Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

Ciclo XXXII

Settore Concorsuale: 07/H1

Settore Scientifico Disciplinare: VET/01

Characterization of Cannabinoid Receptors in the Peripheral Nervous System and in the Gastrointestinal Tract of Mammals of Veterinary Interest

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Esame finale anno 2020

Abstract

Cannabis has always been used to treat gastrointestinal (GI) symptoms and pain. By considering the beneficial effects obtained in human medicine, the research in veterinary medicine has focused on the endocannabinoid system (ECS), developing products to treat inflammatory conditions and nociception. The target of these products are cannabinoid receptors (CBRs), with the two canonical cannabinoid receptor 1 (CB1R) and 2 (CB2R), and different putative cannabinoid receptors, such as G protein coupled receptor 3 (GPR3) and 55 (GPR55), peroxisome proliferator-activated receptors α (PPARα) and γ (PPARγ), transient receptor potential vanniloid (TRPV1) and ankirin (TRPA1), or serotonin receptor (5-HT1a, 5-HT2a or 5-HT3a).

The principal aim of this research was to evaluate the role of CBRs in the GI tract and in the dorsal root ganglia (DRGs) of different species. The first chapter focuses on the ECS and its components. The second chapter focuses on the GI tract, describing its structure, the interaction with the ECS and the principal pathologies affecting the species of interest, in which the ECS could be involved. The third chapter focuses on DRGs, describing the structure, the role in neuroinflammation and the interaction with the ECS.

The experimental studies are divided in:

- experimental studies about the GI tract;
- experimental studies about the DRGs;
- other experimental studies (focus: endoscopy).

The localization of CBRs has been investigated in the GI tract of dog, cat and horse and in canine and equine DRGs. The receptors showed similarities and differences in their distribution, underlining how the ECS modulates its expression adapting to physiological (and pathological) conditions between the different species.

This research could provide an anatomical substrate upon which it would be possible to develop preclinical and clinical studies aimed to investigate and possibly support the therapeutic use of non-psychotropic cannabinoid in veterinary medicine.

Table of contents

Nomenclature

ECS	Endocannabinoid system
CB1R	Cannabinoid receptor 1
CB2R	Cannabinoid receptor 2
GPR55	G protein coupled receptor 55
PPAR	Peroxisome proliferator-activated receptors
TRP	transient receptors potential channel
5-HT	Serotonin
AEA	Anandamide
2-AG	2 arachydonyl-glicerol
PEA	Palmitoylethanolamide
CBD	Cannabidiol
THC	Tetrahydrocannabinol
mm	Muscularis mucosae
LML	Longitudinal muscle layer
CML	Circular muscle layer
ENS	Enteric nervous system
MP	Myenteric plexus
SMP	Submucosal plexus
EGC	Enteric glial cell
SGC	Satellite glial cell
DRG	Dorsal root ganglion
IBD	Inflammatory Bowel disease
CE	Chronic enteropathy

The endocannabinoid system

The endocannabinoid system (ECS) is composed of cannabinoid receptors, their endogenous ligands, and the enzymes involved in endocannabinoids turnover (Stella, 2004; Ligresti et al., 2016; Lu and Anderson, 2017). The term "endocannabinoid system" was first coined by Di Marzo and Fontana in 1995. The etymology of this term derived from *cannabis*, because this phytocompound, as the endocannabinoids, can act on cannabinoid receptors. The medical use of *cannabis* has a long history: thousands of years ago the Chinese and Indian society used it for the control of anxiety and visceral pain (Figure 1). The ECS has great importance for the physiological functions of the organism. It is widely expressed in the central nervous system (CNS), cardiovascular, gastrointestinal, immune and reproductive system and, moreover, it is similar among different species (Maccarrone et al., 2015; Cabral et al., 2015). The ECS is present in mammals, birds, fish, echinoderms and mussels (Salzet et al., 2000). A growing body of evidences indicates that activation of cannabinoid receptors by endogenous, plant-derived, or synthetic cannabinoids may exert beneficial effects on inflammation and visceral pain.

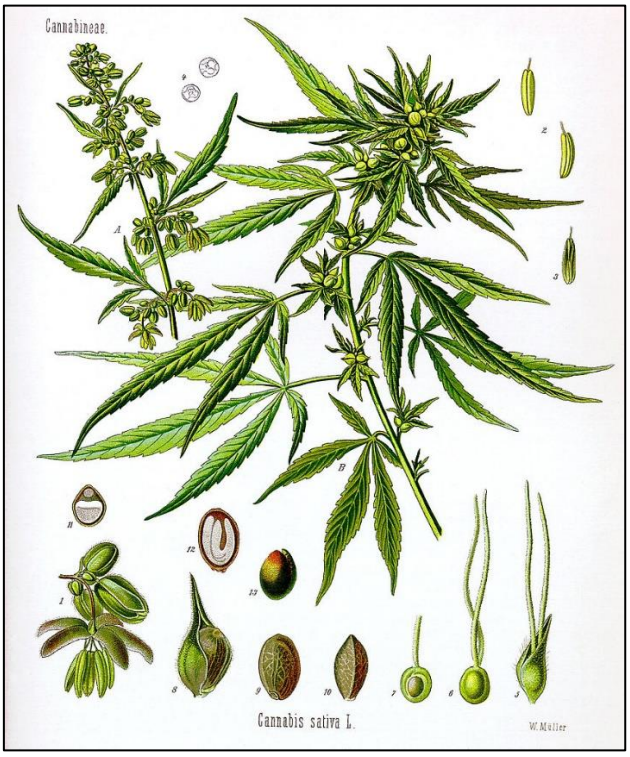

Figure 1: Illustration of Cannabis sativa. Scientific drawing (1887). In Franz Eugen Köhler's Medizinal-Pflantzen. Published and copyrighted by Gera-Untermhaus, FE Köhler. Drawing by W. Müller.

Cannabinoids receptors

G protein-coupled receptors

The G protein-coupled receptors (GPCRs) are the largest family of receptors and the principal target of current therapeutic drugs (Lefkowitz, 2004). In the human genome, about 1000 genes encode such receptors (Fredriksson et al., 2003). GPCRs are involved in all known physiological processes in mammals (Lefkowitz, 2004). A GPCR system is composed of a ligand, a receptor, and a transducer. Stimulated by hormones, neurotransmitters, and lipids, GPCRs change in an "active" conformation that leads to a wide range of intracellular responses (Figure 2) (Hodavance et al., 2016). The most important cannabinoid receptors are part of this family (Maccarrone, 2015). After the discovery of CB1R and CB2R receptors in the Eighties, other G protein-coupled receptors were identified, considered "putative cannabinoid receptor", such as GPR55, GPR3, GPR6, GPR12, GPR18 and GPR119 (Gribble and Reimann, 2016, Ryberg et al., 2007, Morales et al., 2018).

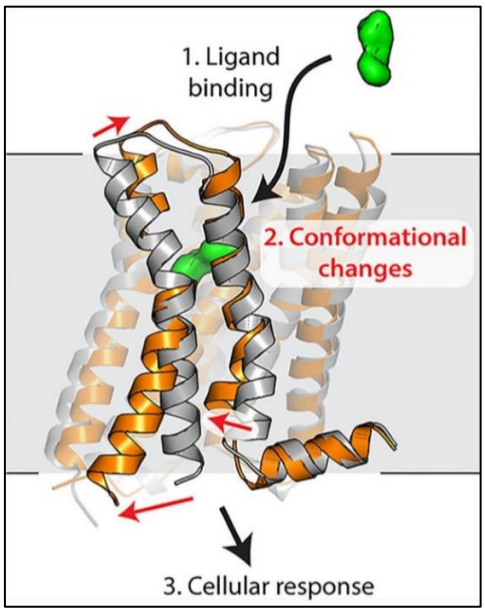

Figure 2: G protein coupled receptor system. Modified from Manglik and Kruse, 2017.

CB1R

The cannabinoid receptor 1 (CB1R) is a G protein-coupled receptor expressed mostly in the CNS and peripheral nervous system (PNS). It is primarly involved in cognition and short-term memory (cerebral cortex and hippocampus) and in motor function and movement (basal ganglia and cerebellum), so the receptor is more concentrated in these areas (Pertwee, 1997; Hu and Mackie, 2015; Freundt-Revilla et al., 2017). CB1 receptor is also present, with a lower concentration, in a variety of peripheral tissues and cells (Pacher et al., 2006). CB1R is involved in feeding behavior: its activation can increase appetite, whereas the inhibition of CB1R suppresses hunger and induces

hypophagia (Terragon and Moreno, 2019). The wide distribution of the CB1R in the CNS limits its properties, due to the psychoactive side effects linked to its activation (Moreira et al., 2009). For example, the CB1R antagonist rimonabant was used for several years for the treatment of obesity, reducing food intake as well as abdominal adiposity and cardiometabolic risk factors (Bermudez-Silva et al., 2010). In 2009, it was banned due to psychiatric side effects, including depression, anxiety and suicidal thoughts (Simon and Cota, 2017).

CB2R

CB2 receptor, another G protein-coupled receptor, is mainly expressed by cells of the hematopoietic and immune systems, such as monocytes, macrophages, and lymphocytes B and T, hihglighting its immunomodulatory properties (Staiano et al., 2005; Pacher et al., 2006; Matias and Di Marzo, 2007). In human medicine, CB2R has a potential as target in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis, atherosclerosis, and Inflammatory Bowel Disease (IBD) (Turcotte et al., 2016). CB2R seems to be involved also in the pathogenesis of IBD: in humans, the CB2-Q63R genetic variant increases the risk of pediatric IBD. This variant is linked to the balance between Th1 and Th2 cells. Children affected by pediatric IBD with the CB2- Q63R variant develop a more severe phenotype of IBD (Strisciuglio et al., 2018). CB2R has recently been identified in neurons and microglia (Malfitano et al., 2014). This receptor seems to be upregulated in different CNS diseases involving microglia and/or astroglia activation, suggesting a possible role of CB2 receptor as pharmacological target in neuroinflammatory diseases (Skaper et al., 2013; Navarro et al., 2016; Cassano et al., 2017; Chen et al., 2017; Freundt-Revilla et al., 2018). The absence of CB2R in the brain result in no psychotropic effects linked to its activation (Dhopeshwarkar and Mackie, 2014). Moreover, recent evidences demonstrate the role of ECS in modulating cell-signaling targets in diabetes mellitus (DM) (Kumawat and Kaur, 2019). The activation of CB2R inhibits the expression of inflammatory cytochines, as tumor necrosis factor alpha (TNF-α), Interleukin 6 (IL-6), Nuclear factor kappa beta (NF-κβ) (Horváth et al., 2012). Moreover, CB2R is present in pancreatic β-cells in human and rat, and can stimulate the secretion of insulin through Ca^{2+} signal regulation (Juan-Picó et al., 2006). In human and rat kidney, CB2 receptor has been localized in glomeruli and tubules (Cakir et al., 2019). Several studies demonstrate that CB2R activation can reduce kidney damage, while CB2R inhibition increase renal damage (Mukhopadhyay et al., 2010; Zoja et al., 2016). Activation of CB2 receptor with CB2R-agonists reduce apoptosis, inflammation, and oxidative stress in the kidney (Cakir et al., 2019).

GPR55

The G protein-coupled receptor 55 (GPR55) was first described in 1999 (Sawzdargo et al., 1999). GPR55 shares 10–14% homology with CB1 and CB2 receptors (Lauckner et al., 2008). It is widely distributed in the enteric nervous system (ENS) of humans and rodents, especially in the myenteric and submucosal plexus (Lin et al., et al., 2011; Ross et al., 2012; Li et al., 2013; Goyal et al., 2017). GPR55 has been identified in a large number of cell types, as macrophages, plasma cells, neutrophils, natural killer, 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; Grill et al., 2019). It is also expressed by microglia, the principal cells in the CNS involved in the innate immune response, playing essential roles in the homeostasis and responses to inflammatory stimuli (Ransohoff and Pery, 2009). An overexpression of microglia is associated with neurodegenerative diseases, as Alzheimer's disease (AD) or Parkinson disease (PD) (Streit et al., 2005). GPR55 antagonists, blocking microglia activation, can potentially provide anti-inflammatory effects (Saliba et al., 2018). As other cannabinoid receptors, GPR55 seems to be involved in human IBD. In inflammed colonic samples of patients affected by Crohn's disease, GPR55 mRNA expression is significantly higher than in patients with Ulcerative Colitis or non-inflammed patients. GPR55 shows strong affinity with palmitoylethanolammide (PEA), a lipid mediator structurally related to the endocannabinoids (Petrosino and Di Marzo, 2016). Otherwise cannabidiol (CBD), another therapeutic phytocannabinoid, acts as an antagonist of the GPR55 (Ligresti et al., 2016), so the function of this receptor is still questioned.

Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors belonging to the family of nuclear hormone receptors (NRs), part of the steroid receptor superfamily (Figure 3) (Berger and Moller, 2002). After interacting with specific ligands, NRs move to the nucleus, modify their structure and regulate gene trascription (Grygiel-Gòrniak, 2014). They act as transcription factors, modulating various metabolic processes, principally lipid and glucose homeostasis (Burstein, 2005; O'Sullivan, 2007; Morales et al., 2017).

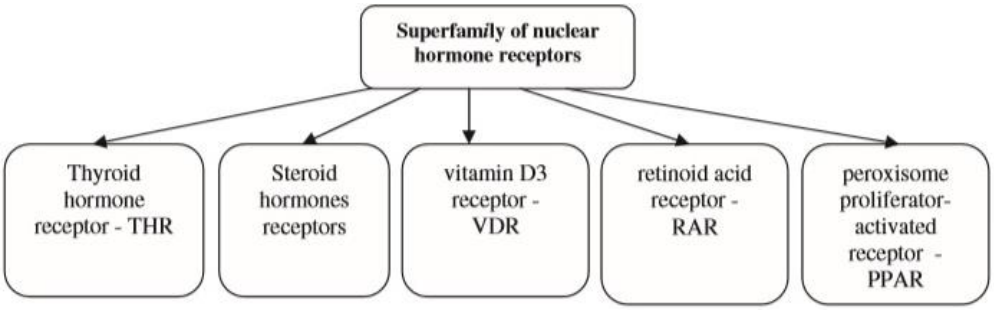

Figure 3: Superfamily of nuclear hormone receptors. Modified from Berger and Moller, 2002.

The structure of PPARs is a three-dimensional structure, composed by a DNA binding domain (N-terminus) and a ligand binding domain (C-terminus) (Grygiel-Gòrniak, 2014). There are different co-activators and co-repressors of PPAR, which can activate or inhibit receptors function (Viswakarma et al., 2010). The family of PPAR comprises PPARα, PPARβ/δ and PPARγ (Berger and Moller, 2002). Natural ligands of PPARs are essential fatty acids (EFA) or eicosanoids, involved in glucose and lipid homeostasis (Krey et al., 1997). Synthetic ligands are, for example, fibrates, involved in the treatment of hypertriglyceridemia, and thiazolidinediones, used in the treatment of DM (Taniguchi et al., 2001).

PPARα

The PPARα receptor is a ligand-activated transcription factor. PPARα can modulate gene expression, playing a key role in glucose and lipid homeostasis and inhibiting inflammation (Naidenow et al., 2016). This receptor is highly expressed in metabolic active tissue like heart, liver, mucosal intestine, skeletal muscle, and brown adipose tissue. In the liver, increased fatty acid concentrations activate PPARα, which uptakes oxidized forms of fatty acids. Oxidation prevents steatosis of the liver, in case of starvation/fasting (Sethi et al., 2001). Administration of PPARα agonists prevents hepatic fibrosis in animal models (Ip et al., 2003). The antinflammatory action of palmitoylethanolamide, a natural fatty acid ethanolamide, is also mediated by the interaction with PPARα. Indeed, PPARα$^{-/-}$ mice display longer inflammatory responses than wild type mice (Lo Verme et al., 2005).

PPARγ

PPARγ is another ligand-activated transcription factor. It is widely expressed in white and brown adipose tissue, spleen and intestine. PPARγ plays a key role in adipogenesis and lipid metabolism, and it is essential for the control of insulin sensitivity (Grygiel-Gòrniak, 2014). The activation of PPARγ balances the secretion of adipocytokines, mediators of insulin action in peripheral tissues (Kintscher and Law, 2005). PPARγ is also present in endothelial and vascular smooth muscle cells, and it is involved in regulation of vascular inflammation and atherosclerosis (Marx et al., 1999). This

receptor seems to have neuroprotective potential in CNS diseases (Hung et al., 2019). The natural agonists of PPARγ are polyunsatured fatty acid (PUFA), but they not always determine an activation of the receptor and consequently a gene transcription (Grygiel-Gòrniak, 2014). This interaction between PUFA and PPARγ seems to regulate cancer development. Indeed, the activation of PPARγ has an apoptotic action on cancer cells, while in vitro activation of PPARα or PPARβ/δ in human cell lines of breast cancer stimulates cell proliferation (Suchanek et al., 2002). PPARγ is a target of CBD, which reduce intestinal inflammation mainly through a modulation of the neuro-immune axis (De Filippis et al., 2011; Couch et al., 2017).

Transient receptors potential channel

The transient receptors potential channel (TRP) are integral membrane proteins that modulate the entrance of ions Ca^{++} in the cells (Morales et al., 2017). Hormones, growth factors and neurotransmitters allow Ca^{++} entry through receptor-activated cation channels. All channels have six transmembrane segments (from S1 to S6), with a pore region (P) between S5 and S6 (Figure 4) (Nilius and Owsianik, 2011). These proteins are conserved in invertebrates and vertebrates. TRP are widely expressed, both in excitable and non-excitable tissues; they are localized mostly in all cellular membranes. The superfamily of TRP contains seven subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin), and TRPN (NOMPC-like, only in invertebrates and fish) (Nilius et al., 2007). Cannabinoids can interact with three subfamilies of TRP: TRPV, TRPA and TRPM. In particular TRPV1, TRPV2, TRPV3, TRPV4, TRPM8 e TRPA1 interact with cannabinoids (De Petrocellis et al., 2008; De Petrocellis et al., 2011; De Petrocellis et al., 2012).

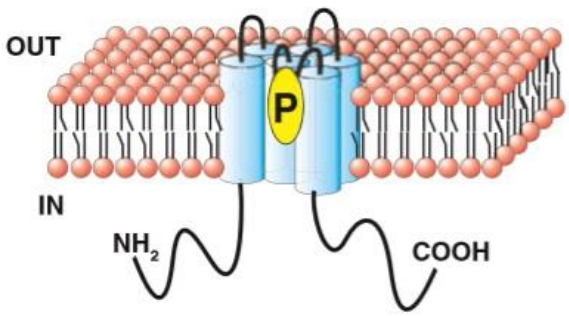

Figure 4: Predicted structural topology of TRP channels. Modified from Nilius and Owsianik, 2011.

TRPV1

The TRPV family contains six members, from TRPV1 to TRPV6. These receptors are tetrameric complexes, and every subunit contains six N-terminal ankyrin repeats (Du et al., 2019). The TRPV1 is located in DRG and trigeminal ganglia, spinal and peripheral nerve terminals, brain, skin, pancreas,

gastrointestinal tract and bladder (Nilius and Owsianik, 2011). In the brain, TRPV1 has been found in dopaminergic neurons of the *substantia nigra*, hippocampal pyramidal neurons, hypothalamic neurons, *locus coeruleus* in the brainstem, and in various layers of the cortex (Mezey et al., 2000). TRPV1 is activated by heat (>43°C), low pH and capsaicin (Caterina et al., 1997). It usually undergoes desensitization by endocannabinoids (Ambrosino et al., 2013; Zygmunt et al., 2013). TRPV1 is involved in different functions, such as thermo-regulation, nociception, pain management or food intake regulation (Nilius and Owsianik, 2011). The stimulation of TRPV1 by caspaicin seems to prevent adipogenesis and obesity in wild type mice (Zhang et al., 2007). In primary sensory neurons, TRPV1 is essential for the development of inflammatory hyperalgesia (Davis et al., 2000). In the gastrointestinal tract, TRPV1 is mainly expressed by the submucosal plexus (SMP), myenteric plexus (MP), *muscolaris mucosae*, gastric mucosal and parietal cells, and gastric antral G cells (Akbar et al., 2010). On gastric functions, TRPV1 acts reducing gastric acid secretion (Hirokuni et al., 2012), accelerating gastric emptying process (Debreceni et al., 1999), increasing mucosal blood flow (Satyanarayana, 2006) and protecting gastric mucosa through the secretion of prostaglandins (PGE_2) and epidermal growth factor (EGF). TRPV1 is involved also in Irritable Bowel Syndrome (IBS), a human functional bowel disorder that causes abdominal pain, abdominal distention, and changes in stool characteristics (Du et al., 2019). Shi et al. (2013) demostrated that TRPV1 expression on nerve fibers was significantly upregulated in colonic samples with IBS, and this upregulation was positively correlated with the severity of abdominal pain.

TRPA1

The TRPA family contains one mammalian receptor, TRPA1 (Nilius et al., 2007). TRPA1 is an ion channel that reacts to mechanical, thermal (cold) and chemical stimulation. It can be activated by different chemical substances present in herbs and spices, like allicin (obtained from garlic), cinnamaldehyde (from cinnamon) or wasabi. It is expressed by nociceptive/thermo-receptive neurons, which detect temperature below -17°. This receptor is also expressed by non-neuronal cells, such as, in human and mice, T cells. Sahoo et al. (2019) found that TRPA1 is expressed at the surface of these immune cells, rather than intracellularly, and it is overexpressed in activated T cells, where it mediates Ca^{++} influx and determines the release of certain citokines (Figure 5). TRPA1 seems to act as a proinflammatory regulators, involved in neurogenic inflammation (Bautista et al., 2013), dermatitis (Liu et al., 2013) and colitis in mice-models (Utsumi et al., 2018). So, TRPA1 inhibition should reduce T cells activation. Otherwise, other studies suggest an anti-inflammatory role of TRPA1, via $CD4^+$ T cells involvement (Bertin et al., 2016). TRPA1, cold-sensitive channel and TRPV1, a heat-sensitive channel, are often co-expressed (Story et al., 2003). The anti-inflammatory, anti-nociceptive and analgesic properties of CBD might be due, in part, to the capability to activate

and/or desensitize both the TRPA1 (De Petrocellis et al., 2008) and the TRPV1 (Bisogno et al., 2001; Ligresti et al., 2016). TRPA1 is involved in the etiology of FEPS (Familial Episodic Pain Sydrome), an autosomal dominant disease that gives upper body pain, provoked by physical stress or fasting (Kremeyer et al., 2010).

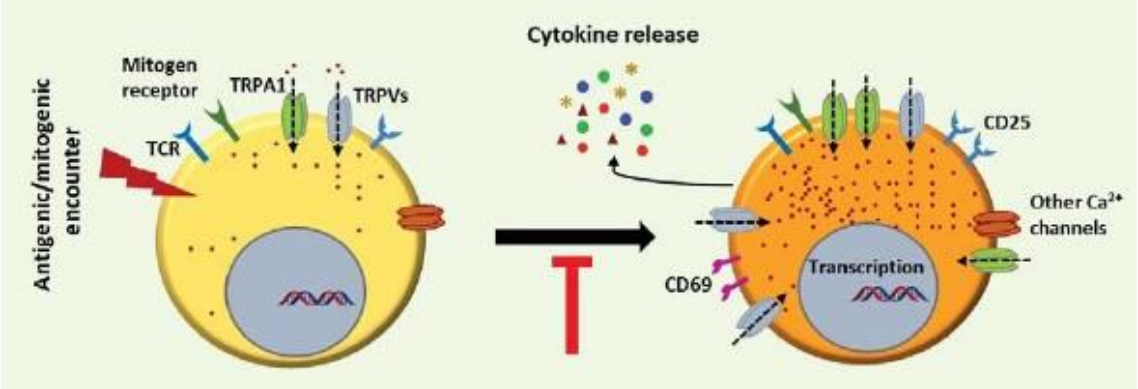

Figure 5: Expression and involvement of TRPA1 in Tcells. Modified from Sahoo et al., 2019.

Serotonin receptors

Serotonin (5-HT) was first discovered by Vittorio Espramer in 1938, isolated from the gastrointestinal tract, and named "enteramine" (Espramer and Boretti, 1950). The name serotonin derives from its vasoconstriction properties ("*sero*" serum and "*tonin*", to constrict). Now, it is one of the most studied chemical messengers for its wide distribution and functions. Serotonin receptors (5-HTR) family comprises seven subtypes, six G protein-coupled receptor and one (5-HT3) ligand-gated cation channel receptor, and 13 receptors (Mc Corvy and Roth, 2015). In the human CNS, almost all the 5-HTRs are expressed (except 5-HT5b), and they are involved in different functions, such as sleep-wake cycle, appetite, emesis, mood, memory or breathing (Ray et al., 2011). Surprisingly, the major quantity of 5-HT in the body is found in the gastrointestinal tract, playing an important role in motility, secretion and gastrointestinal syntoms (Gershon et al., 1990). Cannabinoids can interact with different serotonin receptors, such as 5-HT1a, 5-HT2a and 5-HT3a, as both agonist and antagonist (Russo et al., 2005; Cascio et al., 2010; Cascio et al., 2015 Viñals et al., 2015).

5-HT1aR

5-HT1aR is the most widely distributed of all 5-HT receptors (Pytliak et al., 2011). In the CNS, the highest density of 5-HT1aR was found in areas important for learning and memory, such as the frontal cortex, hippocampus and septum (King et al., 2008). It is expressed as pre-synaptic receptor in the raphe nuclei, where inhibits the release of serotonin at CNS level, and as post-synaptic receptor in different cortical areas, where it modulates the release of dopamine (Altieri et al., 2013). 5-HT1aR is involved in anxiety, and its agonists are getting interest as anti-depressant and anti-psychotic drugs

(Celada et al., 2013). CBD acts as an agonist of 5-HT1aR, with ansiolitic properties (Campos and Guimarães, 2008). Other phytocannabinoids, as cannabigerol (CBG), act as antagonist (Cascio et al., 2010). In an animal model of Alzheimer's disease (AD), 5-HT1aR antagonist (NAD-299) helps to attenuate the neuronal apoptosis, as reported in AD (Shahidi et al., 2019). 5-HT1aR can be found in the gastrointestinal (GI) tract, comprising the myenteric plexus (Pytliak et al., 2011). 5-HT1aR is involved in numerous GI functions, such as secretion and motility. On the stomach, 5-HT1aR agonists act as mucosal protectants, reducing acid and pepsin secretion and increasing adherent mucus production (Farré et al., 1995). These agonists are also involved in inhibition of the gastric motility (Tack et al., 1992), and modulation of colonic motility (Dickson et al., 2010).

Glycine receptors

Glycine receptors are ionotropic receptors that belong to the cys-loop superfamily. These receptors are composed by α (α1 to α4) and β subunits, with a α1 and α3 subunit mainly expressed in the spinal cord, and α2 in the brain (Hejazi et al., 2006). Glycine receptors are involved in pain trasmission and dopamine release, so they play a key role in analgesia and drug addition. They are an important target for nociception at the spinal level (Xiong et al., 2012). Tetrahydrocannabinol (THC) and anandamide (AEA) directly interact with glycine receptors; this interaction contributes to the antinflammatory and analgesic effects of phytocannabinoids in neuropathic diseaes (Morales et al., 2017).

Endocannabinoids

The endocannabinoids are the endogenous ligands of cannabinoid receptors, first isolated in the Nineties (Devane et al., 1995). These molecules are eicosanoids, capable of binding to and activating cannabinoid (Malfitano et al., 2014). Endocannabinoids derived from long chain polyinsatured fatty acids, especially arachidonic acid. Circulating endocannabinoids come from different organs, such as brain, muscle, adipose tissue and circulating cells (Hillard, 2018). Various stimuli can enhance the production of endocannabinoids, and these molecules can have effects on different organs or tissue, as the brain, adipose tissue, liver, gastrointestinal tract or immune system (Figure 6).

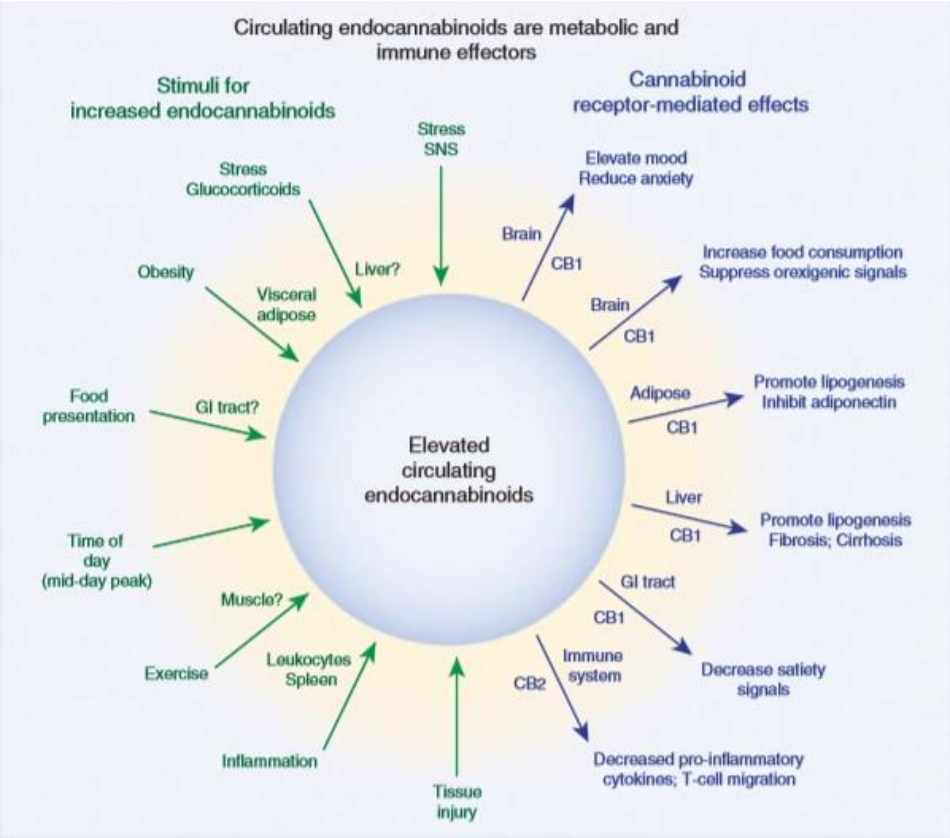

Figure 6: Stimuli and effects of circulating endocannabinoids. Modified from Hillard, 2018.

The principal endocannabinoids are anandamide (N-arachidonylethanolamine or AEA) and 2-AG (2 arachydonyl-glicerol). Other endocannabinoids are 2-arachidonyl-glycerylether (or noladin), O-arachidonoyl-ethanolamine (or virodhamine, or O-AEA), N-arachidonoyl dopamine or (NADA) and other compounds (Pertwee, 2015). Palmitoylethanolamide (PEA) or oleoylethanolamide (OEA) are considered endocannabinoid-like and can directly or indirectly act on cannabinoid receptors (Bradshaw and Walker, 2005).

AEA

Anandamide was the first endocannabinoid to be discovered in porcine brain and belongs to the N-acylethanolamine family (Devane et al., 1992). Anandamide is synthesized on demand from the hydrolysis of a phospholipid precursor, N-arachidonoyl-phosphatidylethanolamine, by a phospolipase D enzyme, in a calcium ion-dependent manner (Di Marzo et al., 1994). The release of AEA is not by vescicles, but via facilitated diffusion through the cell membrane (Mechoulam et al., 1998). AEA is a partial agonist of CB1R and CB2R, with greater affinity for CB1R (De Petrocellis and Di Marzo, 2010). Moreover, it can bind to PPARα (O'Sullivan, 2007), GPR55 (Ryberg et al., 2007) and TRPV1, supposing a possible role of anandamide as "endovanilloid" (Di Marzo et al., 2001; Ross, 2003).

2AG

The 2-AG is a monoacylglicerol, first isolated from rat brain and canine gut (Mechoulam et al., 1995; Sugiura et al., 1995). The production of 2-AG is mediated by diacylglycerol lipase, which converts the phospholipase C (PLC) product diacylglycerol to 2-acylglycerols, including 2-AG and 2-oleoylglycerol (2-OG) (Hillard, 2018). 2AG is a pure agonist of CB1R and CB2R, with greater affinity for CB2R than AEA (Pertwee et al., 2010). As AEA, it can interact with PPARα and GPR55. 2AG is also a metabolic intermediate in lipid synthesis, being the principal source of arachidonic acid in the synthesis of prostaglandin (Nomura et al., 2011).

Palmitoylethanolamide

Palmitoylethanolamide (PEA) is a lipid mediator structurally related to AEA. It was first isolated from egg yolk, soybeans, and peanut meal (Coburn et al., 1954) and then from a variety of food sources (Petrosino et al., 2016). PEA can also be synthesized in the organism, through the hydrolysis of N-palmitoyl-phosphatidyl-ethanolamine by the enzyme N-acyl-phosphatidyl-ethanolamine-selective phospholipase D (NAPE-PLD) (Okamoto et al., 2004). The degradation is mediated by FAAH and NAAA (N-acylethanolamine-hydrolyzing acid amidase) (Ueda et al., 2001). The mechanisms of action of PEA are different. The first method was identified by Rita Levi-Montalcini laboratory, and was named "ALIA" (Autocaoid Local Injury Antagonism) (Aloe et al., 1993). They showed how lipid amides, when administered systematically, could reduce mast cell degranulation, suggesting a strong local anti-inflammatory effect of PEA. PEA can also act directly on different receptors, as PPARα (Lo Verme et al., 2005; Gabrielsson, et al., 2016), GPR55 (Ryberg et al., 2007) and weakly on CB2R. The action on CB2R is principally mediated by an *entourage* effect, reducing the metabolism/ stimulating the synthesis of endocannabinoids AEA and 2-AG (Di Marzo et al., 2001). Finally, PEA acts directly and indirectly on TRPV1 (De Petrocellis and Di Marzo, 2010), increasing the activation of TRPV1 by AEA and 2AG, or activating it through PPARα receptors (Ambrosino et al., 2013). PEA is used both in human and veterinary medicine for its anti-neuroinflammatory, neuroprotective, analgesic, and anti-pruritic properties, and for the action against visceral pain (Re et al., 2007; Gabrielsson et al., 2016; Petrosino and Di Marzo, 2016). The benefits of PEA have been evidenced in several neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), or Multiple Sclerosis (MS). PEA seems to act on neurodegenerative disease modulating the altered expression of proteins in AD or PD and inhibiting pro-apoptotic markers or pro-inflammatory factors (Petrosino and Di Marzo., 2017). The anti-inflammatory properties, as the antipuritic, are principally linked to the ALIA mechanism, with the down regulation of mast cells. The analgesic properties seem to derive from the direct action on PPARα and the

indirect modulation of CB2R (Petrosino and Di Marzo., 2017). A recent study demonstrated that the analgesic effects of PEA are progressive, not linked to age and gender, and not related to the aetiopathogenesis of chronic pain (Paladini et al., 2016).

Phytocannabinoids

Phytocannabinoids are cannabis meroterpenoids and their analogues of plant origin (Hanuš et al., 2016). To date, about 104 phytocannabinoids have been isolated, divided in 11 types: (-) -delta-9-trans-tetrahydrocannabinol (Δ^9-THC), (-) -delta-8-trans-tetrahydrocannabinol (Δ^8-THC), cannabigerol (CBG), cannabichromene (CBC), cannabinol (CBN), cannabidiol (CBD), cannabinodiol (CBDN), cannabielsoin (CBE), cannabicyclol (CBL), cannabitriol (CBT), and myscellaneous-type cannabinoids (Elsohly and Gul, 2014). They have different affinity and function on cannabinoid receptors (Figure 7).

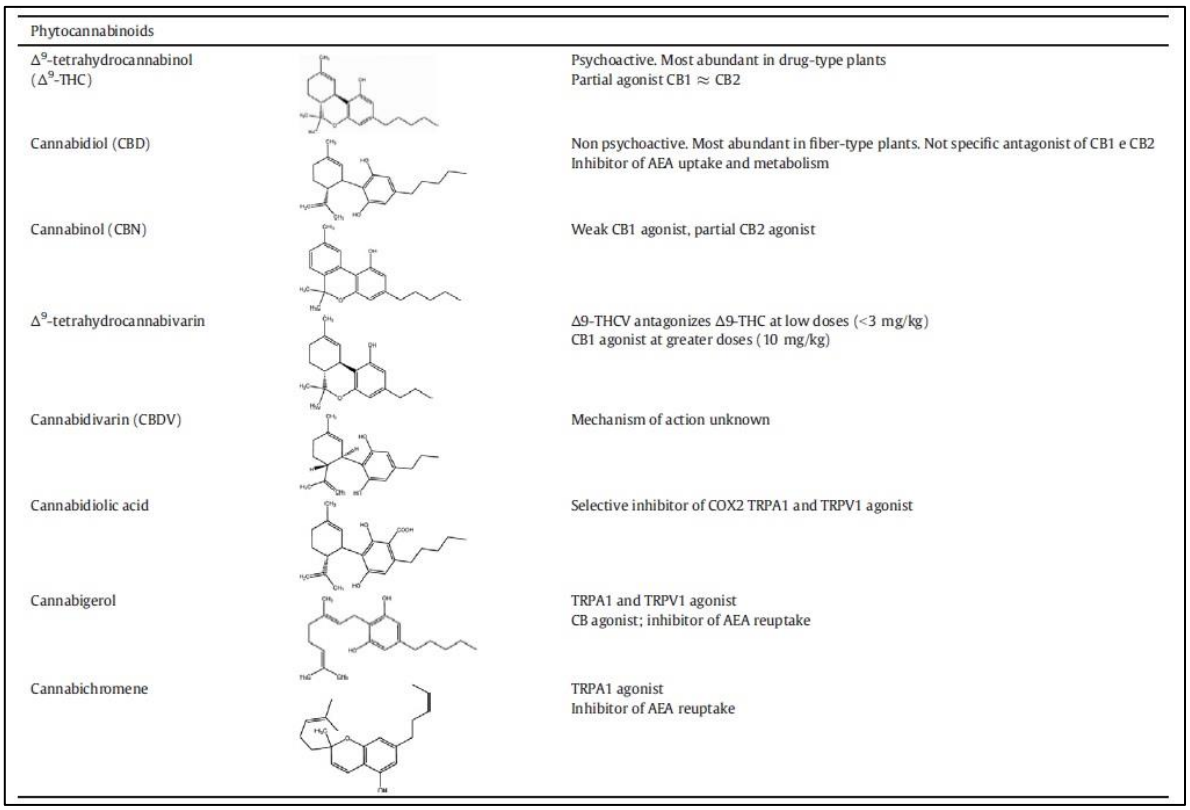

Figure 7: Molecular structure and mechanism of action of phytocannabinoids. Modified from Pisanti et al., 2017.

Δ9-THC is the main compound of *Cannabis sativa*, isolated for the first time in 1964, and well known for its psychoactive side effects (Gaoni and Mechoulam, 1964). Many of the other compounds are non-psychotropic, so they are of extreme interest for their therapeutic properties (Figure 8). The principal non-psycoactive compound in cannabis is Cannabidiol (CBD).

Cannabidiol

Cannabidiol is the second major component of cannabis, after THC, and it is not associated with psychoactivity, alteration of motor function, memory or thermoregulation (Bisogno et al., 2001). For this reason, CBD has been investigated in several models of pathologies, like inflammatory and neurodegenerative diseases, epilepsy, or autoimmune disorders like multiple sclerosis, arthritis, schizophrenia and in neoplastic diseases (Izzo et al., 2009; Pisanti et al., 2017). CBD plays an important role in IBD treatment, through modulation of inflammatory cytokines (Sacerdote et al., 2005), inhibition of mast cells and macrophages recruitment (De Filippis et al., 2011), and reduction of intestinal permeability (Couch et al., 2019). Cannabidiol can act directly on cannabinoid receptors, with low affinity with CB1R and CB2R, or as an *entourage* molecule, both reducing side effects of Δ9-THC (Pisanti et al., 2017). It is also an agonist of TRPV1 (Bisogno et al., 2001), PPARγ and 5-HT1aR (Russo et al., 2005). Moreover, CBD acts on AEA, by inhibiting its uptake and preventing its hydrolisis (Bisogno et al., 2001). CBD can reduce intoxication, sedation and tachycardia induced by THC; on the other hand, it can enhance its analgesic, anti-emetic or anti-carcinogenic properties (Russo and Guy, 2006). So combined preparations with THC and CBD can allow to use higher doses of THC, mantaing the safety for patients. CBD is getting more interest in recent years, as treatment for people unsatisfied by conventional therapies or absence of therapies. CBD is not considered an abuse drug, but the regulation for its sale is not clear. Several products as CBD oil, tinctures and vapor are in commerce, but the absence of regulation exposes to the risk of a poor-quality product, with lower concentration than the effective one, or with biological contamination of the compounds (Pisanti et al., 2017).

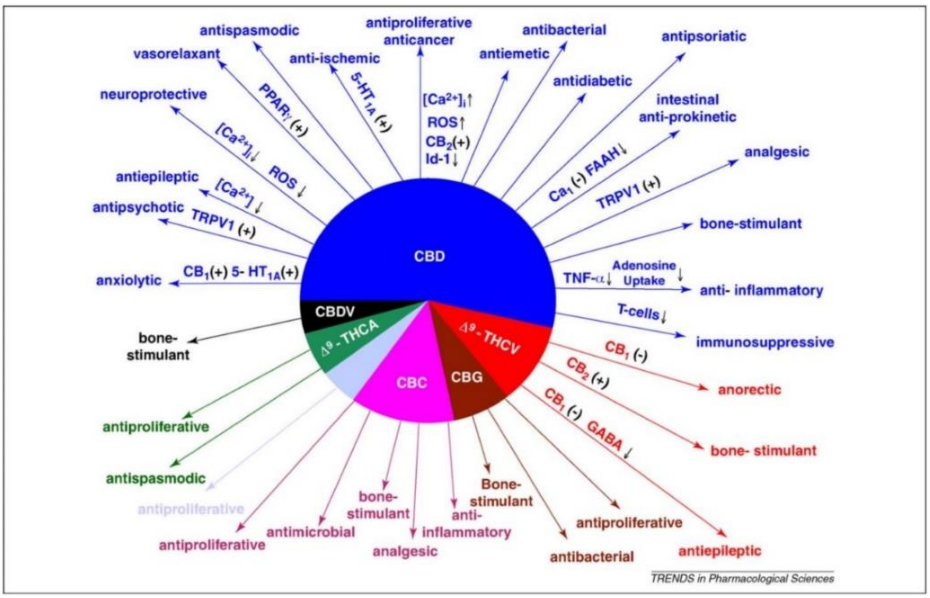

Figure 8: Pharmacological actions of non-psychotropic cannabinoids. Modified from Izzo et al., 2009.

Enzymes for synthesis and degradation

Synthesis

AEA and 2-AG both contain arachidonic acid, but synthesis and degradation are mediated by different enzymes (Pacher et al., 2006). They are synthesized and released *on demand*, in a Ca^{+}- dependent way, following physiological or pathological stimuli (Di Marz o and Deutsch, 1998).
AEA belongs to the family of N-acyl-ethanolamines and is produced from the hydrolysis of the corresponding NAPEs (N-acyl-phosfatidylethanolamines), in a phospholipid-dipendent pathway. The enzyme responsible for this hidrolysis is NAPE-PLD, a phospholipase D selective for NAPE (Schmid and Berdyshev, 2002).
2-AG is principally produced from diacylglycerols (DAGs) hydrolysis by a DAG lipase (DAGL) (Bisogno et al., 2005). There are two isoforms of DAGL: DAGLα and DAGLβ: the first one is predominant in adult CNS; the second plays an important role in 2AG synthesis during immune responses (Hsu et al., 2012).

Degradation

FAAH (fatty acid amide hydrolase) is the principal enzyme involved in anandamide turnover and, in some cases, 2-AG hydrolisis (Cravatt et al., 1996; Cravatt and Lichtman, 2002). FAAH is responsible for the degradation of multiple fatty acide amides, including PEA. It is a membran protein and it has a high degree of conservation between mouse and human (Bisogno et al., 2005). In addition to FAAH, AEA can be degradated via oxidation by COX-2 (cyclooxygenase-2), to create prostamides (Woodward et al., 2008). The third route of degradation is via N-acylethanolamine-hydrolizing acid amidase (Tsuboi et al., 2005). 2-AG can be degradated by FAAH hydrolysis or COX-2 oxidation, but there are other enzymes primarly involved in its degradation, as MAGL (monoacyl-glycerol lipase), alpha/beta domain-containing hydrolase 6 (ABHD6) and alpha/beta domain-containing hydrolase 12 (ABHD12).

The gastrointestinal tract

The digestive system (*systema digestorium*) includes all the organs involved in the digestive processes. In addition to digestion and absorption, due to its continuous contact with the external environment, the digestive system is the largest immunological organ in the body (Denbow, 2015). The gastrointestinal tract is a long tube starting with the oral cavity (*cavum oris*) and terminating with the anal canal (*canalis analis*), and, with the teeth (*dentes*) and the annexed glands, composes the digestive system (Figure 9) (Barone, 2006). All the anatomical references are based on the Nomina Anatomica Veterinaria (Constantinesco et al., 2017).

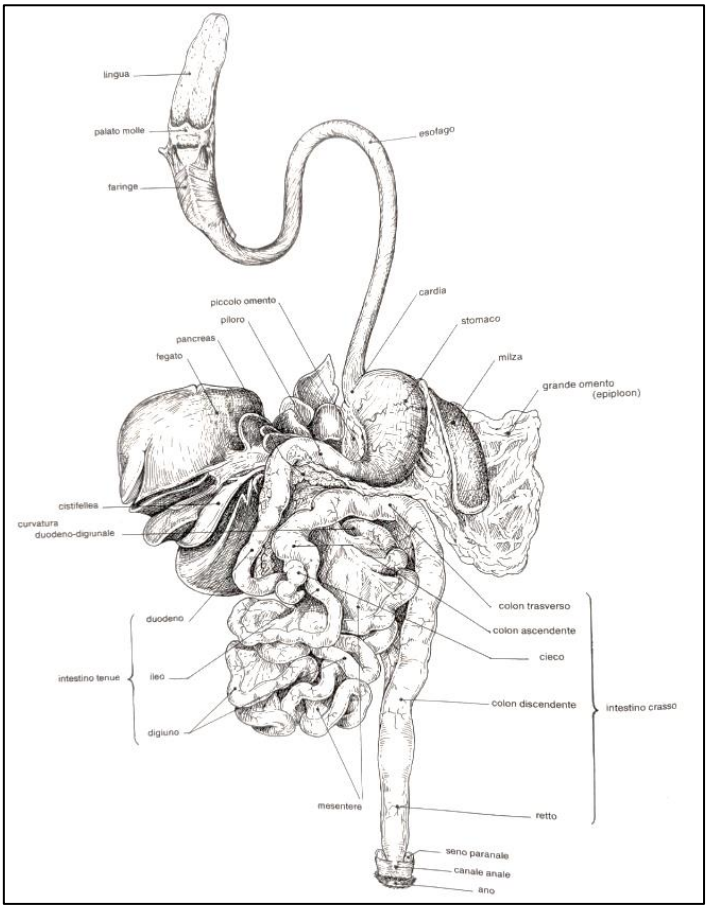

Figure 9: The canine gastrointestinal tract. Modified from Barone, 2006.

Anatomy

From the esophagus (*esophagus*) to the colon (*colon*), the gastrointestinal wall is composed of four layers (from the inner to the outer layer): mucosa (*tunica mucosa*), submucosa (*tela submucosa*), main muscular coat (*tunica muscularis*) and serosa (*tunica serosa*) (Figure 10).

The *tunica mucosa* is composed by the epithelium (*epithelium*), lamina propria (LP, *lamina propria mucosae*) and *muscularis mucosae* (*mm*, *lamina muscularis mucosae*). The epithelium, in close contact with the ingested food, is responsible for the absorption of nutrient, water and electrolytes. The lamina propria is the home of intestinal immunitary cells, i.e. B and T lymphocytes, macrophages, mast cells and dendritic cells. The *mm* is composed of two layers: the inner circular layer and the outer longitudinal layer (Frappier, 2000).

The *tela submucosa* is composed by connective tissue, rich in lymphatic and blood vessels, nerves, immune cells and, in some tracts (esophagus, duodenum), submucosal glands (Gelberg, 2014). Where there is no *mm*, the submucosa, in contact with the lamina propria, becomes a *propria-submucosa*. In the submucosa, there is the Meissner plexus, or submucosal plexus (SMP) (*plexus submucosus*), part of the ENS.

The *tunica muscularis* is made of two parts: the circular muscle layer (CML, *stratum circulare*) and the longitudinal muscle layer (LML, *stratum longitudinale*). The CML is the inner part, and it is responsible for the mixing movements of the intestinal contents, while the external portion (i.e. LML) helps in the progression of the digested food. Between the two muscular layers resides the Auerbach plexus, or myenteric plexus (MP) (*plexus myentericus*), responsible for the contractile actions of the *tunica muscularis* (Collins and Badireddy, 2019).

The *tunica serosa* is the external layer of the gastrointestinal wall, in the portion included in serosal cavities (mediastinum, abdomen and pelvic cavity). It is composed by connective tissue covered by mesothelium, a simple squamous epithelium. In the portions of the gastrointestinal tract outside from serosal cavity (cervical esophagus, retroperitoneal rectum), the external portion is called *tunica adventitia*. This layer is not covered by mesothelium (Frappier et al., 2000).

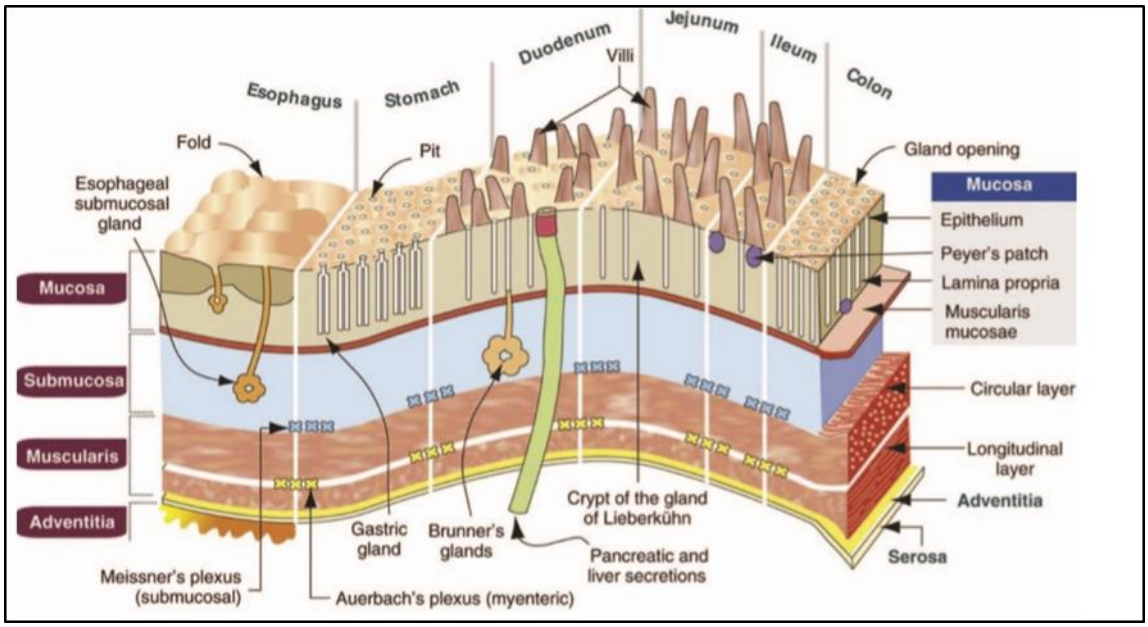

Figure 10: Schematic diagram of the anatomic and histologic organization of the digestive tube. Modified from Kierzenbaum, 2002.

Esophagus

The esophagus is a muscular-membranous tube that connects the pharynx (*pharynx*) with the stomach (*ventriculus*). It starts with a proximal esophageal sphincter and ends with the cardias (ostium cardiacum), the sphincter that controls the passage in the stomach (Barone, 2006). The esophagus, through the course in the neck, is dorsal to the trachea except for the distal part, where it moves to the left (in Carnivores it remains quite dorsal). In the mediastinum, it moves again to a dorsal position and passes to the right of the aorta. Through the esophageal hiatus, the esophagus crosses the diaphragm, and after a small abdominal portion, it becomes stomach (Barone, 2006).

The mucosa is composed of three layers: the stratified epithelium, keratinized in swines, equids, ruminants, rats, and mice and non-keratinized in carnivores and humans; the LP, composed by collagens and elastic fibers; and the *mm*, which is incomplete in the dog and the pig (just in the distal part) (Barone, 2006; Gelberg, 2014). The submucosa is made of connective tissue, rich in elastic fibers and really loose, to allow wide movements of the mucosa (Barone, 2006). Submucosal mucus glands (i.e. Brunner's glands) are present at the pharyngeal junction in equids, ruminants, rabbits and rodents; in the first half of the esophagus and just some sporadic glands in the rest of the esophagus in the pig; throughout the esophagus in dogs and humans (Gelberg, 2014). In the horse, these glands are also present in the third caudal of the esophagus (Chiocchetti et al., 2015). The *tunica muscularis* is composed by the LML and the CML; it includes striated muscle cells partially mixed with smooth cells. Smooth muscle cells replace entirely the striated cells at the cardias in ruminants and dogs and in the last 4-5 cm in the pig. In humans, cats and equids, the change is gradual and it becomes complete

when the esophagus crosses the aorta (Chiocchetti et al., 2015). The *tunica adventitia* wraps the esophagus in the cervical portion, becoming thicker in the thorax; a full serosa is present in the small abdominal portion of the esophagus (Gelberg, 2014).

Stomach

The stomach is the first essential organ for the digestion, where the enzymatic and hydrolytic processes start (Frappier, 2000). It is connected to the esophagus with the cardias, and to the duodenum (*duodenum*) with the pylorus (*pylorus*); it is divided in fundus (*fundus ventriculi*), corpus (*corpus ventriculi)* and antrum (*antrum pyloricum*). It is more developed and dilatable in carnivores than omnivores or herbivores (Barone, 2006). The mucosa is completely glandular in carnivores, humans and rabbits. In equids, pigs ans rodents, the mucosa is just partially grandular; the aglandular portion is covered with stratified epithelium, and is linked to the glandular portion with an irregular linear area called *margo plicatus*. The mucosa has big gastric folds that disappear with the distension of the organ. The surface of the mucosa has diffuse gastric pits (*foveolae gastricae*), which denote entrances to tubular shaped gastric glands, located in the LP. The mucous secrete of the glands protects the mucosa from autolytic processes. The mucosa is divided, based on the kind of glands, in cardial region (*pars cardiac*), fundic region (*fundus ventriculi*) and pyloric region (*antrum pyloricum*) (Frappier, 2000). The cardial region is widely extended in the pig but limited to the *margo plicatus* in other species. These glands have a mucous secrete. In the fundic region, glands are composed by a bottom, a body, a collar, and an isthmus that comes out at the level of the gastric pits. These glands contain mucous cells of the collar (mucocytes), principal cells, and parietal cells in the body and endocrine cells in the bottom. Mucocytes are cuboid cells secreting mucus that covers the collar of the glands. Principal cells are the most numerous in the gastric region, and are responsible of the production of pepsinogen, transformed in pepsin by cloridric acid (HCl) (Frappier, 2000). The latter (HCl) is the product of parietal cells. Endocrine cells provide gastrointestinal hormones, such as gastrin, cholecystokinin or PYY (Fothergill et al., 2019). The pyloric region occupies half of the stomach in carnivores, and the pyloric glands contain typical mucus secreting cells. The submucosa is closely linked to the mucosa, following the gastric folds (Barone, 2006). The *tunica muscularis*, as the other intestinal tracts, includes a LML and CML; the serosa is made up by the visceral peritoneum (Barone, 2006).

Small intestine

The small intestine (*intestinum tenue*) is a fundamental component of the digestive system, which allows the absorption of important nutrients. The small intestine includes duodenum, jejunum

(*jejunum*) and ileum (*ileum*). The duodenum is the first section, connected to the antrum of the stomach by the pylorus. The duodenum surrounds the right lobe of the pancreas (in Carnivores), in the shape of a "C." The duodenum is a “mixing pot”, where the intestinal digestion starts. It receives the chyme from the stomach, pancreatic enzymes to break down the products from the stomach, and bile from the liver for the digestion and absorption of fat from food products. The jejunum is the longest (and the most mobile) portion of the small intestine, and with the ileum is principally involved in absorption. The ileum is the last part of the small intestine; its major absorptive products are vitamin B12 and bile acids (Collins and Badireddy, 2018). The histological structure of the wall is almost similar in all the small intestine, showing just little differences. The serosa consists of loose connective tissue covered by mesothelium (Frappier 2000). The *tunica muscularis* consists of the thin LML (thicker in the horse), and the CML. In the connective tissue between the two layers there is the MP (Frappier, 2000; Collins and Badireddy, 2018). The submucosa is made of connective tissue that contains blood and lymphatics vessels, and the SMP. Brunner’s glands are submucosal mucous glands in dogs and ruminants, serosal glands in equids and sero-mucosal in the cat (Frappier, 2000). All along the small intestine, but especially in the ileum, the submucosa includes the Peyer’s patches, which are aggregated lymphatic nodes. The mucosa is composed by epithelium, LP and *mm*. The mucosa is designed for the maximal absorption, so it is covered by intestinal villi (*villi intestinales*), protrusion of the mucosa that increases the surface area (Collins and Badireddy, 2018). The intestinal glands, also called crypt of Lieberkühn or intestinal crypt, are located between the villi. Villous height and crypt depth decrease aborally. There is a variety of epithelial cell types in the intestine, produced by progenitor cells in the crypts: enterocytes, mucous goblet cells, enteroendocrine cells (EECs), Paneth cells, and M cells (Figure 11) (Leibich, 2012).

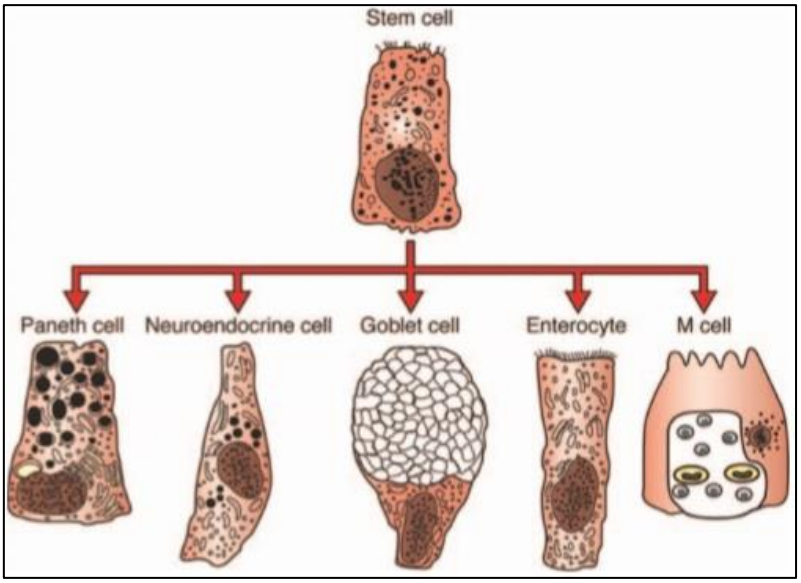

Figure 11: Schematic illustration of the epithelial cell types of the small intestine. Modified from Gelberg, 2014.

Enterocytes are the principal cells of the intestinal mucosa. Microvilli, on the apical surface of enterocytes, increase their surface area, creating the brush border. Mucous goblet cells decrease going from the botton to the apex of the villi, and increase going aborally towards the large intestine (Gelberg, 2014). These cells produce mucus, fundamental to help the progression of the intestinal content, protect the mucosa from bacterial invasion or to avoid autolytic processes (Liebich, 2012). Enteroendocrine cells (EECs) are the source of gastrointestinal hormones, like gastrin, secretin, cholecystokinin, PYY, 5HT or others (Fothergrill and Furness, 2018). Historically, they were named with a letter code, considering the hormone produced or ultrastructural features identified by electron microscopy (Table 1). In the last decades, different studies highlight the fact that EECs usually contain more than one hormone, usually concentrated in separated vescicles (Helander and Fändriks, 2012; Drucker, 2015; Fothergrill and Furness, 2018).

LETTER CODE	NAMED FOR	IDENTIFYING HORMONE (historical view)	Exemple of colocalization
EC (enterochromaffin)	Reaction with chrome salts	5HT	CCK, secretin, tachykinins, motilin, ghrelin, GLP-1, PYY
S	Small vescicles	secretin	Ghrelin, CCK, 5HT
I	Intermediate size vescicles	CCK	Proglucagon, PYY, GIP, ghrelin, secretin, neurotensin
L	Large vescicles	GLP-1 and PYY	GIP, CCK, secretin, ghrelin, 5HT, neurotensin
ECL (EC-like)	Similarity to EC cells	histamine	pancreastatin
X/A	X for unknown product A for similarity to pancreatic A cells	Ghrelin	Secretin, CCK, proglucagon, motilin
D	Similarity to pancreatic D cells	Somatostatin	GIP
K	Vescicles that differentiate from L cells	GIP	GLP-1, secretin, CCK, PYY, somatostatin
N	Neurotensin content	Neurotensin	GLP-1, PYY
G	Gastrin content	Gastrin	Co-expression not investigated adeguately
M	Motilin content	Motilin	5HT, ghrelin

Table 1: The historical naming of enteroendocrine cells. Modified from Fothergrill and Furness, 2018.

Paneth cells are interspersed between the intestinal stem cells in the intestinal glands. Their cytoplasm contains large acidophil granules, rich in lysozyme (Frappier, 2000). M cells are considered as phagocytary cells, able to tie to alimentary, bacterial or viral antigens and to bring them to the closest lymphocytes, causing an immunitary response (Samuelson, 2007). The lamina propria of the mucosa creates the axis of the villi and surrounds the intestinal glands. Inside this layer, there are blood and lymphatic vessels, smooth muscle cells, fibrocytes, lymphocytes, plasmacells, and mast cells (Frappier, 2000). In the central portion of the lamina propria of the villus, there is the central lacteal, a lymphatic capillary that absorbs dietary fats. Two layers of smooth muscular cells, longitudinal and circular, as usually compose the muscular layer (*mm*) of the mucosa.

Large intestine

The large intestine (*intestinum crassum*) begins at the terminal ileum with the cecum (c*ecum*), continues with the colon, rectum (*rectum*), and terminates with the anal canal (Kahai et al., 2018). In

the large intestine there is the absorption of water and electrolyte and the secretion of mucus, to help washing the intestinal contents and extracting the last nutrients (Frappier, 2000; Barone, 2006). The large intestine is more developed in herbivores (especially non-ruminants) than carnivores, to help the demolition of great amount of aliments containing cellulose. The characteristic of the large intestine, preserved in all the tracts, are absence of villi and development of intestinal glands, increase number of goblet cells, and increasing number of lymphatic nodules (*noduli lymphatici*) (Frappier, 2000). The cecum is a fermentation chamber, important in equids and not developed in carnivores. In all its extension, the cecum shows a great number of lymphatic nodules. The colon, as the cecum, is developed in equids and in the pig. The mucosa of the colon is thicker than in the small intestine because of the longer intestinal glands. The rectum is the terminal part of the large intestine. The mucosa is similar to the cecum and colon, with an increased number of goblet cells. The serosa surrounds the rectum in the cranial portion, while the retroperitoneal tract is covered by an adventitia. The anal canal is the termination of the digestive tube that connect the rectum with the outside (Barone, 2006). As highlighted before, the structure of the large intestinal wall is quite similar along its course.The serosa surrounds the large intestine until the cranial portion of the rectum. The inner circular and outer longitudinal muscle layers compose the *tunica muscularis*. The submucosa is similar to the small intestine, except for the cecum and rectum, where it is thicker for the presence of numerous lymphatic nodules. The mucosa shows thick *mm*, a lamina propria rich in lymphocytes, and an epithelium with numerous intestinal glands, longer than in the small intestine and richer in goblet cells that deepen until the *mm*. At the bottom of the glands, numerous stem cells are responsible for the epithelial renewal (Barone, 2006). The anal mucosal drastically changes, showing a stratified squamous epithelium.

The Enteric Nervous System

The enteric nervous system (ENS) is a complex network of neurons and glial cells in the gut wall that controls many functions of the intestinal tract, such as motility, absorption, secretion, and it is involved in pathological processes of the digestive system (Lake and Heuckeroth, 2013). The ENS interacts with the CNS, but in the same way, it can control the digestive functions without relying on commands from the CNS. The enteric nervous system interacts also with the endocrine and immune systems, and has roles in modifying nutrient absorption and maintaining the mucosal barrier (Furness, 2012). In humans, the ENS is composed of 400-600 millions of neurons and even more supporting cells. Nerve cells and glial cells are grouped in small clusters that compose the enteric ganglia, which are connected to each other by nerve fiber bundles (Furness, 2006). There are two major plexuses, the MP and the SMP (Figure 12).

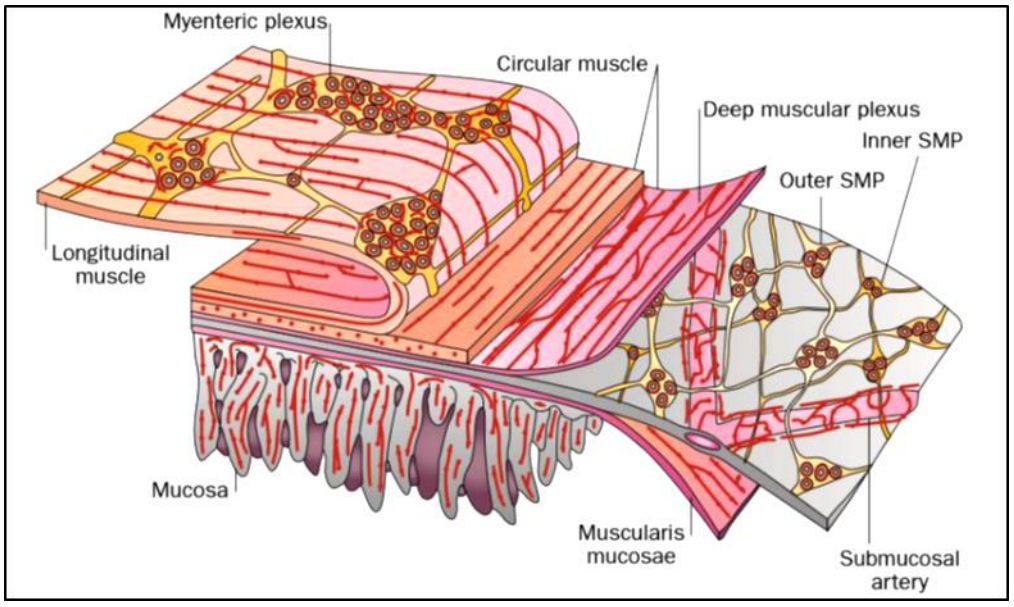

Figure 12: Organization of the ENS in human and medium/large mammals. Modified from Furness, 2012.

The MP is located between the longitudinal and circular layers of the *tunica muscularis*, while the SMP is located in the submucosa, between the *muscularis mucosae* and the CML. MP and SMP are connected between each other by vertical fibers, perpendicular to the CML (Furness, 2006). The MP is continuous around the wall and along the entire gastrointestinal tract, from the upper esophagus to the internal anal sphincter (Figure 13). MP ganglia are larger and more numerous than those within the SMP, and are connected with primary strands, with longitudinal course, which constitute, together with the ganglia, the MP primary plexus. The secondary plexus is composed by nerve fibers parallel to the CML. The tertiary plexus includes the smallest interconnecting strands, which supply the LML. The SMP can be identified in the intestine; sporadic ganglia can be found also in esophageal and gastric submucosa, but they do not constitute a real plexus (Schemann et al., 2001). In the esophagus of the horse, the SMP is well developed and organized in two layers (Chiocchetti et al., 2015). As the MP, the SMP is continuous around the circumference and for all the length of the small and large intestine (Furness, 2006). In the SMP of large mammals, ganglia form different plexuses that lie on distinct layers, constituting an inner and an outer plexus. Briefly, neurons of the MP mainly regulate muscle functions, while SMP neurons should control epithelial functions. However, it is known how some neurons of the outer SMP participate, in large mammals, in the innervation of the CML and LML, while the inner SMP supplies principally the mucosa (Porter et al., 1999). Both plexuses control immune functions, cell proliferation and microcirculation (Schemann et al., 2019).

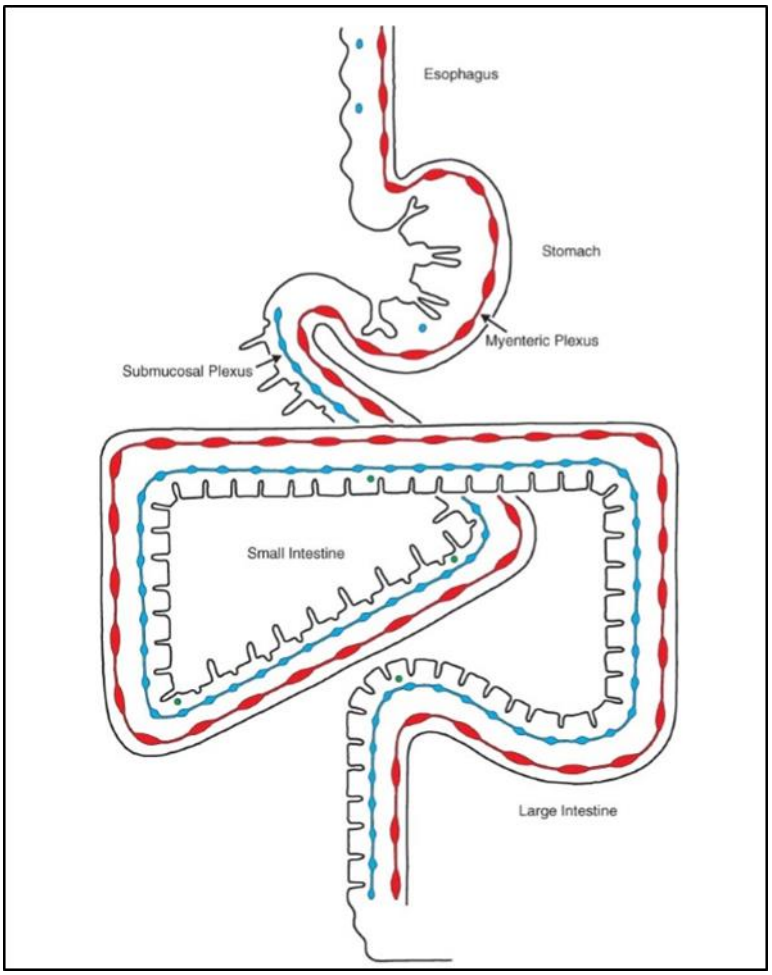

Figure 13: Distribution of enteric ganglia in the tubular digestive tract. Modified from Furness, 2006.

Neurons

Enteric neurons are classified considering their shape, neurochemical code, projections, electrophysiological properties, and function. In 1989, using a methylene blue staining, Dogiel identified three type of neurons, named Dogiel types I, II and III. In the following years, several authors proposed new classifications, arriving at a new classification including type I, II, III, IV, V, VI and VII and "mini-neurons" (Brehmer, 2006). The neurochemical code is the combination of messengers that a neuron contains (Costa et al., 1996). Primary neurotransmitters include Acetylcholine (Ach) and tachykinins (as substance P, SP) in excitatory motor neurons, vasoactive intestinal polypeptide (VIP) and nitric oxide (NO) in inhibitory motor neurons. While primary neurotransmitters are conserved across species and along the gastrointestinal tract, secondary neurotransmitters are different considering the various tract and the different species (Furness, 2006). Examples of primary and secondary neurotransmitter are shown in Table 2.

TYPE OF NEURON	PRIMARY TRANSMITTER	SECONDARY TRANSMITTERS	OTHER NEUROCHEMICAL MARKERS
Enteric excitatory muscle motor neuron	Ach	Tachykinin, enkephalin (presynaptic inhibition)	Calretinin, γ
Enteric inhibitory muscle motor neuron	NO	VIP; ATP carbon monoxide	PACAP, opioids
Ascending interneuron	Ach	Tachikinin, ATP	Calretinin, enkephalin
ChAT, NOS descending interneuron	ATP, Ach	ND	NO, VIP
ChAT, 5-HT descending interneuron	Ach	5HT, ATP	ND
ChAT, somatostatin descending interneuron	Ach	ND	Somatostatin
Intrinsic sensory neuron	Ach, CGRP, tachykinin	ND	Calbindin, calretinin, IB4 binding
Interneurons supplying secretomotor neurons	Ach	ATP, 5HT	ND
Non cholinergic secretomotor neuron	VIP	PACAP	NPY (in most species)
Cholinegic secretomotor neuron	Ach	ND	Calretinin
Motor neuron to gastric cells	GRP, Ach	ND	NPY
Motor neurons to parietal cells	Ach	Potentially VIP	ND
Sympathetic neurons, motility inhibiting	Noradrenaline	ND	NPY in some species
Sympathetic neurons, secretion inhibiting	Noradrenaline	Somatostatin (in guinea pig)	ND
Sympathetic neurons, vasoconstrictor	Noradrenaline, ATP	Potentially NPY	NPY
Intestinofugal neurons to sympathetic ganglia	Ach	VIP	Opioid peptides, CCK, GRP

Table 2: Neurotransmitters in the digestive tract. Modified from Furness, 2012.
Abbreviations: 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; CCK, cholecystokinin; ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide; GRP, gastrin releasing peptide; ND, not determined; NPY, neuropeptide Y; NOS, nitric oxide synthase; PACAP, pituitary adenylyl-cyclase activating peptide; VIP vasoactive intestinal peptide.

Considering their function, enteric neurons can be classified in motor neurons, interneurons, intrinsic primary afferent neurons (IPANs), and intestinofugal primary afferent neurons (IFANs) (Furness, 2003). Motor neurons can be excitatory and inhibitory, and are responsible to innervate gut musculature (LML, CML and *mm*); they are located principally in the MP and, to a lesser extent, in outer and inner SMP (outer> inner) in humans and large mammals (Hens et al., 2001). Enteric interneurons are present in all the gut, but differ among the different tracts (Furness, 2006); these neurons are mainly localized in the MP, but have long projections that extend orally and anally (Bornstein et al., 2004). IPANS are intrinsic primary afferent neurons that respond to different stimuli, such as luminal chemicals, distortion and mechanical stimulation of the mucosa (Furness, 2006). IFANS are intestinofugal primary afferent neurons that have the cell bodies in the gut wall, with their processes going to prevertebral ganglia, where they interact with post-ganglionic sympathetic neurons (Szurszewski et al., 2002). The sympathetic neurons innervated by IFANs are inhibitor neurons for motility and secretion (Furness, 2006).

Enteric glial cells

Enteric glial cells (EGCs) were first observed in 1899 by Dogiel. The word glia derived from the Greek "γλοια", that means "glue" of the enteric nervous system (ENS). EGCs are satellite cells, which represent the largest cell population of the ENS, outnumbering from three to five times enteric neurons (Gabella, 1981; Jessen, 2004; Furness, 2006). EGCs are small cells with a star-like shape, comparable to astrocytes in the CNS. They envelop enteric neuronal cell bodies and axon bundles, and their processes reach the intestinal mucosa (Ruhl, 2005). Differently from neurons, they are not excitable cells, but they communicate through Ca^{++} signalling, and integrate their informations with neurons, immune cells, and other cells of the gastrointestinal tract (Ochoa-Cortes et al., 2016). EGCs can be found in MP and SMP, but also in smooth muscle layers and gut mucosa (Hoff et al., 2008). Considering their localization, EGCs are divided into four types: type I, with a star-shaped morphology within ganglia; type II, more elongated, for interganglionic EGCs; type III for mucosal; and type IV for intramuscular EGCs (Boesmans et al., 2015). Until recently, structural support was considered their main function, but recently it was highlighted their vital role in gut homeostasis (Ochoa-Cortes et al., 2016). In physiological conditions, EGCs are principally involved in neuronal functions (neuroprotection, neuromediator expression, or neuronal renewal) and regulation of intestinal epithelial barrier (IEB) homeostasis. Under pathological conditions, such as inflammation or bacterial stimulation, reactive enteric gliosis can develop. Altered enteric gliosis can both exacerbates intestinal inflammation and protect IEB and neurons from inflammatory processes (Neunlist et al., 2014).

Intertitial cells of Cajal

Interstitial Cajal cells (ICC) were first described by Santiago Ramòn y Cajal in 1889, and he defined them "interstitial neurons" because they were labeled through staining techniques specifically for neurons (as methylene blue or silver impregnation), and were found in the interstitium between nerve endings and smooth muscle cells (Cajal, 1911). ICC are fusiform cells with few processes, with a large oval nucleus containing one or more nucleoli (Faussone-Pellegrini and Thuneberg, 1999). According to Cajal, these cells modulate the contraction of smooth muscle cells of the GI tract. After him, several groups studied these cells, understanding they were not neuronal cells, and defined them as Interstitial Cajal cells. ICC are considered as pace-maker cells of the alimentary tract. Cajal cells are characterised by ability to spontaneously depolarise and create slow waves, generating the basic electrical rhythm of smooth muscle cells. Slow waves migrating from ICC toward myocytes of the longitudinal layer, induce electrotonic energy within internal circular layer. Removal of ICC causes a lack of slow waves in the remaining part of smooth muscle cells (Pasternak et al., 2016). Pacemaker ICC activity starts with periodic release of Ca^{++} from endoplasmatic reticulum, which activates mitochondria to intake ions, generating the potential energy (Ward et al., 2000).

The endocannabinoid system and the GUT

The endocannabinoid system (ECS) participates in many physiological functions of the gut (Taschler et al., 2017). Its role has been demonstrated in regulation of the appetite, intestinal motility, secretion, nausea and emesis, visceral nociception and inflammation (Izzo and Sharkey 2010). The CB1R is involved in the regulation of appetite: CB1 agonists, such as THC, stimulate appetite and promote weight gain (Matias and Di Marzo, 2007). Otherwise, CB1 antagonists, as rimonabant, were used in the treatment of obesity, reducing the ingestion of food and the body weight (Riedel et al., 2009). The PPARα stimulation evokes satiety as an answer to the ingestion of food (Lo Verme et al. 2005). The principal problem with CB1R drugs is the distribution in the CNS, with psychoactive effects. Therefore, in these last years researchers focus on developing new drugs to act selectively in the periphery, avoiding the action on the CNS (Izzo and Sharkey, 2010).

Different cannabinoid receptors, e.g. CB1R, GPR55 and TRPV1, reduce intestinal motility both in physiological and pathological conditions. Their action is principally linked to the presence of CBRs in the muscular layers and in the myenteric and submucosal plexuses, highlighting the importance of the interaction between the ECS and ENS (Pertwee, 2001). The CB1R has also an inhibitory effect on gastrointestinal secretion and on visceral hyperalgesia, as CB2 receptor; this effect is enhanced in inflammatory conditions (Mahmud et al., 2009). Moreover, CBR agonists are used to reduce nausea

and emesis associated with chemotherapy. In animal models, the phytocannabinoid cannabidiol (CBD) has been demonstrated to significantly reduce nausea and vomit (Izzo and Sharkey, 2010). Increasing evidences show that CBR expression and/or the level of endocannabinoids are altered in patients with intestinal diseases, suggesting a possible role of the ECS in intestinal pathophysiology (Izzo and Camilleri, 2009; Alhouayek and Muccioli, 2012). Studies on animal models reinforce the hypothesis that drugs acting on ECS, such as CBR agonists or inhibitors of degradating enzyme FAAH and MAGL (resulting in increased endocannabinoids), can have protective properties from intestinal inflammation (Kimball et al., 2006; Alhouayek et al., 2011). For example, CBD, with low affinity for CB1 and CB2, exerts its protective role in colitis acting both directly on other cannabinoid receptors and inhibiting FAAH (Bisogno et al., 2001). PEA reduces intestinal inflammation and permeability in murine colitis acting via CB2R, GPR55, PPARα and TRPV1 (Esposito et al., 2013; Borrelli et al., 2015).

Chronic enteropathies

Chronic gastrointestinal inflammatory pathologies can affect humans and animals, with deep consequences in the quality of life and, considering productive livestock, in the production. These enteropathies show some common elements in the pathogenesis, clinical presentation or therapies between different species. Therefore, studying chronic enteropathies in animals can be useful both for veterinary and human medicine.

Humans

Inflammatory bowel disease (IBD) is a global healthcare problem, with an increasing incidence in the last years (Xavier and Podolsky, 2007). IBD is a chronic, uncontrolled inflammation of the gastrointestinal tract; although the etiology remains uncertain, the environment, genetic alterations, the intestinal microbiota and the immune system are involved in the pathogenesis (Figure 14) (Zhang and Li, 2014). The two most common subtypes are Crohn's disease (CD) and ulcerative colitis (UC), which present some differences (Hanauer, 2006). CD can affect every part of the GI tract (principally ileum and perianal region), while UC is usually limited to the colon. Moreover, CD inflammatory process is transmural, UC tendentially mucosal. Finally, CD is associated with complications such as fistulas, abscesses or stenosis (Abraham and Cho, 2009).

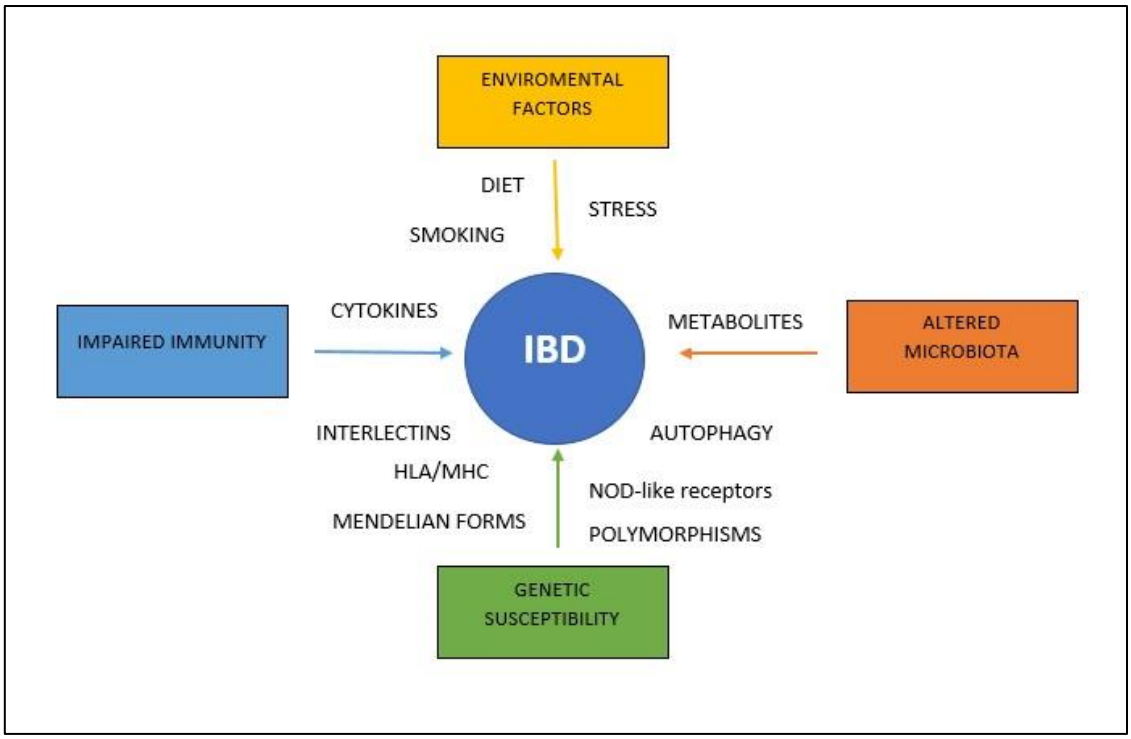

Figure 14: Factors implicated in the pathophysiology of inflammatory bowel disease (IBD). Modified from Karantos and Gazouli, 2011.

Laboratory animals as rats, mice or guinea pigs are often used as model for human pathologies, inducing iatrogenic mucosal inflammation with chemicals. Dextran sulfate sodium (DSS)-induced colitis is a model of Th2 mediated immune response; trinitrobenzene sulfonic acid (TNBS)-induced colitis is more dominated by a Th1 response (Wirtz et al., 2007; Alex et al., 2009). In these experimental models of IBD, the ECS is overexpressed compared to healthy animals (Kimball et al., 2006). Indeed, the tissue levels of CBR are also altered in these experimental models: CB1R is increased in MP neurons of colon of inflamed mice, and blocking CB1 with antagonists increases the severity of DNBS (2,4-dinitrobenzene sulfonic acid) and DSS colitis (Massa et al., 2004). In another study in models of induced-colitis in mice, CB1R and CB2R agonists both reduced inflammation improving disease symptoms and decreasing histological inflammatory scores (Kimball et al., 2006). Analysis of the ECS in biopsies from IBD patients evidences increased AEA levels in mucosal biopsies of UC colons vs control biopsies (D'Argenio et al., 2006). However, another study shows that AEA levels were lower in inflamed mucosa than in controls, with a reduced level of NAPE-PLD (AEA- synthesizing enzyme) and increased FAAH (AEA-degrading enzyme) (Di Sabatino et al., 2011). In the same study CB1R but not CB2R was over-expressed in CD and UC patients; otherwise other studies evidenced an increased CB2R in inflamed tissues (Wright et al., 2005; Mahmud et al., 2009). Although results from literature are often in contrast, different prospective studies found a benefit in the use of cannabinoids for IBD. A prospective placebo-controlled study in 21 patients with CD, consisting in 8 weeks-protocol with cannabis, shows beneficial effects as improved appetite and

sleep in 90% of patients (Naftali et al., 2013). Another study on 100 patients with UC and 191 with CD reveals that 33% of UC subjects and 50% of CD were cannabis-lifetime users, to reduce IBD-related symptoms such as diarrhea and abdominal pain (Longstreth et al., 2006).

In addition to CD and UC, the role of the ECS has been evidenced in different intestinal diseases in humans, as Irritable Bowel Syndrome (IBS), diverticulitis, celiac disease and colon cancer (Table 3) (Lee et al., 2016).

Disease		Diverticulitis	Celiac disease		IBS		IBD			CRC	
		Human	Human	Animal	Human		Human		Animal	Human	Animal
					IBS-D	IBS-C	CD	UC			
Ligands	AEA	Increased	Increased	Increased	No change	No change	Decreased	Increased or Decreased	Increased	Increased	No change
	2-AG	Decreased		Increased	Increased	No change	No change	No change	No change	Increased	Increased
Receptors	CB_1	No change (R,P)	Increased (R,P)		Genetic polymorphism	Genetic polymorphism	Increased (R,P) Genetic polymorphism	No change (R,P) or Increased (P) Genetic polymorphism	Increased (P)	Decreased (R,P)	No change (R)
	CB_2		Increased (R,P) Genetic polymorphism				Increased (P) or No change (R,P)	Increased (P) or No change (R,P)	Increased (R)	Increased (P) or No change (R)	No change (R)
	TRPV1				Increased (P)	Increased (P)					Decreased (R)
Metabolic enzymes (Synthesis)	NAPE-PLD		Increased (R,P)				Decreased activity	Decreased (P) Decreased Activity			
	DAGL							Increased (P)			
Metabolic enzymes (Hydrolysis)	FAAH		No change (R,P)		No change (R) Genetic polymorphism	Decreased (R)	Increased activity	No change (P) Increased activity			No change (R)
	MAGL							Increased (P)			

Table 3: Expression levels of the endocannabinoid system in intestinal diseases. Modified from Lee et al., 2016.

A great body of evidences demonstrated a bidirectional pathway between the gastrointestinal tract and the central nervous system (CNS), both in healthy conditions and neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. Patients affected by AD or PD often show gastrointestinal symptoms. Therefore, investigating the ECS in the GI tract can give benefits to the research in different fields.

Although there are different prospective studies and reviews about the use of cannabinoids for gastrointestinal diseases, the evidences are not enough to suggest these molecules as approved therapies. Their use is nowadays regarded as individual therapeutical trial, based on the effects on nociception and symptoms in every different patient (Häuser et al., 2017).

We performed one study about the ileum of the rat:

- *Localization of cannabinoid receptors in the myenteric plexus of the rat ileum.*

Dogs

Canine chronic enteropathies (CE) were described as IBD until some years ago. The multifactorial etiology (environment, genetic, microbiota and immunity) corresponds to human IBD, but the clinical presentation, with a major involvement of the small intestine (duodenum and ileum), and the treatment (Figure 15) are quiet different (Jergens and Simpson, 2012; Dandrieux, 2016).

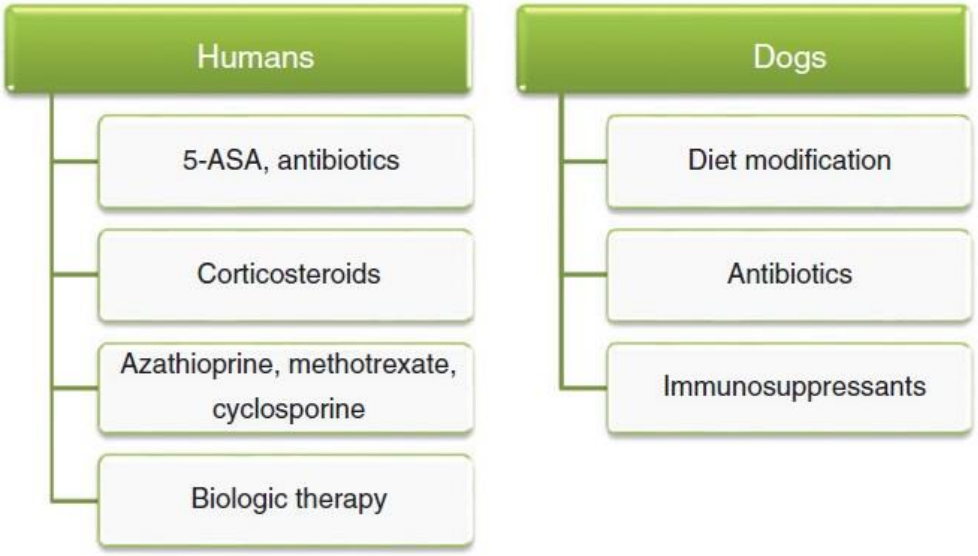

Figure 15: Stepwise medical treatment approach of IBD in humans and CE in dogs. Modified from Dandrieux, 2016. 5-ASA: 5-aminosalicylyc acids compounds; biologic therapy: TNF antagonists, anti-adhesion molecule.

The clinical signs are quiet aspecific: vomit and diarrhea, decreased or increased food intake, weight loss and other gastrointestinal signs that persist for more than 3 weeks defined a chronic pathology. Signalment and medical history can help in evaluating any predisposition, such as breed (Boxer or German Shepherd dogs are exemples of predisposed breeds), age (usually allergies are more frequent in young patients, while "IBD" phenotype is more typical of middle age dogs) or previous gastrointestinal pathologies (e.g. parvovirus, giardiasis) (Cave, 2013). The initial diagnostic protocol (Table 4) must exclude other pathologies, because these symptoms can be referred to gastroenteric, extragastroenteric or metabolic syndrome (Simpson and Jergens, 2011). After the exclusion of other diseases and the identification of a chronic enteropathy, the diagnosis of the phenotype depends on the response to the therapeutic trial.

Initial diagnostic approach to chronic diarrhea	
Integrate signalment, history, and physical examination	Breed predisposition, environment, diet, other clinical signs, localizing findings
Detect endoparasites and enteric pathogens	Fecal analysis (eg, Giardia)
Perform clinicopathologic testing	
Detect non-GI disease	CBC, biochemistry profile, UA, ± TLI, ACTH stimulation test, freeT_4/TSH levels, bile acid levels
Detect/characterize GI disease	Hypoproteinemia, hypocalcemia, hypocholesterolemia, leukopenia, leukocytosis, low cobalamin or folate levels[4]
Perform diagnostic imaging	
Detect non-GI disease	Radiography, ultrasonography of liver, spleen, pancreas, lymph nodes, masses, and effusions
Detect and characterize GI disease	Radiography, ultrasonography[59] to detect obstruction, intussusception, focal masses, thickening, loss of layering, hypoechoic appearance, hyperechoic striations

Abbreviations: ACTH, adrenocorticotropic hormone; CBC, complete blood cell count; T4, levorotatory thyroxine, TSH; thyroid-stimulating hormone, TLI, trypsin like immunoreactivity; UA, urinalysis.

Table 4: Initial diagnostic approach to chronic diarrhea. Modified from Simpson and Jergens, 2011.

The first step is to introduce, for a period of at least two weeks, an exclusive diet, with a novel protein or a hydrolyzed diet (Verlinden et al., 2006; Mandingers et al., 2010). It is known that many dogs respond to diet alone, so it is important to consider it the first-line treatment with mild to moderate symptoms (Dandrieux, 2016). If there is not a complete remission of the symptoms, the second step is usually the association of an antibiotic, to act on the dysbiosis to restore a correct gut microbiota. The first line-antibiotics in CE are tylosine or metronidazole (Westermark et al., 2005). However, the increasing problem of the antibiotic resistance and the uncomplete response of dogs with CE to antibiotics is limiting their use. If these therapies are not sufficient to treat the patients, it is fundamental to pick up gastrointestinal biopsies during a gastro-duodenum-ileal-colonscopy, for histopathological examination. The chronic intestinal inflammation is usually characterized by different degrees (from mild to severe) of lymphoplasmacytic enteritis or eosinophilic enteritis, but it is fundamental to exclude intestinal lymphoma. Typically dogs that do not respond to the change of diet and the antibiotic therapy, need the introduction of an immunosuppressant therapy (Allenspach et al., 2007).

In 2016 Dandrieux proposed a new classification for canine chronic enteropathies, based on the answer to the terapeuthical trial (Allenspach et al., 2007). Canine CE were finally classified in food responsive enteropathy (FRE), antibiotic responsive enteropathy (ARE), immunosoppressant responsive enteropathy (IRE) and non-responsive enteropathy (NRE) (Dandrieux, 2016).

Figure 16: Classification of canine chronic enteropathies based on the response to treatment. Modified from Dandrieux, 2016.

This classification (Figure 16) highlights how a little group of dogs (the top of the pyramid) does not respond to any treatment (NRE), so researchers are continuosly studying for new therapeutic approach, following findings in human medicine. Probiotics, fecal microbiota transplantation or intestinal stem cells (ISCs) are considered a new therapeutical approach during canine CE (Makielski et al., 2019). Probiotics have different action on the GI, including the support of the epithelial barrier, the regulation of the mucosal immune system and the modulation of the microbiota (Jergens, 2010).
Between all the new therapeutical possibilities, cannabinoids are gaining more and more interest, due to their antinflammatory, analgesic and other beneficial properties. Literature about cannabinoids and the dog is still in its infancy. Excluding the CNS distribution, CB1R has been immunohistochemically identified in canine salivary gland cells (Dall'Aglio et al., 2010), hair follicles (Mercati et al., 2012) and skin, in both healthy subjects and dogs with atopic dermatitis (Campora et al., 2012). As CB1R, CB2R were present in hair follicles and skin of healthy and AD dogs; the level of the two receptors in atopic dogs were higher than in healthy subjects. The expression of these receptors on cutaneous mast cells, notoriously enrolled in allergic disease, suggests potential benefits of cannabimimetic compounds, as PEA, in canine allergic cutaneous disease (Campora et al., 2012). A recent randomized, vehicle controlled, double blinded study in atopic Beagles using a topical endocannabinoid membrane transporter inhibitor (WOL067-531) showed a reduction in allergic flares and pruritus without adverse effects (Marsella et al., 2019).
Despite the multiple evidences in human gastrointestinal diseases, there are no studies about the treatment of canine chronic enteropathies with cannabinoids or related compound. For this reason, we decided to analyze the distribution of cannabinoid receptors in the gastrointestinal tract of dogs.

In dogs, we performed one study related to the endocannabinoid system:

- *Localization of cannabinoid receptors CB1, CB2, GPR55, and PPARα in the canine gastrointestinal tract.*

We performed other studies, focusing on chronic enteropathies:

- *The relationship between duodenal enterochromaffin cell distribution and degree of inflammatory bowel disease (IBD) in dogs.*
- *Effect of an extruded animal protein-free diet on fecal microbiota of dogs with food-responsive enteropathy.*
- *Effects of Chronic Enteropathies on VIPergic and Nitrergic Immunoreactive Neurons in the Dog Ileum.*

Cats

Chronic enteropathies in cats include food responsive enteropathy, IBD and intestinal lymphoma (Bottero et al., 2019). Risk factors such as genetic and molecular alterations, diet, and chronic inflammation have been connected to the development of these disorders (Garraway et al., 2018). Chronic intestinal inflammation in cats seems to be linked to an aberrant T cell response to enteric bacteria in predispose subjects. As seen in dogs and humans, environmental factors can stimulate an inflammatory onset or modulate genetic susceptibility to disease (Jergens, 2012). Affected cats are usually middle age, and some breeds as Siamese or other Asian breeds seem to be over-represented (Jergens et al., 1992). Gastrointestinal signs in cats with CE include chronic weight loss, vomiting, disorexia and diarrhea (Garraway et al., 2018). The clinical presentation is often cyclical, with period of remissions and other of exacerbations (Jergens et al., 2012). Trigger factors include dietary indiscretions, exposure to intestinal pathogens or drugs (Jergens, 1999). After the exclusion of infectious, extra GI or metabolic diseases, anatomical abnormalities, the differential diagnosis are FRE, feline IBD and intestinal lymphoma, with some differences in predisposition and clinical presentation (Table 5).

	Feline IBD	Food-responsive enteropathy	Alimentary lymphoma
Signalment	Predominantly middle-aged cats Siamese breed at risk?	Young cats No breed predisposition	Middle-aged to older cats No breed predisposition
Clinical signs of illness	Lethargy, weight loss, inappetence, vomiting, diarrhea	Large bowel diarrhea, ± weight loss, ± cutaneous lesions (alopecia)	Lethargy, weight loss, inappetence, vomiting, diarrhea, ± icterus
Clinical course	Progessive signs or cyclical flares	Progessive signs or cyclical flares	Progessive signs
Physical examination findings	May be normal, ± thickened bowel loops, abdominal pain (cholangitis/pancreatitis)	Often normal, ± alopecia	May be normal, thickened bowel loops, ± palpable masses
Diagnostic evaluation	Rule out non-GI causes for clinical signs; FNA cytology of mesenteric lymph nodes or masses; intestinal biopsy for definitive diagnosis	Rule out GI parasites, perform dietary trial	Rule out non-GI causes for clinical signs; fine-needle aspirate (FNA) cytology of mesenteric lymph nodes or masses; intestinal biopsy for definitive diagnosis
Potential pitfalls of diagnostic testing	False negatives when enlarged lymph nodes are present; differentiation from alimentary lymphoma may be difficult; cholangitis or pancreatitis may be concurrent	None; clinical response to elimination diet confirms diagnosis	False negatives when enlarged lymph nodes are present; differentiation from feline IBD may be difficult; may require immunophenotyping or PCR for confirmation

Table 5: Comparative features of feline IBD, FRE and lymphoma. Modified from Jergens, 2012.

Therefore, the first step, as seen in dogs, is a dietary trial with antigen-restricted or hydrolyzed petfood. In a study including 55 cats with CE, 49% of the patients responded to dietary modifications as primary treatment (Guildford et al., 2001). Differentiating IBD from alimentary lymphoma is more challenging, and requires the acquisition of endoscopic - or better laparotomic - biopsies for histological, immunohistochemical examination or clonality (Evans et al., 2006). The use of antibiotics in feline CE is not well demonstrated as in dogs. The principal antibiotics are, as for canine CE, tylosine or metronidazole. Treatment for feline IBD includes corticosteroids and various immunosuppressive agents, as chlorambucil or ciclosporin (Jergens et al., 2010).

In cats, we performed one study related to the endocannabinoid system:

- *Localization of cannabinoid receptors in the cat gastrointestinal tract.*

We performed a comparison between the dog and the cat:

- *Localization of cannabinoid receptors in the canine and feline gastrointestinal tract.*

Horses

Colic is a frequent digestive disorder of equines and includes different form of abdominal pain (Pilliner and Davies, 2004). It is an important cause of death for horses and a primary health concern of owners (Worku et al., 2017). There could be a breed predisposition: thoroughbreds and Arabs are overrepresented (Traub-Dargatz et al., 2001). Horses suffering for chronic gastrointestinal inflammation usually present recurrent colic, weight loss, poor performances and lethargy (Kalck, 2009). Malabsorption syndrome in horses are classified as IBD. Equine IBD can affect both the small and/or the large intestine, and it is classified considering the mucosal or submucosal infiltration, including granulomatous enteritis (GE), lymphocytic-plasmacytic enterocolitis (LPE), multisystemic eosinophilic epitheliotropic disease (MEED), diffuse eosinophilic enterocolitis (DEE) and proliferative enteritis (PE) (Boshuizen et al., 2018). Colic can be either medical or surgical; it usually involves large colon, followed by small intestine, caecum and small colon (Worku et al., 2017). Usually medical treatment includes fluid therapy, analgesics and antimicrobials (Fielding, 2018). Colic is one of the most dangerous emergency problem for horses and one of the principal cause of death (Curtis et al., 2019). For this reason, also in equine medicine, the research is focusing on novel therapies for management of pain and gastrointestinal diseases, but studies on the evaluation of the ECS in horses are still lacking.

Therefore, in horses, we performed a preliminary study about the endocannabinoid system in the equine gastrointestinal tract:

- *Localization of cannabinoid receptors in the horse ileum.*

Pigs

Gut health in pigs is fundamental in all the stage of growth and development, and have consequences in swine production (Pluske et al., 2018). Infective pathogens are often involved in diarrhea and gastroenteritis in pigs (Burrough, 2017; Rhouma et al., 2017). Stressors associated with breedings and critic period as weaning and post-weaning can affect the structure and the function of the GI tract (Celi et al., 2017). Therefore, management of an adeguate diet, a functional gut barrier, an appropriate microbiota and an effective gut immune system are fundamental to guarantee the gut health. Studies about the porcine gastrointestinal tract have consequences both on veterinary and human medicine. Pigs are omnivorous animals, whose digestive tract is functionally similar to humans. The interaction between the ECS and the gastrointestinal tract in pigs has been poorly investigated. A study in 2000 analyzed the distribution of CB1R in porcine enteric nervous system (Kulkarni-Narla and Brown, 2000). In the porcine ileum and colon, CB1R immunoreactivity was found in neurons and fibers in MP and SMP, often colocalized with ChAT. The inhibitory effect of CB1R on cholinergic neurotransmission in the MP can induce a reduction in peristaltic contractions. The presence of CB1R in SMP neurons and nerve fibers explains the effect of this receptor in mucosal secretory and immune function. Acetylcoline stimulates also chloride secretion in the mucosa of the colon, therefore colocalization between CB1R and ChAT may regulate ion transport across the porcine colonic epithelium. Another study found that the vanilloid receptor TRPV1 was also expressed by myenteric and submucosal neurons and fibers in the porcine ileum (Poonyachoti et al., 2002). Enteric neurons expressing vanilloid receptors, as cannabinoids, may constitute a target for the development of drugs alleviating painful intestinal inflammatory or dysmotility diseases.

In pigs, we performed one study about the gastrointestinal tract:

- *Distribution and co-expression patterns of specific cell markers of enteroendocrine cells in pig gastric epithelium.*

Dorsal root ganglia

Dorsal root ganglion (DRG) looks like the enlargement of a nerve, made by a group of cell bodies (Fletcher, 2009). The DRG contains the cell bodies of primary sensory neurons, responsible for the transduction and modulation of sensory information and the trasmission to the spinal cord (Krames et al., 2015). Different studies in the last years highlighted the important role of DRGs in managing neuropathic pain (Deer et al., 2013; Pope et al., 2013). Therefore, DRGs could be an interesting therapeutical target in neuropathic pain-conditions.

Anatomy

Spinal nerves, composed by afferent sensory dorsal axons (the dorsal root) and motor ventral efferent axons (the ventral root) carry autonomic, motor, and sensory informations between the spinal cord and the periphery (Sheng et al., 2010). When the dorsal sensory root exits the intervertebral neural foramina between two vertebral segments, it forms the dorsal root ganglion (Krames, 2014). The DRG is a collection of cell bodies of neurons surrounded by glial cells; the axons of the DRG sensory cells form the primary afferent sensory nerve. DRG neurons are defined pseudounipolar neurons, because the axon is divided in two branches, one branch extending from the T-junction to the periphery and one branch to the spinal cord. Otherwise, in bipolar cells, the body lies within the path of the axon (Figure 17). Primary afferent neurons could be classified according to various morphological, physiological and biochemical characteristics. An old morphological classification divided neurons into two main groups: ″light″ (L) cells, rich in neurofilament, and the neurofilament-poor ″dark″ (D) cells, according to the appearance at light and electron microscopy. These cells have been further subdivided considering other characteristics, but the most adopted is the subdivision by size (Willis and Coggeshall, 1992). The classification in small and large size neurons has been done in different species, such as mouse, rat, cat and horse (Lawson et al., 1979; Lawson et al., 1984; Lee et al., 1986; Russo et al., 2011). Moreover, DRG neurons can be classified by the characterization of their neurochemical code. The neuronal content is a great marker of the functional activity of primary afferent neurons (Garry et al., 1989).

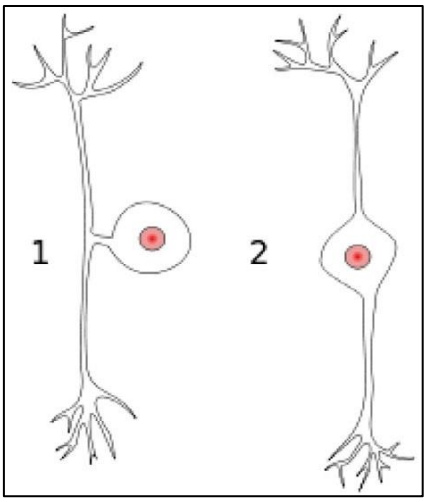

Figure 17: A pseudounipolar sensory neuron (1) and a bipolar cell (2). In a pseudounipolar sensory neuron one axon is divided into two separate branches, one from the periphery to the body and one from the body to the spinal cord. In bipolar cells, the body lies within the path of the axon. Modified from Krames, 2014.

Except of neurons, each DRG contains fibrous tissue, macrophages, nerve fibers, and supporting cells, which are glial cells (Kolesár et al., 2017). The glial cells of the PNS primarily include Schwann cells and satellite glial cells (SGCs).

Schwann cells are the most abundant glial cells in the PNS. They include two major phenotypes, myelinating and non-myelinating Schwann cells (Wey et al., 2019).

Schwann cells, as SGCs, originate from migrating cells of the neural crest, which can convert to different large polarized cell types, such as peripheral neurons, melanocytes or endocrine cells (i.e thyroid C cells or adrenal medulla) (Lobsiger et al., 2002; Mirsky et al., 2008).

Initially, Schwann cells surround the external margins of the axon bundles (Kidd et al., 2013). During maturation, myelinating Schwann cells incorporate larger axons to produce a myelin sheath, otherwise non myelinating Schwann cells embed smaller axons (Pereira et al., 2012; Kidd et al., 2013). Schwann cells support axonal outgrowth by producing a variety of growth factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and glial cell line-derived neurotrophic factor (GDNF) (Kidd et al., 2013).

SGCs surround the cell bodies of DRG neurons (Ballabh et al., 2004). In general, each sensory neuron has its own "envelope", which consists of several SGCs; the neuron and its surrounding SGCs create a morphological and functional unit (Hanani, 2005). Neurons, sending fine processes into invaginations of SGCs, create an extension to their surface area (by 30-40%) and may allow an interaction between the two cell types (Pannese, 2002).

DRG and neuroinflammation

A growing body of literature indicates that DRGs are involved in developing and maintaining neuropathic pain (Figure 18) (Vancamp et al., 2017). In response to tissue inflammation, DRG

produces changes in glial cells, nerve growth factors, chemokines; and produces genetic change and change in ion channels, as an answer to an inflammatory stimulus (Krames, 2014).

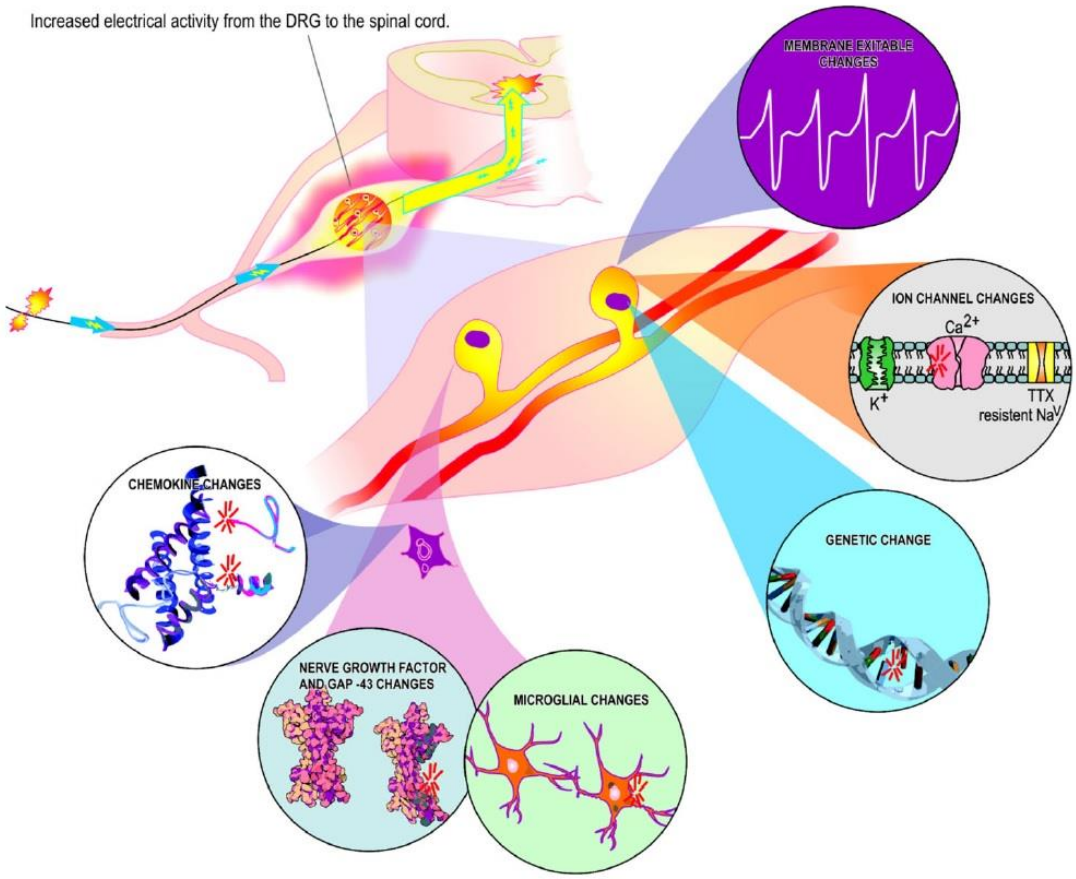

Figure 18: DRG response to tissue inflammation. In response to tissue inflammation or injury of a peripheral afferent fiber, the dorsal root ganglion (DRG) produces changes in glial cells, chemokines, nerve growth factors, gene expression, and ion channels including Na^+ channels, K^+ channels, and Ca^{++} channels. Modified from Krames 2014.

Schwann cells play a key role in the study of neuropathic pain. These cells are involved in the development of allodynia through a MHC class II-mediated mechanism. Schwann cells act as conditional APCs (antigen presenting cells) under inflammatory conditions: MHC class II activation stimulate $CD4^+$ T cells, promoting neuropathic pain (Hartlehnert et al., 2017). TRPA1 expression on Schwann cells seems to be involved in the pathogenesis of neuropathic pain. In mice, TRPA1 silencing in Schwann cells decreased mechanical allodynia and neuroinflammation (De Logu et al., 2017). By the other hand, Schwann cells are also involved in mechanisms of regeneration after injury. These cells both give physical support to the axon and release growth factors for nutriment and myelination of associated axons (Kidd et al., 2013). In pathological conditions, such as sciatic nerve injury, Schwann cells activation stimulates phenotype modulation, proliferation, migration and release of numerous factors, which may promote nerve regeneration (Scheib and Höke, 2013).

SGCs play a pivotal role in pathophysiological processes involving pain and inflammation (Watkins and Mayer 2002). As spinal glial cells (microglia and astrocytes) undergo activation in pain models

(gliosis), SGCs are stimulated after painful injuries, and play a key role in the development of chronic pain (Hanani et al., 2002; Dublin and Hanani, 2007; Chen et al., 2008). In particular, SGCs are involved in the induction and early maintenance of neuropathic pain: their reaction after injuries is estabilished within 4 hours, peaks at one week, and decreased in the first three weeks (Zhang et al., 2009; Liu et al., 2012). Sensory neurons have different receptors for neurotransmitters and hormones (i.e. ATP, glutamate, substance P), which allow them to comunicate each other or with other cell types, especially glial cells (Amir and Devor, 1996). By the other hand, SGCs have receptors for different molecules and can influence neighboring cells, including DRG neurons (Hanani 2005). For example, ATP release from neuronal cell bodies activates P2X7 (ion channel) in SGCs, which release tumor necrosis factor α (TNFα), which acts on surrounding neurons increasing their excitability (Sorkin et al., 1997). In addition, the ATP released by SGCs activates P2X3 receptor in neurons, to trigger peripheral sensitization. Persistent nociceptive activity, or opioid receptors-activation by morphine, results in release of matrix metalloproteinase-9 (MMP-9) from primary sensory neurons, causing the interleukin-1β (IL-1β)-release in SGCs, which elicit neuronal hyperexcitability (Figure 19) (Ji et al., 2013). Therefore, there is a bidirectional communication between neurons and glial cells, which is mediated by gap junctions, and the interaction between these cells (SGC-SGC or SGC-neuron) is enhanced during chronic pain conditions (Dublin and Hanani 2007; Hanani 2012). Neurons in DRGs are completely surrounded by SGCs, forming a tight envelope which functions as a partial barrier between the circulation and the neurons (Hanani, 2015). However, DRGs are devoid of a blood-brain barrier, therefore molecules released from neurons or SGCs can, from the bloodstream, reach and stimulate other DRGs. The role of SGCs seems to be central in neuronal communication: “Glial cells tell the nervous system what to do” (Nedegaard et al., 2003).

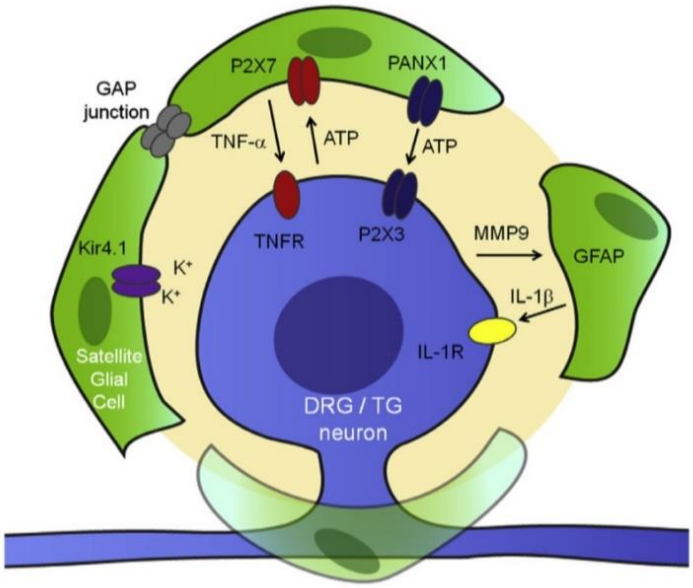

Figure 19: Schematic representation of neuronal-glial interactions in dorsal root and trigeminal ganglia. After painful injury, neurons release adenosine triphosphate (ATP) in neuronal somata, leading to the activation of P2X7 and subsequent release of tumor necrosis factor-α (TNF-α) in satellite glial cells (SGCs). Persistent nociceptive activity results in matrix metalloproteinase-9 (MMP-9) release from neurons, causing the cleavage and release of interleukin-1β (IL-1β) in SGCs. TNF-α and IL-1β bind respective TNFR and IL-1R on sensory neurons to elicit hyperexcitability. Modified from Ji et al., 2013.

Summarizing, neuropathic pain developes from an interaction between peripheral immune system, different cell types (DRG cell bodies and glial cells) and neuronal pathways, so investigating these elements can lead to new prospectives to treat neuropathic pain (Watkins et al., 2001).

DRG and the Endocannabinoid system

Chronic pain (inflammatory or neuropathic) represents a complicated condition that influences the quality of life in both animals and humans. It derives from an injury to the CNS or PNS, resulting in an enhancement of the transmission of pain stimuli. Consequently, painful stimuli are amplified (hyperalgesia) and normal stimuli are perceived as painful (allodynia) (Luongo et al., 2014). In the last years, the DRGs have been identified as a possible target in managing neuropathic pain (Deer et al., 2013; Pope et al., 2013). Among the different therapeutical strategies, the activation of cannabinoid receptors has been supported by various studies in animal models (Goya et al., 2003; Cravatt and Lichtman, 2004; Maione et al., 2006). The distribution of canonical and putative cannabinoid receptors in dorsal root ganglia have been partially investigated.

CB1R has been identified in DRG neurons and SGCs in laboratory rodents, dogs and humans (Sanudo-Pena et al., 1999; Anand et al., 2008; Freundt-Revilla et al., 2018). A recent study observed CB1R-immunoreactivity in 100% of Schwann cells in the canine sciatic nerve (Freundt-Revilla et al., 2017).

CB2 receptor was observed in neurons and glial cells in rodents, dogs and humans (Anand et al., 2008; Stella 2009; Freundt-Revilla et al., 2018; Sánchez-Zavaleta et al., 2018). In a neuropathic pain-model in rats, with unilateral sciatic nerve injury, CB2R protein and mRNA were increased bilaterally in DRG neurons and glial cells when compared with naive animals (Svíženská et al., 2013). The bilateral change can be explain with the propagation of inflammation along the neuraxis, and with the neuroprotective effects of CB2R. Being upregulated during PNS diseases, CB2R may be an interesting target for managing neuropathic pain and neuroinflammation (Navarro et al., 2016).

GPR55-immunoreactivity has been detected only in mice DRG neurons (Lauckner et al., 2008). The authors observed that GPR55 was abundantly expressed in large-diameter DRG neurons – which can be involved in nociception (Neumann et al. 1996; Ruscheweyh et al. 2007) - otherwise small-diameter DRG neurons expressed it at low levels. GPR55 acts on ion channels inhibiting potassium current and increasing intracellular calcium, thus enhancing neuronal excitability. Contrary, CB1R activates some potassium channels, suppressing neuronal excitability (Mackie et al., 1995; Kreitzer et al., 2002). The stimulation of neuronal excitability together with the expression in large-diameter neurons suggest a possible role of GPR55 in the etiopathogenesis of neuropathic and inflammatory pain states.

The role of Peroxisome proliferator-activated receptors (PPARs) in alleviating peripheral neuropathic pain has been confirmed in the last decades. This could be mediated by their anti-inflammatory effects (Morgenweek et al., 2010). PPARα and PPARγ are both expressed in DRG neurons (Dunn et al., 2015; Wu et al., 2017). Aldossary et al. (2019) showed that the analgesic properties of palmitoylethanolamide are - at least partially - mediated by PPARα.

TRPV1-immunoreactivity has been observed in DRG neurons of rodents, pigs and humans (Helliwell et al., 1998; Anand et al., 2008; Obreja et al., 2008; Russo et al., 2013). In rats and pigs, the percentage of positive neurons was higher than in mice (Zwick et al. 2002; Russo et al. 2013). TRPV1 is a cation channel that plays a key role in the transduction of noxious stimuli to the spinal cord (Caterina and Julius, 2001). During chronic inflammatory nociception, TRPV1 expression increases in DRG neurons in rats. This receptor is involved in the pathogenesis of thermal hyperalgesia and mechanical allodynia (Yu et al., 2008).

5-HT1aR has been observed in rat type 1 DRG cells, which resemble nociceptors (Cardenas et al., 1997), in contrast with the results obtained by Pierce et al. (1996). In a study on human DRG, 5-HT1aR mRNA has been detected by PCR in one to four subjects (Pierce et al., 1997). Peripheral 5-HT2aR is involved in the development of inflammatory and neuropathic pain. 5-HT2aR have been identified as a potential therapeutic target for treatment of sciatica in lumbar disc herniation (LDH) in an animal model (Kato et al., 2008). 5-HT2aR antagonists attenuate pain and suppress the expression of 5-HT2aR in the rat DRG (Kato et al., 2015). In the rat, about 40% of lumbar DRG cells

were immunoreactive for 5-HT2aR, principally small to medium-sized cell bodies (Van Steenwinckel et al., 2009). 5-HT3 receptor was expressed by rat DRG cells in culture preparations (Robertson and Bevan, 1991; Smith et al., 1997). Nicholson et al. (2003) used in situ hybridization to detect serotonin receptor mRNA expression in rat dorsal root ganglion neurons. In this study, mRNAs for 5-HT1b, 5-HT1d, 5-HT2a, 5-HT2b, 5-HT3b and 5-HT4 receptors were detected in small, medium and large diameter neurons. Contrary, mRNAs for 5-HT1a, 5-HT1e, 5-HT2c, 5-HT5a, 5-HT5b, 5-HT6 and 5-HT7 receptors were undetectable in these neurons (Nicholson et al., 2003). Serotonin (5-HT) interacts with different 5-HT receptors in pain modulation. Phytocannabinoid, as cannabidiol (CBD), interacts with 5-HT1a, 5-HT2a, and 5-HT3a receptors (Pertwee 2015). CBD seems to act on neuropathic pain conditions through TRPV1 and 5-HT1aR activation (De Gregorio et al., 2019).
Given that the ECS system influences neuronal and immune cell function, both involved in the etiopathogenesis of pain, targeting this system may hold promise as novel analgesic therapy.

For this reason, we developed two anatomical studies to identify the distribution of cannabinoid receptors in dorsal root ganglia:

- *Cellular distribution of canonical and putative cannabinoid receptors in canine cervical dorsal root ganglia*.
- *Localisation of cannabinoid receptors in the equine dorsal root ganglia*.

Experimental studies

Gastrointestinal tract

Experimental studies

Paper published on Histochemistry and Cell Biology (2018) 150(2), 187-205. Modified from:

Localization of cannabinoid receptors CB1, CB2, GPR55, and PPARα in the canine gastrointestinal tract

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Abstract

Introduction - The endocannabinoid system (ECS) is composed of cannabinoid receptors, their endogenous ligands, and the enzymes involved in endocannabinoid turnover. Modulating the activity of the ECS may influence a variety of physiological and pathophysiological processes. A growing body of evidence indicates that activation of cannabinoid receptors by endogenous, plant-derived, or synthetic cannabinoids may exert beneficial effects on gastrointestinal inflammation and visceral pain.

Objectives - The present ex vivo study aimed to investigate immunohistochemically the distribution of cannabinoid receptors CB1, CB2, G protein-coupled receptor 55 (GPR55), and peroxisome proliferation activation receptor alpha (PPARα) in the canine gastrointestinal tract.

Results - CB1 receptor immunoreactivity was observed in the lamina propria and epithelial cells. CB2 receptor immunoreactivity was expressed by lamina propria mast cells and immunocytes, blood vessels, and smooth muscle cells. Faint CB2 receptor immunoreactivity was also observed in neurons and glial cells of the submucosal plexus. GPR55 receptor immunoreactivity was expressed by lamina propria macrophages and smooth muscle cells. PPARα receptor immunoreactivity was expressed by blood vessels, smooth muscle cells, and glial cells of the myenteric plexus.

Conclusions and relevance - Cannabinoid receptors showed a wide distribution in the gastrointestinal tract of the dog. Since cannabinoid receptors have a protective role in inflammatory bowel disease, the present research provides an anatomical basis supporting the therapeutic use of cannabinoid receptor agonists in relieving motility disorders and visceral hypersensitivity in canine acute or chronic enteropathies.

Introduction

The endocannabinoid system (ECS) is composed of cannabinoid receptors, endocannabinoids, and the enzymes for their production and degradation (Stella, 2004; Ligresti et al., 2016; Lu and Anderson, 2017). It classically comprises the cannabinoid receptors types 1 and 2 (CB1R and CB2R),

the endocannabinoids N-arachidonylethanolamine (anandamide; AEA) and 2-arachidonylglycerol (2-AG), and the enzymes responsible for endocannabinoid biosynthesis and degradation (Iannotti et al., 2016). Currently, the definition of the ECS is expanding to include, besides the classical cannabinoid receptors and endocannabinoids, several fatty acid derivatives—the so-called endocannabinoid -like mediators, such as palmitoylethanolamide (PEA)—as well as other non-CB receptors (Kreitzer and Stella, 2009; Iannotti et al., 2016). This is the case, for example, for the G protein-coupled receptor 55 (GPR55), nuclear peroxisome proliferator-activated receptor alpha (PPARα), and transient receptor potential vanilloid type 1 (TRPV1), all of which are currently considered as possible cannabinoid receptors (Brown et al., 2005; Di Marzo et al., 2002; Izzo and Sharkey, 2010; Lauchner et al., 2008; Lin et al., 2011; Petrosino and Di Marzo, 2017; Tuduri et al., 2017). Notably, the ligands of this manifold receptor system often activate more than one target site in conjunction to exert their effect (O'Sullivan, 2016). Thus, the evolving idea of the "endocannabinoidome"—a more complex system including endocannabinoids and endocannabinoid-like mediators, their redundant metabolic enzymes and ''promiscuous'' molecular targets (i.e., receptors)—is increasingly gaining ground (Maione et al., 2013). The ECS is widely expressed in the central nervous system (CNS), cardiovascular, gastrointestinal, immune, and reproductive system (Maccarrone et al., 2015; Cabral et al., 2015). The CB1 receptor is expressed mostly in the CNS and peripheral nerves (Hu and Mackie, 2015; Freundt-Revilla et al., 2017), and the CB2 receptor is mainly expressed in immune cells (Di Marzo and Izzo, 2006). Many neuronal cell types, distributed in several CNS areas, express the CB1 receptor; however, the wide distribution of the CB1 receptor limits its potential as a pharmacological target for CNS diseases, due to the psychoactive side effects associated with CB1 receptor agonists and antagonists (Moreira et al., 2009). A recent study highlighted the expression of the CB2 receptor in neurons and inflammatory non-neuronal cells of the CNS, e.g., microglia (Malfitano et al., 2014). The evidence that the CB2 receptor is upregulated in a variety of CNS diseases characterized by microglia and/or astroglia activation suggests that the CB2 receptor might represent a promising pharmacological target to be considered in diseases characterized by neuroinflammation (Skaper et al., 2013 ; Navarro et al., 2016; Cassano et al., 2017; Chen et al., 2017; Freundt-Revilla et al., 2018). In the gastrointestinal tract (GIT), cannabinoid receptors regulate motility, secretion, sensation, emesis, satiety, and inflammation (Izzo, 2004; Duncan et al., 2005a, Duncan et al., 2005b, Duncan et al., 2008; Storr and Sharkey, 2007; Wright et al., 2008; Sharkey and Wiley, 2016; Lee et al., 2016; Di Patrizio, 2016). The CB1 receptor is expressed by neurons of the enteric nervous system (ENS) of rodents (Duncan et al., 2005a; Duncan et al., 2005b), guinea -pig (Coutts et al., 2002), pig (Kulkarni -Narla et al., 2000), and ferret (Van Sickle et al., 2001). The CB2 receptor may be expressed by macrophages, plasma cells, mast cells, dendritic cells, lymphocytes,

smooth muscle cells, epithelial cells, and enteric neurons (Facci et al., 1995; Duncan et al., 2005b; Wright et al., 2008; Duncan et al.,, 2008; Svensson et al., 2010; Ke et al., 2016). Several investigations suggest that CB1 or CB2 receptors might have a protective role in inflammatory bowel disease (IBD) in humans, and support the potential therapeutic effects of targeting these pathways using pharmacological agents (Izzo, 2004 ; 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). The activation of CB1 receptor reduces emesis, intestinal motility and secretion, and inhibits gastric acid secretion and relaxation of the lower esophageal sphincter (Izzo and Coutts, 2005). Activation of the CB2 receptor in pathological conditions such as inflammatory bowel disease (IBD) or endotoxic inflammation reduces intestinal motility (Izzo, 2004); thus, activation of CB2 receptor seems to represent a novel mechanism for the re-establishment of normal gastrointestinal transit after an inflammatory stimulus. Palmitoylethanolamide (PEA), a lipid mediator structurally related to AEA, is used in human and veterinary medicine for its neuroprotective, anti-neuroinflammatory, analgesic, and antipruritic properties (Re et al., 2007; Gabrielsson et al., 2016; Petrosino and Di Marzo, 2017; Cremon et al., 2017). It was isolated for the first time from lipid fractions of soybeans, egg yolk, and peanut meal and was then found in a wide variety of food sources (data reviewed in Petrosino and Di Marzo, 2017). Several investigators have identified different mode of action of PEA (Iannotti et al., 2016; Petrosino and Di Marzo, 2017), which seems to have a direct effect upon PPARα (Lo Verme et al., 2005a; Lo Verme et al., 2005b; Gabrielsson et al., 2016), GPR55 (Ryberg et al., 2007; Cantarella et al., 2011), and CB2R (Facci et al., 1995). The latter receptor may also be activated through an indirect mechanism (De Petrocellis et al., 2001; Guida et al., 2017). Finally, PEA directly and indirectly acts on TRPV1 (Ho et al., 2008; De Petrocellis and Di Marzo, 2010), a receptor usually expressed by nociceptive dorsal root ganglia sensory neurons (Caterina et al., 1997) that undergoes desensitization by endocannabinoids (Zygmunt et al., 1999; Ambrosino et al., 2013). PEA, which also seems to act favourably on visceral pain (Jaggar et al., 1998; Farquhar- Smith et al., 2002; Gabriels-son et al., 2016), represents a promising natural approach for hypersensitivity management in dogs with intestinal inflammation. A prerequisite for the therapeutic potential of PEA in pathological GIT conditions, such as acute or chronic enteropathies, is the cellular distribution of the receptors. PEA is known to act upon, i.e., the cannabinoid receptors, in different tracts of the canine digestive system. Therefore, the present study aimed to immunohistochemically characterize the cellular expression of CB1, CB2, GPR55, and PPARα receptors in ex-vivo GIT tissues of dogs.

Materials and methods

Animals

Gastrointestinal tissues were collected from three dogs (#1 female, 8-month-old Chihuahua; #2 spayed female, 11-year-old Labrador Retriever, and #3 male, 17-year-old West High-land White Terrier), that did not have a history of gastrointestinal disorders and did not show gross alteration of the gastrointestinal wall. Animals died spontaneously or were euthanized for human reasons due to different diseases and tissues were collected following consent from the owners. 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) did 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 collected within 1 h of death and were longitudinally opened along the pyloric small curvature and intestinal mesenteric border. 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 previously described (Chiocchetti et al., 2015; Giancola et al., 2016, Giancola et al., 2017).

Immunofluorescence

Cryosections were hydrated in phosphate-buffered saline (PBS), and processed for immunostaining. To block non-specific binding, 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 overnight in a humid chamber at RT with a cocktail of primary antibodies (Table 1) diluted in 1.8% NaCl in 0.01M PBS containing 0.1% sodium azide. After washing in PBS (3 × 10 min), the sections were incubated for 1 h at RT in a humid chamber with the secondary antibodies (Table 2) 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, Santa Cruz, CA, USA). Since classic (CB1R and CB2R) and "new" cannabinoid receptors (GPR55 and PPAR-α) might be located on different cellular types, we employed a panel of specific antibodies with the aim of colocalizing the cannabinoid receptors' immunoreactivity on enteric neurons, enteric glial cells (EGCs), mast cells (MCs), macrophages, and plasma cells. In particular, to identify enteric neurons and glial cells, anti-human neuronal protein (HuC/HuD) and

anti-GFAP antibodies were used, respectively. To identify MCs, different antibodies against MC tryptase were used. To identify macrophages, we utilized the anti-ionized calcium binding adapter molecule 1 antibody (IBA1). Plasma cells were identified using the anti-IgA antibodies. Endothelial cells were identified using two different endothelial markers, i.e., the mouse anti-CD31 antibody, and the rabbit anti-Factor VIII-related antigen/von Willebrand factor. To identify enterochromaffin cells, we utilized the anti-serotonin antibody.

Specificity of the primary antibodies

The specificity of the mouse anti-HuC/HuD antibody was recently demonstrated in dog tissues by Western blot analysis (Giancola et al., 2016). The supplier of the anti-CB1 receptor antibody, raised in rabbit against the human CB1 receptor, predicts cross-reactivity with the dog, mouse, and rat antigen. The sequence of canine CB1 protein is homologous (98.3%) to the sequence of human CB1 protein (https:// www.uniprot.org/) (Anday and Mercier 2005). The anti-CB2 receptor antibody was raised in rabbit and directed against residues 200–300 of the rat CB2 receptor. The sequence of the canine CB2 protein is homologous (98.3%) to the sequence of the rat CB2 protein (https://www.uniprot.org/). The anti-CB2 receptor antibody utilized in the present study has already been tested on dog tissues (Campora et al., 2012). Based on the sequence identities, the antibodies against CB1 and CB2 receptors should cross-react with the same antigens in the dog. The antibody anti-GPR55 receptor was raised against a17 amino acid synthetic peptide of human GPR55 receptor. The sequence of canine GPR55 protein is homologous (83.5%) to the sequence of human GPR55 protein (https:// www.uniprot.org/); furthermore, the antibody supplier indicated greater (94%) cross-reactivity of the antibody with the canine GPR55 protein. Taken together, this suggests that this antibody should cross-react with the same antigen in the dog. The anti -PPARα receptor antibody was raised in rabbit against the synthetic peptide of mouse PPAR-α. The sequence of canine PPARα protein is homologous (90%) to the sequence of mouse PPARα protein (https://www.unipr ot.org/). Furthermore, the supplier of the anti-PPARα recep-tor antibody predicted cross-reactivity with the same antigen in the dog.

Despite the presumed or already demonstrated specificity of the anti-cannabinoid receptor (CB1, CB2, GPR55, PPARα) antibodies utilized in the present research on canine tissues, we tested their specificity by Western blot (WB) analysis (Fig. 1). In addition, to confirm that CB1 antibody staining was specific for CB1 protein, the rabbit anti-CB1 antibody was tested on brain cryosections obtained from wild-type mice and mice with congenital deficiency of CB1 (Marsicano et al., 2002). In the cryosections of CB1 null mice, the specific CB1 staining of axons was absent (Supplementary Fig. 1a–f). Furthermore, the rabbit anti-CB1 antibody was tested on wholemount preparations of rat MP

and SMP, in which neurons of both the plexuses showed CB1 receptor immunolabelling (Supplementary Fig. 1g–l). To identify MCs, we utilized the only commercially available anti-dog tryptase antibody (Cloude-Clone, PA B070Ca01, Huston, TX, USA). Since this antibody was raised in rabbit, as were all the anti -cannabinoid receptor antibodies utilized in this study, we colocalized the dog-specific anti-tryptase antibody with two commercially available anti-human tryptase antibodies raised in mouse: Dako,M 7052 (Clone AA1) and Novus Biol (NBP1-40202). The first antibody (clone AA1) has already been used in canine intestinal tissues (Kleinschmidt et al., 2007). The supplier of the second antibody (NBP1-40202) indicated cross-reactivity with canine tryptase (canine tryptase shows 76% homology with human tryptase; http://www.uniprot.org/). The dog-specific rabbit anti-tryptase antibody labelled a greater number of MCs in all GIT layers (data not shown) than those labelled by the human-specific anti-tryptase antibodies, which were not immunolabelled with the other two antibodies (data not shown). Nevertheless, since both the mouse anti-tryptase antibodies labelled a large number of lamina propria MCs (Novus Biol. > Dako), which were also immunolabelled by the dog-specific anti-tryptase antibody, we utilized these two mouse anti-human tryptase antibodies in colocalization studies to identify lamina propria MCs bearing cannabinoid receptors. The goat anti-IgA antibody (Novus Biol., NB100-1028) is directed against porcine IgA; although the specificity of this antibody has not been tested on dog tissues, in the present research we colocalized the goat anti-IgA antibody with the rabbit anti-IgA antibody (Bethyl Lab., A80-103A). The two antibodies co-localized perfectly in the same immunocytes/ plasma cells (data not shown). Thus, in the present study, the goat anti-IgA antibody was utilized to identify immunocytes bearing cannabinoid receptors. The anti-IBA1 antibody should recognize CNS microglia (Pierezan et al., 2014) and macrophages; in the present study the antibody recognized canine gut macrophages (and canine CNS microglia; data not shown). The anti-IBA1 antibody used (Novus Biol. NB100-1028) was raised in goat and is directed against porcine IBA1. Since the dog IBA1 molecule shows 91.2% identity with the porcine one (https:// www.uniprot.org/), it is plausible that this antibody may also recognize canine IBA1. Nevertheless, the specificity of this antibody has not been tested on dog tissues. The antibody anti-CD31, that has been already used in dog tissues (Kader et al., 2001), unequivocally identified the endothelial cells of blood vessels. The specificity of this antibody was tested in a colocalization study with the other endothelial marker, i.e., the anti-Factor VIII-related antigen/ von Willebrand factor antibody (Preziosi et al., 2004). The two antibodies co-localized in the same endothelial cells (data not shown).

Specificity of the secondary antibodies

The specificity of the secondary antibodies was tested by applying them after omission of the primary antibodies. Neither stained cells could be detected after omitting the primary antibodies. In double-immunostaining protocols, control experiments were also carried out to check for non-specific binding of secondary antibodies to the inappropriate primary antibodies by omission of one or other of the first stage reagents. Furthermore, incubation with two primary antibodies followed by only one secondary antibody was carried out to check for the existence of any cross-reactivity between primary and secondary antibodies. Finally, incubation with any single primary antibody followed by the appropriate secondary antibody was also performed to ensure that the labeling pattern for each marker in the double- stained sections was in agreement with that observed in the single-labeled sections. No evidence of nonspecific binding was found.

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

Tissue samples (canine small intestine) were collected, frozen in liquid nitrogen, and stored at − 80°C until sample processing. In addition, the mouse small intestine was utilized as positive control. 50 mg of tissue was homogenized in 500 µl of SDS buffer (Tris–HCl, 62.5 mM; pH 6.8; SDS, 2%; and glycerol, 20%) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Co, St. Louis, MO, USA). Total protein content was determined by Peterson's Modification of Lowry Method using a Protein Assay Kit. Aliquots containing 20 µg of total proteins were separated on Bolt 4–12% bis-Tris Plus (Life Technologies Ltd, Paisley, UK) for 45 min at 165 V. The proteins were then electrophoretically transferred onto a nitrocellulose membrane by a semi-dry system (Trans Turbo Blot Bio-Rad). Non-specific binding on nitrocellulose membranes was blocked with 5% milk powder in PBS-T20 (Phosphate Buffer Saline-0.1% Tween-20) for 1 h at RT. After blocking treatment, the membranes were incubated overnight at 4 °C with the primary antibodies (Table 1) (CB1R 1:500, CB2R 1:1000; GPR55 1:500; PPARα 1:2000) diluted in Tris-buffered saline-T20 (TBS-T20 20 mM Tris–HCl, pH 7.4, 500 mM NaCl, 0.1% T-20). After washes, the blots were incubated with a goat

anti rabbit biotin-conjugate antibody (1:50,000 dilution in TBS-T20, 1 h at RT) and then with a 1:1000 dilution of an anti-biotin horseradish peroxidase (HRP)-linked antibody (40 min at RT). Immunoreactive bands were visualized using chemiluminescent substrate (Clarity Western ECL Substrate Bio Rad), according to the manufacturer's instructions. The intensity of the luminescent signal was acquired by Chemi-doc Instrument and the apparent molecular weight of the resultant bands was analyzed by Quantity One Software (Bio-Rad). Western blot analysis of CB1 revealed a band of ~ 52 kDa (theoretical molecular weight of canine CB1 52,782 kDa; Fig. 1). Western blot analysis of CB2 revealed a band of ~ 40 kDa (theoretical molecular weight of canine CB2 40,107 kDa; Fig. 1). Western blot analysis of GPR55 revealed a band of ~ 35 kDa (theoretical molecular weight of canine GPR55 36,85 kDa; Fig. 1). Western blot of PPARα revealed a band of ~ 52 kDa (theoretical molecular weight of canine PPARα 52,123 kDa; Figure 1). Overall Wb analysis confirmed the specificity of the primary antibodies utilized in the present study.

Results

CB1 immunoreactivity

In the pylorus, small and large intestine, CB1 receptor immunoreactivity was expressed by serotonin-immunoreactive enterochromaffin cells (Fig.2a–f). In the small and large intestine, CB1 immunoreactivity was detected in the cell membrane and cytoplasm of some lamina propria and epithelial cells (Fig. 2g, h). In the ileum of the youngest dog, in which submucosal and mucosal lymphatic nodules were evident, bright CB1 receptor immunoreactivity was identified in unidentified immunocytes localized in the portion of the lymphatic nodules within the lamina propria (Fig. 2i). In the enteric plexuses, faint and punctate CB1 receptor immunoreactivity was observed in some unidentified MP neurons (Fig. 2j–l). CB1 receptor immunoreactivity was undetectable in blood vessels and muscular layers.

CB2 immunoreactivity

CB2 immunoreactivity was widely distributed in all the digestive tracts considered.

Mucosa

The cell membrane of the endothelial and smooth muscle cells of mucosal (Fig. 3a, b) and submucosal (Fig. 3c) blood vessels showed bright CB2 immunoreactivity, above all in the small intestine, which allowed visualization of the blood vascular pattern along the major axis of the villi. When the villi were cut orthogonally, it was possible to observe CB2-immunolabelled vessels arranged like clock numbers around the circumference of the villus (Fig. 3b). Colocalization experiments indicated that

CB2 immunolabelled endothelial cells showed CD31 immunoreactivity; however, there were small areas in which the two different immunolabelling were non-overlapped, indicating that other cellular elements (most likely pericytes) expressed CB2 immunoreactivity (Supplementary Fig. 2). It is of note that some lamina propria tryptase-immunoreactive MCs showed cytoplasmic CB2 immunoreactivity (Fig. 3d–f). CB2 immunoreactivity was also observed in the cytoplasm of some unidentified immunocytes within intestinal lymphatic nodules (Fig. 3g). The cell membrane of smooth muscle cells of the muscularis mucosae (mm) showed bright CB2 immunoreactivity in all the digestive tracts considered (data not shown).

Muscular layers

In the pylorus, CB2 immunoreactivity was observed on the cell membrane of the smooth muscle cells of both layers of the tunica muscularis (Fig. 3j). In the circular muscle layer (CML), there were some patchy distributed clusters of smooth muscle cells that showed stronger immunolabelling. The CB2 immunoreactivity of the muscular layers of the small intestine showed strong immunolabelling, which was attenuated in the colon (small intestine > colon > pylorus) (Fig. 3k–l). In both the intestinal tracts, CB2 immunoreactivity decreased towards the outermost part of the longitudinal muscle layer (LML).

Enteric neurons and glia

In the intestinal submucosal plexus (SMP), there were a few ganglia in which neurons and glial cells showed weak-to-moderate CB2 immunoreactivity (Fig. 3h–i), either on the cell membrane (Fig. 3h) or within the cytoplasm (Fig. 3i). On the contrary, the neurons and glial cells of the myenteric plexus (MP) did not show any CB2 immunoreactivity (Fig. 3j–l).

GPR55 immunoreactivity

GPR55 immunoreactivity was mainly distributed in the mucosa (Fig. 4a–i) and muscular layers (Fig. 4j–l).

Mucosa

A large number of lamina propria and epithelial cells expressed bright GPR55 immunoreactivity (Fig. 4a–c). In the pylorus, there were some thin and elongated enterochromaffin cells, which showed bright nuclear and weaker cytoplasmic GPR55 immunostaining (Fig. 4a). GPR55 immunolabelled enterochromaffin cells were also visible in the intestine, in particular in the colon. Furthermore, epithelial cells of the inner portion of the mucosa also showed diffuse cytoplasmic GPR55-immunolabelling in this tract (Fig. 4b). Co-localization experiments indicated that a large number of lamina propria cells showing cytoplasmic GPR55 immunoreactivity were IBA1-immunoreactive

macrophages (Fig. 4d–f), IgA-immunoreactive plasma cells (Fig. 4g–i), and tryptase-immunoreactive MCs (data not shown).

Muscular layers

The GPR55 receptor distribution showed regional and local differences. In the stomach, GPR55 immunoreactivity was not homogenously distributed and the immunolabelling was more evident in the CML than in the LML (data not shown). In the duodenum, the CML showed faint GPR55-immunolabelling, as did the inner portion of the LML; on the contrary, the outer third of the LML showed very strong GPR55 immunoreactivity (Fig. 4j). In addition, in the ileum, the smooth muscle cells of the outer portion of the LML showed a higher density of the GPR55 receptor, whereas in the colon all the LML expressed bright GPR55 immunoreactivity, with a tendency for increased immunolabelling in its outer portion (Fig. 4k–l). It is noteworthy that the inner portion of the CML (ICML), i.e., the small portion of the CML composed by 6–12 smooth muscle cells facing towards the submucosa (Zelcer and Daniel 1979; Eddinger 2009), showed intense GPR55-immunolabelling (data not shown).

Enteric neurons and glia

No GPR55 immunoreactivity was displayed by enteric neurons or glial cells in the tracts considered.

PPARα immunoreactivity

Bright PPARα immunoreactivity was expressed by lamina propria cells, epithelial cells, blood vessels, smooth muscle cells of the mm and tunica muscularis (Fig. 5a–h), and EGCs (Fig. 6a–i).

Mucosa

In the pylorus, PPARα immunoreactivity was evident in the cytoplasm and nucleus of serotonin-immunoreactive enterochromaffin cells of the deeper portions of the gastric glands (data not shown). In the intestine, the strongest PPARα immunoreactivity was expressed by the mm, from which bundles of smooth muscle cells reached the tips of the villi (Fig. 5a); of note, the muscular cells showed their strongest immunolabelling on the apex of the villi. PPARα immunoreactivity was also expressed by blood vessel endothelial cells (Fig. 5a, b). In the lamina propria of the villi, and in particular in their apex, PPARα immunoreactivity was observed in a network of thin and elongated cellular processes arising from small cells of unknown nature (Fig. 5c); these cells were easily observed when the villi were cut orthogonally to their major axis. Furthermore, some small lamina propria cells with an irregular outline and short cellular processes, which resembled dendritic cells

(Junginger et al., 2014), showed brilliant PPARα- immunolabelling within the cytoplasm (Fig. 5d). In addition, MCs showed cytoplasmic PPARα immunoreactivity (data not shown).

Muscular layers

PPARα receptor, as seen for GPR55 receptor, showed a different distribution in the tunica muscularis and was well represented in both the muscular layers, but more concentrated in the LML, and in particular in its external portion, in the pylorus, duodenum and colon (Fig. 5e, g). In the LML of the ileum, PPARα-immunolabelling was mainly observed concentrated within its outer portion, although there were some ileal tracts in which PPARα receptor were seen in smooth muscle cells scattered in the LML (Fig. 5f). It should be noted that, in contrast to what was observed for GPR55 immunoreactivity, the ICML was PPARα-negative (Fig. 5h). PPARα immunoreactivity was strongly expressed by GFAP-immunoreactive glial cells of the SMP and MP (MP > SMP), whereas the HuC/HuD-immunoreactive neurons were always PPARα-negative (Fig. 6a–i).

Discussion

CB1 receptor

The observation of CB1 receptor immunolabelling of enteric neurons is consistent with data observed in pig, guinea-pig, rat, mouse, and ferret ENS (Kulkarni-Narla and Brown 2000; Van Sickle et al., 2001; Coutts et al., 2002; Storr et al., 2004; Duncan et al., 2005a), in which the CB1 receptor was mainly expressed by cholinergic excitatory motor neurons. In humans, the activity of the CB1 receptor was functionally demonstrated in the ileum by Croci et al. (1998) and CB1 receptor immunoreactivity has been described in enteric neurons and nerve fibres (Wright et al., 2005; Marquez et al., 2009). In the present study, we observed CB1 receptor immunoreactivity in the epithelial cells, including enterochromaffin cells. This is in line with what was observed in human GIT mucosa, where the CB1 receptor was identified on gastric parietal cells, epithelial cells, goblet cells, and enteroendocrine cells (Wright et al., 2005; Pazos et al., 2008; Marquez et al., 2009; Ligresti et al., 2016). It is known that under physiological conditions, the activation of the CB1 receptor reduces gastric acidic secretion and regulates the release of enteroendocrine peptides such as cholecystokinin (Sykaras et al., 2012). The presence of CB1 receptor immunoreactivity in canine small intestine is especially important, given the body of evidences that shows that CB1 receptor in the upper small intestine of rodents controls palatable food intake and overeating in diet-induced obesity (Di Patrizio et al., 2011; Argueta and Di Patrizio, 2017). In addition, the evidence of CB1 receptor immunoreactivity in serotonin expressing enterochromaffin cells of the dog upper gastrointestinal tract may suggest a peripheral mechanism of action of cannabinoids in the modulation

of nausea and vomiting. In fact, it is well known that cannabinoids may inhibit nausea and vomit with a central (and peripheral) action, since CB1 receptor is scattered on neurons of the brainstem nuclei involved in emesis (Ray et al., 2009; Darmani, 2010). The activation of CB1 receptor of 5-HT releasing enterochromaffin cells may limit nausea and vomit by reducing 5-HT release and consequently decreasing the excitability of 5-HT3 receptor of the vagal sensory nerve fibers of the upper gastrointestinal tract (Hu et al., 2007). In the present study, we observed unidentified lamina propria cells expressing CB1 receptor immunoreactivity, which were more concentrated in the small intestine lymphatic nodules. Regarding the expression of CB1 receptor immunoreactivity on epithelial cells, it is interesting to consider that enteric microbiota may regulate the expression of the CB1 receptor on enterocytes, and this in turn may control gut permeability (Muccioli et al., 2010). Consistently, CB1 activation has recently been suggested to play a key role in intestinal mucosa permeability, both in healthy and disease states (Karwad et al., 2017a). The presence of the CB1 receptor on lamina propria cells is consistent with the finding by Marquez et al. (2009), who reported CB1 receptor immunoreactivity in mucosal plasma cells. Moreover, enteric microbiota may regulate the expression of the CB1 receptor on enterocytes, and this in turn may control gut permeability (Muccioli et al., 2010). Consistently CB1 activation has recently been suggested to play a key role in intestinal mucosa permeability, both in healthy and disease states (Karwad et al., 2017a).

CB2 receptor

CB2 receptor immunoreactivity was absent in the MP, whereas it was observed in some neurons and EGCs of the submucosa, in which it showed different degrees of brightness. This is not surprising, since the CB2 receptor although usually expressed by non-neuronal elements such as immunocytes and inflammatory cells has been repeatedly demonstrated in the central (Cabral et al., 2008) and peripheral nervous system, including enteric neurons (Duncan et al., 2005b, Duncan et al., 2008; Wright et al., 2008). To the best of our knowledge, this is the first time that CB2 immunoreactivity has been reported in EGCs. Notably, this could be related to the expression of PPARα on the same cell type (see below). The localization of CB2 receptor in the EGCs is consistent with the expression of CB2 receptor on astrocytes of healthy and inflamed CNS (Sheng et al., 2005; Freundt-Revilla et al., 2018). We observed very strong CB2 immunoreactivity in endothelial and muscular components of enteric blood vessels. Our observations are consistent with the findings by Golech et al. (2004), Ashton et al. (2006), Marquez et al. (2009), and Dowie et al. (2014), who found CB2-immunolabelling on endothelial vascular cells of human and rat CNS. More specifically, our data are reinforced by the findings of Campora et al. (2012), who observed CB2-immunolabelling on canine endothelial cells in skin. In co-localization studies aimed to identify the co-expression of the CD31 -

and CB2- immunoreactivity in endothelial cells, we noted a certain degree of non-overlap between the two markers. This evidence may suggest that endothelial cells, and also pericytes, express CB2 immunoreactivity. This last finding, although not demonstrated by the use of a specific marker for pericytes, is of some importance, because it has been shown that the contraction of pericityes can regulate the vascular flow of the capillaries in the intestine (Wille and Schnorr, 2003) as well in the CNS. Hall et al. (2014) demonstrated that ischaemia evokes capillary constriction by pericytes, which are major regulators of cerebral blood flow. Zong et al. (2017) showed that exogenous CB1 agonist promotes the vasorelaxation of pericyte-containing rat retinal capillaries. Benyó et al. (2016) showed that in certain cerebrovascular pathologies, activation of CB2 receptor (and probably yet unidentified non-CB1/non-CB2 receptors) appears to improve the blood perfusion of the brain via attenuating vascular inflammation. Thus, the expression of the CB2 receptor in dog gastrointestinal vessels (in smooth muscle cells, endothelium and, perhaps, pericytes) may have relevant therapeutic importance in the treatment of acute and chronic enteropathies. In fact, alteration of the microvascular perfusion and adhesion of leukocytes to the endothelium are hallmark events in inflammation. As already demonstrated in rodents (Kinian et al., 2013; Sardinha et al., 2014), it is possible that even in dogs the use of CB2 receptor agonists might protect the gut microcirculation during inflammation. The expression of CB2 immunoreactivity in lamina propria cells was expected, since the presence of this receptor among different classes of immunocytes and inflammatory cells has already been reported (Wright et al., 2008; Izzo and Sharkey, 2010; Gyires and Zádori, 2016; Lee et al., 2016). Notably, as already shown in experimental rodents (Facci et al., 1995), we observed CB2 receptor immunoreactivity in canine mucosal MCs. The finding is of particular interest if one considers that MCs are now recognized to be involved in a number of non-allergic diseases including IBD and food intolerance (Shea-Donohue et al., 2010; Wouters et al., 2016; Bednarska et al., 2017). During intestinal inflammation, MCs release proinflammatory mediators (e.g., tryptase, chymase, and histamine), which recruit and stimulate adjacent MCs, thus amplifying the inflammatory signal (He 2004). CB2 receptor immunoreactivity on canine gut MCs renders them a potential target for CB2 agonists. Although CB2 receptor activation is considered to exert no effect on GIT motility under physiological conditions (Izzo et al., 1999), upregulation during experimental GIT inflammation might be envisaged. Indeed, the CB2 receptor seems to be upregulated in the dog (specifically in the SMP) during chronic enteritis (personal observation of Dr. R. Chiocchetti; Supplementary Fig. 3).

GPR55 receptor

The GPR55 receptor, considered by some as the third cannabinoid receptor (Moriconi et al., 2010), is a G protein-coupled receptor sharing 10–14% homology with CB1 and CB2 receptors (Lauckner

et al., 2008). Although a detailed description of tissues and cells expressing GPR55 is still lacking, a growing body of evidence shows that GPR55 is widely distributed in the ENS of humans and rodents, in particular in the two ganglionated plexuses (Lin et al., 2011; Ross et al., 2012; Li et al., 2013; Goyal et al., 2017). In contrast with these findings, we did not detect the GPR55 receptor in ENS neurons, whereas it was abundantly expressed in smooth muscle cells, possibly playing some role in controlling excitability. The peculiar distribution of GPR55 immunoreactivity in the muscular layers, i.e., the high density of the GPR55 receptor in the ICML (small intestine) and outer portion of the LML (colon), suggests that the GPR55 receptor might be involved in ICML relaxation during inflammatory-induced excessive contraction of the intestinal wall. In fact, as hypothesized by Eddinger (2009), ICML cells seem to be primarily involved in maintaining basal intestinal tone, while the muscle cells of the outer portion of the CML are primarily involved in peristalsis. As specified above, GPR55 receptor immunoreactivity was also well represented in the outer portion of the LML. Although the enteric neurons and interstitial cells of Cajal are determinant for the beginning and coordination of peristalsis, smooth muscle cells of the CML and LML have intrinsic myogenic activity (Huizinga et al., 1998). Due to its role in the regulation (increase) of intracellular calcium levels (Lauckner et al., 2008); the GPR55 receptor may thus play a role in the excitability of these smooth muscle cells. We also found that a large number of lamina propria macrophages, plasma cells, and MCs showed bright GPR55 immunoreactivity (macrophages > plasma cells > MCs). The presence of the GPR55 receptor on macrophages was recently also shown in rodents and humans (Taylor et al., 2015; Lanuti et al., 2015). As reported above, pro-inflammatory mediators released by MCs during intestinal inflammation may induce macrophage accumulation in the basal portion of the lamina propria (He et al., 1997; He and Walls 1998). One could thus speculate that endocannabinoid-related compounds acting on CB2 receptor (MCs and immunocytes) and/or GPR55 receptor (macrophages and MCs) may limit the inflammatory cascade during GIT disturbances.

PPARα receptor

The PPARα receptor is a ligand-activated transcription factor belonging to the superfamily of nuclear hormone receptors. By modulating gene expression, it plays key roles in maintaining glucose and lipid homeostasis and inhibiting inflammation (Naidenow et al., 2016). Activation of the PPARα receptor is known to exert anti-nociceptive and anti-inflammatory effects, even at the gastrointestinal level (Escher et al., 2001; Azuma et al., 2010; Petrosino and Di Marzo, 2017). The strong PPARα immunoreactivity observed in the mm and its mucosal emanations, as well as the peculiar localization of this receptor in the smooth muscle cells of CML and LML, suggests a unique role for this receptor (as seen for GPR55 receptor) in GIT motility. Interestingly, whereas in the LML the distribution of

the PPARα receptor overlapped that of the GPR55 receptor, in the ICML the former seemed to be missing, whereas the latter was widely distributed. At present, we are not able to speculate on the physiological meaning of this different receptor distribution. Although it was not possible to precisely identify the strong PPARα-immunoreactive cells within the lamina propria of the villi, their shape, cytoplasmic projections and distribution are suggestive of mucosal dendritic cells (DCs) (Junginger et al., 2014). Notably, DCs are widely distributed in the digestive system and play a relevant role in innate and adaptive immunity and in the maintenance of tolerance (Svensson et al., 2010). Finally, the expression of PPARα immunoreactivity on cells particularly involved in gut pathophysiology, i.e., the intestinal MCs (Lee et al., 2016; Bischoff, 2016; Wouters et al., 2016), suggests a potential role of PPARα agonists in GIT inflammation. The most intriguing localization of PPARα receptor revealed by our study was at the level of EGCs, i.e., cells that are functionally comparable to CNS astrocytes (Liu et al., 2013; Sharkey, 2015) and able to multifunctionally interact with the epithelium, immune system, nerve fibres, lymphatic and blood vessels (Liu et al., 2013). It has been reported that EGC activation may amplify intestinal inflammation (Cirillo et al., 2011; Ochoa-Cortes et al., 2016) and PPARα agonists mitigate it by reducing the glial expression of S100B protein (Esposito et al., 2014). The robust expression of the PPARα receptor on the muscular and endothelial components of blood vessels suggests a possible role of this receptor in the control of canine GIT blood flow. The hypothesis is sustained by previous observations on the beneficial effects of PPARα agonists on inflammatory responses in vascular smooth muscle cells (Zahradka et al., 2003; Ji et al., 2010) and endothelial cells (Naidenow et al., 2016).

Conclusion

Taken together, the data of the present study show the wide distribution of the cannabinoid receptor ensemble in several cellular types of all layers of the canine GIT. These morphological findings, although not yet supported by physiological or pharmacological evidence, suggest that cannabinoid receptor agonists have a therapeutic potential for controlling gastrointestinal inflammatory conditions and visceral hypersensitivity in this species. The hypothesis is supported by a great deal of evidence on the intestinal protective effects of one of the most studied naturally occurring cannabinoid receptor ligands, PEA (Borrelli et al., 2015). PEA was originally considered to activate the CB2 receptor (Facci et al., 1995; Re et al., 2007; Petrosino et al., 2016), resulting in MCs down-modulation through the so-called ALIA mechanism (Autacoid Local Injury Antagonism) (Aloe et al., 1993; De Filippis et al., 2013; Petrosino and Di Marzo, 2017). Currently, PEA has been shown not only to have a strong affinity for other cannabinoid receptors, like GPR55 (reviewed by Petrosino et al., 2016), but also to reduce intestinal radiation injury in a mast cell-dependent manner (Wang et al., 2014), and to

normalize intestinal motility through a mechanism involving CB1 receptor (Capasso et al., 2014). Furthermore, using both in vitro and in vivo preclinical models of enteropathies, it has been demonstrated that the activation of PPAR-α by PEA results in inhibition of colitis-associated angiogenesis (Sarnelli et al., 2016), modulation of intestinal permeability (Karwad et al., 2017b), improvement of colon inflammation (Esposito et al., 2014), and protection against ischemia/reperfusion-induced intestinal injury (Di Paola et al., 2012). In conclusion, the findings of the present research support the potential therapeutic use of non-psychotropic and safe cannabinoid agonists such as PEA (Nestmann, 2016) in canine intestinal inflammation and may constitute a starting point for future comparative studies on the possible changes in the cannabinoid receptor ensemble during GIT inflammatory conditions in the dog.

Primary antibody	**Host**	**Code**	**Dilution**	**Source**
CB1	Rabbit	Orb10430	1:200	Biorbyt
CB2	Rabbit	AB45942	1:200	Abcam
CD31	Mouse	M0823 Clone JC70A	1:30	Dako
GFAP	Chicken	AB4674	1:800	Abcam
GPR55	Rabbit	NLS6817	1:200	Novus Biol.
Factor VIII	Rabbit	A0082	1:1000	Dako
HuC/HuD	Mouse	A21271	1:200	Life Technologies
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
Tryptase	Rabbit	PAB070Ca01	1:80	Cloude-Clone Corp.
Tryptase	Mouse	NBP1-40202	1:200	Novus Biol.
Tryptase	Mouse	M7052 Clone AA1	1:200	Dako

Table 1 *- Primary antibodies used in the study. Primary antibodies Suppliers: Abcam, Cambridge, UK; Bethyl Laboratories, Montgomery, TX, USA; Biorbyt Ltd., Cambridge, UK; Agilent, Santa Clara, CA, USA; Cloude-Clone Corp., Huston, TX, USA; Dako Cytomation, Golstrup, Denmark; Fitzgerald Industries Int., Inc. Concord, MA, USA; Life Technologies, Carlsbad, CA, USA; Novus Biologicals, Littleton, CO, USA.*

Secondary antibody	Host	Code	Dilution	Source
Anti-mouse IgG Alexa 594	Goat	A11005	1:200	Life Technologies
Anti-mouse IgG Alexa 488	Donkey	20010	1:100	Biotium
Anti-mouse IgG Alexa 594	Donkey	AB150132	1:1000	Abcam
Anti-rabbit IgG FITC	Goat	401314	1:200	Calbiochem-Novabiochem
Anti-rabbit 488	Donkey	AB150073	1:800	Abcam
Anti-goat IgG 594	Donkey	AB150132	1:600	Abcam
Anti-chicken TRITC	Donkey	703-025-155	1:200	Jackson

Table 2 *- Secondary antibodies used in the stud. Secondary antibodies Suppliers: Abcam, Cambridge, UK; Biotium, Inc. Hayward, CA, USA; Calbiochem-Novabiochem, San Diego, CA, USA; Jackson Immuno Research Laboratories, Inc. Baltimore Pike, PA, USA; Life technologies, Carlsbad, CA, USA*

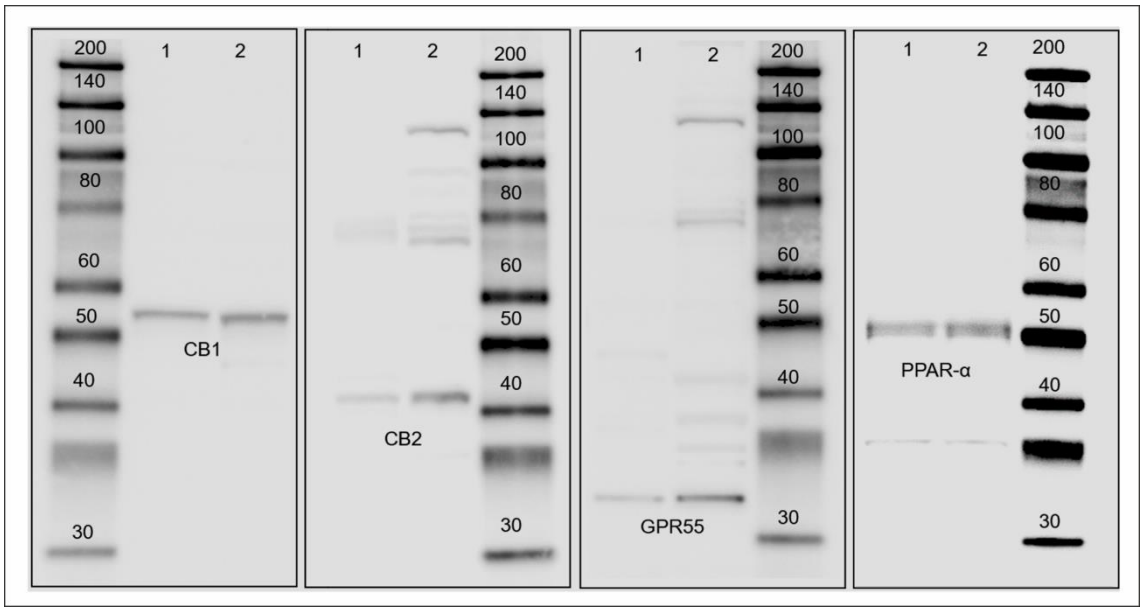


Figure 1 Representative image of Western blots (WB) analysis showing the specificity of the primary antibodies utilized: rabbit anti-cannabinoid receptor 1, rabbit anti-cannabinoid receptor 2, rabbit anti-G protein-coupled receptor 55 (GPR55), and rabbit anti nuclear peroxisome proliferator-activated receptor alpha (PPARα). Each antibody showed a major band close to the theoretical molecular weight. Lane 1 = dog small intestine, lane 2 = mouse small intestine. The images of the different immunoblots were slightly adjusted in brightness and contrast to match their backgrounds.

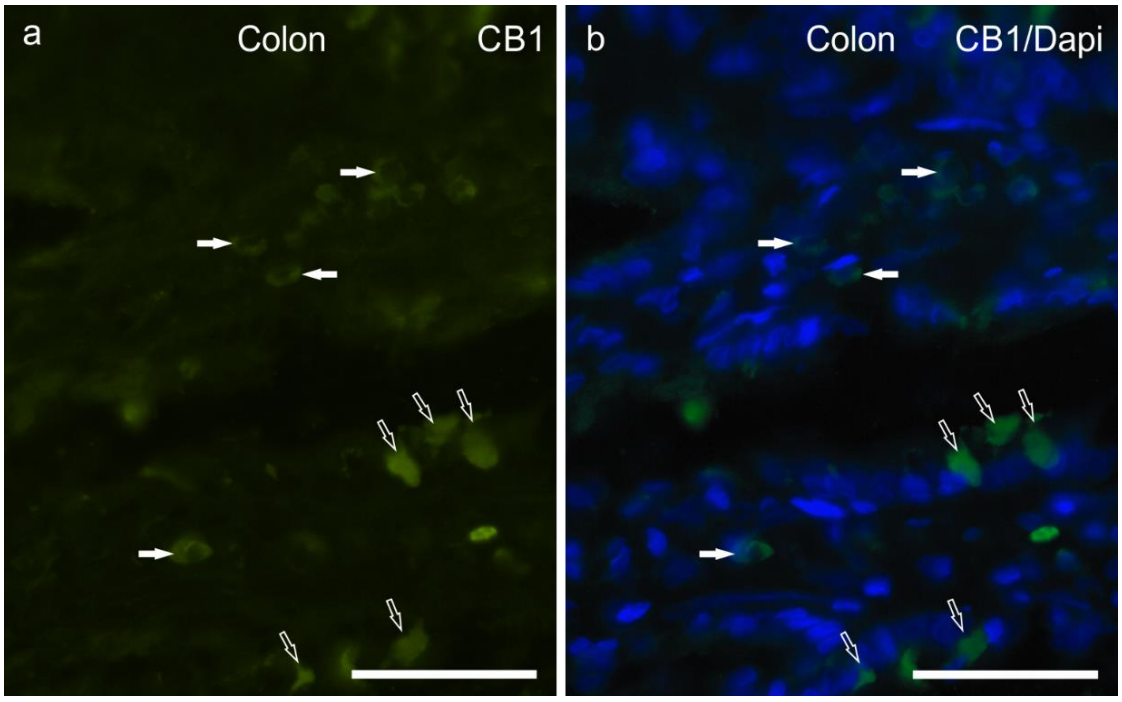


Figure 2. Photomicrograph showing cryosection of the mucosa of dog colon in which some lamina propria (white arrows) and epithelial cells (open arrows) of unknown nature were immunolabelled with the anti-cannabinoid receptor 1 antibody (CB1) (a). In the panel on the right (b), the nuclei of cells were labelled with the nuclear stain DAPI. Scale bar: a-b, 50 μm.

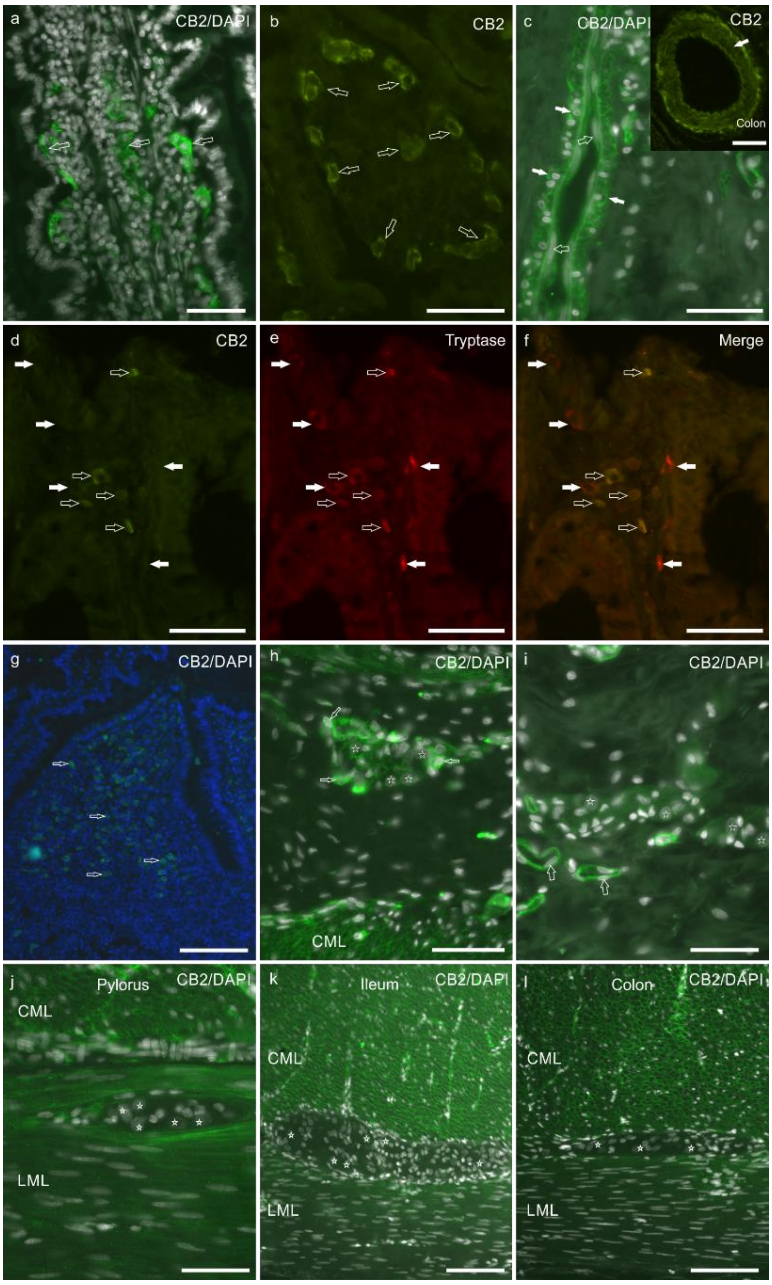


Figure 3. a-i) Photomicrograph showing longitudinal cryosections of dog gastrointestinal tract immunolabelled with the anti-cannabinoid receptor 2 antibody (CB2). In figures a, c, g-i, the cellular nuclei were labelled with the nuclear stain DAPI. Arrows indicate bright CB2-immunolabelled endothelial cells of blood capillaries running along the major axis of the duodenal villus (longitudinal section, a); when the villus was cut orthogonally (b), it was possible to observe CB2-immunolabelled vessels arranged like clock numbers around the circumference of the villus. c) White and open arrows indicate, respectively, the nuclei of smooth muscle and endothelial cells expressing strong CB2 immunoreactivity. In the insert, the white arrow indicates a thick submucosal artery of the colon showing CB2 immunoreactivity. d-e) Open arrows indicate mast cells of the lamina propria of the colon which were immunoreactive for CB2 (d) and tryptase (e); white arrows indicate mast cells which were tryptase-immunoreactive and CB2-negative (f, merged image). g) Arrows indicate some CB2-immunoreactive immunocytes within a duodenal mucosa lymphatic nodule. h) Stars indicate the nuclei of small neurons of the submucosal plexus showing moderate CB2 immunoreactivity on cell membrane. Arrows indicate nuclei of smaller dimension belonging to enteric glial cells showing bright CB2 immunoreactivity. i) Stars indicate the nuclei of submucosal neurons showing faint and diffuse cytoplasmic CB2 immunoreactivity. The arrows indicate the nuclei of endothelial cells, which showed strong CB2-immunolabelling. j-l) Stars indicate the nuclei of myenteric plexus neurons of pylorus (j), ileum (k) and colon (l), which were CB2-negative. On the contrary, the smooth muscle cells of the longitudinal (LML) and circular muscle layers (CML) showed intense CB2 immunoreactivity. Scale bar: a-j, 50 μm; k, l, 100 μm.

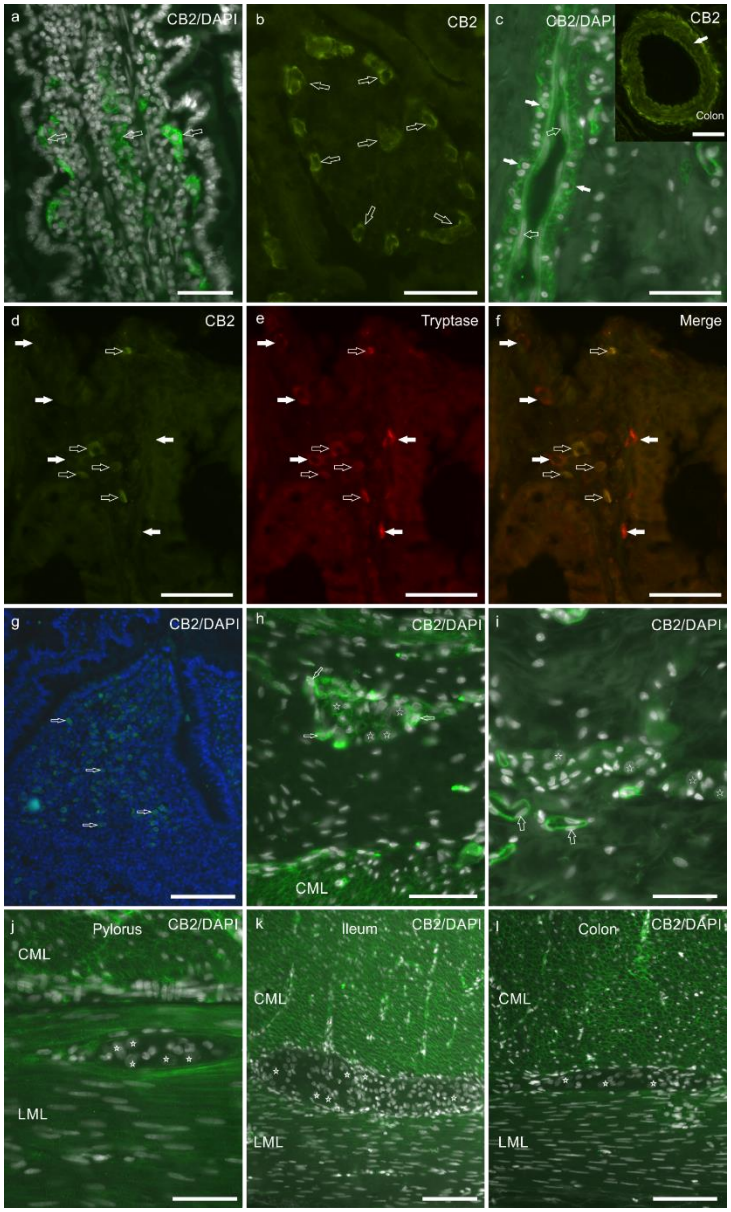


Figure 4. Photomicrograph showing cryosections of dog gastrointestinal tract immunolabelled with the anti-GPR55 antibody. a-c) Small white arrows indicate lamina propria cells of pylorus (a) and colon (b-c) showing bright GPR55 immunoreactivity. The small open arrows (a, b) indicate GPR55-immunolabelled enterochromaffin cells of the pylorus (a) and colon (b). Large open arrows indicate epithelial cells of the inner portion of the mucosa of the colon, which showed diffuse GPR55 immunoreactivity. d-f) The white arrows indicate that GPR55-immunoreactive cells (d) of the duodenal mucosa co-expressed IBA1 immunoreactivity (e), which indicates that these cells were predominantly macrophages. The open arrow indicates GPR55-immunoreactive cells, which were not IBA1-positive. g-i) White arrows indicate lamina propria cells of the colon which co-expressed GPR55- and IgA immunoreactivity, indicating that these cells were plasma cells. Open arrows, on the contrary, indicate GPR55-immunoreactive cells, which were not IgA-positive plasma cells. j-l) Distribution of GPR55-immunolabelling within the circular (CML) and longitudinal muscle layer (LML) of the duodenum (j), ileum (k) (cut in longitudinal sections) and colon (l) (cut in transverse section). Arrows indicate bright GPR55-immunolabelling in the external portions of the small intestine LML (j-k) and in the whole LML of the colon. Scale bar: a-c, g-i, 50 μm; d-f, j-l 100 μm

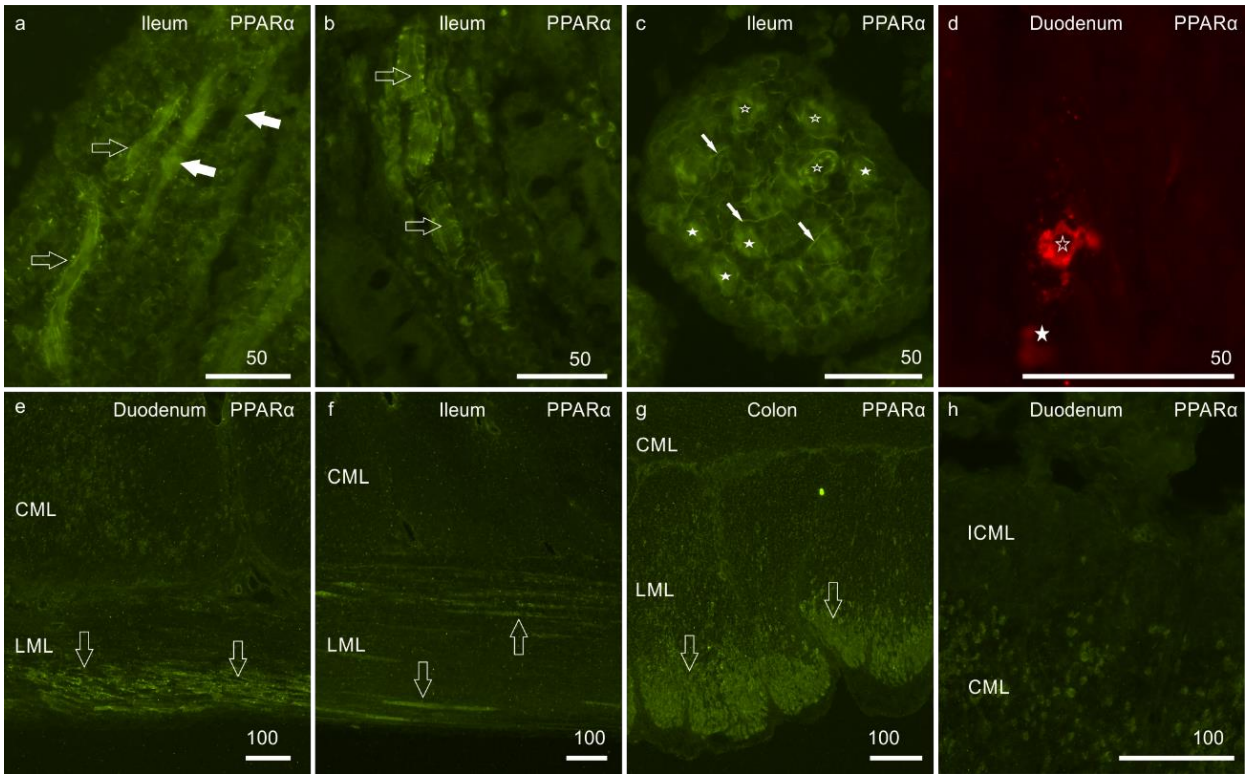


Figure 5. Photomicrograph showing cryosections of dog small and large intestine immunolabelled with the antibody anti-PPARα. a-b) Open arrows and white arrows indicate, respectively, blood vessels and fascicles of smooth muscle in a villus of ileal mucosa showing bright PPARα. c) Arrows indicate thin and PPARα-immunoreactive elongated cellular processes of unknown nature, visible in the apex of a villus cut orthogonally to its major axis. The white stars and open stars indicate, respectively, fascicles of smooth muscle cells and blood vessels showing PPARα immunoreactivity. d) The open star indicates a strong PPARα-immunoreactive lamina propria dendritic-like cell showing strong PPARα immunoreactivity, close to another less visible blurred cell (white star), because it is out of focus. e-h) Distribution of PPARα -immunolabelling within the circular (CML) and longitudinal muscle layer (LML) of the duodenum (e, h), ileum (f) (cut in longitudinal sections) and colon (g) (cut in transverse section). Arrows indicate bright PPARα-immunolabelling in the external portions of the small and large intestine longitudinal muscle layer LML, in which the immunostaining was more evident. In the ileum (f), PPARα-immunoreactive smooth muscle cells could also be scattered throughout the whole thickness of the LML. In the inner portion of the circular muscle layer (ICML) (h), PPARα immunoreactivity was almost undetectable. Scale bar: a-d, 50 μm; e-h, 100 μm.

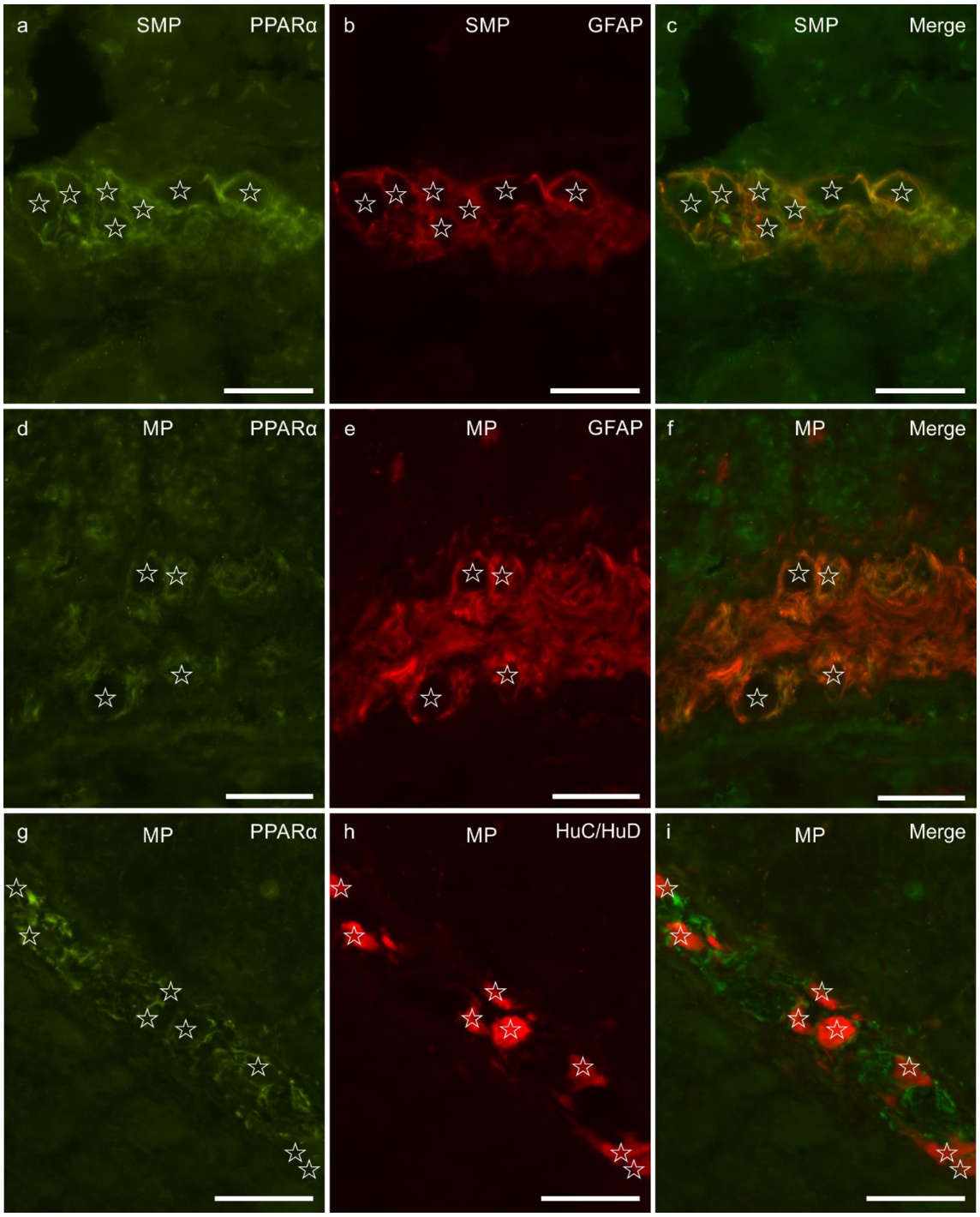
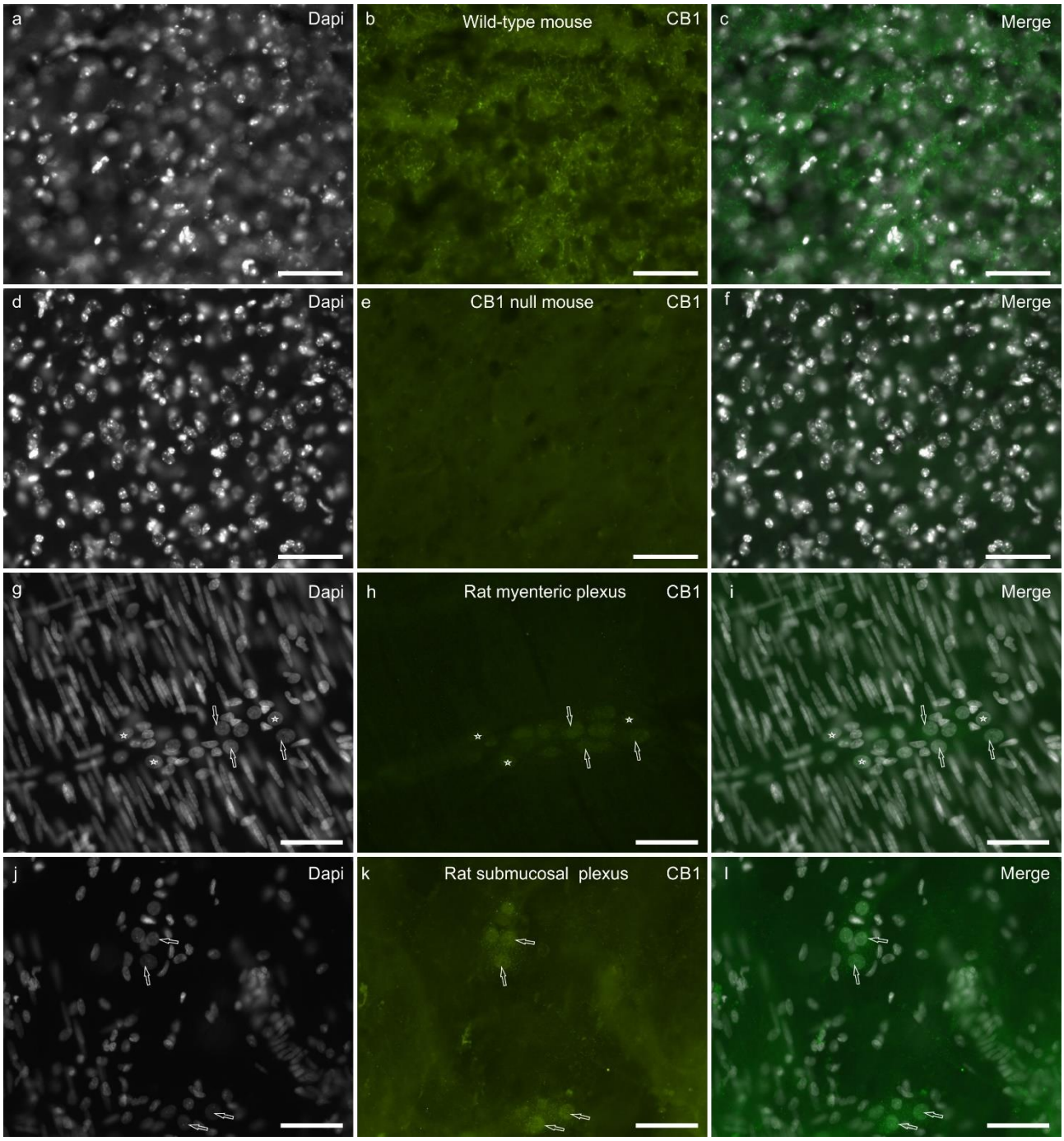
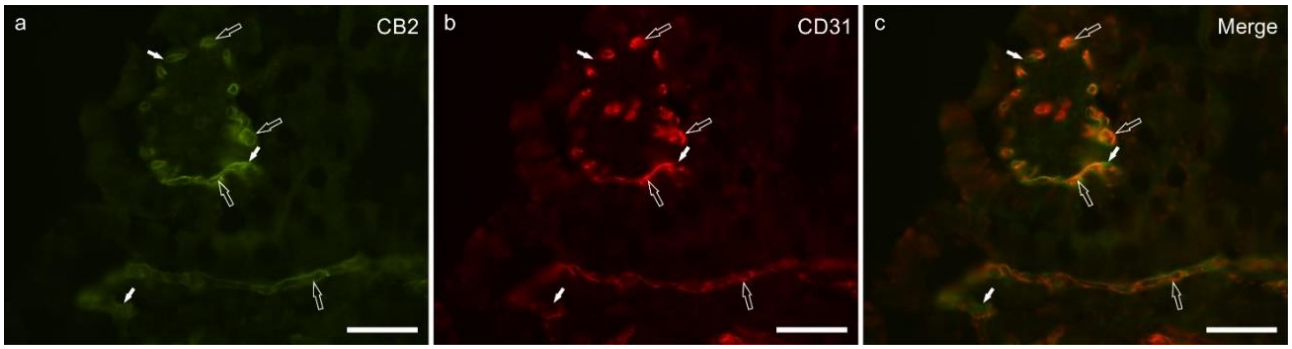


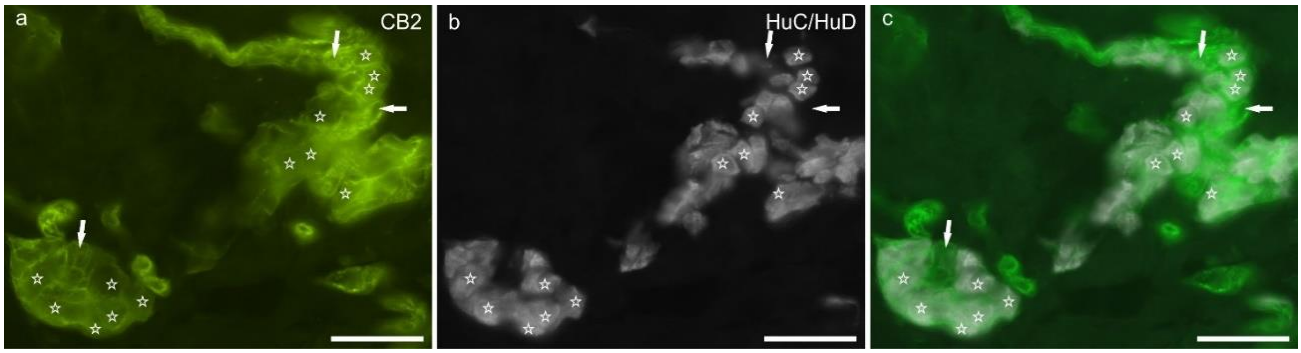
Figure 6. Photomicrograph showing cryosections of submucosal plexus (SMP) (a-c) and myenteric plexus (MP) (d-i) of the dog duodenum, immunolabelled with the anti-PPARα antibody. a-f) Stars indicate SMP (a-c) and MP (d-f) neurons (not visible) encircled by enteric glial cells which were immunoreactive for PPARα (a) and GFAP (b) (c, f, merge images). g-i) Stars indicate MP HuC/HuD immunoreactive neurons (h) which were PPARα-negative (g). On the contrary, a network of PPARα-positive cellular processes belonging to enteric glial cells is visible around HuC/HuD neurons. Scale bar: a-i, 50 μm.



Supplementary Figure 1. Photomicrograph showing cryosections of the neocortex of wild-type mouse (a-c) and CB1 null mouse (d-f), and wholemount preparations of the myenteric (g-i) and submucosal plexus (j-l) of rat ileum in which neurons and nerve fibers showed immunoreactivity for the CB1 receptor. In the cryosection of the wild type mouse, CB1 immunoreactivity was expressed by axonal varicosities (b, c), which were absent in the CB1 null mouse (e, f). In the rat myenteric and submucosal plexuses, CB1 immunoreactivity was expressed by neurons which displayed CB1 immunolabelling also in nucleus (arrows) or only in the nucleus (stars). Scale bar: a-l, 50 µm.



Supplementary Figure 2. Photomicrograph showing cryosections of the dog ileum in which the mucosal blood vessels showed immunoreactivity for the CB2 receptor (a) and platelet endothelial cell adhesion molecule (CD31) (b). Open arrows indicate capillaries in which the two markers were co-localized, as visible in the merge image (c); white arrows indicate small areas in which the two markers were non overlapped, indicating that other cellular types (likely pericytes) showed CB2 immunoreactivity. Scale bar: a-c, 50 µm.



Supplementary Figure 3. Photomicrograph showing a cryosection of the duodenum of a dog with chronic and severe enteritis immunolabelled, in which the anti-cannabinoid receptor 2 (a) and anti-HuC/HuD (pan-neuronal marker) antibodies (b) were co-localized. Stars indicate submucosal plexus neurons showing diffuse and moderate cytoplasmic CB2-immunostaining and bright cell membrane immunolabelling. Arrows indicate bright CB2 immunoreactivity in peri-neuronal enteric glial cells. Scale bar: a-c, 50 µm.

Experimental studies

Student thesis –Paper submitted to Histochemistry and Cell Biology. Modified from:

Localization of cannabinoid receptors in the cat gastrointestinal tract

Agnese Stanzani[1], Giorgia Galiazzo[1], Fiorella Giancola[2], Claudio Tagliavia[1], Margherita De Silva[1], Marco Pietra[1], Federico Fracassi[1], Roberto Chiocchetti[1]

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Abstract

Introduction - A growing body of literature indicates that activation of cannabinoid receptors may exert beneficial effects on gastrointestinal inflammation and visceral hypersensitivity.

Objectives - The present *ex vivo* study was aimed to investigate immunohistochemically the distribution of the canonical cannabinoid receptors CB_1 (CB1R) and CB_2 (CB2R), and the putative cannabinoid receptors G protein-coupled receptor 55 (GPR55), nuclear peroxisome proliferator-activated receptors alpha (PPARα) and gamma (PPARγ), transient receptor potential ankyrin 1 (TRPA1), and serotonin receptor 5-HT1a (5-HT1a) in the gastrointestinal tract of the cat.

Results - CB1R-immunoreactivity (CB1R-IR) was observed in gastric epithelial cells, intestinal enteroendocrine cells (EECs) and goblet cells (Figure 1 a-l), lamina propria mast cells (MCs), and myenteric plexus (MP) neurons (Figure 2 a-f). CB2R-IR was expressed by EECs, enterocytes, and macrophages (Figure 3 a-o). GPR55-IR was expressed by EECs, macrophages, immunocytes, and MP neurons (Figure 4 a-i). PPARα-IR was expressed by parietal cells, immunocytes, smooth muscle cells, and enteroglial cells (Figure 5 a-i). PPARγ-IR was expressed by the nucleus of MP neurons (Figure 5 j-l). TRPA1-IR was expressed by enteric neurons and intestinal goblet cells (Figure 6 a-f). 5-HT1a receptor-IR was expressed by gastrointestinal epithelial cells and gastric smooth muscle cells (Figure 6 g-i).

Conclusions and relevance - Cannabinoid receptors showed a wide distribution in the feline GIT layers. Although not yet confirmed/supported by functional evidences, the present research might represent an anatomical substrate that might be useful to support, in feline species, the therapeutic use of cannabinoids during gastrointestinal inflammatory diseases.

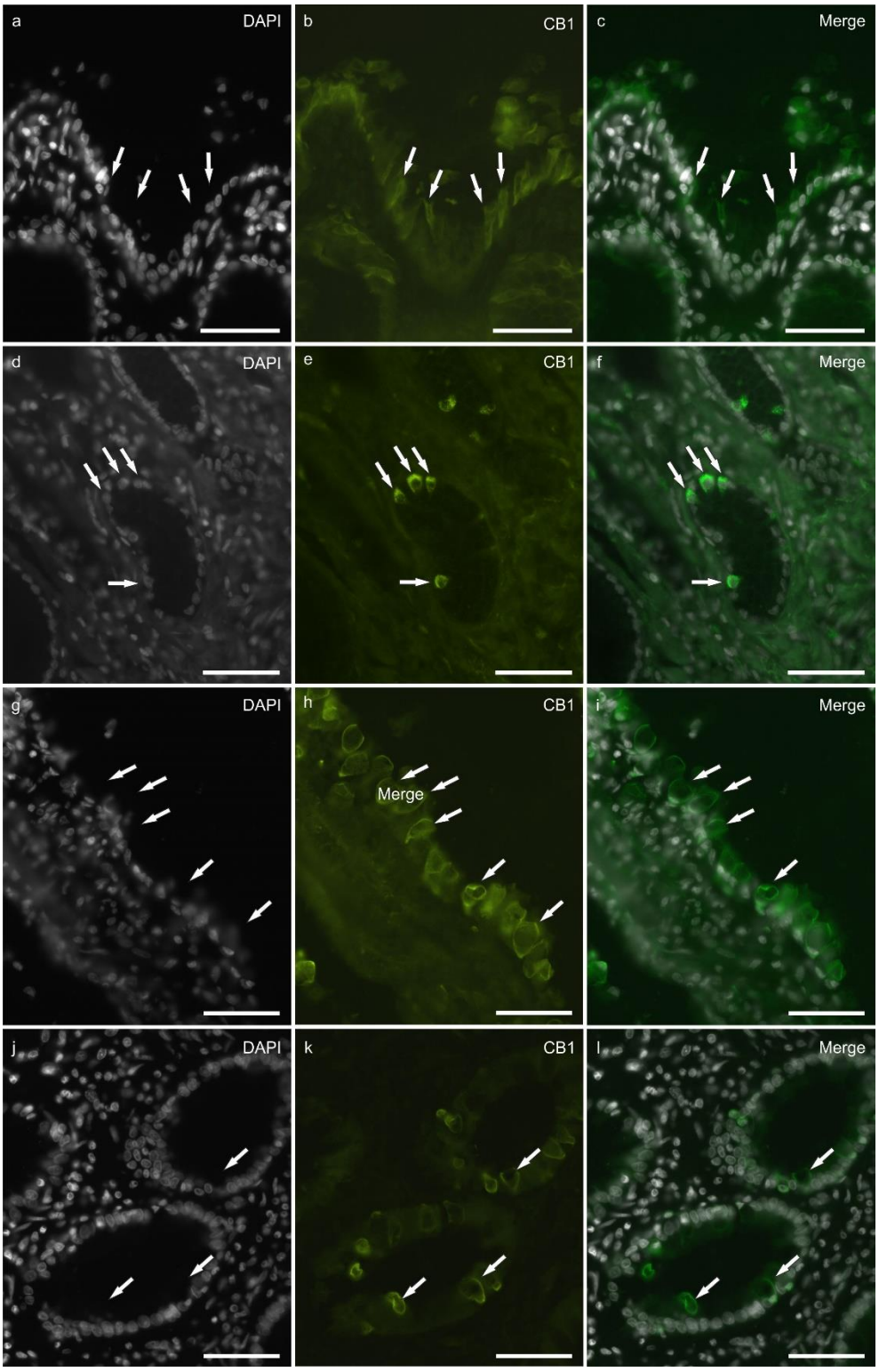

Figure 1(a-l): Photomicrograph showing cryosections of the cat gastrointestinal tract immunolabeled with the antibody anti-cannabinoid receptor 1 (CB1) a-c). Arrows indicate some of the pyloric elongated mucosal cells, which show bright CB1 receptor immunoreactivity. d-f) Arrows indicate four pyloric enteroendocrine cells displaying bright cytoplasmic CB1 receptor immunoreactivity. 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.

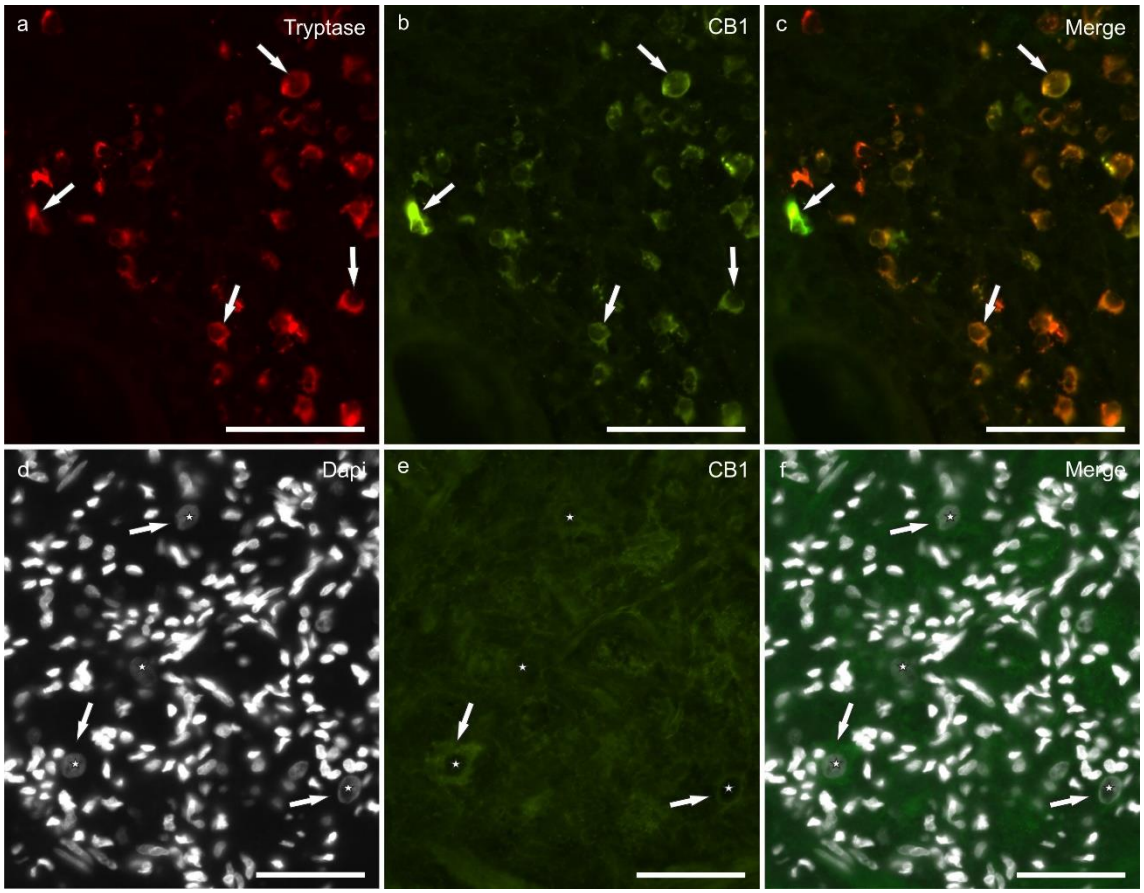

Figure 2(a-f): Photomicrograph showing a cryosection of the cat gastrointestinal tract immunolabeled with the antibody anti-cannabinoid receptor 1 (CB1). Cellular nuclei were labelled with the nuclear stain DAPI. a-c). Arrows indicate four of the numerous lamina propria mast cells co-expressing tryptase (a) and bright CB1 receptor (b) immunoreactivity. In c the merge image. d-f) Some of the myenteric plexus neurons (arrows) showed faint CB1 receptor immunoreactivity. The large nuclei of the enteric neurons were labelled with DAPI. Scale bar: a-f, 50 µm.

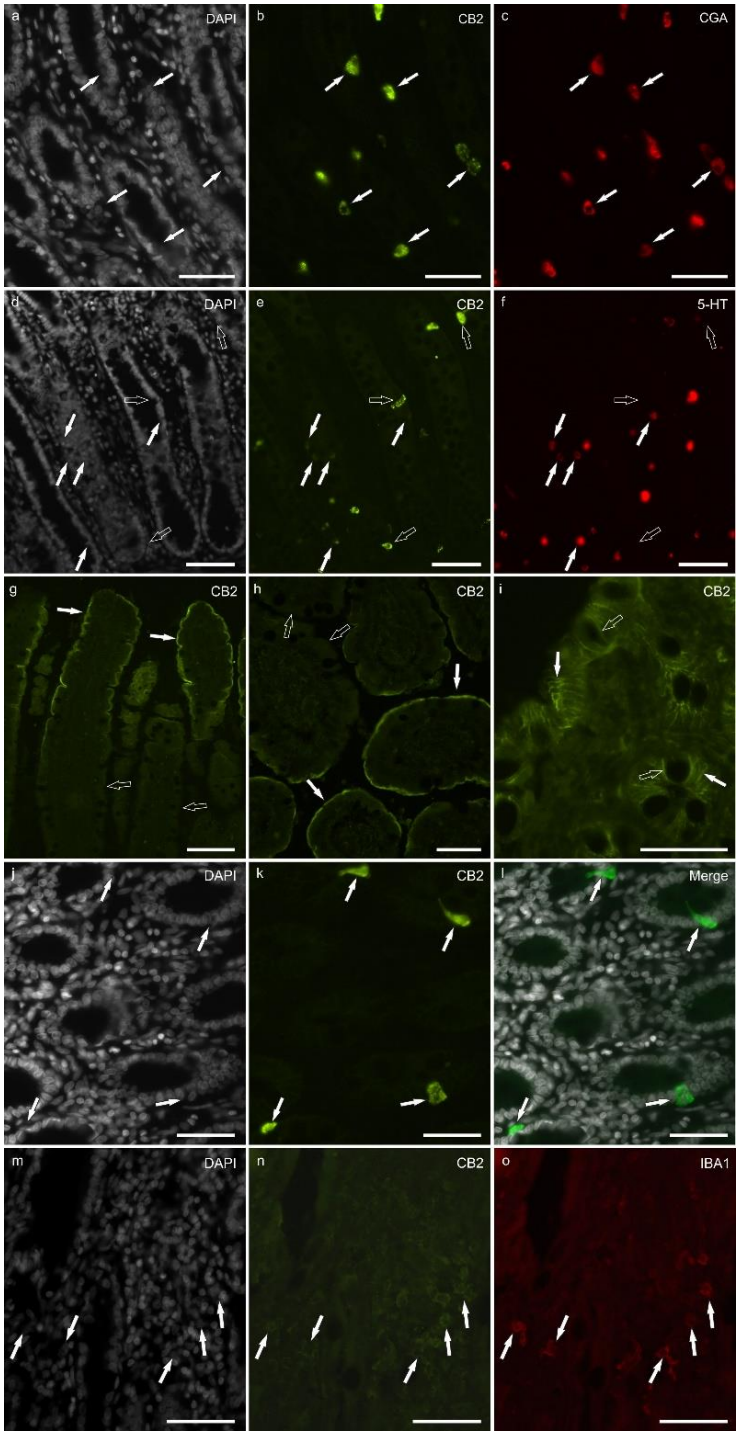

Figure 3(a-o): Photomicrograph showing cryosections of cat gastrointestinal tract immunolabelled with the antibody anti-cannabinoid receptor 2 (CB2). Cellular nuclei were labelled with the nuclear stain DAPI. a-b) Arrows indicate some of the numerous enteroendocrine cells identified with the antibody anti-chromogranin A (CGA) (c) co-expressing bright CB2 receptor immunoreactivity (b) d-f) Arrows indicate some of the numerous enteroendocrine cells identified with the antibody anti-serotonin (5-HT) (c) co-expressing bright CB2 receptor immunoreactivity (b). g-h) In the small intestine of one subject (#2), CB2 receptor immunoreactivity was expressed by the luminal surface of enterocytes distributed along the outer half (apical portion) of the villi (white arrows), whereas in their inner half the epithelial cells were CB2 negative (open arrows). 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-o) The lamina propria macrophages, recognized for their IBA1 immunoreactivity, co-expressed moderate CB2 immunolabelling (arrows). Scale bar: a-f, h-o 50 µm; g, 100 µm.

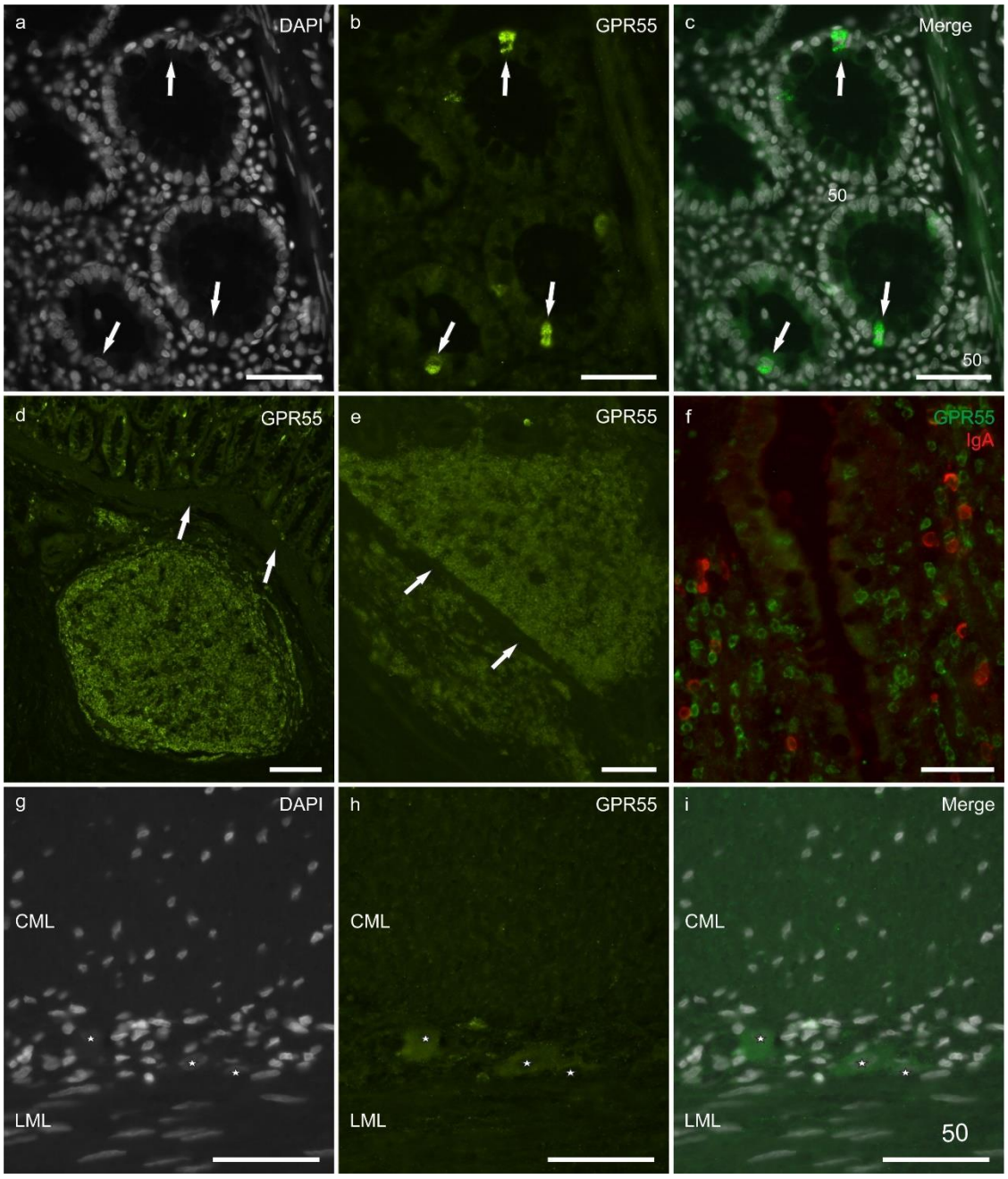

Figure 4 (a-i): Photomicrograph showing cryosections of cat small and large intestine immunolabelled with the antibody anti-GPR55. Cellular nuclei were labelled with the nuclear stain DAPI. a-c) Arrows indicate bright GPR55 receptor immunolabelling (b) of the enteroendocrine cells of the colon. d-e) Intestinal lymphatic nodules in which a large number of immunocytes showed bright GPR55 immunoreactivity. The arrows indicate the muscularis mucosae. f) Lamina propria IgA immunoreactive plasma cells (red color) did not co-express GPR55 immunoreactivity (green color). g-i) Gastrointestinal subsets of myenteric plexus neurons expressed moderate GPR55 immunoreactivity. The white stars indicate three neuronal nuclei. Abbreviations: circular muscle layer (CML); longitudinal muscle layer (LML). Scale bar: a-d, e-i 50 µm; d, 100 µm.

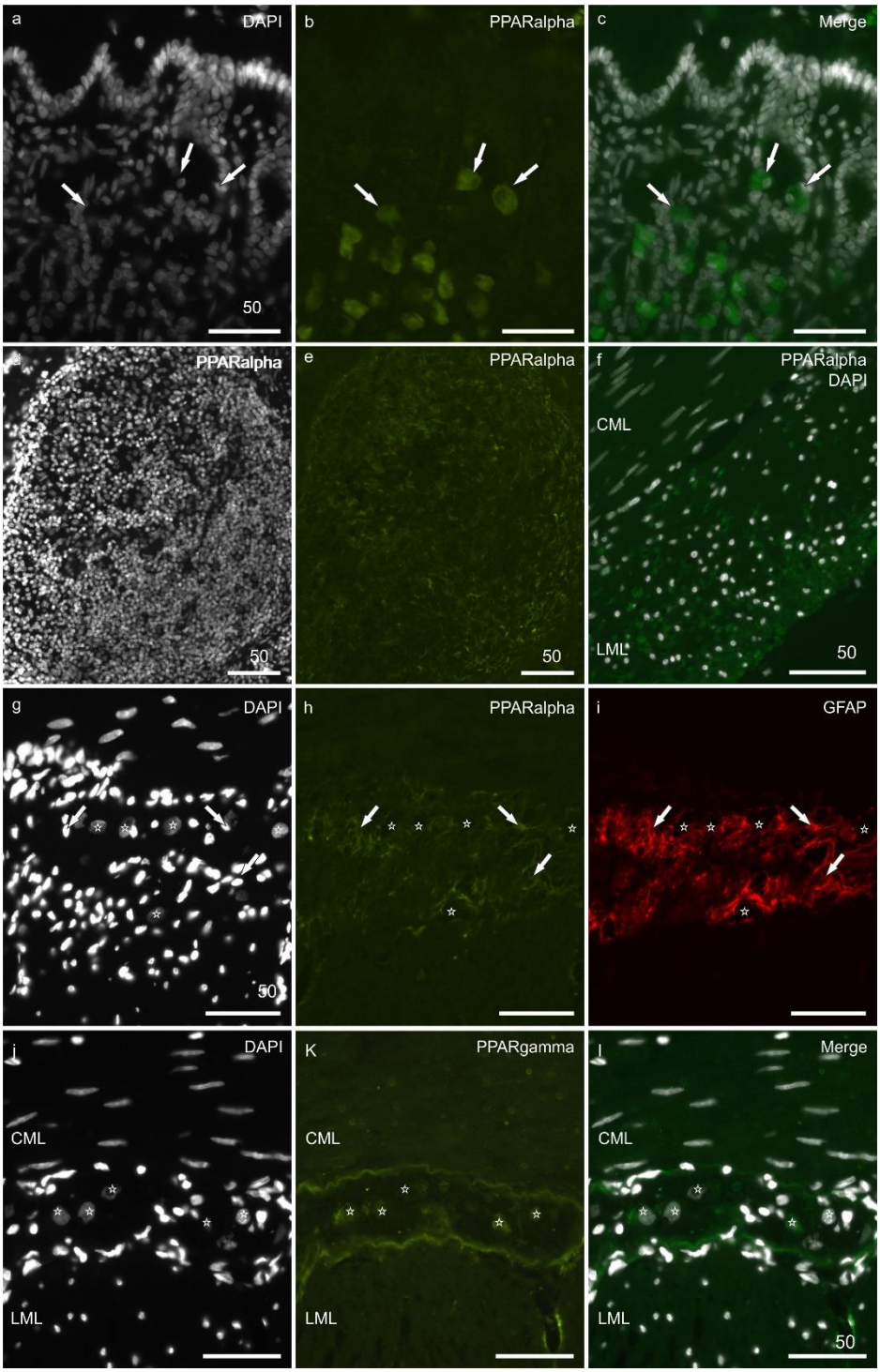

Figure 5 (a-l): Photomicrograph showing cryosections of cat pylorus and intestine immunolabelled with the antibody anti-PPARα (a-i) and PPARγ (j-l). Cellular nuclei were labelled with the nuclear stain DAPI. a-b) Arrows indicate three large pyloric gland cells (putative parietal cells) which showed bright PPARα immunoreactivity. d-e) Intestinal lymph node in which a large percentage of immunocytes showed PPARα immunoreactivity. f) PPARα immunoreactivity was observed also in the smooth muscle cells of the longitudinal muscle layer (LML). Abbreviation: circular muscle layer, CML. g-i) 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. j-l) Stars indicate the nuclei of some myenteric plexus neurons, which showed weak PPARγ immunoreactivity. Abbreviations: circular muscle layer, CML; longitudinal muscle layer (LML). Scale bar: a-l, 50 µm.

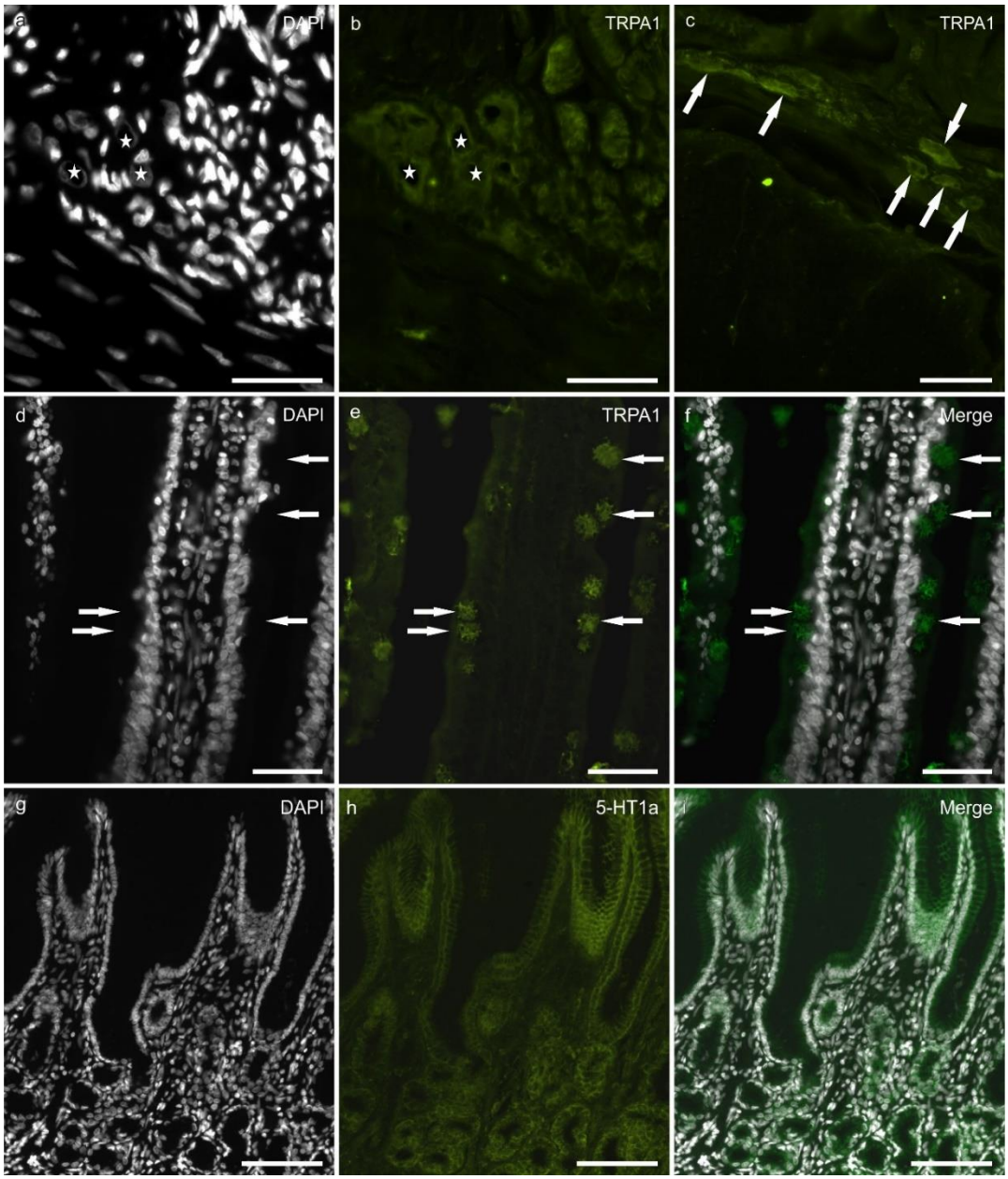

Figure 6 (a-i): Photomicrograph showing cryosections of cat gastrointestinal tract immunolabelled with the antibody anti-TRPA1 (a-f) and 5-HT1a (g-i). Cellular nuclei were labelled with the nuclear stain DAPI. a, b) Stars indicate three nuclei of the pyloric myenteric plexus neurons which showed TRPA1 immunoreactivity. c) Arrows indicate duodenal myenteric plexus neurons TRPA1 immunoreactive. d-f) Arrows indicate small intestinal goblet cells which showed bright TRPA1 immunoreactivity. g-i) Pyloric mucosa in which mucous and glandular cells expressed 5-HT1a immunoreactivity. Scale bar: a-i, 50 µm.

Poster presented at NEUROGASTRO 2019, Lisbon (Portugal). Modified from:

Localization of cannabinoid receptors in the canine and feline gastrointestinal tract

Roberto Chiocchetti[1], Giorgia Galiazzo[1], Agnese Stanzani[1], Fiorella Giancola[2], Claudio Tagliavia[1], Margherita de Silva[1], Federico Fracassi[1], Marco Pietra[1]

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Abstract

Introduction - The endocannabinoid system (ECS) is involved in the control of gastrointestinal inflammation and visceral pain.

Objectives - The present ex vivo study was aimed to investigate the distribution of the canonical [CB1 (CB1R) and CB2 (CB2R)], and putative cannabinoid receptors [G protein-coupled receptor 55 (GPR55), nuclear peroxisome proliferator-activated receptors alpha (PPARα), transient receptor potential ankyrin 1 (TRPA1), and serotonin receptor 5-HT1a (5-HT1aR)] in the gastrointestinal tract of the dog and the cat.

Results - Gastrointestinal tissues (pylorus, duodenum, ileum and distal colon) from four dogs and four cats were collected after spontaneous death or after euthanasia, following owners' consent. Specimens were processed by immunohistochemistry using species-specific primary antibodies. Antibodies targeting enteric neurons, glial cells (EGCs), enteroendocrine cells (EECs), mast cells (MCs), macrophages, and plasma cells were employed in co-localization experiments to identify the cell types expressing cannabinoid receptors. CB1R-immunoreactivity (CB1R-IR) was observed in epithelial and lamina propria (LP) cells, and in myenteric plexus (MP) neurons in both the species. CB2R-IR was expressed by LP MCs and immunocytes, blood vessels and smooth muscle cells in the dog. In the cat it was expressed by EECs, intestinal epithelial cells, and macrophages. GPR55-IR was expressed by LP macrophages and smooth muscle cells in the dog; in the cat it was expressed by EECs, immunocytes and MP neurons. PPARα-IR was expressed by smooth muscle cells and EGCs in both the species. In the cat PPARα-IR was also expressed by immunocytes and gastric parietal cells. TRPA1-IR was expressed by globet cells in both the species and enteric neurons only in the cat. 5-HT1aR-IR was expressed by epithelial cells in the cat and by globet cells, LP cells and MP neurons in the dog (Figure 1).

Conclusion and relevance - The present research provides an anatomical basis supporting the therapeutic use of cannabinoid receptor agonists in relieving motility disorders and visceral hypersensitivity in acute or chronic enteropathies.

Student thesis. Paper ready for submission. Modified from:

Localization of cannabinoid receptors in the horse ileum

Giorgia Galiazzo[1], Fiorella Giancola[2], Claudio Tagliavia[1], Agnese Stanzani[1], Margherita De Silva[1], Alessandro Spadari[1], Riccardo Rinnovati[1], Roberto Chiocchetti[1]

[1]*Department of Veterinary Medical Sciences, University of Bologna, Italy*

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Abstract

Introduction – Colic is a digestive disorder of horses and one of the most dangerous emergency problems in equine medicine. A growing body of literature indicates that activation of cannabinoid receptors could exert beneficial effects on gastrointestinal inflammation and visceral hypersensitivity.

Objectives – The present *ex vivo* study was aimed to investigate immunohistochemically the distribution of the canonical cannabinoid receptors CB_1 (CB1R) and CB_2 (CB2R), and the putative cannabinoid receptors G protein-coupled receptor 55 (GPR55), nuclear peroxisome proliferator-activated receptors alpha (PPARα) and gamma (PPARγ), transient receptor potential ankyrin 1 (TRPA1), transient potential vanilloid receptor 1 (TRPV1) and serotonin receptor 5-HT1a (5-HT1aR) in the horse ileum.

Results – CB1R-immunoreactivity (CB1R-IR) was observed in epithelial cells, myenteric plexus (MP) and submucosal plexus (SMP) neurons, nerve fibers and glial cells. CB2R-IR was expressed by epithelial and lamina propria (LP) cells. GPR55-IR was expressed by enteroendocrine cells (EECs), LP immune cells, smooth muscle cells of the circular muscular layer (CML). PPARα-IR was expressed by smooth muscle cells of the longitudinal muscular layer (LML), endothelial cells of submucosal vessels, MP and SMP glial cells, and elongated cells in proximity to the MP resembling interstitial cells of Cajal (ICCs). PPARγ-IR was expressed in the nuclei of neurons, glial cells, smooth muscle cells, epithelial cells, and LP cells. TRPA1-IR was expressed by enteric neurons and nerve fibers. TRPV1-IR was expressed by MP glial cells and SMP glial cells and neurons. 5-HT1aR-IR was expressed by Paneth cells cells and LP immune cells.

Conclusions and relevance – Cannabinoid receptors showed a wide distribution in the ileum of the horse, although not yet supported by functional evidences. The present research might represent an anatomical substrate that might support further studies about the distribution of cannabinoids receptors during gastrointestinal inflammatory diseases.

Introduction

Cannabinoid receptors regulate different gastrointestinal functions, including motility, emesis, appetite or satiety, both in physiological and pathological conditions (Izzo and Coutts, 2005).
Colic is a digestive disorder of horses, and includes different form of abdominal pain (Pilliner and Davies, 2004). It is one of the most dangerous emergency problem in equine medicine and one of the principal cause of death for horses, so it is a primary health concern of owners (Curtis et al., 2019). For this reason, several companies produce medical marjiuana and cannabinoid receptor agonists to be used in equine medicine, directed principally against somatic and visceral pain, although not yet support by anatomical or functional studies. Cannabidiol (CBD), a non-psychoactive compound found in *cannabis sativa*, seems to be one of the most promising therapeutic substances, due to its analgesic, anti-inflammatory, anti-spasmodic benefits (Mechoulamet al., 2007; Pertwee, 2008).
Phytocannabinoids are known to act on multiple targets, more than CB1 and CB2 receptors. They interact with other G-protein coupled receptors (GPR), nuclear receptors, transient receptor potential (TRP) channels, serotonin (5-HT) receptors and glycine receptors (Morales et al., 2017).
Knowing the cellular distribution of the specific receptors is fundamental to understand the action of a drug. To date, reliable anatomical studies regarding the cellular distribution of cannabinoid receptors in the horse intestinal tract are still lacking. In order to help filling these anatomical gaps, the present ex vivo study was designed to identify, in the equine ileum, the cellular distribution of two canonical cannabinoid receptors, i.e. CB1R and CB2R, and of six different putative cannabinoid receptors, i.e. G protein-coupled receptor 55 (GPR55), nuclear peroxisome proliferator-activated receptor alpha (PPARα) and gamma (PPARγ), transient potential vanilloid receptor 1 (TRPV1), transient potential ankyrin receptor 1 (TRPA1), and serotonin receptor 1a (5-HT1aR).

Material and methods

Aniamals

Ileal samples were collected ex-vivo from six horses (about 1.5 years of age) at the public slaughterhouse. Animals did not show gross alteration of the gastrointestinal wall.
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

Ileal samples (about 10 cm in lenght) were harvested within 30 minutes from death and were longitudinally opened along the mesenteric border. Tissues were then washed in phosphate-buffered saline (PBS), fixed and processed to obtain cryosections (2.0 cm x 0.5 cm) which were later processed for immunohistochemistry, as described in a previous study (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 overnight in a humid chamber at RT with a cocktail of primary antibodies (Table 1) diluted in 1.8% NaCl in 0.01M PBS containing 0.1% sodium azide. After washing in PBS (3 x 10 min), the sections were incubated for 1 h at RT in a humid chamber with the secondary antibodies (Table 2) diluted in PBS. Cryosections were then washed in PBS (3 x 10 min) and mounted in buffered glycerol at pH 8.6 with 4',6-diamidino-2-phenylindole – DAPI- (Santa Cruz Biotechnology, CA, USA).

Specificity of the primary antibodies

The choice of the primary antibodies used in the study was based on the homology of the amino acid sequence between the immunogen of the commercially available antisera and the horse proteins, verified by the "alignement" tool available on the Uniprot database (www.uniprot.org) and the BLAST tool of the National Center for Biotechnology information (NCBI) (www.ncbi.nlm.nih.gov) (Table 3).

Specificity of the secondary antibody

The specificity of the secondary antibody was tested by applying them after omission of the primary antibodies. No stained cells were detected after omitting the primary antibodies.

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).

Results

CB1 receptor - CB1 receptor immunoreactivity was expressed by the epitelium, in particular enterocytes and goblet cells. These cells showed cytoplasmatic and membrane reactivity, otherwise the nuclei were CB1-negative. Neurons and glial cells of the MP and SMP showed CB1R-immunoreactivity, confirmed with colocalization with HuC/HuD for neurons and GFAP for glial cells (Fig. 1a-f; Fig. 2a-d). Some nNOS (nitric oxide synthases)immunoreactive neurons showed CB1R immunoreactivity (Fig. 3a-c). In the muscular layer, near the MP, some nerve fibers showed CB1R-immunoreactivity. Cells surrounding nerve fibers, probably Schwann cells, showed CB1R immunoreactivity (Figure 4 a-c).

CB2 receptor - CB2 receptor immunoreactivity was expressed only in the mucosa, in particular epithelium and LP. In the epithelium, enterocytes and goblet cells showed cytoplasmatic immunoreactivity. In the lamina propria, immune cells showed bright cytoplasmatic immunoreactivity; however, the nature of these cells were not investigated by the co-localization with specific markers (Figure 5 a-d).

GPR55 - GPR55 was expressed in the mucosal, submucosal and muscular layer. In the mucosa, enteroendocrine cells (EECs) showed bright GPR55-immunoreactivity (Figure 6 a-f). Numerous small round immune cells, probably lymphatic cells, showed bright GPR55-immunoractivity. They were distributed in the LP (Figure 6 a-c), in proximity to the *muscularis mucosae*, and concentrated in the Peyer patches (Figure 6 d-f). However, the nature of these cells was not investigated by the use of specific markers fot B or T cells, such as CD79 and CD3, respectively. In the LP, other immune cells showed GPR55-immunoreactivity, likely/probably macrophages, because of the presence of aspecific pigmented granules in their cytoplasm (Fig. 7a-d). Finally, GPR55-immunoreactivity was present in groups of smooth muscle cells in the internal portion of the CML.

PPARα - PPARα-immunoreactivity was brightly expressed by smooth muscle cells and vascular cells. In the muscular layer, numerous cells in the LML were positive for PPARα (Fig. 8 g-i). Along the bundles of nerve fibers, glial cells (probably Schwann cells) showed bright immunoreactivity. Endothelial cells of large submucosal blood vessels showed bright immunoreactivity. Close to the MP, elongated cells between LML and CML, probably interstitial cells of Cajal (ICCs), were PPARα positive. In the ENS, glial cells were strongly positive both in MP and SMP (Fig. 8 a-f).

PPARγ - In the ENS, glial cells and neurons showed bright nuclear immunoreactivity, and weak cytoplasmatic immunoreactivity in some neurons (Fig. 9 a-c). A nuclear marking was also evident in smooth muscle cells, epithelial cells and LP cells (Fig. 9 d-f).

TRPA1 - TRPA1-immunoreactivity was expressed by enteric neurons (nuclei> cytoplasm) and in nerve fibers close to the MP (Fig. 10 a-f).

TRPV1 - TRPV1-immunoreactivity was expressed only in the ENS. In the MP, glial cells showed bright TRPV1-immunoreactivity (Fig. 11 a-c). Otherwise in the SMP, both glial cells and enteric neurons showed immunoreactvity (glial cells > neurons) (Fig. 11 d, e).

5-HT1aR - 5-HT1aR immunoreactivity was observed only in the mucosa. Large cells in the bottom part of intestinal crypts showed bright cytoplasmatic immunoreactivity, mainly in cytoplasmatic granules located in the apycal part of the cells (Fig 12 a-c). The antibody Rb anti Lyzozyme – specific marker for Paneth cells - gives the same pattern as the Rb anti 5-HT1aR, confirming that these cells are the same (Figure 13 a-b). In the LP, immune cells (likely macrophages or mast cells) showed bright 5-HT1aR- immunoreactivity (Fig. 12 d-f).

Discussion

As we recently observed in dogs (Galiazzo et al., 2018), cannabinoid receptors are also widely distributed in horse intestine. CB1R-immunoreactivity in epithelial cells and ENS confirmed the results obtained in rat, mouse, ferret, guinea-pig, pig and dogs (Kulkarni-Narla and Brown, 2000; Van Sickle et al., 2001; Coutts et al., 2002; Duncan et al., 2008; Galiazzo et al., 2018). The presence of CB1R-immunoreactivity in enterocytes and goblet cells may reflect a possible role of the receptor in the regulation of intestinal permeability and enteric cells regeneration. Moreover, the intestinal microbiota can influence the expression of CB1R in the enterocytes (Muccioli et al., 2010). The expression of CB1R in goblet cells indicates that cannabinoid can modulate mucus secretion, probably reducing it. Different studies highlighted the presence of CB1R in enteric neurons, but usually not in nNOS neurons, as we observed in this study. In the horse, nNOS neurons can be inhibitory neurons but also interneurons (Chiocchetti et al., 2009). The presence of CB1R-immunoreactivity in Schwann cells could be linked to a possible role of the receptor in myelinization process and neuronal regeneration (Costa et al., 2005; Freundt-Revilla et al., 2017).

CB2R-immunoreactivity was observed in enterocytes and goblet cells, suggesting a possible role of this receptor in preserving the integrity of the intestinal mucosa (Harvey et al., 2013). The presence of CB2R in immunitary cells is well known, in particular macrophages, mast cells, plasmacells, dendritic cells and lymphocytes (Facci et al., 1995; Wright et al., 2005, Duncan et al., 2008; Svensson et al., 2010). The immunomodulatory effect of cannabinoids is probably mediated by CB2R (Turcotte

et al., 2016). GPR55-immunoreactivity was observed in the ENS of rodents and humans (Ross, 2011; Li et al., 2013), but not of dogs (Galiazzo et al., 2018) neither in the horse. The presence of GPR55 in the enteroendocrine cells can explain a possible role in secretion of intestinal hormons. The expression of GPR55 in immunitary cells, in particular macrophages, has been demonstrated in rodents, humans and dogs (Taylor et al., 2015; Lanuti et al., 2015; Galiazzo et al., 2018) with immunomodulatory effect. Proinflammatory mediators, released by mast cells during intestinal inflammation, cause the accumulation of macrophages in the basal portion of the LP (He et al., 1997; He and Walls, 1998). Therefore, cannabinoids compounds acting on CB2R and/or GPR55, could limit the inflammatory cascade during GI diseases (Esfandyari et al., 2007).

PPARα was widely distributed in the ENS, musculature and vasculature. Mielinic sheath cells, probably Schwann cells, distributed along the bundles of nerve fibers, showed PPARα immunoreactivity. The expression of this receptor in smooth muscle cells of the LML was also observed in the dog (Galiazzo et al., 2018). This receptor could be involved in regulation of intestinal motility also in the horse, reinforced by the presence of PPARα immunoreactivity in ICCs, pacemaker cells of the GI tract (Torihashi et al., 1995). Glial cells surrounding MP and SMP neurons expressed bright PPARα immunoreactivity. These cells interact with the epithelium, immune system, nerve fibers, lymphatic and blood vessels (Sharkey, 2015; Liu et al., 2016).

In this study the presence of PPARγ in the nuclei of MP neurons could be explained with its neuroprotective potential, observed in certain central nervous system diseases (Hung et al., 2019). PPARγ is also involved in the regulation of intestinal homeostasis. Its expression on epithelial cells could be linked to the intestinal microbiota; indeed, butirrate, produced by intestinal microorganisms, is a PPARγ agonist. The activation of PPARγ has been demonstrated to prevent intestinal dysbiosis (Byndloss et al., 2017). PPARγ deletion in animal models seems to be correlated with the development of IBD and colon cancer, underlining a possible antiinflammatory and antineoplastic role of the receptor (Adachi et al., 2006; Varga et al., 2011). PPARγ is a target of CBD, which reduces intestinal inflammation mainly through a modulation of the neuro-immune axis (De Filippis et al., 2011; Couch et al., 2017).

TRPA1, an ion channel, detects specific chemicals in food and transduces mechanical, cold and chemical stimulation. In the present study we observed enteric TRPA1-IR neurons, which is consistent with data obtained on mouse intestine by Poole et al. (2011), who identified TRPA1-immunoreactivity on inhibitory neurons. However, in the present study we did not characterize the phenotype of equine enteric TRPA1-positive neurons.

TRPV1-immunoreactivity was expressed by MP glial cells and SMP glial cells and neurons. The expression of TRPV1-immunoreactivity in enteric neurons was observed in other studies (Anavi-

Goffer and Coutts, 2003; Buckinx et al., 2013). TRPV1 is fundamental in the mediation of heat sensitivity, increased during inflammation. Hyperalgesia and allodinia during inflammation are mediated by TRPV1. In animal models, TRPV1 antagonists attenuated visceral pain (Ghilardi et al., 2005).

We observed 5-HT1aR-immunoreactivity in epithelial cells. Considering the pivotal role of serotonin in regulating gut motility, visceral sensitivity, and fluid secretion via specific receptors, 5-HT1aR may exert a role in cellular homeostasis and secretion in the horse.

Conclusion

This study is the first to describe the distribution of different cannabinoid receptors in the equine ileum. Various cellular elements (epithelial cells, immune cells, neurons and glial cells, and muscular cells) showed immunoreactivity for cannabinoid receptors, highlighting the important role of the endocannabinoid system in the gut homeostasis. However, multiple colocalizations are still missing (i.e. immune cells, enteroendocrine cells, neurons). Considering the importance of gastrointestinal diseases in equine medicine, these results can provide an anatomical basis for further functional and clinical studies on the therapeutic use of non psycothropic cannabinoids for horses.

Primary antibody	Host	Code	Dilution	Source
CB1	Rabbit	ab23703	1:100	abcam
CB2	Rabbit	ab45942	1:200	abcam
GFAP	Chicken	ab4674	1:800	abcam
GPR55	Rabbit	NB110-55498	1:200	Novus Biol.
HuC/HuD	Mouse	A21271	1:200	Life Technologies
Lysozyme	Rabbit	Ab74666	1:2	abcam
PPARα	Rabbit	NB600-636	1:200	Novus Biol.
PPARγ	Rabbit	ab45036	1:300	abcam
5-HT1a	Rabbit	ab85615	1:100	abcam
TRPA1	Rabbit	ab58844	1:100	abcam
TRPV1	Rabbit	ACC-030	1:200	Alomone

Table 1. Primary antibodies used in the study. Primary anti bodies Suppliers: Abcam, Cambridge, UK; Life Technologies, Carlsbad, CA, US; Biotium, Inc. Hayward, CA, USA; Alomone, Jerusalem, Israel.

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-rabbit 555	Goat	ab150078	1:500	abcam
Anti-chicken TRITC	Donkey	703-025-155	1:200	Jackson

Table 2. Secondary antibodies used in the study. Secondary antibodies Suppliers: Abcam, Cambridge, UK; Biotium, Inc. Hayward, CA, USA; Jackson Immuno Research Laboratories, Inc. Baltimore Pike, PA, USA.

Antibody (host)	**Homology between the amino acidic sequences (immunogen and horse)**	**Homology with the immunogen sequence**
Rabbit anti CB1 (Ab23703)	97.88% Human	100%
Rabbit anti CB2 (Ab45942)	80.9% Rat	83.33%
Rabbit anti GPR55 (NB110-55498)	80% Human	78%
Rabbit anti PPARα (NB600-636)	90.81% Mouse	100%
Rabbit anti PPARγ (Ab45036)	92% Human	87.5%
Rabbit anti 5-HT1a (ab85615)	89.3% Rat	99%
Rabbit anti TRPA1 (Ab58844)	82% Rat	100%
Rabbit anti TRPV1 (ACC-030)	85% Rat	87.5%

Table 3. Homology between the AA sequences (between the immunogen and horse) and with the specific sequence of the immunogen of the CBR antibodies used in the study.

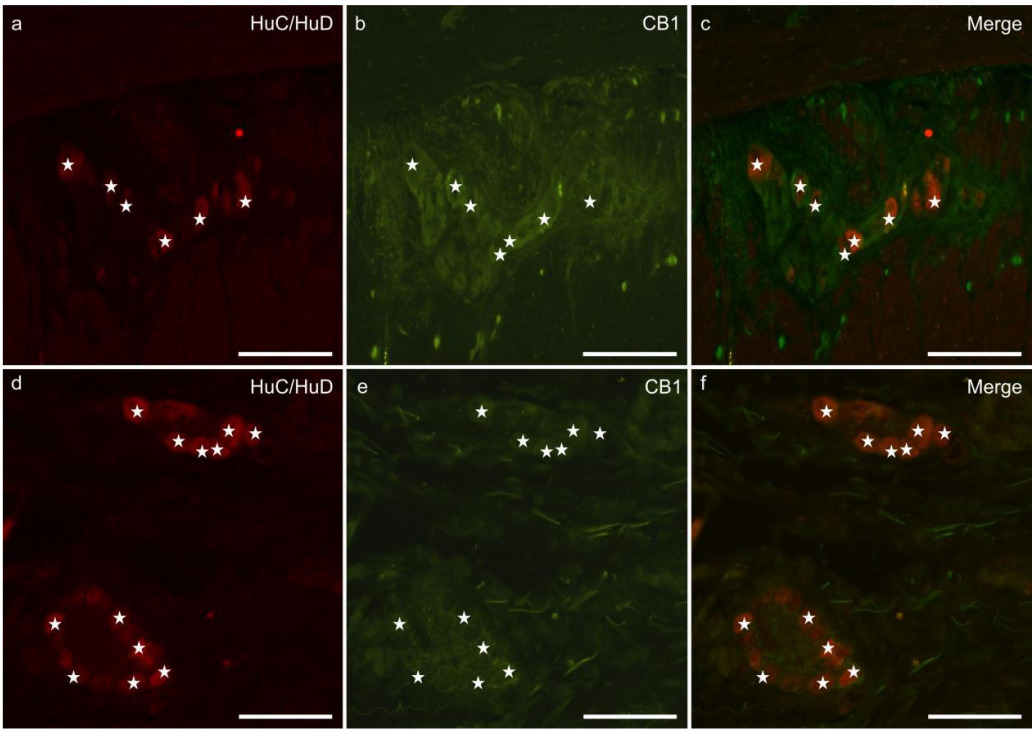

Figure 1 (a-f): Cryosections of equine ileum immunolabeled with the Ab CB1R and the pan-neuronal marker HuC/HuD. Stars indicate neurons in the MP (a-c) and SMP (d-f) which expressed both HuC/HuD (a, d) and CB1R (b, e).(c, f: merging). Scale bar a-f: 50 µm.

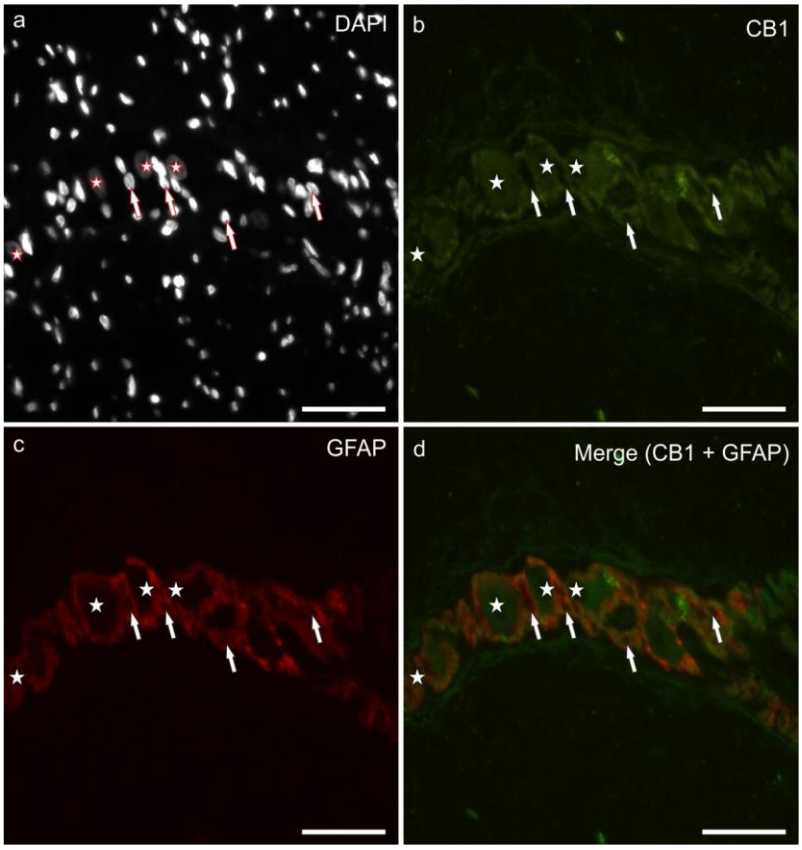

Figure 2 (a-d): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab CB1R and GFAP for glial cells. Stars indicate MP neurons nuclei immunolabeled with the marker DAPI (a), while arrows indicate nuclei of glial cells (c). Both nuclei of neurons and glial cells showed CB1R-immunoreactivity (b, d: merging). Scale bar: a-d, 50 µm

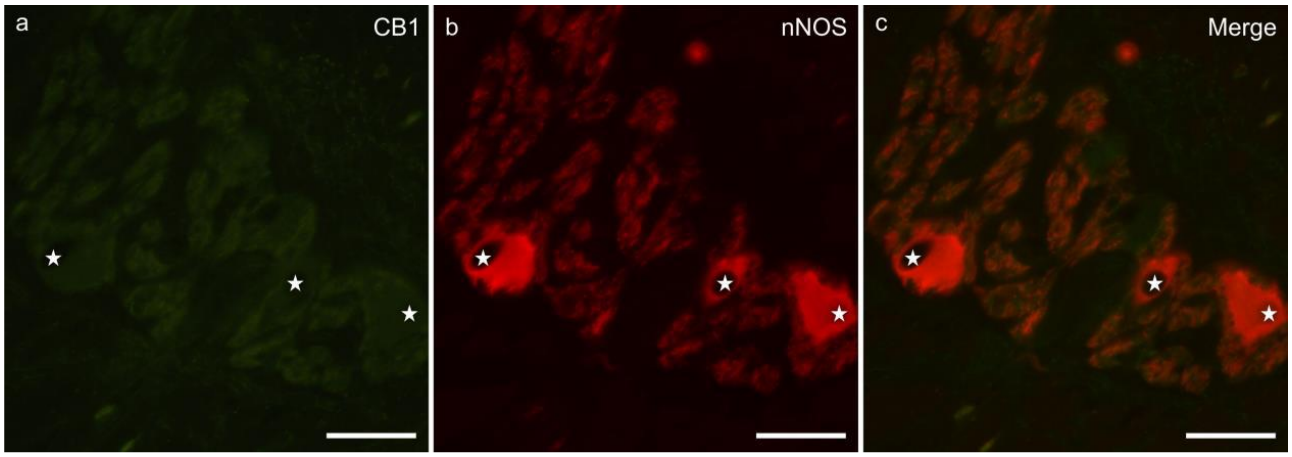

Figure 3 (a-c): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab CB1R (a) and the Ab anti nNOS (b). Stars indicate three MP neurons which co-express CB1R and nNOS (c). Scale bar 50 µm.

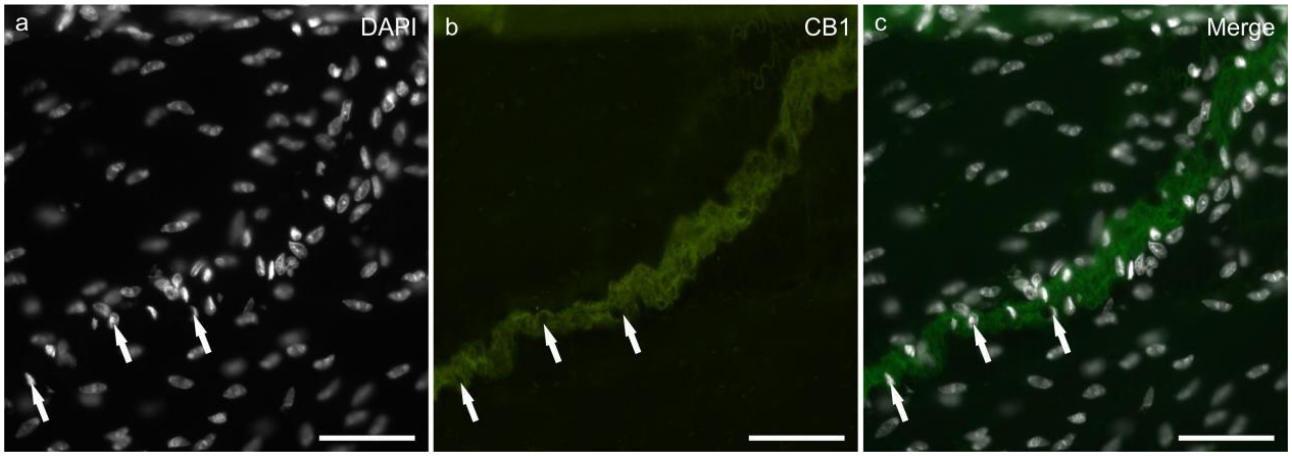

Figure 4 (a-c): Photomicrograph showing a cryosection of equine ileum (muscular layer) immunolabeled with the Ab CB1R. Nuclei are marked with DAPI (a). Stars indicate three nuclei of glial cells, probably Schwann cells, brightly immunolabeled with the ab CB1R.

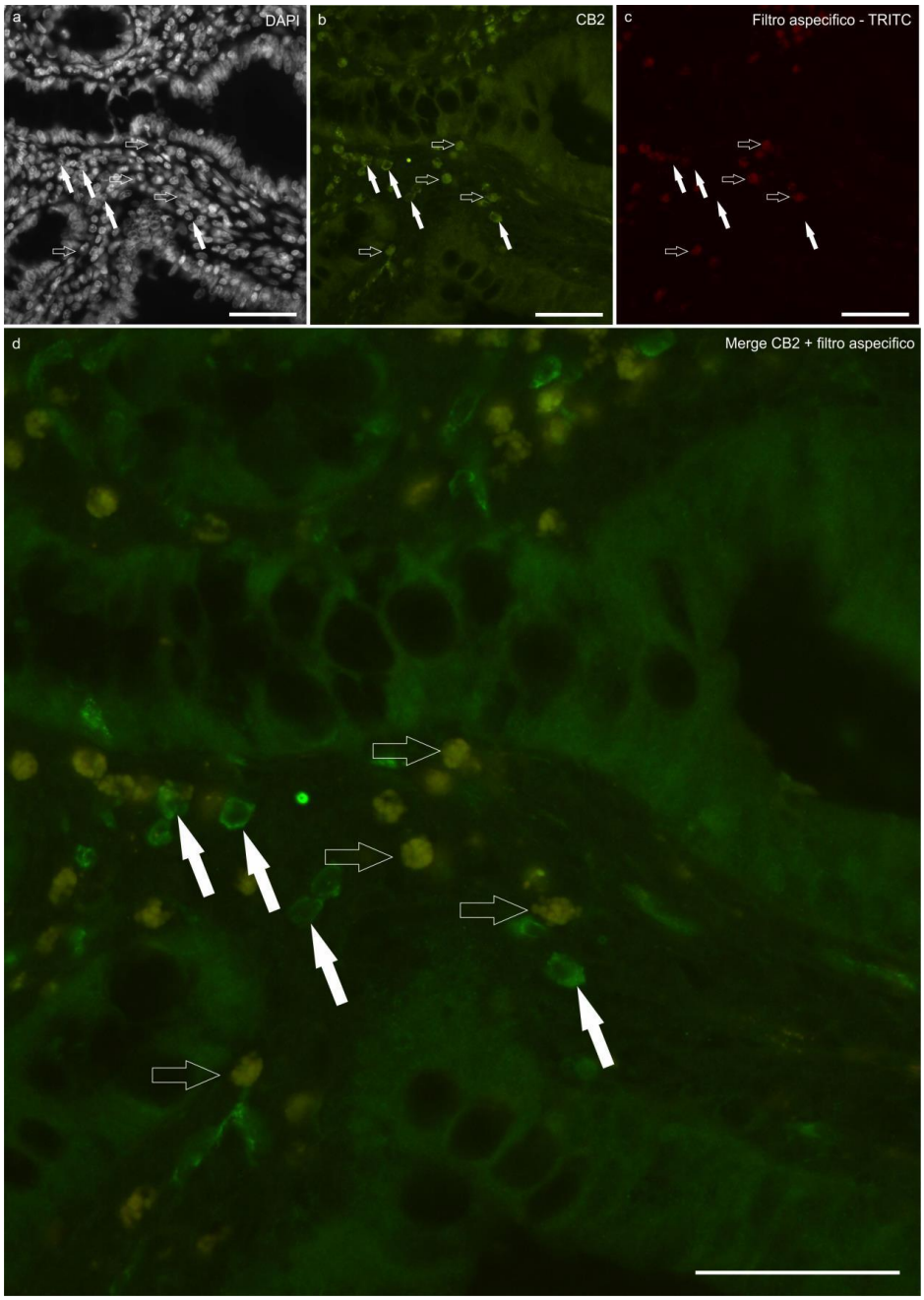

Figure 5 (a-d): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab CB2R. Nuclei are immunomarked with the DAPI (a). Epithelial cells showed diffuse CB2 immunolabelling (b); white arrows indicate positive LP immune cells. Empty arrows indicate cells with autofluorescent granules, immunolabeled also with the red TRITC (aspecific filter, c). Scale bar: a-d, 50 μm.

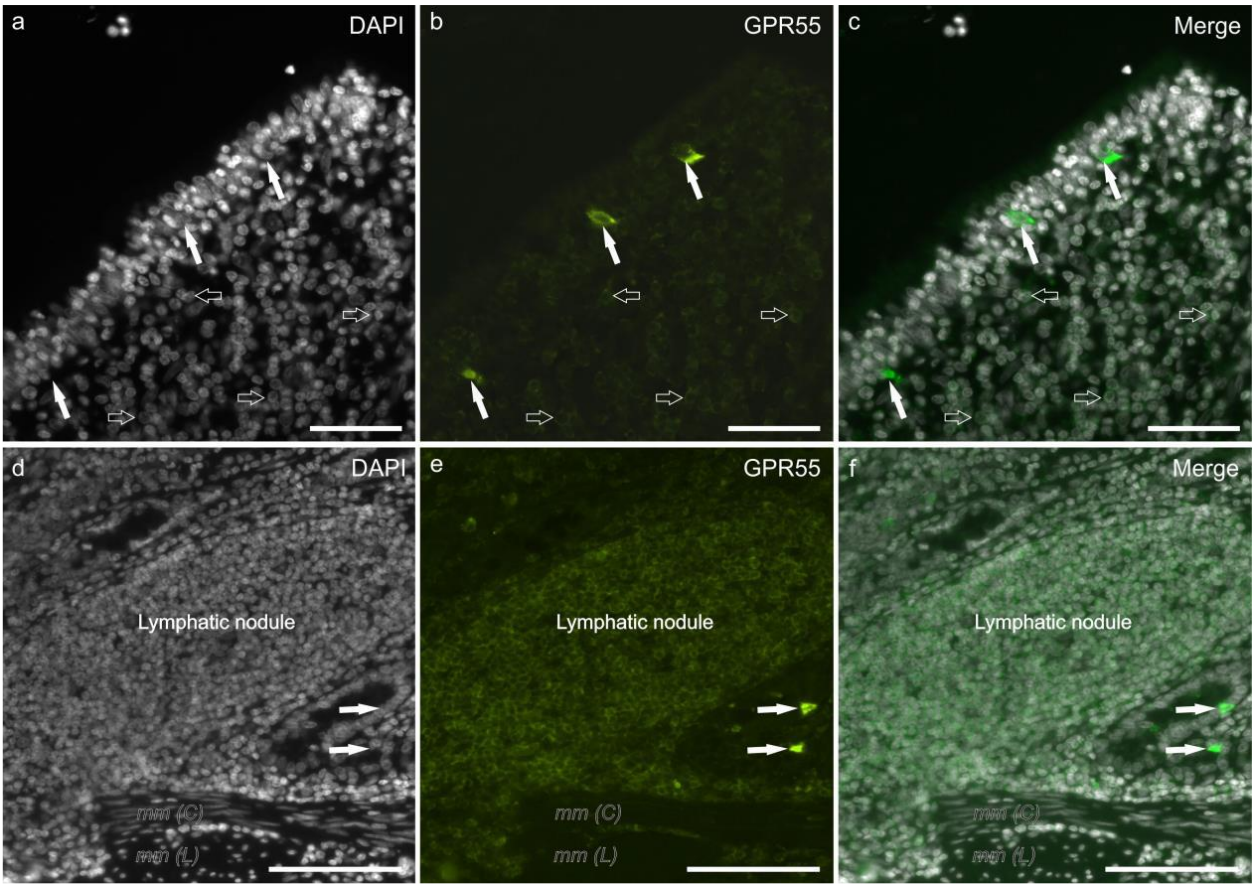

Figure 6 (a-f): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab GPR55. Nuclei are immunolabeled with the marker DAPI (a, d). White stars indicated enteroendocrine cells immunolabeled with GPR55 in the LP (b) and in a lypmphatic nodule (e). Empty arrows indicate some of the numerous positive immune cells (b). Scale bar: a-c, 50 μm; d-f, 100 μm.

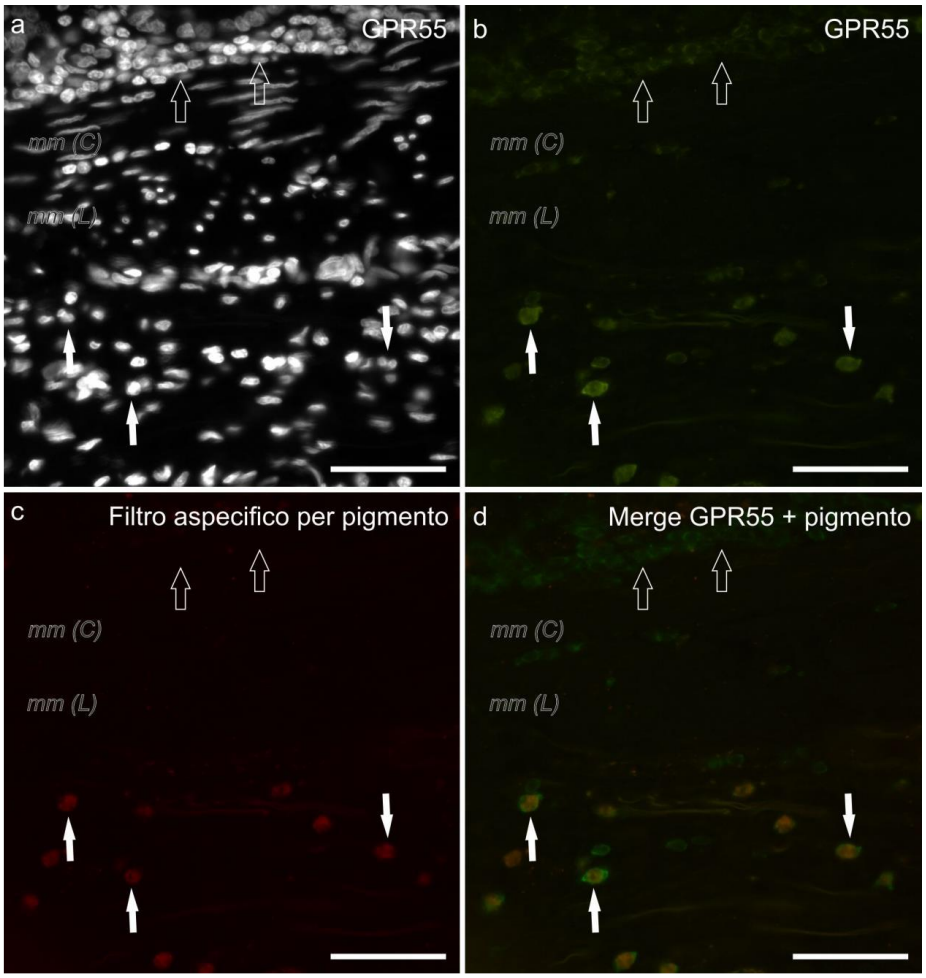

Figure 7 (a-d): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab GPR55. Nuclei are immunolabeled with the marker DAPI (a). White arrows indicate submucosal cells close to the mm, brightly immunolabeled with the Ab anti GPR55 (b). These cells were probably macrophages, because their cytoplasm contained granules with autofluorescent pigment (aspecific filter, c). Empty arrows indicate GPR55 positive immune cells, probably lymphatic cells, in the external portion of the LP, close to the mm. Scale bar 50 µm.

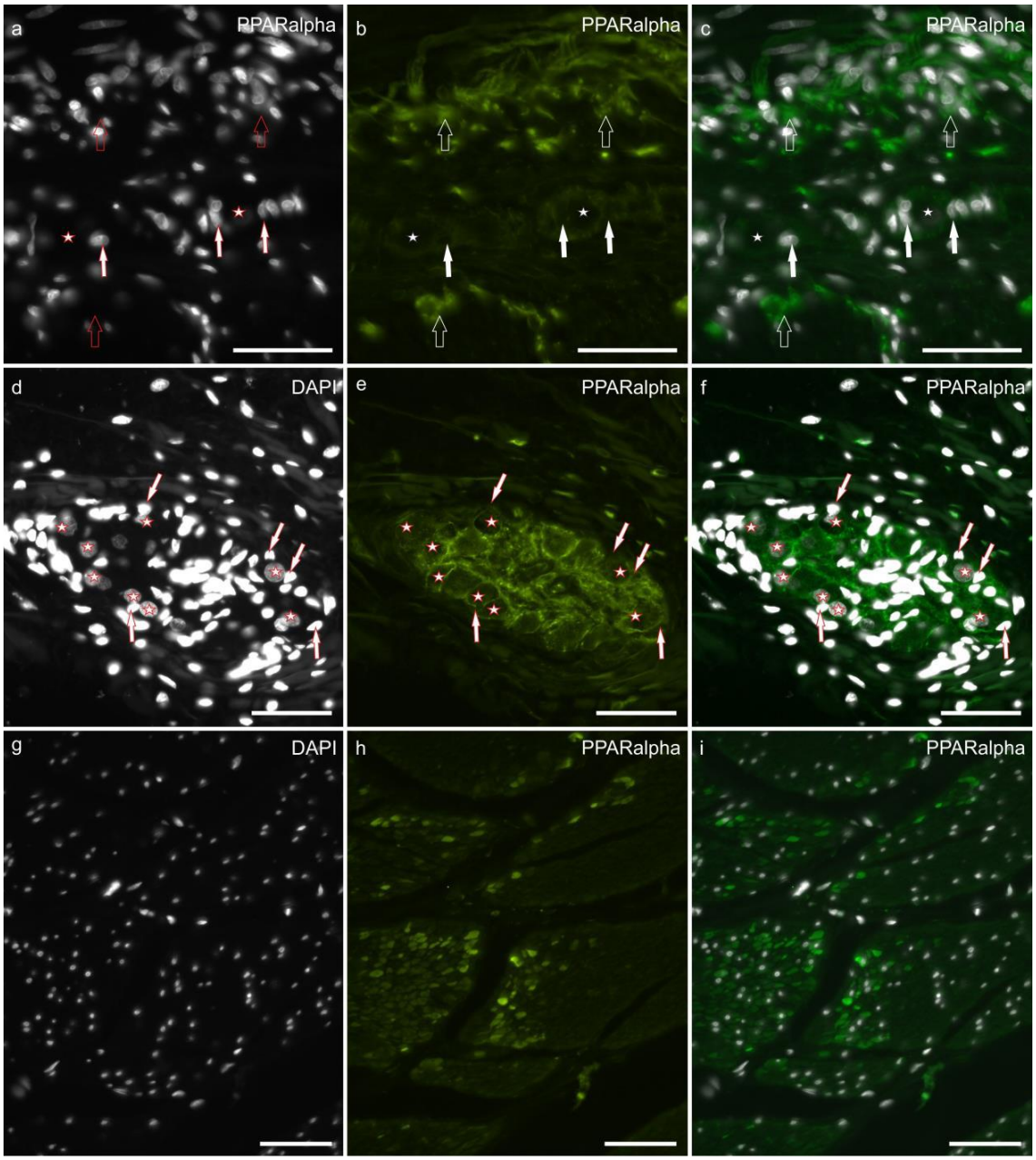

Figure 8 (a-i): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab PPARα. Stars indicate MP (a-c) and SMP (d-f) neurons, surrounded by glial cells immunoreactive for PPARα. White arrows indicate glial cells nuclei. Empty cells indicate presumed interstitial cells of Cajal (ICCs), brightly immunolabelled. Smooth muscle cells of the LML showed bright immunoreactivity for PPARα (g-i). Scale bar: a-i, 50 μm.

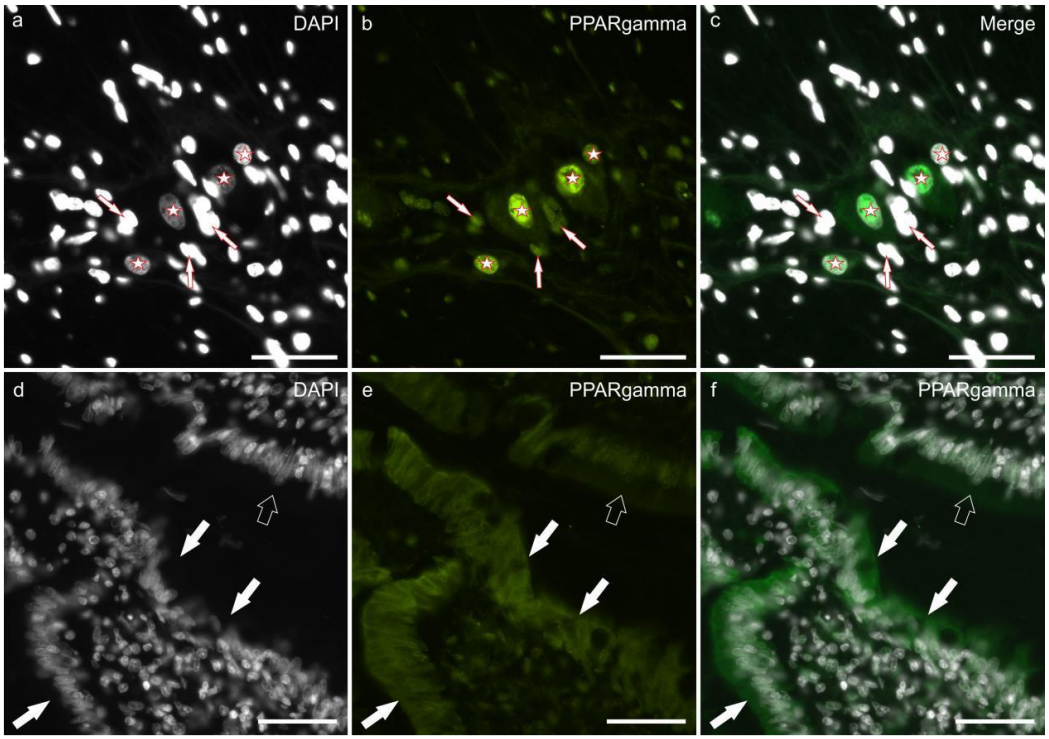

Figure 9 (a-f): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab PPARγ. Stars indicate MP neurons, which showed bright nuclear immunoreactivity for PPARγ. Arrows, indicate the positive nuclei of glial cells. (Figure 6 a-c). Epithelial cells were brightly positive for PPARγ, nuclear (empty arrow) or both nuclear and cytoplasmatic (white arrows) (Figure d-f). Scale bar a-f: 50 µm.

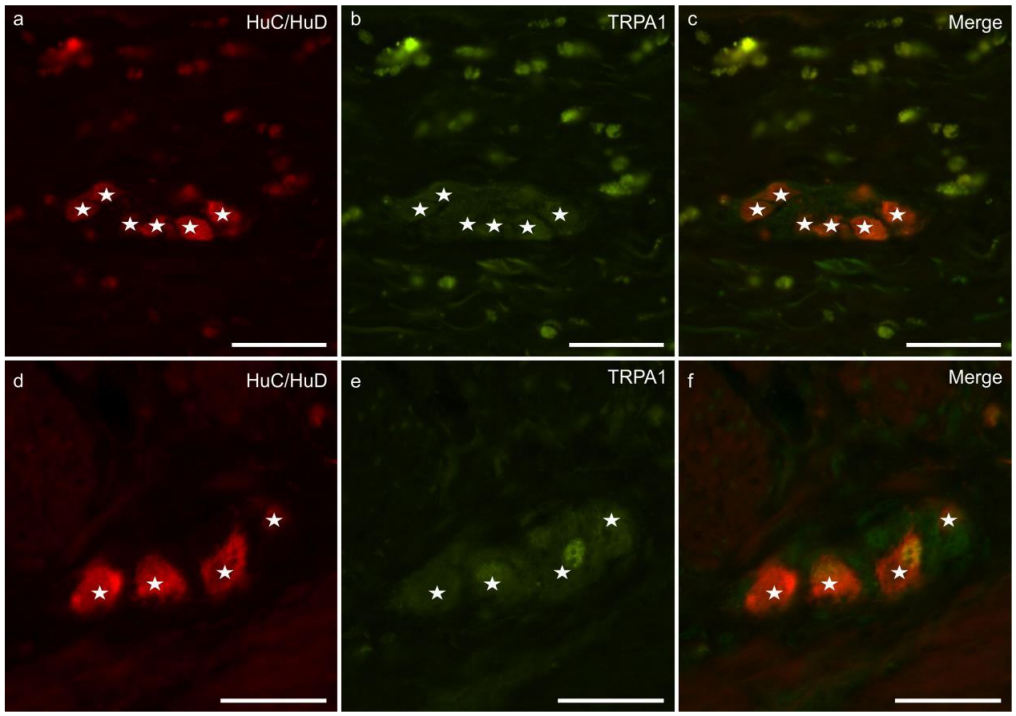

Figure 10 (a-f): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab TRPA1. Stars indicate nuclei of MP (a-c) and SMP (d-f) neurons immunolabeled with the HuC/HuD (pan-neuronal marker) (a, d). TRPA1-immunoreactivity was nuclear and cytoplasmatic in both MP and SMP (b, e). Scale bar a-f: 50 µm.

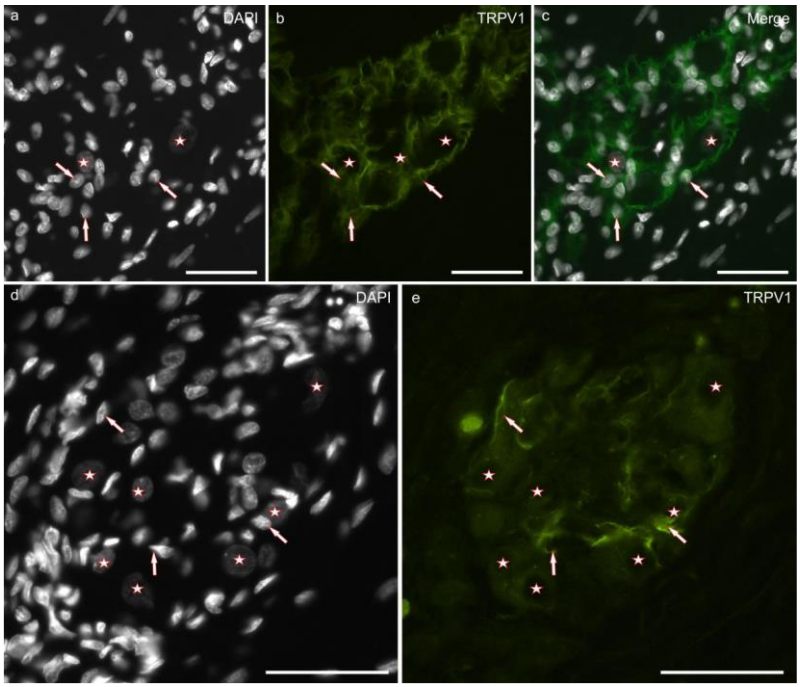

Figure 11 (a-e): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab TRPV1. Stars indicate MP (a-c) and SMP (d, e) neuronal nuclei, TRPV1-negative in the MP (b) but not in the SMP (e). Otherwise, arrows indicate the nuclei of glial cells, brightly immunolabeled both in MP and SMP (b, e). Scale bar: a-e, 50 μm.

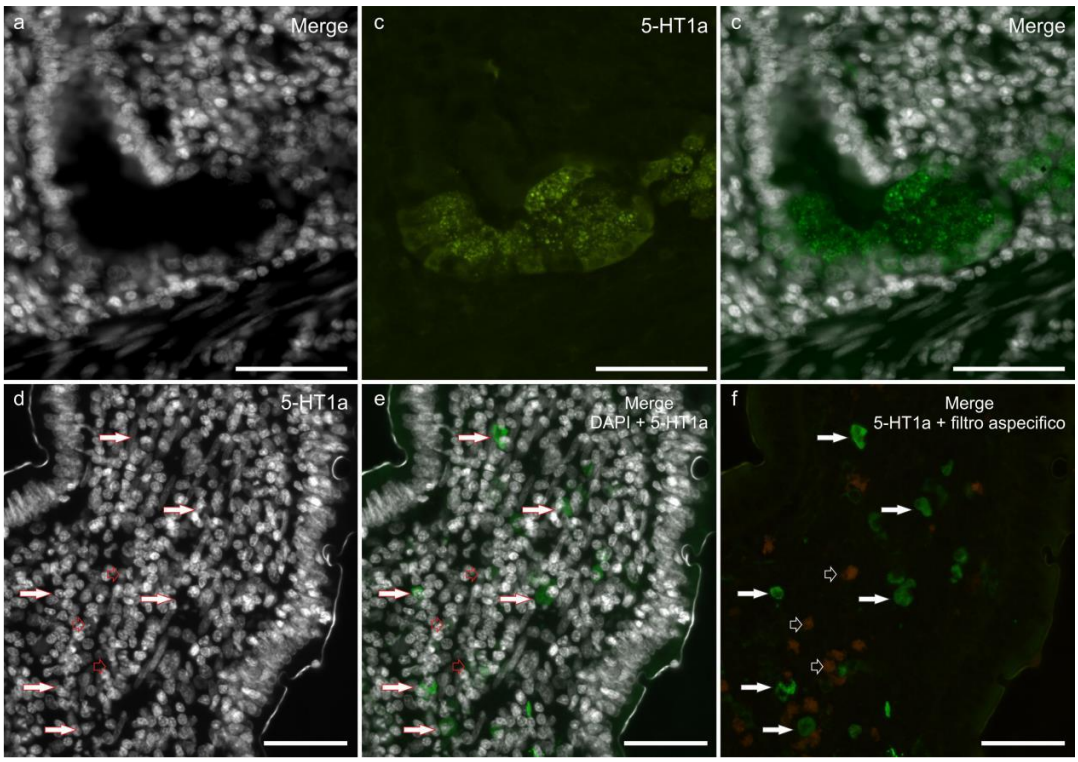

Figure 12 (a-f): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab 5-HT1aR. Big cells in the bottom part of intestinal crypt showed bright cytoplasmatic 5-HT1aR-immunoreactivity, particularly evident in cytoplasmatic granules located in the apycal part of the cells (Paneth cells) (Figure 9 a-c). In the LP of a villus, immune cells showed bright 5-HT1aR- immunoreactivity (d-f). White arrows indicate voluminous immune cells 5-HT1aR-positive (e); empty arrows indicate pigmentated autofluorescent granules, maybe in macrophages, 5-HT1aR negative (e). Scale bar a-f: 50 μm.

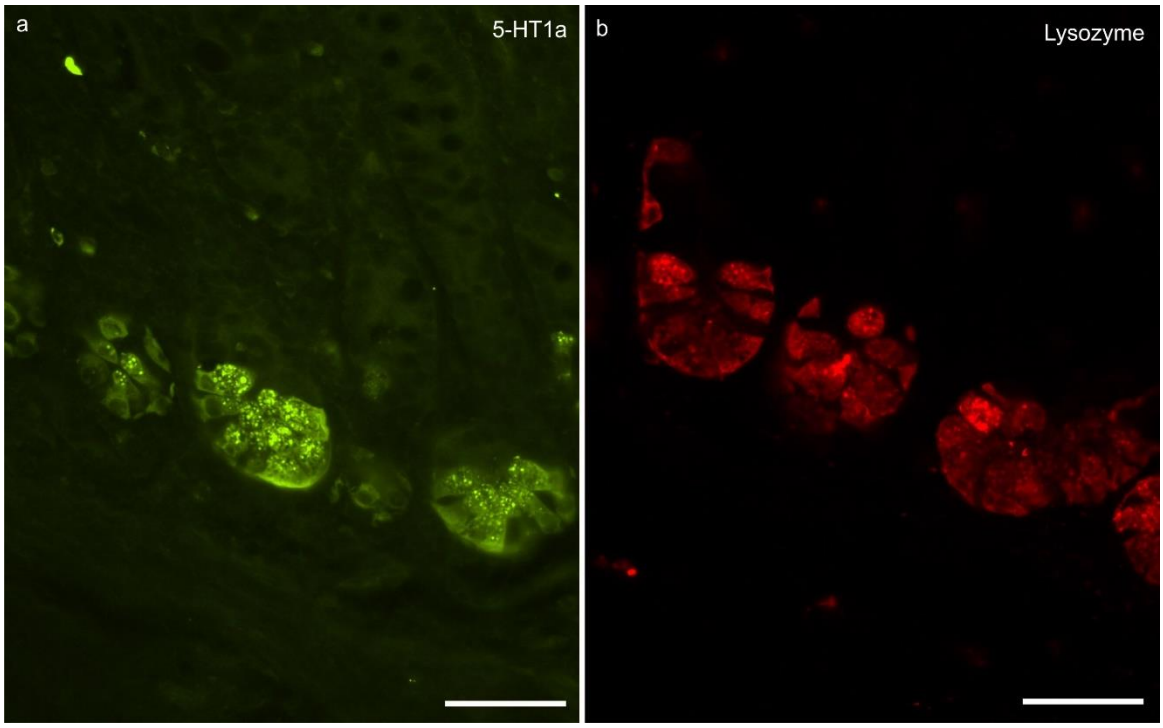

Figure 13 (a-b): Photomicrograph showing a cryosection of equine ileum immunolabeled with the Ab 5-HT1aR (a) and Ab Lysozyme (b). Big cells in the bottom part of intestinal crypt showed bright cytoplasmatic 5-HT1aR-immunoreactivity, particularly in cytoplasmatic granules located in the apycal part of the cells (Paneth cells), with the same pattern showed by the Lysozyme. Scale bar a-b: 50 μm.

In collaboration with the Department of Biomedical and Neuromotor Sciences (University of Bologna) - Preliminary results.

Localization of cannabinoid receptors in the myenteric plexus of the rat ileum

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Abstract

Introduction - The endocannabinoid system (ECS) participates in many digestive processes, such as regulation of the appetite, intestinal motility, secretion, nausea and emesis, visceral nociception and inflammation (Izzo and Sharkey 2010; Taschler et al., 2017). A great body of evidences demonstrated a bidirectional pathway between the gastrointestinal tract and the central nervous system (CNS), both in healthy conditions and neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. The rat-induced synthetic torpor (ST) phenomenon has been demonstrated as an experimental model to resemble neurodegenerative processes (Cerri et al., 2013; Luppi et al., 2019).

Objective – To characterize the cellular distribution of cannabinoid receptors 1 (CB1R) and serotonin 5-HT1a receptor (5-HT1aR) in the myenteric plexus (MP) of the rat (control, CTRL vs ST).

Material and methods – Ex vivo qualitative and quantitative immunohistochemical study on MP wholemount preparations of the ileum of six animals (3 CTRL vs 3 ST). The antibodies used in this study are rat-specific.

Results – Bright CB1R immunoreactivity (CB1R-IR) was expressed by MP neurons (Figure 1 a-b). CB1R- immunoreactive neurons showed Dogiel type II morphology, with smooth outline and long immunolabelled processes In the CTRL, 35±5% of neurons were CB1R immunoreactive (246/683 cells counted, n=3); in the ST 31±4% (200/639 cells counted, n=3). Although the percentages of immunoreactive neurons were similar, the CTRL showed brighter CB1R-IR than the ST. In some animals (both CTRL and ST), clusters of flat cells (likely mesothelial cells), with an irregular shaped nucleus, in close contact between each other, showed bright CB1R-IR.

The 5-HT1aR was analyzed only in CTRL rats; in these rats, 55±8% of MP neurons showed 5-HT1aR-IR (142/ 205 cells counted, n=3) (Figure 2 a-b). The next step will be to evaluate the expression of this receptor also in ST subjects (n=3, counting in progress).

Suggested conclusion – The wide distribution of cannabinoid receptors CB1 and 5-HT1aR in the neurons of the rat MP in both CTRL and ST confirms the importance of the ECS in the functional activity of the GI tract, but further analysis are required to understand the role in neurodegenerative diseases.

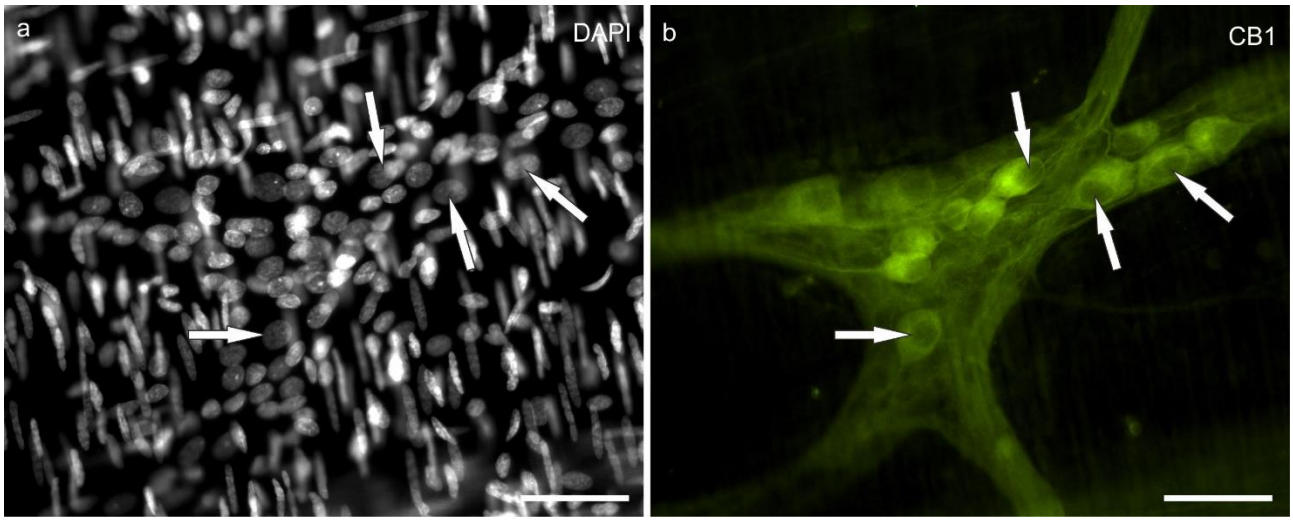

Figure 1 (a-b): Photomicrograph showing cryosections of myenteric plexus (MP) of the rat ileum (CTRL), immunolabelled with the anti-CB1R antibody. The nuclei of cells were labelled with the nuclear stain DAPI (a). MP neurons showed bright CB1R - immunoreactivity (b); arrows indicate some of these neurons. Scale bar: a-b, 50 μm.

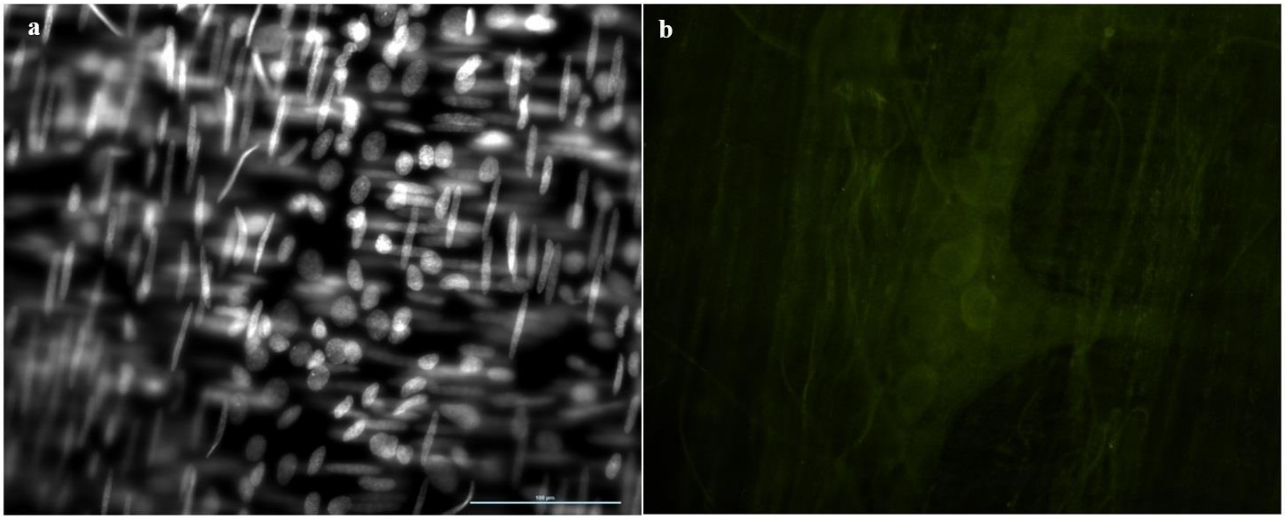

Figure 2 (a-b): Photomicrograph showing cryosections of myenteric plexus (MP) of the rat ileum (CTRL), immunolabelled with the anti-5-HT1aR antibody. The nuclei of cells were labelled with the nuclear stain DAPI (a).MP neurons showed 5-HT1aR - immunoreactivity (b). Scale bar: a-b, 100 μm.

In collaboration with the Digestive Laboratory of Professor John Furness, University of Melbourne (Australia). Paper published on Cell and Tissue Research (2019) 1-13. Modified from:

Distribution and co-expression patterns of specific cell markers of enteroendocrine cells in pig gastric epithelium

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Abstract

Introduction - Although the pig is an accepted model species for human digestive physiology, no previous study of the pig gastric mucosa and gastric enteroendocrine cells has investigated the parallels between pig and human.

Objectives - In this study, we have investigated immunohistochemically markers for each of the classes of gastric endocrine cells, gastrin, ghrelin, somatostatin, 5-hydroxytryptamine, histidine decarboxylase, and PYY cells in pig stomach.

Results - The lining of the proximal stomach consisted of a collar of stratified squamous epithelium surrounded by gastric cardiac glands in the fundus. This differs considerably from human that has only a narrow band of cardiac glands at its entrance, surrounded by a fundic mucosa consisting of oxyntic glands. However, the linings of the corpus and antrum are similar in pig and human. Likewise, the endocrine cell types are similar and similarly distributed in the two species. As in human, gastrin cells were almost exclusively in the antrum, ghrelin cells were most abundant in the oxyntic mucosa and PYY cells were rare. In the pig, 70% of enterochromaffin-like (ECL) cells in the antrum and 95% in the fundus contained 5-hydroxytryptamine (5-HT), higher proportions than in human. Unlike the enteroendocrine of the small intestine, most gastric enteroendocrine cells (EEC) did not contain colocalised hormones.This is similar to human and other species.

Conclusion and relevance - We conclude that the pig stomach has substantial similarity to human, except that the pig has a protective lining at its entrance that may reflect the difference between a pig diet with hard abrasive components and the soft foods consumed by humans.

Introduction

Pigs are commonly used as a translational model of gastrointestinal function, being of similar size and having a comparable gastrointestinal anatomy and physiology with humans (Gonzalez et al.,

2015; Roura et al., 2016). An animal of similar size and physiology is particularly important for the investigation of devices that are being developed to treat gastric conditions, for example vagal or gastric stimulating electrodes and implanted pulse generators for electrical stimulation (Payne et al., 2018). One of the conditions that is amenable to gastric electrical stimulation in some patients is gastroparesis. Unlike most laboratory animal, the pig is a species that vomits (Szelenyi et al., 1994), which is important for testing the utility of electrical stimulation therapy for the treatment of nausea and vomiting that are common in gastroparesis. Moreover, some pathological conditions that are the targets for therapy are controlled by gastric hormones that have significant roles in regulating digestion, metabolism, appetite, and nausea. Of the major gastric hormones, gastrin and histamine are best known for regulating gastric acid secretion, ghrelin stimulates appetite and reduces nausea, and somatostatin has broad counterregulatory effects. 5-HT may have a role in signalling the presence of toxins and initiating expulsion of potentially noxious substances, although it has several other actions (Mawe and Hoffman, 2013; Diwakarla et al., 2017; Martin et al., 2017). PYY is also expressed in some cells of the gastric epithelium, but its roles in the stomach are unclear. Gastrointestinal hormones are stored in enteroendocrine cells (EEC), which were classically defined as discrete cell types that each produces a single hormone. In the last decade, however, studies have revealed a substantial degree of overlap between EEC hormones or their genes in single EEC of the small and large intestines (Fothergill and Furness, 2018). The co-expression patterns of gastric hormones were recently characterised in detail in human (Fakhry et al., 2019) and rat (Hunne et al., 2019). In contrast to the intestine, relatively few EEC in the stomach expressed more than one hormone, with the exception that 5-HT was frequently found in histamine-producing cells. Although the pig stomach is similar to human, there are some significant differences, one being the epithelial structure of the gastric fundus. In humans, the corpus and fundus both contain oxyntic glands, which are characterised by acid-secreting parietal cells and enzyme-secreting chief cells. In contrast, the fundic mucosa in pigs is composed of cardiac glands (Meulengracht, 1935). Cardiac glands can also occur in humans at the gastro-oesophageal junction; however, they are not always observed and their presence has been associated with gastro-oesophageal disease (Lenglinger et al., 2012; Chandrasoma, 2013; Kim et al., 2015). The lining of the rodent fundus is different once again, being covered by non-glandular stratified epithelium. This lining in rodents resembles that of the oesophageal groove, which occurs in pigs but not humans, and is characterised by a lining composed of stratified squamous epithelium. Other aspects of the pig gastric mucosa have yet to be characterised in detail, including the distribution and co-expression patterns of gastric EEC. We have investigated the distributions and patterns of colocalisation of gastrin, ghrelin, 5-HT, somatostatin, PYY, and histamine-producing cells. Histamine-producing cells were identified with an antibody raised against histidine

decarboxylase, an enzyme involved in the synthesis of histamine. We also characterised the anatomy and mucosal structure of the pig stomach.

Methods

Tissue sources and preparation

All procedures were conducted according to the National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee. Large White/Landrace crossbred pigs (30–35 kg females) were from the University of Melbourne School of Agriculture and Food. Pigs were sedated with a xylazil and ketamine mix and euthanised by cardiac injection of pentobarbital sodium (150 mg/kg). Tissues for haematoxylin and eosin staining and immunohistochemistry were removed, opened along the mesenteric border, and pinned flat, mucosa up, without being stretched. Segments were washed with phosphate buffered saline (PBS; 0.15 mol.L−1 NaCl in 0.01 mol. L−1 sodium phosphate buffer, pH 7.2) and fixed at 4 °C overnight with Zamboni's fixative (2% w/v formaldehyde and 0.2% w/v picric acid in 0.1 mol. L−1 sodium phosphate buffer, pH 7.2). Tissues were washed three times with dimethyl sulfoxide and three times with PBS, before being stored in PBS-sucrose-azide (0.1% w/v sodium azide and 30% w/v sucrose in PBS) at 4 °C.

Haematoxylin and eosin staining

Tissue was placed into histology cassettes and dehydrated through graded ethanol to histolene and embedded in paraffin. Sections (5 μm) were cut and stained with haematoxylin and eosin (H&E). Slides were coverslipped with ProLong Diamond (Thermo Fisher) mounting medium. Slides were examined and photographed using an Axioplan microscope (Zeiss, Sydney, Australia).

Immunohistochemistry

Samples for immunohistochemistry were placed in PBS sucrose- azide and OCT compound (Tissue Tek, Elkhart, IN, USA) in a 1:1 ratio overnight before being embedded in 100% OCT and snap frozen in isopentane cooled with liquid nitrogen. Cryosections (12 μm) were cut and air dried for 1 h on SuperFrost Plus® microscope slides (Menzel-Glaser; Thermo Fisher, Scoresby, Vic, Australia). They were then covered with normal horse serum (10% v/v with triton-X in PBS) for 30 min at room temperature and incubated with mixtures of primary antibodies (Table 1) overnight at 4 °C. The preparations were then washed three times with PBS before a 1-h incubation with mixtures of secondary antibodies (Table 1) at room temperature. Sections were washed three times with dH2O and, in some cases, incubated with Hoechst 33258 nuclear staining solution (10 μg/mL bisbenzimide-

blue in dH_2O; Sigma-Aldrich) for 5 min. Slides were washed three times with distilled water before being mounted under coverslips with Dako fluorescence mounting medium (Agilent, Tullamarine, Vic, Australia). Slides were examined and imaged using an Axio Imager microscope (Zeiss), or an LSM800 or LSM880 confocal microscope (Zeiss).

Immunofluorescence image quantification

Sections for cell counts were imaged as tile scans with a nominal optical thickness of 7.7 μm using a × 10 objective on the LSM800 confocal microscope (Zeiss). A 1.5-mm-wide region from each imaged section, which contained the full thickness of the mucosa, was selected for analysis in Fiji (http://imagej.nih.gov/ij/). Cells from each channel were manually circled by one investigator and verified by a second investigator, and were counted as positive if their mean pixel intensity was clearly above a threshold determined from the background fluorescence. The total mucosal area was also measured in order to determine the cell density (positive cells per mm^2 of mucosa). The number of positive cells in the luminal, middle, and submucosal portions of the mucosa was also determined. Data are presented as mean ± SEM, for n = 3 animals.

Results

Anatomy of the pig stomach

The pig has a single chambered stomach similar in shape to human. The pig stomach was cut open along the greater curvature to reveal the gastric lining (Fig. 1). On gross inspection, a distinctive collar of epithelium with an irregular surface was observed around the oesophageal junction. On the lesser curvature, this extended to the boundary of the antrum as the oesophageal groove. The mucosa of the remainder of the stomach had large folds (rugae). The fundus, corpus, and antrum were distinguishable by position and colour (Fig. 1). A prominent swelling, the torus pyloricus, occurs in the stomach on the lesser curvature, adjacent to the gastro-duodenal junction, and there is a diverticulum of the fundus, on the greater curvature, adjacent to the oesophagus. Ten regions were selected for histological analysis by haematoxylin and eosin (H&E) staining (Fig. 2). The epithelial lining of the peri-oesophageal collar and groove was stratified without a cornified surface, thus being similar to the lining of the oesophagus, and was around 0.5 mm thick (Fig. 2b, c). There were subepithelial papillae, similar to those seen in the skin. The muscle layer was approximately 5 mm thick near the gastro-oesophageal junction but was thinner towards the antrum. The fundus mucosa was about 0.5 mm thick and consisted of cardiac glands (Fig. 2a). These were branched glands with mucous cells lining the parts near the gastric lumen, while the deeper branches were lined with a simple columnar epithelium. The fundic diverticulum formed a deep distendable pocket with a narrow

entrance, adjacent to the esophago-gastric junction. The mucosal lining of the diverticulum was composed of cardiac glands, similar to the rest of the fundus (Fig. 2d). The mucosa of the gastric corpus consisted of closely packed straight tubular oxyntic glands. The corpus mucosa was relatively thick, being around 1.5 mm, although the muscle layer was amongst the thinnest of the regions investigated (approximately 2 mm). Pyloric glands in the antrum and pyloric regions of the pig stomach were convoluted, similar to the cardiac glands found in the pig fundus. The luminal ends of the glands were branched and dominated by mucus cells (Fig. 2k). Being around 1 mm thick, the antral and pyloric mucosa was thinner than the corpus mucosa but thicker than the fundic mucosa. The muscle was especially thick in these regions, reaching around 8 mm in the mid antrum. The torus pyloricus is a bulging fibro-muscular structure in the pig stomach on the lesser curvature adjacent to the gastro-duodenal junction (Fig. 1) and accordingly had the thickest wall.

Localisation and morphology of enteroendocrine cells in the pig gastric mucosa

The gastric fundus, corpus, and antrum were examined for ghrelin, somatostatin, 5-HT, PYY, HDC, and gastrin immunoreactivity (Fig. 3). Cells immunoreactivity for each marker were identified in all gastric regions examined, except that gastrin cells were extremely rare in the fundus and corpus, and PYY was uncommon in all three regions. Cell density was quantified for each of these markers (Fig. 5a), and the localisation of EEC within the mucosa was determined as the proportion of EEC that was within the submucosal, middle, or luminal thirds of the mucosa (Fig. 5b). Ghrelin cells were the most abundant EEC type observed in the fundus and the corpus (31 ± 5 and 67 ± 4 cells/mm^2 respectively) but were relatively less common in the antrum compared with most other markers investigated (16 ± 1 cells/mm^2; Fig. 5a). In the fundus, ghrelin cells were primarily localised in or near the submucosal third of the mucosa, whereas in the corpus and antrum, they were more evenly distributed between the middle and submucosal thirds (Fig. 5b). In the corpus and fundus, ghrelin cells were round or ovoid in shape and were closed, meaning they were not in contact with the lumen (Fig. 4a). Some of these cells possessed short thin processes.In the antrum, ghrelin cells were frequently flaskshaped, although whether the apical extremities of these cells were in contact with the lumen was unclear (Fig. 4b). Somatostatin (SST) cells were most abundant in the antrum, followed by the corpus and the fundus (76 ± 8, 34 ± 3, and 18 ± 5 cells/mm^2 respectively; (Fig. 5a). SST cells were fairly evenly distributed in the corpus mucosa and slightly more concentrated in the middle third of the antrum, whereas in the fundus, they were skewed towards the submucosal side (Fig. 5b). In the corpus and fundus, SST cells were typically round or ovoid closed cells (Fig. 4e), whereas cells were generally flask-like or irregular in shape in the antrum and open to the lumen (Fig. 4f). Examples of small thin basal processes on some SST cells were observed in all three regions. Gastrin cells were the most

abundant type of EEC in the antrum (118 ± 15 cells/mm^2), whereas they were extremely rare in the corpus and fundus (fewer than 1 cell/mm^2; Fig.5a). Gastrin cells were more frequent in or near the middle third of the antrum mucosa (Fig. 5b). These were generally flask-shaped open cells (Fig. 4c). Basal processes of gastrin cells were not observed. HDC cells were most abundant in the antrum but were also common in the fundus and corpus (47 ± 7, 18 ± 3, and 16 ± 3 cells/mm^2 respectively; Fig. 5a). HDC cells were predominantly localised to the submucosal third of the fundus mucosa, fairly evenly distributed in the corpus mucosa, and concentrated in or near the middle third of the antrum mucosa (Fig. 5b). In the corpus, HDC cells were generally round or ovoid closed cells (Fig. 4d). In contrast, cells in the fundus and antrum were a mixture of cell shapes, including round, ovoid, and flaskshaped. In the antrum, flask-shaped cells were most common. 5-HTcells were most abundant in the antrum but were also found in significant numbers in the corpus and fundus (41 ± 5, 19 ± 3, and 18 ± 3 cells/mm^2 respectively; Fig. 5a). As with ghrelin and SST, 5-HT cells were concentrated in the basal third of the fundus mucosa. In the corpus, cells were fairly evenly distributed, whereas 5-HT cells in the antrum were predominantly localised in or near the middle third, with a tendency to be closer to the submucosal rather than luminal side (Fig. 5b). LikeHDC, a mixture of cell shapeswas observed in the fundus and antrum, whereas cells in the corpus were generally round or ovoid. Some thin processes were evident (Fig. 4g). PYY cells were very rare, especially in the corpus (fewer than 1 cell/mm^2 in the corpus; Fig. 5a). PYY cells were generally round or ovoid (Fig. 4h), although some flask-shaped cells were observed in the antrum.

Colocalisation of EEC markers

Colocalisation was assessed between all combinations of hormones, except for HDC and PYY since our only effective antibodies against these peptides were both raised in rabbit and PYY cells were rare. Furthermore, since gastrin cells were extremely rare in the corpus and fundus, we did not quantify the colocalisation of gastrin with hormones in these regions. Qualitatively, we did not observe much overlap of gastrin with other hormones in the corpus and fundus, except for rare cells containing gastrin and 5-HTor gastrin and PYYin the corpus. Overlap between gastric hormones was generally low (Fig. 7). One significant exception to this was between 5-HT and HDC (Fig. 6). A substantial degree of colocalisation occurred in all regions investigated. For example, 83 ± 2% of 5-HT cells contained HDC in the fundus, corresponding to 94 ± 3% of HDC cells containing 5-HT. Although PYY cells were extremely rare throughout the pig stomach, a relatively high proportion of these cells, about 50%, contained other hormones, including ghrelin, somatostatin, 5-HT, and gastrin.

Localisation of parietal cells and ECL cells

Histamine is known to act in a paracrine fashion to promote acid secretion from parietal cells in the corpus (Soll and Walsh, 1979). In the present study, however, HDC cells were more abundant in the antrum than the corpus (Fig. 8 a, b), which led us to investigate whetherHDC cells were situated near parietal cells in the antrum. However, parietal cells were extremely rare or absent in pig gastric antrum and not related to ECL cells, whereas they were abundant in the corpus (Fig. 8 a′, b′). In contrast, ghrelin cells, which are abundant in the corpus, were in close proximity to parietal cells, some adjacent cells forming close associations, as seen in other species (Fakhry et al., 2019; Hunne et al., 2019).

Discussion

The pig gastric mucosa shares many similarities with human, including similar mucosal architecture in the corpus and antrum. However, in contrast to humans, where oxyntic glands cover the mucosa of both corpus and fundus, the lining of the pig fundus consists of mucus cell-dominated cardiac glands. Furthermore, a collar, around the oesophageal entrance, and the oesophageal groove in pigs were characterised by a thick stratified squamous epithelium. Oxyntic glands were tightly packed long tubular structures, whereas the cardiac and pyloric glands were branched, convoluted, and less dense, as observed by Meulengracht (1935) in pigs. Thus, it seems that the entrance to the pig stomach is protected against abrasion by a thick epithelium and adjacent to this is an epithelium with similar appearance to the human cardiac glands that secrete watery fluid and mucus. The cardiac gland secretion can be assumed to have moistening and lubricating effects. The presence of a thick protective epithelium at the entrance to the stomach and the adjacent cardiac glands with their numerous mucus cells may be in response to the varied diets of pigs in their natural environment that can include dry, hard, and abrasive foods. This contrasts with humans, whose diets over 1000s of years have been dominated by soft processed foods (Furness et al., 2015). Once food passes this protective zone in the pig, it enters an environment very similar to the human stomach with glands of the corpus and antrum being almost indistinguishable between the two species. Cells immunoreactive for ghrelin, somatostatin, 5-HT, PYY, HDC, and gastrin was identified in all gastric regions examined, although gastrin cells were extremely rare in the fundus and corpus, and PYY was uncommon in all three regions. These observations were on female pigs. It should be noted that EEC populations may differ between genders, for example, in the colon, 5-HT cell abundance during oestrus is 30% greater than in pro-oestrus or in males (Balasuriya et al., 2016). Unlike the small intestine where colocalisation of hormones is observed in the majority of EEC (Egerod et al., 2012; Habib et al., 2012; Sykaras et al., 2014; Cho et al., 2015; Fothergill et al., 2017), very little

colocalisation was seen in pig gastric EEC. One significant exception to this is that 5-HT and HDC (a marker of histamine producing ECL cells) were generally co-expressed in all three gastric regions investigated. These results are similar to findings in human oxyntic mucosa, where the only significant overlap observed was between 5-HT and pancreastatin (an alternative marker of histamine-producing cells), although the overlap involved a significantly smaller proportion of cells in the human (Fakhry et al., 2019). In rat, 5-HTand HDC were also frequently colocalised in the antrum, but overlap was rare in the corpus (Hunne et al., 2019). Colocalisation of other hormones was observed in fewer than 5% of ghrelin, somatostatin, or gastrin cells.

Ghrelin cells

Ghrelin cells were most abundant in the oxyntic mucosa, which is consistent with findings in the rat and human (Date et al., 2000; Rindi et al., 2002; Hunne et al., 2019). In the corpus, ghrelin cells were round or ovoid closed cells, meaning they were not in contact with the lumen. This is also consistent with findings in both rat and human (Date et al., 2000; Dornonville De La Cour et al., 2001; Fakhry et al., 2019; Hunne et al., 2019). However, in the antrum, cells were frequently flask-shaped, which is often indicative that the cell is in contact with the lumen. This is contrary to the literature describing ghrelin cells. However, there were no clear examples of the apical ends of these cells reaching all the way to the lumen, so it is possible that these are closed cells, despite the flask-shaped morphology. Gastric ghrelin has an important role in stimulating appetite, and it also increases gastric emptying in humans and laboratory animals (Levin et al., 2006; Kojima and Kangawa, 2010; Avau et al., 2013). In pigs, the relationship between ghrelin and feeding behaviour is less obvious than in other mammals. Plasma ghrelin is elevated in fasting pigs and is reduced by feeding; however, administration of ghrelin did not alter food intake but did increase weight gain in weaner and grower pigs fed ad libitum (Salfen et al., 2004; Lents et al., 2016). Thus, in pigs, ghrelin has a similar distribution as in other mammals, being dominant in the stomach, with lesser amounts in the upper small intestine (Vitari et al., 2012), but seems to have a stronger effect on metabolism than on appetite.

Gastrin cells

Consistent with other species, gastrin cells were extremely rare in the corpus and fundus but were abundant in the gastric antrum. These cells were generally flask-shaped open cells, which relates to their role in sensing luminal contents (Rehfeld et al., 2007). Gastrin cells were clustered within the middle third of the mucosa in contrast to rat gastrin cells which are concentrated in a band near the base of the mucosa (Hunne et al., 2019). Gastrin's major role is to promote acid secretion in the stomach (Feldman et al., 1978; Eysselein et al., 1984). This is achieved by stimulating histamine

secretion, which in turn promotes acid secretion from parietal cells (Friis-Hansen, 2002). Furthermore, gastrin promotes the expression and activity of histidine decarboxylase, the enzyme responsible for producing histamine, and promotes the development of ECL cells and parietal cells (Sandvik et al., 1994; Friis-Hansen et al., 1998). This relationship is interesting given that histamineproducing cells were most abundant in the pig antrum, in contrast to rat and human where they are related to oxyntic glands; the antral ECL cells in pigs are well situated for interactions with gastrin cells, but not with parietal cells.

Somatostatin cells

Somatostatin cells were more abundant in the antrum than the corpus, which is also observed in the rat (Hunne et al., 2019). However, this contrasts with the human, where somatostatin cell density is higher in the corpus (Kasacka et al., 2012; Choi et al., 2014). Somatostatin cells in the rat and human frequently possessed large basal processes, which in the antrum appear to selectively connect with gastrin cells (Larsson et al., 1979; Fakhry et al., 2019; Hunne et al., 2019). This relationship is consistent with the physiological role of somatostatin to provide a negative feedback control of gastrin secretion, thereby limiting acidification of the antrum in human and in animal models (Schubert et al., 1988; Chuang et al., 1993; Vuyyuru et al., 1997; Schubert and Peura, 2008), including in the pig (Holst et al., 1992). Thus, it is surprising that prominent basal processes of somatostatin cells were not observed in the pig. Although small thin processes were sometimes seen, these did not appear to extend to any particular cell type. The antral cells are generally of the open type and respond to acid in the lumen as well as neural signalling and gut hormones, including CCK, GIP, GLP-1 and secretin (Schubert et al., 1988; Gribble et al., 2018). Somatostatin cells in the corpus were round or ovoid. This is consistent with the literature which suggests that oxyntic SST cells are typically closed type and are predominantly regulated by neural and hormonal signalling (Schubert et al., 1988; Gribble et al., 2018). Somatostatin inhibits acid production and histamine release (Schubert et al., 1988; Vuyyuru et al., 1995). SST cells associated with oxyntic glands are tonically active between meals, providing a basal inhibition of gastric acid secretion. These SST cells are temporarily inhibited following the ingestion of food, providing time for gastrin to promote gastric acid release (Li, 2003; Gribble et al., 2018).

Histamine and 5-HT cells

5-HTcells were more abundant in the antrum than the corpus, consistent with human, rat, and mouse (Ito et al., 1986; Reynaud et al., 2016; Hunne et al., 2019). HDC cells were also more common in pig antrum than the corpus, which contrasts to the rat and human where they are significantly more

abundant in the corpus (Choi et al., 2014; Hunne et al., 2019). Over 70%ofHDC cells contained 5-HTin pig antrum, similar to rat antrum where 65% of HDC cells contained 5-HT (Choi et al., 2014; Hunne et al., 2019). In pig corpus, a high proportion of HDC cells also contained 5-HT (80%), which contrasts with both rat corpus (1%) and human corpus (11%) (Choi et al., 2014; Hunne et al., 2019). HDC cell morphology also differed between species, generally being round or ovoid in the pig corpus, whereas they are elongated, flattened cells at the bases of the epithelial cell layer in rat corpus (Håkanson et al., 1986; Hunne et al., 2019). From this, we can infer that a population of cells in which 5-HTand histamine are colocalised occurs in pig, rat, and human, but that the pig seems to lack a large population of HDC-positive 5-HT-negative cells (‘classical’ ECL cells) in the corpus. The overlap between histamine and 5-HT in all species is peculiar given that histamine promotes acid secretion whereas 5-HT inhibits acid secretion (Canfield and Spencer, 1983; LePard et al., 1996). Given histamine’s role in promoting acid secretion from parietal cells (Friis-Hansen, 2002), it is peculiar that ECL cells were sparse in the corpus. On the other hand, ECL cells were common in the antrum, where histamine is unlikely to act on parietal cells, which were rare or absent in this region. The roles of histamine in the antrum are not resolved.

Concluding remarks

In many respects, the pig stomach is very similar to human. It is similar in size and shape and, like human, has prominent mucosal rugae. One difference is the thick protective layered epithelium and the mixed mucus and simple columnar (cardiac) glands at and beyond the gastric entrance. The difference here may reflect differences in the physical properties of typical pig and human food, as discussed above. Beyond the gastric fundus, the human and pig corpus and antrum are remarkably similar, suggesting similar gastric digestive physiology. In both species, ghrelin, somatostatin, 5-HT, PYY, HDC, and gastrin EEC are present with similarities in distributions and cell types, for example gastrin cells are extremely rare in the fundus and corpus, PYY cells are uncommon in all three regions, and ghrelin cells are numerous in the corpus. Some quantitative differences were noted, for example the greater proportion of somatostatin and histamine (ECL) cells in the antrum of pig, whereas they are more abundant in the corpus of human. Similar to human and rat, colocalisation of the peptide hormones was rare.

Primary antibody	**Host**	**Code**	**Dilution**	**Source**
5-HT	Goat	#20079	1:5000	Incstar
5-HT	Rabbit	#20080	1:2000	Immunostar
CCK/gastrin	Mouse	28.2	1:2700	Gift from Drs JH Walsh and H. Wong, UCLA (Kovacs et al., 1997)
Gastrin	Rabbit	#8007	1:3000	Gift from Dr JF Rehfeld
Ghrelin	Chicken	Ab15861	1:800	Abcam
Ghrelin	Rabbit	#RY1601	1:5000	Mizutani et al., (2009)
H^+/K^+ ATPase	Mouse	#12.18	1:200	Smolka et al., 2000
HDC	Rabbit	#16045	1:2000	Progen Biotechnick GmbH
PYY	Rabbit	HPA010973	1:100	Sigma- Aldrich
Somatostatin	Mouse	#S895	1:1000	Buchan et al., 1985

Table 1. Primary antibodies used in the study.

Secondary antibody	**Host**	**Code**	**Dilution**	**Source**
Anti-chicken IgG Alexa Fluor® 647	Donkey	#703-605-155	1:400	Jackson laboratories
Anti-Goat IgG Alexa Fluor® 555	Donkey	A21432	1:400	Molecular Probes
Anti-mouse IgG Alexa Fluor® 555	Donkey	Ab150110	1:500	Abcam
Anti-mouse IgG Alexa Fluor® 647	Donkey	A31571	1:1000	Molecular Probes
Anti-rabbit IgG Alexa Fluor® 555	Donkey	Ab150070	1:1000	Abcam
Anti-rabbit IgG Alexa Fluor® 647	Donkey	A31573	1:1000	Molecular Probes
Anti-sheep IgG Alexa Fluor® 647	Donkey	#A21448	1:500	Molecular Probes

Table2: Secondary antibodies used in the present study.

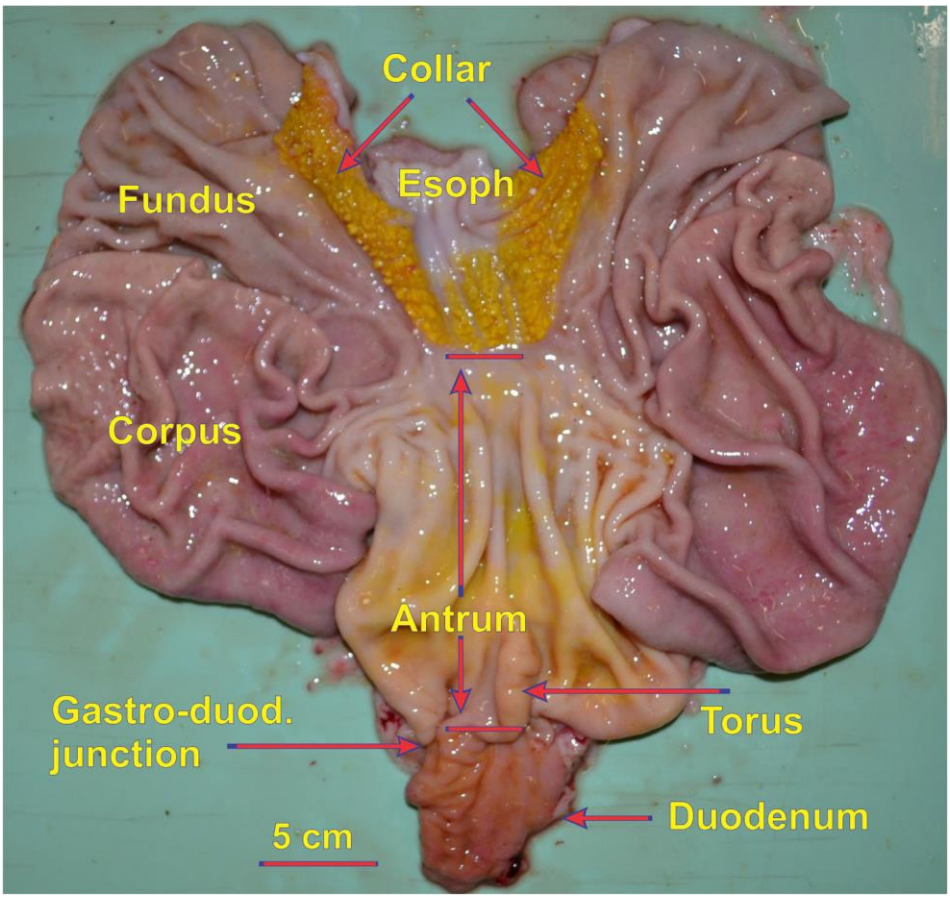

Figure 1 Photograph of the stomach from a 35-kg pig opened along the greater curvature to reveal the gastric lining. The regions are indicated. Esoph. indicates the gastric end of the oesophagus. The arrow adjacent to "Torus" indicates the torus pyloricus (point of arrow is on the torus). The arrows next to 'Antrum' indicate the extent of the antrum along the lesser curvature. Note that the antrum extends to the collar of stratified squamous epithelium that surrounds to oesophageal entry to the stomach.

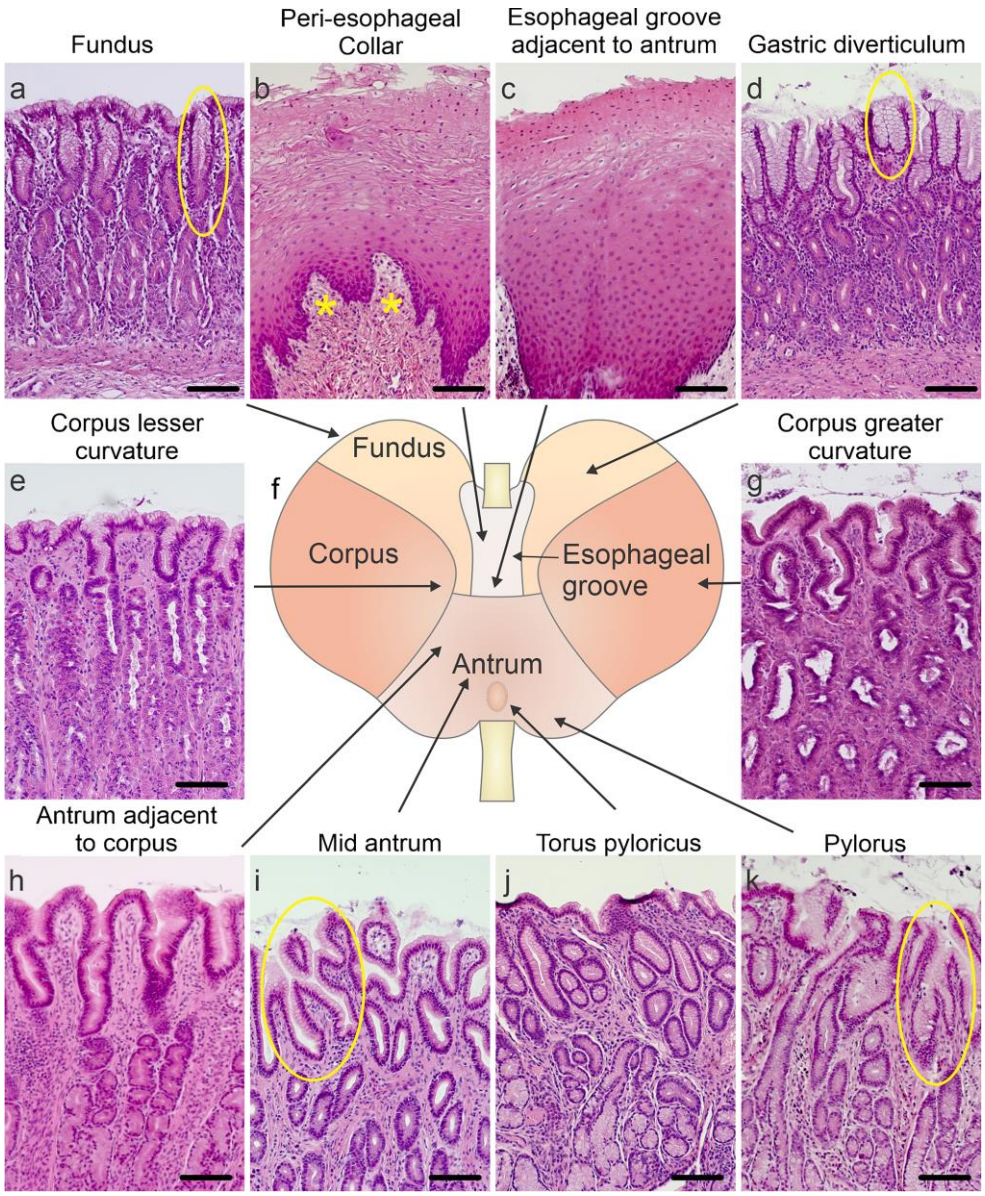

Figure 2: Histological appearance of the pig gastric mucosa stained with haematoxylin and eosin (H&E) and diagram of the pig stomach indicating the regions sampled for histological analysis (f). The fundus (a), including the fundic diverticulum (d), was lined with cardiac glands that had prominent gastric pits lined with mucus cells (circled). A collar of thick non-keratinised stratified squamous epithelium surrounded the oesophageal entrance (asterisks mark dermal papillae) that continued as the oesophageal groove (b, c). The antrum and pylorus (h–j) were lined by an epithelium characterised by branched glands (examples circled). Note that the full thickness of the mucosa is not shown for thicker regions of mucosa such as the corpus. Scale bars are 100 µm

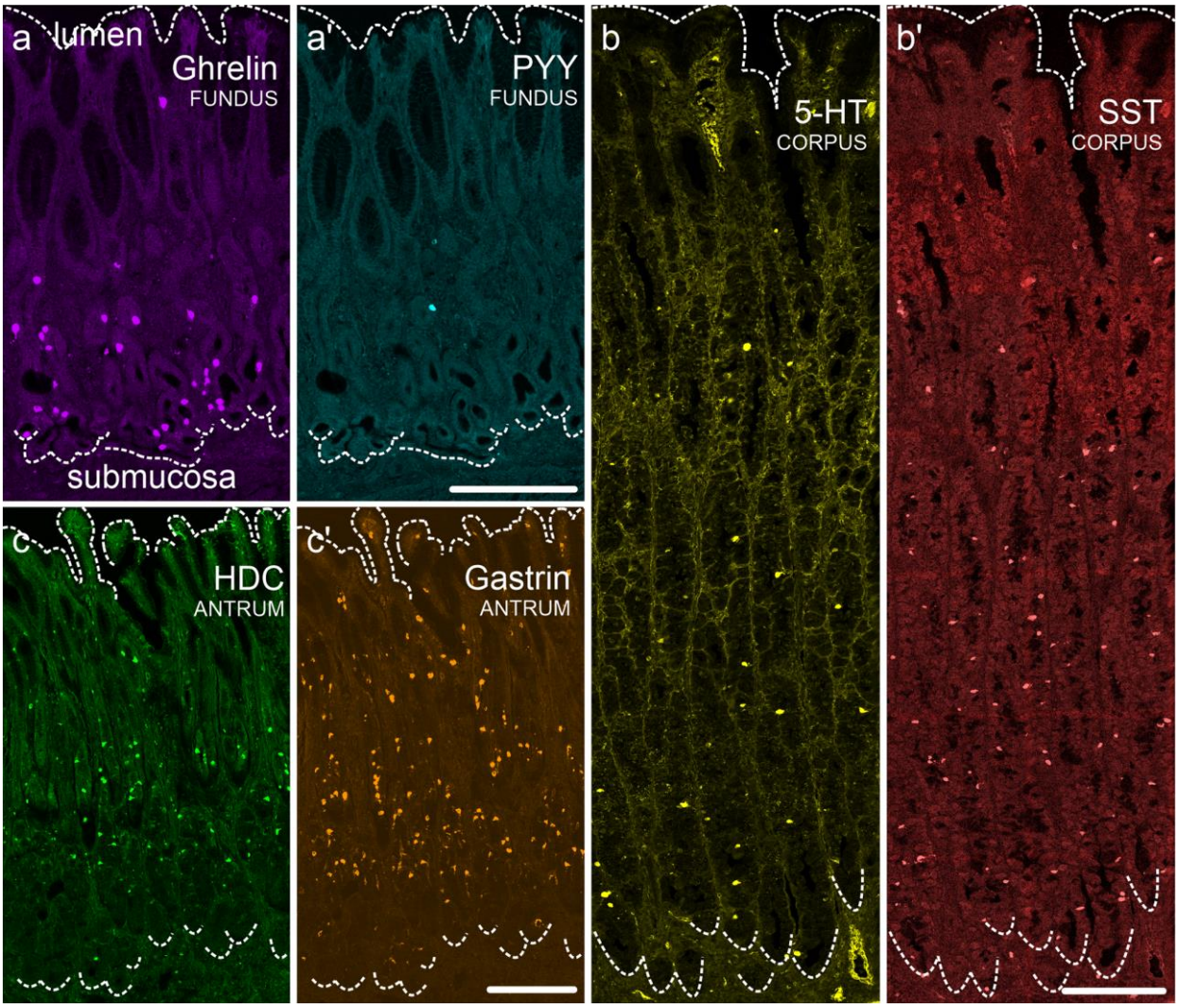

Figure 3 (a-c'): EEC immunoreactivity for ghrelin (a) and PYY (a') in gastric fundus, 5-HT (b) and somatostatin (b') in gastric corpus, and HDC (c) and gastrin (c') in gastric antrum. The bases of the glands and the surface of the mucosa are marked with dotted white lines. The image is oriented with the submucosal (basal) ends of the glands at the bottom of the image. Scale bars: 200 μm.

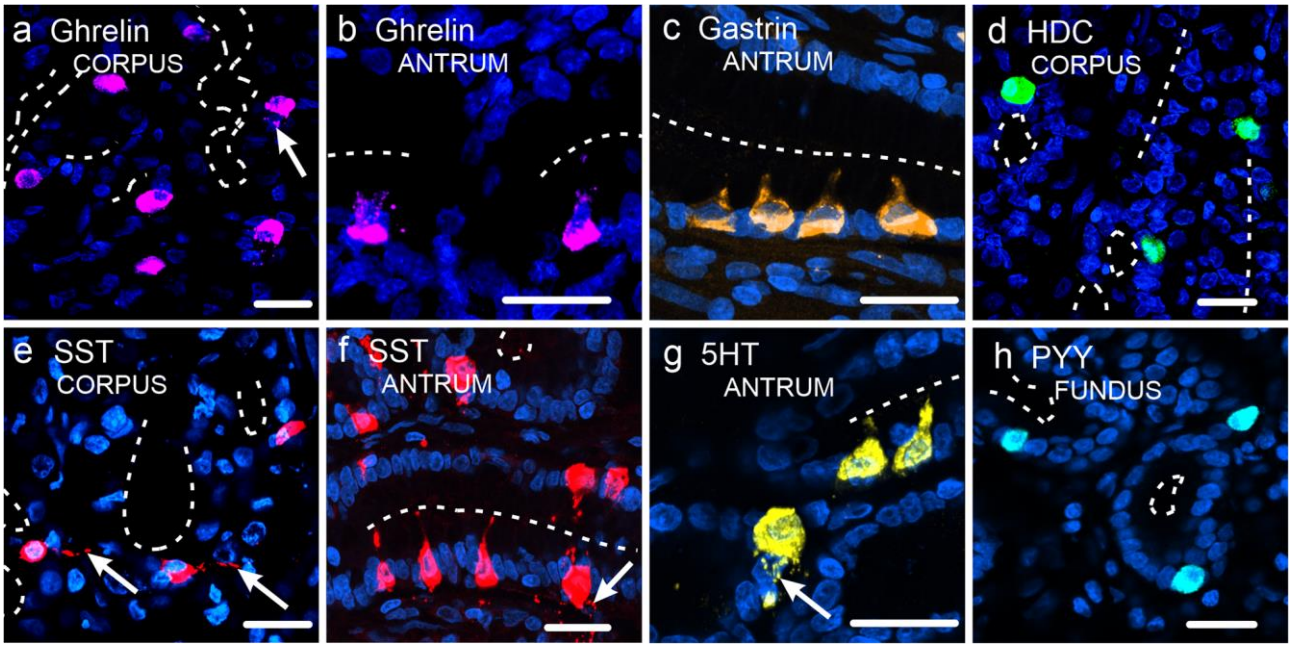

Figure 4 (a-h): EEC morphologies and relationships. Examples of cells immunoreactive for ghrelin (a, b), gastrin (c), HDC (d; a marker for histamine-producing cells), somatostatin (SST) (e, f), 5-HT (g), and PYY (h). The luminal surfaces of the epithelial cells forming the glands are marked with dotted white lines. Arrows indicate small basal processes in a, e, f, and g. Scale bars: 20 μm

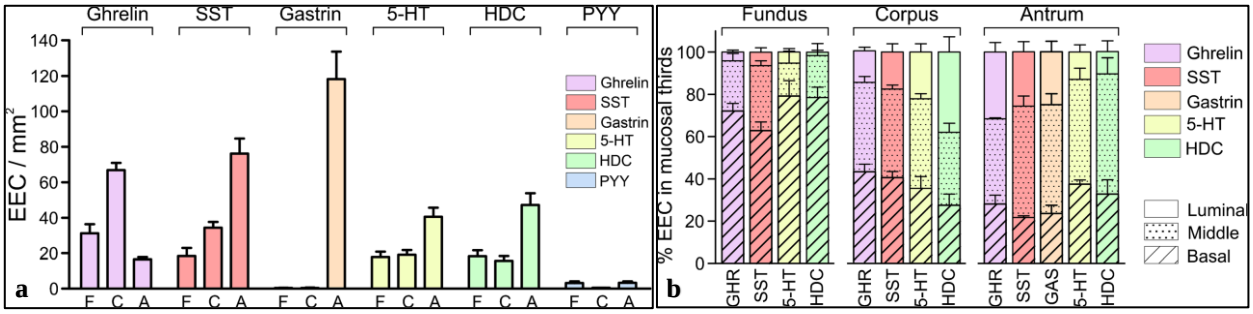

Figure 5 (a, b):a) Cell density of gastric EEC in the gastric fundus (F), corpus (C), and antrum (A). Numbers of cells counted were around 100 cells or more per pig per region, except for the rare PYY cells in all regions and rare gastrin cells in the corpus and fundus.b) Distribution of EEC across the width of the mucosa in pig gastric fundus, corpus, and antrum. The proportion of EEC immunoreactivity for each hormone marker (indicated below each column) situated in the submucosal third of the mucosa is indicated by a striped pattern, cells in the middle third are indicated by a spotted pattern, and cells in the luminal third are indicated with no pattern. Due to their rarity, the distribution of gastrin cells in the fundus and corpus and PYY cells in all regions was not accurately determined.

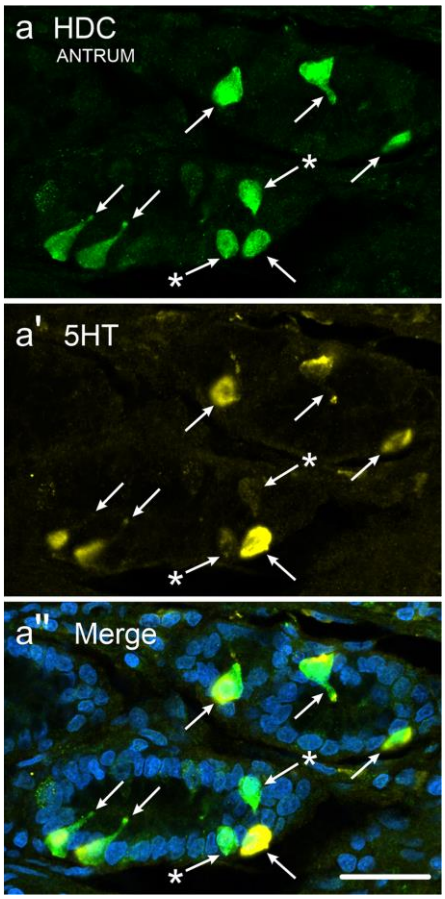

Figure 6 (a-a''): Examples of cells showing colocalisation of HDC (a) and 5-HT (a'). Colour merge images also show Hoechst nuclear staining in blue. Most cells contain both hormones (indicated by an arrow), but two cells are immunoreactive for HDC and not 5-HT (indicated by an arrow with an asterisk). Scale bar is 20 µm.

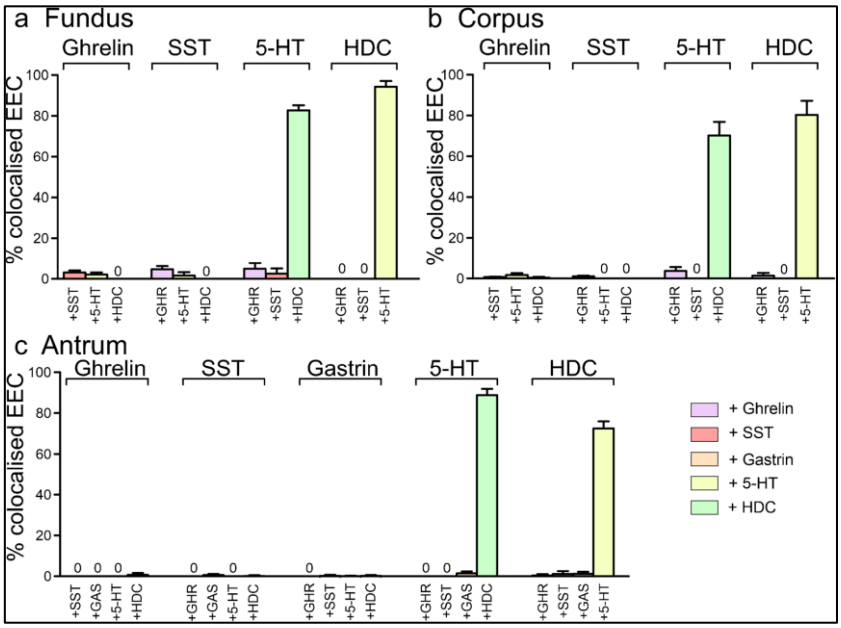

Figure 7 (a-c): Quantitation of overlaps between hormones in pig gastric fundus (a), corpus (b), and antrum (c). Colocalisation of two hormones is expressed as a percentage of cell immunoreactivity for the marker indicated at the top of each group of columns. 0= no colocalisation.

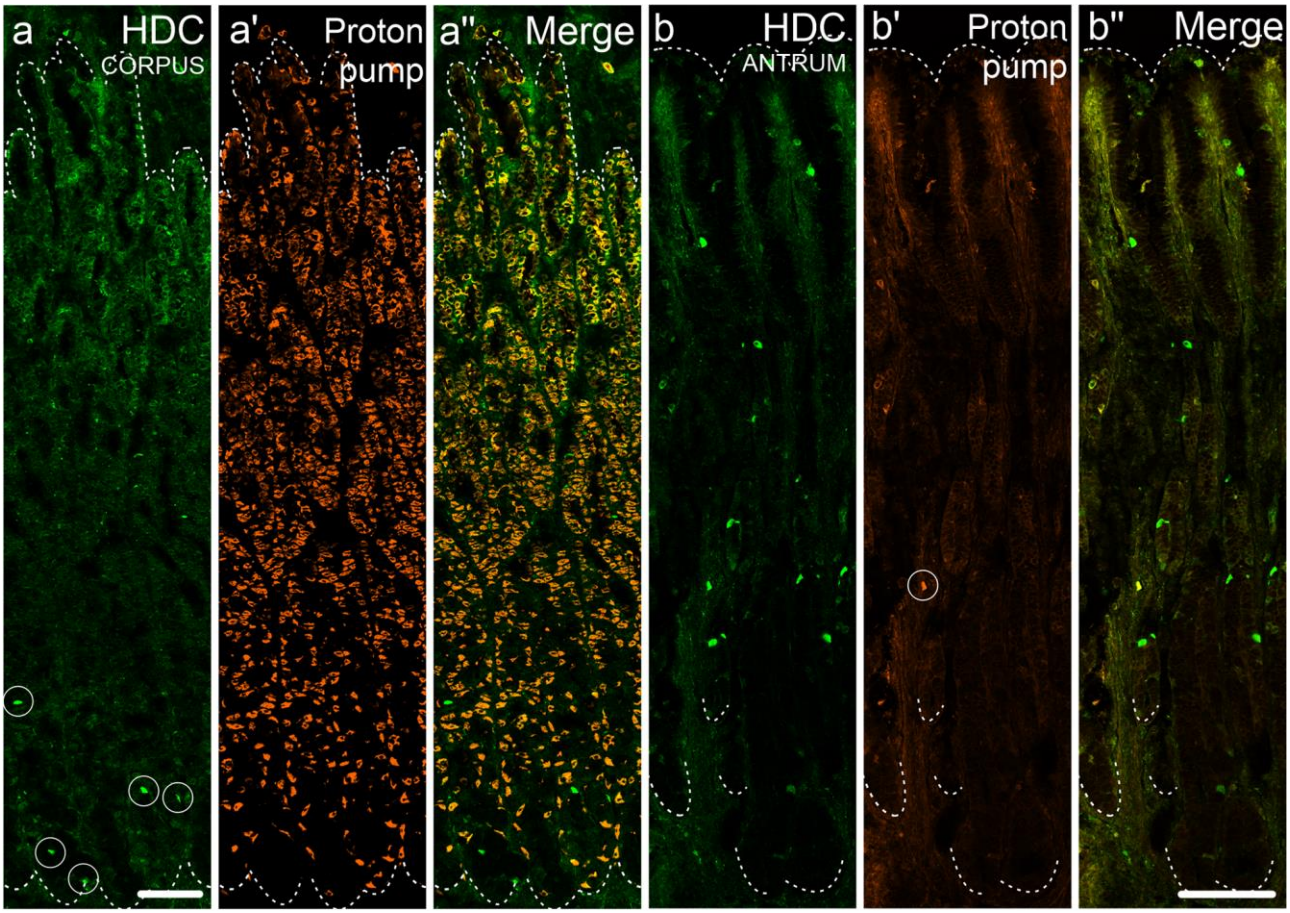

Figure 8 (a-b''): The relationship between parietal cells and ECL cells in the gastric corpus (a), and the gastric antrum (b). ECL cells (circled in a) are stained with an anti-HDC antibody and parietal cells (circled in b') are marked by anti-proton pump (H+/K+ ATPase). The bases of the glands and the surface of the mucosa are marked with dotted white lines. The image is oriented with the base of the glands at the bottom of the image. Scale bars: 100 μm.

Paper published on the Israel Journal of Veterinary Medicine (2017, 72 (3): 22-27. Modified from:

The Relationship between Duodenal Enterochromaffin Cell Distribution and degree of Inflammatory Bowel Disease (IBD) In Dogs

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Abstract

Introduction - Despite numerous studies carried out over the last 15 years in veterinary medicine, the pathogenesis of canine Inflammatory Bowel Disease (IBD) has still not been completely elucidated. In particular, unlike what has been demonstrated in human medicine, the influence of serotonin on clinical signs in canine IBD has not yet been clarified.

Objective - The objective of this paper has been to seek a possible correlation between duodenal epithelial distribution of serotonin-producing cells (enterochromaffin cells) and disease-grading parameters (clinical, clinico-pathological, endoscopic and histopathological) in dogs with IBD. The medical records of dogs with a diagnosis of IBD were retrospectively reviewed and 21 client-owned dogs with a diagnosis of IBD were registered. Clinical score (by Canine Chronic Enteropathy Clinical Activity Index), laboratory examinations (albumin, total cholesterol, folate, cobalamin), endoscopic score and histopathological score, were compared by regression analysis with duodenal enterochromaffin cell percentage.

Results - The study results suggested a relationship between a decrease in folate absorption and an increase in duodenal enterochromaffin cell percentage (regression equation y=16.89-6.14x; coefficient of determination r2= 0.7; significant level: P=0.007). However, no significant relationship was evidenced between duodenal enterochromaffin cell percentage and the other analyzed variables.

Conclusion and relevance – Further researchs are required to improve our understanding of the involvement of 5-HT in the pathogenesis of canine IBD, evaluating if SERT activity is related with IBD severity, and therefore if the decrease in 5-HT reuptake is linked to nociception and clinical signs in these patients.

In collaboration with the Gastrointestinal Laboratory, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University (Texas). Paper published on the Journal of Veterinary Internal Medicine (2018), 32(6), 1903-1910.Modified from:

Effect of an extruded animal protein-free diet on fecal microbiota of dogs with food-responsive enteropathy

Francesca Bresciani[1], Yasushi Minamoto[2], Jan S. Suchodolski[2], Giorgia Galiazzo[1], Carla Giuditta Vecchiato[1], Carlo Pinna[1], Giacomo Biagi[1], Marco Pietra[1]

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Abstract

Introduction - Dietary interventions are thought to modify gut microbial communities in healthy individuals. In dogs with chronic enteropathies, resolution of dysbiosis, along with remission of clinical signs, is expected with treatment.

Objective - To evaluate changes in the fecal microbiota in dogs with foodresponsive chronic enteropathy (FRE) and in healthy control (HC) dogs before and after an elimination dietary trial with an animal protein-free diet (APFD). Fecal microbiota was analyzed by Illumina 16S rRNA sequencing and quantitative polymerase chain reaction (PCR).

Results - A significantly lower bacterial alpha-diversity was observed in dogs with FRE compared with HC dogs at baseline, and compared with FRE dogs after the trial. Distinct microbial communities were observed in dogs with FRE at baseline compared with HC dogs at baseline and compared with dogs with FRE after the trial. Microbial communities still were different in FRE dogs after the trial compared with HC dogs at baseline. In HC dogs, the fecal microbiota did not show a significant modification after administration of the APFD.

Conclusion and relevance - Our results suggest that, in FRE dogs, treatment with the APFD led to a partial recovery of the fecal microbiota by significantly increasing microbiota richness, which was significantly closer to a healthy microbiota after the treatment. In contrast, no changes were detected in the fecal microbiota of HC dogs fed the same APFD.

Paper published on EC GASTROENTEROLOGY AND DIGESTIVE SYSTEM (2018) 5.6 415-425.
Modified from:

Effects of Chronic Enteropathies on VIPergic and Nitrergic Immunoreactive Neurons in the Dog Ileum

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Abstract

Introduction - The enteric nervous system (ENS) comprises a huge amount of neurons and nerve fibers interposed between the two muscular layers of the tunica muscularis and in the submucosa. Neuropeptides produced by the ENS neurons act as neurotransmitters/neuromodulators, which control intestinal motility and mucosal functions, and play a crucial role also in the regulation of inflammatory processes via cross talk with the local immune system. A growing body of evidence indicates that the gastrointestinal inflammatory response damages the enteric neurons themselves, thus resulting in deregulations in gut motility and mucosal functions.

Objective - The purpose of this study was to evaluate quantitatively enteric neurons immunoreactive for the vasoactive intestinal polypeptide (VIP) and neuronal nitric oxide synthase (nNOS) in the myenteric (MP) and submucosal (SMP) plexus of the ileum of dogs without (CTRL-dogs, n= 6) and with spontaneous chronic enteritis (inflamed dogs, INF-dogs, n=10). In addition, the percentage of nNOS immunoreactive neurons co-expressing VIP immunoreactivity (and vice versa) was evaluated. Data were expressed as mean ± standard deviation.

Results - In the myenteric plexus of INF-dogs, the percentage of VIPergic neurons (16 ± 7%) was significantly greater than that observed in the CTRL-dogs (8 ± 3%) (P = 0,022). Conversely, in the submucosal plexus of CTRL- and INF-dogs the percentages of VIPergic neurons were similar (31 ± 9% and 30 ± 11%, respectively; P = 0,786). In the myenteric plexus of INF-dogs, the percentage of nitrergic neurons (24 ± 5%) showed only a tendency to decrease in comparison to that evaluated in the CTRL-dogs (29 ± 5%) (P= 0.138); also in the submucosal plexus the percentages of nitrergic neurons of CTRL-dogs (8 ± 5%) and INF-dogs (7 ± 2%) did not show meaningful differences (P = 0.884). Co-localization studies indicated that also the percentages of nitrergic neurons co-expressing VIP immunoreactivity did not change between CTRL- and INF-dogs in the MP (23 ± 12% and 24 ± 10%, respectively; P = 0.935) and SMP (26 ± 16% and 23 ± 15%, respectively; P = 0.810).

Conclusion and relevance - This is the first quantitative study about the VIPergic and nitrergic neurons harbored in the in MP and SMP of the canine ileum and the first comparison between these subclasses of neurons in dogs with and without chronic enteritis. Our findings showed significant neuroplasticity only of myenteric VIP immunoreactive neurons during chronic enteritis, which may influence intestinal motility.

Dorsal Root Ganglia

Paper published on Frontiers in Veterinary Science (2019) 6:313 doi:10.3389/fvets.2019.00313
Modified from:

Cellular Distribution of Canonical and Putative Cannabinoid Receptors in Canine Cervical Dorsal Root Ganglia

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Abstract

Introduction - Growing evidence indicates cannabinoid receptors as potential therapeutic targets for chronic pain. Consequently, there is an increasing interest in developing cannabinoid receptor agonists for treating human and veterinary pain. To better understand the actions of a drug, it is of paramount importance to know the cellular distribution of its specific receptor(s).
Objective - The distribution of canonical and putative cannabinoid receptors in the peripheral and central nervous system of dogs is still in its infancy. In order to help filling this anatomical gap, the present *ex vivo* study has been designed to identify the cellular sites of cannabinoid and cannabinoid-related receptors in canine spinal ganglia. In particular, the cellular distribution of the cannabinoid receptors type 1 and 2 (CB_1 and CB_2) and putative cannabinoid receptors G protein-coupled receptor 55 (GPR55), nuclear peroxisome proliferator-activated receptor alpha (PPARα), and transient receptor potential vanilloid type 1 (TRPV1) have been immunohistochemically investigated in the C6-C8 cervical ganglia of dogs.
Results - About 50% of the neuronal population displayed weak to moderate CB_1 receptor and TRPV1 immunoreactivity, while all of them were CB_2-positive and nearly 40% also expressed GPR55 immunolabeling. Schwann cells, blood vessel smooth muscle cells, and pericyte-like cells all expressed CB_2 receptor immunoreactivity, endothelial cells being also PPARα-positive. All the satellite glial cells (SGCs) displayed bright GPR55 receptor immunoreactivity. In half of the study dogs, SGCs were also PPARα-positive, and limited to older dogs displayed TRPV1 immunoreactivity.
Conclusion and relevance - The present study may represent a morphological substrate to consider in order to develop therapeutic strategies against chronic pain.

Introduction

Spinal ganglia, also referred to as dorsal root ganglia (DRG), contain the cell bodies of pseudounipolar primary sensory neurons, which are surrounded by a layer of satellite glial cells (SGCs), also called amphicytes because of their position around each neuron. Chronic pain, both inflammatory and neuropathic, is associated with hyperexcitability of DRG cellular elements and their down-modulation could thereby decrease pain (Krames, 2015). A growing body of literature suggests that cannabinoid receptors play a critical role in nociception through central and peripheral mechanisms (Hohman et al., 1995; Martin et al., 1996; Tsou et al., 1996; Calignano et al., 1998; Richardson et al., 1998; Stella, 2010; Davis, 2014). Recent studies have shed some light on the expression of cannabinoid receptors on neurons and glial cells of the canine nervous system (Pirone et al., 2016; Freundt-Revilla et al., 2017; Freundt-Revilla et al., 2018). In particular, CB1 receptor was observed in central nervous system (CNS) neurons (Pirone et al., 2016) and in DRG neurons and glial cells (Freundt-Revilla et al., 2017), whereas CB2 receptor was found in glial cells (astrocytes) of the spinal cord (Freundt-Revilla et al., 2018).

In addition to the known canonical (i.e. prototypical) cannabinoid receptors CB1 and CB2, other receptors, such as G protein-coupled receptor 55 (GPR55), nuclear peroxisome proliferator-activated receptor alpha (PPARα), and transient receptor potential vanilloid type 1 (TRPV1) are currently considered putative cannabinoid receptors (Petrosino and Di Marzo, 2017; Pertwee et al., 2010; Yang et al., 2016).

The anti-nociceptive potential of the endocannabinoid system (Donvito et al., 2018) has prompted the development of therapeutic cannabinoid receptors agonists or medical marjiuana to be used in pets in order to treat chronic pain. The clinical/medical properties of botanical and synthetic cannabinoids in the management of neuropathic pain, allodynia, and chronic non-cancer pain have been recently reviewed (Pergolizzi et al., 2018). Methodological challenges (quali-quantitative variability in cannabinoid content of cannabis plant extracts, inconsistent dosing) as well as acute and chronic impacts on cognition, immune and cardiovascular system are still unsolved issues associated with the therapeutic use of phytocannabinoids (Sachs et al., 2015; Bonn-Miller et al., 2017; Pavlovic et al., 2018; Carcieri et al, 2018). This is why many research efforts are currently focused on body's own cannabinoids (i.e. endocannabinoids) and related physiological compounds, acting through canonical and putative cannabinoid receptors (Donvito et al., 2018; Skaper et al., 2015).

Although there is a growing interest in the subject, reliable anatomical studies regarding the cellular distribution of cannabinoid receptors in the canine central and peripheral nervous system (PNS) are still lacking. In order to help filling this anatomical gap, the present ex vivo study

immunohistochemically investigated the cellular distribution of the cannabinoid and cannabinoid-related receptors CB1, CB2, GPR55, PPARα, and TRPV1 in cervical DRG of pet dogs.

Material and Methods

Animals

Cervical sensory ganglia and related spinal cord were collected from eight dogs (Table 1). None of them had history of neurological disorders and any gross changes of the spinal cord and vertebral canal. Dogs died spontaneously or were euthanized for human reasons due to different diseases and tissues were collected following owner's 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.

Since the suppliers of the antibodies employed in the present study state them to rat-specific (CB2 and TRPV1) or react with rat tissues (CB1, PPARα), rat cervical sensory ganglia were used for comparison purposes (authorization no. 112/2018-PR of 12 February 2018). The distribution of the study receptors in subclasses of rat sensory neurons was out of the scope of the present study, and was not evaluated.

Tissue collection

Tissue Samples (C6-C8 DRG) were collected within 1 hour from death through a dorsal laminectomy. DRG were localized by counting them from the last cervical spinal nerve (C8) located just cranial to the first rib. C6-C8 cervical DRG were selected for the present study because of technical and pathophysiological implications, i.e. large size, involvement in chronic pain (caused by cervical disk herniation and vertebral column instability), presence of all the subsets of sensory neurons activated by mechanical, thermal and nociceptive inputs from the forelegs. Once removed from the spinal cord, DRG were fixed for 12 hours in 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.2) at 4°C. Tissues were subsequently rinsed overnight in phosphate-buffered saline (PBS; 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) and stored at 4°C in PBS containing 30% sucrose and sodium azide (0.1%). The following day, the tissues were transferred to a mixture of PBS–30% sucrose–azide and Optimal Cutting Temperature (OCT) compound (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) at a ratio of 1:1 for an additional 24 hours before being embedded in 100% OCT in Cryomold® (Sakura Finetek Europe). The sections were prepared by freezing the tissues in

isopentane cooled in liquid nitrogen. Serial longitudinal sections (14-16 µm thick) of C6-C8 DRG were cut on a cryostat, and mounted on polylysinated slides.

Immunofluorescence

Cryosections were hydrated in phosphate-buffered saline (PBS) and processed for immunostaining. To block non-specific bindings, the sections were incubated in a solution containing 20% normal goat or donkey serum (Colorado Serum Co., Denver, CO, USA), 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 overnight in a humid chamber at RT with a cocktail of primary antibodies (Table 2) diluted in 1.8% NaCl in 0.01M PBS containing 0.1% sodium azide. After washing in PBS (3 x 10 min), the sections 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 x 10 min) and mounted in buffered glycerol at pH 8.6.

Cellular nuclei were identified with the DAPI Fluorishield (F6057-20ML, Sigma Aldrich, Milan, Italy, Europe), DRG neurons were identified with the blue fluorescent Nissl staining solution (NeuroTrace®, # N-21479, Molecular Probes, Eugene, OR, USA; dilution 1:200). Satellite glial cells were identified with a polyclonal chicken anti-glial fibrillary acid protein (GFAP) antiserum. Schwann cells were identified with a polyclonal chicken anti-myelin Protein Zero (P0) antiserum. Since CB2 receptor may also be expressed by blood vessels (Kunos et al., 2002; Lípez-Miranda et al., 2008; Galiazzo et al., 2018), the endothelial cells were recognized with two different antibodies, i.e. the mouse anti-CD31 antibody (Kader et al., 2001; Ren et al., 2002), and the rabbit anti-Factor VIII-related antigen/von Willebrand factor (Preziosi et al., 2004), herein referred to as FVIII-Rag.

In order to determine the proportion of neurons immunoreactive for each of the markers, sections subjected to single immunohistochemistry for cannabinoid receptors were counterstained with blue fluorescent Nissl stain solution (NeuroTrace®, see above) following the manufacturer's instructions. At least one hundred Nissl stained neurons were counted for each marker. Data were collected from preparations obtained from at least three animals (n=3). The percentage of immunopositive neurons was expressed as mean ± standard deviation.

Specificity of the primary antibodies

The specificity of the anti-cannabinoid receptors CB1, CB2 and PPARα antibodies in dog tissues has been recently tested by Western blot (Wb) analysis on canine intestinal tissues (Galiazzo et al., 2018). In the present study we used the antibody anti-human GPR55 (NB110-55498; Novus Bio) which, based on sequence identity (85%), is predicted to cross-react also with canine tissues. However, we tested its specificity on canine tissue by Wb analysis.

To identify TRPV1 immunoreactive neurons, we utilized two different antisera raised in rabbit (Alomone, ACC-030) and goat (Santa Cruz, c12498), directed against two different portions of the rat TRPV1. The immunogen of the rabbit anti-TRPV1 (Alomone) was the peptide [(C)EDAEVFK DSMVPGEK] (824–838) of rat TRPV1. The immunogen of the goat anti-VR1 antibody (Santa Cruz) was a synthetic peptide [PHIFTTRSRTRLFGKGDSE(C)] (38–57) from N-terminus of the rat TRPV1. The manufacturer's datasheets for both the anti-TRPV1 antibodies state that the antibodies are specific only for rodents (mouse and rat) and human DRG neurons. The specificity of the goat anti-VR1 antibody has been tested on canine tissues with Wb (Vercelli et al., 2015). Thus, we tested the specificity of the two antibodies on rat and canine DRG cryosections beforehand, by using a double-staining protocol. On rat DRG cryosections, the anti-TRPV1 antibody raised in rabbit (Alomone) and the anti-VR1 antibody raised in goat, showed full correspondence within the same neurons, which appeared brightly labeled, providing additional value to the specificity of both the anti-TRPV1 antibodies (data not shown). As observed in porcine DRG (Russo et al., 2013), only the rabbit anti-TRPV1 antibody identified TRPV1-immunoreactivity in the canine ganglia. However, the specificity of the rabbit anti-TRPV1 antibody was not tested on canine tissues by Wb.

The specificity of the endothelial markers antibodies (anti-CD31 and anti FVIII-Rag) was tested by using a double-staining protocol. Both antibodies recognized the same endothelial cells; however, the antibody anti-CD31 showed a sharper and more delicate immunolabeling of the cells (data not shown). For this reason, the anti-CD31 antibody was used as endothelial marker.

The specificity of the anti-myelin marker protein zero (P0) antiserum was tested by using a double-staining protocol. The anti-P0 antiserum was co-localized with the anti-S100 antiserum; both the myelin markers were co-localized in all the Schwann cells (data not shown).

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

Tissue sample (small intestine/jejunum) was collected, frozen in liquid nitrogen and stored at -80°C until sample processing. 100 mg of tissue were homogenized in 1 ml of SDS buffer (Tris-HCl, 62.5 mM; pH 6.8; SDS, 2%; and glycerol, 20%) supplemented with a protease inhibitor cocktail (Sigma-

Aldrich, Co, St. Louis, MO, USA). Total protein content was determined by Peterson's Modification of Lowry Method using a Protein Assay Kit. 20 μg of total proteins were separated on NuPage4–12% bis-Tris Gel (Life Technologies Ltd, Paisley, UK) for 30 minutes at 200V. The proteins were then electrophoretically transferred onto a nitrocellulose membrane by a semi-dry system (Trans Turbo Blot Bio -Rad). Non-specific bindings on nitrocellulose membrane were blocked with 5% milk powder in PBS-T20 (Phosphate Buffer Saline-0.1% Tween-20) for 1 h at room temperature. After blocking treatment, the membrane was incubated overnight at 4°C with the primary antibodies (GPR55 NB110-55498), 1:500 diluted in PBS added with 1,5% of milk. After washes, the blot was incubated with a goat anti rabbit biotin-conjugate antibody (1:50,000 dilution in TBS-T20, 1 h at RT) and then with a 1:1000 dilution of an anti-biotin horseradish peroxidase (HRP)-linked antibody (40 min at RT). Immunoreactive bands were visualized using chemiluminescent substrate (Clarity Western ECL Substrate Bio Rad), according to the manufacturer's instructions. The intensity of the luminescent signal was acquired by Chemidoc Instrument (Bio Rad) and the apparent molecular weight of the resultant bands was analyzed by Quantity One Software (Bio-Rad). Western blot analysis of GPR55 revealed a single band of expected molecular weight (~ 40 kDa) (Fig. 1).

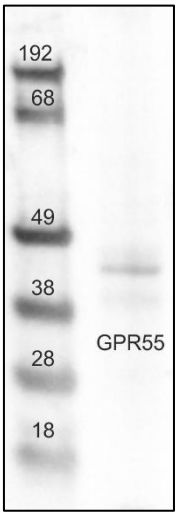


Figure 1: Representative image of Western blots (WB) analysis showing the specificity of the primary antibody rabbit anti-G protein-coupled receptor 55 (GPR55). The antibody revealed a single band of expected molecular weight (~ 40 kDa). The images of the different immunoblots were slightly adjusted in brightness and contrast to match their backgrounds.

Results

CB1 receptor immunoreactivity – About half neuronal population (55±6%; 278/507 counted sensory neurons, n= 4) displayed weak to moderate cytoplasmic CB1 receptor immunoreactivity (Fig. 2 a-d). CB1 receptor immunoreactivity was occasionally observed in SGCs, although it could be confused with background. This finding is partially consistent with observation in the rat DRG, in which

neurons and SGCs expressed CB1 receptor immunoreactivity also in the nuclei (neurons>SGCs) (Supplementary Fig. 1 a-c).

CB2 receptor immunoreactivity – CB2 receptor immunoreactivity was brightly expressed by Schwann cells and cells surrounding blood capillaries (most likely pericytes) (Fig. 3 a-l), while smooth muscle cells of blood vessels showed moderate CB2 receptor immunolabeling (Supplementary Fig. 2). SGCs did not display CB2 receptor immunolabeling (Fig. 3 a-f). Faint CB2 immunolabeling was expressed by the nuclei of all the DRG neurons (Fig. 3 d, f). GFAP immunostaining was stronger at the periphery of the ganglia, while CB2 receptor immunoreactivity was stronger in the central portion of the ganglia (data not shown). The expression of the CB2 receptor on Schwann cells depicted the path of nerve fibres, rolling between neurons before abandoning the ganglion at its central and peripheral pole (Fig. 3 g-i). In the oldest subjects, the CB2 receptor immunolabeling was less intense than in the younger dogs (data not shown). The co-localization of CB2 receptor with the myelin marker P0 showed that both the markers were expressed by all Schwann cells (Supplementary Fig. 3 a-d). CB2 receptor immunoreactivity was brightly expressed by pericyte-like cells (Fig. 3 j-l). The co-localization study between CB2 receptor and the endothelial marker CD31 showed that the endothelium was CB2 receptor negative whereas the vascular smooth muscle cells showed faint CB2 receptor immunoreactivity (Fig. 3 j-l). The CB2 receptor immunolabeling was also observed within the neuronal nuclei of the rat DRG, whereas Schwann cells and blood vessels were CB2 receptor negative (Supplementary Fig. 1 d-f).

GPR55 immunoreactivity –Bright GPR55 immunoreactivity, with grainy appearance, was expressed by all (GFAP positive and GFAP negative) SGCs (Fig. 4 a-f). Also a percentage of different size sensory neurons (38±14%; 214/542 cells counted, n=3) showed faint to moderate GPR55 immunolabeling (Fig. 4 d-f). This finding is consistent with that obtained in neurons and SGCs of the rat DRG (Supplementary Fig. 1 g-i).

PPARα immunoreactivity – PPARα immunoreactivity was expressed by SGCs (Fig. 4 g-i) and endothelial cells of blood vessels (data not shown). Quite surprisingly, four out of eight dogs did not show PPARα immunoreactivity. In the remainders, all the SGCs were PPARα-positive. These data are partially consistent with those obtained in rat DRG, in which also the neuronal cytoplasm showed faint PPARα immunoreactivity (Supplementary Fig. 1 j-l).

TRPV1 immunoreactivity – TRPV1 immunoreactivity was unevenly distributed and highly variable within the study cases. In the younger subjects, it was limited to different size neurons (and neuronal processes) while in older dogs, TRPV1 immunolabeling was expressed also by SGCs (Fig. 5 a-f). In all the subjects, the brightest TRPV1 immunolabeling was displayed by small neurons. The percentage of TRPV1 immunoreactive neurons was 55±11% (563/1017 cells counted, n=4). In the

rat DRG, TRPV1 immunolabeling was expressed only by the cytoplasm of a subset of sensory neurons and nerve fibers (Supplementary Fig. 1 m-o).

The results of the cellular distribution and intensity of the immunolabeling in the canine DRG are summarized in Table 4.

Discussion

The present study showed the expression of canonical and putative cannabinoid receptors in different cellular elements of canine cervical DRG, such as neurons (CB1 and GPR55), SGCs (GPR55 and CB1), Schwann cells and muscle cells of blood vessels (CB2). These findings further substantiate the hypothesis that endogenous ligands, e.g. endocannabinoids and related compounds, may play important roles in modulating the responses associated with hyperexcitability of DRG, such as chronic pain (Krames, 2015). While the role of DRG in pain physiology (i.e., on the crossroads between PNS and CNS) is well established (Woodhams et al., 2017), much less is known about its active involvement in processing chronic pain (Krames, 2015; Berta et al., 2017). Given the involvement of the endocannabinoid system in pain modulation (Woodhams et al., 2017; Donvito et al., 2018; Guerrero-Alba et al., 2019), our findings may help to shed new light on this challenging issue.

CB1 and CB2 receptors - The expression of CB1 receptor in DRG neurons and SGCs is in agreement with previous studies in laboratory rodents (Sanudo-Pena et al., 1999), humans (Anand et al., 2008) and dogs (Freundt-Revilla et al., 2018). However, the neuronal subpopulation expressing CB1 receptors (i.e., small sensory neurons) was different from a previous in situ hybridization study by Hohmann and Herkenham (1999) who found medium-and large-sized cells in rat DRG to predominantly express CB1 receptor mRNA. Although, in the present study, the area of DRG neurons was not measured, it is possible to state with some confidence that, in the rat DRG, CB1 receptor immunoreactivity was expressed also by large-sized neurons.

The expression of faint CB2 receptor immunolabeling in neurons and its absence in SGCs of canine DRG, partially agrees with previous findings in laboratory rodents, where only very weak immunoflorescence was found in basal conditions (Svíženská et al., 2013). Although CB2 receptor was considered lacking in neurons and glial cells, recent literature highlights its expression in these cell types (Sánchez-Zavaleta et al., 2018; Stella, 2009), even in humans (Anand et al., 2008) and dogs (Fernández-Trapero et al., 2017; Freundt-Revilla et al., 2018). Similarly to CB1 (Mitrirattanakul et al., 2006), CB2 receptor is upregulated in a variety of PNS and CNS diseases and is suggested as a promising pharmacological target in the management of chronic pain and neuroinflammation (Skaper et al., 2013; Svíženská et al., 2013; Navarro et al., 2016; Cassano et al., 2017). At present we are not

able to explain the presence of the CB receptors in neuronal nuclei of canine (CB2 receptor) and rat (CB1 and CB2 receptors) DRG. The study on the subcellular distribution and function of cannabinoid receptors is still expanding. The nuclear envelope, which is a part of the endoplasmic reticulum, may be one of the sources of nuclear Ca2+; Currie et al. (2008) identified the expression of CB1 and CB2 receptors on the nuclear membrane of cardiac muscle cells and demonstrated that these receptors, when activated by anandamide, can (negatively) modulate nuclear Ca2+ release and, very likely, gene transcription.

To the best of our knowledge, this is the first time that CB2 receptor immunoreactivity in Schwann cells has been reported. Up to now, endocannabinoid receptor immunolabeling of Schwann cells was limited to CB1, which was shown in about 100% of this cell type in the canine sciatic nerve (Freundt-Revilla et al., 2017). Besides forming the myelin sheath, Schwann cells orchestrate much of the regenerative response that occurs after nerve injury in order to restore nerve function (Glenn and Talbot, 2013). The expression of CB1 (Freundt-Revilla et al., 2017) and CB2 receptors (present study) in Schwann cells could thus support the neuroprotective and/or neuroreparative role suggested for cannabinoids and related compounds in the PNS (Svíženská et al., 2013; Truini et al., 2011).

The presence of thin interneuronal GFAP-negative cellular processes expressing CB2 receptor-immunoreactivity is at present not easy to interpret. These CB2 receptor immunoreactive slender evaginations might belong to GFAP-negative SGCs (Tongtako et al., 2017) or to a different type of DRG glial cells, i.e. pericyte-like satellite cells (Wyburn, 1958; Bunge et al., 1967). Also the presence of different cell types with elongated cellular processes immunoreactive for CB2 receptor, such as fibroblasts and histiocytes (Bunge et al., 1967; Tongtako et al., 2017), cannot be excluded.

Some considerations are needed when dealing with DRG blood vessels. First, little information is available and it mainly refers to laboratory rodents. Second, blood-nerve barrier is lacking in intact DRG (Jacobs et al., 1978) and fenestrations together with open intercellular junctions characterize ganglionic vessels (Anzil et al., 1976; Bush et al., 1991). Although the sheath of SGCs is considered to control the traffic of substances from blood to ganglionic neurons - thus functionally substituting for the vascular barrier (Pannese, 2010) - circulating signalling molecules are allowed to diffuse into the microenvironment of DRG. This was recently confirmed by Svíženská et al. (2013), who demonstrated that sciatic nerve injury induces bilateral increase of CB2 receptor (both protein and mRNA) in lumbar L4–L5 as well as cervical C7–C8 DRG.

In the present study we detected CD31 and FVIII-RAg immunoreactivity in a small proportion of DRG vessels, mostly confined to the periphery of the ganglion rather than among sensory neurons. The finding is quite unexpected, since the endothelial marker CD31 allowed to trace an extensive network of blood vessels in the mouse L4 DRG, that was found to encapsulate and encircle sensory

neurons (Jemenez-Andrade et al., 2008). The paucity of vascularization of canine DRG did not seem to depend on methodological issues since the antibody anti-CD31 was recently found to perfectly label the endothelium of canine blood vessels, at least in the intestinal mucosa (Galiazzo et al., 2018). In the present study CB2 receptor immunoreactivity was limited to smooth muscle cells of blood vessels, being absent from CD31-positive endothelium, differently from what observed in canine intestinal (Galiazzo et al., 2018) and skin blood vessels (Campora et al., 2012), or human brain endothelium (Zhang et al., 2011). One possible explanation for this discrepancy might be the well known regional distribution of the cannabinoid receptors in blood vessels (Stanley and O'Sullivan, 2014). Indeed, CB2 receptor immunoreactivity of vascular smooth vessels was recently detected in bovine pancreas (Dall'Aglio et al., 2017) and mice skin (Zheng et al., 2012). Endocannabinoids exert a prohomeostatic function on vascular biology through complex mechanisms often involving canonical as well as putative cannabinoid receptors (e.g., TRPV1 and GPR55, Ho and Kelly, 2017). In particular, vasodilating effect occurs at different cellular site, i.e., nerves, endothelial cells, vascular smooth muscle cells, perycites (Benyó et al., 2016), employing different receptors and leading to nitric oxide release (Ho and Kelly, 2017).

GPR55 – The GPR55 represents a novel target for various cannabinoids (Morales and Reggio, 2017). Strong expression of GPR55 immunoreactivity in different size neurons and SGCs was found in the present study. GPR55 immunoreactivity was expressed also by GFAP negative SGCs; a recent study showed that GFAP recognizes up to 89% of all SGCs of the canine DRG (Tongtako et al., 2017). This finding indicates that GPR55 might be utilized as canine SGCs marker. In the present study, a similar pattern of GPR55 immunoreactivity has been observed also in the neurons and SGCs of rat DRG. This is a relatively new finding, since up to now GRP55 immunoreactivity has been detected only in the neuronal component of DRG (Lauckner et al., 2008). Consistently, the GPR55 immunoreactivity in medium- and large-sized DRG neurons as detected here agrees with the finding of Lauckner et al. (2008), who observed strong GPR55 signal in mice DRG large neurons. Interestingly, large sensory neurons may mediate inflammatory and neuropathic pain hypersensitivity by switching their phenotype and expressing the nociceptive neurotransmitter Subtance P (Neumann et al., 1996; Ruscheweyh et al., 2007). It is noteworthy to recall that some phytocannabinoids, e.g. Δ9-tetrahydrocannabinol (THC), cannabidiol, synthetic cannabinoids (AM251 and O-1602), as well as palmitoylethanolamide (PEA) have been described as GPR55 ligands (Stella, 2010; Kramar et al., 2017).

Although further functional investigations are necessary, GPR55 immunoreactivity in both SGCs and neurons as detected in the present study likely may suggest a relevant role of this receptor in neuron-SGCs crosstalk, which is currently considered a critical component of neuroinflammatory changes

eventually leading to chronic pain (Cairns et al., 2015; Hanani, 2012;Iwata et al., 2017; Skaper et al., 2018).

PPARα – The PPARα is a ligand-activated transcription factor belonging to the superfamily of nuclear hormone receptors. By modulating gene expression, it plays key roles in maintaining glucose and lipid homeostasis and inhibiting inflammation (Naidenow et al., 2016). The PPARα activation has also been shown to induce rapid, cellular changes without requiring transcription (Lo Verme et al., 2005). In the present study PPARα immunoreactivity has been detected in the canine SGCs and endothelial cells. In the comparative study on rat DRG, we observed bright PPARα immunoreactive SGCs, whereas neurons were faintly immunolabeled. These findings are in line with previous data on the expression of PPARα in mice DRG (Lo Verme et al., 2006; D'Agostino et al., 2009; Khasabova et al., 2012) and canine gastrointestinal tract (Galiazzo et al., 2018). The ganglia of four out of eight dogs did not show PPARα immunoreactivity. At present we do not have any clear explanation for this discrepancy. No apparent correlation with any particular factor (e.g., age or cause of death) was found. Nonetheless, we cannot exclude that it was due to an undetected subclinical state, given that metabolic disorder, for example, is associated with significantly decreased spinal PPARα expression (Wang et al., 2014).

TRPV1 – The TRPV1 is a ligand-gated nonselective cation channel usually expressed by peptidergic nociceptors of rodents (Zwick et al., 2002; Yu et al., 2008) and large mammals (Russo et al., 2013) as well as nonpeptidergic nociceptors (Tominaga et al., 1998; Breese et al., 2005). The TRPV1 is activated by heat (>43°C), low pH and capsaicin (Caterina et al., 1997) and desensitized by endocannabinoids (Zygmunt et al., 1999; Ambrosino et al., 2013).

In accordance with previous studies in rodent and human DRG (Caterina et al., 1997; Helliwell et al., 1998; Hoffman et al., 2010; Anand et al., 2008) we have observed diffuse TRPV1 immunoreactivity in neurons of canine DRG, with the brightest immunolabeling being displayed by small size neurons. This latter finding agreed with the study of Binzen et al. (1996), who found TRPV1 to be mainly expressed in small-sized neurons of rat DRG, the vast majority of which co-expressed CB1 receptors. Our comparative study on rat DRG confirmed that the brightest TRPV1 immunoreactivity was mainly expressed by small neurons. Moreover, SGCs from two old dogs were also brightly immunolabeled, in accordance with TRPV1 expression by DRG glial cells (Doly et al., 2004).

To the best of our knowledge no information is yet available about the influence of age on neuronal and/or glial expression of TRPV1, however one could tentatively speculate that aging itself has an impact on pain pathophysiology through changes in the pain involved receptor TRPV1. Actually, increased expression of TRPV1 was recently observed in rat DRG after neuropathic pain induction (Chukyo et al., 2018). Marrone et al. (2017) reported TRPV1 immunoreactivity in microglial cells

rather than neurons of the mice brain areas. Moreover, they showed that in mice suffering from neuropathic pain, TRPV1 was also functionally expressed in cortical neurons. Together with the present morphological data, the findings by Marrone et al. (2017) indicate that TRPV1 might be a key player of glia-neuron communication.

Recent studies have shown that TRPV1 is desensitized by a number of cannabinoids, including THC, cannabinol, synthetic cannabinoid WIN 55,212-2, AEA, rimonabant (Stella, 2010) as well as PEA (Ho et al., 2008; De Petrocellis and Di Marzo, 2010; Ambrosino et al., 2013; Aldossary et al., 2019). This ability is very important as TRPV1 channel desensitization is considered to be responsible for analgesic and anti-inflammatory effects (Marrone et al., 2017).

A limitation of the study is the lack of unquestionable specificity test of the employed TRPV1 antibody in dog tissue. The TRPV1 has been cloned and functionally characterized from different species, including dogs. Peptide alignment of the dog TRPV1 orthologue with other species of the TRPV1 family revealed a high degree of sequence homology (human, 89.1%; rat, 87.5%; mouse, 83.3%) (Phelps et al., 2005). Actually, the antibody performs well in an optimized IHC assay, binding the indicated target, not only in dog tissue (TRPV1 immunolabeled SGCs were observed also in cat and horse cervical DRG, while in small rodents and guinea-pig the TRPV1 immunoreactivity was always limited to DRG neurons – RC personal observation). Thus, since the dog was proposed as a good model for studying the role of TRPV1 in inflammatory diseases and nociception and the effects of TRPV1 antagonists in humans (Phelps et al. 2005), additional molecular analysis, such as knockout cell lines and Western blot (assuming the IHC-based antibody also works in Western blots), might be necessary to strength the results of TRPV1 immunolabeling, and to increase confidence for the validity in the dog.

Conclusion

The present study highlighted the expression of canonical and putative cannabinoid receptors on different DRG cell types, in particular neurons and glial cells (SGCs and Schwann cells). Given the key role of DRG elements and cannabinoid receptors in the pathophysiology of chronic pain, targeting and modulating these receptors, possibly through a multifaceted approach, may become a novel way to manage pain in veterinary patients.

Controls	**Breed**	**Gender**	**Age**	**Cause of death**
Dog 1	Chihuahua	F	8 mo	Head trauma
Dog 2	Great Dane	M	2 yr	Peritonitis/ intussusception
Dog 3	Pitbull	M	13 yr	Splenic neoplasia, skin neoplasia
Dog 4	Mongrel	M	11 yr	Mast cell tumor
Dog 5	Mongrel	F	11 yr	Mast cell tumor, Cushing's syndrome
Dog 6	Mongrel	M	14 yr	Gastric dilatation-volvulus
Dog 7	Lagotto Romagnolo	FS	10 yr	Thymoma
Dog 8	Cane Corso italiano	F	8 yr	Gastric tumor

Table 1 Clinico-pathological data of the dogs included in the present research (M, male; F, female. FS, female spayed).

Primary antibody	Host	Code	Dilution	Source
CB1	Rabbit	Orb10430	1:200	Biorbyt
CB2	Rabbit	AB45942	1:200	Abcam
CD31	Mouse	M0823 Clone JC70A	1:30	Dako
GFAP	Chicken	AB4674	1:800	Abcam
GPR55	Rabbit	NB110-55498	1:200	Novus Biol.
Factor VIII	Rabbit	A0082	1:1000	Dako
PPARα	Rabbit	NB600-636	1:200	Novus Biol.
TRPV1(VR1)	Rabbit	ACC-030	1:200	Alomone

Table 2: Primary antibodies used in the present research. Primary antibodies Suppliers: Abcam, Cambridge, UK; Alomone, Jerusalem, Israel Biorbyt Ltd., Cambridge, UK; Dako, Carpinteria, CA, USA; Novus Biologicals, Littleton, CO, USA.

Secondary antibody	Host	Code	Dilution	Source
Anti-rabbit IgG FITC	Goat	401314	1:200	Calbiochem-Novabiochem
Anti-rabbit 488	Donkey	AB150073	1:800	Abcam
Anti-chicken TRITC	Donkey	703-025-155	1:200	Jackson

Table 3: Secondary antibodies used in the present research. Secondary antibodies Suppliers: Abcam, Cambridge, UK; Biotium, Inc. Hayward, CA, USA; Calbiochem-Novabiochem, San Diego, CA, USA; Jackson Immuno Research Laboratories, Inc. Baltimore Pike, PA, USA.

Cervical dorsal root ganglion	**CB1**	**CB2**	**GPR55**	**PPARα**	**TRPV1**
Neurons N, nucleus; C, Citoplasm	C ++	N +	C ++	-	C+/+++
Satellite glial cells	C +	-	C +++	C ++	C +++
Schwann cells	-	+++	-	-	-
Blood vessels E, endothelium; M, smooth muscle cells	-	E +++ M ++	-	E ++	-

Table 4: Semiquantitative evaluation of the density of CB1, CB2, GPR55, PPARa, and TRPV1 receptors immunoreactivity in different cellular elements (neurons, satellite glial cells, Schwann cells, blood vessels) of the canine C8 cervical dorsal root ganglia.

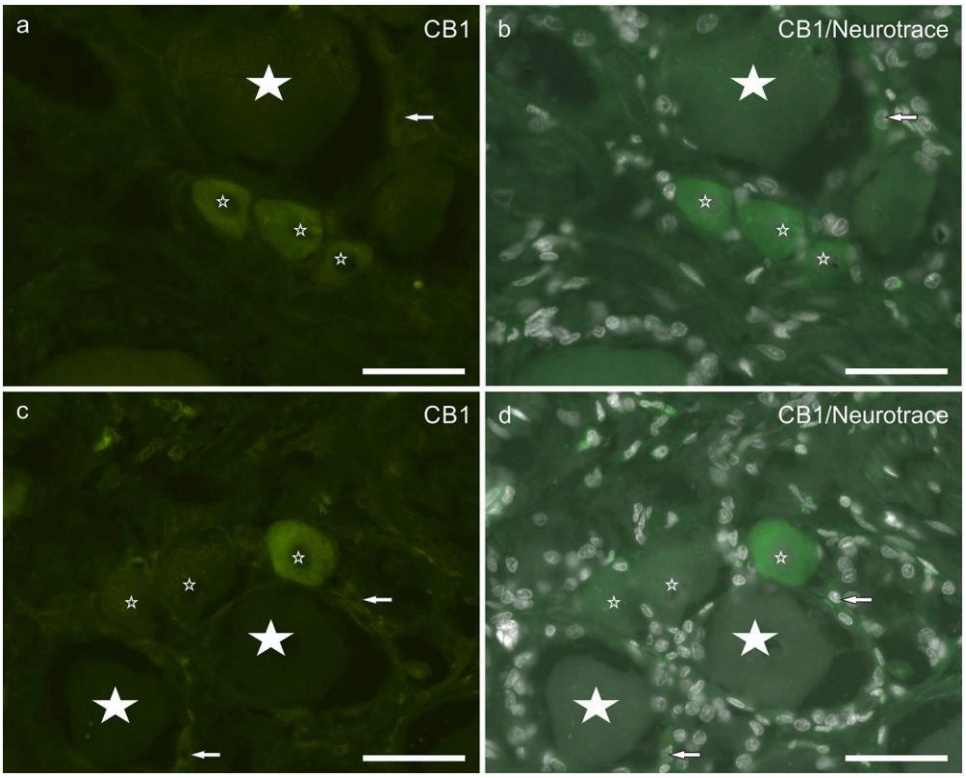


Figure 2 (a-d): Photomicrographs of cryosections of canine cervical (C8) dorsal root ganglion showing cannabinoid receptor 1 (CB1) immunoreactivity. Small stars indicate small neurons showing CB1 receptor weak to moderate immunoreactivity. Large stars indicate CB1 receptor negative neurons. Arrows indicate satellite glial cellsshowing weak CB1 receptor immunoreactivity. Bar: a–d = 50μm.

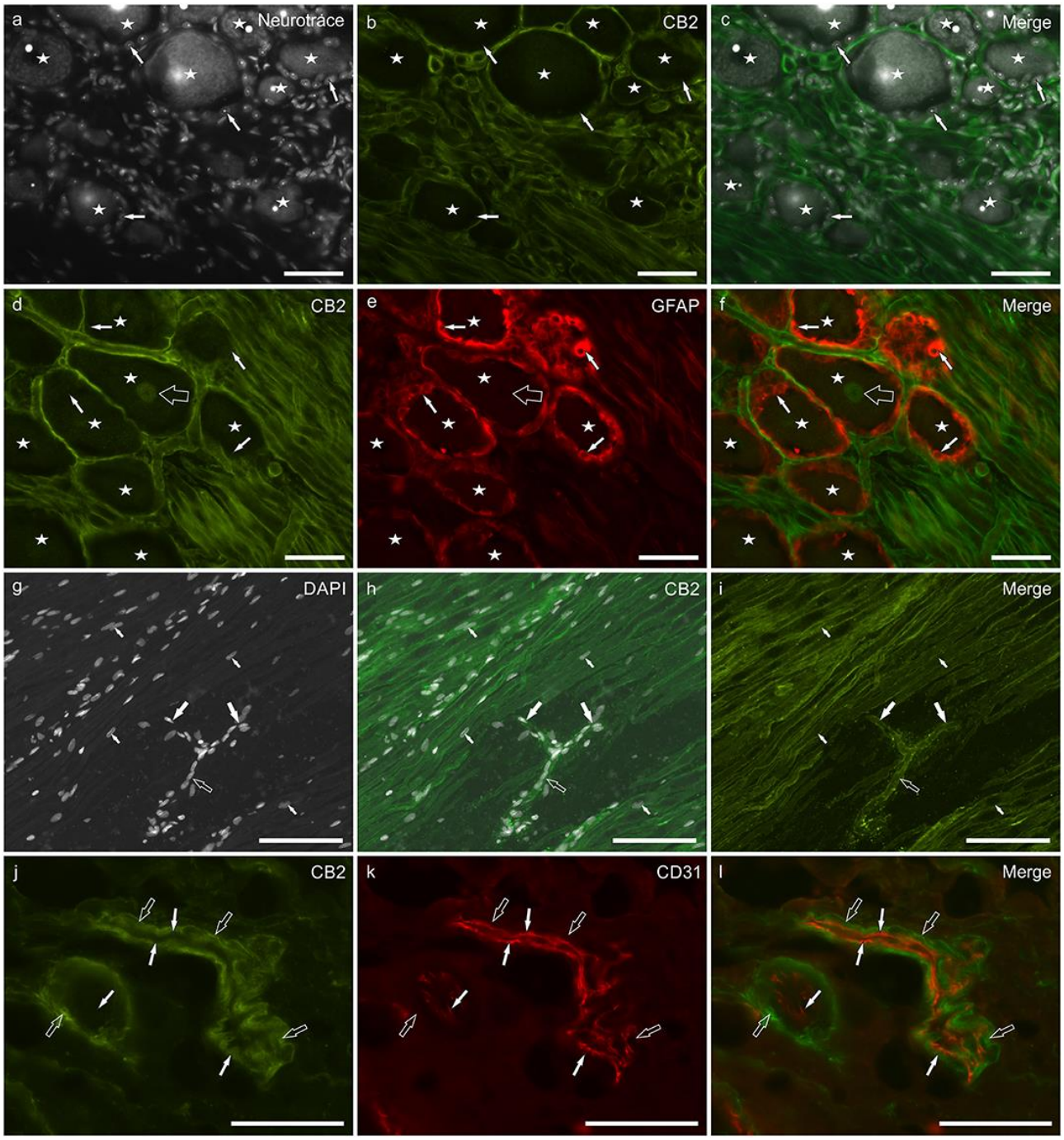


Fig. 3 (a-l): Photomicrographs of cryosections of canine cervical (C8) dorsal root ganglion showing cannabinoid receptor 2- (CB2), glial fibrillary acidic protein- (GFAP), and CD31-immunoreactivity. a-c) Stars indicate NeuroTrace labelled (a) dorsal root ganglion sensory neurons which were CB2 receptor negative (b), as well as the satellite glial cells (white arrows). d-f) Stars indicate sensory neurons encircled by satellite glial cells (white arrows) which were GFAP-immunoreactive (e) and CB2 receptor negative. CB2 receptor immunoreactivity was expressed by Schwann cells and neuronal nuclei (open arrow). g-i) The empty arrow indicates one neuronal axon that bifurcates (T-junction) in its central and peripheral portions (large white arrows). The small arrows indicate the nuclei of Schwann cells. j-l) Open arrows indicate smooth muscle cells (vessel on the left) and pericyte-like cells (elongated and thin blood vessel on the right) showing CB2 receptor immunoreactivity (j). White arrows indicate endothelial cells showing CD31 immunoreactivity (k). Bar: a-f, j-l = 50 µm; g-i = 100 µm.

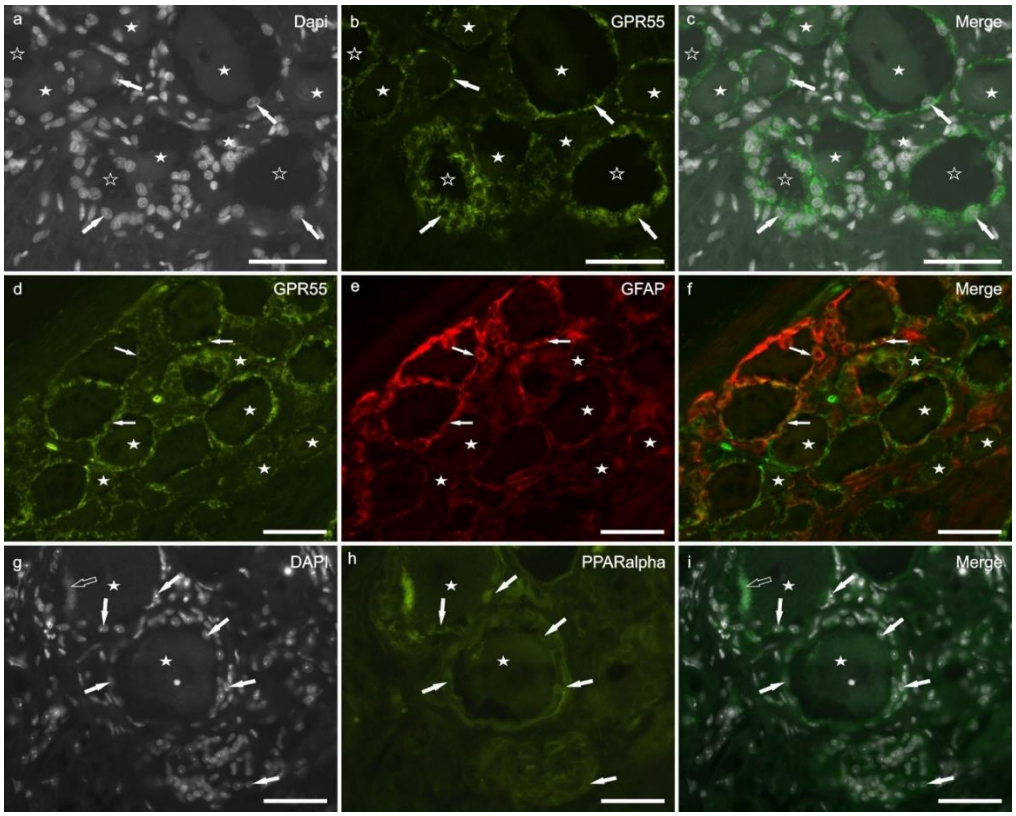


Figure 4 (a-i): Photomicrographs of cryosections of canine cervical (C8) dorsal root ganglion showing GPR55 (a–f) and PPARalpha (g–i) immunolabeling. (a–c) Arrows indicate the Neurotrace-labeled nuclei of satellite glial cells (a) which showed bright GPR55 immunolabelling (b). White stars indicate unlabeled sensory neurons; open stars indicate empty spaces in which sensory neurons were no more evident. (d–f) White arrows indicate satellite glial cells which co-expressed bright GPR55- (d) and glial fibrillary acidic protein (GFAP) immunoreactivity; open arrows indicate SGCs which were GPR55 immunoreactive and GFAP negative (e). Stars indicate sensory neurons of different dimension, which expressed faint –to-moderate GPR55 immunoreactivity. (g–i) White arrows indicate the Neurotrace labeled nuclei of SGCs which showed PPARalpha immunoreactivity (h). Open arrows indicate autofluorescent pigment. Bar: a–i = 50μm.

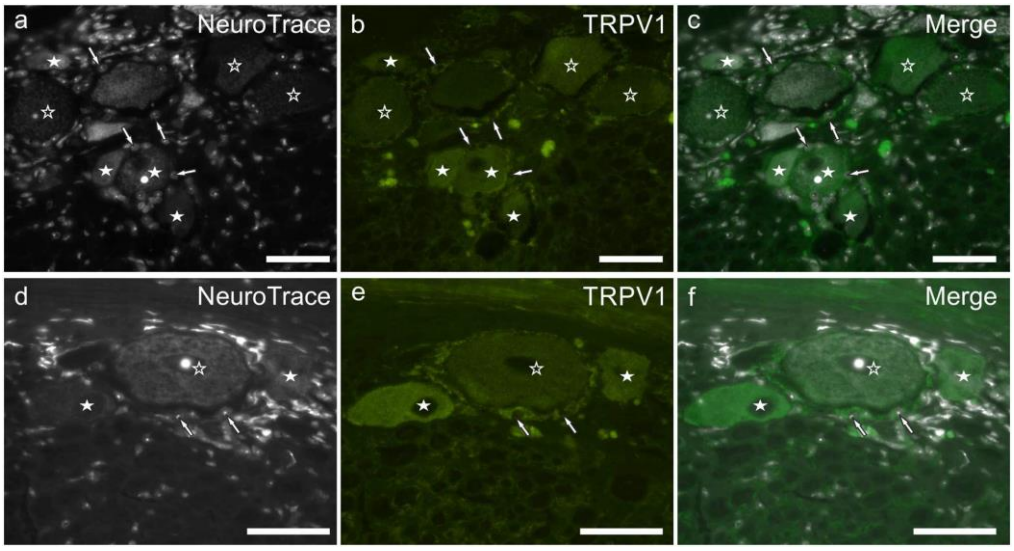
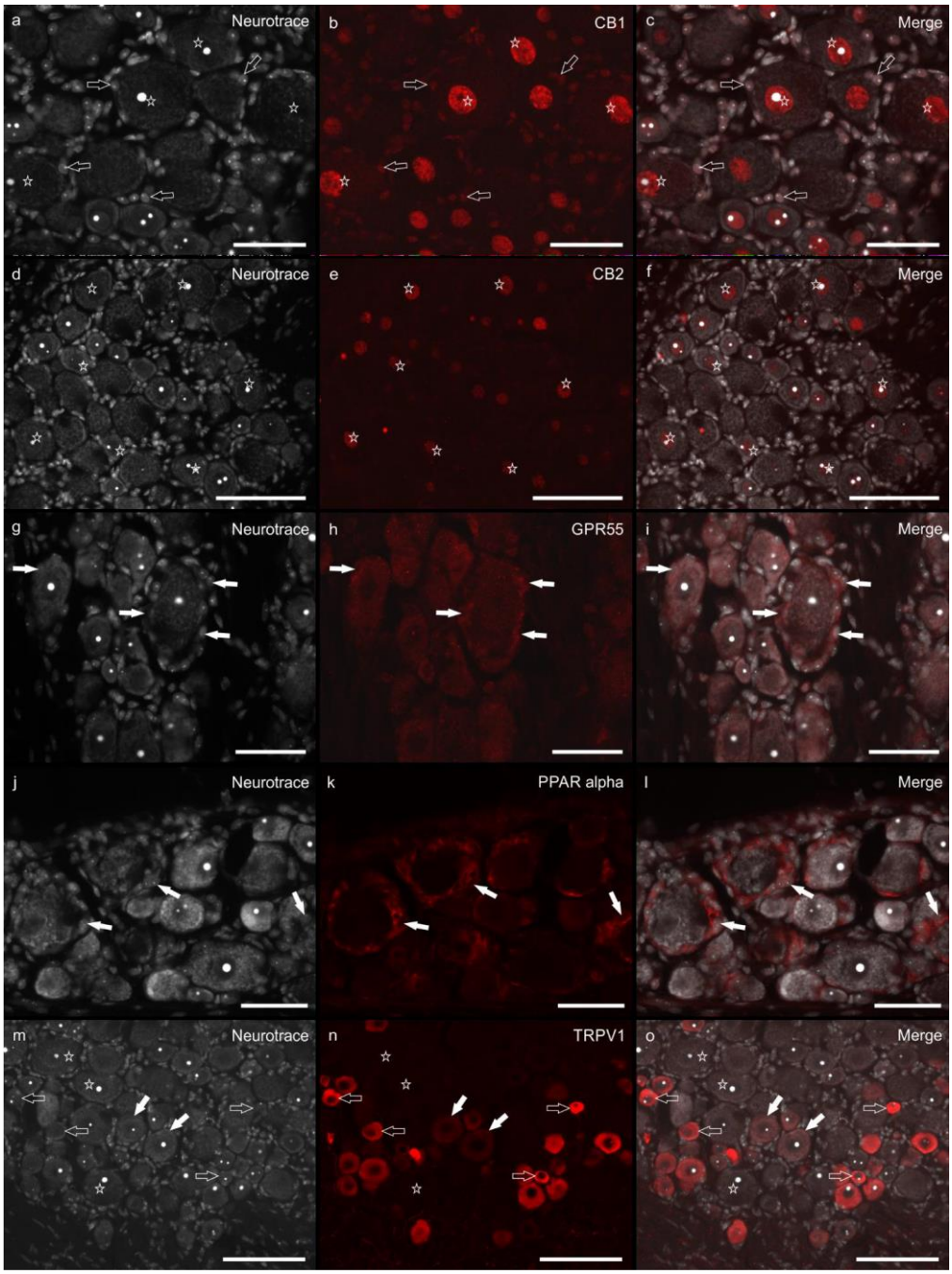
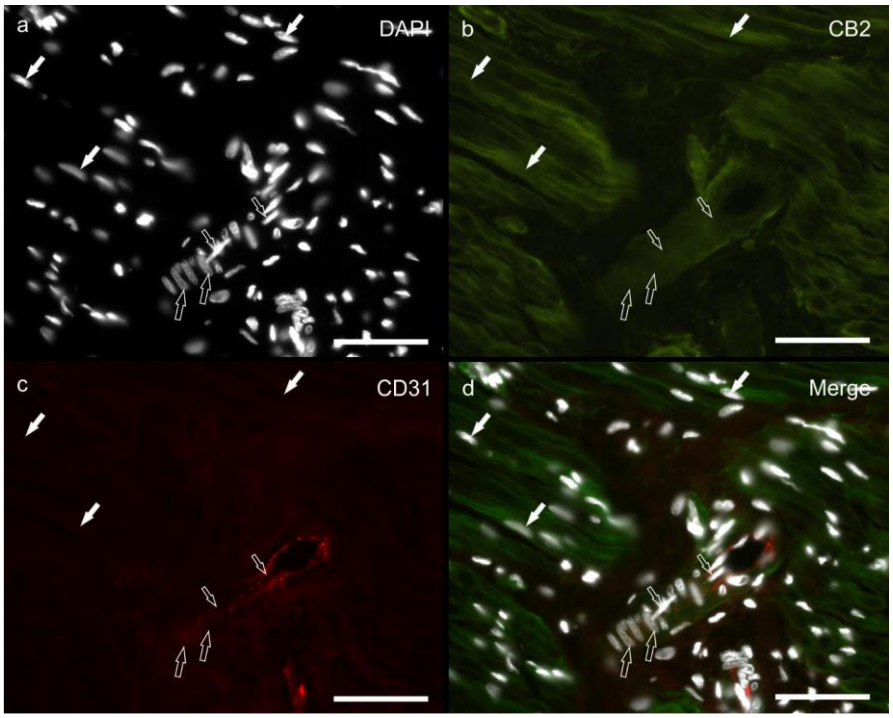


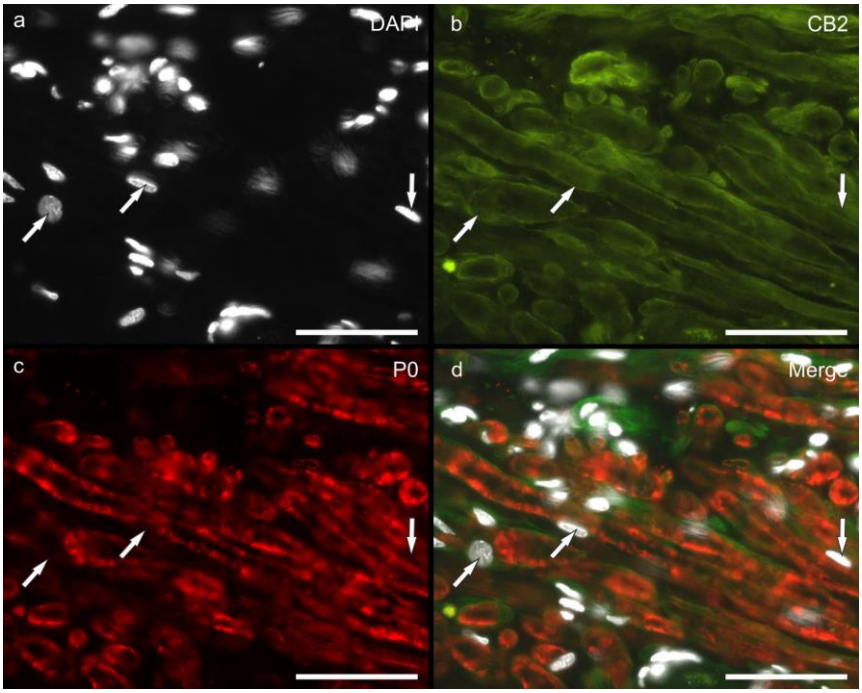
Figure 5 (a-f): Photomicrographs of cryosections of the C8 cervical dorsal root ganglia belonging to two aged dogs showing transient receptor potential vanilloid type 1 (TRPV1) immunoreactivity. White stars indicate neurons showing bright TRPV1 immunoreactivity, while open stars indicate larger neurons showing weaker TRPV1 immunoreactivity. Arrows indicate the Neurotrace labeled nuclei of satellite glial cells showing bright TRPV1 immunolabeling (b, e). Bar: a–f = 50μm.



Supplementary Figure 1(a-o): Photomicrographs of cryosections of rat cervical (C8) dorsal root ganglion showing CB1 (a-c), CB2 (d-f), GPR55 (g-i), PPARalpha (j-l), and TRPV1 (m-o) immunolabeling. a-c) CB1 immunoreactivity was brightly expressed by the nuclei of sensory neurons (stars), whereas the nuclei of satellite glial cells (arrows) showed weaker CB1 immunolabeling. The neuronal cytoplasm showed, on the contrary, weak or undetectable CB1 receptor immunoreactivity d-f) Stars indicate some of the nuclei of the sensory neurons expressing CB2 immunoreactivity. g-i) Sensory neurons expressing weak to moderate GPR55 immunoreactivity; arrows indicate the nuclei of some satellite glial cells which expressed brighter GPR55 immunoreactivity. j-l) Arrows indicate the nuclei of SGCs expressing bright PPARalpha immunoreactivity. m-o) Arrows indicate sensory neurons expressing bright TRPV1 immunoreactivity. Larger neurons were TRPV1 negative (stars) or showed weaker TRPV1 immunolabeling (white stars). Scale Bar: a-c, g-l = 50 µm; d-f, m-o = 100 µm.



Supplementary Figure 2: a-d) Photomicrographs of cryosections of canine cervical (C8) dorsal root ganglion (DRG) showing cannabinoid receptor 2- (CB2) and endothelial marker CD31 immunoreactivity. Large and empty arrows indicate the DAPI labelled nuclei of vascular smooth muscle cells (a), which showed weak CB2 receptor immunoreactivity (b). The small empty arrows indicate the nuclei of CD31 immunoreactive endothelial cells (c). White arrows indicate the nuclei of CB2 receptor immunoreactive Schwann cells (b) Scale Bar: a-d= 50 μm



Supplementary Figure 3 (a-d): Supplementary Fig. 3. a-d) Photomicrographs of cryosections of canine cervical (C8) dorsal root ganglion (DRG) showing cannabinoid receptor 2- (CB2) and myelin protein zero (P0) immunoreactivity. Arrows indicate the nuclei of Schwann cells showing co-localization between CB2 receptor and P0 immunoreactivity. Scale Bar: a-d= 50 μm

Experimental studies

Paper ready for submission. Modified from:

Localisation of cannabinoid receptors in the equine dorsal root ganglia

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Abstract

Introduction – The activation of cannabinoid receptors by endogenous, plant-derived or synthetic cannabinoids may exert beneficial effects on somatic and visceral pain perception.

Objectives – The aim of this study was to localise the cellular distribution of nine canonical and putative cannabinoid receptors [cannabinoid receptors 1 (CB1R) and 2 (CB2R), G protein-coupled receptor 3 (GPR3) and 55 (GPR55), nuclear peroxisome proliferator-activated receptor alpha (PPARα) and gamma (PPARγ), transient receptor potential vanilloid type 1 (TRPV1) and ankyrin 1 (TRPA1), serotonin 5-HT1a receptor (5-HT1aR)] in the equine cervical dorsal root ganglia. Ex vivo qualitative and quantitative immunohistochemical study.

Results – All the receptors were expressed by neurons, SGCs, or both cellular types. The neurons showed immunoreactivity for CB1R (100%), CB2R (88±18%), GPR3 (66±16%), GPR55 (64±16%), PPARα (100%), PPARγ (100%), TRPV1 (66±18%), TRPA1 (73±12%), and 5-HT1aR (83±7%). Neuronal processes showed CB1R and TRPA1 immunoreactivity. The SGCs showed immunoreactivity for CB2R, GPR55, PPARα, PPARγ, TRPA1, and 5-HT1aR.

Conclusions and relevance - The present study highlighted the expression of cannabinoid receptors in the DRG neurons and/or glial cells. Given the key role of DRG elements and cannabinoid receptors in the pathophysiology of chronic pain, these findings could support the use of cannabinoid agonists in the horse with chronic pain and encourage the development of new drugs to manage neuropathic pain in equine medicine.

Introduction

A growing body of literature indicates that the activation of cannabinoid receptors by endogenous, plant-derived or synthetic cannabinoids may exert beneficial effects on inflammatory and neuropathic pain perception (Ligresti et al., 2016). Their scientific evidence has prompted several companies to produce medical marjiuana and cannabinoid receptor agonists to also be used in equine medicine to

treat different forms of somatic and visceral pain. For instance, cannabidiol (CBD), a non-psychoactive compound found in cannabis sativa, seems to be one of the most promising therapeutic substances, due to its numerous health-related benefits, including analgesic, anti-inflammatory, anti-spasmodic and anti-anxiety benefits (Mechoulam et al., 2007; Pertwee 2008).

For many years, it was assumed that the beneficial effects of the cannabinoids were mediated by cannabinoid receptors 1 (CB1R) and 2 (CB2R). However, it is currently known that phytocannabinoids may act on multiple targets. These compounds have been shown to interact with other G-protein coupled receptors, nuclear receptors, transient receptor potential (TRP) channels, serotonin receptors and glycine receptors, among others (Morales et al. 2017). In particular, CBD, which shows indirect interaction with CB1R and CB2R, seems to be involved in the modulation of receptors outside the endocannabinoid system, such as the serotoninergic 5-HT1a receptor (5-HT1aR), and the transient receptors potential vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1), the latter two being excitatory ion channels expressed by the sensory neurons mediating somatic and visceral pain (Ligresti et al. 2016).

As a general rule, to better understand the actions of a drug, it would be of extreme importance to know the cellular distribution of its specific receptors. To date, reliable anatomical studies regarding the cellular distribution of cannabinoid receptors in the horse central and peripheral nervous system are still lacking. In order to help filling these anatomical gaps, the present ex vivo study was designed to identify, in the equine dorsal root ganglia (DRG), the cellular distribution of two canonical cannabinoid receptors, i.e. CB1R and CB2R, and of seven putative cannabinoid receptors, i.e. G protein-coupled receptor 3 (GPR3) and 55 (GPR55), nuclear peroxisome proliferator-activated receptor alpha (PPARα) and gamma (PPARγ), TRPV1 and TRPA1, and 5-HT1aR.

Material and Methods

Animals

The cervical (C6-C8) dorsal root ganglia were collected from six horses (1.5 years of age) at the public slaughterhouse, following the division of the trunk of the animals into two half-carcasses. The tissues were fixed and processed to obtain cryosections as described elsewhere (Russo et al. 2011).

Immunofluorescence

The cryosections were hydrated in phosphate–buffered saline (PBS) and processed for immunostaining. To block non-specific bindings, the sections were incubated in a solution containing 20% normal goat serum, 0.5% Triton X-100b and bovine serum albumin (1%) in PBS for 1 h at room temperature (RT). The cryosections were incubated overnight in a humid chamber at RT with primary

antibodies (Table 1) diluted in 1.8% NaCl in 0.01M PBS containing 0.1% sodium azide. After washing in PBS (3 x 10 min), the sections were incubated for 1 h at RT in a humid chamber with the secondary antibody [goat F(ab)2 anti-Rabbit FITC; ab98430] diluted in PBS. After washing in PBS (3 x 10 min) to identify the DRG neurons and the SGCs, the sections, single-stained with each marker studied, were counterstained with Blue fluorescent Nissl stain solution (NeuroTrace®, # N-21479, dilution 1:200). The cryosections were then washed in PBS (3 x 10 min) and mounted in buffered glycerol at pH 8.6.

In order to determine the proportion of neurons immunoreactive for each of the studied marker, at least one hundred Nissl stained neurons were counted for each marker. Data were collected from preparations obtained from at least three animals (n=3). The percentages of immunopositive neurons were expressed as mean ± standard deviation.

Specificity of the primary antibodies

The choice of the primary antibodies utilised in the study was based on the homology of the amino acid sequence between the immunogen of the commercially available antisera and the horse proteins, verified by the "alignement" tool available on the Uniprot database (www.uniprot.org) and the BLAST tool of the National Center for Biotechnology information (NCBI) (www.ncbi.nlm.nih.gov). Details are summarized in Table 2.

Specificity of the secondary antibody

The specificity of the secondary antibody was tested by applying them after omission of the primary antibodies. No stained cells were detected after omitting the primary antibodies.

Fluorescence microscopy

The preparations were examined, and the images were recorded and adjusted as described elsewhere (Giancola et al. 2017).

Results

CB1R immunoreactivity

Bright CB1R immunoreactivity (CB1R-IR) was displayed, with different degrees of intensity, by the cytoplasm (and nucleolus) of all sensory neurons (100%; 604/604 cells counted, n=4) (Figure 1 a-c); no distinction of CB1R immunolabelling was observed among neurons of different sizes. The nerve processes also showed CB1R-IR, although it was weaker than that observed in the neuronal somata. Also the SGCs showed faint CB1R-immunolabeling.

CB2R immunoreactivity

The CB2R-IR was expressed by the majority of neurons (88±18%; 597/678 cells counted, n=3) and all SGCs. In general, there was an inverse correlation between the brightness of CB2R immunoreactivity in neurons and SGCs. In addition, small-to-medium-sized neurons showed brighter granular CB2R cytoplasmic immunolabeling, in comparison with largest ones, wich were encircled by strongly labeled SGCs (Fig. 1 d-f). Large and faintly CB2R labeled neurons were about 40% of the total neuronal population, whereas the brighter and smaller neurons accounted for about 60%.

TRPA1 immunoreactivity

The TRPA1-IR was expressed by the cytoplasm and nucleus of both the neurons (73±12%; 385/551 cells counted, n=3) and the SGCs, and by the nerve processes (Fig. 2 a-c). Of the nerve processes, thin and unmyelinated nerve fibres showed brighter TRPA1 immunolabelling than large myelinated nerve fibers.

TRPV1 immunoreactivity

The TRPV1-IR was expressed by the cytoplasm of the sensory neurons (66±18%; 356/570 cells counted, n=3) (Fig. 2 d-f). Whilein some horses it was challenging to establish whether the SGCs showed TRPV1-IR or not due to the presence of a faint signal, in other subjects SGCs showed a bright TRPV1 labeling. When looking at TRPV1-IR in terms of signal intensity, different-sized neurons did not show any apparent differences. TRPV1 was brightly expressed also by nerve fibers.

PPARα immunoreactivity

The PPARα-IR was brightly displayed by the cytoplasm of the SGCs, whereas in the cytoplasm of the sensory neurons (100%; 456/456 cells counted, *n*=4) it was very weak (Fig. 2 g-i). The endothelium of blood vessels showed bright PPARα-IR (data not shown).

PPARγ immunoreactivity

The PPARγ-IR was brightly expressed by the nuclei of all the neurons; the nuclei of SGCs also showed moderate PPARγ immunolabelling (Fig. 2 j-l).

GPR3 immunoreactivity

Faint and granular GPR3-IR was displayed, albeit with different degrees, by the cytoplasm of a subset of neurons (66±16%; 474/740 cells counted, n=3) (Fig. 3 a-c).

GPR55 immunoreactivity

The GPR55-IR was expressed, with different degrees, by the cytoplasm of a subset of neurons (64±16%; 293/469 cells counted, n=4). In addition, GPR55-IR was also displayed by some SGCs (Fig. 3 d-f) and perineuronal cellular elements (probably macrophages) (data not shown).

5-HT1aR immunoreactivity

The 5-HT1aR-IR was expressed by the cytoplasm of the sensory neurons (83±7%; 367/437 cells counted, n=3) and the SGCs (Fig. 3 g-i), and by the Schwann cells (Fig. 3 j-l).

Discussion

Cannabinoid receptors may play a critical role in nociception by means of central and peripheral mechanisms (Hohmann et al., 1995; Calignano et al., 1998; Richardson et al., 1998; Stella 2010; Ligresti et al., 2016; Pergolizzi et al., 2018).

To the best of our knowledge, this is the first immunohistochemical study regarding the distribution of cannabinoid receptors in the equine DRG. The observation of a prodigious level of cannabinoid receptors in different cellular elements of the equine DRG, such as neurons, SGCs and Schwann cells, indicated that endocannabinoids and cannabinoid agonists may play a notable role in pain transmission, inflammation, myelination and, possibly, neuroprotection. However, at present it is not possible to know whether the elevated expression of these receptors in the horse peripheral sensory pathways corresponds to effective functional activity.

Despite their apparent simplicity and the total lack of synaptic contacts, the DRG sensory neurons are the site of a certain degree of processing of sensitive information (Hanani 2012; Krames 2015). In fact, the perykaria of primary sensory neurons show specific receptors for several neurotransmitters and may release extracellular neurotransmitters, such as glutamate, adenosine triphosphate (ATP), Substance P (SP) and calcitonin gene-related peptide (CGRP), which can change the membrane potential of the neighboring sensory neurons and also activate the SGCs (Kung et al., 2013). Conversely, the SGCs can modulate the activation of the nociceptive neurons by means of the release of ATP and other neuromodulators, cytokines, chemokines, and proteases (Zhang et al., 2007; Ohara et al., 2009; Ji et al., 2013). Thus, the SGCs also play a pivotal role in neurotransmission and pain regulation, and their release of small molecules could contribute to the sensitisation of pain transmission nociceptors. The observation that several types of cannabinoid receptors are expressed by horse DRG neurons and SGCs indicated the relevant role of these receptors in the neuron-SGC synergy.

Cannabinoid CB1R is widely expressed throughout the nociceptive system and its activation by endogenous or exogenous cannabinoids modulates the neurotransmitter release. The expression of

CB1R-IR in the horse DRG is in line with data obtained in laboratory rodents and dogs (Hohmann and Herkenham 1999; Ross et al., 2001; Freundt-Revilla et al., 2017), which show that CB1R is mainly expressed in the myelinated fibres of the DRG neurons (Hohmann and Herkenham 1999; Ross et al., 2001; Bridges et al., 2003;Freundt-Revilla et al., 2017) and co-localise with CGRP and TRPV1, at least in rodents (Hohmann and Herkenham 1999; Ahluwalia et al., 2000; Bridges et al., 2003).
Cannabidiol acts as an "indirect" CB1R/CB2R agonist by inhibiting the enzymatic hydrolysis of the endogenous cannabinoid anandamide (AEA) (Bisogno et al., 2001) which is an endogenous agonist of TRPV1 (Muller et al., 2019). The data of the present study may also support some analgesic effects of natural and synthetic cannabinoids in the horse, mainly for the treatment of neuropathic pain (Pergolizzi et al., 2018).
The CB2R was initially presumed to be well represented in the immune system (Pacher et al., 2011) and was absent in the central nervous system (CNS), whereas recent literature points out its expression in the astrocytes, microglia and neurons of the CNS (Hsieh et al., 2011; Malfitano et al., 2014; Freundt-Revilla et al., 2018), and also in the nociceptive sensory neurons (Ross et al., 2001; Anand et al., 2008; Svízenská et al., 2013). Since the CB2R is upregulated in a variety of CNS neuroinflammatory diseases, characterised by microglia and/or astroglia activation, it might represent a promising pharmacological target. (Chen et al. 2012). It has been shown that, when nerve damage occurs, the CB2R is upregulated in the DRG and the superficial laminae of the dorsal horn of the spinal cord (Wotherspoon et al., 2005; Anand et al., 2008; Svízenská et al., 2013), and functional studies regarding sensory neurons have pointed to an antinociceptive role of the CB2R (Burston and Woodhams 2014).
In the present study, bright CB2R-IR was observed in the SGCs and a weaker signal in the sensory neurons. The presence of CB2R-IR in the DRG neurons suggested that the CB2R agonists could modulate pain transmission by means of glial and neuronal action. These findings appeare somewhat useful for the peripheral modulation and treatment of painful sensation in the horse. Cannabidiol increases the levels of endocannabinoid 2-arachidonoylglycerol (2-AG) which is an endogenous agonist of the CB1R and the primary endogenous ligand for the CB2R (Stella et al., 1997).
Concerning the TRP channels (a group of membrane proteins involved in the transduction of chemical and physical stimuli including pressure, temperature and pain) (Wu et al., 2010; Moran et al., 2011; Morales et al., 2017), the data obtained in the present study demonstrated that, in the horse DRG, TRPV1 was expressed by the sensory neurons, and TRPA1 by the sensory neurons and also SGCs, with bright immunolabelling in thin unmyelinated nerve fibres. This is, in part, in line with previous studies in rodents, showing that the DRG neurons co-expressing TRPV1, substance P, and CGRP also express TRPA1 (Story et al., 2003; Bautista et al., 2005; Kobayashi et al., 2005). The TRPV1 is

activated by a multitude of endogenous and exogenous chemical agents, such as capsaicin, an active ingredient contained in chili pepper (Caterina et al., 1997) and its analogs, and by different phytocannabinoids, such as CBD (Di Marzo and De Petrocellis 2010; Caterina 2014). Moreover, the TRPV1 is also activated by high temperatures (T> 42° C), due for example to local inflammation and osmotic changes, such as acid pH which also develops inflammation (Nagy et al., 2014). The TRPA1 is required for normal mechano- and chemosensory functions in specific subsets of vagal, splanchnic, and pelvic afferents (Brierley et al., 2009). The TRPA1 also mediates somatic and visceral pain in response to a stimulation of chemical, mechanical or thermal origin (McNamara et al., 2007; Wang et al., 2019), and can be desensitised by different mechanisms (Akopian et al., 2007).

The TRPA1 is closely associated with the TRPV1 and, together, they are pain and neurogenic inflammation players in terms of both expression and function (Anand et al., 2008; Huang et al., 2019). The endogenous presynaptic TRPA1 and TRPV1 activity at the spinal level contributes to increased nociceptive input from the primary sensory nerves to the dorsal horn neurons in inflammatory pain (Huang et al., 2019). The anti-inflammatory, anti-nociceptive and analgesic effects of CBD might be due, in part, to the capability of phytocannabinoids to activate and desensitise the TRPA1 (De Petrocellis et al., 2008) and the TRPV1 (Bisogno et al., 2001; Costa et al., 2007; Ligresti et al., 2016). All together, these evidences strongly supported the hypothesis that, also in the horse, the TRPV1 and the TRPA1 may exert a pivotal role in pain and neurogenic inflammation.

Peroxisomal proliferation receptors (PPARs) belong to the family of intranuclear receptors which act as transcription factors, modulating different physiological functions. Once activated by their ligand, PPARs induce the expression of hundreds of genes in each cell type (Issemann and Green 1990). However, their activation has also been shown to result in rapid cellular changes which do not require transcription, including reduction of inflammation (Lo Verme et al., 2005; O'Sullivan 2007). Recent studies have shown that cannabinoids activated PPARs (Burstein 2005; O'Sullivan 2007; Morales et al., 2017), and that this activation is associated with some of the pain-relieving, anti-inflammatory and neuroprotective properties of cannabinoids. In the present study, PPARα-IR was observed in the SGCs and PPARγ-IR in the sensory neurons and SGCs. Both these types of immunolabelling suggested that, also in the horse, phytocannabinoids may offer prospects for the treatment of painful somatic and visceral diseases by acting on these receptors.

Another finding of this study was the expression of 5-HT1aR in the cytoplasm of the sensory neurons, SGCs and Schwann cells. It is well established that serotonin (5-HT) exerts a pivotal role in sensory information processing (Richardson 1990). At the level of the spinal cord, 5-HT is primarily released from the descending bulbospinal serotonergic neurons and causes analgesia by inhibiting dorsal horn neuronal responses to noxious stimuli by means of the activation of the 5-HT1aR (Liu et al., 2002).

In addition, the activation of the 5-HT1aR inhibits glutamate release from the sensory neurons, reducing pain transmission (Haleem et al., 2018). Functional studies have suggested the presence of the 5-HT1aR in the DRG and its role in nociception (Todorovic and Anderson 1992). The 5-HT1aR-IR was observed in both the neurons and the SGCs of the horse DRG, in line with the presence of this receptor on glial cells (Miyazaki and Asanuma 2016). Thus, it is plausible to consider that, in the horse, the 5-HT1aR might play a role in pain perception/modulation. Clinical studies have indicated that CBD also interacts with the serotonin 5-HT1aR and exerts analgesic and anxiolytic effects (De Gregorio et al., 2019). It has been shown that, under certain conditions of experimentally induced nociception, different receptors (5-HT1a, 5-HT3, TRPA1) are simultaneously activated (Krimon et al., 2013; Fischer et al., 2017); thus, CBD may reduce nociception by simultaneously desensitising these receptors.

The orphan receptor GPR3 is phylogenetically related to the cannabinoid receptors and is considered to be a novel molecular target for CBD (Laun et al., 2019). The GPR3 is expressed in the CNS and has been implicated in the health and disease states of the brain. The GPR3 alters emotional behavior, is involved in the development of neuropathic pain and regulates morphine-induced antinociception (Ruiz-Medina et al., 2011). The GPR3 expression also seems to be involved in neurite outgrowth (Tanaka et al., 2007). A subset of neuronal cell bodies of the horse DRG showed GPR3-IR.

The GPR55, considered the third cannabinoid receptor (Moriconi et al., 2010), in the present study was expressed by a DRG neurons and glial cells; its presence in both SGCs and neurons likely indicates a relevant role of this receptor in neuron-SGCs crosstalk. Studies in mice indicate a pronocioceptive role of GRP55 in DRG neurons (Lauckner et al., 2008). It is worth noting that CBD acts as a GPR55 antagonist.

The study of the phenotype (neurochemical code) of the sensory neurons expressing cannabinoid receptors was not the aim of the present study; thus, additional studies are needed to better characterise these receptors and their potential therapeutic effects in the horse.

Conclusions and relevance

Canonical and putative cannabinoid receptors had a wide distribution in the sensory neurons and SGCs of the horse DRG, with a close functional relationship between the sensory neurons and the SGCs in the peripheral processing of nociceptive imputs. These findings represented an important anatomical basis upon which it would be possible to continue with other preclinical and clinical studies aimed at investigating and possibly supporting the specific therapeutic uses of non-psychotropic cannabinoid agonists against noxius stimulation in horses.

Primary antibody	**Host**	**Code**	**Dilution**	**Source**
CB1R	Rabbit	ab23703	1:100	abcam
CB2R	Rabbit	AB45942	1:200	abcam
GPR3	Rabbit	ab106589	1:300	abcam
GPR55	Rabbit	NB110-55498	1:200	Novus Biol.
PPARα	Rabbit	NB600-636	1:200	Novus Biol.
PPARγ	Rabbit	ab45036	1:300	abcam
5-HT1aR	Rabbit	ab85615	1:100	abcam
TRPA1	Rabbit	ab58844	1:100	abcam
TRPV1	Rabbit	ACC-030	1:200	Alomone

Table 1 - Primary antibodies used in the study. Primary antibodies Suppliers: abcam, Cambridge, UK; Alomone, Jerusalem, Israel; Novus Biologicals, Littleton, CO, USA.

Antibody(host)	**Homology between the amino acidic sequences (immunogen and horse)**	**Homology with the immunogen sequence**
Rabbit anti CB1 (Ab23703)	97.88% Human	100%
Rabbit anti CB2 (Ab45942)	80.9% Rat	83.33%
Rabbit anti GPR3 (Ab106589)	94.24 % Human	84.62%
Rabbit anti GPR55 (NB110-55498)	80% Human	78%
Rabbit anti PPARα (NB600-636)	90.81% Mouse	100%
Rabbit anti PPARγ (Ab45036)	92% Human	87.5%
Rabbit anti 5-HT1a (ab85615)	89.3% Rat	99%
Rabbit anti TRPA1 (Ab58844)	82% Rat	100%
Rabbit anti TRPV1 (ACC-030)	85% Rat	87.5%

Table 2: Homology between the AA sequences (betweenthe host and horse) and with the specific sequence of the immunogen of the CBR antibodies used in the study

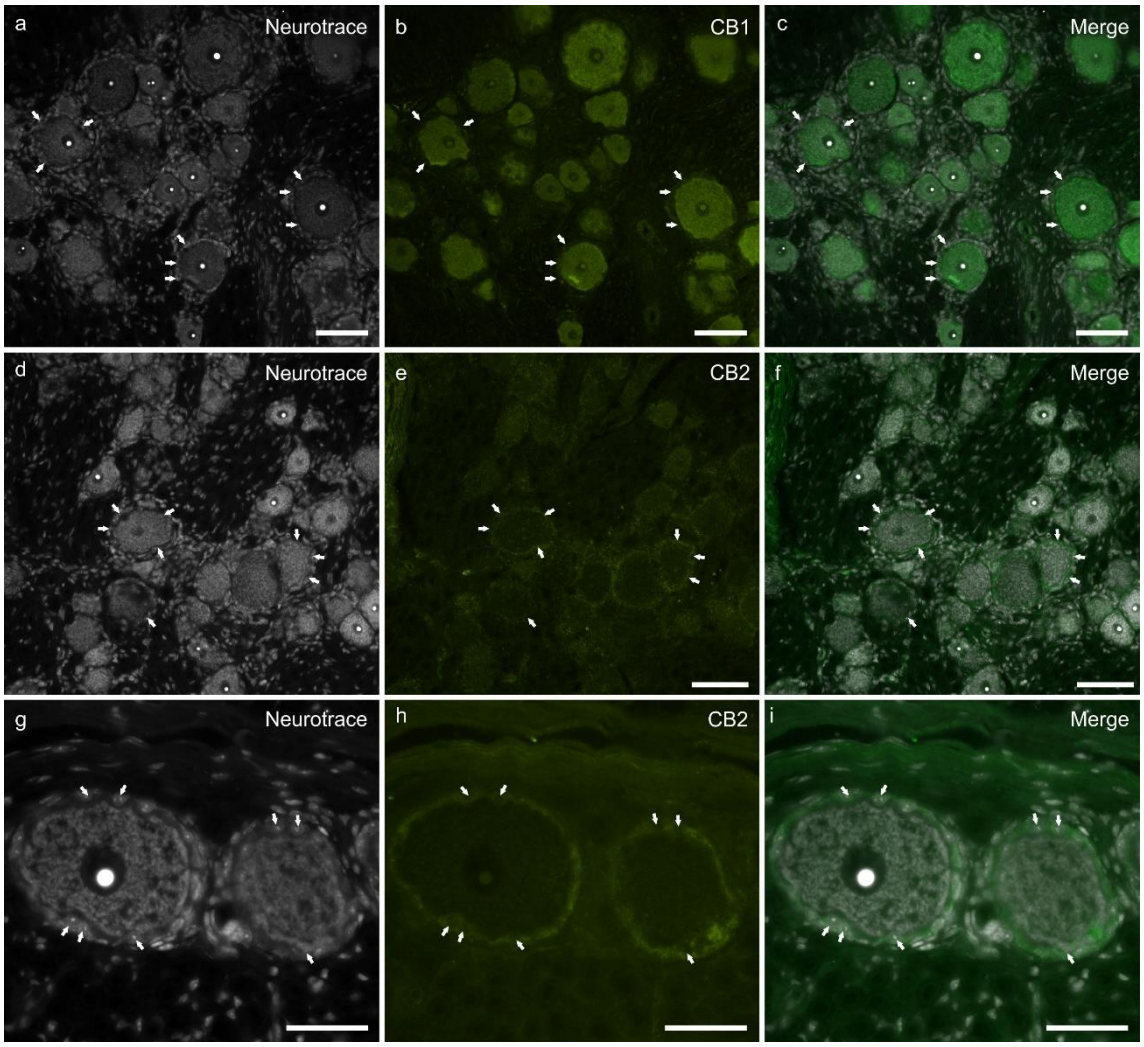

Figure 1 (a-i): Photomicrographs of cryosections of a horse cervical (C8) dorsal root ganglion showing cannabinoid receptor 1 (CB1R) (a-c) and cannabinoid receptor 2 (CB2R) (d-i) immunoreactivity (IR). a-c) CB1R-IR was expressed by the sensory neurons whereas the satellite glial cells, whose nuclei are indicated by arrows, were CB1R-negative. d-i) Arrows indicate satellite glial cells which were CB2R immunoreactive. Sensory neurons, in particular the smallest ones, showed very faint granular CB2R immunolabelling. Scale bar = 50µm

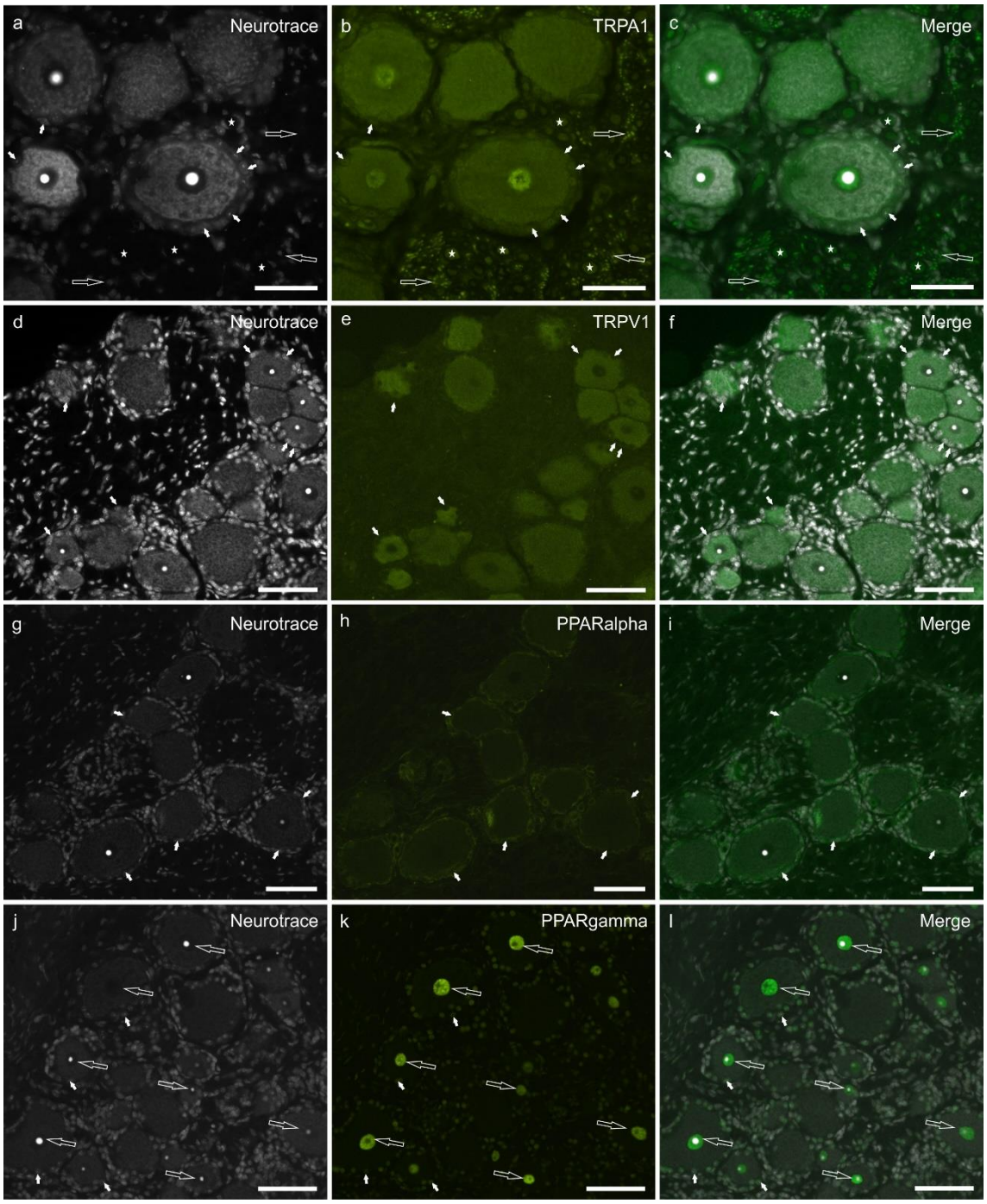

Fig. 2 (a-l). Photomicrographs of cryosections of a horse cervical (C8) dorsal root ganglion showing TRPA1-(a-c), TRPV1- (d-f), PPARα- (g-i), and PPARγ- (j-l) immunoreactivity (IR). a-c) Sensory neurons and satellite glial cells (small arrows) expressed TRPA1-IR. Large arrows indicate groups of amyelinic sensory fibres which showed very bright TRPA1-IR, whereas the nerve fibres with a larger diameter (stars) showed a weaker immunostaining. d-e) Only the sensory neurons were TRPV1 immunoreractive whereas satellite glial cells (arrows) were TRPV1-negative. g-i) Only the satellite glial cells (arrows) showed PPARα-IR. j-l) Bright PPARγ-IR was expressed by neuronal nuclei (large arrows) whereas the nuclei of the glial cells (small arrows) showed fainter immunolabelling. Scale bar = 50µm

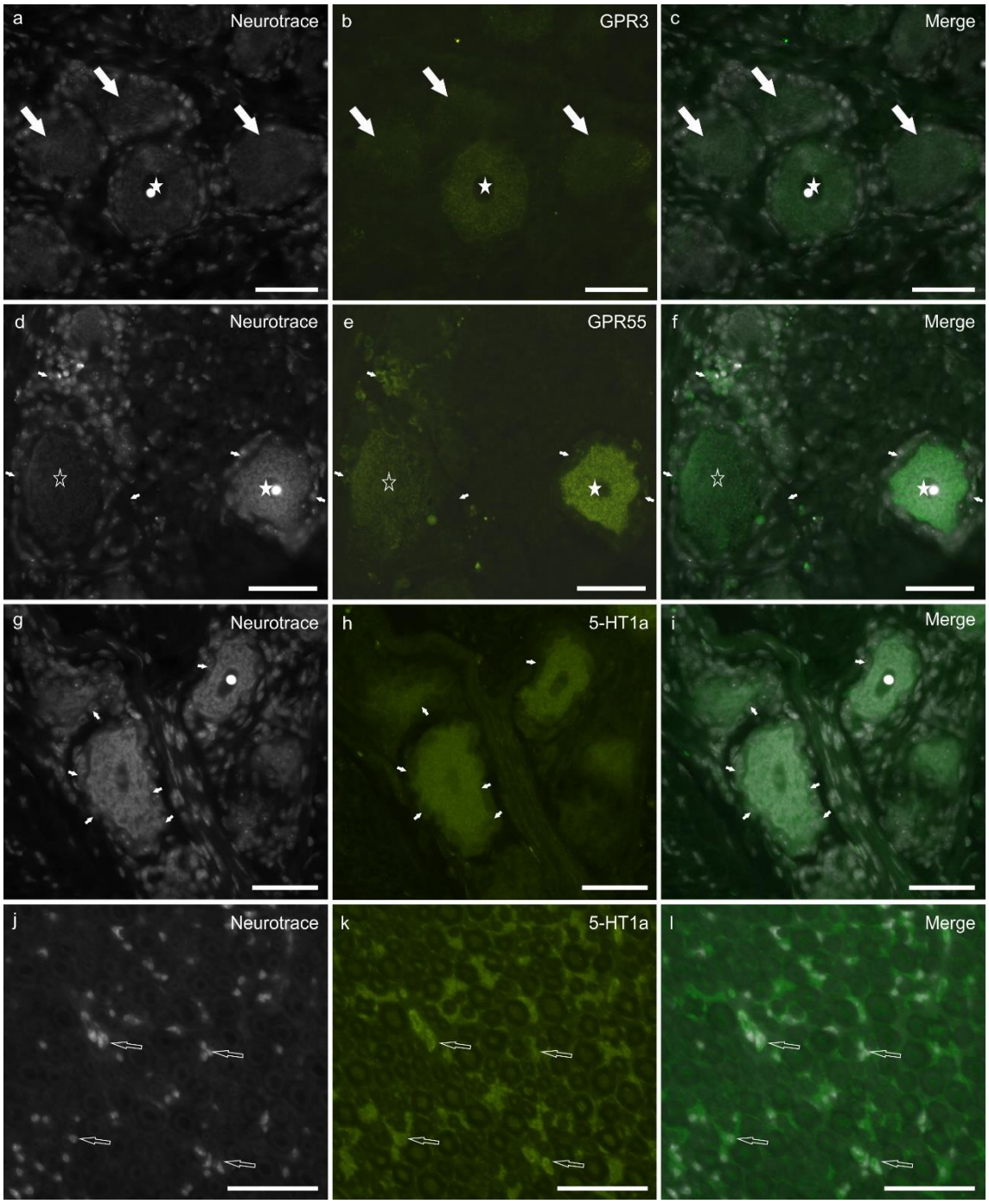

Fig. 3 (a-l) Photomicrographs of cryosections of a horse cervical (C8) dorsal root ganglion showing GPR3-(a-c), GPR55 (d-f) and 5-HT1a receptor- (g-i) immunoreactivity (IR). a-c) Three arrows indicate the sensory neurons expressing weak GPR3-IR. The star indicates the nucleus of one large sensory neuron expressing moderate GPR3-IR. d-f) White star indicate a sensory neurons expressing bright GPR55-IR, whereas open star indicate a neurons with weaker immunolabeling. Arrows indicate some SGCs showing GPR55-IR. g-i) Sensory neurons expressed bright 5-HT1a receptor-IR; satellite glial cells (arrows) were also moderately 5-HT1a receptor-IR. j-l) The Schwann cells (arrows) showed 5-HT1a receptor-IR. Scale bar = 50 µm.

Other experimental studies

Endoscopy

Paper published on Veterinarni medicine (2017), 62(11), 614-619. Modified from:

A rare case of nasal osteoma in a dog: a case report

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Abstract

A 35-month-old female German shepherd weighing 33.2 kg was referred to our department with a 10-month history of sneezing and left nasal swelling. On clinical examination, the dog showed deformity of the left nasal plane in the absence of any cutaneous lesions or nasal discharge, and presented with nasal snoring noises during both the inspiratory and expiratory phases. The patency of nasal cavities was evaluated using the cotton swab test, and was found to be preserved only on the right side. Endoscopic, radiographic and computed tomographic examination revealed an osteoproductive lesion that distorted the nasal, maxillar and frontal bones, completely occupying the left nasal cavity and frontal sinuses, resulting in destruction of the nasal septum and invasion of the contralateral nasal cavity. Five bioptic samples of the mass were collected from the cutaneous surface using a 9G Jamshidi bone marrow bioptic instrument. Histologically, the lesion consisted of a non-encapsulated, multilobulated mass composed of dense coalescing trabeculae of well-differentiated bone, which was lined by osteogenic cells. The morphology was suggestive of nasal osteoma. Due to the large size of the mass, evidenced by computed tomography, chronic systemic nonsteroidal anti-inflammatory treatment with carprofen was proposed instead of surgery. The description of this case is useful for veterinarians, who should consider osteoma as a possible differential diagnosis for nasal tumours.

In collaboration with the Gastrointestinal Laboratory, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University (Texas). Abstract presented to the ACVIM Forum (2017).

Ileal and colonic mucosal microbiota in dogs with steroid responsive chronic enteropathies

Francesca Bresciani[1], Yasushi Minamoto[2], Jan S. Suchodolski[2], Giorgia Galiazzo[1], Carla Giuditta Vecchiato[1], Carlo Pinna[1], Giacomo Biagi[1], Marco Pietra[1]

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Abstract

Introduction - Exact etiology for inflammatory chronic enteropathies in dogs remains unknown. Accumulating evidence suggests a pivotal role for intestinal dysbiosis in disease pathogenesis. Many studies have evaluated the alteration of fecal microbiota in canine chronic gastrointestinal (GI) disease, and less research is focused on mucosal microbiota, especially in the ileum and colon.

Objective - The objectives of the current study were to evaluate ileal and colonic mucosal microbiota in dogs with steroid responsive enteropathy (SRE) before and after 4 months of treatment, and to compare them to control dogs (CD). A total of 10 dogs diagnosed with SRE were enrolled. Complete GI endoscopy was performed and samples were collected by a cytology brush at diagnosis (SRE-Baseline, n=10) and after 4 month of treatment (SRE-After, n=8). Oral laxative and 2-4 water enemas were performed before endoscopy. A total of 6 CD that were euthanized for reasons unrelated to this study, with no GI disease, were included. Samples from CD were obtained during necropsy within 3 hours of death. Mucosal genomic DNA was extracted and used for Illumina sequencing of 16S rRNA genes. Sequence data were analyzed using the QIIME pipeline. Statistical significance was set at $p < 0.05$.

Results - Clinical signs improved significantly after 4 month of treatment in SRE, but no improvement was seen on endoscopic or histological evaluation. Significant differences in microbial communities between SRE baseline and CD were observed in the colon (ANOSIM p=0.002), but not in the ileum (ANOSIM p=0.180). In dogs with SRE, both ileal and colonic microbial communities remained similar after 4 month of treatment (ANOSIM p=0.189 and p=0.637, respectively), and were different from CD (ANOSIM p=0.001 and p=0.004, respectively).

Conclusion and relevance - Results of this study suggest that the mucosal microbiota in the colon of dogs with SRE is different from that of CD. Although clinical signs improved, colonic mucosal dysbiosis was still present after 4 months of treatment.

This project was presented as poster at the 28th ECVIM-CA Congress, Rotterdam 2018

Endoscopic bronchial anatomy in the dog

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Abstract

Introduction - Bronchoscopy is an important diagnostic procedure for the evaluation of many respiratory diseases and the removal of foreign bodies. During bronchoscopy, it is essential to know precisely the bronchial topography, in order to recognize abnormal anatomy or pathological changes. Few authors treat bronchial anatomy: currently, the endoscopic anatomy is based on a paper of 1986. However, the appearance of bronchial division during a canine endoscopy seems to differ from the proposed map. Moreover, it seems that some subjects present individual differences from the standard topography.

Objective - The aim of this study was to obtain a description of topographic anatomy and morphometric value of the canine bronchial tree, to introduce a new standardised nomenclature and to draw a correct bronchial map. Twelve dogs, different in age, sex and breed, which died spontaneously for reasons other than pulmonary diseases, were included in the study. They were distinguished by weight in three groups: small (<10 kg), medium (10-25 kg), and large (>25 kg) size. All the subjects were examined endoscopically in a systematic manner with a flexible endoscope (Ø 6mm). After that, on the same lungs, casts of polyurethane foam2 were made and diameter and length of the bronchial branches were measured. Furthermore, to name the structures and to draw the bronchial map, we defined them by looking at their direction and position.

Results - The casts confirmed the orientation, the branching pattern and the topographic relationship of the bronchial system seen during bronchoscopy (Figure 1). However, even with reduced case studies, individual variables are present. The morphometric examination allowed us to obtain a mean value of diameter (Table 1) and length (Table 2) of bronchi for each group of weight and to confirm the monopodial branching system. Due to their diameter, principal bronchi were accessible in all groups, while lobar bronchi were accessible in medium and large size dogs, not always in small size group. Segmental bronchi were always accessible only in large size dogs.

Conclusion and relevance - In conclusion, this study allowed the identification of bronchial architecture with a new map and the definition of a nomenclature for the first three series of bronchial division. The morphometric examination provided accurate references useful in diagnostic imaging, especially during bronchoscopy.

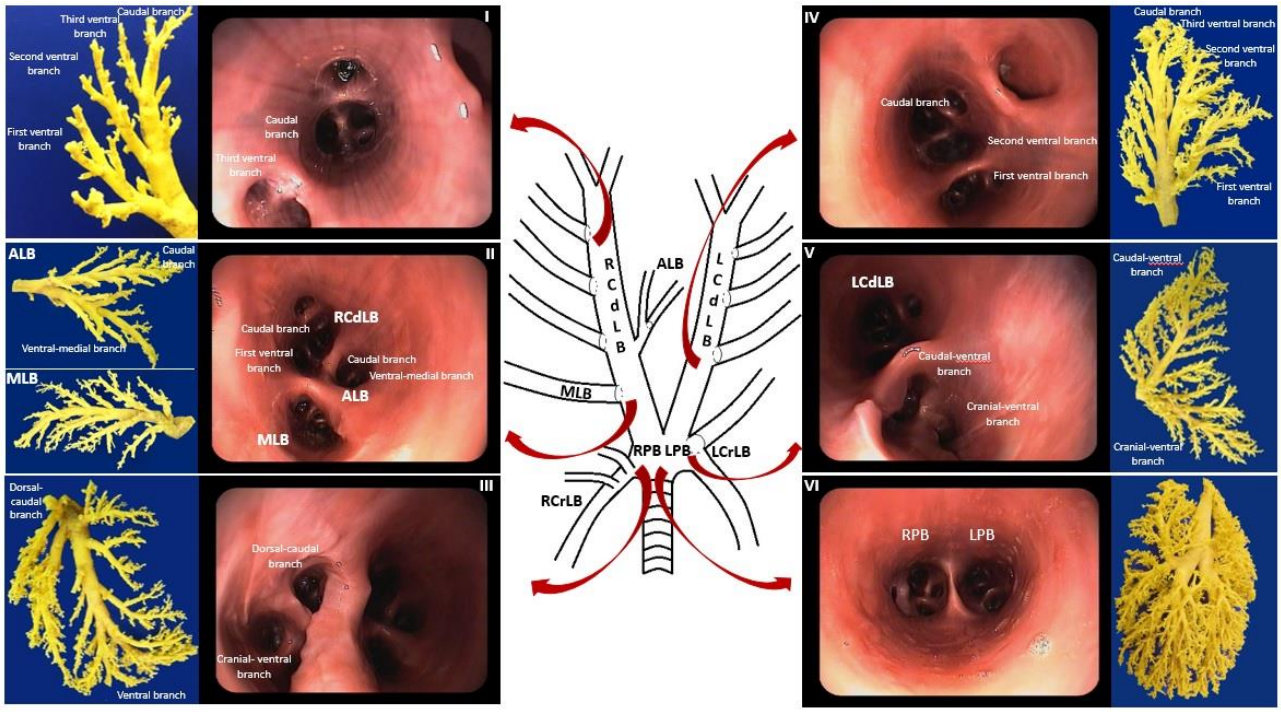
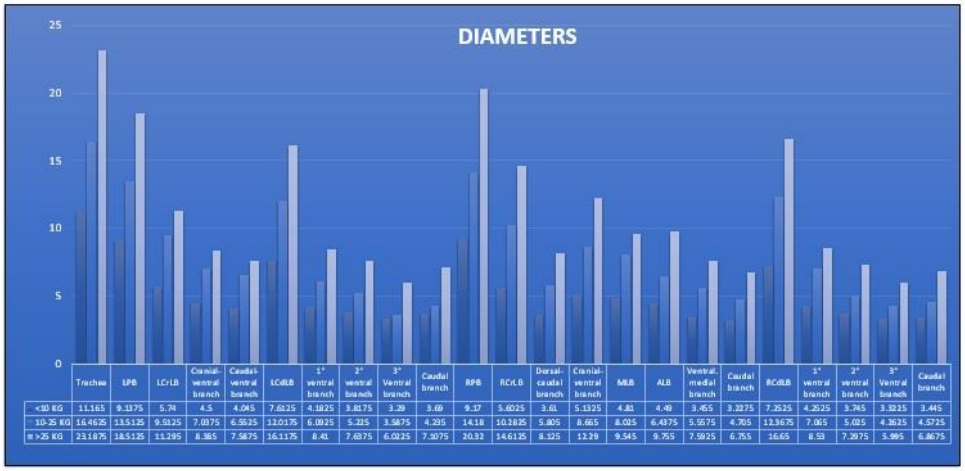
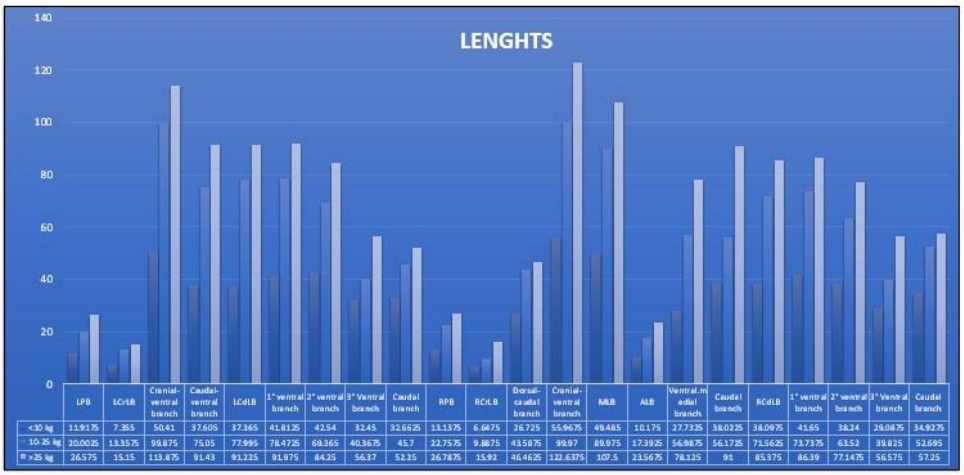


Figure 1 (I-VI). Endoscopic images and corresponding cast portions. The trachea ends at the tracheal bifurcation with a right principal bronchus (RPB) and a left principal bronchus (LPB)VI, which diverges laterally more than the RPB, confirming the monopodial branching system. The RPB gives off a right cranial lobar bronchus (RCrLB), a middle lobar bronchus (MLB), an accessory lobar bronchus (ALB) and finally a right caudal lobar bronchus (RCdLB), to the corresponding right lung lobes. The RCrLB gives off a dorsal-caudal branch, and then continues with a cranial- ventral branchIII. The MLB originates from the ventral floor of the RPB and releases a series of segmental bronchi, while the ALB divides in two main branches: a ventral-medial and a caudal branchII. The RCdLB gives off three ventral branches and then continues as caudal branchI. The LPB gives off the left cranial lobar bronchus (LCrLB), divided in a cranial-ventral and caudal-ventral branchesV, while the left caudal lobar bronchus (LCdLB) behaves as the RCdLBIV.



	Trachea	LPB	LCrLB	Cranial-ventral branch	Caudal-ventral branch	LCdLB	1° ventral branch	2° ventral branch	3° Ventral branch	Caudal branch	RPB	RCrLB	Dorsal-caudal branch	Cranial-ventral branch	MLB	ALB	Ventral.medial branch	Caudal branch	RCdLB	1° ventral branch	2° ventral branch	3° Ventral branch	Caudal branch
<10 KG	11.165	9.1375	5.74	4.5	4.045	7.6125	4.1825	3.8175	3.29	3.69	9.17	5.6025	3.61	5.1325	4.81	4.49	3.455	3.3275	7.2525	4.2525	3.745	3.3225	3.445
10-25 KG	16.4625	13.5125	9.5125	7.0375	6.5525	12.0175	6.0925	5.225	3.5875	4.225	14.18	10.2825	5.805	8.665	8.025	6.4375	5.5575	4.705	12.3675	7.065	5.025	4.2625	4.5725
>25 KG	23.1875	18.5125	11.295	8.385	7.5875	16.1175	8.41	7.6375	6.0225	7.3075	20.32	14.6125	8.125	12.29	9.545	9.755	7.5925	6.755	16.65	8.53	7.2975	5.995	6.8675

Table 1. Mean value of bronchi diameter (mm) of the three groups of dogs.



	LPB	LCrLB	Cranial-ventral branch	Caudal-ventral branch	LCdLB	1° ventral branch	2° ventral branch	3° Ventral branch	Caudal branch	RPB	RCrLB	Dorsal-caudal branch	Cranial-ventral branch	MLB	ALB	Ventral.medial branch	Caudal branch	RCdLB	1° ventral branch	2° ventral branch	3° Ventral branch	Caudal branch
<10 kg	11.9175	7.355	50.41	37.605	37.365	41.8125	42.54	32.45	32.6625	13.1375	6.6475	26.725	55.9675	49.485	10.175	27.7325	38.0225	38.0975	45.65	38.24	29.0875	34.9275
10-25 kg	20.0025	13.3575	98.875	75.05	77.995	78.4725	69.365	40.3675	45.7	22.7575	9.8875	43.5875	99.97	86.975	17.3925	56.9875	56.1725	71.5625	73.7375	63.52	39.825	52.695
>25 kg	26.575	15.15	113.875	91.43	91.225	91.975	84.25	56.37	52.25	26.7875	15.92	46.4625	122.6375	107.5	23.5675	78.125	91	85.375	86.39	77.1475	56.575	57.25

Table 2. Mean value of bronchi lenght (mm) of the three groups of dogs.

This project was presented as poster at the 29th ECVIM-CA Congress, Milano 19th–21th September 2019. Paper ready for submission. Modified from:

Water immersion vs air insufflation in canine duodenal endoscopy: is the future underwater?

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Abstract

Introduction - Endoscopy is a routinary approach for canine enteropathy. Randomized controlled trials in human colonscopy suggest that the introduction of warm-to touch water to distend the intestinal lumen, instead of air, decreases pain and increases the visualization of mucosal texture.

Objective - To compare air insufflation (AI) and water immersion (WI) during duodenoscopy in anesthetized dogs (n=25), in order to evaluate differences in nociception and in the quality of mucosal visualization. To evaluate cardiocirculatory differences, heart rate and arterial blood pressure were measured. A random sequence of AI or WI was applied and the same image of the descending duodenum was recorded with AI and WI (Figure 1). Every image was subjected to a texture analysis and to a subjective blind evaluation by three expert endoscopists. The distribution of the data was evaluated with a D'Agostino and Pearson omnibus normality test. A T test for paired data was applied for the image analysis, while a Fleiss' Kappa test was applied for the subjective evaluation. Anesthesiological paramethers were compared with a Friedman test.

Results - The subjective evaluation identified the WI images as qualitatively better (fixed-marginal K =0.74; range 0.61 – 0.80), otherwise no significant differences were evidenced by applying texture analysis. Anesthesiological parameters between AI and WI did not show any significant difference.

Conclusions and relevance - The results of the study highlight how the painful answer does not change between AI and WI (Figure 2 a-d), maybe influenced by the good control of nociception given by the anesthesia. The insufflation of water instead of air, during duodenoscopy in dogs, can provide an increase in the quality of the endoscopic images, confirmed by a subjective analysis but not by texture analysis, without side effects.

a b

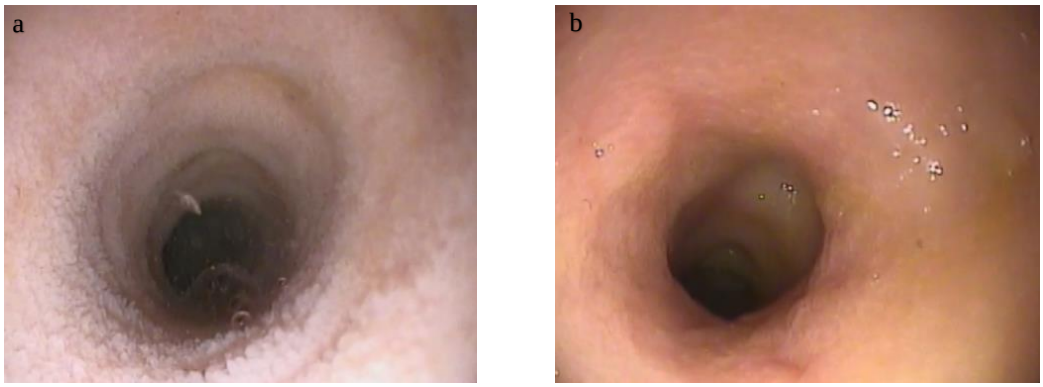

Figure 1: Images of the descending duodenum with the two methods: water immersion (a) and air insufflation (b).

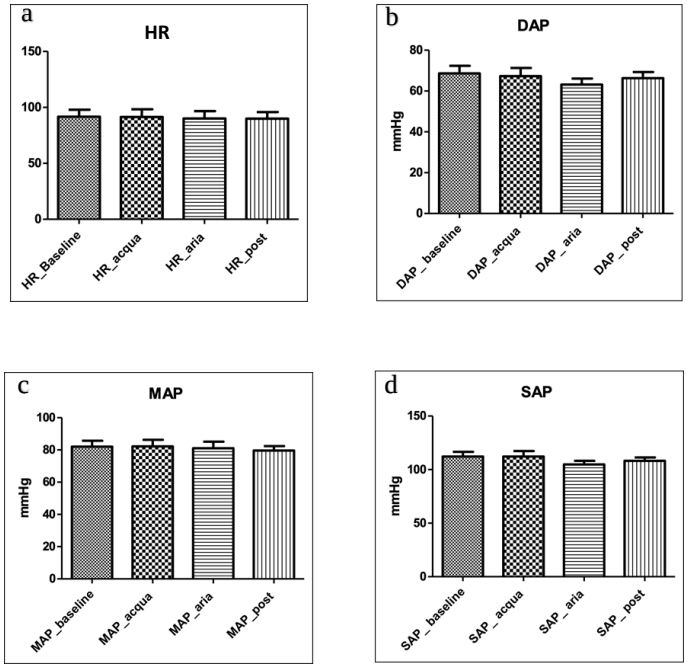

Figure 2 (a-d): Results of the evaluation of the cardiovascular parameters (a: HR: heart rate; b: DAP: diastolic arterial pressure; c: MAP: mean arterial pressure; d: SAP: systolic arterial pressure). The parameters were analyzed, every 5 minutes, for 30 minutes from time of intubation, and divided in 4 steps. On the X-axis are represented the four steps to compare: baseline (from time of intubation to application of the first method), water (WI method), air (AI method) and post (from the end of the application of the second method to the end of the 30 minutes). On the Y axis is represented for HR the mean value of the frequence in beats per minute (BPM) for each step, and for pressure the mean value in mmHg for each step. The significance was set at P value < 0,05. There was not any significant result for cardiovascular parameters.

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