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Research article

Morphological elucidation of short-chain fatty acid receptor GPR41-positive enteric sensory neurons in the colon of mice with dextran sulfate sodium-induced colitis

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ABSTRACT

Although the etiology of inflammatory bowel disease (IBD) remains unclear, it has generally been accepted that abnormalities in the intestinal immune system and dysbiosis of the gut microbiota are involved in the pathology of IBD. Recently, short-chain fatty acids (SCFAs) produced by gut microbiota were reported to maintain intestinal homeostasis through their receptors, such as GPR41. However, there are contradictory reports about the role of GPR41 in intestinal inflammation. Consequently, the roles of GPR41 in dysbiosis induced by intestinal inflammation remain unclear. Thus, we investigated the distribution of GPR41 in the colonic mucosa of mice with dextran sulfate sodium (DSS)-induced colitis. GPR41-immunoreactive fibrous structures were observed in the colonic lamina propria and muscularis layer of normal mice. In addition, GPR41-immunoreactive fibrous structures partly colocalized with calcitonin gene-related peptide (CGRP; a neurotransmitter of cholinergic enteric sensory neurons)-immunoreactive nerve fibers in the colonic lamina propria, indicating that GPR41 is expressed in cholinergic intrinsic sensory neurons. Furthermore, both GPR41-immunoreactivities and CGRP-immunoreactivities were significantly increased in the lamina propria of the colon in mice with DSS-induced colitis. Interestingly, GPR41-immunoreactivities were often found in close proximity to F4/80$^+$ macrophages in the colonic mucosa of normal mice, and their frequency was elevated in the colonic mucosa of mice with DSS-induced colitis. Therefore, the crosstalk between SCFA-sensing intrinsic sensory neurons and macrophages might be involved in the pathology of acute colitis.

1. Introduction

The gut microbiota is a pivotal contributor to human health. Some of its effects are at least partly mediated by short-chain fatty acids (SCFAs), consisting mainly of acetate, propionate, and butyrate produced by the microbiota [1, 2]. Recently, SCFAs have been shown to regulate various functions through the activation of SCFA-sensing G protein-coupled receptors, such as GPR41 and GPR43 [3]. GPR41 and GPR43 are thought to mediate the interaction between the host and the gut microbiota. Both GPR41 and GPR43 have been reported to be coupled with Gαi/o family of G proteins. Furthermore, Inoue et al. have demonstrated that SCFAs stimulate the release of noradrenaline from primary cultured sympathetic neurons via GPR41 signaling pathway of Gβγ-PLCβ3-ERK1/2-synapsin 2 [4].

To date, there have been several studies on the pathological roles of GPR41 and GPR43 in chronic inflammatory disorders such as colitis, asthma and arthritis using knockout mice [3]. In the gut, it has also been reported using GPR41-mRFP and GPR43-mRFP transgenic mice that GPR41 and GPR43 are expressed in enteroendocrine cells as sensors for SCFAs [5]. Furthermore, GPR41 is also expressed in cell bodies of enteric neurons but cannot be detected in nerve fibers in the mucosa and enteric ganglia [5]. However, studies have shown conflicting evidence as to whether GPR41 and GPR43 are protective or causative in intestinal inflammation, and many questions regarding the functions of GPR41 and GPR43 remain unanswered [6].

Inflammatory bowel disease (IBD) is a chronic relapsing disease of the gastrointestinal tract that includes ulcerative colitis and Crohn's disease. Genome-wide association studies have revealed that more than 200

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genes associated with susceptibility to IBD are involved in mediating the host response to the gut microbiota [7]. Although the etiology of IBD remains unclear, it has generally been accepted that abnormalities of the intestinal immune system and dysbiosis of the gut microbiota are involved in the pathogenesis of IBD. The dextran sulfate sodium (DSS)-induced acute colitis model is the most widely used animal model of IBD and causes dysbiosis, which is usually experienced in IBD patients [8]. In DSS-induced colitis, intestinal macrophages in the inflamed colonic mucosa respond to microbial stimulation and produce large amounts of proinflammatory cytokines, which further cause inflammation and damage in the colon [9]. We have already reported that the proportion of intestinal F4/80$^+$ macrophages is increased in the mouse colon in response to DSS treatment [10]. However, DSS-induced colitis studies related to dysbiosis have rarely been carried out.

Therefore, from the viewpoints of the interaction between the host and the gut microbiota and the interaction between the enteric nervous system and the enteric mucosal immune system, we investigated the localization of GPR41-IRs, enteric nerve fibers and F4/80$^+$ macrophages in the colonic mucosa of mice with DSS-induced colitis to elucidate the pathophysiological roles of GPR41 in intestinal inflammation.

2. Material and methods

2.1. Animal

Male BALB/c mice aged 8 weeks and male C57BL/6 mice aged 10–12 weeks were purchased from SLC (Shizuoka, Japan). All of the animal care procedures and experiments were approved by the Animal Experiment Committee at the University of Toyama (authorization No. A2018 INM-3).

2.2. DSS-induced colitis model

BALB/c mice were treated with 3% DSS (36–50 kDa; MP Biomedicals, Santa Ana, CA, USA) in the drinking water for 7 days [10]. To assess the severity of colitis, body weight, stool consistency, and blood in the stool were monitored daily. The disease activity index (DAI) was the average of 2 parameters: diarrhea (0, normal; 1, soft stools; 2, loose stools; 3, mild diarrhea; 4; severe diarrhea) and blood in the stool (0, normal; 1, faint bleeding; 2, slight bleeding; 3, gross bleeding; 4, severe bleeding). Whole colons were excised on day 7 after DSS administration.

2.3. Measurement of IL-1β and IL-6 mRNA expression

Cytokine mRNA expression was measured in the excised distal colon as described previously [10]. Briefly, real-time PCR amplification of IL-1β, IL-6 and GAPDH was performed using TB Green Premix EX Taq (Takara Bio, Ohtsu, Japan). The levels of target mRNAs were normalized to those of GAPDH as an internal control for each sample. The primer sequences are shown in Table 1.

2.4. Antibiotic-induced gut dysbiosis model (ABX model)

C57BL/6 mice received an oral antibiotic cocktail for 10 days [11]. The antibiotic cocktail consisted of ampicillin (1 mg/mL), vancomycin (0.5 mg/mL), metronidazole (1 mg/mL), gentamicin (1 mg/mL) and neomycin (1 mg/mL). Mice were sacrificed on day 10 after antibiotic treatment. Each cecum was carefully dissected and weighed.

2.5. Immunohistochemical and histological analysis

Immunohistochemistry and histology were performed according to the procedure described in previous reports [12]. Briefly, the excised distal colons were fixed with 4% paraformaldehyde for 24 h at 4 °C. The frozen sections (10 μm) for histology were then routinely stained with hematoxylin and eosin (H&E). The frozen sections (30 μm) for immunohistochemistry were exposed to a rabbit anti-human GPR41 antibody (ab236654, Abcam, Cambridge, MA, USA), goat anti-rat CGRP antibody (ab36001, Abcam) or rat anti-mouse F4/80 (MCA497GA, BioRad, Hercules, CA, USA) for 12–18 h and then incubated with the appropriate secondary antibodies for 2 h (Alexa 488 donkey anti-rabbit IgG), Cy3 donkey anti-rabbit IgG, Cy3 donkey anti-goat IgG or Alexa 488 donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The immunostained sections were examined using a confocal microscope (LSM700 & LSM780, Carl Zeiss, Oberkochen, Germany). To avoid selection bias, three representative preparations were selected from each mouse colon and then three mucosal sites of each preparation were quantitatively analyzed using open-source software ImageJ (ImageJ bundled with 64-bit Java 1.8.0_172; a Java-based image processing program developed at the NIH) [13].

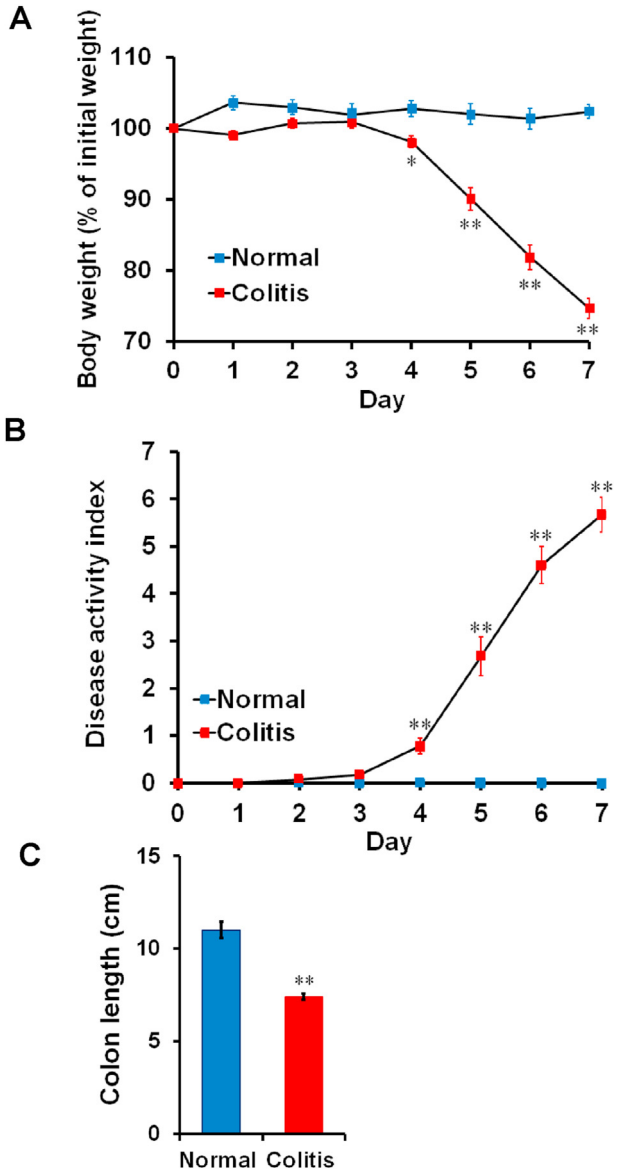

Figure 1. DSS-induced colitis model. Treatment with 3% DSS in BALB/c mice for 7 days (A) induced body weight loss, (B) increased the disease activity index (diarrhea and rectal bleeding; DAI) score, (C) shortened the colon. The data are presented as the mean ± SE of 7 mice, *P < 0.05 and **P < 0.01 compared with normal mice by Student's t-test.

Table 1. Primer sequences.

Gene	Primer sequence, 5′-3′	
	Forward	Reverse
Gapdh	TGACCACAGTCCATGCCATC	GACGGACACATTGGGGGTAG
Il-1β	CTGTGTCTTTCCCGTGGACC	CAGCTCATATGGGTCCGACA
Il-6	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC

2.6. Statistical analysis

The data are presented as the mean ± SE. Statistical analyses were performed using an unpaired Student's t-test. Probability (P) values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. DSS-induced colitis model

To generate dysbiosis conditions related to IBD, we used a DSS-induced colitis model with BALB/c mice. In mice with colitis, body weight loss, diarrhea and rectal bleeding first appeared on day 4 after starting DSS treatment. Significant body weight loss was observed on day 7 in the mice with colitis (Figure 1A, colitis mice: 78.7 ± 1.9% of initial body weight; normal mice: 102.4 ± 1.0% of initial body weight, $n = 7$, $P < 0.01$). The DAI score (Figure 1B, 3.4 ± 0.2, $n = 7$, $P < 0.01$), calculated from the diarrhea score and rectal bleeding score on day 7 indicated severe colitis. The colons of mice with colitis were significantly shortened compared to those of normal mice (Figure 1C, colitis mice: 7.9 ± 0.2 cm; normal mice: 11.9 ± 0.4 cm, $n = 7$, $P < 0.01$). The expression of IL-1β and IL-6 mRNA on day 7 was significantly upregulated in the colons of mice with colitis compared with those of normal mice (IL-1β: colitis mice: 17.4 ± 5.4, normal mice: 1.0 ± 0.3, $n = 7$, $P < 0.05$; IL-6: colitis mice: 377.6 ± 164.2, normal mice: 1.0 ± 0.6, $n = 7$, $P < 0.05$). Destruction of epithelial integrity and crypt architecture was observed on day 7 in the colons of mice with colitis.

3.2. GPR41 expression in the colon of mice with DSS-induced colitis

According to a previous report using GPR41-mRFP transgenic mice, GPR41 is expressed in the cell somas of submucosal neurons and myenteric neurons but not nerve fibers in the mucosa and enteric ganglia [5].

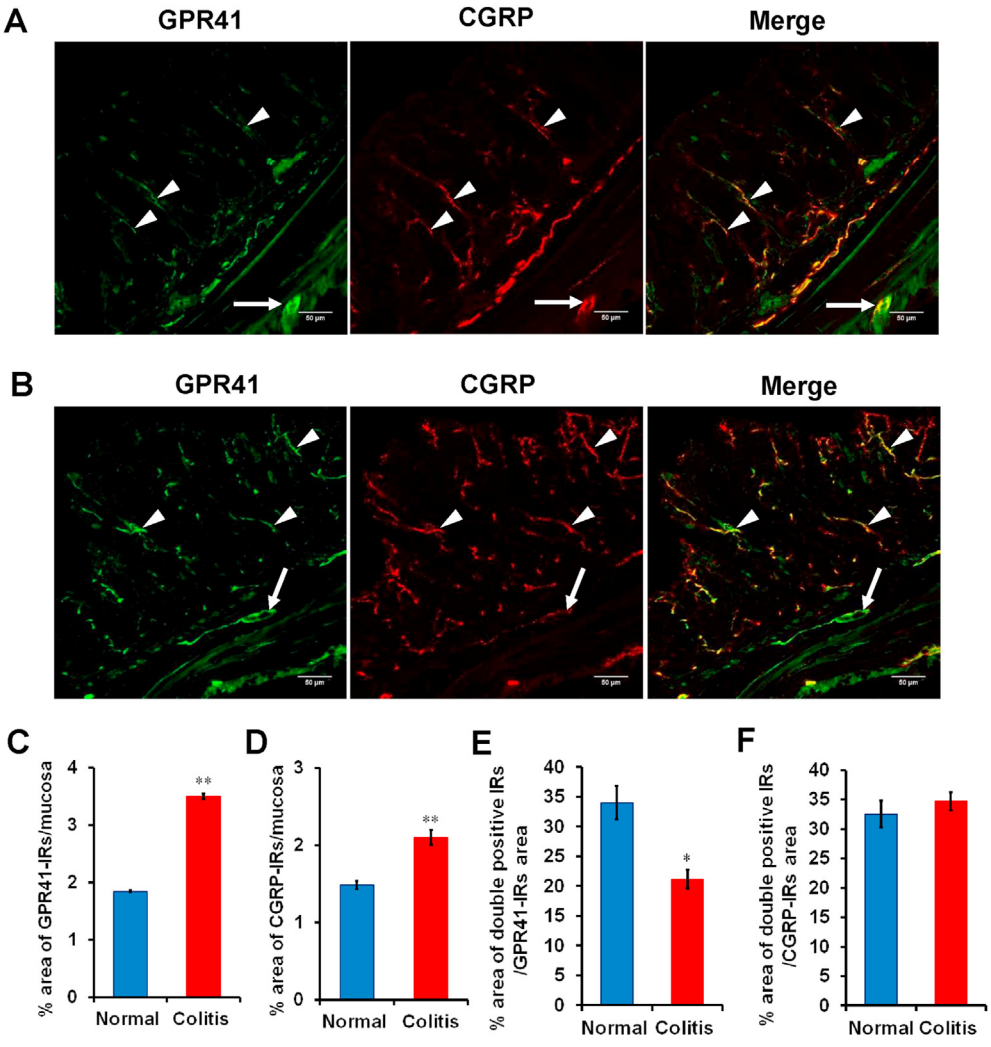

Figure 2. GPR41 and CGRP expression in the colon of mice with DSS-induced colitis. Treatment with 3% DSS elevated GPR41-IRs and CGRP-IRs in the mouse colon. (A) Representative images of GPR41-IRs, CGRP-IRs and colocalization of GPR41 and CGRP in the colon of normal mice, (B) representative images of GPR41-IRs, CGRP-IRs and colocalization of GPR41-IRs and CGRP-IRs in the colon of mice with colitis, (C) quantitative analysis of GPR41-IRs in the lamina propria of the mouse colon, (D) quantitative analysis of CGRP-IRs in the lamina propria of the mouse colon, (E) quantitative analysis of the ratio of double-positive IRs for GPR41 and CGRP to GPR41-IRs in the lamina propria of the mouse colon and (F) quantitative analysis of the ratio of double-positive IRs for GPR41 and CGRP to CGRP-IRs in the lamina propria of the mouse colon. Arrowheads: nerve fibers; arrow: neurons. The data are presented as the mean ± SE of 5 mice, *$P < 0.05$; **$P < 0.01$ compared with normal mice by Student's t-test.

In addition, there is no report about GPR41 in enteric nerve fibers using immunohistochemistry. By contrast, in the present study, GPR41-IRs were observed with immunohistochemistry in the nerve-like fibrous structures (Figure 2A and B: arrowheads) of both the lamina propria and muscularis layer and in the cell somas of enteric neurons (Figure 2A and B: arrow), indicating that GPR41 is expressed in both nerve fibers and cell somas of enteric neurons, especially myenteric neurons. A plausible explanation for the discrepancy between the present immunohistochemical data using anti-GPR41 antibody (ab236654) and the previous data using GPR41-mRFP transgenic mouse is that immunohistochemistry using antibodies against GPR41 was much more sensitive and accurate than GPR41 reporter mouse method to detect GPR41 in the nerve fibers of the mouse colonic mucosa. Furthermore, GPR41-IRs significantly increased approximately 2-fold in the colonic lamina propria of mice with colitis compared to that of normal mice (Figure 2C, $n = 5$, $P < 0.01$).

3.3. CGRP expression in the colon of mice with DSS-induced colitis

There is no information available concerning which type of enteric neuron GPR41 is located in. The enteric nervous system, composed of neurons, such as intrinsic sensory neurons, interneurons and excitatory and motor neurons [14], forms intrinsic neural circuits and plays an essential role in the maintenance of gut integrity, and these functions largely depend on rapid alarms from sensory neurons in the gut [15]. CGRP is presumed to be a neurotransmitter in cholinergic intrinsic sensory neurons in the mouse enteric nervous system [12, 14]. It is well known that the local release of CGRP from terminals of sensory neurons is critical for the development of neurogenic inflammation. Furthermore, our previous study indicates that CGRP functions in the intestine as a mediator between the nervous system and the immune system in a murine food allergy (FA) model [12].

CGRP-IRs were observed in nerve fibers of the colonic lamina propria (Figure 2A and B: arrowheads) and enteric neurons (Figure 2A and B: arrow). CGRP expression in the colonic lamina propria was significantly upregulated by 41.4 ± 8.9% in the mice with colitis compared to the normal mice (Figure 2D, $n = 5$, $P < 0.01$). These results are in close agreement with our previous study showing that CGRP-immunoreactive nerve fibers juxtaposed with mucosal mast cells are specifically increased in the colonic mucosa of FA mice [12].

DSS-induced colitis reportedly causes bacterial overgrowth in the colonic lumen with commensal enterobacteria such as *Escherichia coli* with potentially proinflammatory properties [16]. Toll-like receptor-4, which recognizes lipopolysaccharide (membrane component of gram-negative bacteria), is reportedly expressed in myenteric neurons of the mouse colon [17], suggesting that enteric neural circuits might be directly activated by enteric bacteria. One possible explanation for the increased GPR41 and CGRP expression is the activation of enteric neurons, especially sensory neurons, by easy penetration of SCFAs and enteric bacteria into the lamina propria through colitis-induced disruption of the epithelial barrier function in the colon of mice with colitis.

3.4. Colocalization of GPR41 and CGRP in the colonic lamina propria in DSS-induced colitis model

GPR41-IRs partly colocalized with CGRP-IRs in the colonic lamina propria and muscularis layer of normal mice (Figure 2A) and the ratio of double-positive IRs to GPR41-IRs or to CGRP-IRs in the colonic lamina propria was 34.0 ± 2.8% (Figure 2A and E) or 32.5 ± 2.3% (Figure 2A and F) in the normal mice ($n = 5$), indicating that GPR41 is located in the nerve fibers and cell somas of cholinergic intrinsic sensory neurons in the mouse colon.

The ratio of double-positive IR to GPR41-IRs in the colonic lamina propria was significantly reduced in the mice with colitis (Figure 2E; colitis mice: 21.2 ± 1.6%; normal mice: 34.0 ± 2.8%, $n = 5$, $P < 0.05$), whereas the ratio of double-positive IRs to CGRP-IRs was not affected by DSS treatment (Figure 2F). These results suggest that GPR41 expression in nerve fibers of enteric neurons other than sensory neurons is upregulated by DSS treatment, which may cause a disturbance of intrinsic neural circuits and subsequently result in disorders of colonic motility such as diarrhea that are typically observed in colitis mice.

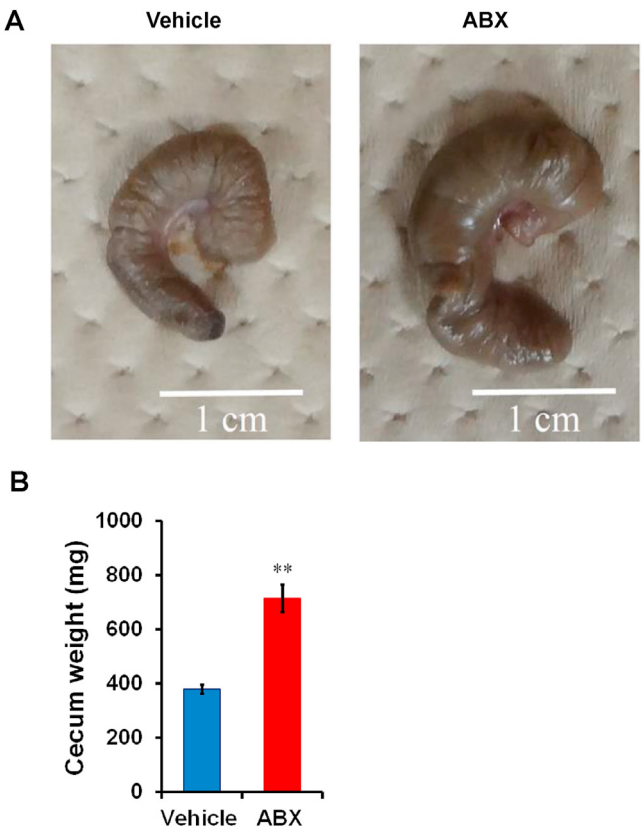

Figure 3. Antibiotic-induced gut dysbiosis. Antibiotic treatment for 10 days resulted in a massive increase in cecal weight and size in mice. (A, B) Effect of antibiotic treatment for 10 days on the mouse cecum. The data are presented as the mean ± SE of 7 mice, **$P < 0.01$ compared with normal mice by Student's t-test.

3.5. Expression of GPR41 and CGRP in the colonic lamina propria in ABX model

ABX model has been frequently used to study the interaction between the gut microbiota and the host in terms of gut homeostasis, luminal signaling and metabolism and is considered to be a more reasonable model than the germ-free mouse model for studying the causality of microbiota-dependent effects.

Antibiotic treatment for 10 days significantly enlarged the mouse cecum (Figure 3A and B, ABX mice: 712.9 ± 50.5 mg; vehicle-treated mice: 378.6 ± 16.8 mg, $n = 7$, $P < 0.01$), indicating dysbiosis of the gut microbiota [18, 19]. However, antibiotic treatment failed to affect the length of the small intestine and colon, and to cause antibiotic-induced diarrhea. GPR41-IRs were significantly decreased by 20.8 ± 3.4% in the colonic lamina propria of ABX mice compared to that of vehicle-treated mice (Figure 4A, B and C, $n = 6–7$, $P < 0.01$). Down regulation of GPR41 expression in the colonic lamina propria is probably attributed to a reduction in the number of enteric bacteria and alteration of the gut microbiota composition after antibiotic treatment. Zarrinpar et al. demonstrated that in normal mice, the gut microbiota composition is dominated by Firmicutes species (butyrate-producing bacteria) and Bacteroidetes species, whereas after antibiotic treatment, the gut microbiota composition is shifted to Proteobacteria [20]. These changes are also accompanied by significantly decreased SCFAs, which probably resulted in downregulation of GPR41 expression.

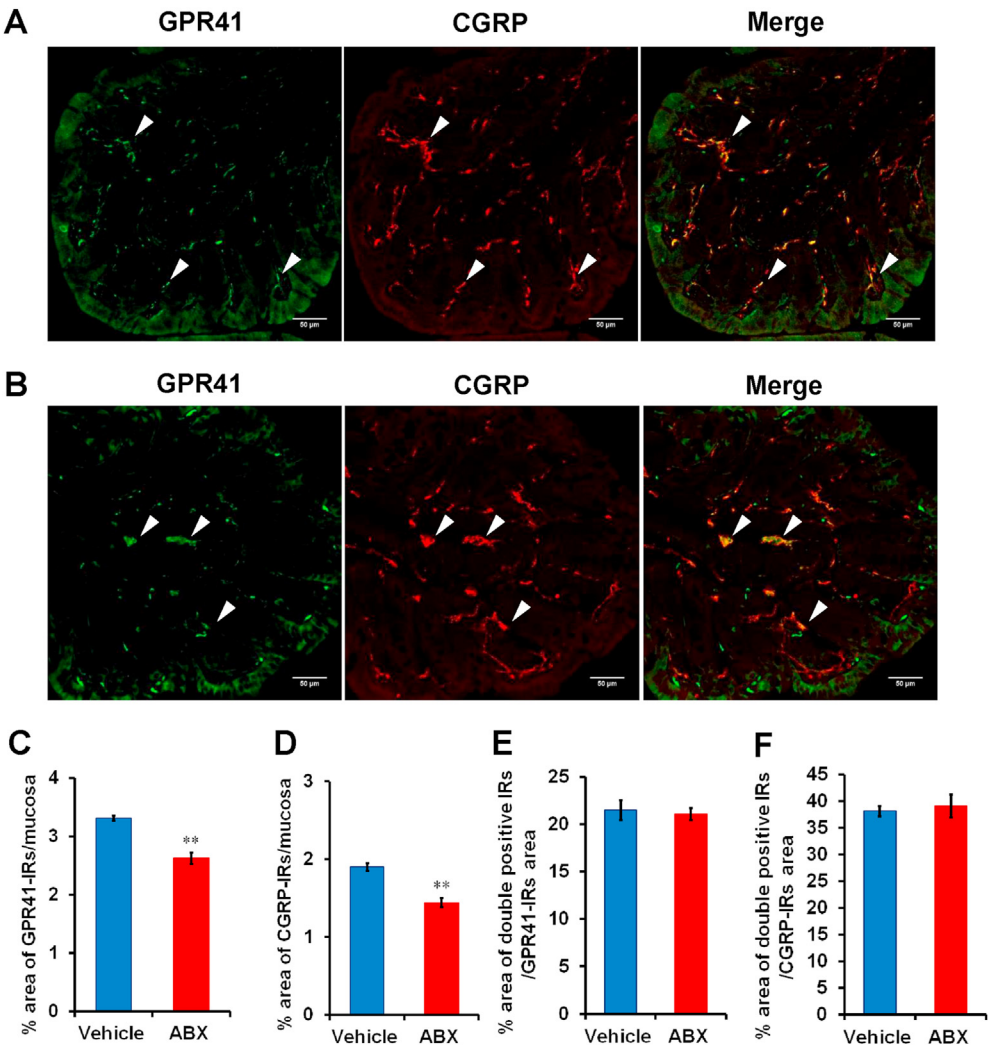

Figure 4. GPR41 and CGRP expression in the ABX mouse colon. Effect of antibiotic treatment for 10 days on GPR41-IRs and CGRP-IRs in the mouse colon. (A) Representative images of GPR41-IRs, CGRP-IRs and colocalization of GPR41 and CGRP in the colon of vehicle-treated mice, (B) representative images of GPR41-IRs, CGRP-IRs and colocalization of GPR41-IRs and CGRP-IRs in the colon of ABX-treated mice, (C) quantitative analysis of GPR41-IRs in the lamina propria of the mouse colon, (D) quantitative analysis of CGRP-IRs in the lamina propria of the mouse colon, (E) quantitative analysis of the ratio of double-positive IRs for GPR41 and CGRP to GPR41-IRs in the lamina propria of the mouse colon and (F) quantitative analysis of the ratio of double-positive IRs for GPR41 and CGRP to CGRP-IRs in the lamina propria of the mouse colon. Arrowhead: nerve fibers. The data are presented as the mean ± SE of 6–7 mice, **P < 0.01 compared with normal mice by Student's t-test.

CGRP expression in the colonic lamina propria was significantly reduced by 24.2 ± 3.6% in ABX mice compared to vehicle-treated mice (Figure 4A, B and D, n = 6–7, P < 0.01). GPR41-IRs colocalized with CGRP-IRs in the colonic lamina propria of normal mice and ABX mice (Figure 4A, B, E and F). The ratio of double-positive IRs to GPR41-IRs or CGRP IRs was unaffected by antibiotic treatment, indicating that antibiotic treatment did not affect the composition of either GPR41-positive nerve fibers or CGRP-positive sensory nerve fibers and, therefore, did not cause antibiotic-induced diarrhea. These results suggest that antibiotic-induced dysbiosis may not influence enteric neural circuits.

3.6. Morphological interaction between macrophages and GPR41-positive nerve fibers in the colonic mucosa of mice with DSS-induced colitis

To date, the mechanisms and pathways whereby GPR41 may be involved in the pathology of enteric immune diseases remain obscure.

We found that intestinal F4/80$^+$ macrophages were located in the colonic lamina propria of mice with colitis and that the proportion of intestinal macrophages was significantly increased in the inflamed colonic mucosa after DSS treatment compared to that of normal mice (Figure 5A, C and D, n = 3, **P < 0.01), as we previously reported [10]. GPR41-immunoreactive nerve fibers were found in the colonic lamina propria of mice with colitis (Figure 5C and D), many of which were juxtaposed with F4/80$^+$ macrophages (Figure 5C and D). The number of F4/80$^+$ macrophages in close proximity to GPR41-immunoreactive nerve fibers was significantly elevated (Figure 5B, n = 3, *P < 0.05) in the inflamed colonic mucosa after DSS treatment (Figure 5D) compared to that of normal mice (Figure 5C).

We already reported that CGRP-positive intrinsic sensory neurons, through neuro-immune interactions with mucosal mast cells, are deeply involved in the pathology of food allergies [12, 21, 22]. Furthermore, microbiota-driven crosstalk between muscularis macrophages and enteric neurons reportedly regulates gastrointestinal motility [23]. Taken together, these results suggest that the neuro-immune interaction of GRP41-positive intrinsic sensory neurons and F4/80$^+$ macrophages in the colonic lamina propria might be involved in the maintenance of intestinal homeostasis and the pathology of intestinal immune diseases accompanied by dysbiosis.

4. Conclusion

In conclusion, the network of microbiota, SCFA-sensing intrinsic sensory neurons and mucosal macrophages in the gut should be tightly regulated to maintain intestinal homeostasis. Disruption of this network caused by noxious environmental stimuli such as inflammation can affect sophisticated gut ecosystems consisting of the enteric immune system, enteric nervous system and microbiota, leading to the chronic activation of the intestinal immune responses and resulting in intestinal disorders, such as IBD.

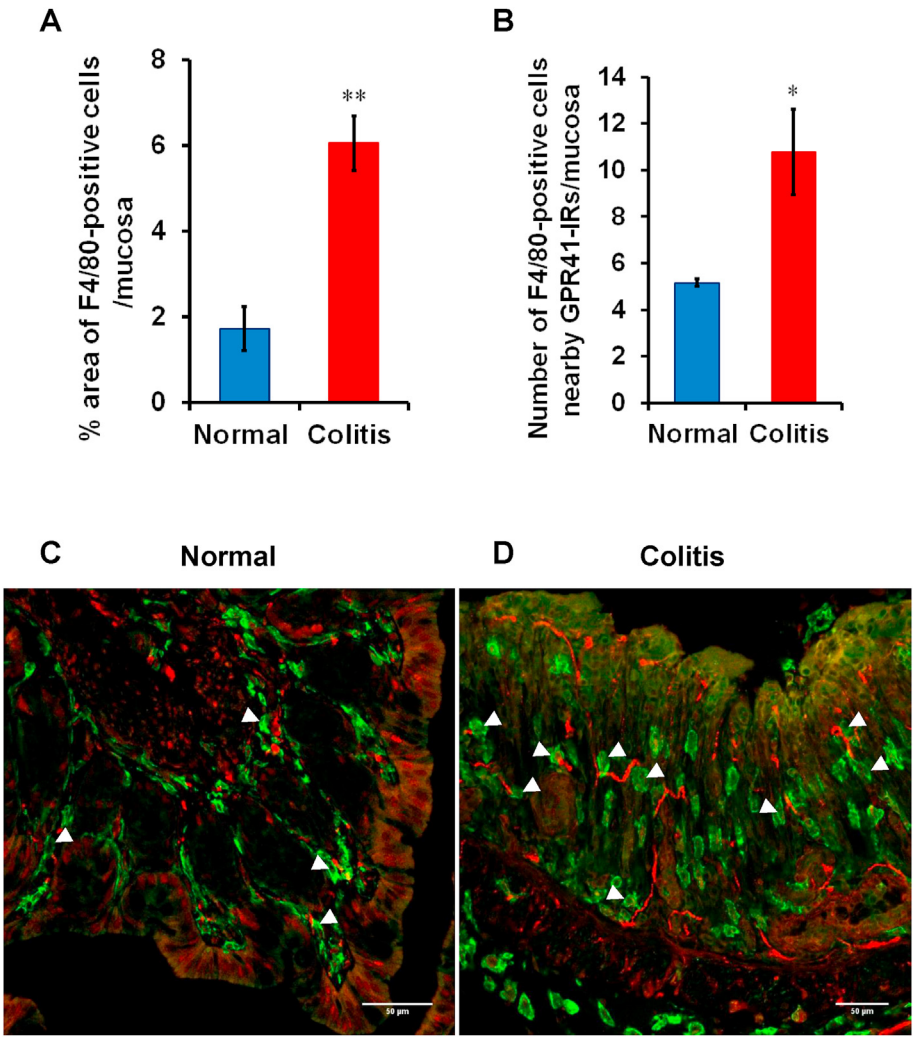

Figure 5. Morphological proximity between GPR41-positive nerve fibers and F4/80^{+} macrophages in the colon of mice with DSS-induced colitis. Treatment with 3% DSS increased GPR41-IRs and F4/80-IRs in the colon of mice with colitis. (A) Quantitative analysis of F4/80 + macrophages in the colon. (B, C, D) The number of F4/80 + macrophages located in close proximity to GPR41-immunoreactive nerve fibers (arrowhead) was significantly elevated in the inflamed colonic mucosa after DSS treatment compared to that of normal mice. The data are presented as the mean ± SE of 3 mice, *P < 0.05; **P < 0.01 compared with normal mice by Student's *t*-test.

Declarations

Author contribution statement

M. Kadowaki: Conceived and designed the experiments; Wrote the paper.

A. Hertati: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

S. Hayashi: Analyzed and interpreted the data; Wrote the paper.

R. Kato and K. Miyata: Performed the experiments; Contributed reagents, materials, analysis tools or data.

H. Ogata: Performed the experiments.

T. Yamamoto: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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