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EC GASTROENTEROLOGY AND DIGESTIVE SYSTEM

Research Article

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

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Received: March 23, 2018; **Published:** May 14, 2018

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) and with spontaneous chronic enteritis (inflamed dogs, INF). In addition, the percentage of nNOS immunoreactive neurons co-expressing VIP immunoreactivity (and *vice versa*) was evaluated.

Methods and Material: Animal tissues were collected from the ileum of six control (CTRL) dogs (none had evident gastrointestinal disorders) and ten INF-dogs with chronic enteritis of the ileum. All the enteric neurons, VIPergic and nitrergic neurons were immunohistochemically identified with the anti-HuC/HuD, anti-VIP, and anti-nNOS antibodies, respectively. VIP- and nNOS-immunoreactive neurons were immunohistochemically quantified as a relative percentages, in consideration of the total number of HuC/HuD neurons. 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: 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.

Keywords: *Enteric Nervous System; Ileum; Immunohistochemistry; Neuronal Nitric Oxide Synthase; Vasoactive Intestinal Peptide*

Abbreviations

CML: Circular Muscular Layer; ENS: Enteric Nervous System; IBD: Inflammatory Bowel Disease; GIT: Gastrointestinal Tract; LML: Longitudinal Muscle Layer; *mm: Muscularis mucosae*; MP: Myenteric Plexus; nNOS: Neuronal Nitric Oxide Synthase; SMP: Submucosal Plexus; SP: Substance P; VIP: Vasoactive Intestinal Peptide; WB: Western Blot

Introduction

The regulation of the gastrointestinal tract (GIT) is based on a complex interaction between the parasympathetic, the sympathetic and the enteric nervous system (ENS). Nevertheless, the intestinal motility and secretion are controlled primarily by the ENS and secondarily

by the extrinsic nervous system [1]. The ENS, which consists of millions of neurons harbored in the wall of the digestive system from the esophagus to the inner anal sphincter, consists of neurons which have been classified by their morphology, functions, connections, phenotype, etc [1]. The enteric neurons form two main ganglionated plexuses: the myenteric plexus (MP), mainly regulating muscle activity, and submucosal plexus (SMP), mainly regulating mucosal functions. In MP and SMP ganglia, sensory neurons, smooth muscle motor neurons, interneurons, vasodilator, and secretomotor neurons are organized into functional reflex circuits. The gastrointestinal peristalsis and secretion are triggered by intramural sensory neurons responsive to mechanical and/or chemical stimuli. Once exited, the ENS sensory neurons activate muscle motor neurons (excitatory and inhibitory) and secretomotor/vasodilator neurons [1]. The excitatory neurons mainly release acetylcholine and substance P (SP), whereas the inhibitory neurons mainly release nitric oxide and vasoactive intestinal peptide (VIP). Submucosal secretomotor neurons utilize the vasoactive intestinal polypeptide (VIP) as main neurotransmitter. The different classes of neurons act in synergy, as musicians of a large orchestra, in order to perform the physiological gastrointestinal functions such as peristalsis and mucosal activities. If some musicians of an orchestra are stoning, they may alter the performance of the whole orchestra; in the same way, if one (or more) subclass of neurons is somehow "sabotaged" or hyperstimulated, dysfunction of the physiology of the digestive system can occur.

During chronic enteritis, the ENS may be a target of inflammation, which may induce neuronal alteration (degeneration or hyperplasia), alterations of neurotransmitters content (synthesis and release), and which may contribute to impair the secretory and motor gastrointestinal functions [1,2]. In turn, neuronal damage and alterations in neural circuitry can influence the intestinal inflammation through secretion of neuropeptides [3-6], which interact with the immune system, playing an important role in the chronic enteritis [7,8].

In this regard, the purpose of the present study was to provide quantitative information on canine MP and SMP neurons of the ileum immunoreactive for the vasoactive intestinal polypeptide (VIP) and neuronal nitric oxide synthase (nNOS) in control dogs and in dogs with chronic enteropathies. These two categories of neurons represent two subclasses of a certain numerical relief in both the plexuses and any variation of their proportions may be correlated with disorders of the gastrointestinal motility and mucosal secretion. Furthermore, we quantified the percentage of nitrergic neurons co-expressing VIPergic phenotype (and *vice versa*).

Materials and Methods

Animals tissues were collected from six control dogs (CTRL-dogs) (none had evident gastrointestinal disorders) (Table 1) and ten dogs with spontaneous enteritis (ileitis) (INF-dogs) (Table 2). The enteritis was diagnosed through a documented clinical history or necroscopy, and confirmed by pathologist refertations (Table 2).

Controls	Breed	Gender	Age	Cause of death
CTRL 1	German Sheperd	M[a]	10 yr	Euthanasia due to progressive physical deterioration
CTRL 2	German Sheperd	M	10 yr	Heart haemangiosarcoma
CTRL 3	Boxer	M[a]	8 yr	Cardiovascular disease
CTRL 4	German Sheperd	M	10 yr	Cardiovascular disease
CTRL 5	Chihuahua	F	8 mo	Head trauma
CTRL 6	Half-breed	M[a]	11yr	Euthanasia due to pulmonary metastasis (haemangiosarcoma)

Table 1: *Clinico-pathological data of the control dogs included in the present research.*

Abbreviations: *M: Male; F: Female; M[a]: Male Neutered*

All animals died spontaneously or were euthanized, then their tissues were collected following owner permissions. According to 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.

Tissue collection

The entire small and large intestine was removed within 2h after each animal's death and longitudinally cut open along the mesenteric border. The intestine of CTRL dogs did not present apparent mucosal hyperemia or inflammatory lesions, against the hyperemia of the ileum of INF-dogs or mild peritonitis. The ileum were treated to obtain tangential and longitudinal cryosections, as described elsewhere [9,10]. Specimens from all the subjects were processed for histology and immunohistochemistry.

Histology

Longitudinal cryosections from ileum of CTRL- and INF-dogs were stained using hematoxylin and eosin (H&E) for histological examination. Ileal sections of the control dogs showed a normal histology (absence of inflammatory infiltrate). The histopathological features related to the INF-dogs tissues are listed in table 2.

Inflamed	Breed	Gender	Age	Cause of death	Histopathology
INF 1	Great Dane	F	2 yrs	Peritonitis due to gastric dilatation volvulus	severe chronic lymphoplasmacytic enteritis
INF 2	Hovawart	M	11 yrs	Degenerative myelopathy	Moderate chronic lymphoplasmacytic enteritis
INF 3	Belgian Sheperd	M	9 yrs	Euthanasia due to paraplegia	Moderate chronic eosinophilic enteritis
INF 4	Golden Retriever	M	8 yrs	Olfactory bulb neoplasia	Moderate chronic lymphoplasmacytic enteritis
INF 5	Half-breed	F	11 yrs	Cushing, mast cells tumor	Moderate chronic lymphoplasmacytic and eosinophilic enteritis
INF 6	Half-breed	F	8 yrs	Oedema lungs, protein losing enteropathy	Severe chronic lymphoplasmacytic enteritis/intestinal mucosal lymphoma
INF 7	Labrador	F	11 yrs	AKI/CKD and sepsis	Moderate/severe acute lymphoplasmacytic enteritis
INF 8	Pinscher	M	3yrs	AKI/CKD	Moderate/severe acute eosinophilic enteritis
INF 9	Hovawart	M	12 yrs	Euthanasia due to paraplegia	Moderate chronic lymphoplasmacytic enteritis
INF 10	Cane Corso	M	8 yrs	Euthanasia due to metastasis	Moderate acute catarrhal enteritis

Table 2: *Clinico-pathological data of dogs with gastrointestinal inflammation included in the present research and histopathological report.*

Abbreviations: *M: Male; F: Female*

Immunohistochemistry

Double labelling studies were carried out by an indirect immunofluorescence method. Tissue specimens were incubated in 20% normal serum (obtained from the same species used to produce the secondary antibodies) in PBS containing 0.5% Triton X-100 for 1h at room temperature (RT), in order to reduce the background and permeabilize the tissues to the antisera. Tissues were then incubated at 4°C in a humid chamber overnight in a mixture of two primary antibodies (Table 3a) diluted in a suitable medium (1.8% NaCl in 0.01 M phosphate buffer containing 0.1% Na-azide). After washing in PBS (3 x 10 minutes), the tissues were incubated for 1h at RT in a humid chamber in a mixture of two secondary Antibodies (Table 3b) diluted in PBS. Tissues were then washed in PBS (3 x 10 minutes) and mounted in buffered glycerol pH 8.6.

a) Primary antibody	Host	Code	Dilution	Source
HuC/HuD	Mouse	A21271	1:200	Life Technologies[a]
nNOS nNOS	Mouse Rabbit	SC5302 AB5380	1:800 1:500	Santa Cruz Merk Milipore
VIP VIP	Rabbit Mouse	7913 55	1:2500 1:2000	Sternini UCLA Sternini UCLA
b) Secondary antibody	**Host**	**Code**	**Dilution**	**Source**
Anti-mouse IgG 594	Goat	A11005	1:200	Molecular probes
Anti-mouse IgG 488	Donkey	20010	1:100	Biotium
Anti-rabbit IgG FITC	Goat	401314	1:200	Calbiochem-Novabiochem
Anti-rabbit IgG 594	Goat	AB150132	1:200	Molecular Probes

Table 3: *Primary and secondary antibodies used in the study.*

Suppliers of primary antibodies: Life Technologies, California, USA; Merck Millipore, Merck KGaA, Germany, Europe; Santa Cruz Biotechnology,California, USA; Sigma Aldrich, Italy, Europe.

Abbreviations: *HuC/HuD: Human Neuronal Protein; nNOS: Neuronal Nitric Oxide Synthase; VIP: Vasoactive Intestinal Peptide.*

The antibody anti-human neuronal protein (HuC/HuD) was utilized as a pan-neuronal marker to identify all the enteric neurons. VIPergic and nitrergic immunoreactive neurons were immunohistochemically identified by the use of anti-VIP and anti-nNOS antibodies, respectively.

Specificity of the primary antibodies

The primary antibodies mouse anti-HuC/HuD, mouse anti-nNOS, and rabbit anti-nNOS utilized in the present research, have been previously tested for their specificity on canine tissues by Western blot (WB) analysis [9]. The specificity of the antibody rabbit anti-VIP has been already tested on dog tissues [11]. In addition, the rabbit anti-VIP antibody was tested in double immunolabeling with another anti-VIP antibody raised in mouse [12]; both the antisera labelled the same neuronal cell bodies and fibers (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 neurons nor nerve fibers could be detected after omitting the primary antibodies. In double-immunostaining protocols, control experiments were also carried out to check for nonspecific 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 142 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).

Quantitative analysis

The proportions of neurons that were immunoreactive for a particular chemical marker and that were also positive for other neurochemicals were determined by examining fluorescently labelled, double-stained preparations. Neurons were first located by the presence of a fluorophore that labelled one antigen and then the filter was switched to determine whether or not the neuron was labelled for a second antigen, located with a fluorophore of a different color. In this way, proportions of neurons labelled for pairs of antigens were determined. The percentage of neurons that were immunoreactive for a particular marker and that were also immunoreactive for a second neurochemical marker was calculated and expressed as mean ± SD, with n being the number of animals considered.

For counting the percentages of VIP immunoreactive neurons on total MP and SMP neuronal population, 200 HuC/HuD-IR ileal neurons were counted at least from each animal (n = number of dogs considered). For counting the percentages of VIPergic neurons co-expressing immunoreactivity for the enzyme neuronal nitric oxide synthase (and *vice versa*) in both the enteric plexuses, smaller number of neurons and subjects were considered.

Statistical analysis

Data related to the percentages of HuC/HuD immunoreactive neurons co-expressing VIP- and nNOS immunoreactivity, as well as the percentages of nNOS immunoreactive neurons co-expressing VIP immunoreactivity (and *vice versa*) were compared with those obtained in the CTRL-dogs.

Due to the small numbers of subjects, the Mann-Whitney non parametric test was used to analyze the differences between the two groups of dogs (CTRL vs. INF). The level of significance was set at $P < 0.05$. All analyses and graphical representations were performed using a commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software Inc., La Jolla, CA, USA).

Results and Discussion

Results

VIP immunoreactive neurons: In general, VIP immunoreactivity was stronger in the SMP neurons than in the MP ones, which showed faint-to moderate immunolabelling. In the submucosa, some neurons, located close to the deep muscular plexus, showed bright VIP immunoreactivity. Since a great number of publications qualitatively characterized VIP-and nNOS-immunoreactive neurons and nerve fibers in the dog GIT, in this paragraph only the quantitative findings will be analyzed.

In total, 7543 HuC/HuD- and 1608 VIP-immunoreactive neurons were counted. More in detail, in CTRL- and INF-dogs, 2508 and 5035 HuC/HuD immunoreactive neurons were considered, respectively, whereas VIP immunoreactive neurons were 487 and 1121, respectively.

Myenteric plexus: In CTRL-dogs, VIP immunoreactive neurons represented 8 ± 3% (123/1403 cells, n = 5) of the total neuronal population. In INF-dogs, this percentage was meaningful greater, representing 16 ± 7% (394/2743 cells, n = 10) of all MP neurons (P = 0,022).

Submucosal plexus: In CTRL-dogs, VIP immunoreactive neurons were widely represented, and constituted 31 ± 9% (364/1105 cells; n = 6) of the total neuronal population; in INF-dogs, a similar percentage, i.e. 30 ± 11% (727/2292 cells, n = 10) of VIP immunoreactive neurons were counted (P = 0,786). The figure 1 graphycally reasumes the numeric values of VIPergic neurons in the MP (Figure 1A) and SMP (Figure 1B) of CTRL- and INF-dogs.

Nitrergic neurons: In total, 6360 HuC/HuD- and 1429 nNOS-immunoreactive neurons were counted.

More in detail, in CTRL- and INF-dogs, 2800 and 2031 HuC/HuD immunoreactive neurons were considered, respectively, whereas nNOS immunoreactive neurons were 832 and 597, respectively.

Myenteric plexus: In the myenteric plexus of INF-dogs, the percentage of nitrergic neurons (24 ± 5%) (528/2031 cells; n = 7) showed a not significant trend (P = 0,138) to decrease, in comparison to that evaluated in the CTRL-dogs (29 ± 5%) (795/2800 cells; n = 6).

Submucosal plexus: In the submucosal plexus the percentages of nitrergic neurons of CTRL-dogs (8 ± 5%) (40/527 cells; n = 4) and INF-dogs (7 ± 2%) (69/1007 cells; n = 4) did not show meaningful differences (P = 0.884). The figure 1 graphycally reasumes the numeric values of nNOS immunoreactive neurons in the MP (Figure 1C) and SMP (Figure 1D) of CTRL- and INF-dogs.

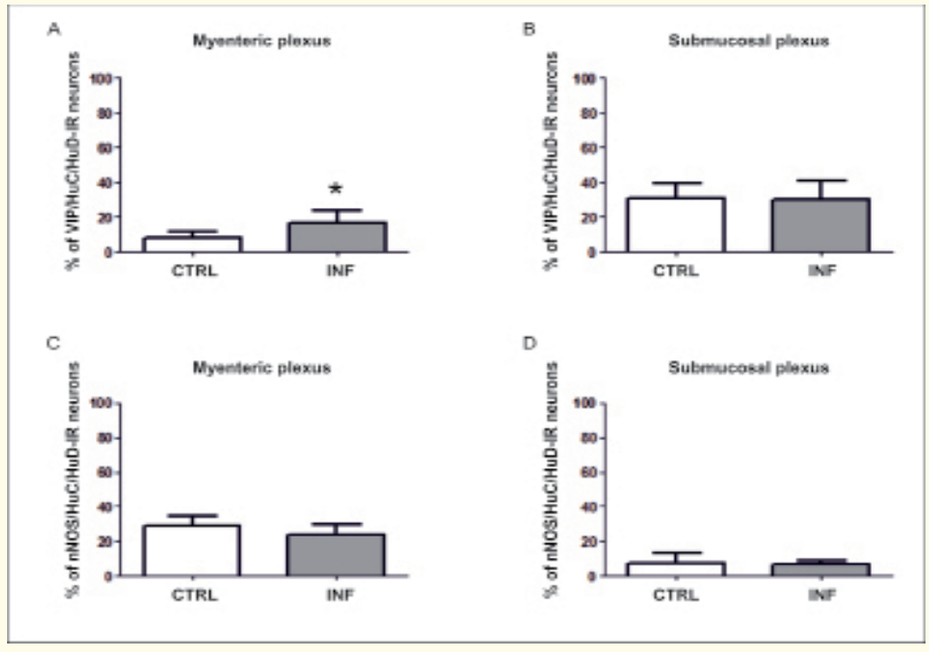

Figure 1: *Graphical representation of the percentages (mean ± standard deviation) of neurons immunoreactive (IR) for the vasoactive intestinal peptide (VIP) (A, B) and neuronal nitric oxide synhase (nNOS) (C, D) in the myenteric and submucosal plexus of the ileum of control dogs (CTRL) and dogs with intestinal inflammation (INF).*

Co-localization of nNOS- and VIP-immunoreactivity in CTRL- and INF-dogs: In the myenteric plexus of CTRL- and INF-dogs similar percentages, respectively 23 ± 12% (124/453 cells, n = 7) and 24 ± 10% (89/383 cells, n = 5) of nNOS immunoreactive neurons co-expressed VIP immunoreactivity (P = 0.935) (Figure 2A-2C). The percentages of VIP immunoreactive neurons co-expressing nitrergic phenotype were 86 ± 15% (128/143 cells, n = 6) and 70 ± 35% (83/119 cells, n = 5) in CTRL- and INF-dogs, respectively (P = 0,784).

In the submucosal plexus of CTRL- and INF-dogs, 26 ± 16% (23/151 cells, n = 6) and 23 ± 15% (51/292 cells, n = 6), respectively, of nitrergic neurons showed VIP immunoreactivity (P = 8095) (Figure 2D-2F). In CTRL-dogs, 15 ± 8% of VIPergic neurons (23/185 cells, n = 6) co-expressed nNOS immunoreactivity; in INF-dogs these neurons showed wide variability, ranging from 0% to 51% (average = 16%; 39/210 cells, n = 5) (P = 0.537). The figure 2 graphycally reasumes the numeric values of nNOS immunoreactive neurons co-localizing VIP immunoreactivity (and vice versa) in the MP (Figure 2A, 2B) and SMP (Figure 2C, 2D) of CTRL- and INF-dogs.

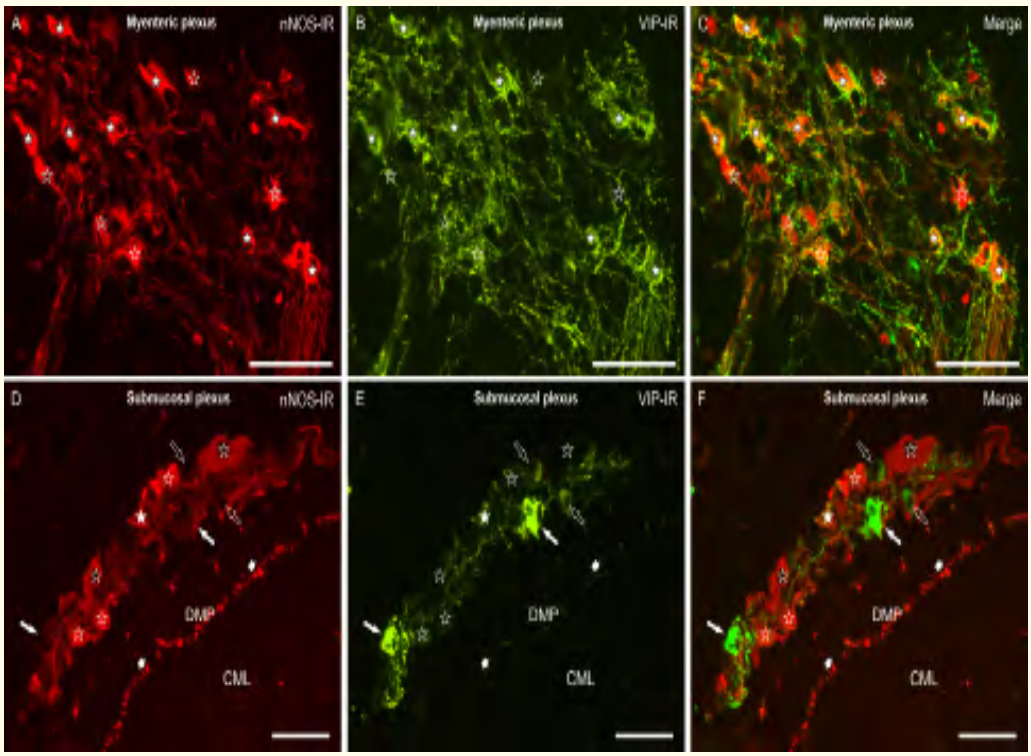

Figure 2: *Micrographs showing tangential (A-C) and longitudinal (D-F) cryosections of the dog ileum in which neurons showed immunoreactivity for the neuronal nitric oxide synthase (nNOS) (nNOS-IR) (A, D) and vasoactive intestinal peptide (VIP-IR) (B, E). A-C) White stars indicate myenteric plexus neurons co-expressing nNOS- and VIP-immunoreactivity. Open stars indicate nitrergic neurons which were VIP-negative. D-F) Open stars indicate submucosal plexus nitrergic neurons which were VIP-negative. The white star indicates a small submucosal plexus neuron co-expressing nNOS- and VIP-immunoreactivity. Among VIP-ergic submucosal neurons, there were cell body somata showing strong (white arrows) and weak (open arrows) VIP immunoreactivity. The short arrows indicate the deep muscular plexus (DMP) distributed in the inner portion of the circular muscle layer (CML); it is possible to observe that nNOS-immunoreactive (nNOS-IR) nerve fibers were strongly represented in the DMP, whereas VIP-IR nerve fibers were poorly represented. C and D: merged images.*
Bar = 50 µm.

Discussion

A great amount of articles have qualitatively characterized VIP- and nNOS-immunoreactive neurons and nerve fibers in the dog gastrointestinal tract [1,9,11,13-27]. From all the available substantial literature, it was possible to summarize the following distinctive features: 1) Myenteric plexus nNOS immunoreactive neurons are inhibitory motor neurons; 2) A portion (not yet quantified in the dog, before the present study) of nNOS immunoreactive MP inhibitory motor neurons co-expresses VIP immunoreactivity; 3) The axons of VIP- and nNOS-immunoreactive motor neurons are anally directed; 4) VIP- and nNOS-immunoreactive neurons innervate both the layer of the *tunica muscularis*; 5) Myenteric plexus VIP immunoreactive neurons may be also intestinofugal sensory neurons; 6) Since the submucosa of the stomach lacks enteric ganglia, gastric myenteric plexus VIPergic neurons innervate the mucosa; 7) In the intestine, submucosal VIP immunoreactive neurons mainly innervate the mucosa and a small subset of neurons innervates the smooth cells of the inner CML and *muscularis mucosae*.

In the present study we observed that VIPergic myenteric plexus neurons were, in proportion, about a quarter compared to nitrergic neurons, while the great majority of VIPergic neurons synthesize also nitric oxide as neurotransmitter; our data are consistent with those obtained in the past [23,24].

In the submucosa, VIPergic neurons were in prevalent percentage respect to nitrergic ones and only a few VIPergic neurons, distributed in the outer layer of the submucosal plexus (closer to the CML), co-expressed nNOS immunoreactivity. It is well known that, in large mammals, the submucosal plexus is bilayered and that the outer SMP ganglia contain neurons innervating the CML and showing a phenotype similar to that of MP neurons. Submucosal VIP/nNOS neurons innervate also the *muscularis mucosae* [1,16,19] and mediate its relaxation also in the dog [28,29]. Nevertheless, a great percentage of submucosal VIPergic neurons lacks nNOS immunoreactivity; these neurons should be non-cholinergic secretomotor/vasodilator neurons innervating the mucosa, as observed in the guinea-pig [1]. In the present study, however, we did not co-localize VIP with markers of cholinergic neurons (for instance, choline acetyltransferase, ChAT or its peripheral isoform, pChAT). VIPergic neurons appear to be involved in epithelial ion transport, barrier functions, gastric secretion, hemodynamic regulation, and immune response [3,30,31]. VIP may promote secretory functions via its intestinal receptor (vasoactive intestinal peptide receptor 1, VPAC1) [32] through cAMP; however, VIP can also have antisecretory effects by inhibiting cyclooxygenase-2 (COX-2) and NF-κB. In addition, VIP may reduce paracellular permeability and decrease epithelial permeability via regulation of tight junction proteins [33].

Functional studies carried out on the dog mucosa indicate that VIP may cause capillary dilatation, slight increase the protein output, and close intercellular spaces. The action of VIP on the mucosa of dog jejunum was even compared to that of cholera toxin [34]. The prosecretory role of VIP has been demonstrated recently also in Parkinson patients, in which the reduction of submucosal VIP immunoreactive neurons and VIP receptors may induce severe constipation during pathology [35].

To the best of our knowledge, the literature does not offer any data on the effects of neuropeptides on immune system of the dog mucosa. The mucosal lamina propria has an extensive network of nerve fibers, which release neuropeptides such as VIP and Substance P to act on immune and inflammatory cells. In fact, subpopulations of T cells, B cells, monocytes macrophages, and several other immunologically cells have the ability to recognize and respond to these neuropeptide signals [36-38].

While there is a general acknowledgment/agreement about the anti-inflammatory effect of VIP [38], there are conflicting evidences regarding the significance of VIP in intestinal inflammation [3,5,33,39]. Some data demonstrate also a pro-inflammatory role for VIP in murine colitis [40] and in human inflammatory bowel disease (IBD) [41]; in murine experimental colitis it has been shown a meaningful increase of the number of VIP receptors in the inflammatory mucosa [42]. In patients with IBS, a mechanism VIP- and mast cells- dependent has been demonstrated in translocation of commensal and pathogenic live bacterial in colonic epithelium [43].

In the present study, we observed a meaningful increased percentage of VIP immunoreactive neurons in the MP of INF-dogs, whereas in the SMP the neurons did not vary considerably between groups of dogs.

Contrary to what observed for VIP neurons, whose percentage meaningful increased in the myenteric plexus of the INF-dogs ileum, the percentage of nitrergic neurons did not show significant variations, although it showed a tendency to decrease.This finding was unexpected, because in contrast with previous findings related to the sensibility of nitrergic neuronal subpopulation to intestinal inflammation [44], also secondary to diabetes mellitus, as showed recently by the present research group [9].

Nitric oxide (NO) and VIP are co-transmitters released in parallel from enteric inhibitory neurons. Other candidate inhibitory transmitters responsible for gastrointestinal smooth muscle inhibition that have been identified: adenosine triphosphate (ATP), pituitary adenyl-

ate cyclase-activating peptide (PACAP), carbon monoxide (CO), protease-activated receptors (PARs), hydrogen sulfide (H_2S), neurotensin (NT) and beta-nicotinamide adenine dinucleotide (b-NAD) [45]. VIP is stored in vesicles in the nerve endings, while nitric oxide (NO) is synthetized on demand by the nNOS. At the presynaptic level, VIP and NO can induce each others release: VIP induced NO release, and NO facilitated VIP release [46]. At the postsynaptic level, many studies support that VIP and NO are parallel co-transmitters, whereby VIP is the principle neurotransmitter, acting partially via a VPAC receptor and the adenylate cyclase/cAMP pathway. NO acts via the guanylate cyclase/3'5' cyclic guanosine monophosphate pathway. Considering the pre and post synaptic interactions between VIP and nNOS, it is therefore plausible that the increased proportion of VIP immunoreactive neurons (and the slight decreased proportion of nNOS immunoreactive neurons) might anyhow affect the "rhythm" of the GIT peristalsis, which often happens in dogs with chronic enteritis.

To represent the possible effects of chronic enteritis on the ENS, and to to provide an example on how some substances released during intestinal inflammation can involve VIP-and nNOS-immunoreactive neurons, we can refer, although in an absolutely speculative way, to the inflammatory cascade triggered by the intrleukin 1 beta (IL-1b), which is released also during dog enteritis [47,48]. In fact, in the inflamed bowel of guinea-pig, it has been shown that IL-1b activates MP nitrergic and SMP VIPergic neurons, leading to disturbances in motility and secretion [49].

Collectively, the responses evoked by neuropeptides is characterized as "neurogenic inflammation" [50]. The effects of neuropeptides last long because of the feedback regulation between neuropeptide release and inflammatory mediators release from immune cells [33]. The enteric hyperinnervation can actively drive intestinal inflammation, thus emphasizing the relevance of neurogenic inflammation in the gut [51]. Abnormalities of the ENS might therefore contribute to the pathogenesis of inflammatory bowel disease.

Conclusion

This is the first quantitative study about the VIPergic and nitrergic neurons harbored in the ileum of the canine MP and SMP 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 potentially influence intestinal motility (and mucosal functions).

The increased percentage of the VIPergic neurons in dogs with chronic enteropathies could have a protective meaning. The IBD in human patients, as well as chronic enteritis in domestic dogs, are chronic relapsing disorders of the intestine, with increasing incidence worldwide both in human and animal patients [32]. At present, the management of chronic enteropathies is an unmet medical need due to the ineffectiveness of currently available drugs in treating human and animal patients, and there is strong demand for novel therapeutics.

Further studies are needed to better understand the nature of neuropeptide signaling in human IBD and mammals' chronic enteritis. Animal models of colitis, neuropeptide knockout animals, and neuropeptide agonists and antagonists may be critical to this process [52]; all these investigations are certainly more achievable on laboratory rodents, whereas are much more difficult and questionable ethical procedures on domestic carnivores. Clarification of the molecular mechanisms of action of VIP on immune and inflammatory reactions will likely yield new treatment options in the future [5], as recently demonstrated in mice [32].

Acknowledgment

We thank Prof. Catia Sternini (UCLA, Los Angeles) for the generous gift of the two anti-VIP antibodies used for this study.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Volume 5 Issue 6 June 2018