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Alterations in enteric calcitonin gene-related peptide in patients with colonic diverticular disease
CGRP in diverticular disease

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ABSTRACT

Diverticular disease (DD) is one of the most prevalent diseases of the large bowel. Lately, imbalance of neuromuscular transmission has been recognized as a major etiological factor for DD. Neuronal calcitonin gene-related peptide (CGRP) is a potent gastrointestinal smooth muscle relaxant shown to have a widespread effect within the alimentary tract. Nevertheless, CGRPergic innervation of the enteric ganglia has never been considered in the context of motility impairment observed in DD patients.

Changes in CGRP and calcitonin receptor-like receptor (CRLR) abundance within enteric ganglia were investigated in sigmoid samples from symptomatic and asymptomatic DD patients using quantitative fluorescence microscopy. CGRP effect on gastrointestinal smooth muscle was investigated using organ bath technique.

We found CGRP levels within the enteric ganglia to be declined by up to 52% in symptomatic DD patients. Conversely, CRLR within the enteric ganglia was upregulated by 41% in symptomatic DD. Longitudinal smooth muscle displayed an elevated (+10.5%) relaxant effect to the exogenous application of CGRP in colonic strips from symptomatic DD patients. Samples from asymptomatic DD patients consistently showed intermediate values across different experiments.

In conclusion, the present study demonstrates that CGRPergic signaling is subject to alteration in DD. Our results suggest that a hypersensitization mechanism to gradually decreasing levels of CGRP-IR nerve fibers takes place during DD progression. Alterations to CGRPergic signaling in DD disease may have implications for physiological abnormalities associated with colonic DD.

1. Introduction

Formation of a diverticulum, a sac-like protrusion of the mucosa and submucosa through the muscular layers, is regarded as the onset of colonic diverticular disease (DD). With high prevalence in the elderly population, DD is one of the most common diseases of the large bowel (Reichert and Lammert, 2015). Despite its ever-growing burden on the national health systems, high prevalence, and complicated clinical management, over the years DD has drawn relatively little research effort and has repeatedly been named the “neglected disease” in scholarly literature (Kruis et al., 2012; Tursi, 2016).

The etiology of colonic DD is known to be a multifactorial process which involves both environmental factors and genetic predisposition (Von Rahden and Germer, 2012). Furthermore, pathogenesis of DD is accompanied by structural remodeling and functional alterations of the colon. An eminent cause of diverticula formation appears to be altered colonic motility, as DD patients display increased intraluminal pressure profiles in the sigmoid (Arfwidsson et al., 1964; Painter, 1964; Parks and Connell, 1969), along with numerous changes to colonic musculature (Alvarez-Berdugo et al., 2015; Gallego et al., 2013; Hellwig et al.,

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2014) and the enteric nervous system (ENS) (Bassotti et al., 2015; Böttner et al., 2013; Wedel et al., 2010).

In large mammals the ENS is composed of three distinct intrinsic ganglionated plexuses (the myenteric (MP) – Auerbach's, the outer submucous (OSP) – Schabadasch's, and the inner submucous (ISP) – Meissner's), where MP and OSP maintain intestinal motility, while OSP and ISP governs the epithelial functions (Brehmer et al., 2010; Hansen, 2003; Timmermans et al., 2001).

An array of colonic motor dysfunctions are linked with distinct abnormalities of the ENS (De Giorgio and Camilleri, 2004; Di Nardo et al., 2008). In case of DD, it is established that colonic smooth muscle becomes hypersensitive to cholinergic stimulation (Alvarez-Berdugo et al., 2015; Golder et al., 2003) and loses its capacity to relax to sodium nitroprusside (SNP), a NO donor (Golder et al., 2007). Thus, an imbalance in neuromuscular transmission is suspect to impair gastrointestinal motility observed in DD patients.

Calcitonin gene-related peptide (CGRP) is a potent smooth muscle relaxant involved in multiple physiological processes throughout the body (Russell et al., 2014). CGRP acts through a heteromeric receptor composed of a G-protein coupled receptor called calcitonin receptor-like receptor (CRLR) and a receptor activity-modifying protein 1 (RAMP1) (Hay and Pioszak, 2016). Within the digestive system, CGRP fibers innervate a multitude of targets (epithelia, muscle cells, neuronal elements) (Sternini et al., 1992), providing a morphological basis for the range of biological activities exerted by this peptide. In the alimentary tract CGRP exerts multidirectional action and has a prominent role in sensory and pain conduction (Russell et al., 2014), vasodilation (Nuki et al., 1993), immune response (Assas et al., 2014), absorption and secretory activity (Barada et al., 2000). Major targets of CGRPergic innervation are the intrinsic plexuses (Cottrell et al., 2012; Makowska and Gonkowski, 2018; Sternini et al., 1992), and in animal studies, CGRP was demonstrated to induce peristaltic reflexes (Grider, 1994, 2003; Grider et al., 2006), increase the peristaltic threshold (Holzer et al., 1989), relax intestinal smooth muscle cells (Katsoulis and Conlon, 1989; Takaki et al., 1989), induce phasic contractile activity (Holzer et al., 1989; Maggi et al., 1996, 1997) and excite myenteric neurons (Palmer et al., 1986). Regardless, the importance of CGRP in mediating gastrointestinal motor activity is mostly overlooked in recent reviews.

One downstream target of CRLR activation is neuronal NO synthase (NOS1), known to be subject to alteration in DD (Espin et al., 2014; Golder et al., 2007; Tomita et al., 2000). We hypothesized that CGRP signaling might be affected to counteract changes in NO production in a negative feedback loop. Information about CGRP in the human gastrointestinal tract is limited, and changes in CGRP innervation has never been considered in a context of motility impairment observed in DD. Thus, we set out to investigate CGRP innervation in sigmoid colon samples of DD patients using quantitative fluorescence microscopy and in vitro organ-bath technique.

2. Methods and materials

2.1. Patients and tissue samples

Control samples were obtained from patients undergoing surgery for non-obstructing colorectal carcinoma, who did not have symptoms of clinical motility disorders or previous episodes of symptomatic complicated or uncomplicated DD. This type of operation was a source of both control and asymptomatic diverticular disease (ADD) samples if diverticula were found to be present in these patients. Tissue specimens for the symptomatic diverticular disease (SDD) group were obtained from patients who underwent sigmoid resection or left hemicolectomy for symptomatic DD. Patients were operated after recurrent attacks of diverticulitis by elective surgery (Table 1).

Segments were taken from macroscopically normal regions of colon cancer patients, or in patients with diverticulitis, from the apparently normal area adjacent to the diverticulum. Diverticula containing areas that displayed altered colonic wall anatomy due to transmural mucosal/submucosal outpouchings or signs of inflammation and fibrotic scaring were excluded from tissue sampling. Colon segments were collected in the operating room, immediately placed at 4 °C pre-aerated (95% O_2, 5% CO_2) Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 11 mM glucose).

Table 1
Patient characteristics.

Group	n	Gender	Age, years (range)	BMI (range)
Control	11	5F/6M	64 (35–87)	25.18 (21.18–31.11)
ADD	10	6F/4M	62 (40–76)	27.59 (22.94–33.67)
SDD	10	7F/3M	62 (39–80)	26.11 (22.72–31.64)

ADD – asymptomatic diverticular disease; SDD – symptomatic diverticular disease; BMI – body mass index.

All experimental procedures with the human samples were approved by the Kaunas Regional Biomedical Research Ethics Committee, Kaunas, Lithuania (Permission number: BE-2-10) in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Immunohistochemistry

Following resection, tissue was submerged in 4% PFA solution (Sigma-Aldrich) for 150 min in RT. Later, samples were rinsed 3 × 10 min in PBS (0.01 M) and cut into 10 × 10 mm pieces. Segments were cryoprotected by immersion in 25% sucrose (Sigma-Aldrich) and 0.05% sodium azide (Carl-Roth) solution overnight in 4 °C. Next day, samples were embedded in OCT compound (Shandon™ Cryomatrix™, Thermo Fisher Scientific) and serially sectioned to obtain 16 µm full-thickness sections made along both longitudinal and circular muscle axes, changing the orientation of the mounted sample in the cryostat (CryoStar NX70, Thermo Fisher Scientific, USA). Sections were made no < 1 mm apart from one another to prevent measuring the same ganglia in adjacent sections. Sections were mounted onto microscope slides, air-dried and stored at −20 °C until use.

Sections were rehydrated by successive rinses in PBS and permeabilized in 0.5% Triton X-100 (Carl Roth) and 10% DMSO (Carl Roth) solution for 1 h in RT. Later, samples were rinsed and incubated in 5% NDS (Jackson ImmunoResearch Laboratories) for 1 h to block non-specific binding. Double immunohistochemical staining was carried out by incubating samples in primary antisera (Table 2) overnight at 4 °C. Next day, samples were rinsed in PBS and incubated in an appropriate combination of secondary antibodies (Table 2) for 4 h in RT. For fluorescence quantification experiments, pan-neuronal marker (PGP 9.5) was used in combination with antibodies against CGRP, CRLR or RAMP1. Finally, specimens were rinsed in PBS and cover-slipped using Vectashield® mounting medium (Vector Laboratories, USA).

Double staining with primary antibodies from the same host species was performed as detailed by Balen et al. (2008). Briefly, samples were incubated (in sequence) with first primary antibody (either *rb* anti-CRLR or *rb* anti-RAMP1, 1:500) overnight in 4 °C, rinsed in PBS and incubated with first secondary antibody (anti-*rb* Cy3, 1:500) for 4 h in RT. Later, samples were rinsed in PBS and incubated with the second primary antibody (*rb* anti-VIP, 1:1000) overnight at 4 °C. Next day, samples were rinsed in PBS and incubated for 20 min in second secondary antibody (anti-*rb* AF488, 1:50).

For all antibodies used in this study, negative controls were processed as outlined above except that either the primary or the secondary antibody was omitted. In all trials this eliminated detection of histofluorescence. Single positive controls were processed for all primary antibodies used in the study. In all trials, the signal of single positive controls was indistinguishable to that of experimental samples.

Table 2
Primary and secondary antibodies used in the study.

Antigen	Host	Type	Dilution	Source	Cat. #
Primary					
CGRP	Mouse	Monoclonal	1:1000	Abcam[a]	Ab 10987
RAMP1	Rabbit	Polyclonal	1:1000	Bioss[b]	BS-1567R
CRLR	Rabbit	Polyclonal	1:1000	Bioss[b]	BS-1860R
PGP 9.5	Mouse	Monoclonal	1:1000	Abcam[a]	Ab 72911
PGP 9.5	Rabbit	Polyclonal	1:1000	Bio-Rad[c]	7863-0504
NOS1	Rabbit	Monoclonal	1:1000	Abcam[a]	EP 1855Y
NOS1	Mouse	Monoclonal	1:500	Santa Cruz[d]	SC-5302
VIP	Rabbit	Polyclonal	1:1000	Chemicon[e]	AB982
Secondary					
Anti-Rabbit Cy3	Donkey	Polyclonal	1:500	Millipore[f]	AP182C
Rabbit AF488	Donkey	Polyclonal	1:500	Invitrogen[g]	A21206
Mouse Cy3	Donkey	Polyclonal	1:500	Millipore[f]	AP192C
Mouse AF488	Donkey	Polyclonal	1:300	Invitrogen[g]	A21202

[a] Abcam, Cambridge, UK.
[b] Bioss Antibodies Inc., Woburn, Massachusetts, USA.
[c] Bio-Rad (Formerly AbD Serotec), Kidlington, UK.
[d] Santa Cruz biotechonology, Dallas, Texas, USA.
[e] Chemicon International, Temecula, California, USA.
[f] Millipore Corp., Temecula, California, USA.
[g] Invitrogen, Ltd., Paisley, UK.

A full list of the chemicals and reagents used in the study can be found in the supplement material Table A.1.

2.3. Microscopy and image analysis

Amount of CGRP-IR nerve fibers and CRLR/RAMP1-IR neuronal somata and nerve fibers in the enteric ganglia was estimated using quantitative fluorescence microscopy method in accordance with Waters (2009). Samples were kept in the dark at 4 °C. Images were acquired no later than one week following the sample preparation to avoid fluorophore bleaching. Fluorescent images of intrinsic neural plexuses were acquired using Zeiss AxioImager Z1 wide-field microscope (Carl Zeiss, Germany) equipped with AxioCam MRm Rev.3 digital camera. The objective used in the study was 40 × /0.9 EC Plan NeoFluar and images were captured using AxioVision Rel.4.8.2 software (Carl Zeiss, Germany). The fluorescent light source was HXP 120 V illuminator using 38HE (EX 470/40, EM 525/50) and 43HE (EX 550/25, EM 605/70) filter set.

Images of enteric ganglia of myenteric (Auerbach), outer submucosal (Schabadasch) and inner submucosal (Meissner) plexuses were captured in three dimensions throughout the section. Varying number of images were made that would capture 10–20 autonomic ganglia of each enteric plexus (this was done due to smaller size of the submucosal ganglia; since magnification (objective) was kept constant, some images contained several submucosal ganglia leading to a fewer images being made for that neural plexus). Each image consisted of a Z-projection composed of 10 focal planes (2-D images) made at 1 μm increment. Exposure interval that would not result in pixel saturation was selected during the pilot experiments. Images were taken at a fixed exposure time (800 ms) for the channel of interest for all images used for quantification.

It must be noted that CGRP-IR signal investigated in the present study represents primary afferent nerve fibers of either intrinsic or extrinsic origin, composed of numerous smooth and varicose axons, and intraganglionic laminar endings (IGLEs) (Furness et al., 2004). IGLEs are complex branching nerve endings that give rise to flat (laminar) expansions within the enteric ganglia (Zagorodnyuk et al., 2001).

Image analysis was performed in ImageJ/Fiji software. In brief, images were loaded into Fiji as a stack and Z-projected using average intensity projection. Fluorescence intensity was determined by selecting the region of interest (ROI) in PGP 9.5 signal view (selecting boundaries of the enteric ganglion) and measuring the densitometric fluorescence intensity (IntDen) within the ROI in the channel of interest (containing CGRP/RAMP1/CRLR signal). The obtained value was background corrected by subtracting the size of ROI multiplied by the average background intensity value of the image. Since CGRP-IR neurons occurred inconsistently and were absent in majority of the samples, ganglia containing CGRP-IR neuronal bodies were excluded from the analysis. This was done to prevent irregular sampling due to the large proportional area of CGRP-IR cell bodies that would positively shift FI measurements.

To correct for potential inconsistencies of the fluorescent lighting and optical shading effects, the resulting intensity values are reported as a fraction of a fluorescence standard as described by Model and Burkhardt (2001) Rose Bengal (Sigma-Aldrich) (0.25 g mL^{-1}) was used as a fluorescence standard and a series of images of this reference were made each time before imaging the colonic sections. The original (background corrected) intensity value was divided by the average reference value of the fluorescent standard to obtain the final normalized fluorescence intensity value in arbitrary units (AU).

Images for illustrations were generated using Zeiss LSM 700 laser-scanning confocal microscope equipped with a dual T-PMT sensor and using 40 × /1.4 Plan Apochromat and 60 × /1.46 αPlan Apochromat oil immersion objectives in ZEN Black SP1 2010 software (Carl Zeiss, Germany). Confocal images were processed into final figures by adjusting image size, brightness, and contrast using Photoshop CS6 (Adobe Systems, San Jose, USA).

2.4. In vitro contractility experiments

Following resection, tissue was placed in cold-aerated Krebs-Henseleit solution (4 °C) where the musculature was separated from the mucosa. Then, colonic musculature was cut into 2 × 10 mm strips orientated along longitudinal or circular muscle axes. Intestinal muscle strips were submerged in warm (37 °C) aerated Krebs-Henseleit solution in individual 25 mL wells (Radnoti organ bath, AD instruments Pty, AU) anchored by a metallic hook at the lower end and attached by silk suture to a force transducer for isometric recording of muscular activity (PowerLab®, AD Instruments Pty). The muscular strips were pre-stretched to a passive tension of 35–40 mN and allowed to equilibrate for at least 1 h, replacing Krebs-Henseleit solution every 15 min, until a stable baseline was established.

Bethanechol (10^{-4} M) was added and a contact period of up to 5 min was allowed to obtain a maximal reference contraction. Human CGRP (10^{-7} M) was then added and a contact period of 15 min was allowed until the relaxation response would plateau. Then SNP (10^{-3} M) was added to induce maximal reference relaxation response. In a subset of experiments, tetrodotoxin (10^{-7} M) (TTX) were administered 15 min before the addition of bethanechol.

Relaxation response to CGRP was calculated within the range of maximal reference contraction and maximal reference relaxation for every recording. The magnitude of the response is expressed as a percentage of the maximal reference relaxation to SNP. Untreated strips were run in parallel with strips subjected to investigational compounds. Each strip was only subjected to a single set of drugs used in experimental protocol and all strips at one experimental run were taken from the same individual.

2.5. Statistical analysis

The distribution of data was inspected using histograms and data normality was confirmed with the Shapiro-Wilk test. Differences in the data were assessed using unpaired Student's *t*-test with Welch's correction or Mann–Whitney *U* test where appropriate (GraphPad Prism 6, San Diego, USA). $p < 0.05$ was used as a threshold for statistical significance. Data are presented as mean ± SEM if not stated otherwise. Changes in CGRP, CRLR and RAMP1 abundance is expressed as ± %

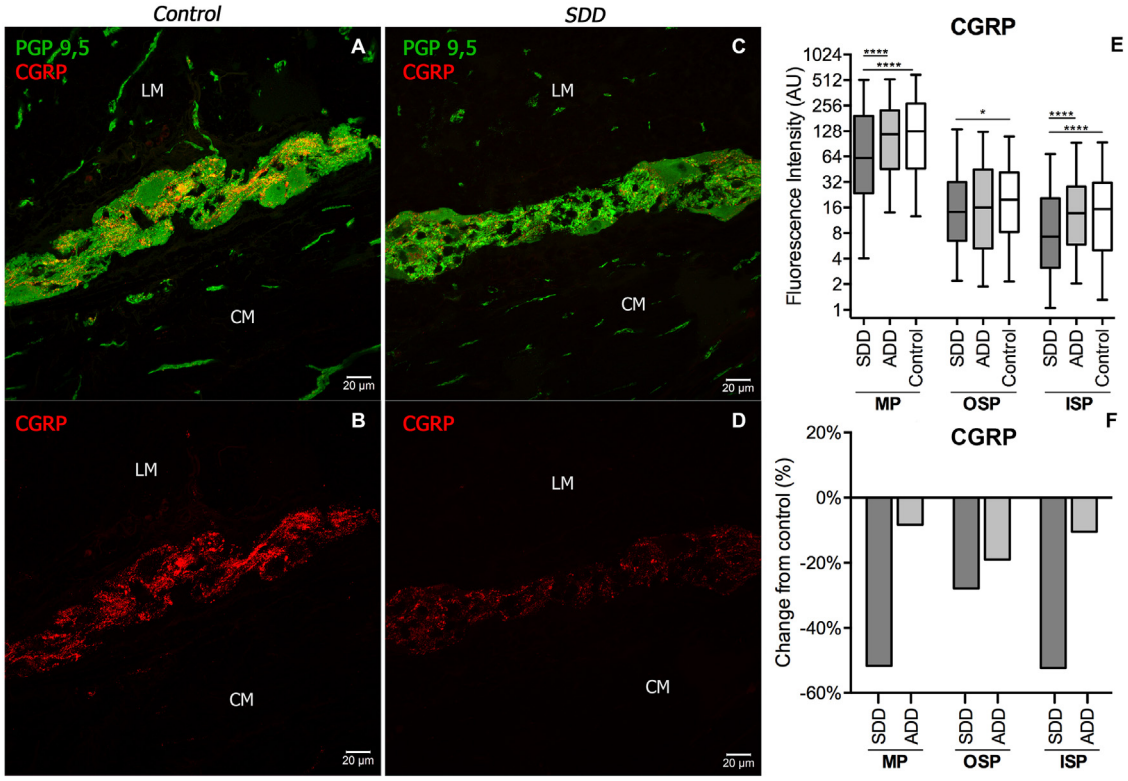

Fig. 1. CGRP-IR nerve fibers in a myenteric ganglion of DD patients. (A–D) Confocal micrographs demonstrating differential abundance of CGRP-IR fibers within myenteric ganglion of control (A–B) and SDD (C–D) patients. (E) Quantification of FI within the ENS of DD patients. Whiskers – 5–95th percentile. *–p < 0.05, ****–p < 0.001 (Mann-Whitney *U* test). Log2 scale. (F) Percentage change from median control value. LM – longitudinal muscles, CM – circular muscles.

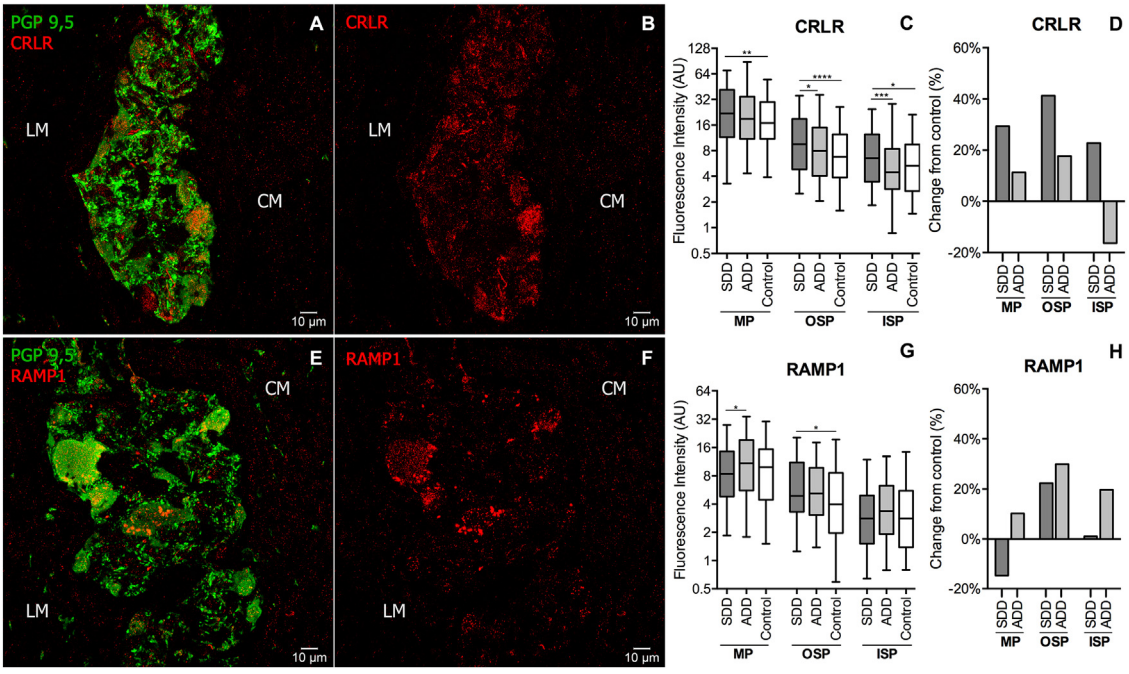

Fig. 2. CRLR-IR and RAMP1-IR nerve structures within the enteric ganglia of DD patients. (A–B, E–F) Confocal micrographs showing CRLR and RAMP1 within the myenteric ganglia. Note the highest accumulations of CRLR-IR and RAMP1-IR fluorescence signal in the neuronal somata and in lesser amount dispersed in the neuropil. (C–D) Quantification of CRLR-IR structures FI within the MP, OSP and ISP ganglia of DD patients. (G–H) Quantification of RAMP1-IR structures abundance within the ENS of DD patients. Whiskers – 5–95 percentile. *–p < 0.05, **–p < 0.01, ***–p < 0.005, ****–p < 0.001 (Mann-Whitney *U* test). Log2 scale. (D, H) Percentage change from median control value. LM – longitudinal muscles, CM – circular muscles.

from median control value.

3. Results

3.1. CGRP is decreased in SDD

CGRP-IR nerve fibers were situated throughout the colonic sections with the primary target of innervation being the intrinsic plexuses. The majority of CGRP-IR fibers were located in the vicinity of enteric neurons within the myenteric (MP) and both submucosal plexuses (OSP, ISP). CGRP-IR fibers entangled intestinal glands and abundantly innervated the mucosa, whereas only sparse, minute CGRP-IR nerve fibers were located in the muscular layers (Fig. 1B).

Seldom, enteric ganglia contained CGRP-IR neurons in which case that ganglion was excluded from further analysis (Figs. A.1–A.2).

In control samples, CGRP-IR nerve fibers were six-fold more abundant in the ganglia of MP compared to SP ganglia. The lowest fluorescence intensity levels of CGRP-IR nerve fibers were found in the ISP. The levels were similar within the enteric ganglia of the OSP. (Fig. 1E, Table A.2). The ratio of CGRP-IR nerve fibers between MP, OSP and ISP plexuses was constant between the experimental groups.

Quantification of fluorescence intensity revealed that abundance of CGRP-IR nerve fibers to be decreased within the enteric ganglia of sigmoid colon of DD patients (Fig. 1). This change was most evident within the MP where CGRP-IR nerve fibers amount in SDD patients was half that of the control group (Fig. 1A–D). When we compared relative change from median control value (Δ), we found that in MP ganglia CGRP-IR nerve fibers were decreased by 51.7% ($p < 0.0001$, Mann-Whitney *U* test) in SDD.

While that in OSP and ISP, CGRP-IR nerve fibers decreased by 27.8% ($p = 0.04$) and 52.4% ($p < 0.0001$), respectively. ADD patients had intermediate values throughout the enteric plexuses (MP: Δ-8.31%, $p = 0.326$; OSP: Δ-19%, $p = 0.407$; ISP: Δ-10.5%, $p = 0.804$) that did not differ from that of control (Fig. 1E–F, Table A.2).

3.2. CRLR-IR nerve structures are increased in SDD

CRLR-IR structures had a granular appearance, with the majority of the signal contained within neuronal cell bodies (Fig. 2A–B). In control samples, CRLR-IR nerve structures were was 2.5 times more abundant in the MP compared to SP and similar in quantity between OSP and ISP (Fig. 2C). In this regard, abundance of CRLR-IR nerve structures was similar to that of amount of CGRP-IR nerve fibers.

Amount of CRLR-IR structures was increased within the enteric ganglia of SDD patients (Fig. 2C–D, Table A.2). Compared to CGRP-IR nerve fibers, this reflected an opposite trend between the experimental groups. The greatest increase of 41.3% ($p < 0.0001$) was found in the OSP, whilst in MP and ISP amount of CRLR-IR nerve structures was increased by 29.3% ($p = 0.008$) and 22.7% ($p = 0.022$), respectively. Abundance of CRLR-IR structures in ADD (MP: Δ + 11.3%, $p = 0.078$; OSP: Δ + 17.6%, $p = 0.415$; ISP: Δ-16.3%, $p = 0.243$) again had intermediate values across the experimental groups.

Signal of RAMP1-IR structures mirrored CRLR-IR with regards to its localization. We found expression of RAMP1-IR structures not to differ statistically between SDD and control patients within MP and ISP ganglia (MP: −14.7%, $p = 0.67$; ISP: Δ + 0.11%, $p = 0.81$) (Fig. 2G–H, Table A.2), whereas that in OSP was increased by 22.3% ($p = 0.04$).

3.3. Relaxation response to CGRP is increased in longitudinal muscle of SDD patients

Exogenous application of CGRP induced a tonic relaxation response in both circular and longitudinal smooth muscle strips of the human sigmoid colon (Fig. 3A). We have observed no phasic contractile activity induced by CGRP application. Application of TTX had no effect on the CGRP induced response. Longitudinal muscles of DD patients displayed an increased relaxation response to exogenous CGRP stimulation (Fig. 3A–B, Table A.3). In control human sigmoid colon samples CGRP induced 69.5% of maximum reference relaxation in longitudinal muscle and 73% in circular muscle (Fig. 3B). CGRP induced relaxation was increased by 4.54% ($p = 0.677$) (unpaired Welch's *t*-test) and 10.5% ($p = 0.033$) in ADD and SDD longitudinal smooth muscle strips, respectively. CGRP induced relaxation was decreased in the circular muscle layer (SDD: −2.44%, $p = 0.536$; ADD: −6.99%, $p = 0.149$) but did not differ statistically between the experimental groups.

3.4. Association between CGRP and NOS1/VIP

To elucidate the role of CGRP in smooth muscle relaxation we performed double immunohistochemical staining for CGRP, CRLR or RAMP1 and either NOS1 or VIP (Figs. 4, 5). CGRP-IR fibers were found in close association with both NOS1-IR and VIP-IR neurons within the enteric ganglia of human sigmoid colon samples. Both VIP-inergic and nitrergic neurons within human ENS were found to express CRLR (Figs. 4C–D, 5C–D) and RAMP1 thus subject to CGRP activation. NOS1-IR neurons were predominantly located within the MP, whereas VIP-IR neurons were predominantly situated throughout OSP and ISP ganglia.

4. Discussion

The key findings of the present study are that CGRPergic signaling is subjected to alteration in DD where (1) the amount of CGRP-IR nerve fibers is decreased and (2) CRLR-IR structures are increased within the enteric ganglia of the sigmoid colon. (3) Moreover, the longitudinal smooth muscle of the sigmoid colon displays an elevated response to exogenous application of CGRP in DD patients.

CGRP is involved in an array of physiological processes throughout the alimentary tract. These include a role in nociception, immune response (Assas et al., 2014), secretion (Ahren and Pettersson, 1990; Esfandyari et al., 2000) and gastrointestinal motility (Grider, 1994; Maggi et al., 1997). Therefore, declining levels of CGRP may be suspected to consequence in a range of detrimental downstream effects within the GI tract. It must be noted that the present study was designed with an emphasis on CGRP involvement in smooth muscle relaxation, but effects on other associated functions cannot be out ruled.

To elucidate the scope of these effects, we investigated the abundance of our proteins of interest within different plexuses of the ENS, i.e., myenteric (MP), outer (OSP) and inner (ISP) submucosal plexuses. Apposed to experimental rodents, large mammals harbour two interconnected but morphologically and functionally distinct intrinsic submucous networks: one located close to the muscularis mucosae (ISP), the other near the circular layer of the external muscle (OSP) (Timmermans et al., 2001). Main distinction being the distribution pattern and neurochemical profile of the ganglionic cells, it is regarded that MP and OSP together maintain the intestinal motility, while ISP governs the absorption/secretory function (Brehmer et al., 2010). Some authors suggest that in the human intestine, even a third intermediate submucous nerve network can be discerned (Hoyle and Burnstock, 1989). It must be noted, neurochemical similarities between the intermediate plexus and either the inner or outer plexus in the human small and large intestine, appear to be region-dependent (Timmermans et al., 2001), second to being difficult to identify morphologically (Brehmer et al., 2010). For this reason, intermediate submucosal plexus was not investigated in the present study.

A semi-quantitative approach used in this study allowed estimation of protein levels with precise anatomical localization and enabled quantification of differential protein expression within different ENS plexuses in the same sample. This revealed the primary target of CGRP innervation to be the MP. The ratio of abundance of CGRP-IR nerve fibers between different plexuses was consistent between experimental groups. In our view, these results reflect (at least by quantitative means) CGRP involvement in distinct functional roles associated with MP and

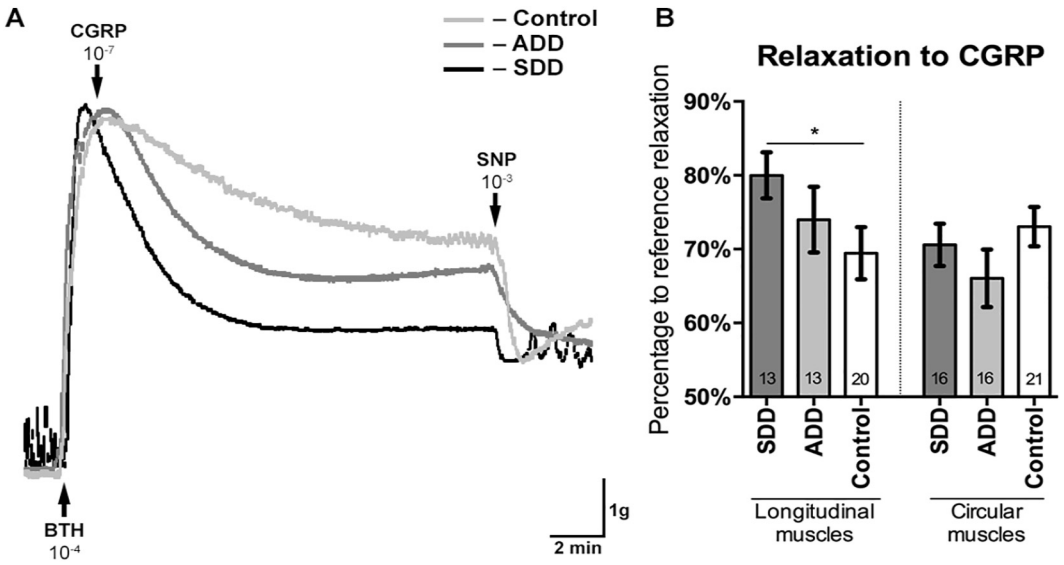

Fig. 3. Smooth muscle relaxation to CGRP. (A) Representative curves demonstrating CGRP induced tonic relaxation of longitudinal smooth muscle. (B) Quantification of relaxation response to CGRP. *–p = 0.033 (unpaired Welch's *t*-test). Mean ± SEM. Numbers indicate repeats. n(SDD) = 6, n(ADD) = 6, n (Control) = 10.

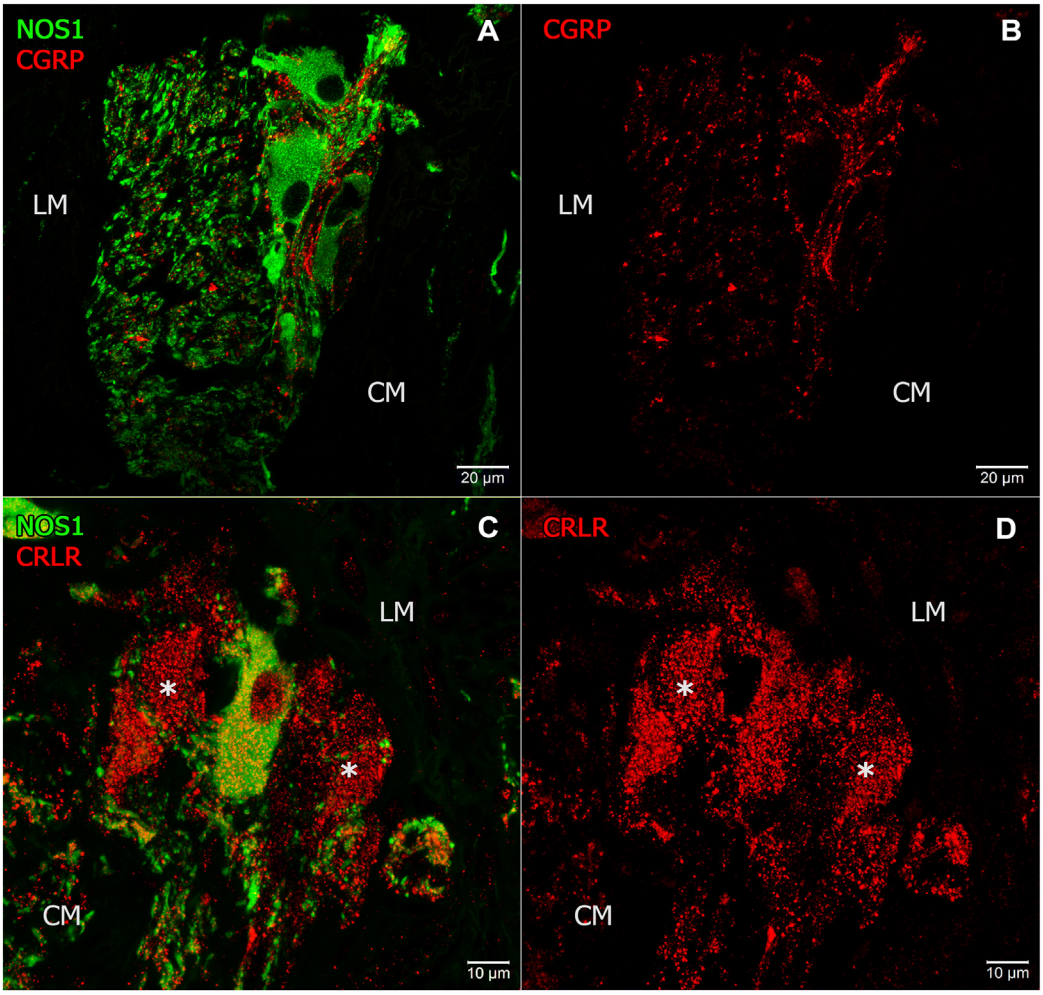

Fig. 4. CGRP-IR nerve fibers innervating nitrergic components within ENS. (A–B) CGRP-IR fibers entangling nitrergic neurons within myenteric ganglion. (C–D) CLRL-IR structures in the myenteric ganglion are mainly accumulated in the neuronal somata and in lesser extent in the ganglion neuropil. Note that some neuronal somata (*) are strongly positive for CRLR, but are not labelled by NOS1. LM – longitudinal muscles, CM – circular muscles.

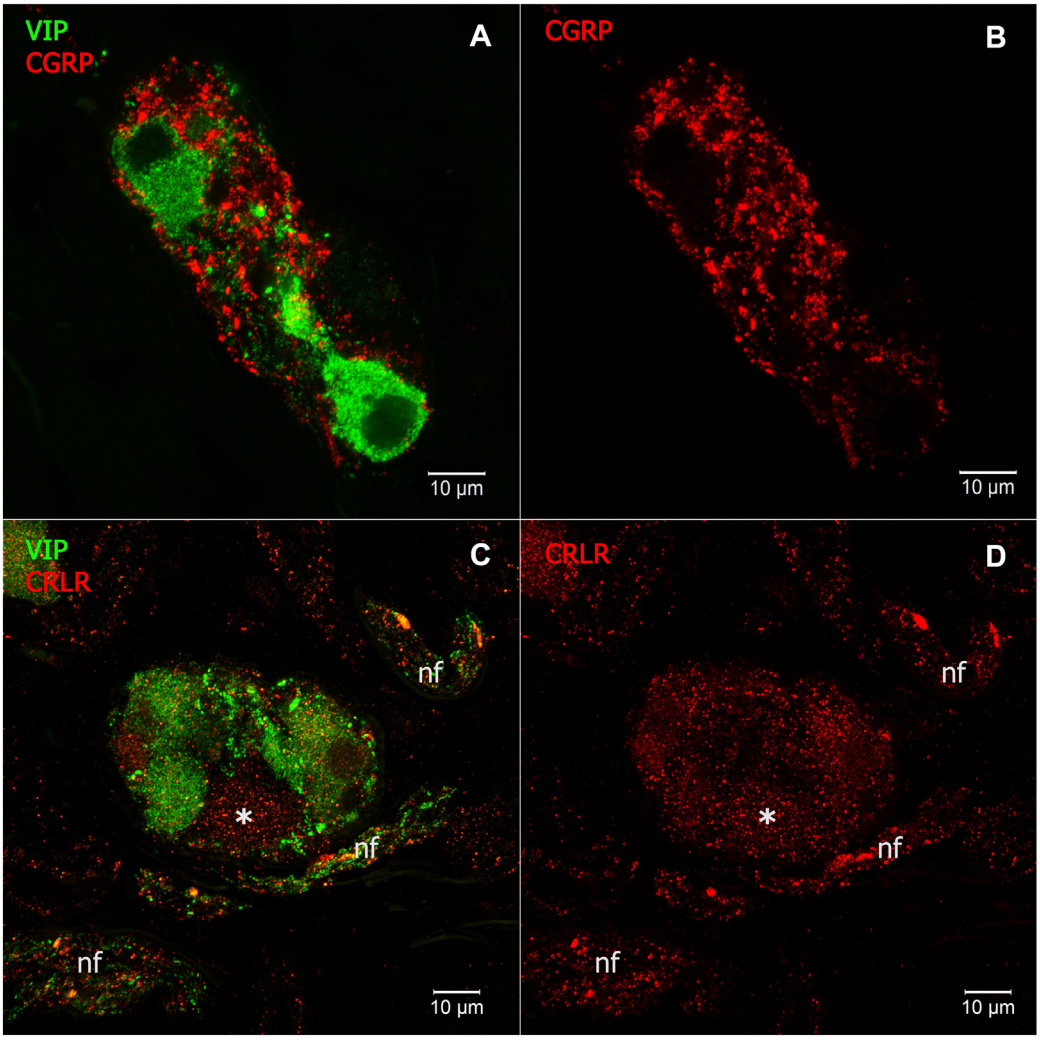

Fig. 5. CGRP-IR nerve fibers innervating VIP-IR components within ENS. (A–B) CGRP-IR fibers in close association with VIP-IR neurons residing in the submucosal plexus. (C–D) CRLR-IR structures in the submucosal ganglion are mainly accumulated in the neuronal somata and in lesser extent in the ganglion neuropil. Note that some neuronal somata (*) are positive for CRLR, but are not labelled by VIP. LM – longitudinal muscles, CM – circular muscles, nf – nerve fibers.

SP ganglia.

In the present study, CGRP-IR nerve fibers innervated the colonic tissue as previously described in canines (Sternini et al., 1992), but different than that detailed in the human small intestine (Timmermans et al., 1992) or swine descending colon (Makowska et al., 2017; Makowska and Gonkowski, 2018). In regards that we rarely detected CGRP-IR ganglionic cells and never identified a ganglion composed solely of CGRP-IR neurons as detailed by Timmermans et al. (1992). Similarly, descending colon of a swine model has been reported to contain numerous CGRP-IR neurons which made up to 25% of the total cell count in the MP, and up to 40% in the ISP (Makowska et al., 2017). This suggests that the exact function of CGRP may not only be region dependent, but also display a degree of interspecific differences. Whereas CRLR signal was contained within the neuronal cell bodies of all enteric ganglia as previously described by Cottrell (Cottrell et al., 2012).

CGRP is involved in the generation of GI motility patterns in a number of ways. Extrinsic and intrinsic sensory pathways which mediate peristaltic response to muscle stretch and mucosal stimulation were shown to utilize CGRP as a sensory neurotransmitter (Grider, 1994). Mechanism of this effect was elucidated showing that the peristaltic reflex is initiated by mucosal release of 5-HT and subsequent activation of 5-HT4 receptors on CGRP sensory neurons that in turn relay the signal via interneurons to VIP/NOS inhibitory and acetylcholine/tachykinin excitatory motor neurons (Grider, 2003; Grider and Piland, 2007). Potentially, declined levels of CGRP could interfere with this pathway leading to an imbalance of stimulatory and inhibitory input driving the peristaltic reflex in DD. Finding of high amount of CGRP-IR nerve fibers within the MP is consistent with functional studies showing CGRP role in gastrointestinal motility. Conversely, a profound decline of CGRP-IR nerve fibers in the MP ganglia of DD patients would be consistent with the altered motor function associated with DD. Moreover, Holzer reported that CGRP inhibited peristalsis by increasing the intraluminal pressure required to trigger peristaltic waves in the guinea pig (Holzer et al., 1989). Thus it could be hypothesized that reduction in CGRP expression could lead to lowering of the peristaltic threshold eliciting enhanced propagated high-amplitude contractions, intraluminal high-pressure zones, and excessive segmental contractions observed in the colonic musculature of DD patients (Bassotti et al., 2001, 2005; Painter, 1964; Parks and Connell, 1969). However, this hypothesis requires further functional assessment that could not be provided in the present study.

Given that CGRP is a potent smooth muscle relaxant (Katsoulis and Conlon, 1989; Maggi et al., 1996; Takaki et al., 1989) we hypothesized that declined levels of CGRP-IR nerve fibers observed in DD could have a direct effect on colonic smooth muscle relaxation. Smooth muscle contractility results revealed an elevated relaxant effect of CGRP in the longitudinal smooth muscle strips of sigmoid colon of SDD patients.

This could be explained by our subsequent finding that amount of CRLR-IR structures was upregulated in the enteric ganglia of SDD patients. Taken together these findings suggest that a local hypersensitization mechanism might take place to counteract gradually increasing smooth muscle tone. Moreover, ADD patients had transitional values across the experimental groups thus a gradual decrease of CGRP could be anticipated. This would imply that decreasing levels of CGRP-IR nerve fibers might play a role in the progression of DD and coincide with the emergence of DD symptoms. This trend was present across all enteric plexuses investigated in the study.

CGRP acts through a heteromeric receptor composed of a G-protein coupled receptor (CRLR) and a receptor activity-modifying protein 1 (RAMP1). RAMPs are required to chaperone CRLR to the cell surface, and heteromeric RAMP1-CRLR complex act as receptors for CGRP (Hay and Pioszak, 2016). We hypothesized that sensitization for CGRP could take place by upregulation of either CRLR or RAMP1. However, in the present study we did not find any clear trend in RAMP1 expression within MP and ISP ganglia of DD patients. However, RAMP1-IR structures were upregulated in the OSP which was in line with increased abundance of CRLR-IR structures indicating an increase of both components necessary for CGRP sensitization.

Both VIP and NO are the primary relaxant agents of the intestinal musculature (Van Geldre and Lefebvre, 2004). In the vasculature, CGRP receptor activation precedes NO release (Russell et al., 2014). Potentially, an analogous mechanism of relaxation is employed within the GI tract. Here, we immunohistochemically demonstrate CGRP-IR nerve fibers to be in close association with VIP-ergic and nitrergic components, suggesting that the relaxant effect of CGRP may act through downstream NOS1-IR and VIP-IR neuronal activation. Furthermore, differential localization of NOS1-IR and VIP-IR neurons within the ENS may suggest a different mechanism of relaxation/inhibition by which MP and OSP govern GI motility. Although, in the present study the CGRP induced relaxation response was TTX insensitive. Previous studies reported TTX to enhance the relaxant effect of CGRP by excitatory component inhibition (Maggi et al., 1996). These results suggest that smooth muscle relaxation reported in the present study might be due to secondary activation of ATP-sensitive potassium channels located on smooth muscle cells (Russell et al., 2014).

It is known that enteric neuropathies tend to preferentially affect neurons containing NOS1 (Rivera et al., 2011). It was demonstrated that enteric neuropathy is an underlying trait of DD (Wedel et al., 2010), and several other studies showed altered NOS1 expression and contractility responses to NO in SDD (Golder et al., 2007; Tomita et al., 2000). Furthermore, Espin reported increased NO-mediated responses together with upregulated NOS1 mRNA levels in ADD (Espin et al., 2014). These findings suggest that NOS1 overexpression at the early stages of DD may lead to excessive production of NO and commence neuropathic processes within the ENS. This explanation is coherent with a number of studies reporting other neurotransmitters to be declined in DD (Böttner et al., 2013; Golder et al., 2003, 2007; Tomita et al., 1993). However, the significance of any given ENS component for the intestinal motor disturbances observed in DD patients remains elusive as the detrimental effects of all associated factors are likely to take place simultaneously. It is feasible that decreased amount of CGRP-IR nerve fibers may reflect an overall trend of gut denervation in DD, further establishing an imbalance of neuro-muscular transmission as a major etiological factor of colonic DD.

It has been suggested that nerve tissue remodeling and neuropathic processes observed in DD are driven by inflammation of the enteric plexuses (Bassotti et al., 2015; Humes et al., 2012; Simpson et al., 2009; Wedel et al., 2010). In this study, SDD patients were operated on after recurrent episodes of diverticulitis thus it can be argued that the decrease in the abundance of CGRP-IR nerve fibers within the enteric ganglia is evoked by inflammatory processes of the diseased state. It is known that inflammation initially destroys nerves endings, and may lead to long term changes in gene expression and protein levels, which may represent changes observed in the present study. That being said, it must be noted that these considerations warrants additional experiments to reveal mechanistic basis of these findings.

Limitation of the present study was restricted functional assessment of the array of physiological responses mediated by CGRP. Here we were only able to investigate the relaxant effect elicited by CGRP and further studies elucidating alternative mechanisms are requisite.

5. Conclusions

Taken together, our results show that CGRP signaling is subject to alteration in symptomatic DD disease. These findings further demonstrate that an imbalance in neuro-muscular transmission is a major etiological factor of colonic DD. Lastly, our results suggest that CGRP is an important molecular mediator in DD and may have further implications for targeted disease prevention or treatment strategies which need to be evaluated in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.autneu.2018.09.006.

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Abbreviations

ADD: asymptomatic diverticular disease
CGRP: calcitonin gene-related peptide
CRLR: calcitonin receptor-like receptor
DD: diverticular disease
ENS: enteric nervous system
FI: fluorescence intensity
IR: immunoreactive
ISP: inner submucosal (Meissner's) plexus
MP: myenteric (Auerbach's) plexus
NO: nitric oxide
NOS1: neuronal nitric oxide synthase
OSP: outer submucosal (Schabadasch's) plexus
PGP 9.5: protein gene-product 9.5 (pan-neuronal marker)
RAMP1: receptor activity modifying protein 1
SDD: symptomatic diverticular disease (diverticulitis)
SNP: sodium nitroprusside
TTX: tetrodotoxin
VIP: vasoactive intestinal peptide