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Research Paper

NR2B-containing NMDA receptors contribute to diarrhea-predominant irritable bowel syndrome

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ABSTRACT

Colonic mucosal N-methyl-D-aspartate receptors (NMDARs) contribute to visceral hypersensitivity in diarrhea-predominant irritable bowel syndrome (IBS-D) by increasing the production of brain-derived neurotrophic factor (BDNF). We investigated which of the multiple NMDAR subtypes and BDNF isoforms are responsible for this effect. Immunohistochemistry and Western blotting were used to detect levels of colonic mucosal NR2A-D subunits and the precursor (proBDNF) and mature (mBDNF) forms of BDNF in 67 participants. Only NR2B subunit expression was elevated in the colonic mucosa of IBS-D patients, in parallel with increased total BDNF and proBDNF expression. Expression of 15 kDa mBDNF was not detected in the colonic mucosa. NR2B, total BDNF and proBDNF levels correlated with abdominal pain scores. Quantitative real-time PCR and Western blotting showed that NMDAR activation substantially induced total BDNF/proBDNF expression in HT-29 cells, while the NMDAR inhibitor MK-801 and the NR2B subunit antagonist Ro25-6981 each completely blocked these effects. Thus, colonic mucosal NR2B-containing NMDARs may contribute to visceral hypersensitivity in IBS-D patients by upregulating BDNF, especially proBDNF.

INTRODUCTION

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder, and involves abdominal pain, discomfort or bloating associated with changes in bowel habits [1]. IBS affects 9.8–12.8% of the population [2] and markedly reduces the quality of life [3]. In the Rome III criteria, four subtypes of IBS are described: diarrhea-predominant IBS (IBS-D), IBS with constipation, mixed-type IBS, and unsubtyped IBS. Among these subtypes, the diarrhea subtype especially impairs patients' quality of life by limiting their diet and their ability to travel or eat out [4]. Visceral hypersensitivity has been recognized as a crucial mechanism in IBS-D [5].

N-methyl-D-aspartate receptors (NMDARs) are heterotetramers that require the assembly of two NMDA receptor 1 (NR1) subunits with either two NR2 subunits (NR2A, NR2B, NR2C and NR2D) or a mixture of NR2 and NR3 subunits (NR3A and NR3B) [6, 7]. The variety of subunit combinations results in a large number of NMDAR subtypes, and the NR2 (A-D) subunits are major determinants of the functional heterogeneity of the receptors [8]. Previous studies have demonstrated that mucosal NMDARs in the colon contribute to the visceral

hypersensitivity in IBS by increasing the production of brain-derived neurotrophic factor (BDNF) [9]. Therefore, mucosal NMDAR-targeting drugs could be developed as effective therapies. However, NMDARs have multiple functions in the gastrointestinal tract. Loss of NR2D-containing receptors by siRNA knockdown reduced vascular endothelial cell migration, tube formation and transmigration [10], suggesting that the complete inhibition of NMDARs could have serious side effects. The targeted inhibition of NMDAR subtypes could avoid the side effects of NMDAR guided therapies for IBS. However, it remains unclear which NMDAR subtype contributes to the visceral hypersensitivity in IBS.

BDNF is a neurotrophin with multiple biological effects in the nervous system, including neuronal survival, synaptic plasticity and pain [11–13]. Previous studies have demonstrated that BDNF expression is markedly elevated in the colonic mucosa of IBS-D patients and contributes to colonic hypersensitivity [14–16]. Like other neurotrophins, BDNF is initially synthesized as a precursor [17–19]. The precursor of BDNF (proBDNF) is not merely an intermediate in the synthesis of mature BDNF (mBDNF), but rather has its own biological functions [20]. In the central nervous system, proBDNF binds to its receptor, p75 neurotrophin receptor ($p75^{NTR}$), and exerts some functions that oppose the functions of mBDNF. For example, in contrast to mBDNF, proBDNF can promote neuronal apoptosis and negatively regulate spine density [20, 21]. However, the expression of proBDNF and mBDNF have not yet been measured in the colonic mucosa of patients with IBS-D.

In this study, we assessed which of the NMDAR subtypes and BDNF isoforms in the colonic mucosa contribute to the visceral hypersensitivity in IBS-D.

RESULTS

Subjects

In total, 32 IBS-D patients (15 females, mean age 46.47 ± 14.84 years) and 35 control subjects (20 females, mean age 52.09 ± 10.55 years) were enrolled in this study (Table 1). The IBS-D and control groups did not differ significantly in age ($P = 0.077$) or sex ($P = 0.401$). On the whole, IBS-D patients had significantly higher abdominal pain/discomfort severity ($P < 0.0001$) and frequency ($P < 0.0001$) scores than healthy controls.

Only NR2B subunit expression was elevated in the colonic mucosa of IBS-D patients

Immunohistochemical staining revealed NR2 (A-D) subunit immunoreactivity primarily in colonic epithelial cells (Figure 1A). Quantification indicated that the immunohistochemical staining for NR2B was stronger in IBS-D patients (0.034 ± 0.011 integrated optical density [IOD]/area) than in healthy controls (0.028 ± 0.010 IOD/area; $P = 0.048$). However, there were no differences in NR2A, NR2C or NR2D expression between the two groups (Figure 1B).

Western blotting revealed that NR2B levels were 1.5-fold greater in IBS-D patients (87.49 ± 47.42%) than in healthy controls (58.15 ± 29.45%, $P = 0.003$). There were no significant differences between the IBS-D patients and controls in the expression of NR2A (33.36 ± 26.94% vs. 39.67 ± 43.34%, $P = 0.481$), NR2C (42.06 ± 17.68% vs. 39.92 ± 15.48%, $P = 0.599$) or NR2D (66.80 ± 49.59% vs. 64.33 ± 46.83%, $P = 0.835$) (Figure 1C and 1D).

Mucosal BDNF expression was elevated in IBS-D patients and correlated with NR2B expression

As reported previously [9], mucosal BDNF expression was higher in IBS-D patients than in healthy controls (139.00 ± 11.90% vs. 91.89 ± 5.98%, $P < 0.001$; Supplementary Figure 1A). BDNF expression correlated positively with NR2B expression in the mucosa (healthy controls: $r = 0.335$, $P = 0.050$; IBS-D patients: $r = 0.407$, $P = 0.021$; all subjects: $r = 0.550$, $P < 0.001$; Figure 2A).

Mucosal BDNF and NR2B levels correlated with abdominal pain scores

Mucosal NR2B levels correlated significantly with the severity of abdominal pain/discomfort (controls: $r = 0.404$, $P = 0.016$; IBS-D patients: $r = 0.630$, $P < 0.001$; all subjects: $r = 0.720$, $P < 0.001$; Figure 2B) and the frequency of abdominal pain/discomfort (controls: $r = 0.586$, $P < 0.001$; IBS-D patients: $r = 0.459$, $P = 0.008$; all subjects: $r = 0.712$, $P < 0.001$; Figure 2B). Mucosal BDNF levels also correlated with abdominal pain scores (Supplementary Figure 1C).

BDNF expression was induced by an NMDAR agonist

NMDA, alone or in combination with D-serine, can activate NMDARs [7, 22, 23]. As shown in Figure 3A, activating the NMDARs with NMDA (with/without D-serine) increased *BDNF* mRNA expression in HT29 cells. In HT29 cells exposed to 10 μM D-serine, NMDA dose-dependently upregulated *BDNF* (Figure 3A). Likewise, treatment of HT29 cells with NMDA and D-serine increased BDNF protein levels, which declined to control levels by 24 hours (Figure 3B and 3C).

MK-801 and Ro25-6981 each completely blocked BDNF expression in cells treated with NMDA and D-serine

MK-801 (a selective and high-affinity NMDAR antagonist) and Ro25-6981 (a selective and high-affinity

Table 1: Demographics and baseline characteristics of the groups

Patient group	Age (y) Mean ± SD	Gender M/F	BMI (kg/m²) Mean ± SD	Severity of abdominal pain/discomfort	Frequency of abdominal pain/discomfort
HC	52.09 ± 10.55	15/20	24.70 ± 3.57	0.14 ± 0.36	0.23 ± 0.43
IBS-D	46.47 ± 14.84	17/15	24.39 ± 3.40	2.16 ± 0.88	2.81 ± 1.06
P value	0.077	0.401	0.718	<0.001	<0.001

HC, healthy control; IBS-D, diarrhea-predominant irritable bowel syndrome; y, years; M, male; F, female; BMI, body mass index.

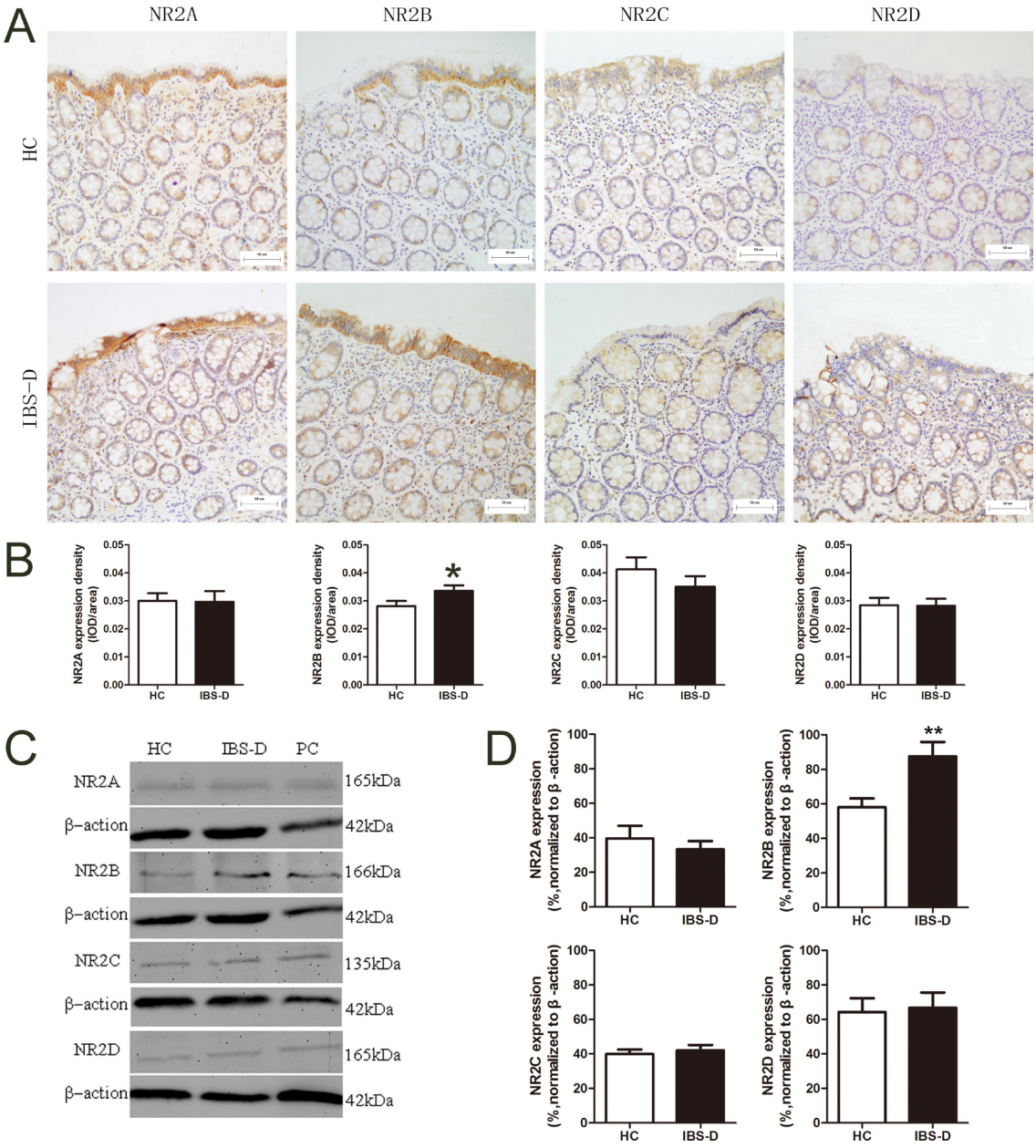

Figure 1: Only NR2B subunit expression was elevated in the colonic mucosa of IBS-D patients. (**A**) Representative images of NR2 (A–D)-immunoreactive cells in colonic mucosal tissues from healthy controls (HC) or diarrhea-predominant irritable bowel syndrome (IBS-D) patients. Magnification 20×, scale bar 50 μm. (**B**) Results of immunohistochemical analysis. (**C**) Representative bands of Western blots from HC, IBS-D patients or the positive control (PC, mouse brain tissue). (**D**) Results of Western blot analysis. $n = 35$ for HC, $n = 32$ for IBS-D. Data are presented as means ± SDs. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control (Student's *t* test).

NR2B subunit antagonist) have been used extensively to block NMDARs and the NR2B subunit, respectively [24–27]. Preincubation of HT29 cells with MK-801 or Ro25-6981 abolished the effect of NMDA and D-serine on *BDNF* mRNA expression (Figure 4A), indicating that the receptor containing the NR2B subunit was responsible for *BDNF* mRNA expression.

Likewise, while BDNF protein expression in HT29 cells doubled following NMDA and D-serine exposure, it was reduced to the control level when the NMDARs were blocked with MK-801 and when NR2B was blocked with Ro25-6981 (Figure 4B and 4C).

Mucosal proBDNF levels were elevated in IBS-D patients and correlated with NR2B levels

Immunohistochemical staining revealed proBDNF immunoreactivity primarily in colonic epithelial cells (Figure 5A). Quantification demonstrated that immunohistochemical staining for proBDNF was stronger in IBS-D patients (0.041 ± 0.002 IOD/area) than in healthy controls (0.031 ± 0.002 IOD/area; $P < 0.001$) (Figure 5B). Western blotting indicated that mucosal proBDNF levels were substantially higher in IBS-D patients than in healthy controls (72.19 ± 30.25% vs. 55.93 ± 26.82%, $P = 0.023$, Figure 5C). ProBDNF expression correlated with NR2B expression (healthy controls: $r = 0.385$, $P = 0.022$; IBS-D patients: $r = 0.547$, $P = 0.002$; all subjects: $r = 0.623$, $P < 0.001$; Figure 5D).

ProBDNF levels correlated with abdominal pain scores

In all the patient groups, mucosal proBDNF expression correlated with the severity of abdominal pain/discomfort (controls: $r = 0.340$, $P = 0.046$; IBS-D

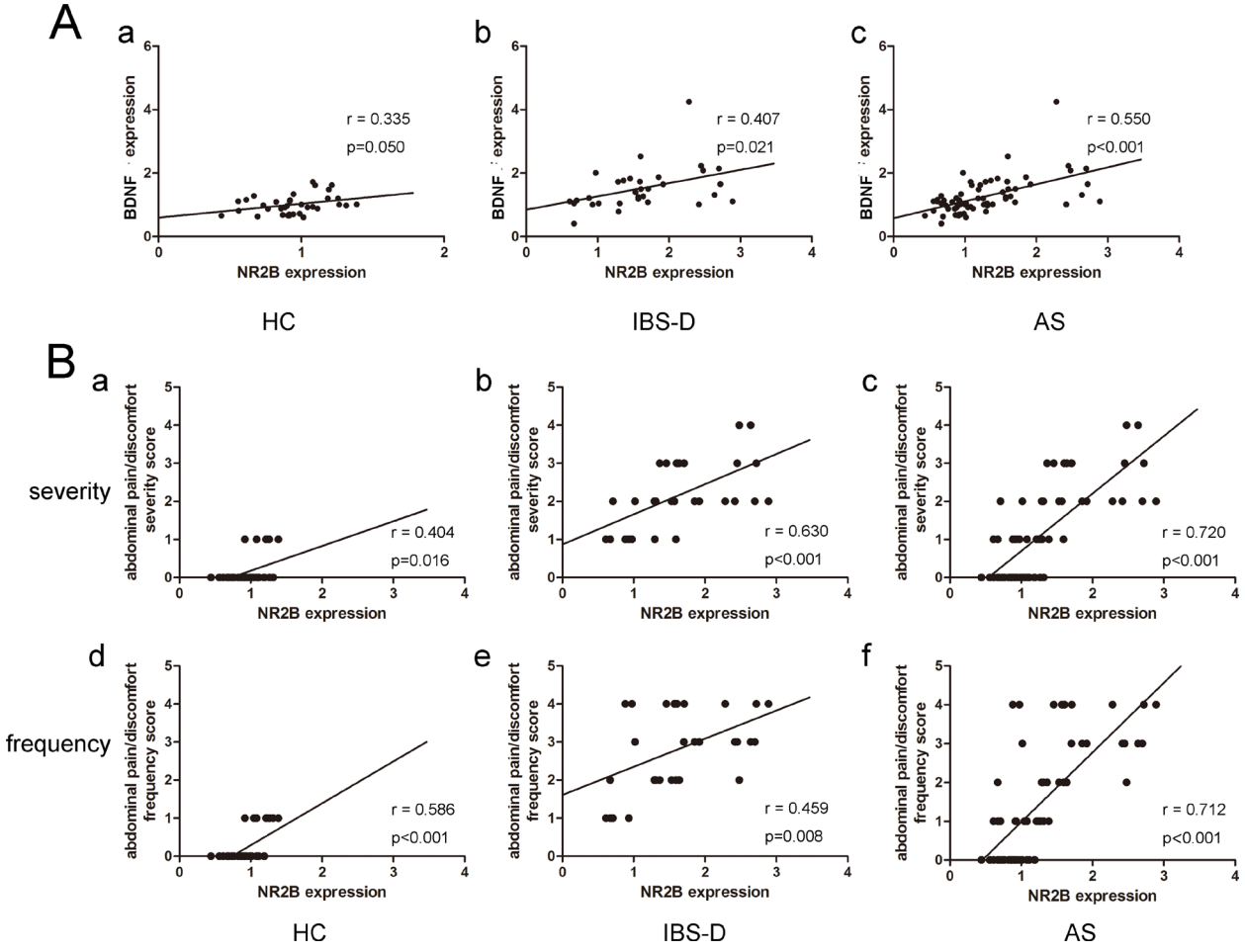

Figure 2: Mucosal NR2B levels correlated with BDNF levels and abdominal pain scores. (A) Pearson's correlation between colonic mucosal NR2B and BDNF protein levels in (a) healthy controls (HC, $r = 0.335$, $P = 0.050$), (b) IBS-D patients ($r = 0.407$, $P = 0.021$) and (c) all subjects (AS, $r = 0.550$, $P < 0.001$). **(B)** Scatter plots depicting the correlation between NR2B levels and the severity of abdominal pain/discomfort in (a) healthy controls ($r = 0.404$, $P = 0.016$), (b) IBS-D patients ($r = 0.630$, $P < 0.001$) and (c) all subjects ($r = 0.720$, $P < 0.001$), and the correlation between NR2B levels and the frequency of abdominal pain/discomfort in (d) healthy controls ($r = 0.586$, $P < 0.001$), (e) IBS-D patients ($r = 0.459$, $P = 0.008$) and (f) all subjects ($r = 0.712$, $P < 0.001$). Correlation analysis was performed with Spearman's rank correlation.

patients: $r = 0.653$, $P < 0.001$; all subjects: $r = 0.638$, $P < 0.001$; Figure 5E) and the frequency of abdominal pain/discomfort (controls: $r = 0.418$, $P = 0.013$; IBS-D patients: $r = 0.572$, $P = 0.001$; all subjects: $r = 0.655$, $P < 0.001$; Figure 5E).

NR2B activation induced proBDNF protein expression

After the addition of NMDA with/without D-serine to HT29 cells, proBDNF expression increased, and then decreased to the control level by 24 hours (Figure 6A and 6B). Likewise, while proBDNF expression in HT29 cells increased 1.9-fold following NMDA and D-serine exposure, it decreased to the control level when NMDA receptor activation was inhibited with MK-801 or Ro25-6981 (Figure 6C and 6D).

Mucosal mBDNF expression at 15 kDa was not detected

As shown in Supplementary Figure 1B, a band at 15 kDa corresponding to mBDNF was observed in brain tissue, but not in the colonic mucosa.

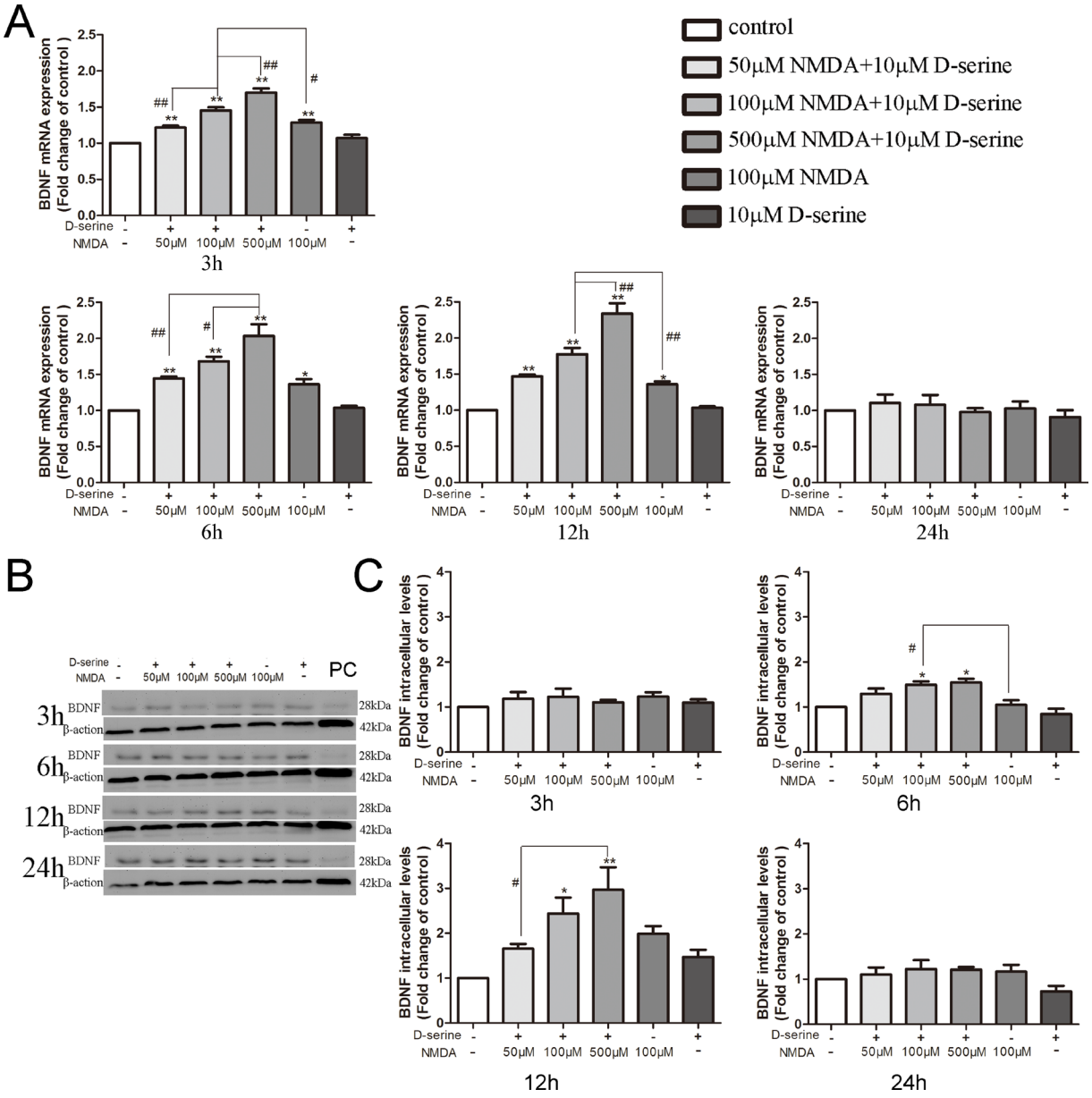

Figure 3: BDNF expression was induced by NMDAR agonist. (**A**) *BDNF* mRNA expression at 3, 6, 12 and 24 hours with different concentrations of NMDA and/or D-serine($n \geq 5$). (**B**) Representative Western blot bands after the addition of NMDA and/or D-serine. PC: positive control. (**C**) Results of densitometric analysis ($n = 3$). Data are presented as means ± SDs. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; NS: not significant (one-way ANOVA, Tukey tests).

DISCUSSION

Previous studies in three kinds of research subjects (clinical patients, mice and cultured cells) have demonstrated that the activation of colonic mucosal NMDARs contributes to the visceral hypersensitivity in IBS by increasing the production of BDNF [9, 28]. In the present study, we confirmed that the NR2 (A-D) subunits were expressed in the colonic mucosa, but we found that only NR2B subunit expression was elevated in IBS-D patients. NR2B expression correlated positively with abdominal pain scores and BDNF expression. Accordingly, both an NMDAR antagonist and an NR2B subunit antagonist completely abolished the induction of BDNF expression by an NMDAR agonist, indicating that the receptor containing the NR2B subunit was responsible for BDNF expression. These findings confirmed the critical involvement of the NR2B subunit in visceral hypersensitivity.

A growing number of people suffer from IBS, but the pathophysiological mechanism of IBS is complex and poorly understood. In addition, conventional drugs are still limited in clinical practice because of their side effects [29, 30], and some drugs have even been withdrawn from the market due to severe adverse events [31, 32]. The development of novel medications that act locally in the gastrointestinal tract is a promising way to overcome this problem. A recent study demonstrated that NMDARs were markedly upregulated in the colonic mucosa of IBS patients and contributed to colonic hypersensitivity [9]. A colon-targeted NMDAR antagonist would be a new approach to attenuating abdominal pain in IBS patients. However, NMDARs exist as multiple subtypes and have multiple biological functions in the gastrointestinal tract. Thus, targeted inhibition of an NMDAR subtype would be a better way to avoid side effects.

NMDARs have primarily been studied as neuronal ionotropic glutamate receptors, and have multiple functions in the nervous system [7]. All NMDAR subtypes are thought to combine two copies of the obligatory NR1 subunit with two copies of NR2 and/or NR3 subunits. The subunit composition of an NMDAR determines its receptor subtype. To date, seven different subunits have been identified, which fall into three subfamilies: the NR1 subunit, four NR2 subunits (NR2A, NR2B, NR2C and NR2D) and a pair of NR3 subunits (NR3A and NR3B) [8]. Among these subunits, the four NR2 subunits are major determinants of the functional heterogeneity of the receptors. Thus, we investigated the effects of the NMDAR subtypes by studying the NR2 subunits.

In the nervous system, NMDARs containing the NR2B subunit are critical for anterior cingulate cortex sensitization and visceral pain responses in viscerally hypersensitive rats [33]. In our study, we confirmed that the NR2B NMDA receptor subtype in the colonic mucosa was responsible for the pathogenesis of visceral hypersensitivity in IBS-D, as it increased the production of BDNF. The administration of an NR2B antagonist could inhibit visceral hypersensitivity in the central nervous system and the colon at the same time.

BDNF is a neurotrophin with multiple functions. Members of the neurotrophin family are initially synthesized as 31-35-kDa precursors, which are

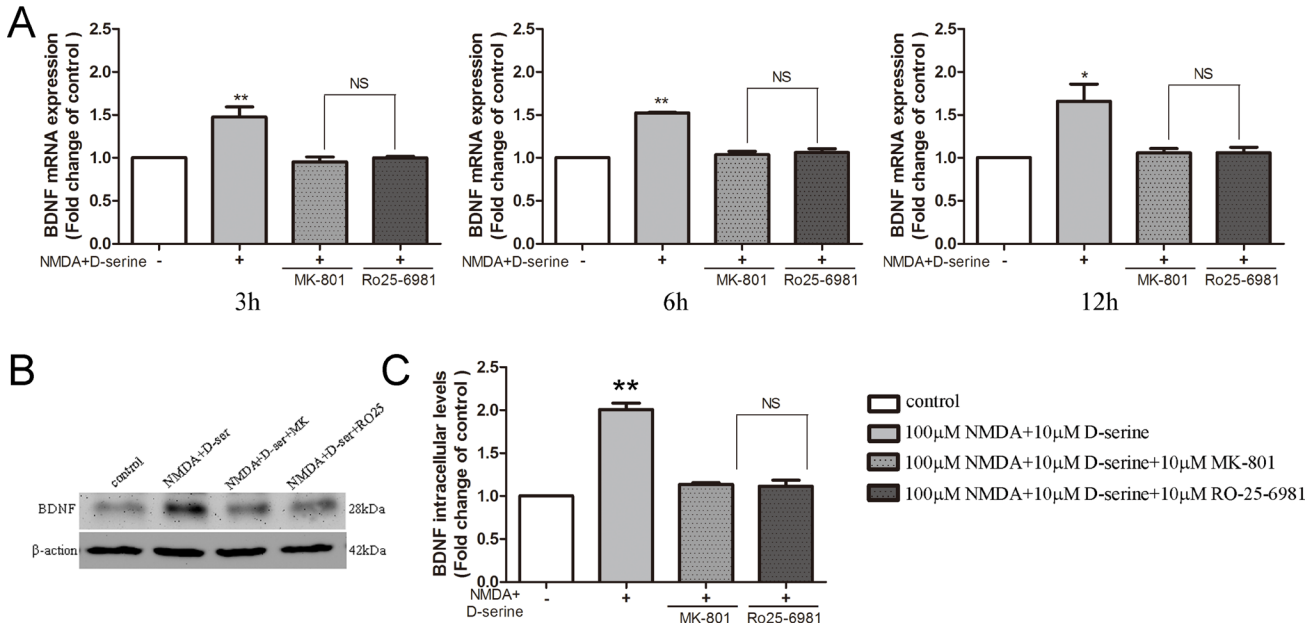

Figure 4: MK-801 and Ro25-6981 each completely blocked BDNF expression. (**A**) MK-801 or Ro25-6981 was used to block the NR1 subunit or the NR2B subunit, respectively. Changes in *BDNF* mRNA expression were measured by qPCR ($n \geq 5$). (**B**) Representative Western blot bands after the addition of NMDA, D-serine, MK-801 or Ro25-6981. (**C**) Results of densitometric analysis ($n = 3$). Data are presented as means ± SDs. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; NS: not significant (one-way ANOVA, Tukey tests).

subsequently cleaved, yielding mature 13.2–15.9-kDa neurotrophins [34]. In accordance with previous studies [35, 36], three bands were evident on our Western blot membrane: the 35-kDa proBDNF, the 28-kDa mixture, and the 15-kDa mBDNF. The band at 28 kDa corresponded to truncated proBDNF and the mBDNF dimer [14, 15].

In the central nervous system, mBDNF promotes pain through central sensitization; thus, neutralizing the increased spinal mBDNF could attenuate pain processing [37, 38]. However, peripheral proBDNF, but not mBDNF, exacerbates inflammatory, visceral, surgical pain in the peripheral nervous system [39]. To investigate the function

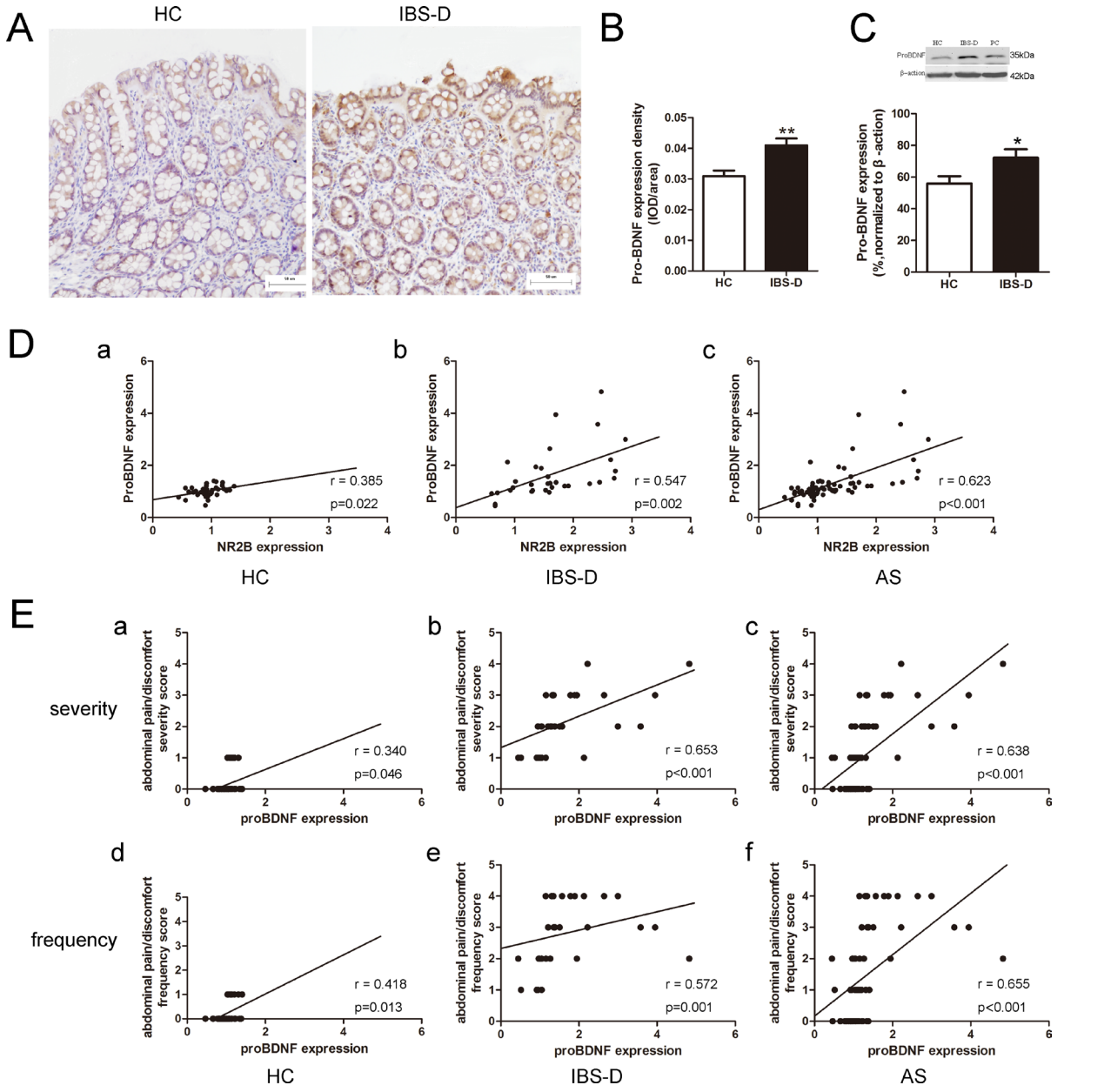

Figure 5: Mucosal proBDNF expression was elevated in IBS-D patients and correlated with NR2B levels and abdominal pain scores. (**A**) Representative images of proBDNF-immunoreactive cells in colonic mucosal tissues from healthy controls (HC) or diarrhea-predominant irritable bowel syndrome (IBS-D) patients. Magnification 20×, scale bar 50 μm. (**B**) Results of immunohistochemical analysis. (**C**) Western blot analysis of proBDNF in mucosal biopsies. PC: positive control. (**D**) Pearson's correlation between colonic mucosal NR2B and proBDNF protein levels in (a) healthy controls ($r = 0.385$, $P = 0.022$), (b) IBS-D patients ($r = 0.547$, $P = 0.002$) and (c) all subjects (AS, $r = 0.623$, $P < 0.001$). (**E**) Scatter plots depicting the correlation between proBDNF levels and the severity of abdominal pain/discomfort in (a) control subjects ($r = 0.340$, $P = 0.046$), (b) IBS-D patients ($r = 0.653$, $P < 0.001$) and (c) all subjects ($r = 0.638$, $P < 0.001$), and the correlation between proBDNF levels and the frequency of abdominal pain/discomfort in (d) control subjects ($r = 0.418$, $P = 0.013$), (e) IBS-D patients ($r = 0.572$, $P = 0.001$) and (f) all subjects ($r = 0.655$, $P < 0.001$). $n = 35$ for HC, $n = 32$ for IBS-D. Data are presented as means ± SDs. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control (Student's t test).

of BDNF in the gastrointestinal tract, we examined proBDNF and mBDNF expression in the colonic mucosa. ProBDNF expression was upregulated in the colonic mucosa of the IBS-D group and correlated with abdominal pain scores. However, mBDNF expression at 15 kDa was not detected in the colonic mucosa, while a band was detected in the positive control. These findings suggested that proBDNF may exacerbate visceral pain in the gastrointestinal tract. However, our study was a preliminary evaluation, and further research in this area is warranted.

In the peripheral tissues, proBDNF secretion by inflammatory cells correlates highly with pain [39, 40]. In addition, low-grade inflammation is involved in the pathogenesis of IBS. However, upon its upregulation, proBDNF was highly distributed in colonic epithelial cells. Thus, it is unlikely that proBDNF contributes to abdominal pain in IBS as an inflammatory mediator.

In recent years, NMDARs and BDNF have been discovered to induce visceral pain hypersensitivity [41–44]. It is well known that NMDARs regulate BDNF expression and secretion [45–47]. BDNF participates in colitis-induced spinal central sensitization by upregulating NR1 phosphorylation [48], suggesting that BDNF modulates NMDAR activity in the spinal cord. The interaction between NMDARs and BDNF in visceral hypersensitivity should be further studied.

In neuronal and vascular cells, protein metabolites such as amino acids and ammonia can activate NMDARs [7, 22]. However, in the colonic mucosa, it is unclear which component of feces induces the expression of BDNF by activating NMDARs. Moreover, NMDARs may contribute to the pathogenesis of IBS through other mechanisms. NMDARs regulate diverse processes such as apoptosis, immune responses, arterial dilation, etc. [22, 49–51]. A previous study demonstrated that NMDARs linked ammonia with gastric epithelial cell death [27]. Multiple mechanisms contribute to IBS symptoms, including altered visceral sensitivity and pain processing, motility disorders, altered microbiota, low-grade inflammation and increased mucosal permeability [52].

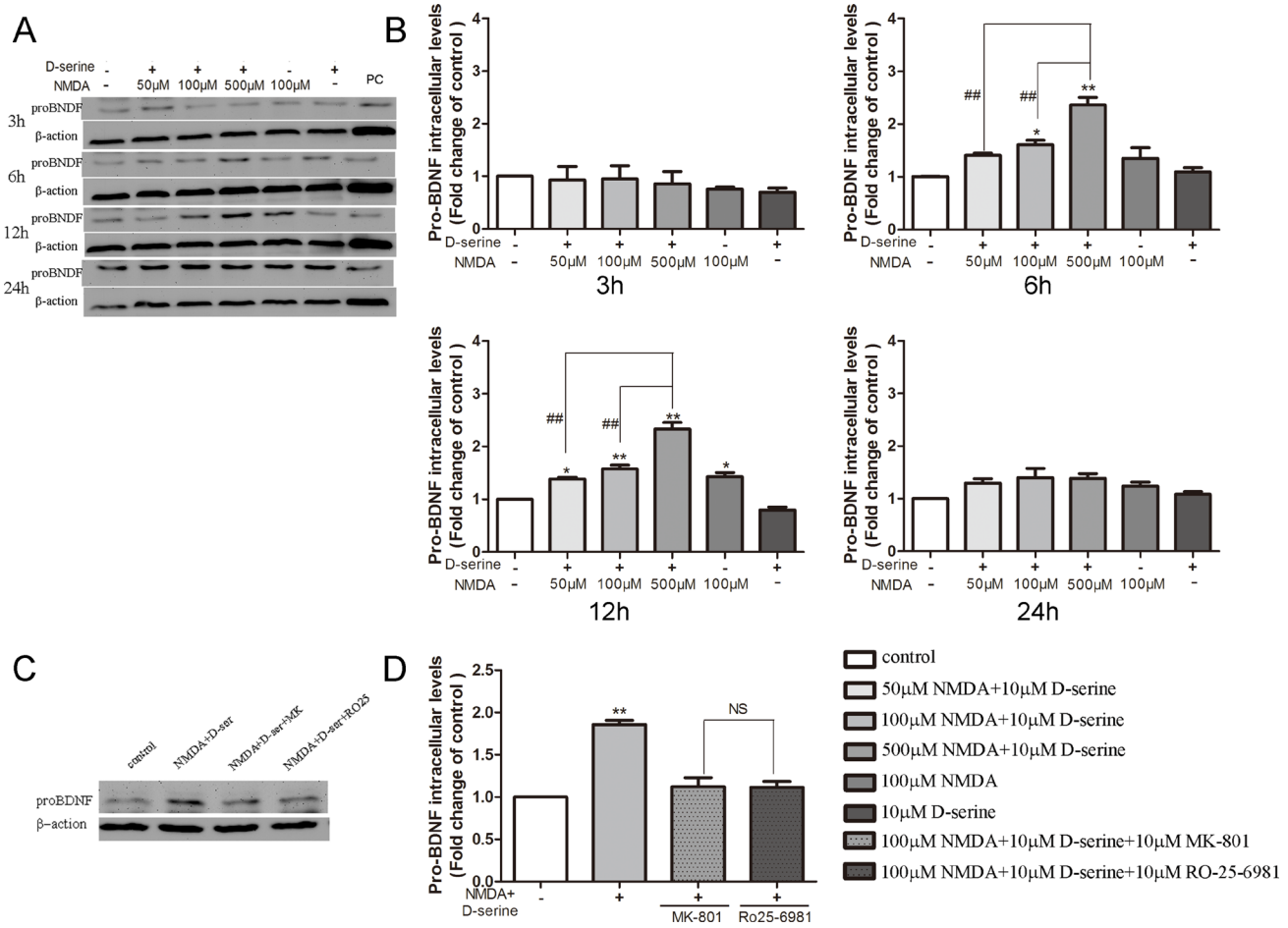

Figure 6: ProBDNF protein expression in HT29 cells was induced by NMDARs. (**A, C**) Representative Western blot bands after the addition of NMDA, D-serine, MK-801 or Ro25-6981. (**B, D**) Results of densitometric analysis. Data are presented as means ± SDs ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{\#\#}P < 0.01$; NS: not significant (one-way ANOVA, Tukey tests). PC: positive control.

Excessive apoptosis of intestinal epithelial cells increases mucosal permeability [53]. It remains unclear whether NMDARs contribute to epithelial barrier dysfunction in IBS-D.

In summary, we demonstrated that the NR2B NMDA receptor subtype in the colonic mucosa contributes to the visceral hypersensitivity in IBS-D by increasing the production of BDNF, especially proBDNF. Thus, NR2B and proBDNF could be considered as potential therapeutic targets for IBS.

MATERIALS AND METHODS

Patients

The protocol for this study was approved by the Ethics Committee of Qilu Hospital, Shandong University (NO.KYLL-2013-087), and was registered at ClinicalTrials.gov (ID: NCT02512146). The diagnosis of IBS-D was made by an experienced gastroenterologist based on the Rome III criteria [54]. The healthy controls were patients undergoing endoscopies for polyps and cancer screening, with negative results. The exclusion criteria were as follows: a history of abdominal surgery; alarm symptoms such as anemia, gastrointestinal bleeding, marked weight loss or abdominal masses; coagulopathy, serious organic disease or impaired cardiac, liver or renal function; pregnancy or breastfeeding; and unwillingness to sign the informed consent form. Human mucosal biopsies were obtained from the rectosigmoid junction of patients undergoing colonoscopies at the Qilu Hospital of Shandong University (Shandong, China). The evaluation included a detailed history, routine blood analysis, tests of blood clotting, thyroid and liver function, an abdominal ultrasound, a colonoscopy and a mucosal biopsy (to exclude microscopic colitis).

Assessment of abdominal pain/discomfort

IBS-D patients were asked to score their abdominal symptoms over the previous two weeks using a validated questionnaire described previously [9, 15, 55]. According to its impact on the patient's daily activities, the severity of abdominal pain/discomfort was graded from 0 (absent) to 4 (extremely severe, precluding daily activities). The frequency of abdominal pain/discomfort was graded from 0 (absent) to 4 (daily), according to the frequency of symptoms per week.

Immunohistochemistry

Paraffin-embedded sections (4 μm) of human mucosal biopsies were collected on gelatin-coated slides. Tissue sections were deparaffinized in xylene and hydrated in gradient alcohol. After being steamed in sodium citrate buffer (10 mM sodium citrate, pH 6.0) for 20 min at 92–98°C for antigen retrieval, the sections were treated with hydrogen peroxide (3% v/v) for 20 min to quench the endogenous peroxidase activity. Thereafter, the slides were blocked with normal goat serum (Zhongshan Gold Bridge, Beijing, China) and incubated with primary antibodies overnight at 4°C. The primary antibodies were detected with a biotin-streptavidin horseradish peroxidase detection system (Zhongshan Gold Bridge, Beijing, China) and diaminobenzidine as the chromogen [9, 56]. For the negative control tissue sections, the primary antibody was replaced with phosphate-buffered saline. Sections from mouse brain tissue were used as a positive control. The primary antibodies used in this study were as follows: anti-NR2A (1:100; Abcam, Cambridge, UK), anti-NR2B (1:200; Abcam), anti-NR2C (1:250; Santa Cruz Biotechnology, CA, USA), anti-NR2D (1:150; Santa Cruz Biotechnology) or anti-proBDNF (1:30; Santa Cruz Biotechnology). Images were obtained under a light microscope (Olympus, BX53), and four 40× TIFF-format images of randomly selected non-overlapping fields from each section were analyzed in a blinded manner. Image-pro Plus 6.0 software was used to calculate the average IOD per stained area (IOD/area; μm^2) for positive staining.

Western blot

Protein lysates were prepared with radioimmunoprecipitation plus buffer (Solarbio, Beijing, China) supplemented with phenylmethane sulfonyl fluoride (Solarbio). Briefly, 50 μg of total protein as determined with a bicinchoninic acid protein assay kit (Beyotime, Shanghai, China) was electrophoretically separated on a 10% sodium dodecyl sulfate polyacrylamide gel (Beyotime) and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After being blocked with 5% bovine serum albumin in Tris-buffered saline-0.1% Tween 20 buffer, the membrane was incubated with a rabbit anti-NR2A antibody (1:800; Abcam), mouse anti-NR2B antibody (1:1200; Abcam), rabbit anti-NR2C antibody (1:400; Santa Cruz Biotechnology), rabbit anti-NR2D antibody (1:100; Santa Cruz Biotechnology), rabbit anti-BDNF antibody (1:1000; Abcam), or mouse anti-β-actin antibody (1:1000; Zhongshan Gold Bridge). The membrane was then incubated with a peroxidase-conjugated secondary antibody (1:5000; Zhongshan Gold Bridge) and scanned with image acquisition and analysis software (UVP Bioimaging, Upland, CA, USA). The density of the bands was analyzed with ImageJ (Version 1.48d; NIH).

Cell culture

HT29 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). The cells were kept at 37°C

in a humidified 5% CO_2 atmosphere. HT29 cells were seeded in 6- or 12-well plates for 36 hours and serum-starved for 24 hours. Under serum starvation conditions, the cells were preincubated for 30 minutes with 10 μM MK-801 (Sigma Aldrich, Oakville, ON, Canada) or 10 μM Ro25-6981 (Tocris Bioscience, Ellisville, MO, USA) and then incubated for 24 hours with 100 μM NMDA (Sigma Aldrich) and 10 μM D-serine (Sigma Aldrich). Cells were harvested for the measurement of BDNF/proBDNF levels by Western blotting and/or quantitative real-time PCR (qPCR).

qPCR

Total RNA was isolated manually with an RNAprep pure cell/bacteria kit (TIANGEN, Beijing, China) and was reverse transcribed into cDNA with a ReverTra Ace® qPCR RT kit (TOYOBO, Osaka, Japan) in a Mastercycler thermal cycler (Bioer, Hangzhou, China). Then, qPCR was performed with SYBR® green real-time PCR master mix (TOYOBO, Osaka, Japan) in a real-time PCR instrument (StepOnePlus; Applied Biosystems, America). The primers for human *BDNF* were as follows: forward 5′-TGAGGACCAGAAAGTTCGGC-3′ and reverse 5′-GAGGCTCCAAAGGCACTTGA-3′. *BDNF* mRNA levels were calculated by the ΔΔCt method, normalized to human β-actin levels and expressed as fold-changes relative to the control.

Statistical analysis

All statistical analyses were performed with SPSS statistical software (version 19.0; SPSS Inc., Chicago, IL, USA). The mean values of quantitative variables were compared between the two groups by Student's *t*-test. Data from multiple groups were analyzed by one-way analysis of variance (ANOVA), followed by multiple comparisons with Tukey tests. Correlations between protein levels were analyzed with Pearson's correlation coefficients. Correlations between protein levels and abdominal pain/discomfort scores were assessed by Spearman's rank correlation. All data are presented as mean values ± standard deviations (SDs). Differences between means at a level of $P \leq 0.05$ were considered to be significant.

Abbreviations

NMDA: N-methyl-D-aspartate; NMDARs: NMDA receptors; IBS-D: diarrhea-predominant irritable bowel syndrome; BDNF: brain-derived neurotrophic factor; proBDNF: the precursor of BDNF; mBDNF: mature BDNF.

Author contributions

Wenxue Zhang carried out the experimental work and drafted the manuscript, and Xiuli Zuo edited the manuscript. Wenxue Zhang, Dongyan Zhao and Xin Long evaluated the immunostained slides and cooperated in the experimental work. Wenxue Zhang, Qingqing Qi, Peng Wang and Lixiang Li conceived the study, participated in its design and edited the manuscript. Wenxue Zhang, Yueyue Li and Yanbo Yu collected clinical parameters for this study. Wenxue Zhang and Yiyuan Sun analyzed and interpreted the data. Xiuli Zuo and Yanqing Li provided infrastructural and financial support for this study. Zhen Li edited the manuscript. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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