

Activating metabotropic glutamate receptor-7 attenuates visceral hypersensitivity in neonatal maternally separated rats

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Abstract. Increasing evidence has indicated that metabotropic glutamate receptor-7 (mGluR7) is an important target for reducing anxiety and stress-associated behaviours. Notably, mood disorders exhibit high levels of comorbidity with gastrointestinal dysfunction; however, the role of mGluR7 outside of the central nervous system is currently unknown. Activating mGluR7 likely increases colonic secretory function. Therefore, the present study aimed to evaluate the possible effects of mGluR7 on the visceral hypersensitivity of irritable bowel syndrome (IBS) in rats. The expression levels of mGluR7 were assessed in the colon tissues of rats with neonatal maternal separation (NMS)-induced visceral hypersensitivity using reverse transcription-quantitative polymerase chain reaction, western blotting and immunohistochemistry. In addition, the mGluR7 agonist AMN082 (3 or 10 mg/kg; i.p.) was administered 1 h prior to the visceral hypersensitivity test, and the effects of AMN082 were then observed on the nuclear factor (NF)- κ B signalling pathway. The mRNA and protein expression levels of mGluR7 were upregulated in the colon mucosa of NMS rats compared with in normal control rats. Notably, administration of AMN082 (10 mg/kg) attenuated colorectal distension (CRD)-induced visceral hypersensitivity in NMS rats. In addition, interleukin-10 and transforming growth factor- β mRNA expression levels were upregulated, whereas

interferon- γ mRNA expression levels were downregulated in the NMS + AMN082 group compared with in NMS rats. The number of cluster of differentiation 3⁺ T cells in the intestinal mucosa and myeloperoxidase activity were decreased in NMS + AMN082 rats. Furthermore, AMN082 treatment reduced the protein expression levels of phosphorylated-NF- κ B in the colon tissue of NMS rats. These results indicated that activation of mGluR7 may attenuate CRD-induced visceral hypersensitivity in experimental IBS and reduce the abnormal immune cytokine response. In addition, it was suggested that the role of AMN082 in modulating the inflammatory response may be partially associated with inhibiting NF- κ B activation. These data suggested that targeting mGluR7 may be useful in the treatment of stress-associated IBS.

Introduction

Irritable bowel syndrome (IBS) is a highly prevalent functional bowel disorder, which is characterised by the presence of abdominal pain or discomfort, an alteration in bowel habits, and diarrhoea and constipation without any structural cause. The aetiology of IBS predominantly includes genetics, motility, visceral hypersensitivity, diet, infection and inflammation, alteration of intestinal flora and psychosocial factors (1,2). Neonatal maternal separation (NMS) is a form of early-life stress, which has been consistently used in rats to develop experimental visceral hypersensitivity (3,4). Visceral hypersensitivity has been widely considered a biological marker of IBS; however, the underlying mechanism remains unclear.

L-Glutamate is the major excitatory neurotransmitter in the central nervous system. The actions of glutamate are modulated by ionotropic receptors and metabotropic glutamate receptors (mGluRs) (5). Based on sequence homology and signal transduction mechanisms, mGluRs have been divided into groups I, II and III; mGluR4, mGluR6, mGluR7 and mGluR8 are group III mGluRs (5,6). These receptors can modulate the effect and release of glutamate in the central nervous system (7). In addition, increasing evidence has indicated that mGluRs are expressed in the periphery, such as the gastrointestinal system. The glutamatergic system has also been implicated in the pathophysiology of depression and anxiety; antidepressants have been reported to inhibit glutamate release, a phenomenon mirrored by activation of group III

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mGluRs (6,8). As a member of the group III mGluRs, mGluR7 is of particular interest because it is an important target for reducing anxiety and stress-associated behaviours (8,9). Selective activation of G-protein-coupled mGluR7 elicits anxiolytic-like effects in mice by modulating γ -aminobutyric acid-ergic neurotransmission (10). Furthermore, activating mGluR7 by AMN082 (a selective agonist of mGluR7) induces a reduction in immobility in a forced swim test and tail suspension test (11,12). Notably, mood disorders exhibit high comorbidity with gastrointestinal dysfunction (13), and a high frequency of IBS symptoms is observed in patients with panic disorder, generalised anxiety disorder and major depressive disorder (14). Therefore, dysregulation of mGluR7 activity may be considered a significant factor underlying stress-induced IBS. AMN082 is a recently discovered selective mGluR7 agonist, which induces an increase in faecal water content in stress-induced defecation (6); however, to the best of our knowledge, the contribution of mGluR7 to early-life stress-induced visceral hypersensitivity in IBS remains unexplored. Therefore, the present study aimed to investigate a possible functional role of mGluR7 in the colon by assessing agonist-induced alterations in visceral hypersensitivity.

Several 5-hydroxytryptamine receptors (5-HTRs) are expressed in the gut, including 5-HT_{1A}R, 5-HT_{2A}R, 5-HT_{2B}R, 5-HT₃R, 5-HT₄R and 5-HT₇R. Alterations in the levels of 5-HT have been observed in experimental models of colitis and in patients with IBS; however, the results are varied (15,16). Nitric oxide (NO) is a gaseous messenger that serves an essential role in the physiology and pathophysiology of the gastrointestinal tract. NO is synthesised by NO synthase (NOS), which is classified into neuronal NOS, endothelial NOS and inducible NOS (iNOS). It has been reported that activation of the NO pathway alleviates the symptoms of IBS; Paragomi *et al* demonstrated that the NOS inhibitor L-NAME reverses the antinociceptive effects of sodium hydrogen sulphide on colorectal distension in a chemically induced model of IBS in rats (17). These findings indicated that NO may serve a protective role against IBS; however, the expression levels of NO in the rectum and plasma in IBS are not consistent (3,17). In addition, the development of IBS is associated with low-grade inflammation, and nuclear factor (NF)- κ B is a critical transcription factor for the inflammatory response (18). Anxiety-depression status may elevate interleukin (IL)-1 β and IL-10 levels in patients with IBS (19), which leads to the occurrence or aggravation of IBS. The present study demonstrated that mGluR7 may serve an important role in visceral hypersensitivity by modulating the function of NOS or inflammatory factors, thereby attenuating visceral hypersensitivity in IBS. The results indicated that there was no difference in 5-HTRs in any of the groups; therefore, alterations in NOS and inflammatory factors were discussed in detail.

Materials and methods

Animals and NMS. All animal protocols were approved by the Animal Care and Use Committee at the Tongji University School of Medicine (Shanghai, China) and were conducted according to the National Institutes of Health (NIH) Guidelines for the Care and Use of Animals in Research (NIH Publication No. 85-23, revised 1996) (20). Seven 15-day

pregnant Sprague-Dawley rats were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were maintained under a 12-h light/dark cycle with free access to food and water at 22°C and 50% relative humidity.

All Sprague-Dawley pups (weight 5–6 g) were divided into three groups: Normal control (NC) group, neonatal maternal separation (NMS) group and NMS + AMN082 group ($n=6$ /group). The pups were maintained under a 12-h light/dark cycle with free access to food and water at 22°C and 50% relative humidity. Pups in the NMS and NMS + AMN082 groups were separated from their dams and placed into individual cages in an adjacent room for 3 h (09:00–12:00) on postnatal day (P)2–14 (date of birth is designated P0). The pups were subsequently returned to the cages of their dams for the remaining time. The rats in the NC group were allowed to remain in standard cages with their dams. Pups were weaned on P22; only male rats were used in the present study, in order to avoid alterations associated with hormonal cycles. Each cage housed five rats; visceral hypersensitivity was measured after 8 weeks of feeding after weaning.

Behavioural testing to measure visceral hypersensitivity
Abdominal withdrawal reflex (AWR). Visceral hypersensitivity responses to colorectal distension (CRD) were measured by recording AWR scores, as described previously (3,10). Prior to testing, the rats were fasted and subjected to water deprivation, in order to reduce faeces. The rats were anaesthetized with isoflurane (1.5%) in a sealed cage (21), and a 6-cm deflated latex balloon was inserted into the distal colon up to 1 cm from the anal verge. After a 15-min recovery period, measurements of the visceral pain threshold were recorded when various pressure was applied to the abdominal walls by inflating the balloon (20, 40, 60 and 80 mmHg). Three measurements for each animal were taken at 5-min recovery intervals. A previously reported standard was used to evaluate the AWR score: 0, no response to given pressure; 1, slight head movement without abdominal muscle contraction; 2, contraction of the abdominal muscles; 3, lifting of the abdominal wall; and 4, body arching and lifting of pelvic structures (22).

Electromyography (EMG). EMG was also used to quantitatively measure visceral hypersensitivity at week 8. The surgical procedures for EMG were performed as previously described (23,24). Rats were deeply anaesthetized with 30–40 mg/kg (3–4 mg/ml) 3% sodium pentobarbital (i.p.), no signs of toxicity were observed. A pair of EMG electrodes was surgically implanted in the lower left abdominal area to expose the external oblique abdominal musculature, and the electrode was tunnelled subcutaneously, exteriorised and secured at the back of the neck for EMG recording. Rats were allowed to recover for ≥ 5 days. Fasting and water deprivation, isoflurane anaesthesia and insertion of the deflated latex balloon were conducted as in the AWR test. Subsequently, the electrodes were connected to a BL-420F Data Acquisition & Analysis system (Chengdu Techman Software Co., Ltd., Chengdu, China), and to record the EMG signal, various pressure was applied to the abdominal walls by inflating the balloon (20, 40, 60 and 80 mmHg). The procedure was repeated three times and the EMG signals were recorded. Alterations in the EMG signal response to CRD were determined by calculating the

Table I. Sequences of primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer	Reverse primer
IL-10	5'-agtggagcaggtgaagaatga-3'	5'-cacgtaggcttctatgcagttg-3'
TGF- β 1	5'-tggagcctggacacacagta-3'	5'-tagtagacgatgggcagtg-3'
IFN- γ	5'-tcacgaatcgacactgat-3'	5'-ggatctgtgggttgcacc-3'
iNOS	5'-gagacaggaagtcggaagc-3'	5'-gtgtgaaggcgtagctgaa-3'
eNOS	5'-ttagctgtgctggcataca-3'	5'-ttgagttggctcatcattg-3'
nNOS	5'-tcattagcaatgaccgaagc-3'	5'-aacattggaagacctggg-3'
5-HT _{1A} R	5'-gatctcgctcacttggtca-3'	5'-aaagcggcgaagtgagta-3'
5-HT _{2A} R	5'-aagccacctgtgtgtgagt-3'	5'-tgtgatggaccgttgaag-3'
5-HT ₃ R	5'-atgactgctcagccatggga-3'	5'-tggtggtggaagaggctat-3'
5-HT ₄ R	5'-tggtttcggtgctggtgaatg-3'	5'-tgatgctgtggtgagtaggaca-3'
5-HT ₇ R	5'-atcacgagaccctcacgta-3'	5'-tctgagccatccgaagaga-3'
mGluR7	5'-tgtgagccctgtgatggata-3'	5'-ccagtttgatgattgggatg-3'
GAPDH	5'-agatccacaacggatacatt-3'	5'-tcctcaagattgtcagcaa-3'

5-HT, 5-hydroxytryptamine; 5-HT_{1A}R, 5-HT 1A receptor; 5-HT_{2A}R, 5-HT 2A receptor; 5-HT₃R, 5-HT 3 receptor; 5-HT₄R, 5-HT 4 receptor; 5-HT₇R, 5-HT 7 receptor; eNOS, endothelial nitric oxide synthase; IFN- γ , interferon- γ ; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; mGluR7, metabotropic glutamate receptor-7; nNOS, neuronal nitric oxide synthase; TGF- β 1, transforming growth factor- β 1.

changes in the area under the curve (AUC) of raw EMG amplitude responses to CRD, based on the formula Δ AUC (AUC during CRD-AUC before CRD) (3,21).

The selective mGluR7 agonist AMN082 (cat. no. 2385; Tocris Bioscience, Bristol, UK) was dispersed in a suspension of 0.5% methylcellulose (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and was administered intraperitoneally (3 or 10 mg/kg) to NMS rats in the NMS + AMN082 group 1 h prior to AWR and EMG testing. The same volume of PBS (vehicle control) was administered to rats in the NMS group. Doses were chosen based on previous experiments indicating behavioural and physiological alterations in response to this range (6,25).

Tissue preparation. Rats were anaesthetized by 3% pentobarbital sodium (30 mg/kg i.p.) immediately following completion of the CRD study. The distal colons were dissected before the rats were sacrificed by decapitation. The colon samples were immediately frozen in liquid nitrogen, and stored at -70°C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the colon tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. RNA was reverse-transcribed to cDNA using a PrimeScript™ RT reagent kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. mRNA transcripts were analysed by qPCR using SYBR® Premix Ex Taq™ (Takara Bio, Inc.) on an Applied Biosystems StepOne/StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed for 40 cycles, as follows: Initial denaturation at 95°C for 10 min, denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. The primers were designed and purchased from Sangon Biotech Co., Ltd. (Shanghai, China), and the sequences are shown

in Table I. GAPDH was used as a reference gene, and relative gene expression was determined using the $2^{-\Delta\Delta C_q}$ method (26).

Western blotting. Proteins were extracted from colon tissues using radioimmunoprecipitation assay buffer supplemented with protease inhibitors (Shanghai Shenggong Co., Ltd., Shanghai, China) and protein concentration was measured using the Bicinchoninic Acid Protein Assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Subsequently, proteins (50 μ g) were separated by 10% SDS-PAGE and were transferred to a nitrocellulose membrane (Whatman; GE Healthcare Life Sciences, Little Chalfont, UK). The membrane was then blocked with 5% skimmed milk at room temperature for 1 h and was incubated overnight at 4°C with anti-mGluR7 antibodies (1:1,000, rabbit anti-rat, cat. no. NB-91787; Novus Biologicals, LLC, Littleton, CO, USA) or antibodies against total NF- κ B (p65, 1:1,000, rabbit anti-rat, cat. no. 9936S), phosphorylated (p)-NF- κ B (p65, 1:1,000, rabbit anti-rat, cat. no. 9936S) or β -actin (1:1,000, rabbit anti-rat, cat. no. 8457) (all from Cell Signaling Technology, Inc., Danvers, MA, USA). The membrane was then incubated with an IRDye® 800CW-conjugated secondary antibody (1:2,000, cat. no. A80-195P; Rockland Immunochemicals, Inc., Limerick, PA, USA) for 1 h at room temperature. Images were acquired using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). The blots were semi-quantified by grey value analysis (ImageJ 1.50i; NIH, Bethesda, MA, USA).

Immunohistochemistry. Colon tissues were fixed in 4% formalin overnight at 4°C; subsequently, paraffin-embedded colon tissues (length, 1 cm; width, 0.5 cm) were mounted on slides, deparaffinised with xylene (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and rehydrated with 1.5% H₂O₂ in PBS for 30 min at room temperature. Tissues were

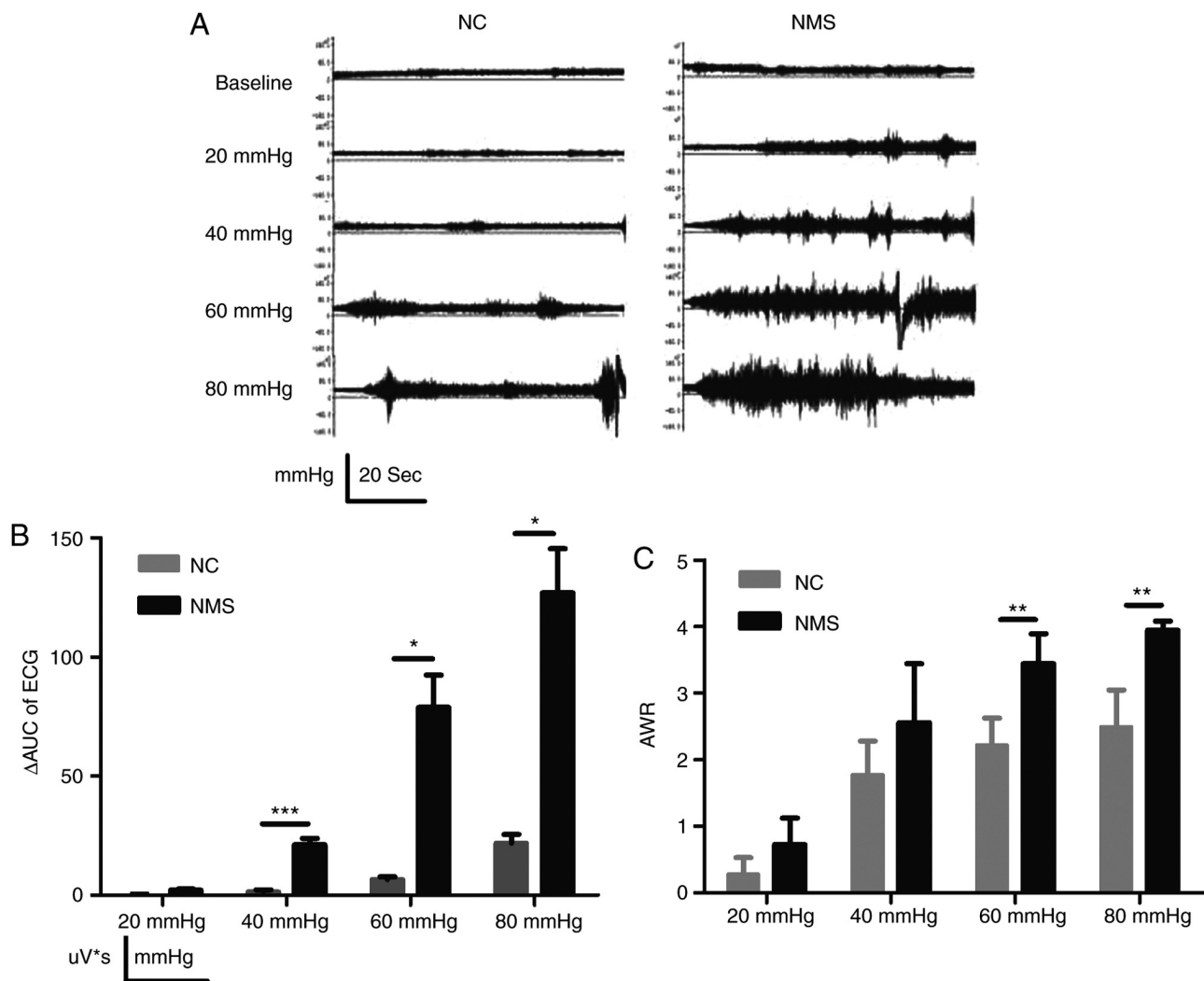


Figure 1. Visceral hypersensitivity of adult male rats. Results were analysed between the NC and NMS groups. (A) EMG signal recordings of an NC rat and an NMS rat in response to CRD. (B) Statistical analysis of the EMG amplitudes of the NC and NMS groups using the Δ AUC formula (AUC during CRD-AUC before CRD). (C) Statistical analysis of the AWR scores of the NC and NMS groups. Data were analysed by Student's t-test and are expressed as the means \pm standard error of the mean (number of rats=6). * P <0.05 ** P <0.01, *** P <0.001. AUC, area under the curve; AWR, abdominal withdrawal reflex; CRD, colorectal distension; EMG, electromyography; NC, normal control; NMS, neonatal maternal separation.

incubated overnight at 4°C with anti-mGluR7 antibody (1:500, rabbit anti-rat, cat. no. NB-91787; Novus Biologicals LLC), or antibodies against cluster of differentiation (CD)3 (1:100, rabbit anti-rat, cat. no. ab16669) CD68 (1:200, mouse anti-rat, cat. no. ab31630) or myeloperoxidase (MPO; 1:50, rabbit anti-rat, cat. no. ab9535) (Abcam, Cambridge, UK). Secondary goat anti-rabbit antibodies or goat anti-mouse antibodies (1:200, cat. nos. A0277 and A0286; Beyotime Institute of Biotechnology, Shanghai, China) were then added to the tissues for 1 h at room temperature. After being incubated with streptavidin-conjugated horseradish peroxidase (HRP) [Rabbit Specific HRP/DAB (ABC) Detection IHC kit, cat. no. ab64261; Abcam] for 10 min at room temperature, sections were stained with DAB for 1-10 min at room temperature (1:2; Gene Tech Biotechnology Co., Ltd., Shanghai, China); sections were then counterstained with haematoxylin for 0.5-2 min at room temperature. The sections were visualised under an Olympus BX41-32P02-FLB3 microscope (Olympus Corporation, Tokyo, Japan). Images were captured using FluoView software (FV1000, Olympus Corporation).

Statistical analysis. The experiments were repeated three times, and all data are presented as the means \pm standard error of the mean. Statistical analyses were performed using SPSS 19.0 (IBM SPSS, Armonk, NY, USA). Statistical significance of differences between two groups was determined with Student's t-test, whereas multiple groups were analysed by one-way analysis of variance followed by the least significant difference test or Dunnett's T3 test. P <0.05 was considered to indicate a statistically significant difference.

Results

Early NMS causes CRD-induced visceral hypersensitivity in rats. Initially, pain threshold in response to CRD was determined using the AWR test and EMG. Electromyogram activity in the NMS group was enhanced compared with in the NC group (Fig. 1A). To quantitatively measure EMG, Δ AUC was used. Compared with in the NC group, the Δ AUC in the NMS group was significantly increased at CRDs of 40, 60 and 80 mmHg (P <0.001, P <0.05 and P <0.05, respectively;

Fig. 1B). AWR in the NMS group was significantly higher compared with in the NC group at CRDs of 60 and 80 mmHg ($P<0.01$ and $P<0.01$, respectively; Fig. 1C). These findings indicated that NMS could simulate negative emotional events during early IBS, and the model successfully induced visceral hypersensitivity.

mGluR7 expression is significantly increased in the colons of rats with visceral hypersensitivity. The expression levels of mGluR7 were increased in the colon tissues of NMS rats compared with in the NC group, as determined by immunohistochemistry. Notably, staining was primarily observed in the mucosa, including a portion of cells at the surface epithelium, as well as in the lamina propria, with particularly strong staining in the bottom of the crypts (Fig. 2A). The mRNA and protein expression levels of mGluR7 were increased in the colon tissues of NMS rats compared with in the NC group ($P<0.05$; Fig. 2B and C). A significant difference was detected between the two groups ($P<0.05$; Fig. 2D). These findings indicated that mGluR7 expression was increased in the colons of rats with visceral hypersensitivity; therefore, IBS episodes may be associated with mGluR7 expression.

Selective mGluR7 agonist AMN082 (10 mg/kg) attenuates CRD-induced visceral hypersensitivity in NMS rats. The effects of AMN082 on alterations in mGluR7 expression and visceral hypersensitivity were detected. Following treatment with AMN082, the protein expression levels of mGluR7 were increased (Fig. 3A). The electromyogram activity in the NMS + AMN082 (10 mg/kg) group was attenuated compared with in the NMS group (Fig. 3B). Compared with in the NMS group, the Δ AUC in the NMS + AMN082 (10 mg/kg) group was significantly decreased at CRDs of 40, 60 and 80 mmHg ($P<0.01$, $P<0.01$ and $P<0.05$, respectively; Fig. 3C). No significant alteration was observed between the NMS and NMS + AMN082 (3 mg/kg) groups. Furthermore, the AWR in the NMS + AMN082 (10 mg/kg) group was significantly reduced compared with in the NMS group at CRDs of 60 and 80 mmHg ($P<0.01$ and $P<0.05$, respectively; Fig. 3D). At a dose of 3 mg/kg, the AWR results in the NMS + AMN082 group were not significantly different compared with in the NMS group. These findings suggested that 10 mg/kg AMN082 significantly activated mGluR7 and attenuated CRD-induced visceral hypersensitivity in NMS rats.

AMN082 (10 mg/kg) suppresses the expression levels of pro-inflammatory cytokines and increases the expression levels of anti-inflammatory cytokines in colon tissues of NMS rats. Since 10 mg/kg AMN082 significantly attenuated CRD-induced visceral hypersensitivity in NMS rats, the present study explored the underlying mechanisms. The mRNA expression levels of the anti-inflammatory cytokines IL-10 and TGF- β 1, the pro-inflammatory cytokine IFN- γ , NOS and 5-HTs were detected in colon tissues by RT-qPCR (Fig. 4). IL-10 was decreased in the NMS group compared with in the NC group ($P<0.01$), whereas IFN- γ was increased ($P<0.05$). Notably, treatment with 10 mg/kg AMN082 significantly increased the release of IL-10 ($P<0.01$) and TGF- β 1 ($P<0.01$), and suppressed the release of IFN- γ ($P<0.05$). NOS and 5-HTs exhibited almost no change in expression;

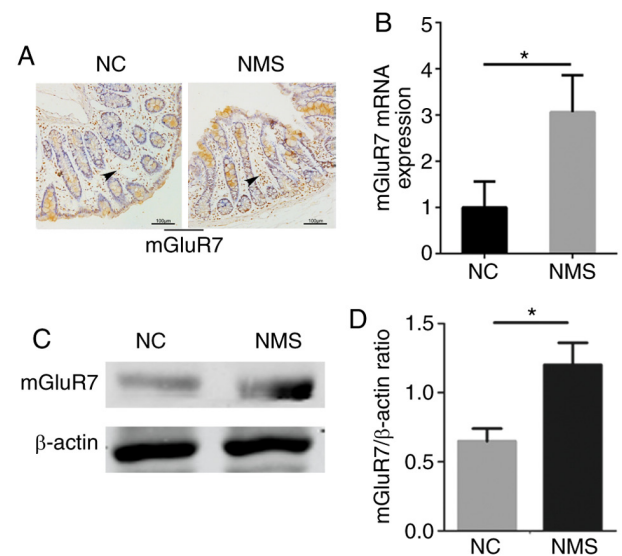


Figure 2. mGluR7 expression in the colon of rats in the NC and NMS groups. (A) Immunohistochemistry of mGluR7 in rat colons. Staining in the mucosa (black arrowheads) was compared between the NC and NMS group; there was no difference beyond the submucosa (magnification, $\times 200$). (B) mRNA expression levels of mGluR7 in the rat colons. (C) Protein expression levels of mGluR7 in the rat colons. (D) Grey values were used in the semi-quantitative analysis of western blotting results; mGluR7/ β -actin ratio was used to indicate relative protein expression levels of mGluR7. Data were analysed by Student's t-test and are expressed as the means \pm standard error of the mean (number of rats=6). * $P<0.05$. mGluR7, metabotropic glutamate receptor-7; NC, normal control; NMS, neonatal maternal separation.

however, iNOS was significantly decreased in the NMS group compared with in the NC group ($P<0.05$). Since the release of inflammatory cytokines is an indicator of the inflammatory response, these results indicated that AMN082 may inhibit inflammation in the colons of NMS rats.

AMN082 (10 mg/kg) reduces the expression levels of MPO and the number of CD3⁺ cells in the intestinal mucosa of NMS rats. MPO enzymatic activity is an index of neutrophil infiltration in colon tissue; and CD3⁺ is a specific membrane surface molecule on T lymphocytes, which is often used as a marker of T lymphocytes; CD68 is a marker of macrophages or monocytes. Both T cells and macrophages participate in regulating the intestinal immune system (27,28); therefore, MPO, CD3 and CD68 were detected in the present study (Fig. 5). NMS rats exhibited increased MPO expression levels, and CD3 and CD68 infiltration in colon tissue compared with the NC group, and staining was primarily observed in the mucosa (Fig. 5A, B, D, E, G and H). Notably, CD68 staining was the least obvious. In addition, the NMS + AMN082 (10 mg/kg) group exhibited markedly decreased CD3⁺ T cell infiltration compared with the NMS group (Fig. 5B and C). Similarly, administration of 10 mg/kg AMN082 markedly reduced MPO expression compared with the NMS group (Fig. 5H and I); there was no difference in CD68 expression in the NMS + AMN082 (10 mg/kg) group compared with the NMS group (Fig. 5E and F).

AMN082 (10 mg/kg) inhibits activation of NF- κ B in the colon of NMS rats. NF- κ B is a critical transcription factor in the inflammatory response. This protein functions as

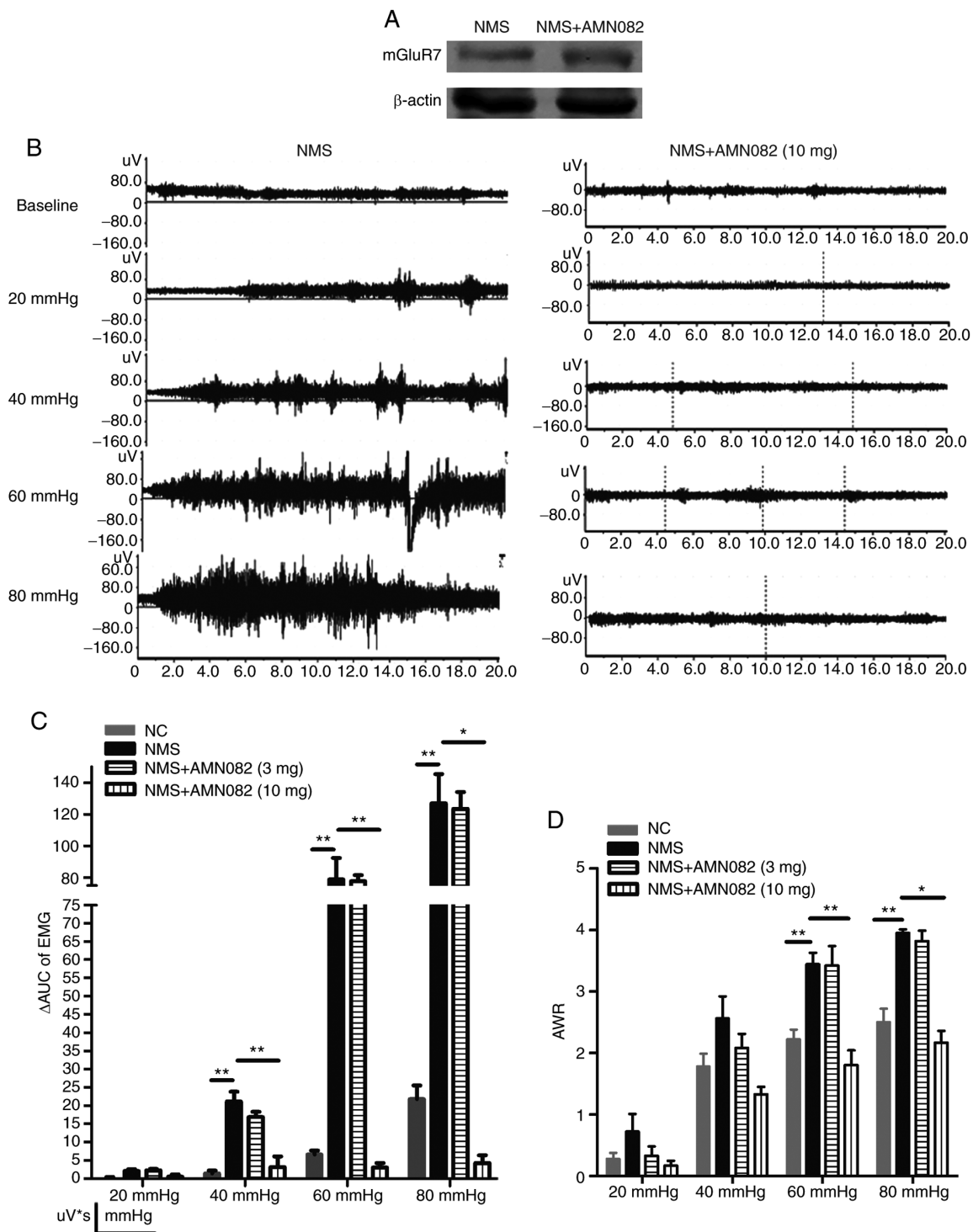


Figure 3. Effects of AMN082 on visceral hypersensitivity in NMS rats. AMN082 (3 or 10 mg/kg) was administered intraperitoneally 1 h prior to the tests. (A) Protein expression levels of mGluR7 in the colon samples obtained from rats in the NMS and NMS + AMN082 (10 mg/kg) groups. (B) EMG signal recordings of an NMS rat and an NMS + AMN082 (10 mg/kg) rat in response to CRD. (C) Statistical analysis of the EMG amplitude among the groups. (D) Statistical analysis of the AWR scores among the groups. AWR data were analysed by one-way ANOVA and least significant difference test; EMG data were analysed by one-way ANOVA and Dunnett's T3 test. Data are expressed as the means \pm standard error of the mean (number of rats=6). * P <0.05, ** P <0.01. ANOVA, analysis of variance; AUC, area under the curve; AWR, abdominal withdrawal reflex; CRD, colorectal distension; EMG, electromyography; mGluR7, metabotropic glutamate receptor-7; NMS, neonatal maternal separation.

a pro-inflammatory factor that participates in the pathophysiology of intestinal inflammatory diseases (29). Intestinal inflammation may provide an initial stimulus for a persistent state of visceral hypersensitivity (30). To investigate the mechanism underlying the anti-inflammatory activity of AMN082,

the effects of AMN082 on activation of the NF- κ B pathway in rats were investigated. Alterations in the expression levels of p-NF- κ B p65 in the colons of NMS rats were evaluated by western blotting (Fig. 6A); p-NF- κ B p65 was upregulated in NMS rats compared with in the NC group. Conversely,

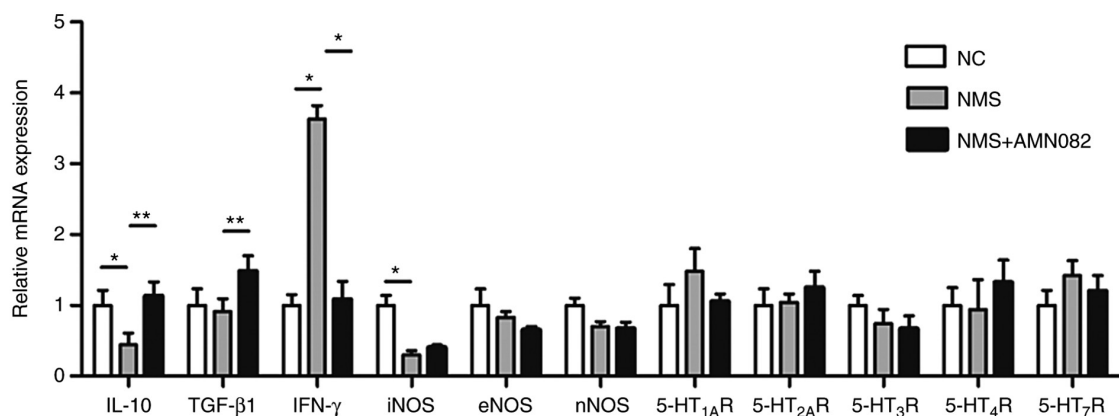


Figure 4. Effects of AMN082 (10 mg/kg) on the mRNA expression levels of 5-HT receptors, NOS and inflammatory cytokines. IL-10 and TGF- β 1 data were analysed by one-way ANOVA and least significant difference test; iNOS and IFN- γ data were analysed by one-way ANOVA and Dunnett's T3 test. Data are expressed as the means \pm standard error of the mean (number of rats=6). * P <0.05, ** P <0.01. 5-HT, 5-hydroxytryptamine; 5-HT_{1A}R, 5-HT 1A receptor; 5-HT_{2A}R, 5-HT 2A receptor; 5-HT₃R, 5-HT 3 receptor; 5-HT₄R, 5-HT 4 receptor; 5-HT₇R, 5-HT 7 receptor; ANOVA, analysis of variance; eNOS, endothelial nitric oxide synthase; IFN- γ , interferon- γ ; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; NC, normal control; NMS, neonatal maternal separation; nNOS, neuronal nitric oxide synthase; TGF- β 1, transforming growth factor- β 1.

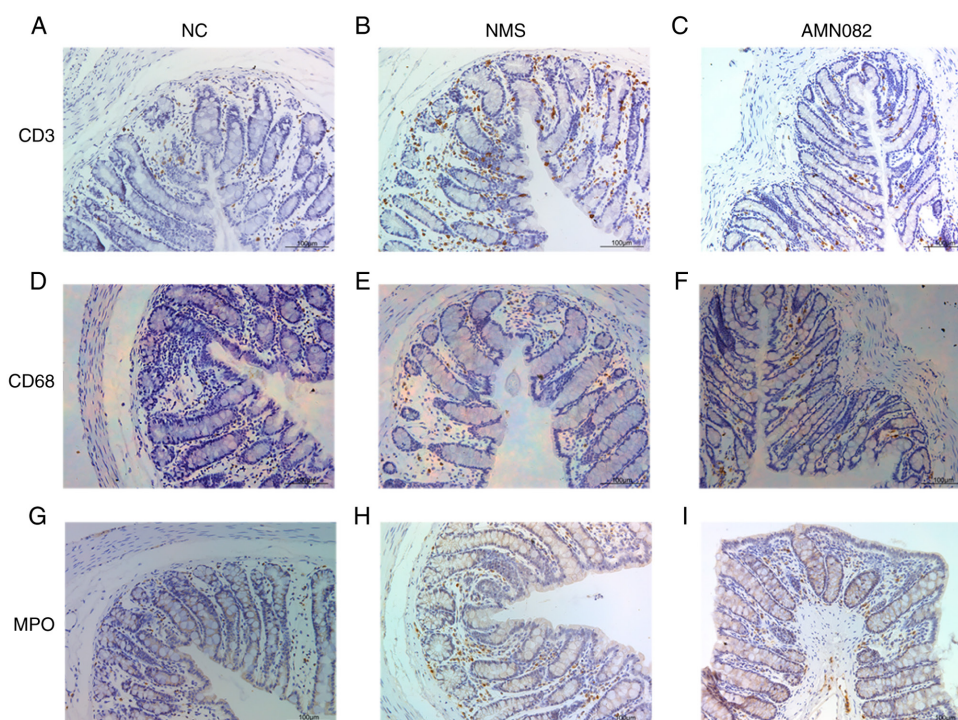


Figure 5. Effects of AMN082 (10 mg/kg) on inflammatory infiltration into the colon mucosa. The infiltration of neutrophils, macrophage cells and CD3⁺ T cells was detected by immunohistochemistry in the colons of rats in the NC, NMS and NMS + AMN082 (10 mg/kg) groups. (A-C) CD3 staining; (D-F) CD68 staining; and (G-I) MPO staining (magnification, \times 200). Positive cells were stained yellow or brown-yellow, and the distribution of positive cells in the colon was mainly localised in the lamina propria of the mucosa and scattered around the intestinal gland. CD, cluster of differentiation; MPO, myeloperoxidase; NC, normal control; NMS, neonatal maternal separation.

AMN082 treatment significantly reduced p-NF- κ B expression compared with in the NMS group (Fig. 6B; P <0.05).

Discussion

The present study demonstrated that early NMS induced visceral hypersensitivity in rats and increased the expression of mGluR7 in the colon. However, selective activation of mGluR7 via AMN082 (10 mg/kg) attenuated CRD-induced visceral hypersensitivity. In addition, AMN082 modulated

the immune response in NMS rats by regulating the balance between pro-inflammatory factors and anti-inflammatory factors, and suppressing low-grade inflammation via the reduction of NF- κ B activation.

A strong body of evidence supports the role of glutamate as a primary neurotransmitter in the vagal circuitry, which is involved in key gastrointestinal functions (31). In addition, mGluRs appear to be relevant not only for the modulation of gastrointestinal vagovagal reflexes, but also for the process of digestion as a whole (8). mGluR4 has been detected in

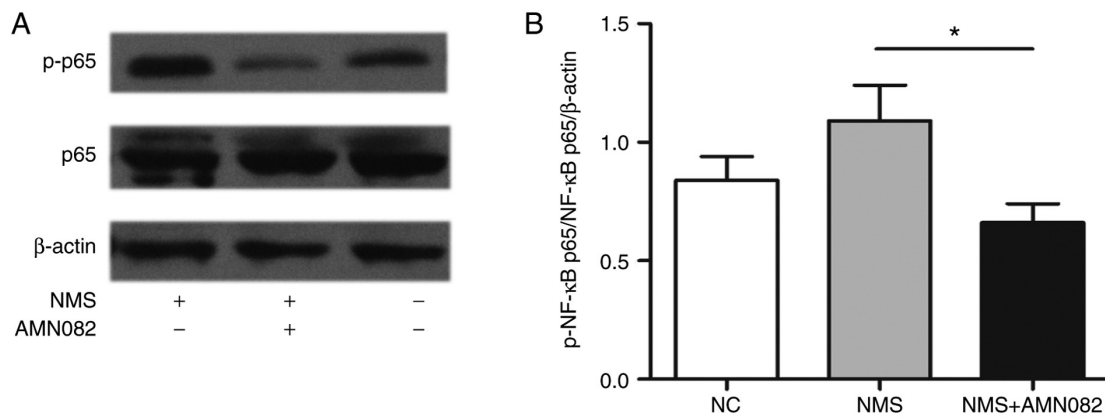


Figure 6. AMN082 inhibits activation of NF- κ B in the colon of NMS rats. (A) Western blot analysis of p-NF- κ B p65 protein expression levels. (B) Protein expression levels of p-NF- κ B p65 were semi-quantified by grey value analysis. Data were analysed by one-way analysis of variance followed by least significant difference test. Data are expressed as the means \pm standard error of the mean (number of rats=6). * P <0.05. NC, normal control; NF- κ B, nuclear factor- κ B; NMS, neonatal maternal separation; p-, phosphorylated.

normal human colon epithelium (32), and an accelerating effect on guinea pig colon motility and longitudinal muscle contractions has been observed upon application of mGluR8 agonists on isolated tissue (33). Furthermore, mGluR7 mRNA and protein are expressed in the mouse colon mucosa, and treatment with the selective mGluR7 agonist AMN082 induces an increase in faecal water content in stress-induced defecation (6). Previous evidence has indicated that overexpression of the N-methyl-D-aspartate receptor (an ionotropic receptor) serves an important role in the formation of post-inflammatory visceral hypersensitivity (34). Therefore, it was hypothesised that mGluR7 may be relevant to visceral hypersensitivity. The majority of studies regarding IBS and AMN082 (mGlu7 receptor agonist) have used animal models; AMN082 is active in the central nervous system via direct central injection, and is also active outside of the central nervous system via oral or intraperitoneal injection (6,35,36). The present study demonstrated that mGluR7 expression was enhanced in NMS rats, as determined by assessing immunohistochemistry, mRNA levels and protein levels. Therefore, it was suggested that mGluR7 may have an important role in the visceral hypersensitivity of IBS; these results were similar to those of Julio-Pieper *et al* (6). A high frequency of IBS symptoms has been detected in patients with panic disorder, generalised anxiety disorder and major depressive disorder, and another study reported that mGluR7-knockout animals display an anxious phenotype (8). The present study confirmed that activating mGluR7 attenuated CRD-induced visceral hypersensitivity. This conclusion was consistent with previous literature reports, and represented a breakthrough in understanding the association between mGluR7 and the pathogenesis of IBS.

It has been reported that the abnormal perception of visceral stimuli, or visceral hypersensitivity, is an important underlying mechanism of IBS, and intestinal inflammation may provide an initial stimulus for a persistent state of visceral hypersensitivity (31). Patients with post-infectious IBS exhibit no signs of overt inflammation but exhibit persistent minor increases in epithelial T lymphocytes and mast cells (37), thus suggesting that long-term inflammatory alterations may be responsible for colonic hypersensitivity. Furthermore, it has

been indicated that a T helper (Th)1/Th2 immune imbalance is closely associated with patients with IBS with diarrhoea (D-IBS); IFN- γ is a Th1-mediated cytokine that promotes the inflammatory response, whereas IL-10 is a Th2-mediated cytokine that inhibits the inflammatory response (38). In IL-10-deficient mice, the majority of animals suffer from chronic enterocolitis, thus suggesting that IL-10 is an essential immunoregulator in the intestinal tract (39). TGF- β 1 is one of the most common members of the TGF- β family, which possesses an anti-inflammatory effect. Furthermore, NF- κ B is an important transcription factor that is mainly involved in inflammatory and immune responses. NF- κ B-dependent inflammatory mediators, including IL-6, are associated with the maternal separation model of IBS, and the downstream activation of extracellular signal-regulated kinase (ERK), Janus kinase (JAK)-signal transducer and activator of transcription (STAT) and NF- κ B signalling cascades (40). Furthermore, stimulating the production of pro-inflammatory cytokines, including IL-1 β and tumour necrosis factor (TNF)- α , contributes to visceral hypersensitivity through the Toll-like receptor 4 (TLR4)/myeloid differentiation primary response 88/NF- κ B signalling pathway in the spinal cord in a neonatal colonic irritation rat model (41). In addition, NO is involved in inhibition of the transmission and perception of visceral hypersensitivity (42). A previous study revealed that ioglitzone (hypoglycemic drug) reduces visceral hypersensitivity, increases nociceptive thresholds, NO production and iNOS activity in D-IBS rats (17). In the present study, iNOS expression was decreased in the NMS group, which could further reduce the synthesis of NO, resulting in enhanced intestinal motility and increased visceral hypersensitivity. Similar to the aforementioned findings, a decrease in iNOS expression may be associated with the occurrence of visceral hypersensitivity in IBS.

In the present study, decreased IFN- γ , and increased TGF- β 1 and IL-10 levels were observed in the intestinal mucosa of AMN082-treated rats. Furthermore, the expression levels of CD3 and MPO were decreased in the intestinal mucosa of AMN082-treated rats. These results indicated that activating mGluR7 may result in an imbalance of Th1/Th2 immunity. Upregulated IL-10 reduces antigen-specific human

T-cell proliferation by diminishing the antigen-presenting capacity of monocytes (43), which then inhibits the secretion of TNF- α and results in the inhibition of NF- κ B pathway activation. A previous report suggested that during acute infection of IBS, TGF- β 1 is increased in the muscle layer (44); therefore, in AMN082-treated NMS rats, upregulated TGF- β 1 may inhibit the maturation of dendritic cells and Th1 activity, and enhance Th2 activity. Conversely, downregulated IFN- γ can reduce the activation of macrophages and inhibit Th1 activity, also resulting in the inhibition of NF- κ B pathway activation. The decreased expression of NF- κ B in AMN082-treated NMS rats is consistent with the aforementioned conclusions. When NF- κ B is activated various downstream inflammatory cytokines are produced. These findings indicated that activating mGluR7 may reduce NF- κ B-associated amplification of the inflammatory response, thereby protecting the intestinal mucosal barrier and reducing intestinal permeability. Subsequently, the number of antigens that pass through the intestine and immune cell interactions are reduced. When the initial inflammatory stimulus is weakened, the persistent state of visceral hypersensitivity may be relieved.

The present study did not assess the mGluR7 signalling pathway *in vitro*; therefore, further studies may be conducted using a cell model and mGluR7 small interfering RNA. To examine the effects of mGluR7 more detail, it would be beneficial to determine the effects of mGluR7 gene knockdown or antagonism in rats. In addition, the association between mGluR7 and other characteristics of IBS, such as diarrhoea and constipation, should be evaluated; in these future studies, stool characteristics, including stool particles and faecal water content, may be detected. Other IBS-associated inflammatory signalling pathways, including ERK, JAK-STAT, TLR4 and NF- κ B signalling cascades (40,41), may also have a relationship with altered mGluR7 in rats with visceral hypersensitivity; further studies are required to evaluate this.

Stressful life events and experimental stress exacerbate symptoms and visceral hypersensitivity in patients with functional gastrointestinal disorders, such as IBS (13). In conclusion, the present study supported the idea of a complex interaction between the gastrointestinal tract and the brain, namely, the brain-gut axis. The present data demonstrated that mGluR7 may have an important role in attenuating visceral hypersensitivity in IBS, in addition to regulating the central components of chronic stress, and regulating fluid and electrolyte transport in the intestine. The mechanisms underlying these effects may be associated with inhibition of NF- κ B activation and reduced inflammation.

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Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LMS and YBL were involved in the conception and design, and conducted most of the study. JHX and QYW assisted with the immunohistochemistry, western blotting and RT-qPCR experiments. LMS was involved in the acquisition, analysis and interpretation of data, and drafted the article. FL and JD made contributions to the conception and design of the study, and analysed and interpreted the data; they were also involved in revising the manuscript critically for important intellectual content and gave final approval of the version to be published. All authors participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work. The final manuscript was read and approved by all authors.

Ethics approval and consent to participate

All animal experiments were performed following approval by the Animal Care and Use Committee of Tongji University School of Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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