ORIGINAL ARTICLE

Brain-derived neurotrophic factor contributes to abdominal pain in irritable bowel syndrome

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ABSTRACT

Objective Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, may play a critical role in many chronic pain conditions. The possible involvement of BDNF in the altered gut sensation in patients with irritable bowel syndrome (IBS) was investigated in the present study.

Methods Rectosigmoid biopsies were collected from 40 patients with IBS fulfilling the Rome II criteria and 21 healthy controls. Abdominal pain was quantified by a validated questionnaire. The presence of BDNF and nerve fibres in the mucosa was assessed by immunohistochemistry. The structure of mucosal nerve fibres was assessed by transmission electron microscopy. Mucosal BDNF release was measured by ELISA and correlated with abdominal pain scores. Animal studies using $BDNF^{+/-}$ mice were carried out to evaluate visceral sensitivity, mucosal nerve fibre density and ultrastructural changes. Alterations of visceral sensitivity and TrkB expression in dorsal root ganglia were examined in $BDNF^{+/+}$ mice following different doses of BDNF administration.

Results Biopsies from patients with IBS revealed a significant upregulation of BDNF ($p=0.003$), as compared with controls. Total nerve fibres were also substantially increased in patients with IBS. Electron microscopy showed ultrastructural damage on the mucosal nerve fibres (eg, swollen mitochondria and nerve axons). Elevated BDNF release was significantly correlated with the abdominal pain scores. Meanwhile, abdominal withdrawal reflex scores to colorectal distension and mucosal protein gene product 9.5 immunoreactivity were significantly lowered in $BDNF^{+/-}$ than in $BDNF^{+/+}$ mice. Electron microscopy showed degenerative changes on the mucosal nerve fibres in $BDNF^{+/-}$ mice. Exogenous BDNF induced an obvious dose-dependent increase in TrkB expression in dorsal root ganglia and dose-dependent decrease in threshold pressure in $BDNF^{+/+}$ mice.

Conclusions The increased expression of BDNF in colonic mucosa, together with the structural alterations of mucosal innervation, may contribute to the visceral hyperalgesia in IBS.

Significance of this study

What is already known on this subject?

- Brain-derived neurotrophic factor (BDNF) has already been shown to play a role as a neuromodulator of gastrointestinal motor and sensory function
- BDNF has been confirmed to play a critical role in chronic pain conditions
- BDNF is expressed in the enteric nervous system and gut mucosa of various species, including humans
- Abdominal pain/discomfort is the most likely complaint to result in medical consultation for irritable bowel syndrome (IBS) and is considered a clinical hallmark of the disease

What are the new findings?

- Patients with IBS showed a significant upregulation of BDNF in the intestinal mucosa as compared with controls
- Colonic BDNF expression correlated with abdominal pain
- Structural alterations of the nerve bundles in the colonic mucosa may contribute to abdominal pain in patients with IBS

How might it impact on clinical practice in the foreseeable future?

- The increased expression of BDNF was demonstrated in the colonic mucosa of patients with IBS, together with structural alterations of mucosal innervation. Hence, it suggests a mechanism that might contribute to the pathophysiology of pain in IBS and may provide useful insights into potential treatments for this condition.

INTRODUCTION

Irritable bowel syndrome (IBS) is a common intestinal disorder characterised by recurrent abdominal pain or discomfort associated with disturbance in bowel habits.[1] Abdominal pain/discomfort is the most likely complaint to result in medical consultation for IBS[2] and is considered a clinical hallmark of the disease.[3] Although the pathogenesis of the increased pain sensitivity in IBS is not fully understood, several mechanisms have been proposed,[4] including (1) the enhanced perception of the intestinal signal in the brain, (2) hypersensitivity of dorsal horn neurons in the central limb of the visceral afferent system and (3) hyperexcitability of primary visceral afferent fibres at the end-organ level.

The molecular mechanisms of the end-organ hypersensitivity in IBS have been increasingly studied. Brain-derived neurotrophic factor (BDNF), originally known for its effects on the development

and regeneration of nervous system, has aroused attention because of its critical roles in chronic pain conditions.[5–7] In humans, BDNF is overexpressed in chronic pancreatitis and the expression is associated with pain in these patients.[8] Significant increases of BDNF in dorsal root ganglia (DRGs) and colonic mucosa after colitis, which may contribute to the colitis-associated hyperalgesia, have been reported.[9 10] Furthermore, BDNF evoked noxious heat-induced hyperalgesia when injected locally into the hind paw of rats.[11] BDNF is expressed in the enteric nervous system (ENS) and gut mucosa of various species, including humans.[12–15] These studies raise the possibility that endogenous BDNF may have a pathophysiological role in the altered gut sensation in IBS.

In humans, the ENS is composed of neurons and glial cells organised in ganglionated and aganglionated plexuses that control the physiological functions of the bowel.[16–18] The plexuses are present in the muscle, in the submucosa and in the lamina propria of the mucosa.[16 19 20] In the colon, mucosal nerve fibres form a plexus that is generally denser near the crypts in the deeper part of the lamina propria.[21] A number of observations have supported the structural alterations of the ENS in inflammatory bowel disease,[22–24] which likely lead to the altered sensory perception and bowel motility even in the quiescent phase of the disease. Further, high concentrations of BDNF are detected in the adult murine colon,[25] which implies that the factor may be essential for the maintenance and plasticity of the adult ENS. However, no studies have specifically addressed any involvement of BDNF-induced ENS plasticity in colonic biopsies of patients with IBS.

In the present study, we aimed to investigate the possible alterations in the expression of BDNF in the colonic mucosa of patients with IBS. We also evaluated the association of BDNF expression and abdominal pain in IBS and parameters known to be related to the altered sensation, such as mucosal nerve fibre density and ultrastructural alterations. Animal studies were carried out to further confirm the involvement of BDNF in the visceral pain.

MATERIALS AND METHODS

Subjects

A total of 40 patients with IBS (23 women and 17 men; age 50.2±13.5 years) and 21 control subjects (12 women and 9 men; age 50.3±12.6 years) participated in the study. This study was approved by the clinical ethical committee of the Qilu Hospital of Shandong University, and all participants gave their written informed consent before participation.

The diagnosis of IBS was based on the Rome II criteria,[3] and patients were further subclassified as having IBS that was diarrhoea-predominant (IBS-D, 11 women and 10 men; age 50.0±10.7 years) or constipation-predominant (IBS-C, 12 women and 7 men; age 50.5±16.4 years). Controls were selected from patients undergoing colonoscopy for polyps and cancer surveillance; all received negative results. Patients were excluded if they were receiving non-steroidal anti-inflammatory drugs or other anti-inflammatory drugs (including mast cell stabilisers, histamine antagonists, probiotics, immunosuppressants and steroids) or on pain medications; had undergone major abdominal surgery; or had any organic syndrome, including coeliac disease, allergic diseases and psychiatric disorders as assessed by history taking, appropriate consultations and laboratory tests. Female participants with associated and/or concomitant symptoms such as irritable bladder, chronic pelvic pain syndrome, dysmenorrhoea and other painful gynaecological disorders (eg, endometriosis) were also excluded.

All participants underwent colonoscopy following a standard bowel preparation with polyethylene glycol, and water enema was used to cleanse stool if necessary. All specimens were taken from the rectosigmoid junction to standardise the site of sampling. In all cases, two biopsies were used for routine H&E histology and immunohistochemistry. Another two biopsies were obtained for BDNF release assays. In addition, two additional specimens were taken for electron microscopy.

Questionnaires

Patients were asked to score the frequency and severity of their abdominal symptoms over the last 2 weeks by using a validated questionnaire.[26] The severity of abdominal pain/discomfort was graded 0–4 according to the impact on patients' daily activities: 0, absent; 1, mild (not influencing activities); 2, relevant (diverting from but not urging modification of activities); 3, severe (influencing activities markedly enough to urge modifications); 4, extremely severe (precluding daily activities). The frequency of abdominal pain/discomfort was graded 0–4 according to the following scale: 0, absent; 1, up to 1 day/week; 2, 2 or 3 days/week; 3, 4–6 days/week; 4, daily. Besides, the Hospital Anxiety and Depression Scale was given to each subject to complete.[27]

Histology and immunohistochemistry

Histopathological and immunohistological studies were performed on paraffin-embedded, 4-μm-thick sections. For the latter, following antigen unmasking, sections were incubated overnight at 4°C with rabbit anti-protein gene product (PGP) 9.5 (1/400, Bioworld, Atlanta, Georgia, USA), or rabbit anti-BDNF (1/450, Santa Cruz Biotechnology, Santa Cruz, California, USA), and then incubated at room temperature for 2 h with horseradish-peroxidase-conjugated anti-rabbit secondary antibody (1/200, Zhongshan Gold Bridge, Beijing, China). Subsequent visualisation involved use of diaminobenzidine as a chromogen. Slides were then counterstained with haematoxylin and viewed under a light microscope (Olympus Bx51).

For each tissue section, five non-overlapping fields chosen at random were scanned. PGP9.5-immunoreactive areas per square millimeter of mucosa were quantified by use of the Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, Maryland, USA) under a 40× objective. For BDNF analysis, immunostaining was measured by density of mucosal staining area with the above software under a 20× objective. All sections were inspected independently by two blinded observers, and the mean values of the readings were used for final analysis.

Transmission electron microscopy

Biopsy specimens for transmission electron microscopy (TEM) were fixed in cacodylate-buffered 2.5% glutaraldehyde solution, postfixed with osmium tetroxide and embedded in araldite. Toluidine-blue-stained semithin sections were screened under an optical microscope to observe the enteric nerves in mucosa. Following this, ultrathin sections were double stained with uranyl acetate and lead citrate and observed under a JEOL CX1200 electron microscope by two blinded observers.

ELISA

Mucosal samples were initially homogenised in a prepared ice-cold 100-mM Tris–HCl buffer, pH 7.0, containing a cocktail of protease inhibitors (Beyotime, Shanghai, China) supplemented with 1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 13 000 g for 15 min at 4°C. Levels of BDNF in supernatants were measured by specific ELISA kits (Promega,

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Table 1 The clinical characteristics of the study population

	Subjects			IBS subtype		
	IBS (n=40)	Control (n=21)	p Value	IBS-D (n=21)	IBS-C (n=19)	p Value
Sex (F/M)	23/17	12/9	0.98	11/10	12/7	0.49
Age (years)	50.2 (13.5)	50.3 (12.6)	0.99	50.0 (10.7)	50.5 (16.4)	0.90
IBS duration (years)	6.6 (3.6)	–	–	6.3 (2.8)	6.9 (4.4)	0.59
Number of bowel movements per week	–	6.6 (0.9)	–	14.5 (4.3)	3.2 (0.9)	<0.001
Severity of abdominal pain/discomfort	2.3 (0.8)	0.4 (0.7)	<0.001	2.4 (0.8)	2.2 (0.5)	0.35
Frequency of abdominal pain/discomfort	2.1 (0.7)	0.3 (0.5)	<0.001	2.2 (0.7)	2.1 (0.6)	0.44
HADS anxiety scores	5.5 (2–8)	5 (2–7)	0.17	6 (2–8)	5 (2–8)	0.98
HADS depression scores	3.5 (1–6)	3 (1–5)	0.18	4 (2–5)	3 (1–6)	0.36

Quantitative data are expressed as mean (SD) or median (range).
F, female; HADS, Hospital Anxiety and Depression Scale; IBS, irritable bowel syndrome; IBS-D, IBS with diarrhoea; IBS-C, IBS with constipation; M, male.

Madison, Wisconsin, USA). The detection range for this assay was 7.8–500 pg/ml. Each sample was analysed in duplicate. The final concentrations of BDNF were normalised to that of total protein content. The intra-assay and interassay coefficients of variation were 2.6% and 4.2%, respectively.

Animals

Animal care and treatment were in accordance with the guidelines of the International Association for the Study of Pain. Heterozygous $BDNF^{+/-}$ mice (C57Bl/6 background) and wild-type $BDNF^{+/+}$ littermates were generous gifts from the Neurobiology Laboratory of Shandong University. Animals were housed under conditions of controlled temperature (20±1°C), hygrometry (50±5%) and lighting (lights on from 07:00 to 19:00). All experiments were performed with adult male mice at 4 months of age.

Experimental protocol

The first series of experiments were conducted on $BDNF^{+/-}$ mice and $BDNF^{+/+}$ littermates. Visceral sensitivity was evaluated by measuring behavioural responses of abdominal withdrawal reflex (AWR) to colorectal distension (CRD) in each animal group, and the effect of BDNF knock-out on the growth of mucosal nerve fibres in the colon was assessed by TEM.

In the second series of experiments, four groups of mice received BDNF (PeproTech, Rocky Hill, Connecticut, USA) in 0.1% bovine serum albumin (BSA) at doses of 0.1, 1 and 10 ng/mice, or its vehicle (0.1% BSA), intraperitoneally (0.3 ml/mice), 30 min before CRD. In addition, the threshold intensity of CRD was recorded in all animal groups. The threshold intensity was determined as the pressure inducing the first abdominal contraction and consequent interruption of the cycle. After the examination of visceral response, western blotting analysis was applied to investigate the expression alterations of TrkB in the DRGs.

CRD and AWR scoring

Briefly, mice were lightly sedated with halothane while a flexible latex balloon (1.5 cm in length, 1 cm in diameter) was inserted intra-anally into the descending colon 1 cm proximal to the anus. The balloon was secured in place by taping the attached tubing to the tail. The animals were allowed to recover 30 min fully from the halothane anaesthesia. During the test, the mice were placed inside a restraint chamber (4.5 cm in diameter,

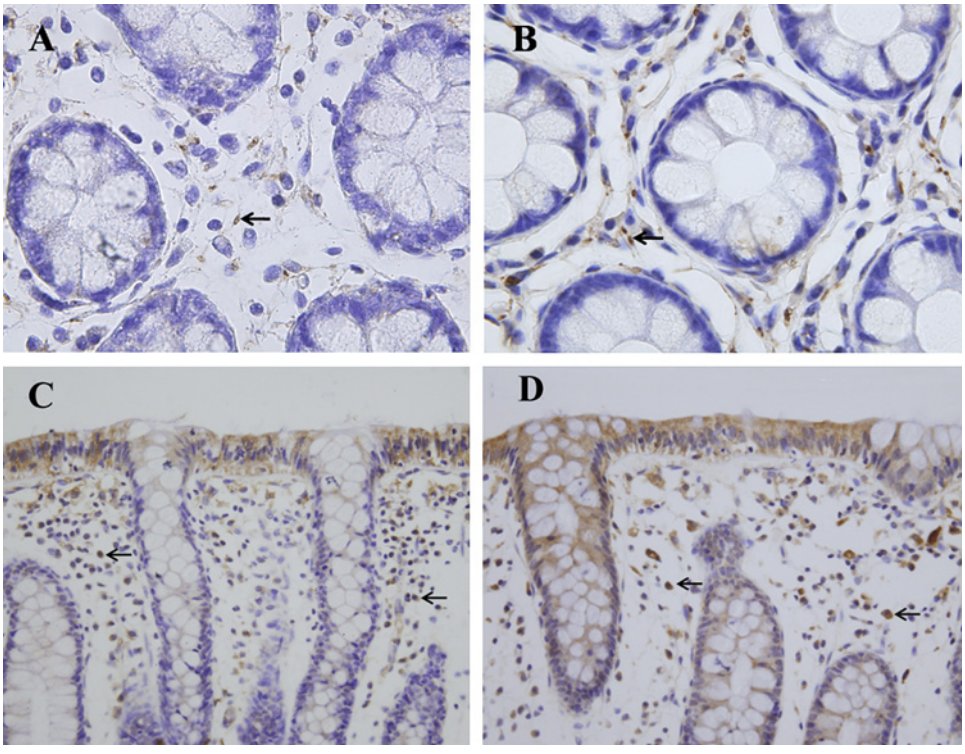

Figure 1 (A,B) Representative photomicrographs showing PGP9.5-immunoreactive fibres (arrow) in the colonic mucosa of a control subject (A) and a patient with IBS (B). Magnification 40×. (C,D) Representative photomicrographs showing BDNF immunoreactivity in mucosal epithelial cells and lamina propria cells from the biopsy of a control subject (C) and a patient with IBS (D). The typical lamina propria cells exhibiting positive BDNF immunoreactivity are indicated by arrows. Magnification 20×. BDNF, brain-derived neurotrophic factor; IBS, irritable bowel syndrome; PGP, protein gene product.

Table 2 Quantification of immunoreactivity in colonic biopsies from patients with IBS and from controls

	Subjects			IBS subtype		
	IBS (n=40)	Control (n=21)	p Value	IBS-D (n=21)	IBS-C (n=19)	p Value
PGP9.5	4.2 (2.9–5.7)	2.3 (1.5–3.2)	<0.01	4.2 (3.2–5.8)	3.9 (2.7–6.0)	0.55
BDNF	23.5 (20.1–27.2)	17.4 (14.5–20.4)	<0.01	25.3 (20.6–28.3)	22.6 (19.2–25.0)	0.10

Values are medians with IQR in parentheses.
Both PGP9.5 and BDNF are expressed as percentage of area.
Significance of differences was determined by using the Mann–Whitney U test.
BDNF, brain-derived neurotrophic factor; IBS, irritable bowel syndrome; IBS-C, IBS with constipation; IBS-D, IBS with diarrhoea; PGP, protein gene product.

10 cm in length) in which they could not escape or turn around. The mice were accustomed to this procedure for 1 day before CRD in order to minimise stress reactions during the experiments.[28 29] For measuring the threshold intensity, the colorectal balloon was progressively inflated with an increment of 5 mm Hg until the pain behaviour displayed. For measuring the AWR, the balloon was rapidly inflated to constant pressure (15, 30, 45 and 60 mm Hg). The AWR scores were graded on a scale of 0–4:[30] 0, no behavioural response to CRD; 1, brief head movement followed by immobility; 2, contraction of abdominal muscles; 3, lifting of abdomen; 4, body arching and lifting of pelvic structures. All the measurements were observed by two blinded observers and performed in triplicate.

Western blotting analysis

Proteins from bilateral DRGs of BDNF-treated mice were prepared and quantified by routine process. Proteins were separated and electrotransferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, California, USA). The membrane was incubated with rabbit anti-TrkB (1/500, Bioworld) primary antibody at 4°C overnight and, subsequently, horseradish-peroxidase-conjugated anti-rabbit secondary antibody (1:2000, Zhongshan Gold Bridge) for 2 h. The bands were detected by an enhanced chemiluminescence technique (Amersham, Buckinghamshire, UK) on Kodak biomax light film. Data were expressed as the ratios of TrkB to β-actin band intensity (1:3000, Abcam, Cambridge, UK).

ELISA in mice

Mucosal samples from $BDNF^{+/-}$ mice and $BDNF^{+/+}$ mice were collected. Mucosal BDNF levels were measured by specific ELISA kit (Promega). Each sample was analysed in duplicate.

Immunofluorescence

Colon tissues from $BDNF^{+/-}$ mice and $BDNF^{+/+}$ mice were processed for PGP9.5 immunofluorescence. Briefly, sections were incubated overnight at 4°C with rabbit anti-PGP9.5 (1/500, Bioworld) and then incubated with Alexa Fluor 488-conjugated donkey anti-rabbit antibodies (1/1000, Invitrogen, Cergy Pontoise, France) for 1.5 h at room temperature. Sections were then examined with a fluorescence microscope (Optiphot; Nikon, Japan) equipped with separate filters. PGP9.5-immunoreactive areas per square millimeter of mucosa were quantified using the Image-Pro Plus 5.0. Analysis were done on five fields of eight $BDNF^{+/-}$ mice and eight $BDNF^{+/+}$ mice.

TEM in mice

Mucosal samples from $BDNF^{+/-}$ mice and $BDNF^{+/+}$ mice were processed as mentioned above and studied with a JEOL CX1200 electron microscope by two blinded observers.

Statistical analysis

All data given were expressed as mean values±SD or medians (IQR). Univariate analysis of the characteristics of the population involved the χ^2 test, independent Student t test or Mann–Whitney non-parametric test as applicable. Statistical analysis of photomicrographic differences involved the Mann–Whitney non-parametric test or independent Student t test. Correlations between two parameters were assessed by Spearman rank correlation. Results of AWR scores were analysed by the Mann–Whitney test. One-way analysis of variance was performed to compare the thresholds measured among the four groups. Differences were considered significant at $p<0.05$.

RESULTS

Group characteristics

The clinical characteristics of control subjects and patients with IBS are described in table 1. The groups were not statistically different for age or sex. Among the included 40 patients with IBS, 52.5% (n=21) and 47.5% (n=19) were considered IBS-D and IBS-C, respectively. The mean duration of symptoms was similar between IBS subgroups. The mean pain intensity was moderate for patients with IBS and significantly higher than that for controls. No significant differences are found between male and female patients with IBS in terms of abdominal pain/discomfort severity and frequency scores (supplemental table 1). The anxiety subscale scores and depression subscale scores between the IBS group and the control group were not statistically different. The colonic mucosa was macroscopically normal in all subjects.

Immunohistochemistry

PGP9.5-immunoreactive nerve fibres were seen scattered throughout the mucosa in all the specimens (figure 1A,B). The median area occupied by PGP9.5-immunoreactive fibres was greater in patients with IBS than in control subjects (IBS: median 4.2, IQR 2.9–5.7; control: 2.3, 1.5–3.2, $p<0.001$).

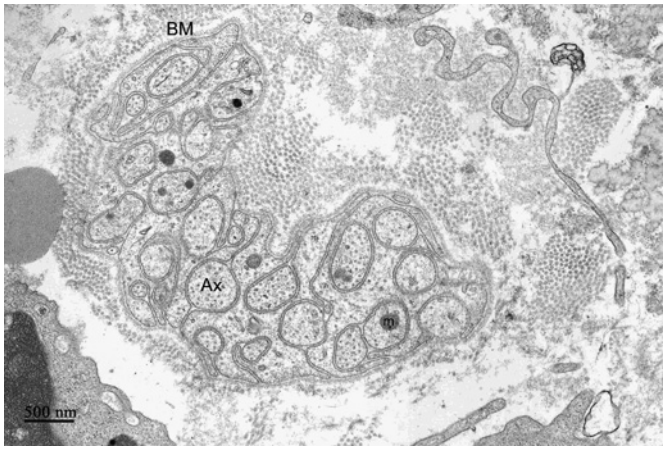

Figure 2 Electron micrograph showing the ultrastructure of nerve axons in the colonic mucosa of control subjects. Ax, axon; BM, basement membrane; m, mitochondrion.

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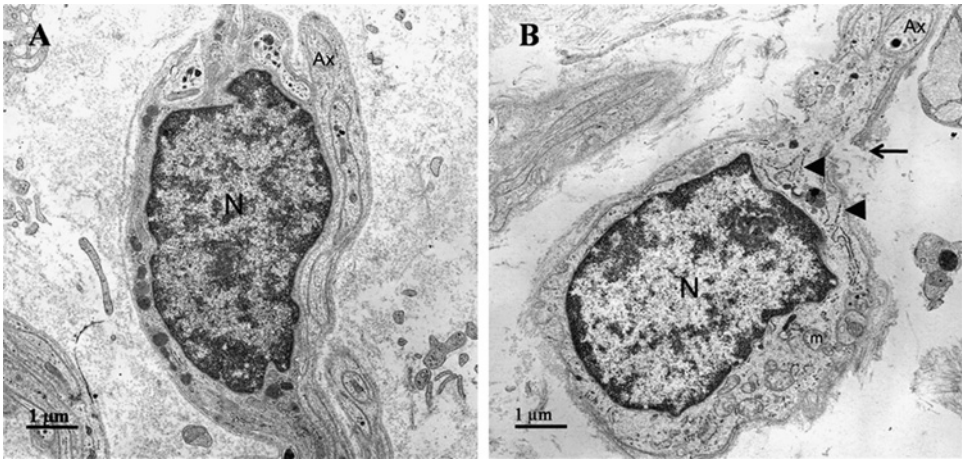

Figure 3 Electron micrographs showing the ultrastructure of Schwann cells in the colonic mucosa of control subjects (A) and patients with IBS (B). Damage is demonstrated by dilated rough endothelial reticulum (arrowheads), swollen mitochondria, disrupted basement membrane (arrow) and decreased electron density. Ax, axon; IBS, irritable bowel syndrome; m, mitochondrion; N, nucleus.

Furthermore, there was no statistically significant difference in the median density of colonic mucosal nerve fibres between the IBS subgroups (IBS-D: median 4.2, IQR 3.2–5.8; IBS-C: 3.9, 2.7–6.0, p=0.55) (table 2).

BDNF-like immunoreactivity was abundant in mucosal epithelial cells and lamina propria cells (figure 1C,D). Quantification revealed that the expression of BDNF in the colonic mucosa was significantly elevated in the patients with IBS than in controls (IBS: median 23.5, IQR 20.1–27.2; control: 17.4, 14.5–20.4, p<0.01) (table 2). With further analysis of IBS subgroups on BDNF expression, no significant difference was found between patients with IBS-D and those with IBS-C (IBS-D: median 25.3, IQR 20.6–28.3; IBS-C: 22.6, 19.2–25.0, p=0.10).

TEM of nerve bundles

In the mucosal lamina propria region of control subjects, axon chambers typically showed a round or elliptical cross section. Axon chambers were filled with microtubules, microfilaments, variable numbers of mitochondria and lucent or dense-core vesicles. The nerve bundles were separated by a well-defined basement membrane (figure 2). Schwann cells exhibited an oval nucleus with condensed heterochromatin mainly distributed in the periphery. The perinuclear cytoplasm was electron-dense and contained abundant mitochondria and endoplasmic reticulum (figure 3A).

Injury was demonstrated in both the nerve bundles and the perinuclear cytoplasm in the biopsies from patients with IBS. Nerve axons appeared swollen, lucent and sometimes with membrane-bound vacuoles. Also, the arrangement of microtubules and microfilaments inside the axon chambers was obviously disrupted. The basement membrane surrounding the nerve bundles was thickened, with a fuzzy border and sometimes even disrupted (figure 4). The cytoplasm of Schwann cells exhibited significantly decreased electron density, with dilated rough endothelial reticulum fragments and swollen mitochondria (figure 3B).

Measurement of BDNF in intestinal mucosa

Patients with IBS showed a significant upregulation of BDNF in the intestinal mucosa as compared with controls (IBS: 2.96±0.96 ng/mg protein vs control: 2.19±0.85 ng/mg protein, p=0.003) (figure 5). The IBS subtypes did not differ in BDNF in intestinal mucosa (IBS-C: 2.66±0.71 ng/mg protein vs IBS-D: 3.23±1.09 ng/mg protein, p=0.051). The level of BDNF was higher but not significantly in patients with IBS-C than in controls (p=0.1). The levels of BDNF were similar between male and female participants in the control group or the IBS group (supplemental table 2).

Correlation analysis

In patients with IBS, a significant correlation was found between mucosal BDNF levels and both severity and frequency of abdominal pain/discomfort (r=0.57, p<0.001 and r=0.46, p=0.003, respectively) but not in the control group (r=0.01, p=0.98 and r=0.06, p=0.81, respectively) (figure 6A–D). For all patients and control subjects, the BDNF levels also correlated statistically with the severity and frequency of abdominal pain/discomfort (r=0.53, p<0.001 and r=0.54, p<0.001, respectively) (figure 6E,F).

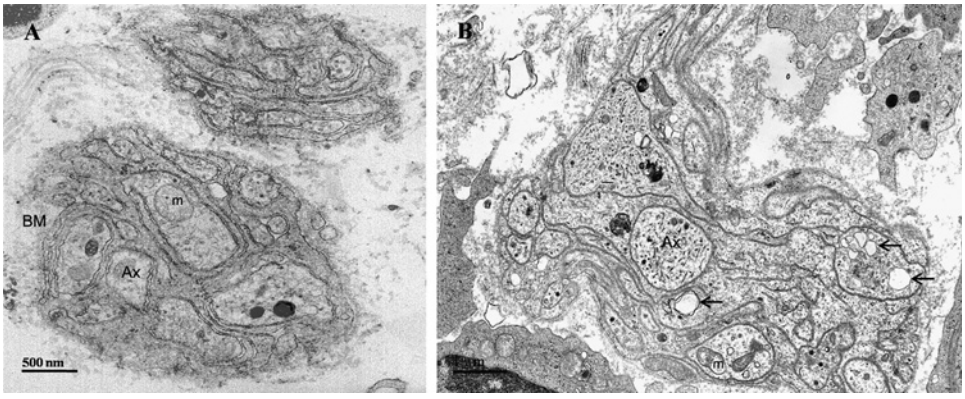

Figure 4 Electron micrographs showing the ultrastructure of nerve axons in the colonic mucosa of patients with IBS (A,B). Damage is demonstrated by disrupted neurotubules, swollen and lucent axon chambers, swollen mitochondria, membrane-bound vacuoles (arrows) and fuzzy borders of the basement membrane. Ax, axon; BM, basement membrane; IBS, irritable bowel syndrome; m, mitochondrion.

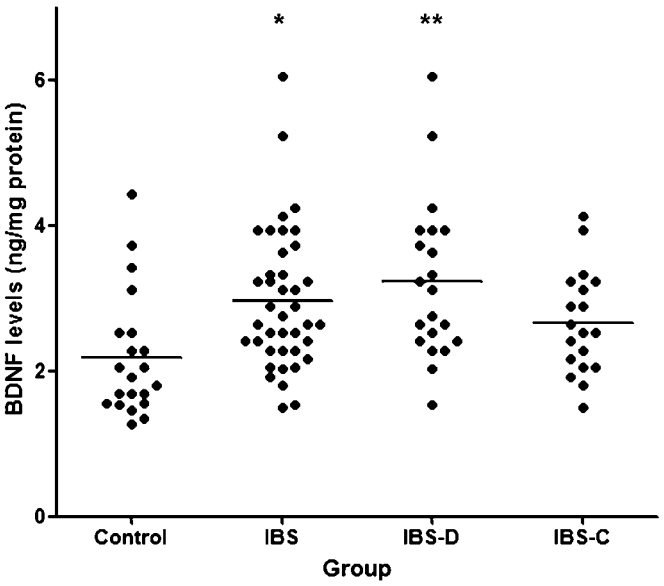

Figure 5 Expression of BDNF in colonic biopsies of patients with IBS (n=40) and control subjects (n=21). Patients with IBS are also shown according to IBS subtypes: IBS-D (n=21) and IBS-C (n=19). *p=0.003 versus control subjects. **p=0.001 versus control subjects. No significant difference exists between IBS subtypes (NS, p=0.051). The horizontal line represents the mean value for each group. BDNF, brain-derived neurotrophic factor; IBS, irritable bowel syndrome; IBS-C, IBS with constipation; IBS-D, IBS with diarrhoea.

Behaviour study of mice

$BDNF^{+/-}$ and $BDNF^{+/+}$ mice did not differ in visceral response to CRD at distension pressures ≤30 mm Hg. However, $BDNF^{+/-}$ mice showed significant lower AWR scores than $BDNF^{+/+}$ mice at 45 and 60 mm Hg distension pressures (*p=0.03, **p<0.001) (figure 7A).

The threshold pressure in control mice was 21.8±2.2 mm Hg. Intraperitoneal injection of BDNF (0.1–10 ng/mice) induced a significant dose-dependent decrease in threshold pressure compared with control mice. With a dose of 0.1 ng/mice, the decrease was not statistically significant (19.8±2.4 mm Hg vs 21.8±2.2 mm Hg, p=0.23). For BDNF at 1 and 10 ng/mice, the threshold pressure was 15.4±3.4 mm Hg and 12.6±1.9 mm Hg, respectively (**p<0.01, ***p<0.001) (figure 7B).

Mucosal BDNF level in mice

The colonic mucosal BDNF level in $BDNF^{+/-}$ mice was approximately half that in $BDNF^{+/+}$ mice (1.76±0.42 ng/mg protein vs 3.30±0.82 ng/mg protein, *p<0.01).

Immunofluorescence

The mucosal surface of PGP9.5 immunoreactivity was significantly lower in $BDNF^{+/-}$ mice than in $BDNF^{+/+}$ mice ($(2.42\pm0.31)\times10^3$ $\mu m^2/mm^2$ vs $(3.11\pm0.27)\times10^3$ $\mu m^2/mm^2$, *p<0.01) (figure 7C–E).

Western blotting analysis

Densitometric analysis of the bands revealed that compared to controls, the levels of TrkB receptor were significantly increased

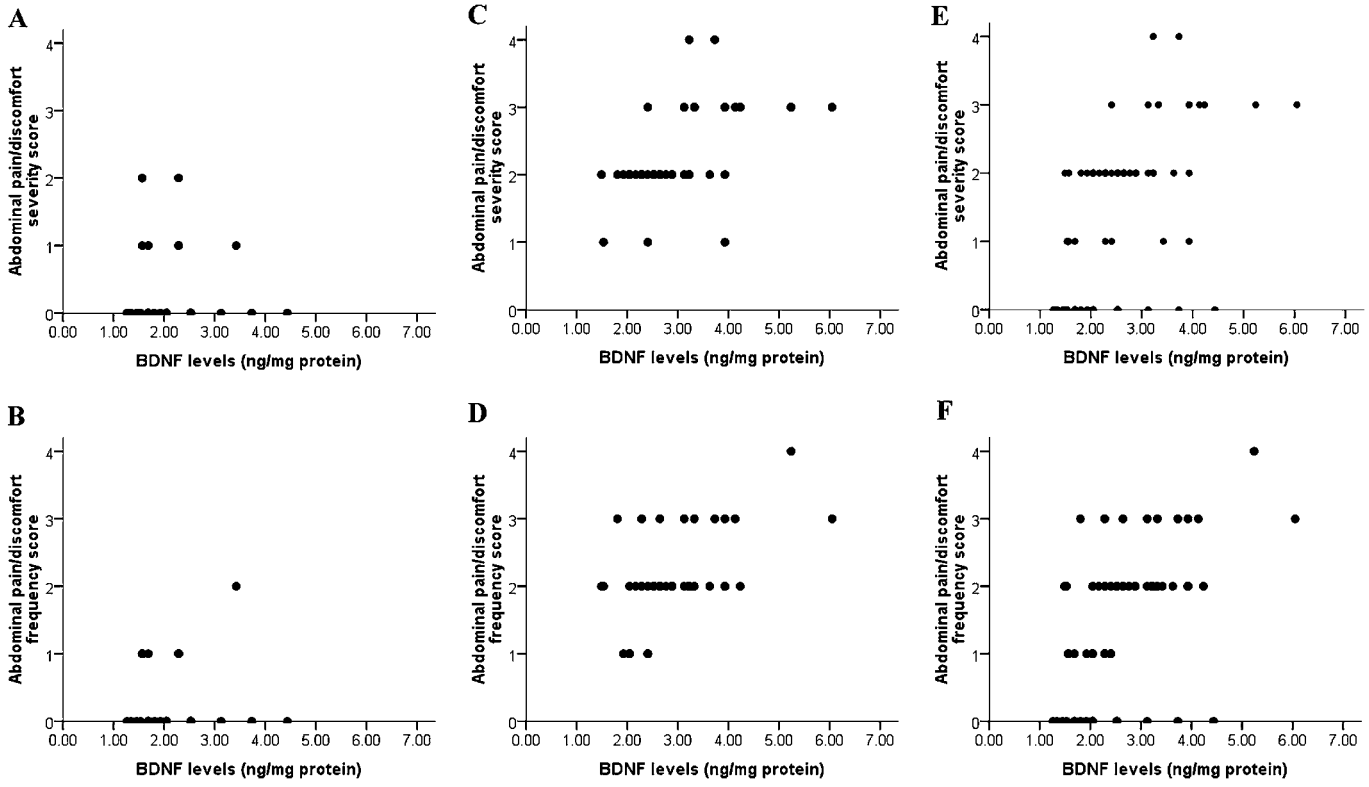

Figure 6 Correlation between severity and frequency of abdominal pain/discomfort and BDNF levels in the colonic mucosa of control subjects, patients with IBS and all subjects (controls and patients with IBS), respectively. Scatter plot showing the correlation between BDNF levels and (A) severity of abdominal pain/discomfort in control subjects (Spearman correlation r=0.01, p=0.98), (B) frequency of abdominal pain/discomfort in control subjects (Spearman correlation r=0.06, p=0.81), (C) severity of abdominal pain/discomfort in patients with IBS (Spearman correlation r=0.57, p<0.001), (D) frequency of abdominal pain/discomfort in patients with IBS (Spearman correlation r=0.46, p=0.003), (E) severity of abdominal pain/discomfort in all subjects (Spearman correlation r=0.53, p<0.001) and (F) frequency of abdominal pain/discomfort in all subjects (Spearman correlation r=0.54, p<0.001). BDNF, brain-derived neurotrophic factor; IBS, irritable bowel syndrome.

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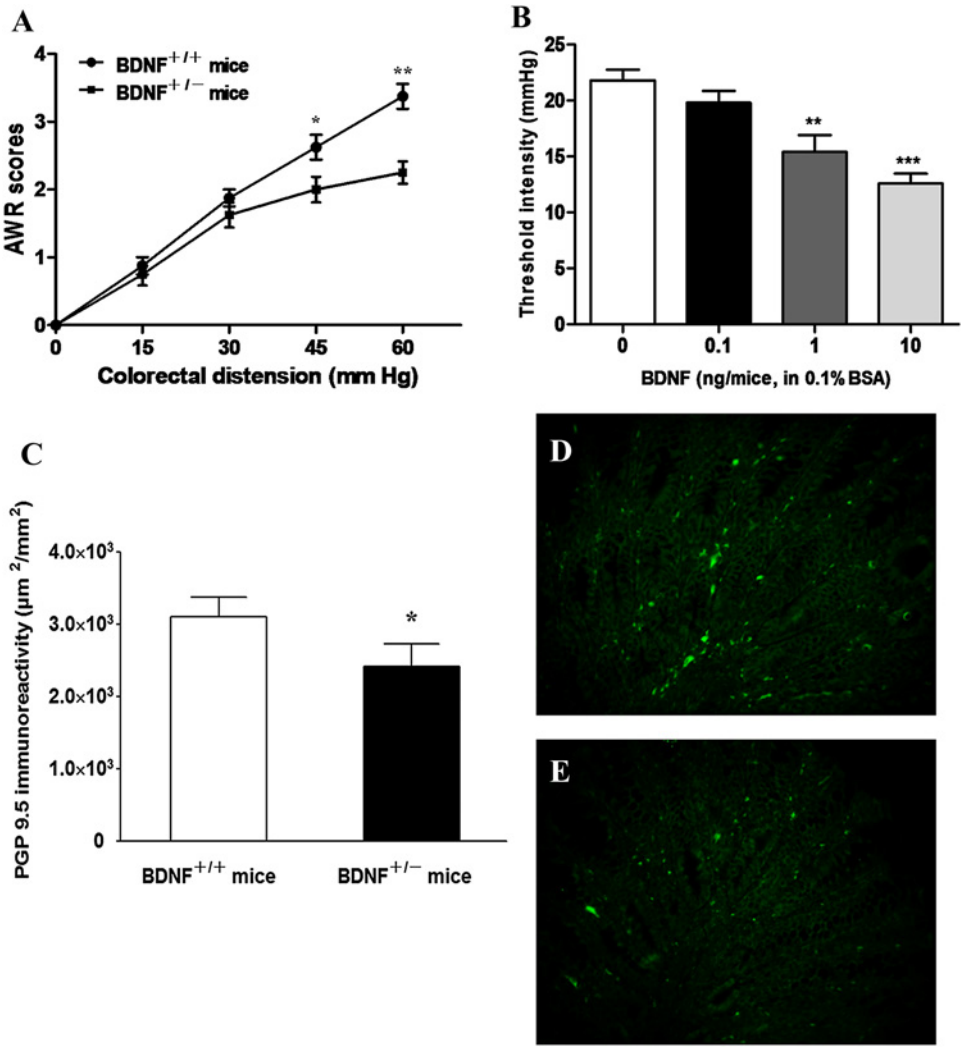

Figure 7 (A) Visceral response to CRD was evaluated by AWR scores. Lower AWR scores were observed in BDNF$^{+/-}$ mice with a CRD pressure of 45 and 60 mm Hg but not with 15 and 30 mm Hg. *p=0.03, **p<0.001. n=8 per group. (B) Effect of exogenous BDNF on colonic reaction threshold of mice in response to CRD. Intraperitoneal injection of BDNF (0—10 ng/mice in 0.1% BSA) induced a significant dose-dependent decrease in threshold pressure compared with control mice. **p<0.01, ***p<0.001. n=5 per group. (C) Quantification of PGP9.5 immunoreactivity in BDNF$^{+/+}$ mice and BDNF$^{+/-}$ mice. *p<0.01. n=8 per group. (D) Representative photomicrographs showing PGP9.5-immunoreactive fibres in the colonic mucosa of BDNF$^{+/+}$ mice. Magnification 20×. (E) Representative photomicrographs showing PGP9.5-immunoreactive fibres in the colonic mucosa of BDNF$^{+/-}$ mice. Magnification 20×. AWR, abdominal withdrawal reflex; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CRD, colorectal distension; PGP, protein gene product.

in DRGs after intraperitoneal injection of BDNF at the dose of 1 and 10 ng/mice (*p<0.05, **p<0.01) (figure 8).

TEM of nerve bundles in mice

In the mucosal lamina propria of BDNF$^{+/+}$ mice, the nerve axons were filled with variable numbers of mitochondria and large amounts of lucent or dense-core synapse vesicles (figure 9). Schwann cells exhibited typical ultrastructural features (figure 10A).

In BDNF$^{+/-}$ mice, degenerative changes of nerve axons and Schwann cells were observed. Swollen or lucent mitochondria were present in the nerve axons. The number of the lucent or dense-core synapse vesicles was much lesser. Further, lamellar bodies were identified inside the nerve axons (figure 11). The nuclei of Schwann cells were elongated and much smaller in size. Swollen mitochondria existed in the cytoplasm of Schwann cells (figure 10B).

DISCUSSION

In the present study, we investigated the possible involvement of BDNF in the altered gut sensation in IBS. Biopsies from patients with IBS revealed a significant upregulation of BDNF as compared with controls. The enhanced expression of BDNF was closely correlated with the degree of abdominal pain in IBS. Furthermore, the upregulation of BDNF may also play a role in the structural alterations of mucosal nerve fibres in patients with IBS.

BDNF has aroused recent attention because of its critical role in inflammatory pain states.[31 32] Neutralisation of BDNF by injecting anti-BDNF antibody intrathecally could effectively reduce the mechanical allodynia induced by surgical incision.[6] Besides, the application of exogenous BDNF to the corium surface of the skin sensitises individual nociceptors to noxious heat.[11] Although the role of BDNF in many forms of hyperalgesia is well documented,[33] little is known about the physiological role BDNF plays in the gut of patients with IBS. The present study showed increased BDNF release by the colonic mucosa of patients with IBS and a correlation with severity and frequency of perceived abdominal painful sensations, which may suggest a basis for IBS symptoms. The use of BDNF$^{+/-}$ mice clearly revealed that BDNF deficiency leads to lower visceral sensitisation to CRD. Moreover, exogenous BDNF induced a significant decrease in distension thresholds in mice. A mechanistic interpretation of the rapid hyperalgesic effect of BDNF is currently lacking. It is noteworthy that exogenous BDNF could augment the peristaltic reflex by promoting the release of 5-HT (5-hydroxytryptamine) and CGRP (Calcitonin gene-related peptide) in the rat colon.[34] Thus, we hypothesised that intraperitoneal injection of BDNF might act on the TrkB receptor in DRG and consequently promote the release of sensory mediators. Moreover, BDNF has been shown to enhance neuronal responsiveness to neurotransmitters such as 5-HT and substance P.[15]

Mucosal epithelial cells are a potential source of BDNF in the intestine of guinea pig.[15] During mucosal stimulation, these

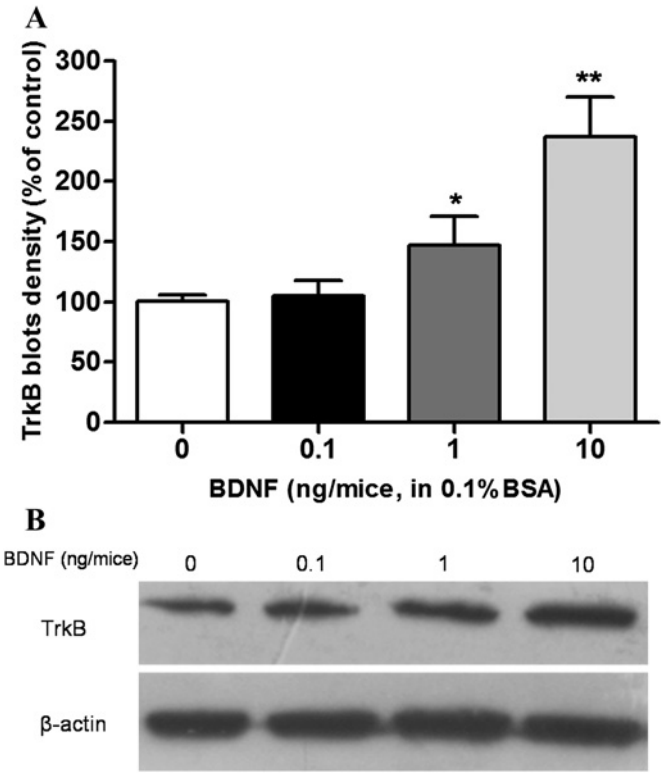

Figure 8 Effect of exogenous BDNF on the expression of TrkB receptor in the DRG of BDNF$^{+/+}$ mice. (A) The levels of TrkB receptor were significantly increased in DRGs after intraperitoneal injection of BDNF at the dose of 1 and 10 ng/mice. (B) Representative western blotting for TrkB in extracts from isolated DRGs after intraperitoneal injection of BDNF. Data are expressed as normalised density to β-actin, mean with the SE, n=5 in each group. *p<0.05, **p<0.01. BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglion.

BDNF-containing epithelial cells are suggested to be the major source of endogenous BDNF release from the gut in rats.[34] Consistent with these observations, we showed that BDNF immunoreactions could be detected in the epithelial layers of colonic specimens in humans. In our study, immunoreaction for BDNF was also shown in lamina propria cells of the mucosal biopsies. These data support the notion that the BDNF-containing mucosal cells in the colon could contribute to the generation of pain in patients with IBS by regulating the release of BDNF.

Mucosal nerve fibre density assessed by PGP9.5 was increased in maternally deprived rats with characteristics of IBS.[35] In line

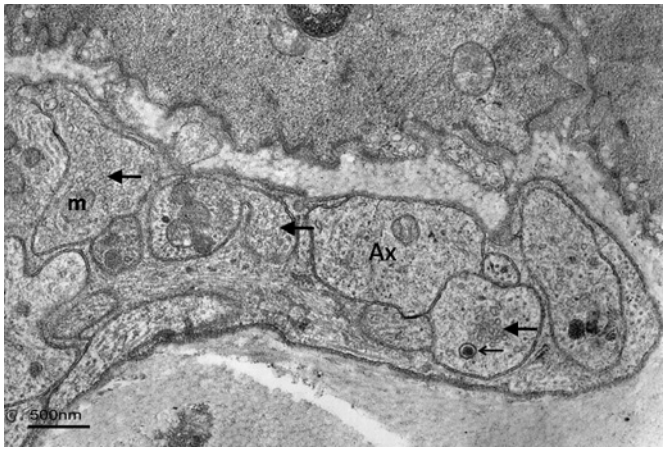

Figure 9 Electron micrograph showing the ultrastructure of nerve axons in the colonic mucosa of BDNF$^{+/+}$ mice. The nerve axons are filled with variable numbers of mitochondria and large amounts of lucent (small arrow) or dense-core synapse vesicles (large arrows). Ax, axon; BDNF, brain-derived neurotrophic factor; m, mitochondrion.

with the animal data, our study showed a marked increase of mucosal nerve fibres in colonic biopsies from patients with IBS. Tissue analysis of patients with inflammatory bowel disease showed the existence of neural hypertrophy and nerve process proliferation, which seemed to be coupled with the increased production of neurotrophins in lamina propria and epithelial cells.[21] The aetiology of increased total nerve fibres immunoreactive to PGP9.5 in IBS is unclear but could be attributed to the increased release of BDNF in the colonic mucosa. BDNF plays a crucial role in the maintenance and survival of visceral sensory neurons during development.[36 37] In addition, intrathecal infusion of BDNF antiserum prevented sprouting of spinal noradrenergic fibres after peripheral nerve injury in rats.[38] In accordance with these previous studies, we demonstrated that the mucosal nerve fibre density was significantly lower in BDNF$^{+/-}$ mice than in BDNF$^{+/+}$ mice.

The physiological changes in visceral sensitivity might also be associated with ultrastructural alterations in the nerve bundles of the mucosal lamina propria in patients with IBS. For example, the disarrangement of microtubules and microfilaments in the axon chambers may induce damage in the axonal transportation and impairment of neuron functions.[39] The exact causes for the ultrastructural damage on the nerve bundles are largely unknown. Evidence indicated that mast cells lay in closer vicinity to nerve fibres supplying the gut mucosa in IBS.[26] Furthermore, increased release of mast cell mediators, including

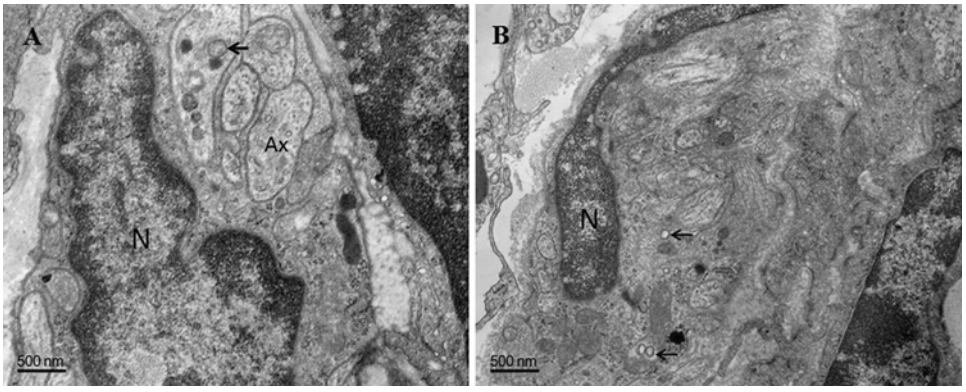

Figure 10 Electron micrographs showing ultrastructure of Schwann cells in the colonic mucosa of BDNF$^{+/+}$ mice (A) and BDNF$^{+/-}$ mice (B). The large arrow indicates normal mitochondria. Degenerative changes of Schwann cells are demonstrated in BDNF$^{+/-}$ mice. The nuclei are elongated and much smaller in size. Swollen mitochondria (small arrows) exist in the cytoplasm of Schwann cells in BDNF$^{+/-}$ mice. Ax, axon; BDNF, brain-derived neurotrophic factor; N, nucleus.

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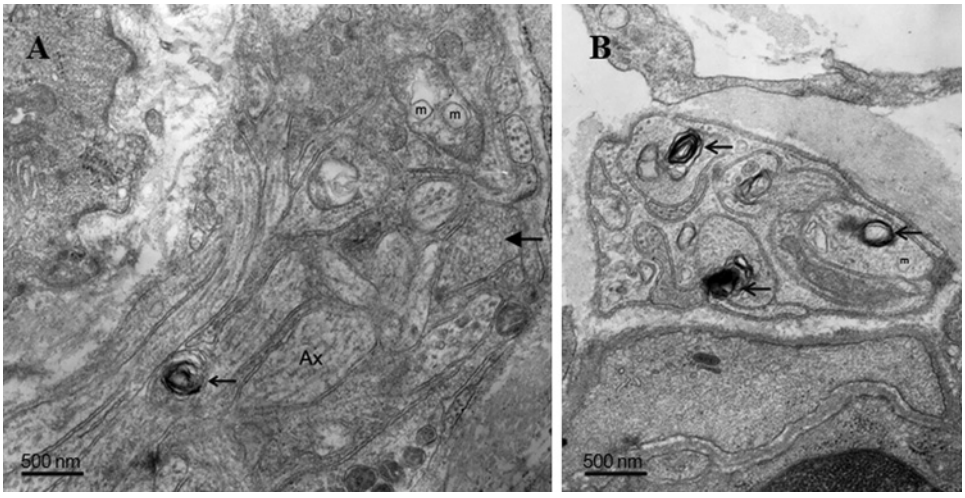

Figure 11 Electron micrographs showing the ultrastructure of nerve axons in the colonic mucosa of BDNF$^{+/-}$ mice (A,B). The nerve axons appear to have decreased electron density, with swollen or lucent mitochondria and less lucent or dense-core synapse vesicles (large arrow). Further, lamellar bodies are identified inside the nerve axons (small arrows). Ax, axon; BDNF, brain-derived neurotrophic factor; m, mitochondrion.

tryptase and histamine, was shown by the colonic mucosa of patients with IBS.[28 40 41] Taken together, these data suggest that the abnormal crosstalk between the immune and nervous system in the gut wall may contribute to the structural abnormalities of the mucosal innervation in patients with IBS. Furthermore, degenerative changes of nerve axons and Schwann cells were observed in BDNF$^{+/-}$ mice. These findings support the view that deficiency of BDNF may cause the impairment of mucosal nerve fibres and consequently result in an abnormal response to CRD.

Our ELISA data showed that the mucosal BDNF level was much higher in patients with IBS-D than in healthy controls. These data agree with the knowledge that BDNF has a potent excitatory effect on the colon. For example, recombinant human BDNF dose-dependently accelerated colonic transit, thus leading to increased frequency of bowel movements in healthy subjects and in patients with constipation.[42] However, of note, we found a higher level of BDNF in patients with IBS-C than in controls, although the difference was not statistically significant (p=0.1). This finding was difficult to compare and reconcile with those previous findings. In constipated patients, the upregulated BDNF may also induce excessive segmental contractile colonic motor activity and ultimately lead to a slow, rather than an accelerated, colonic transit. Indeed, markedly elevated mucosal BDNF levels were found in patients with ulcerative colitis, regardless of bowel habit.[10]

In this study, we used the AWR scoring system to study the behavioural response to CRD. The advantage of AWR over the visceromotor reflex is that the AWR is free of the direct abdominal muscle stimulation induced by implantation of electrodes and therefore eliminates somatic sensitisation.[30 43]

This study has several limitations. First, in keeping with the Rome II criteria, the symptom questionnaire did not discriminate between pain and discomfort. Second, BDNF has been confirmed to be upregulated during the inflammatory processes. Hence, patients with postinfective IBS were excluded to avoid the probable bias related to colonic mucosa BDNF expression. Finally, due to methodological limitations, our TEM studies are qualitative in nature and colonic sensory nerve fibres have not been specifically identified.

In conclusion, the increased expression of BDNF was demonstrated in the colonic mucosa of patients with IBS, together with structural alterations of mucosal innervation. Animal studies using BDNF$^{+/+}$ mice and BDNF$^{+/-}$ mice provided further support for the involvement of BDNF in the visceral sensitivity. Our findings suggest a mechanism that might contribute to the pathophysiology of pain in IBS and may provide useful insights into potential treatments for this condition.

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethical approval was obtained from the clinical ethical committee of the Qilu Hospital of Shandong University.

Contributors Yan-Bo Yu: analysis and interpretation of data, drafting of the manuscript, statistical analysis and technical and material support. Xiu-Li Zuo: study supervision, participant enrolment, critical revision of the manuscript and supplementary experiment support. Qiu-Jie Zhao: analysis and interpretation of data, acquisition of data and technical and material support. Fei-Xue Chen: analysis and interpretation of data and technical support. Jing Yang: acquisition of data, analysis and interpretation of data and technical support. Yan-Yan Dong: participant enrolment and technical and material support. Peng Wang: acquisition of data, statistical analysis, technical support and participant enrolment. Yan-Qing Li: study concept and design, critical revision of the manuscript, obtained funding, study supervision and final approval of the version to be published.

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