016/j.vetimm.2010.05.005

526 Kulkarni-Narla, A., Brown, D.R., 2000. Localization of CB1-cannabinoid receptor immunoreactivity in the
527 porcine enteric nervous system. Cell Tissue Res 302. https://doi.org/10.1007/s004410000261

528 Langlois, X., Mestikawy, S., Arpin, M., Triller, A., Hamon, M., Darmon, M., 1996. Differential addressing of
529 5-HT1A and 5-HT1B receptors in transfected LLC-PK1 epithelial cells: a model of receptor targeting in
530 neurons. Neuroscience 74. https://doi.org/10.1016/0306-4522(96)00234-5

531 Lanuti, M., Talamonti, E., Maccarrone, M., Chiurchiù, V., 2015. Correction: activation of GPR55 receptors
532 exacerbates oxLDL-induced lipid accumulation and inflammatory responses, while reducing cholesterol efflux
533 from human macrophages. PLoS ONE 10. https://doi.org/10.1371/journal.pone.0131850

534 Lauckner, J.E., Jensen, J.B., Chen, H.Y., Lu, H.C., Hille, B., Mackie, K., 2008. GPR55 is a cannabinoid
535 receptor that increases intracellular calcium and inhibits M current. Proc Natl Acad Sci USA 105.
536 https://doi.org/10.1073/pnas.0711278105

Laun, A.S., Shrader, S.H., Brown, K.J., Song, Z.H., 2019. GPR3, GPR6, and GPR12 as novel molecular targets: their biological functions and interaction with cannabidiol. Acta Pharmacol Sin 40. https://doi.org/10.1038/s41401-018-0031-9

Laun, A.S., Song, Z.H., 2017. GPR3 and GPR6, novel molecular targets for cannabidiol. Biochem Biophys Res Commun 490. https://doi.org/10.1016/j.bbrc.2017.05.165

Lee, Y., Jo, J., Chung, H.Y., Pothoulakis, C., Im, E., 2016. Endocannabinoids in the gastrointestinal tract. Am J Physiol Gastrointest Liver Physiol 311. https://doi.org/10.1152/ajpgi.00294.2015

Li, C., Bowe, J.E., Jones, P.M., Persaud, S.J., 2010. Expression and function of cannabinoid receptors in mouse islets. Islets 2. https://doi.org/10.4161/isl.2.5.12729

Li, K., Fichna, J., Schicho, R., Saur, D., Bashashati, M., Mackie, K., Li, Y., Zimmer, A., Göke, B., Sharkey, K.A., Storr, M., 2013. A role for O-1602 and G protein-coupled receptor GPR55 in the control of colonic motility in mice. Neuropharmacology 71. https://doi.org/10.1016/j.neuropharm.2013.03.029

Ligresti, A., Petrocellis, L., Marzo, V., 2016. From phytocannabinoids to cannabinoid receptors and endocannabinoids: pleiotropic physiological and pathological roles through complex pharmacology. Physiol Rev 96. https://doi.org/10.1152/physrev.00002.2016

Lin, X.H., Yuece, B., Li, Y.Y., Feng, Y.J., Feng, J.Y., Yu, L.Y., Li, K., Li, Y.N., Storr, M., 2011. A novel CB receptor GPR55 and its ligands are involved in regulation of gut movement in rodents. Neurogastroenterol Motil 23. https://doi.org/10.1111/j.1365-2982.2011.01742.x

Liu, B., Song, S., Ruz-Maldonado, I., Pingitore, A., Huang, G.C., Baker, D., Jones, P.M., Persaud, S.J., 2016. GPR55-dependent stimulation of insulin secretion from isolated mouse and human islets of Langerhans. Diabetes Obes Metab 18. https://doi.org/10.1111/dom.12780

Liu, Y.A., Chung, Y.C., Pan, S.T., Shen, M.Y., Hou, Y.C., Peng, S.J., Pasricha, P.J., Tang, S.C., 2013. 3-D imaging, illustration, and quantitation of enteric glial network in transparent human colon mucosa. Neurogastroenterol Motil 25. https://doi.org/10.1111/nmo.12115

Lo Verme, J., Fu, J., Astarita, G., Rana, G., Russo, R., Calignano, A., Piomelli, D., 2005a. The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. Mol Pharmacol 67. https://doi.org/10.1124/mol.104.006353

Lo Verme, J., Rana, G., Russo, R., Calignano, A., Piomelli, D., 2005b. The search for the palmitoylethanolamide receptor. Life Sci 77. https://doi.org/10.1016/j.lfs.2005.05.012

Marquéz, L., Suárez, J., Iglesias, M., Bermudez-Silva, F.J., Rodríguez de Fonseca, F., Andreu, M., 2009. Ulcerative colitis induces changes on the expression of the endocannabinoid system in the human colonic tissue. PLoS ONE 4. https://doi.org/10.1371/journal.pone.0006893

Marshall, J.S., 2004. Mast-cell responses to pathogens. Nat Rev Immunol 4. https://doi.org/10.1038/nri1460

Marzo, V., Izzo, A.A., 2006. Endocannabinoid overactivity and intestinal inflammation. Gut 55. https://doi.org/10.1136/gut.2005.090472

Marzo, V., Petrocellis, L., Fezza, F., Ligresti, A., Bisogno, T., 2002. Anandamide receptors. Prostaglandins Leukot Essent Fat Acids 66. https://doi.org/10.1054/plef.2001.0349

Marzo, V., Piscitelli, F., 2011. Gut feelings about the endocannabinoid system. Neurogastroenterol Motil 23. https://doi.org/10.1111/j.1365-2982.2011.01689.x

Mechoulam, R., Peters, M., Murillo-Rodriguez, E., Hanus, L.O., 2007. Cannabidiol-recent advances. Chem Biodivers 4. https://doi.org/10.1002/cbdv.200790147

Miragliotta, V., Ricci, P.L., Albanese, F., Pirone, A., Tognotti, D., Abramo, F., 2018. Cannabinoid receptor types 1 and 2 and peroxisome proliferator-activated receptor-α: distribution in the skin of clinically healthy cats and cats with hypersensitivity dermatitis. Vet Dermatol 29. https://doi.org/10.1111/vde.12658

Morales, P., Hurst, D.P., Reggio, P.H., 2017. Molecular targets of the phytocannabinoids: a complex picture. Prog Chem Org Nat Prod 103. https://doi.org/10.1007/978-3-319-45541-9_4

Moss, C.E., Marsh, W.J., Parker, H.E., Ogunnowo-Bada, E., Riches, C.H., Habib, A.M., Evans, M.L., Gribble, F.M., Reimann, F., 2012. Somatostatin receptor 5 and cannabinoid receptor 1 activation inhibit secretion of glucose-dependent insulinotropic polypeptide from intestinal K cells in rodents. Diabetologia 55. https://doi.org/10.1007/s00125-012-2663-5

Ochoa-Cortes, F., Turco, F., Linan-Rico, A., Soghomonyan, S., Whitaker, E., Wehner, S., Cuomo, R., Christofi, F.L., 2016. Enteric glial cells: a new frontier in neurogastroenterology and clinical target for inflammatory bowel diseases. Inflamm Bowel Dis 22. https://doi.org/10.1097/MIB.0000000000000667

Owyang, C., Heldsinger, A., 2011. Vagal control of satiety and hormonal regulation of appetite. J Neurogastroenterol Motil 17. https://doi.org/10.5056/jnm.2011.17.4.338

Patrizio, N.V., 2016. Endocannabinoids in the gut. Cannabis Cannabinoid Res 1. https://doi.org/10.1089/can.2016.0001

Pazos, M.R., Tolón, R.M., Benito, C., Rodríguez, C.F., Gorgojo, J.J., Nevado, M., Alvarez, M., Arias, F., Almodóvar, F., Fernández, M.T., Lledó, J.L., González, S., Fernández-Ruiz, J.J., Romero, J., 2008. Cannabinoid CB1 receptors are expressed by parietal cells of the human gastric mucosa. J Histochem Cytochem 56. https://doi.org/10.1369/jhc.2008.950741

Pertwee, R.G., 2015. Endocannabinoids and their pharmacological actions. Handb Exp Pharmacol 231. https://doi.org/10.1007/978-3-319-20825-1_1

Pertwee, R.G., 2001. Cannabinoids and the gastrointestinal tract. Gut 48. https://doi.org/10.1136/gut.48.6.859

Petrosino, S., Marzo, V., 2016. The pharmacology of palmitoylethanolamide and first data on the therapeutic efficacy of some of its new formulations. Br J Pharmacol 174. https://doi.org/10.1111/bph.13580

Pierezan, F., Mansell, J., Ambrus, A., Rodrigues Hoffmann, A., 2014. Immunohistochemical expression of ionized calcium binding adapter molecule 1 in cutaneous histiocytic proliferative, neoplastic and inflammatory disorders of dogs and cats. J Comp Pathol 151. https://doi.org/10.1016/j.jcpa.2014.07.003

Poole, D.P., Pelayo, J.C., Cattaruzza, F., Kuo, Y.-. M., Gai, G., Chiu, J.V., Bron, R., Furness, J.B., Grady, E.F., Bunnett, N.W., 2011. Transient receptor potential ankyrin 1 is expressed by inhibitory motoneurons of the mouse intestine. Gastroenterology 141. https://doi.org/10.1053/j.gastro.2011.04.049

Re, G., Barbero, R., Miolo, A., Marzo, V., 2007. Palmitoylethanolamide, endocannabinoids and related cannabimimetic compounds in protection against tissue inflammation and pain: potential use in companion animals. Vet J 173. https://doi.org/10.1016/j.tvjl.2005.10.003

Rigano, D., Sirignano, C., Taglialatela-Scafati, O., 2017. The potential of natural products for targeting PPARα. Acta Pharm Sin B 7. https://doi.org/10.1016/j.apsb.2017.05.005

Ross, R.A., 2011. l-α-lysophosphatidylinositol meets GPR55: a deadly relationship. Trends Pharmacol Sci 32. https://doi.org/10.1016/j.tips.2011.01.005

Russo, E.B., 2018. Cannabis therapeutics and the future of neurology. Front Integr Neurosci 12. https://doi.org/10.3389/fnint.2018.00051

Ryberg, E., Larsson, N., Sjögren, S., Hjorth, S., Hermansson, N.O., Leonova, J., Elebring, T., Nilsson, K., Drmota, T., Greasley, P.J., 2007. The orphan receptor GPR55 is a novel cannabinoid receptor. Br J Pharmacol 152. https://doi.org/10.1038/sj.bjp.0707460

Sadeghinezhad, J., Sorteni, C., Guardo, G., D'Agostino, C., Agrimi, U., Nonno, R., Chiocchetti, R., 2013. Neurochemistry of myenteric plexus neurons of bank vole (Myodesglareolus) ileum. Res Vet Sci 95. https://doi.org/10.1016/j.rvsc.2013.07.028

Samson, M.T., Small-Howard, A., Shimoda, L.M., Koblan-Huberson, M., Stokes, A.J., Turner, H., 2003. Differential roles of CB1 and CB2 cannabinoid receptors in mast cells. J Immunol 170. https://doi.org/10.4049/jimmunol.170.10.4953

Schubert, M.L., 2016. Gastric acid secretion. Curr Opin Gastroenterol 32. https://doi.org/10.1097/MOG.0000000000000308

Sharkey, K.A., 2015. Emerging roles for enteric glia in gastrointestinal disorders. J Clin Investig 125. https://doi.org/10.1172/JCI76303

Sharkey, K.A., Wiley, J.W., 2016. Getting into the weed: the role of the endocannabinoid system in the brain–gut axis. Gastroenterology 151. https://doi.org/10.1053/j.gastro.2016.04.015

Shea-Donohue, T., Stiltz, J., Zhao, A., Notari, L., 2010. Mast cells. Curr Gastroenterol Rep 12. https://doi.org/10.1007/s11894-010-0132-1

Sickle, M.D., Oland, L.D., Ho, W., Hillard, C.J., Mackie, K., Davison, J.S., Sharkey, K.A., 2001. Cannabinoids inhibit emesis through CB1 receptors in the brainstem of the ferret. Gastroenterology 121. https://doi.org/10.1053/gast.2001.28466

Stančić, A., Jandl, K., Hasenöhrl, C., Reichmann, F., Marsche, G., Schuligoi, R., Heinemann, A., Storr, M., Schicho, R., 2015. The GPR55 antagonist CID16020046 protects against intestinal inflammation. Neurogastroenterol Motil 27. https://doi.org/10.1111/nmo.12639

Storr, M., Sibaev, A., Marsicano, G., Lutz, B., Schusdziarra, V., Timmermans, J.P., Allescher, H.D., 2004. Cannabinoid receptor type 1 modulates excitatory and inhibitory neurotransmission in mouse colon. Am J Physiol Gastrointest Liver Physiol 286. https://doi.org/10.1152/ajpgi.00148.2003

Storr, M.A., Sharkey, K.A., 2007. The endocannabinoid system and gut–brain signalling. Curr Opin Pharmacol 7. https://doi.org/10.1016/j.coph.2007.08.008

Svensson, M., Chen, P., Hammarfjord, O., 2010. Dendritic cell regulation by cannabinoid-based drugs. Pharmaceuticals 3. https://doi.org/10.3390/ph3082733

Sykaras, A.G., Demenis, C., Case, R.M., McLaughlin, J.T., Smith, C.P., 2012. Duodenal enteroendocrine I-cells contain mRNA transcripts encoding key endocannabinoid and fatty acid receptors. PLoS ONE 7. https://doi.org/10.1371/journal.pone.0042373

Taylor, L., Christou, I., Kapellos, T.S., Buchan, A., Brodermann, M.H., Gianella-Borradori, M., Russel, A., Igbal, A.J., Greaves, D.R., 2015. Primary macrophage chemotaxis induced by cannabinoid receptor 2 agonists occurs independently of the CB2 receptor. Sci Rep 5. https://doi.org/10.1038/srep10682

Thomas, A., Baillie, G.L., Phillips, A.M., Razdan, R.K., Ross, R.A., Pertwee, R.G., 2007. Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. Br J Pharmacol 150. https://doi.org/10.1038/sj.bjp.0707133

Tudurí, E., Imbernon, M., Hernández-Bautista, R., Tojo, M., Fernø, J., Diéguez, C., Nogueiras, R., 2017. GPR55: a new promising target for metabolism? J Mol Endocrinol 58. https://doi.org/10.1530/JME-16-0253

Uranga, J.A., Vera, G., Abalo, R., 2018. Cannabinoid pharmacology and therapy in gut disorders. Biochem Pharmacol 157. https://doi.org/10.1016/j.bcp.2018.07.048

Walls, A.F., He, S., Buckley, M.G., McEuen, A.R., 2001. Roles of the mast cell and basophil in asthma. Clin Exp Allergy 1. https://doi.org/10.1046/j.1472-9725.2001.00009.x

Wouters, M.M., Vicario, M., Santos, J., 2016. The role of mast cells in functional GI disorders. Gut 65. https://doi.org/10.1136/gutjnl-2015-309151

Wright, K., Rooney, N., Feeney, M., Tate, J., Robertson, D., Welham, M., Ward, S., 2005. Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. Gastroenterology 129. https://doi.org/10.1053/j.gastro.2005.05.026

Wright, K.L., Duncan, M., Sharkey, K.A., 2008. Cannabinoid CB2 receptors in the gastrointestinal tract: a regulatory system in states of inflammation. Br J Pharmacol 153. https://doi.org/10.1038/sj.bjp.0707486

Yang, Y.Y., Hsieh, S.L., Lee, P.C., Yeh, Y.C., Lee, K.C., Hsieh, Y.C., Wang, Y.W., Lee, T.Y., Huang, Y.H., Chan, C.C., Lin, H.C., 2014. Long-term cannabinoid type 2 receptor agonist therapy decreases bacterial translocation in rats with cirrhosis and ascites. J Hepatol 61. https://doi.org/10.1016/j.jhep.2014.05.049

Zhang, L., Song, J., Hou, X., 2016. Mast cells and irritable bowel syndrome: from the bench to the bedside. J Neurogastroenterol Motil 22. https://doi.org/10.5056/jnm15137

Ziring, D., Wei, B., Velazquez, P., Schrage, M., Buckley, N.E., Braun, J., 2006. Formation of B and T cell subsets require the cannabinoid receptor CB2. Immunogenetics 58. https://doi.org/10.1007/s00251-006-0138-x

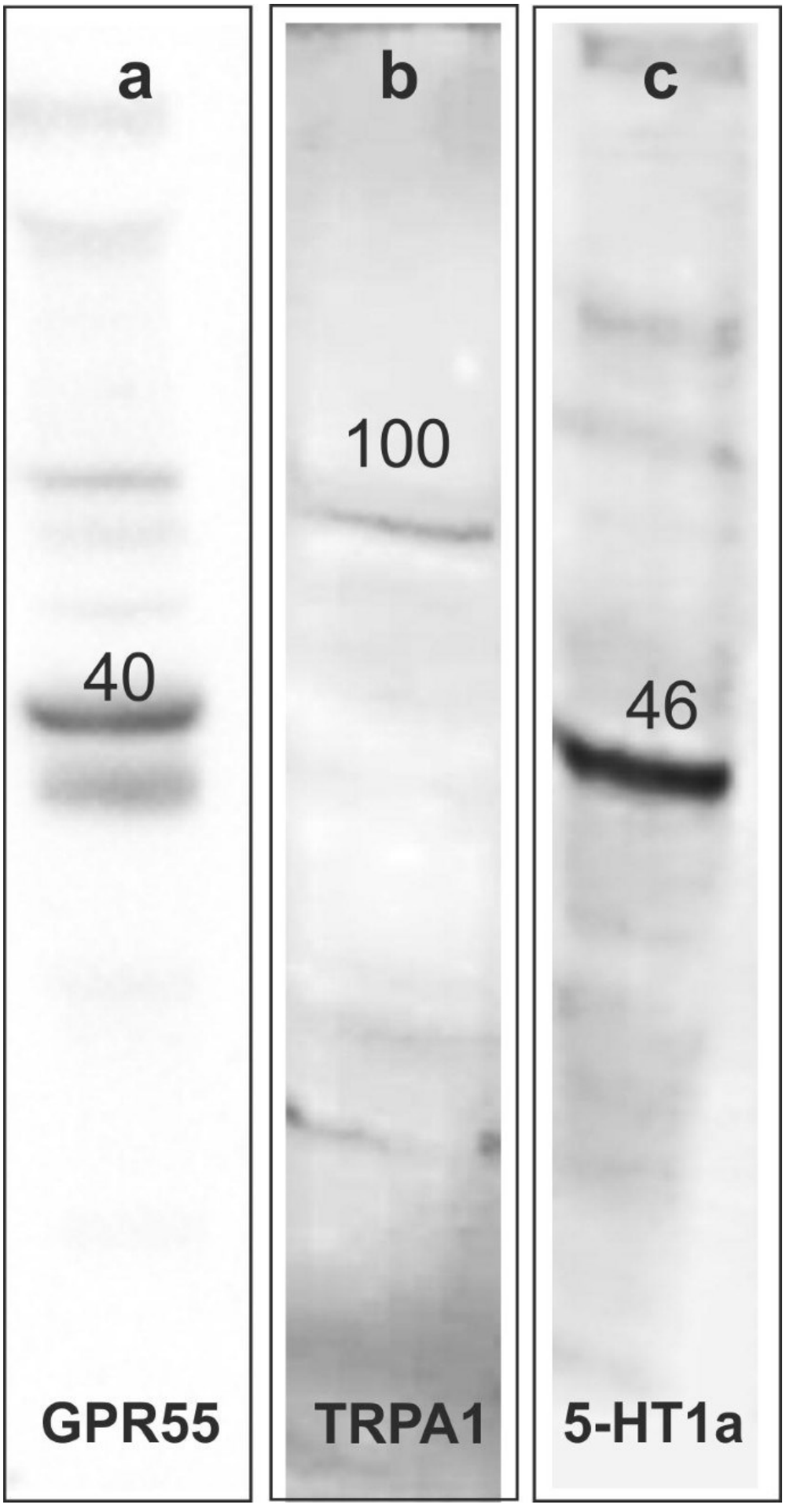

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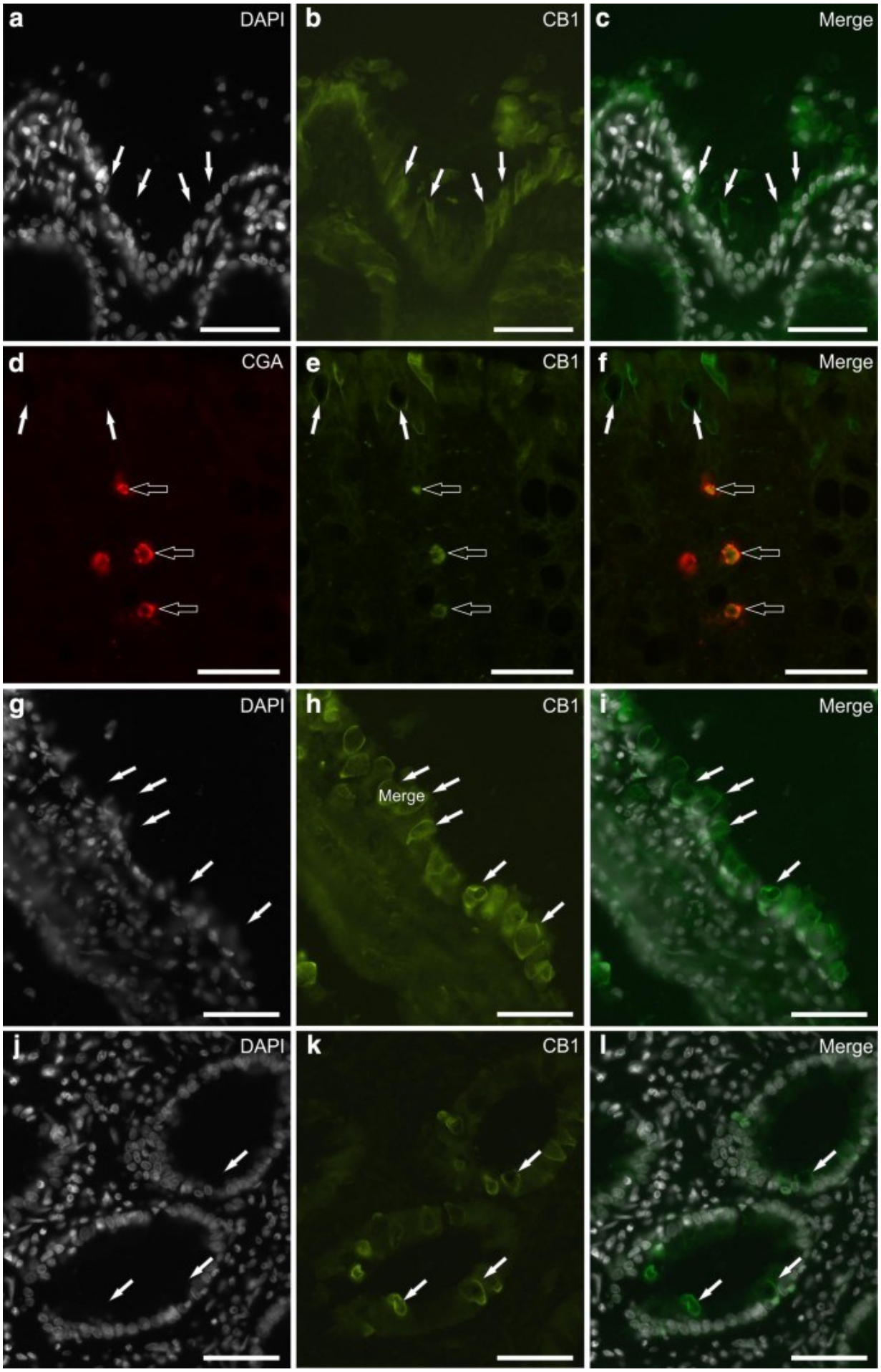
FIG 1. Western blot (Wb) analysis showing the specificity of the following primary antibodies utilized: a rabbit anti-G protein-coupled receptor 55 (GPR55); b rabbit anti-transient receptor potential ankyrin 1 (TRPA1); c rabbit anti-serotonin receptor 5-HT1a (5-HT1a); a the antibody anti-GPR55 showed a major band close to the theoretical molecular weight (about 40 kDa). b The antibody anti-TRPA1 showed a major band close to the theoretical molecular weight (100 kDa). c The antibody anti-5-HT1a receptor showed a major band close to the theoretical molecular weight (46 kDa). The numbers on each line indicates the molecular weight. The images of the immunoblots were slightly adjusted in brightness and contrast to match their backgrounds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i
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j
DAPI
k
CB1
l
Merge

686

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

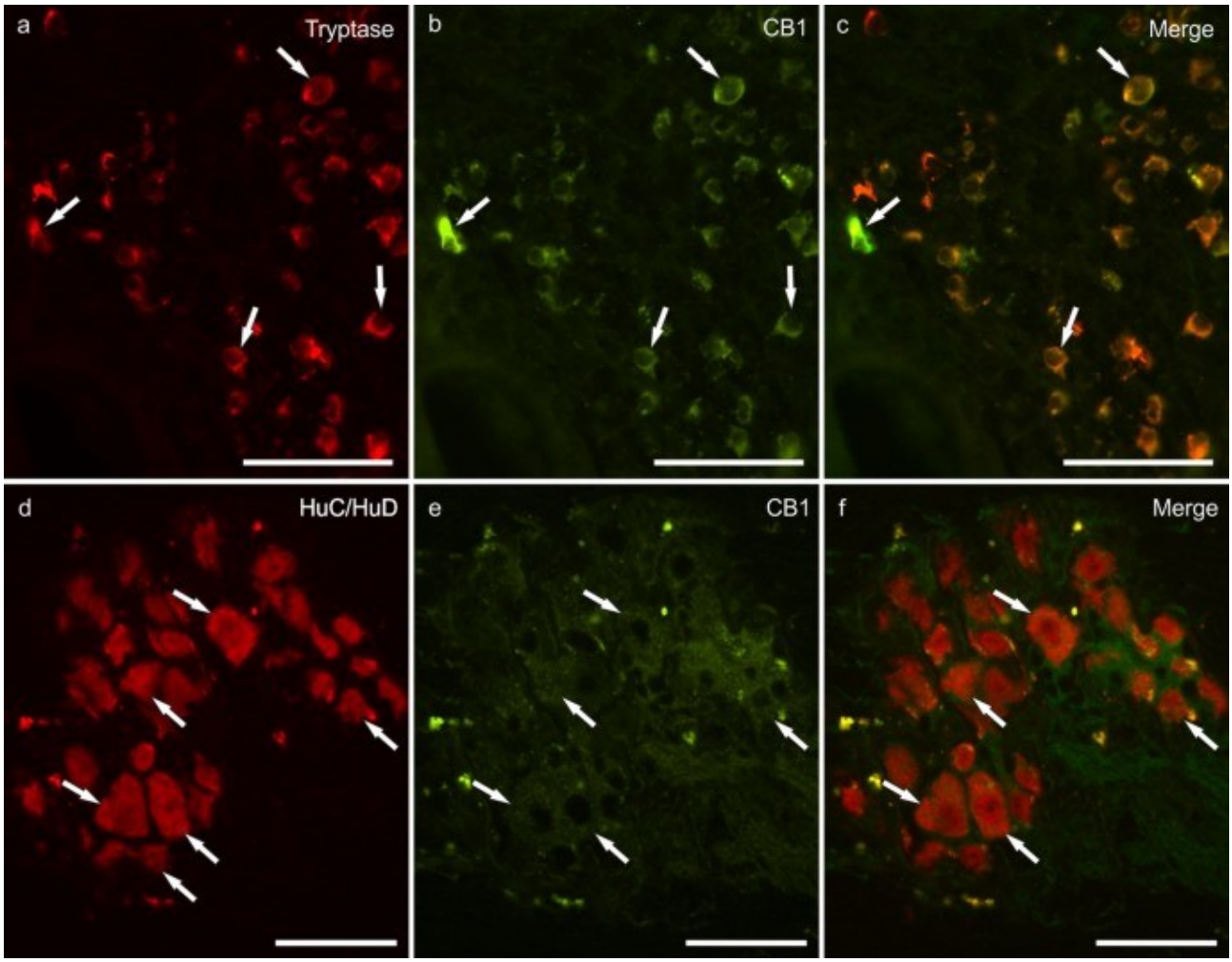

FIG. 3. Photomicrograph showing cryosections of the cat gastrointestinal tract immunolabeled with the antibody anti-cannabinoid receptor 1 (CB1). Cellular nuclei were labelled with the nuclear stain DAPI. Arrows indicate four of the numerous lamina propria mast cells co-expressing tryptase (a) and bright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

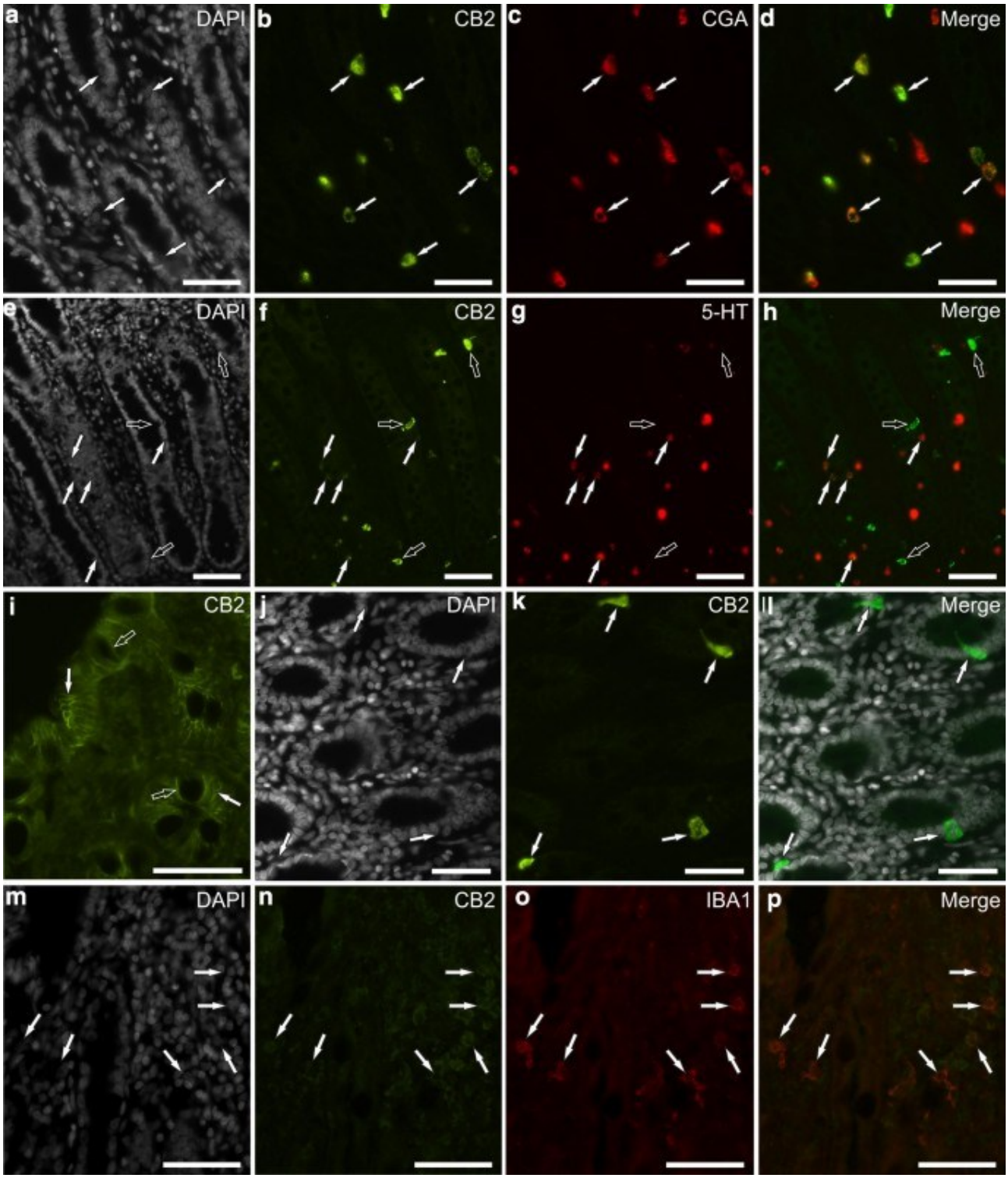

FIG. 4. a–p Photomicrograph showing cryosections of cat intestinal tract immunolabelled with the antibody anti-cannabinoid receptor 2 (CB2). Cellular nuclei were labelled with the nuclear stain DAPI. a–d Arrows indicate some of the numerous enteroendocrine cells identified with the antibody anti-chromogranin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

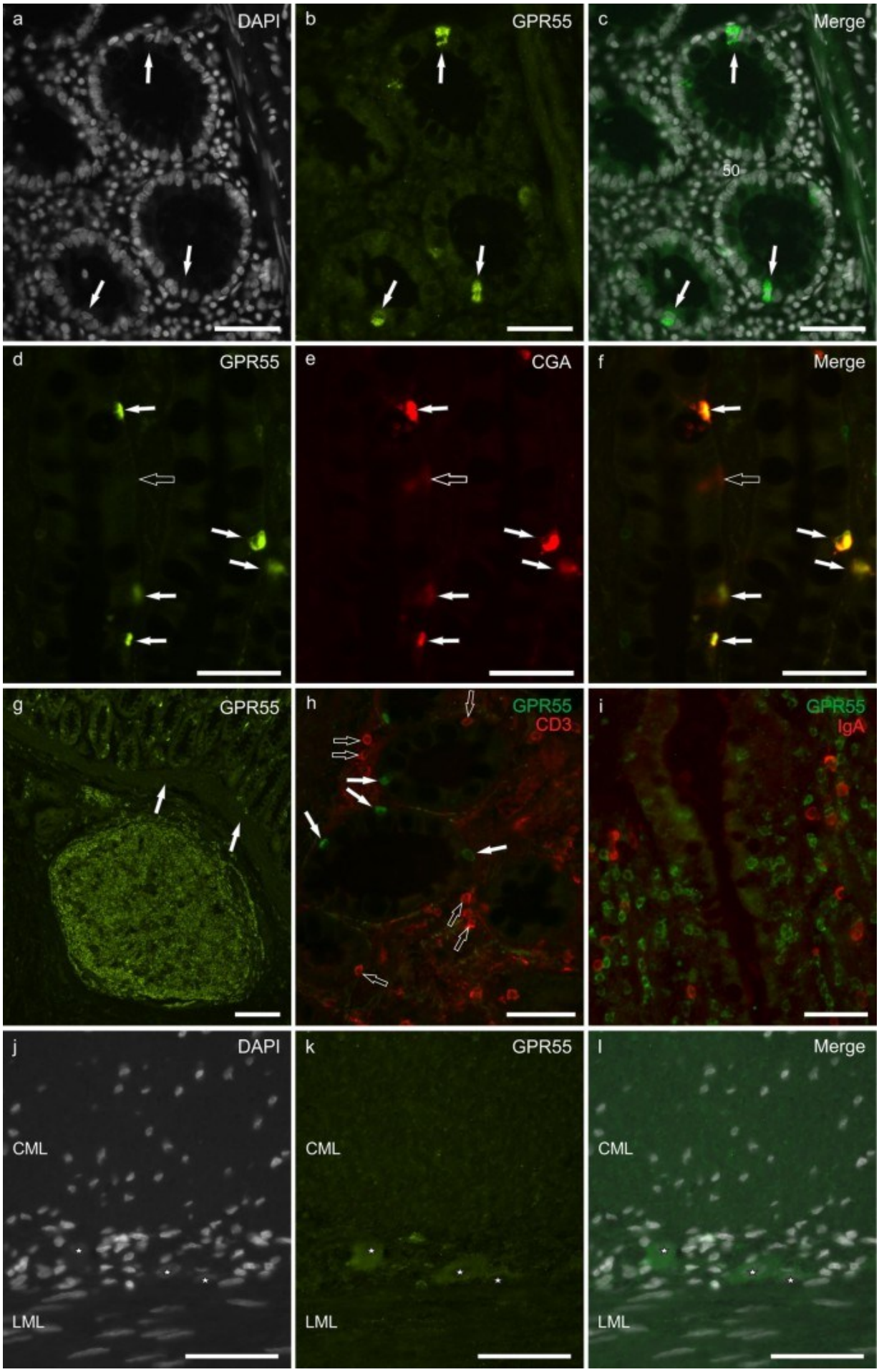
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FIG. 5. Photomicrograph showing cryosections of cat small and large intestine immunolabelled with the antibody anti-GPR55. Cellular nuclei were labelled with the nuclear stain DAPI. a–c Arrows indicate bright GPR55 immunolabelling (b) of the enteroendocrine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

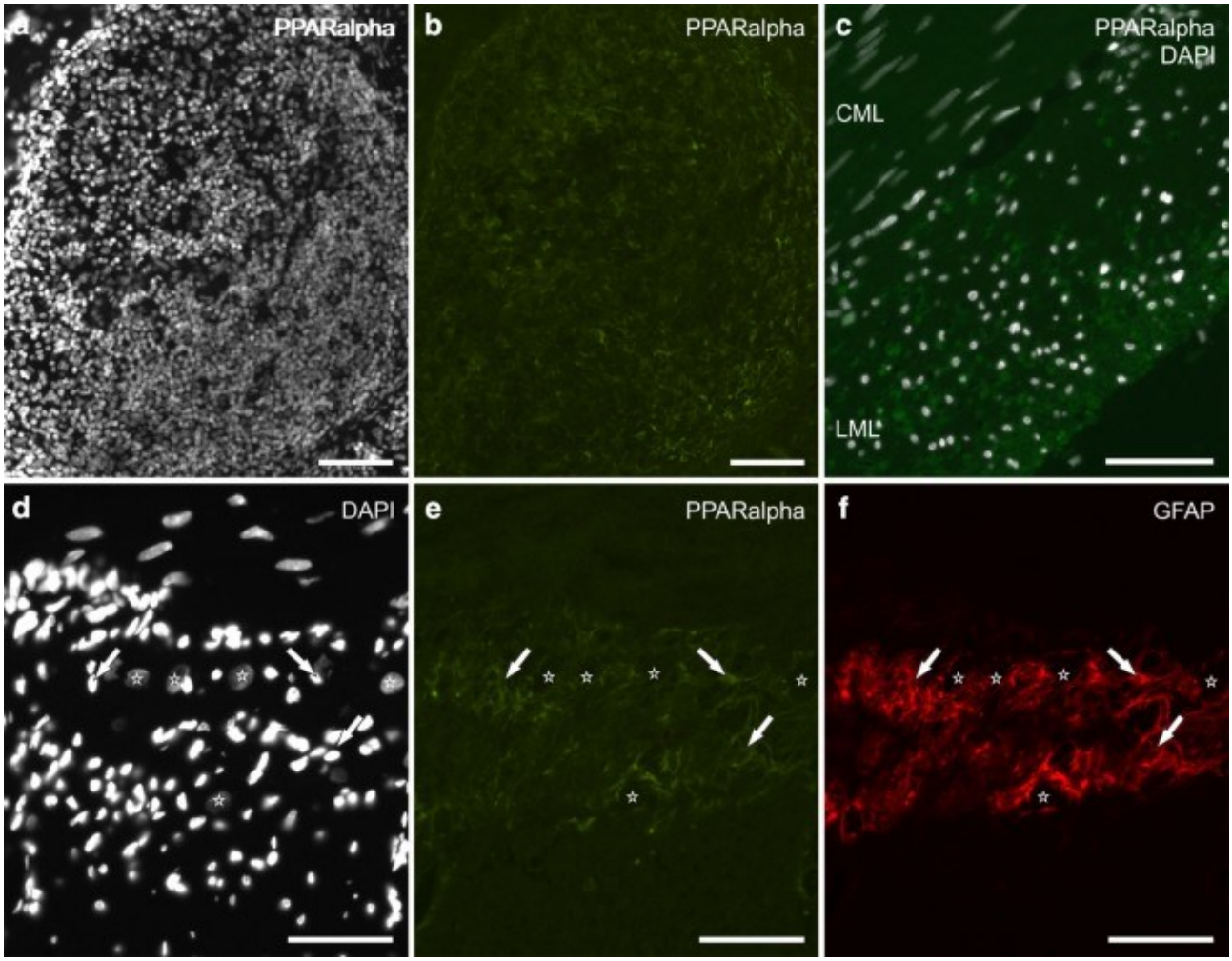
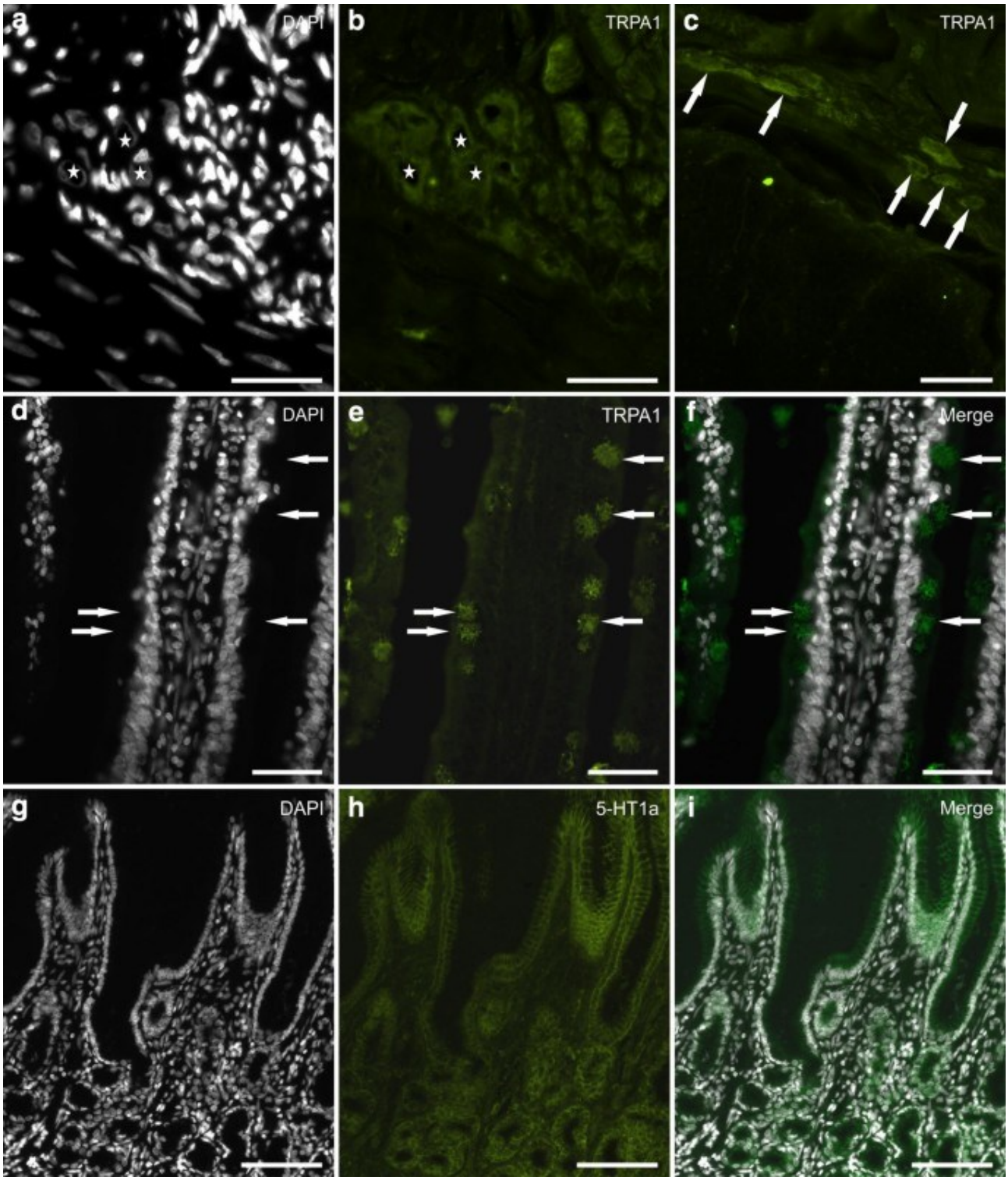


FIG. 6. Photomicrograph showing cryosections of cat intestine immunolabelled with the antibody anti-PPARα (a–f). Cellular nuclei were labelled with the nuclear stain DAPI. a, b Intestinal lymph node in which a large percentage of immunocytes showed PPARα immunoreactivity. c PPARα immunoreactivity was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



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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 μm

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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)

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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.
PPARα	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

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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.
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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.
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