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NERVE FIBER OUTGROWTH IS INCREASED IN THE INTESTINAL MUCOSA OF PATIENTS WITH IRRITABLE BOWEL SYNDROME

Short Title: IBS and neuroplasticity.

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Abbreviations used in this paper: Brain derived neurotrophic factor, BDNF; irritable bowel syndrome, IBS; nerve growth factor, NGF; growth associated protein 43, GAP43; neuronal specific enolase, NSE; retinoic acid, RA; transient receptor potential vanilloid type-1, TRPV-1; tyrosine kinase receptor A, NTRK1.

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ABSTRACT

BACKGROUND & AIMS: Mediators released by the intestinal mucosa of patients with irritable bowel syndrome (IBS) affect the function of enteric and extrinsic sensory nerves, which can contribute to the development of symptoms. Little is known about the effects of mucosal mediators on intestinal neuroplasticity. We investigated how these mediators affect the phenotypes of colonic mucosa nerve fibers, neuron differentiation, and fiber outgrowth.

METHODS: We analyzed mucosal biopsy samples collected from 101 patients with IBS and 23 asymptomatic healthy individuals (controls). We measured levels of neuronal specific enolase (NSE), growth-associated protein 43 (GAP43), nerve growth factor (NGF), and tyrosine kinase receptor A (NTRK1) by immunohistochemitry and ELISA. Primary rat enteric neurons and human SH-SY5Y cells were incubated with supernatants from the mucosal biopsies and analyzed by morphometric and PCR analyses.

RESULTS: Compared with mucosal tissues of controls, mucosa from patients with IBS had a significant increase in the area of lamina propria occupied by NSE-positive (57.7% increase) and GAP43-positive fibers (56.1% increase) and staining density of NGF (89.3% increase) ($P<.05$ for all). Levels of NGF protein were also increased in tissues from patients with IBS vs controls (18% increase; $P=.16$) along with levels of NTRK1 (64% increase; $P<.05$). Mucosal supernatants from tissues of patients with IBS induced higher levels of neuritogenesis in primary culture of enteric neurons, compared with controls, and more NGF-dependent neuronal sprouting in SH-SY5Y cells.

CONCLUSIONS: Nerve fiber density and sprouting, as well as expression of NGF and NTRK1, are significantly increased in mucosal tissues of patients with IBS. Mucosal mediators participate to these neuroplastic changes.

KEYWORDS: mast cells, enteric nerves, nerve growth factor, growth-associated protein 43

INTRODUCTION

The irritable bowel syndrome (IBS) is characterized by abdominal pain/discomfort and bowel habit changes occurring in the absence of an organic cause. IBS represents one of the most common gastrointestinal disorders with an overall prevalence estimated around 10-15 % in Europe and in the United States.[1] The pathogenesis of IBS remains poorly understood but data suggest that symptoms may arise as a variable combination of psychological factors (e.g., anxiety and depression), changes in intestinal motor function and increased visceral perception.[2] There has been recent remarkable advancement in the understanding of the basic mechanisms underlying bowel dysfunction in patients with IBS.[2] The fact that a subgroup of patients develops IBS following an acute episode of bacterial or viral gastroenteritis strongly supports the involvement of microbiological/organic factors in the pathogenesis of IBS.[3] Attention has also been directed to the complex interplay between gut microbiota,[4] mucosal barrier,[5] enteroendocrine cells,[6] immunocytes and enteric nerves.[7,8] Abnormal release of bioactive factors in the intestinal milieu, including serotonin,[9] histamine and mast cell tryptase,[10, 11] has been shown to impact on gut nerve intrinsic/extrinsic activity in a receptor-dependent manner. Mucosal extracts obtained from the colon of IBS patients adoptively transferred to naïve animals or human tissues increased intestinal submucous neuron excitability,[12] mesenteric sensory nerve activity,[11] and visceral or somatic sensitivity.[13, 14] The long-term effect of these and other neuroactive substances on the enteric nervous system has yet to be studied, but may induce nerve plasticity with potential consequences for bowel physiology and symptom generation. A previous study by Akbar et al. showed that the overall density of mucosal innervation as well as substance P and transient receptor potential vanilloid type-1 (TRPV-1) nerve staining was increased in patients with IBS compared with controls.[15] Interestingly, TRPV-1 staining was significantly correlated with abdominal pain perception.[15] A previous study supports the concept that neurotrophic factors contribute to visceral hypersensitivity in IBS.[16] NGF evokes nerve fiber growth and pain transmission through its interaction with the preferred receptor tyrosine kinase receptor A (NTRK1).[17] Mucosal mast cells

may be key players in this scenario since their increased vicinity to nerve terminals correlates with the severity and frequency of abdominal pain in IBS.[10] In addition, mast cells are proficient natural producers of factors affecting nerve function and growth, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF).[18, 19,20] Furthermore, mucosal mast cell infiltration in the colon was associated with an NGF-dependent increase in nerve fiber density and synaptogenesis in the neonatal maternal deprivation model of IBS.[21]

We hypothesized that increased tissue infiltration of mast cells in the colonic mucosa of IBS patients induces neuroplastic changes and neuronal sprouting via the release of NGF. In order to test this hypothesis we assessed in patients with IBS and controls, nerve fiber density, nerve sprouting (as assessed by the quantification of mucosal growth associated protein [GAP]43), NGF amount and the preferential NGF receptor NTRK1 expression. We also evaluated the impact of IBS mucosal mediators and NGF on neuronal differentiation in a human neuroblastoma cell line and in primary cultures of rat enteric nervous system.

MATERIALS and METHODS

Subjects and patients

IBS patients were diagnosed according to Rome III criteria.[1] Controls were recruited by public advertisement or from asymptomatic patients undergoing colonoscopy for colorectal cancer screening or follow-up of polyposis. All patients and subjects included in the study were seen at the Department of Medical and Surgical Sciences of the University of Bologna. We enrolled a total of 23 controls (12 F; mean age 42.9 years, range 20-70 years) and 101 Rome III IBS patients (67 F; mean age 40 years, range 18-69 years). All the experiments involved samples from at least 5 controls and 10 IBS patients. To reduce the number of experimental conditions and in order to identify a clear-cut bowel habit phenotype, we included mainly patients with IBS with diarrhea (IBS-D) (n=54) and with constipation (IBS-C) (n=40). Only for the experiments with primary cultures of rat myenteric neurons (see below and Supplementary material), we included also 7 mixed IBS patients (IBS-M). Exclusion criteria comprised the use of NSAIDs, corticosteroids and mast cell stabilizers, tricyclic antidepressant or serotonin selective reuptake inhibitors, major abdominal surgery, celiac disease (excluded by detection of anti-transglutaminase and anti-endomysial antibodies), allergic diseases (family and personal history and specific anti-IgE antibodies), asthma and other organic or severe psychiatric disorders as assessed by history taking and appropriate consultations and laboratory tests. Patients and controls gave written informed consent and the study protocol was approved by the local Ethic Committee and conducted in accordance with the Declaration of Helsinki.

In all cases we obtained 10 mucosal biopsies from the proximal descending colon during colonoscopy. Two biopsies were fixed in buffered 10% formalin and processed for H&E histology to exclude microscopic colitis and to perform immunohistochemistry (see below). Four biopsies

were used to obtain mucosal mediators (see below) and four biopsies were used for protein extraction.

Mucosal mediator release

Spontaneous release of mucosal mediators was obtained using a previously described method.[10] Briefly, upon removal, 4 mucosal biopsies were rapidly immersed in hard plastic tubes containing 1 ml of Hank's solution (Gibco BRL-Life Technologies, Carlsbad, CA) heated at 37℃ and continuously oxygenated with a mixture of 95% O_2 and 5% CO_2. After 25 min incubation, samples were centrifuged at 200 *g* for 10 min and 400-600 µl of the bathing solution were collected, filtered with a 0.22 µm syringe filter and stored at -20˚C. At the end of the release experiment, biopsies were blotted and weighed.

NGF protein assay

NGF content was quantified in homogenized colonic biopsies using an enzyme immunoassay kit according to the manufacturer's instructions (Emax Immuno Assay System, Promega, Milano, Italy). The NGF ImmunoAssay System is designed for sensitive and specific detection of NGF in an antibody sandwich format (less than 3% cross-reactivity with other neurotrophic factors). For the NGF assay, biopsy specimens from 17 controls and 46 patients (14 IBS-C, and 32 IBS-D) with IBS were homogenized using tissue protein extraction reagent with the addition of a protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions and stored at -80℃ until assay. Protein content was measured using a Comassie blue-based colorimetric assay (Bradford assay, Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry

Immunohistochemistry was performed by application of validated protocols in use in our laboratory (Supplementary Material). The quantification of immunoreactivity was performed by 2 operators in a blind fashion using a Leitz (Weitzlar, Germany) Orthoplan microscope (25X objective) equipped with a computer-assisted analysis system (Cytometrica software; C&V, Bologna, Italy), by slight modifications of a previously published method[10] (Supplementary Material).

Western blot analyses

Protein expression of GAP43 was evaluated by Western blot analysis in rat primary cultures of myenteric neurons exposed to controls or IBS supernatants (Supplementary Material).

Primary cultures of rat myenteric neurons and SH-SY5Y

Primary cultures of rat enteric nervous system were obtained using previously validated methods (Supplementary Material). For morphometric analyses on primary cultures, cells were plated on glass coverslips and were maintained for 24 hours in Neurobasal-B27 medium containing biopsy supernatants from controls or IBS patients diluted 1/2. Cells were then fixed in 4% paraformaldehyde, immunostained for tubulin, beta III (Tuj1) and analyzed for neuronal morphometry. For protein expression analyses, cells were plated in 24-well dishes and maintained in culture at 37°C in a humidified chamber with 95% O_2 : 5% CO_2 for 14 days. Half of the medium was replaced every 24 hours. On day 13, cultures were incubated with biopsy supernatants from controls or IBS patients in duplicate for 48 hours (with renewal at 24 hours). Then, cells were lysed in RIPA buffer (Millipore, Guyancourt, France) containing 2 mM sodium orthovanadate and proteases inhibitors (Roche, Meylan, France).

SH-SY5Y cells were obtained from the European Collection of Cell Cultures (Salisbury, UK) and grown as detailed in Supplementary Materials. SH-SY5Y were exposed to fresh medium containing supernatants (diluted 1/10 v/v with culture medium) or Hank's solution (diluted 1/10 v/v with culture medium) for 96 hours. Cells treated with Hank's solution were used as controls; retinoic acid (RA, 10 μM, Sigma-Aldrich, Milan, Italy) was used as positive control for neuronal differentiation.[22] The medium containing supernatants, Hank's or RA was replaced after 48 hours. The experimental protocol for NGF responses in SH-SY5Y, NGF immune-blocking experiments is reported as Supplementary Material.

Quantitative real time RT-PCR

The effect of IBS/control supernatants on *GAP43* gene expression was evaluated by real-time PCR. SH-SY5Y cells were incubated for 48 hours with control/IBS supernatants or RA (10 μM as positive control) and then RNA was extracted using RNeasy mini kit (Qiagen, Milan, IT). Further details are described in Supplementary Material.

Statistical analysis

Data are reported as mean values ± SE. All data were analyzed by means of the Mann–Whitney U and two way ANOVA followed by Bonferroni post-hoc test or Dunn post-test (for experiments with primary enteric neurons), as appropriated. All statistical analyses were performed using SPSS for Windows version 13 0 (SPSS Inc, Chicago, IL, USA). Two-tailed *P* values less than 0.05 were considered significant.

RESULTS

Mucosal nerve fibers and GAP43

Nerve fibers were identified in the colonic mucosa with immunohistochemistry using an antibody against the pan-neuronal marker NSE. In both controls and IBS patients, cross-sectioned neuronal fibers were identified in the *lamina propria* surrounding mucosal crypts (Figure 1*A*). Quantitative analysis showed a 57.7% significant increase in the nerve fiber density in patients with IBS compared with controls (Figure 1*A*, *P<.05*). In order to assess if the increased neuronal fiber density of IBS was associated with markers for neuronal outgrowth, we stained mucosal biopsies with an anti-GAP43 antibody. GAP43 staining in the *lamina propria* was localized to structures resembling nerve fibers with a spotted type distribution around colonic crypts (Figure 1*B*). In ancillary experiments we established that GAP43 staining was localized in neuronal fibers as shown by GAP43/PGP 9.5 colocalization and absence of GAP43 in S-100β (glial marker) positive structures (data not shown). Compared with controls, the density of GAP43 positive fibers of IBS patients was increased by 56.1% (Figure 1*B*; *P<.05*).

NGF

To explore potential mechanisms underlying the increase of neuronal markers, we assessed NGF and NTRK1 expression in the colonic mucosa. The overall staining density of NGF was increased by 89.3% in patients with IBS compared to controls (Figure 2*A*; *P<.05*). NGF was expressed in different elements within the *lamina propria*. Most of the NGF staining was expressed in cell bodies resembling immunocytes. Based on previous knowledge indicating that mast cells are proficient producers of NGF,[18] we performed NGF and tryptase double labelling immunofluorecence experiments. The results showed that the vast majority of NGF staining was from tryptase-positive

mast cells (Figure 2*C*). NGF was occasionally expressed by enteroendcrine cells (chromogranin A positive). We found no colocalization of NGF immunoreactivity with other immune cells, including eosinophils (eotaxin-1-positive) and macrophages (CD68-positive) (data not shown). The concentration of NGF protein in mucosal biopsies was increased by 18% in IBS samples as compared with controls, although this did not reach statistical significance (12.1±0.7 *vs*. 10.3±0.7, Figure 2*B; P=.16*).

NTRK1

We assessed the expression of NTRK1 receptors in the human colon with quantitative immunohistochemistry. Compared with controls, the expression of NTRK1 was markedly increased (193.8% increase) in mucosal biopsies obtained from IBS patients (Figure 3*A*). Staining for NTRK1 colocalized with NSE positive neuronal elements (Figure 3*B*). Neuronal expression of NTRK1 was also confirmed in experiments performed on human colonic specimens obtained from surgical resections (Figure 3*C*). In addition to neurons, NTRK1 was also expressed by cell elements within the *lamina propria* resembling the shape and size of mast cells. Double staining experiments confirmed the expression of NTRK1 in a substantial proportion of tryptase positive mast cells (Figure 3*D*).

Impact of mucosal supernatants on enteric neurons outgrowth

To test whether mediators present in the mucosal milieu could promote neuronal outgrowth, primary cultures of rat myenteric plexus were incubated with mucosal supernatants from controls or IBS patients (Fig. 4*A, B*). Compared with neurons treated with control supernatants, the percentage of enteric neurons containing more than one neurite was increased after 24 hours incubation with IBS supernatants (56.7± 6.9% of total neurons in controls vs. 72.3± 2.1 in IBS,

P<.01). We next asked whether the mucosal supernatants from IBS-C, IBS-D and IBS-M subgroup of patients were equally potent to stimulate neuronal process outgrowth (Fig. 4*C*). We found that the number of neurons containing more than one neurite was increased in similar proportion by the supernatant derived from each IBS subgroups (56.7±6.9% of total neurons in controls *vs*. 73.8±2.5%, 70.5±3.5% and 78.0±2.8% of total neurons in IBS-C, IBS-D and IBS-M, respectively; **P<.05* as compared to controls). In addition, the effect of IBS supernatants on neurite elongation was analyzed by counting the number of long neurites defined by a length ≥ 130 µm (Fig. 4*D, E*). Although supernatants derived from IBS-D and IBS-M patients induced a trend towards an increase in the number of long neurites, the values did not reach statistical difference when compared to controls. However, the number of long neurites in IBS-M subgroup showed an increase when compared to IBS-C subgroup (Fig. 4*D, E*; % of neurites ≥ 130 µm is 5.0±1.5 for IBS-C and 15.4±2.6 for IBS-M, *P<.05*). The area of the neuronal cell body remained unchanged upon IBS supernatant treatment (data not shown). Altogether, these results suggest that the IBS-derived supernatants from all subgroups exert a trophic effect on the emergence of neuronal processes (increased number of neurites in all subgroups), but that differences exist in between groups regarding their ability to stimulate neurite elongation.

Impact of mucosal supernatants on SH-SY5Y differentiation

The effects of mucosal supernatants on neuritogenesis found in primary cultures of rat enteric neurons might be indirectly mediated by non-neuronal cell types also present in this culture system, such as glial cells. To test whether mucosal supernatants exert a cell-autonomous effect on neurons, we examined the impact of IBS mucosal supernatants on nerve differentiation and outgrowth in a neuronal cell line of human origin, SH-SY5Y. Figure 5 shows representative pictures of SH-SY5Y grown for 96 hours and immunolabelled for GAP43. The staining was localized to neuronal cell bodies and neuronal processes (Figure 5*A*). Neurons exposed to RA (10 µM) were

used as internal positive control of neuronal differentiation[21] (Figure 5*B*). Compared with neurons exposed to Hank's medium, used as negative control, RA evoked a 33% elongation of neuronal processes and increased neuronal process contacts (Figure 5*B*). Compared with negative controls, neurons treated with RA showed a lower total number of neurons, indicative of increased differentiation. SH-SY5Y exposed to supernatants obtained from patients with IBS showed a 13,6% significant increase in neurite outgrowth over that obtained with samples from controls (Figure 5*C-E*, *P<.001*). This effect is about half to that obtained with RA (23.6% increase, *P<.001*; Figure 5*B*). The number of differentiated cells induced by the treatment with IBS supernatants was similar to that induced by RA and increased by 18.7 % over that obtained after exposure of SH-SY5Y to control supernatants (Figure 5F, *P<.001*). There was no significant difference in the total number of neurites per cell in the different experimental groups.

We tested if NGF contained in mucosal supernatants of IBS samples could be involved in the observed neuronal differentiation. Neuronal sprouting induced by IBS supernatants was similar to that induced by NGF 50 ng/ml. Neuronal sprouting was NGF-dependent, as it was significantly inhibited by NGF neutralization with anti-NGF antibody, resulting in a neuronal differentiation comparable with that obtained in negative controls (i.e., cells treated with Hank's medium) (Figure 6).

GAP43 expression in rat myenteric neurons and SH-SY5Y

In order to confirm if the effect of mucosal supernatants seen on SH-SY5Y could be evoked also in enteric nerves, we assessed the expression of GAP43 protein in rat myenteric cultured neurons exposed to IBS supernatants. Rat primary enteric neuron cultures were preferred to human cultures based on our laboratory expertise. Western blot analysis showed that IBS supernatants evoked a 66% increase in GAP43 protein expression in myenteric neurons compared with controls (1.31±0.1 *vs*. 2.17±0.2, *P<.05*; Figure 7*A,B*). Compared to control supernatants, the treatment of

SH-SY5Y cells with IBS supernatants induced a 30% increase of GAP43 mRNA expression (2.63±1.0 vs. 4.05±0.8, *P=.39*; Figure 7*C*). RA induced a significant increase of GAP43 mRNA expression compared to controls (10.22±0.9 vs. 2.63±1.0, *P<.05*) and IBS (10.22±0.9 vs. 4.05±0.8, P<.01) supernatants (Figure 7*C*).

DISCUSSION

In this study we demonstrated that compared with controls, patients with IBS showed a higher density of mucosal nerve fibers and increased nerve outgrowth. These changes were accompanied with increased expression of NGF and NTRK1. Mediators from IBS biopsies evoked greater neurite elongation in rat enteric neurons and in human SH-SY5Y cells, suggesting a cell-autonomous mechanism underlying neuritogenesis. NGF participated to the SH-SY5Y neurite elongation.

In line with previous data,[15, 16, 23] we found a marked (57.7%) increase of NSE positive fibers in the colonic mucosa of IBS patients compared with controls. The increased neuronal sprouting was linked to a higher expression of the neuronal membrane phospho-protein localized in growth cones projecting from the neuronal *soma* GAP43.[24] GAP43 is involved in the polymerization of actin monomers leading to neurite elongation and is commonly used as a marker of neuronal sprouting. Although the contribution of these changes in the pathophysiology of IBS remains unknown, they could be implicated in the pathogenesis of visceral hypersensitivity. Indeed, neuroplastic changes are frequently associated with tissue dysfunction and the development and persistence of pain.[25] Accordingly, previous data showed that mucosal biopsies of patients with IBS had a higher immunoreactivity for TRPV-1.[15] GAP43 may also directly contribute to visceral hypersensitivity as its inhibition with propentofylline inhibits neuropatic pain in animal models.[26]

Several lines of evidence suggest that neuro-immune interactions play a key role in the pathophysiology and symptom generation in IBS.[27] Increased mucosal infiltration of mast cells has been previously demonstrated by us and others.[28] In addition, colonic mast cells in close vicinity to

mucosal innervation correlated with the frequency and severity of abdominal pain.[10] Mast cell proteases and histamine released from mucosal biopsies of patients with IBS, but not controls evoked activation of rat sensory fibers.[11] Proteases obtained from IBS patients induced proteinase receptor-2-dependent activation of pain pathways in mice.[13] In addition, colonic low-grade immune activation correlated with TRPV-1 immunoreactivity and with the severity of abdominal pain.[15]

Our results showed that NGF staining in the intestinal mucosa of IBS patients was significantly increased compared to controls. Although we found increased protein levels of NGF in intestinal biopsies of patients with IBS compared with controls, the values did not reach statistical significance. Nonetheless, NGF levels in IBS-C vs. controls were borderline significant. This could be ascribed, at least in part, to the heterogeneity of IBS population as we found some samples with high levels of NGF while other showed levels comparable with those found in controls, or a type two error as the power analysis yielded a 71% value. Alternatively, the sensitivity of our NGF assay was too low to detect small differences in the other IBS subgroups. NGF is a key mediator involved in the immune-neural signaling for several reasons. Mast cells are proficient producers of NGF[18] and physiological studies suggest that this neurotrophin is involved in the maintenance of mast cell hyperplasia and activation.[29] In addition, NGF is a key stimulus for neuronal sprouting and is a key mediator involved in pain transmission.[30] Upon its release into the tissue, NGF evokes the excitability of sensory nociceptive fibers by altering the expression of sodium channels, increasing the expression of certain receptors such as TRPV1 and key sensory neuropeptides involved in the transmission of pain stimuli.[32] These results are in line with previous evidence indicating that compared to controls, IBS patients have increased mucosal levels of neurotrophins, including BDNF in adult patients[16] and NGF in a pediatric population of IBS patients.[31] Our results showed that most NGF staining was present in mucosal mast cells, as demonstrated by overt colocalization of NGF on tryptase positive cells, but not on other immune cells (e.g., eosinophils and macrophages). The implication of mast cell-derived NGF is also supported by studies in the

maternal deprivation rat model of IBS. In this model NGF released by mast cells was identified as a key mechanism underlying the nerve sprouting occurring 14 weeks after separation.[21]

We postulate that NGF produced by mast cells would act on neurotrophin receptors located on nerve fibers, leading to nerve sprouting. NGF acts on its preferential receptor NTRK1; NGF-NTRK1 coupling leads to the endosomic internalization of the ligand-receptor complex, which promotes neurite elongation, predominantly via RAS/RAF/MEK/MAPK pathways. In addition, NTRK1 accelerates neuronal differentiation by increased transcription of GAP43.[17] To provide the biological plausibility of a potential effect of NGF on neuronal fibers in IBS, here we showed that NTRK1 is widely expressed on neuronal fibers both in the mucosa and within the myenteric and submucous plexus of human colon. In addition to the expression of NTRK1 on nerve fibers, we found a significant NTRK1 staining on mucosal mast cells as demonstrated by the colocalization of tryptase positive mast cells and NTRK1 immunoreactivity. This is in keeping with previous studies indicating that mast cells express both NGF and NTRK1 on cellular surface.[32] Data suggest that mast cell-derived NGF maybe also involved in the maintenance of mast cell hyperplasia and activation via up-regulation of NTRK1 expression by mast cells.[33]

We showed that cellular differentiation and neurite formation were markedly increased in cultures of rat enteric neurons and in a human neuronal cell line exposed to IBS supernatants in comparison to those treated with controls. While IBS-derived supernatants from the three subgroups IBS-C, IBS-D and IBS-M exerted a similar stimulatory effect on neurite emergence, differences were observed regarding neurite elongation between IBS-D or IBS-M and IBS-C subgroups. These results suggest that the composition of mucosal supernatants in mediators involved in neuronal outgrowth exhibit a differential profile according to IBS subgroups. In the human neuronal cell line, the differentiation evoked by IBS supernatants was analogous to that

induced by RA, a positive control widely applied in neuronal differentiation.[22] Although the mucosal supernatants contain many mediators which can have both positive and negative effects on neuronal differentiation, our results suggest that at least part of the effect was related to NGF. Indeed, the neutralization of NGF activity with a monoclonal antibody abolished neurite elongation. Our data confirm that the impact of IBS supernatants is not restricted to a neuronal cell line derived from central nervous system neurons. Indeed, we showed that rat primary myenteric neuron cultures exposed to IBS supernatants showed an increased protein expression of GAP43. In addition, these data provide direct evidence that IBS supernatants evoke the expression of a key molecule (i.e., GAP43) involved in neuronal sprouting which could be relevant for the increased neuronal fiber density of IBS. Further experiments testing the effects of mucosal supernatants on human enteric neurons are now required.

In conclusion, our results suggest that an abnormal mucosal milieu and neuro-immune interactions play a role in the pathophysiology of intestinal dysfunction and pain transmission of IBS patients. The results presented here are complementary to the previous evidence indicating that IBS mucosal biopsy supernatants evoke fast sensitization of rodent[11, 13, 14] and human[12] neurons, and support the concept that the mediators of IBS induce long-lasting neuroplastic changes. Further experiments ere now required to further investigate this hypothesis.

Figure legends

Figure 1. NSE and GAP43 immunoreactivity in colonic specimens from controls (HC) and patients with IBS. Representative example of cross section of the colonic mucosa from HC and IBS patients stained with the pan-neuronal marker NSE (*A*). Most IBS samples showed an increased density of NSE positive neuronal fibers, as confirmed by quantitative analysis, showing a 57.7% significant increase over HC (**P<.05*).

Representative example of cross section from colonic mucosa from HC and IBS patients stained with GAP43 (*B*). We observed a significant increase in GAP43 positive fibers in patients with IBS *vs.* HC (56.1%, **P<.05*). Data are presented as percentage of immunoreactive fibers on total area of *lamina propria* (LP). Scale bar: 50 µm.

Figure 2. NGF immunoreactivity in the colonic mucosa of controls (HC) and patients with IBS. An 89.3% increase in NGF immunoreactivity was observed in lamina propria cells of patients with IBS compared to HC (*A*) (**P<.05*). Results are presented as percentage of immunoreactive cells on total area of *lamina propria* (LP). Results of ELISA analysis showing NGF content in biopsies from HC and patients with IBS (*B*). NGF concentrations in patients with IBS showed a 18% increase although did not reached statistical significance (*P=.16*). NGF and tryptase immunoreactivity and colocalization in colonic mucosa of HC (*C*). NGF was expressed in several *lamina propria* cells; many of these cells expressed tryptase positivity, demonstrating that mast cells were an important source of this growth factor. Scale bar: 50 µm.

Figure 3. NTRK1 immunoreactivity in the colonic mucosa of controls (HC) and patients with IBS. An increase of 193.8% in NTRK1 immunoreactivity was observed in patients with IBS over HC (*A,* **P<.05*). Results are presented as percentage of immunoreactive cells on square mm (sqmm) of

lamina propria (LP). NTRK1 was expressed by NSE positive neurons and nerve fibers in the colonic mucosa and in the myenteric plexus. NTRK1 and NSE immunoreactivity and colocalization in colonic mucosal biopsies (*B*), and in the myenteric plexus of colonic biopsies (*C*). Interestingly, NTRK1 was also expressed by some tryptase positive mucosal mast cells (*D*). Scale bar: 50 µm.

Figure 4. IBS supernatants stimulate neurite formation in rat primary culture of myenteric plexus. (*A*) Tuj1 immunostaining of enteric neurons treated for 24 hours with supernatants from controls (HC), IBS-C, IBS-D or IBS-M patients. Neurite outgrowth data are presented as a percentage of neurons with more than one neurite (*B*) and as percentage of neurites longer than 130 µm (*C*) from 8 HC, 13 IBS-C, 14 IBS-D and 7 IBS-M supernatants. **P<.05* compared to HC (B) and for IBS-C vs IBS-M (C). (D) No difference in the neuronal cell body area was observed in the two experimental groups. Scale bar: 15µm.

Figure 5. GAP43 immunoreactivity in SH-SY5Y (*A)*. Cells treated with Hank's solution were considered as control (basal conditions). Supernatants from patients with IBS induced significant neurite outgrowth *vs*. controls (HC), inducing a neuronal sprouting similar to that of RA. Results of morphometric analysis of SH-SY5Y differentiation after exposure to RA 10 µM, HC, IBS supernatant or Hank's medium. Neurite outgrowth data (*B*) are presented as a percentage on control; neuronal differentiation (*C*) and number of neurites per cells (*D*) are presented as a percentage on a total of 100 cells analyzed. IBS-induced neurite outgrowth and neuronal differentiation were respectively 13.6% (*B*) and 18.7% (*C*) over HC (§*P<.001*). RA induced a 23.6% increase of neurite outgrowth (*B*) and a 23% increase of cellular differentiation (*C*) (***P<.001*). No differences in the number of neurites were observed in the different experimental groups (*D*). Scale bar: 20 µm.

Figure 6. Effect of NGF contained in mucosal supernatants of IBS samples on neuronal differentiation. Compared to controls (HC), IBS supernatants induced a marked increase of percentage of neuronal differentiation (***P<.001*). This effect was NGF-dependent, as it was significantly inhibited (40.5% decrease) by NGF neutralization with anti-NGF antibody (****P<.001*).

Figure 7. GAP43 expression in rat myenteric neurons and SH-SY5Y cells exposed to IBS or controls (HC) supernatants. Representative pictures of GAP43 Western Blot, with protein molecular weights reported on the right (*A*). Western blot analysis showed that IBS supernatants evoked a 66% increase in GAP43 protein expression compared with HC (*B*) (*P<.05*). The levels of relative GAP43 gene expression are reported as fold difference ($2^{-\Delta\Delta Ct}$). SH-SY5Y cells treated with Hank's buffer were used as calibrator. GAP43 expression was increased of 30% by the treatment with IBS compared to HC supernatants (*P=.39*). RA treatment induced a significant increase in GAP43 expression both compared to HC (*P<.05) and IBS (**P<.01) supernatants (C).

Reference List

1. Longstreth GF, Thompson WG, Chey WD, et al. Functional bowel disorders. Gastroenterology 2006;130:1480-91.

2. Camilleri M. Peripheral mechanisms in irritable bowel syndrome. N Engl J Med 2012;367:1626-35.

3. Spiller R, Garsed K. Postinfectious Irritable Bowel Syndrome. Gastroenterology 2009;136:1979-1988.

4. **Simrén M, Barbara G, Flint HJ, Spiegel BM, Spiller RC, Vanner S, Verdu EF, Whorwell PJ, Zoetendal EG; Rome Foundation Committee.** Intestinal microbiota in functional bowel disorders: a Rome foundation report. Gut 2013;62:159-76.

5. Camilleri M, Lasch K, Zhou W. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. The confluence of increased permeability, inflammation, and pain in irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 2012;303:G775-85.

6. Spiller R. Serotonin and GI clinical disorders. Neuropharmacology 2008;55:1072-80.

7. Spiller RC. Inflammation as a basis for functional GI disorders. Best Pract Res Clin Gastroenterol 2004;18:641-61.

8. Barbara G, Cremon C, De Giorgio R, et al. Mechanisms underlying visceral hypersensitivity in irritable bowel syndrome. Curr Gastroenterol Rep 2011;13:308-15.

9. Cremon C, Carini G, Wang B, et al. Intestinal serotonin release, sensory neuron activation, and abdominal pain in irritable bowel syndrome. Am J Gastroenterol 2011;106:1290-8.

10. Barbara G, Stanghellini V, De Giorgio R, et al. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. Gastroenterology 2004;126:693-702.

11. Barbara G, Wang B, Stanghellini V, et al. Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. Gastroenterology 2007;132:26-37.

12. Buhner S, Li Q, Vignali S, et al. Activation of human enteric neurons by supernatants of colonic biopsy specimens from patients with irritable bowel syndrome. Gastroenterology 2009;137:1425-34.

13. Cenac N, Andrews CN, Holzhausen M, et al. Role for protease activity in visceral pain in irritable bowel syndrome. J Clin Invest 2007;117:636-47.

14. Valdez-Morales EE, Overington J, Guerrero-Alba R, et al. Sensitization of peripheral sensory nerves by mediators from colonic biopsies of diarrhea-predominant irritable bowel syndrome patients: a role for PAR2. Am J Gastroenterol 2013;108:1634-43.

15. Akbar A, Yiangou Y, Facer P, et al. Increased capsaicin receptor TRPV1-expressing sensory fibres in irritable bowel syndrome and their correlation with abdominal pain. Gut 2008;57:923-9.

16. **Yu YB, Zuo XL,** Zhao QJ, et al. Brain-derived neurotrophic factor contributes to abdominal pain in irritable bowel syndrome. Gut 2012;61:685-94.

17. Diolaiti D, Bernardoni R, Trazzi S, et al. Functional cooperation between TrkA and p75(NTR) accelerates neuronal differentiation by increased transcription of GAP-43 and p21(CIP/WAF) genes via ERK1/2 and AP-1 activities. Exp Cell Res 2007;313:2980-92.

18. Leon A, Buriani A, Dal Toso R, et al. Mast cells synthesize, store, and release nerve growth factor. Proc Natl Acad Sci U S A 1994;91:3739-43.

19. Levi-Montalcini R, Skaper SD, Dal Toso R, et al. Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci 1996;19:514-20.

20. Skaper SD, Pollock M, Facci L. Mast cells differentially express and release active high molecular weight neurotrophins. Brain Res Mol Brain Res 2001;97:177-85.

21. Barreau F, Salvador-Cartier C, Houdeau E, et al. Long-term alterations of colonic nerve-mast cell interactions induced by neonatal maternal deprivation in rats. Gut 2008;57:582-90.

22. Di Toro R, Baiula M, Spampinato S. Expression of the repressor element-1 silencing transcription factor (REST) is influenced by insulin-like growth factor-I in differentiating human neuroblastoma cells. Eur J Neurosci 2005;21:46-58.

23. Wang LH, Fang XC, Pan GZ. Bacillary dysentery as a causative factor of irritable bowel syndrome and its pathogenesis. Gut 2004;53:1096-101.

24. Benowitz LI, Routtenberg A. GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci 1997;20:84-91.

25. Demir IE, Schafer KH, Tieftrunk E, et al. Neural plasticity in the gastrointestinal tract: chronic inflammation, neurotrophic signals, and hypersensitivity. Acta Neuropathol 2013;125:491-509.

26. Wu F, Miao X, Chen J, et al. Inhibition of GAP-43 by propentofylline in a rat model of neuropathic pain. Int J Clin Exp Pathol 2013;6:1516-22.

27. Barbara G, Cremon C, Carini G, et al. The immune system in irritable bowel syndrome. J Neurogastroenterol Motil 2011;17:349-59.

28. Matricon J, Meleine M, Gelot A, et al. Review article: Associations between immune activation, intestinal permeability and the irritable bowel syndrome. Aliment Pharmacol Ther 2012;36:1009-31.

29. Aloe L. Nerve growth factor and neuroimmune responses: basic and clinical observations. Arch Physiol Biochem 2001;109:354-6.

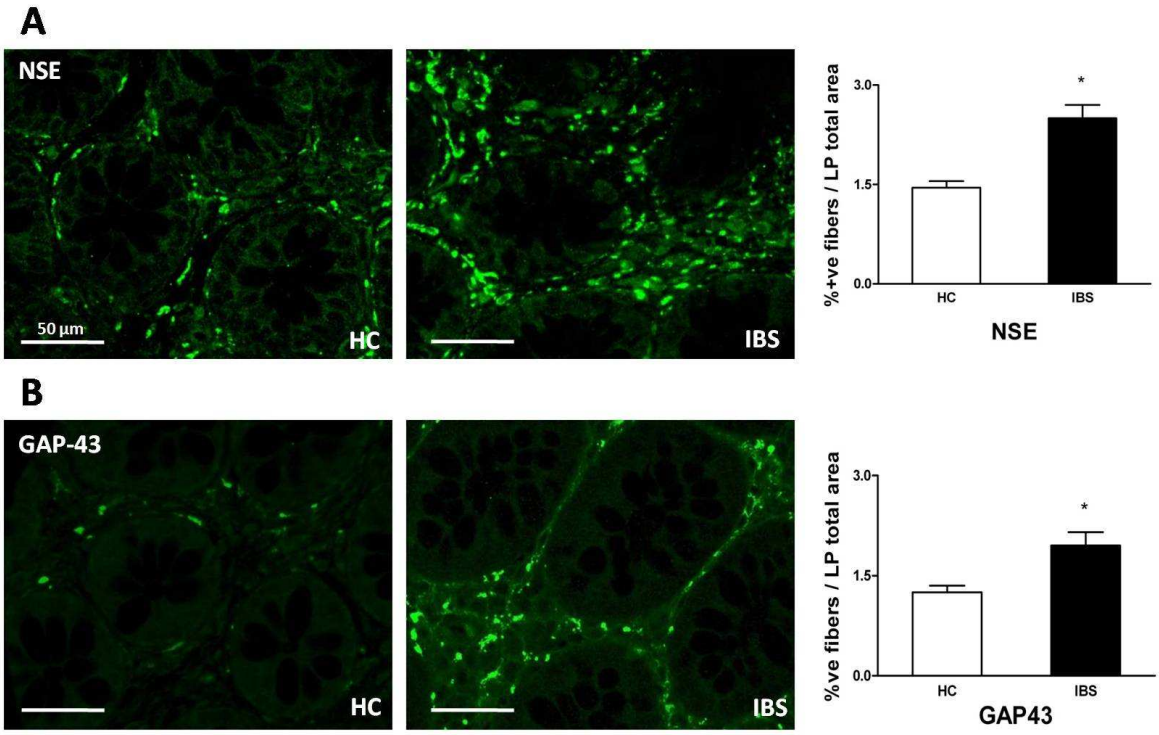
30. Shu XQ, Mendell LM. Neurotrophins and hyperalgesia. Proc Natl Acad Sci U S A 1999;96:7693-6.

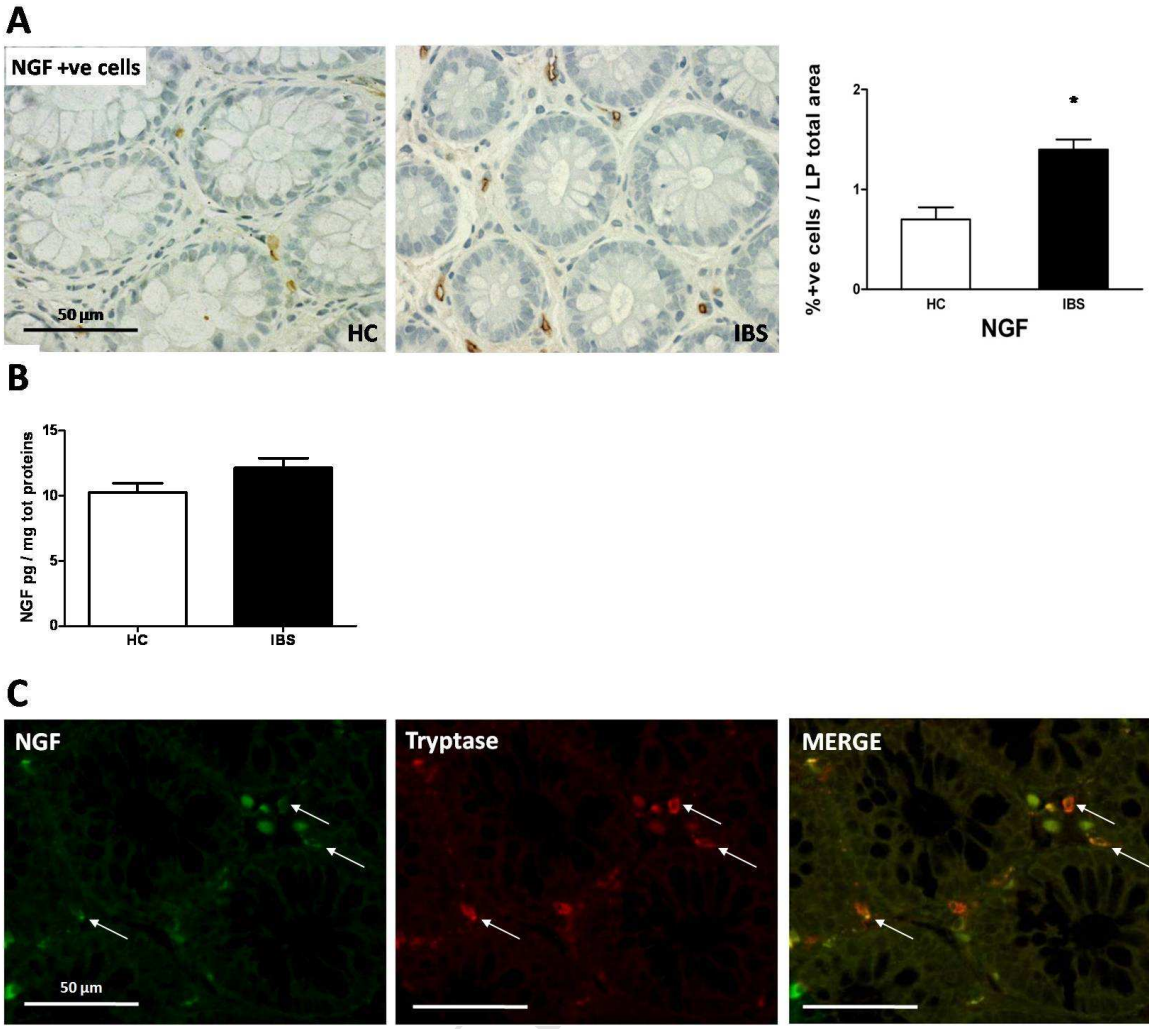
31. Willot S, Gauthier C, Patey N, et al. Nerve growth factor content is increased in the rectal mucosa of children with diarrhea-predominant irritable bowel syndrome. Neurogastroenterol Motil 2012;24:734-9, e347.

32. di Mola FF, Friess H, Zhu ZW, et al. Nerve growth factor and Trk high affinity receptor (TrkA) gene expression in inflammatory bowel disease. Gut 2000;46:670-9.

33. Welker P, Grabbe J, Gibbs B, et al. Nerve growth factor-beta induces mast-cell marker expression during in vitro culture of human umbilical cord blood cells. Immunology 2000;99:418-26.

Author names in bold designate shared co-first authors.





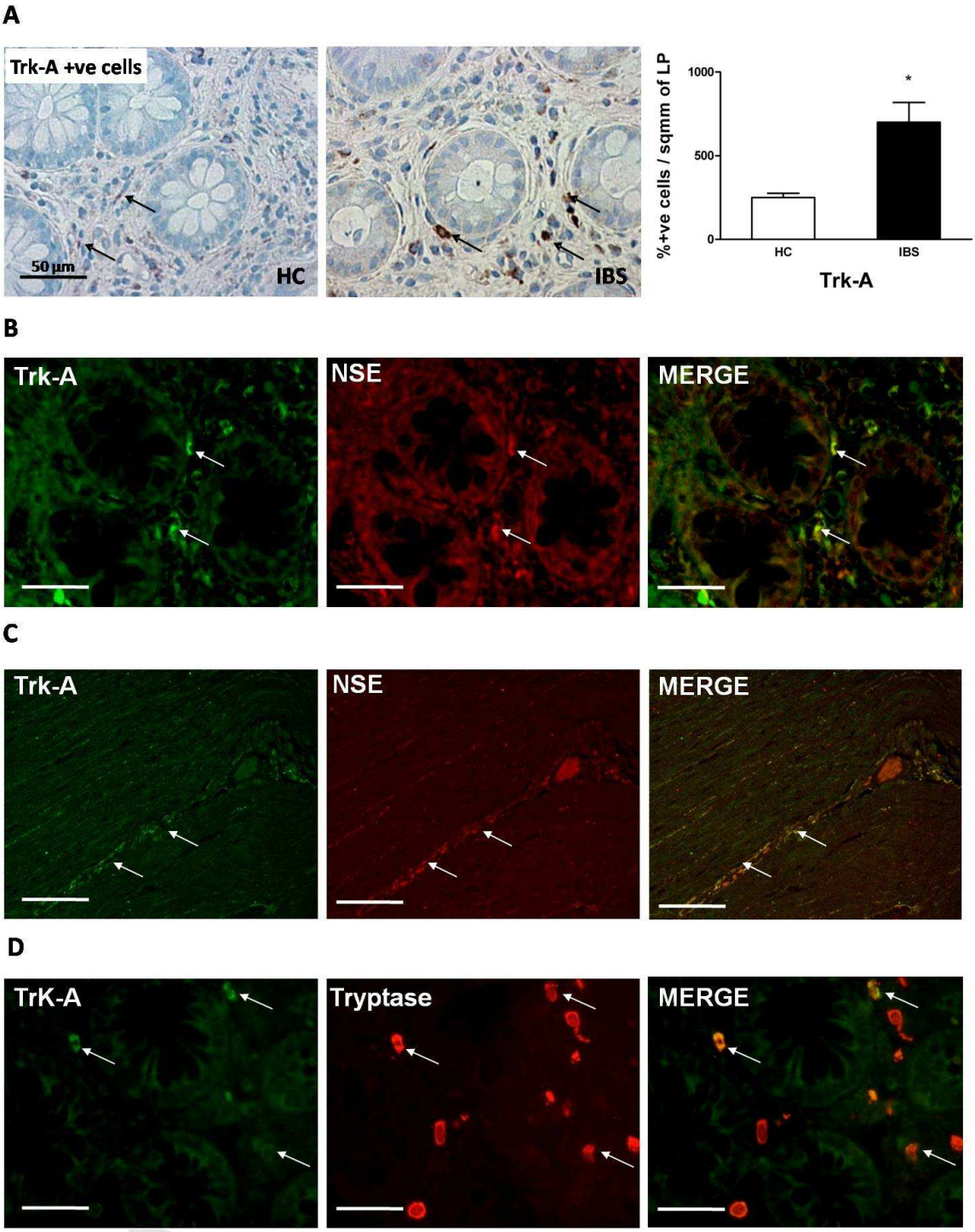

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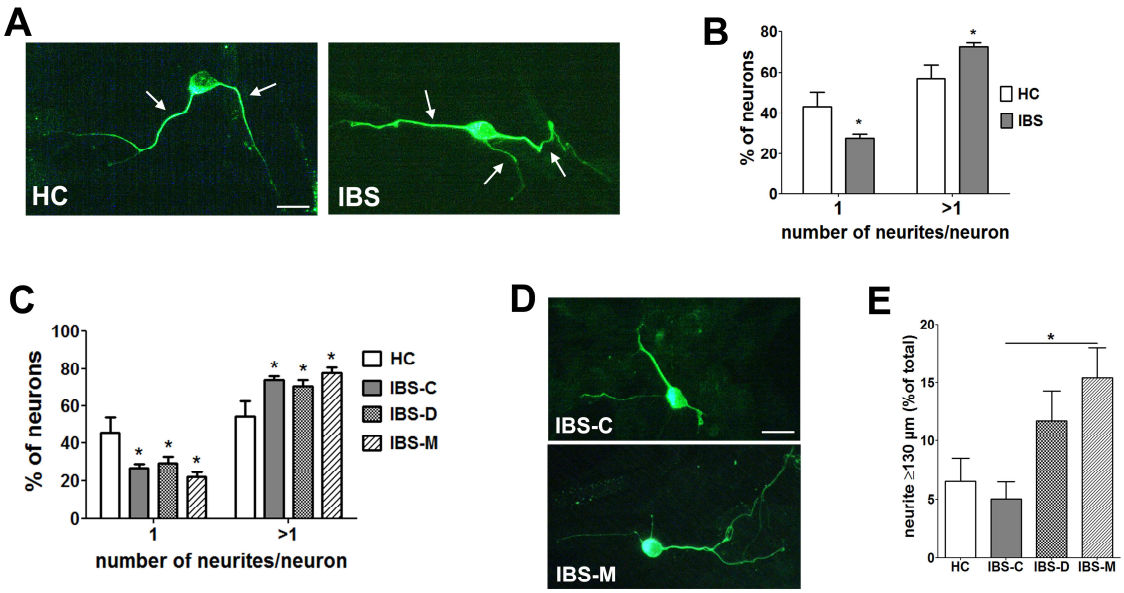
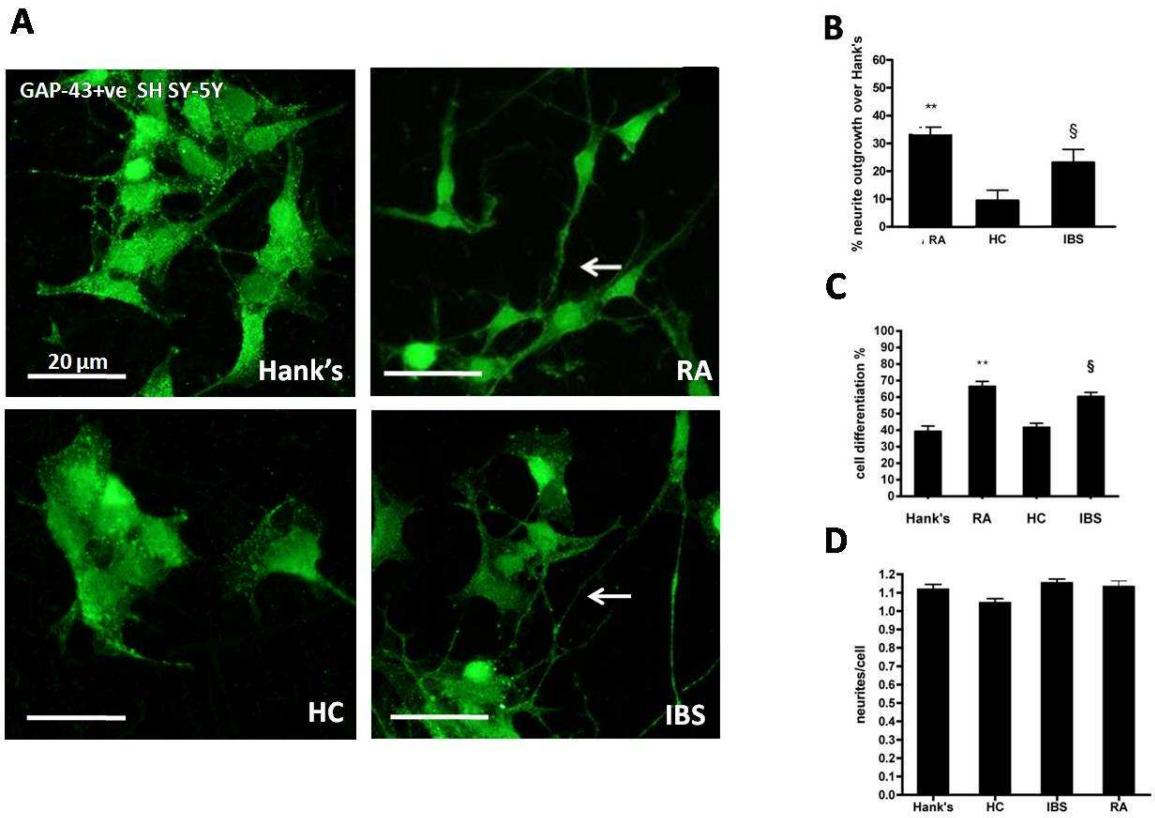
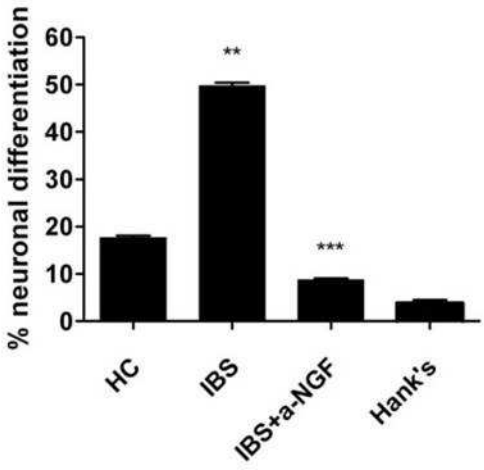
Figure 4







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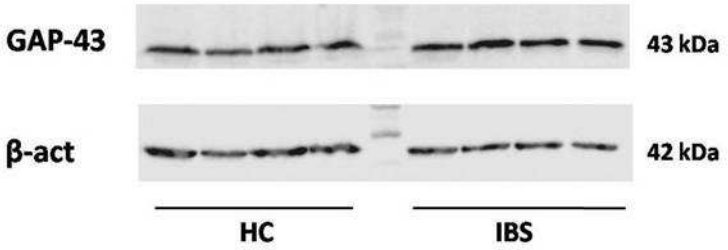

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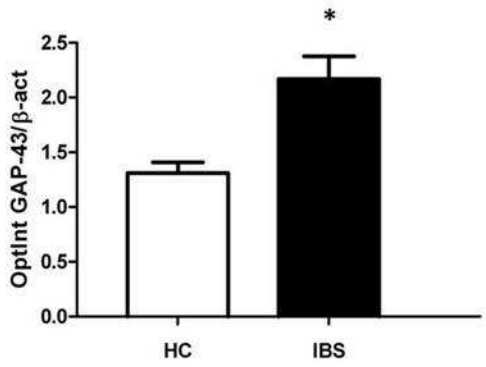

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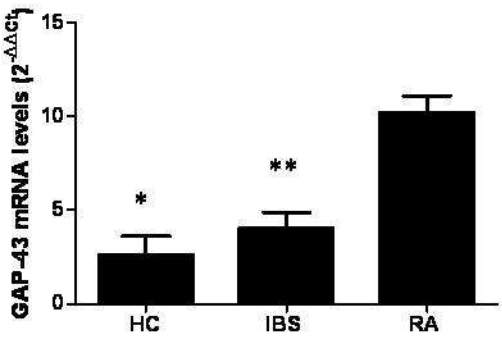

SUPPLEMENTARY MATERIAL

Materials & Methods

Immunohistochemistry and immunofluorescence

Colonic biopsies (15 controls; 15 IBS-C; 14 IBS-D) were fixed in cold neutral 10% formalin and embedded in paraffin. 3-4 µm-thick sections were cut by microtome and serially mounted on glasses; sections were deparaffined (twice, in Bioclear for 15 min), rehydrated in graded ethanol (100% twice, 95% and 70%, for 5 min each) and washed 5 min in distilled water. For immunohistochemistry/immunofluorescence experiments the following antibodies were used: mouse polyclonal anti-NSE (1/200, Sigma-Aldrich, Milan, Italy); rabbit polyclonal anti-GAP43 (1/400, Chemicon-Millipore, Billerica, MA); rabbit polyclonal anti-NGF (1/15.000, Santa Cruz Biotechnology Inc, Santa Cruz, CA); rabbit polyclonal anti-NTRK1; (1/350, Santa Cruz Biotechnology Inc, Santa Cruz, CA); mouse monoclonal anti-tryptase (1/2.000, Sigma-Aldrich, Milan, Italy). For immunohistochemistry, sections were sequentially treated as follows: 1% hydrogen peroxide in methanol to block endogenous peroxidases; overnight primary antibody incubation in a humid chamber at 4 °C; rinsing with PBS and incubation at room temperature with anti-rabbit or anti-mouse secondary antibody as appropriate, Envision System labeled polymer-HRP (DakoCytomation, Glostrup, Denmark), and finally with 3,3'-diaminobenzidine tetrahydrocloride (DakoCytomation, Glostrup, Denmark). Each sample was evaluated in duplicate.

For immunofluorescence, sections were washed three times with PBS and then incubated with 10% normal donkey serum in PBS containing 0.1% Triton® X-100 to block nonspecific binding. Sections were incubated with primary antibody (single or double labeling) in a humid chamber at 4 °C overnight, rinsed with PBS, and incubated at room temperature with goat antimouse TRITC (1/800, Sigma-Aldrich, Milan, Italy) or goat anti-rabbit FITC antibodies (1/100, Sigma) for 2 h. Specimens were mounted with Mowiol® 4–88 reagent (Chemicon- Millipore, Billerica, MA) and examined by fluorescence microscope (ECLIPSE 90i; Nikon Instruments) and representative photomicrographs were taken by DS-5M digital camera (Nikon Instruments).

Quantification of immunohistochemistry.

Briefly, colonic mucosa sections were randomly sampled with the aid of a grid (0.5 mm squares) located below the slide. Once the immunoreactive threshold was set, the computer program binarized the image and selected nerve fibers with staining intensity sufficiently distinct from the background. Briefly, this method adapts morphometric point-counting technique to quantify the area covered by the different types of nerve fibers in the *lamina propria*. In ancillary studies we have demonstrated a high intra-observer and inter-observer reproducibility of the method.[10] This method has the advantage over conventional pathological evaluations to minimize bias and maximize accuracy in counting.[10] The results are expressed as percentage of dots landing on a given structure, and represent the percentage area of *lamina propria* occupied by nerve fibers.

Characterization of neural elements in the *lamina propria* by immunofluorescence

Tissues from patients with IBS were collected and processed as described previously using the following primary antibodies: rabbit polyclonal anti-GAP43 (1/400, Chemicon-Millipore, Billerica, MA), mouse monoclonal anti-Choline Acetyltransferase (ChAT, 1/50, Santa Cruz Biotechnology Inc, Santa Cruz, CA); mouse anti-Vasoactive Intestinal Peptide (VIP, 1/2000; kindly provided by Prof. C. Sternini); rat anti-substance P (1/200; Fitzgerald, Concord, CA); goat anti- transient receptor potential cation channel, subfamily V, member 1 (TRPV-1, 1/50, Santa Cruz Biotechnology Inc, Santa Cruz, CA). Sections were incubated for 2 h at room temperature and then incubated for 1 h with the following secondary antibodies: Alexa Fluor® 488 goat anti-rabbit polyclonal antibody (1/2000, Life Technologies, Carlsbad, CA); Cy3® sheep anti-mouse polyclonal antibody (1/1000, Abcam, Cambridge, UK); Alexa Fluor® 555 donkey Anti-Goat polyclonal antibody (1/1000, Life Technologies); Alexa Fluor® 594 goat anti-rat polyclonal antibody (1/1000, Life Technologies).

SH-SY5Y neurons

Cells were grown as monolayer in MEM and Ham's F12 (1/1) medium supplemented with 10% (v/v) heat-inactivated FBS (60 ℃, 30 min; Gibco), L-glutamine (2 mM; Sigma Chemicals, St. Louis,

MO), 1% non-essential amino acids (Gibco) and 1% antibiotic-antimycotic (Gibco) solution at 37℃ in a humidified chamber with 95% O_2 : 5% CO_2.

Cells were detached with trypsine and viability was determined by propidium iodide staining. Cells were counted with a Burker chamber and seeded at a concentration of 1X6.000 cells/cm² on glass poly-ornithine-coated coverslips in 6-cm dishes (for immunocytochemistry and morphology) and then exposed to various experimental conditions as reported below. Cells were used after a minimum of 12 passages to minimize inter-subline differentiation and were maintained until the 22nd passage.

Cells were visualized in an inverted phase contrast microscope (Eclipse TS100; Nikon Instruments) and photographed with a DS-2MV digital sight camera (Nikon Instruments). We performed morphometric analysis on captured images according to a previously published method.[20]

Processes that were at least two cell diameters in length were considered neurites and the results were expressed as percentage of long neurite-bearing neurons (differentiated cells). The length of neurites, taken as the distance from the tip of a process to the middle of the cell soma, was also scored. Neurites were quantified by counting at least 100 cells in randomly chosen fields (20X magnification) for each treatment group. At least four independent experiments were conducted; each supernatant (7 controls, 10 IBS-C, 3 IBS-D) was tested in duplicate.

Primary cultures of rat myenteric neurons

Pregnant Sprague–Dawley rats were purchased (CERJ, LeGenest St Isle, France) and manipulated in compliance with the French institutional guidelines. These procedures were approved by the local institutional animal research committee (Agreement E. 44011; INSERM, Nantes, France) and by the Ethical Committee for Animal Experiments of the KU Leuven (Agreement P06077), Belgium. Every effort was made to minimize animal suffering and the number of animals used. Pregnant rats were killed by an overdose of CO_2 followed by severing the carotid arteries. The embryos (E15; 35–45 per isolation from 3 pregnant rats) were removed and killed by decapitation. Then, the small intestines of embryos were removed and finely diced in

HBSS (Sigma, Saint Quentin Fallavier, France). Tissue fragments were collected in 5 ml of medium (DMEM–F12 11 medium) and digested at 37° C f or 15 min in 0.1% trypsin (Sigma). The trypsin reaction was stopped by adding 10 ml of medium containing 10% fetal calf serum and then treated by DNAse I (.01%; Sigma) for 10 min at 37° C. After triturating with a 10 ml pipette, cells were centrifuged at 106 *g* for 10 min. For morphometric analyses, cells were plated at a density of $2x10^5$ cells/cm^2 on glass coverslips coated with 1mg/ml poly-L-lysine (Sigma) and were transferred 3h later to a dish containing a glial feeder layer in Neurobasal-B27 medium containing biopsy supernatants from controls or IBS patients diluted ½. After 24h, cells were fixed in 4% paraformaldehyde and immunostained for the neuronal marker β3-tubulin (Tuj1, 1/1000, Sigma). The number and length of neurites emerging from the neuronal cell body were measured with the ImageJ software. Differentiated neurites, as opposed to filopodia, were defined as processes with a length ≥ 5µm. Ten to fifteen neurons were analyzed per patient supernatant for 8 controls, 13 IBS-C, 14 IBS-D and 7 IBS-M patients. For RNA and protein expression studies, cells were plated in 24-well plates previously coated for 6 h with a solution of gelatin (0.5%; Sigma) in sterile phosphate buffered saline (PBS). After 24 h, the medium was replaced with a serum-free medium (DMEM–F12 1/1 containing 1% of N-2 supplement (Life Technologies, CergyPontoise, France).

Experimental protocol for NGF responses in SH-SY5Y and NGF immune-blocking

In order to assess the role of NGF in neuronal sprouting, preliminary experiments were performed to evaluate the effect of increasing concentrations of NGF (0, 5, 10, 20, 50 ng/ml, ImmunoTools, Friesoythe; Germany) on SH-SY5Y. NGF 50 ng/ml was chosen as positive control for NGF-mediated neuronal differentiation. Cells were exposed to fresh medium containing controls/IBS supernatants (1/10 v/v) or NGF 50 ng/ml with or without the addition of anti-NGF antibody 750 ng/ml (Santa Cruz Biotechnology Inc, Santa Cruz, CA) incubated for the entire duration of treatment. Each supernatant (9 controls, 5 IBS-C, and 6 IBS-D) was tested in duplicate. Antibody concentration was chosen on the basis of previously validated experimental protocols. Treatments were repeated every 48 hours. Cells grown on poly-L-lysine-coated coverslips, were fixed with 4% paraformaldehyde for 1 hour at 4˚C, rinsed with PBS and then processed for immunofluorescence

with mouse monoclonal anti-GAP43 antibody (working dilution, 1:100; Chemicon- Millipore, Billerica, MA).

Quantitative real time RT-PCR

Each supernatant (5 controls, 5 IBS-C and 5 IBS-D) was assayed in duplicate; three independent experiments were conducted.

Reverse transcription was performed using QuantiTect Reverse Transcription Kit (Qiagen, Milan, IT) in a final reaction volume of 20 µl, following the manufacturer's recommendations. Real-time PCR reactions were carried out using SYBR Green Quantitect PCR kit (Qiagen, Milan, IT) on a Real Time PCR ICycler (Bio-Rad Laboratories, Hercules, CA) in a final volume of 25 µl. *GAP43* mRNA expression was normalized to the reference gene *β-actin*, using the following primers: β-actin, forward 5'-CATGTTTGAGACCTTCAACAC-3'; reverse 5'-CCAGGAAGGAAGGCTGGAA-3'; GAP43, forward 5'-AGCCAAGGAGGAGCCTAAAC-3'; reverse 5'-TCAGGCATGTTCTTGGTCAG-3'. Amplification conditions were: 15 min at 95℃ followed by 40 cycles of 15 sec at 95℃, 30 sec at 54℃ and 30 sec at 72℃.

A negative control for PCR reaction (1 µl of water instead of cDNA) and a no-reverse transcription control were added in each real-time PCR plate. Each sample was run in duplicate and the mean threshold cycle (Ct) was determined from the two runs.

SH-SY5Y cells treated with Hank's buffer were used as calibrator and relative gene expression was calculated as ΔΔCt.[23] The levels of *GAP43* gene expression were expressed as fold difference ($2^{-\Delta\Delta Ct}$).

Western blot analysis

Protein concentration was determined using a Comassie blue-based colorimetric assay (Bradford method, Quick Start™ Bradford Protein Assay, Bio-Rad) and the protein-dye complex absorbance was read using a spectrophotometer at 595 nm. Bovine serum albumin (BSA) was used as standard. Protein samples (10 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membranes (Whatman®,

Dassel, Germany) using blotting solution containing 50 mM Tris, 200 mM glycine and 10% vol/vol methanol for 2 hours and 30 min at 350 mA. Membrane were then blocked in 5% non-fat milk in T-PBS (10% PBS, 0.1% Tween 20®; Sigma) for 1 hour at room temperature and exposed to mouse anti- GAP43 (1/1000; Chemicon- Millipore, Billerica, MA) primary antibody overnight at 4℃. Membranes were washed three times for 10 minutes with T-PBS and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1/2000; Chemicon-Millipore, Billerica, MA) for 1 h and 30 min Blots were developed with Novex®ECL HRP Chemiluminescent Substrate Reagent Kit (Life Technologies) following the manufacturer's protocol; the same blots were stripped of bound antibodies with stripping buffer at room temperature (100 mM β-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mMTris-HCl, pH 6.7) and re-probed with mouse monoclonal anti-β-actin (1/2000; Chemicon- Millipore, Billerica, MA) and anti-mouse HRP-conjugated (1/10000; DAKO Denmark) secondary antibody following the same procedures for GAP43 detection. Images were digitally acquired by a ChemiDoc™ MP System, band intensity was analyzed through Image Lab™ software (Bio-Rad)**.**

Results

Neural elements in the *lamina propria*

We performed qualitative experiments to identify neural elements in the *lamina propria* of IBS patients showing increased nerve outgrowth. To this purpose, we performed double staining experiments of GAP43 with ChAT, VIP, Substance P and TRPV-1 to include a spectrum of secretomotor (ChAT, VIP), intrinsic (VIP), extrinsic (TRPV-1) and sensory (substance P and TRPV-1) fibers. GAP43 immunoreactivity was detected in all these subpopulation of nerve elements (Figure 8).

Figure Legend

Figure 8. GAP43, ChAT, VIP, substance P, TRPV-1, immunoreactivity in the colonic mucosa of IBS patients. Double staining of GAP43 and ChAT (A), VIP (B), substance P (C) and TRPV-1 (D) revealed a colocalization of GAP43 with all the marker analyzed. Scale bar: 20 µm.