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## ORIGINAL ARTICLE

# BDNF Exacerbates Visceral Hypersensitivity through Hippocampal TrkB-CRF Signaling in Early-Life Stress

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## SUMMARY

***Background:*** **Irritable bowel syndrome (IBS) associated with early-life stress (ELS) commonly manifests as anxiety and visceral hypersensitivity. However, the pathogenic mechanisms underlying these effects are not fully understood. This study aims to investigate the role of brain-derived neurotrophic factor (BDNF) as a key mediator of ELS-induced changes through the brain-gut axis.**

***Methods:*** **A Sprague-Dawley male maternal separation (MS) rat model was used to induce anxiety and visceral hypersensitivity associated with ELS. BDNF levels were measured in the limbic system (cingulate gyrus, amygdala, and hippocampus) and serum. The correlation between BDNF levels, anxiety, and visceral hypersensitivity was analyzed. Corticotropin-releasing factor (CRF) expression in the hippocampus and the extent of visceral hypersensitivity were assessed in control, MS, and MS+K252a (a BDNF receptor antagonist) groups.**

***Results:*** **MS rats exhibited higher levels of anxiety and visceral hypersensitivity compared to controls. BDNF production in the hippocampus was elevated in MS rats and positively correlated with anxiety ($r = -0.78$, $p < 0.05$) and visceral hypersensitivity ($r = 0.93$, $p < 0.01$). CRF expression, a key mediator of stress and visceral hypersensitivity, was also increased in the hippocampus of MS rats. Inhibition of BDNF signaling using K252a reduced CRF expression and alleviated visceral hypersensitivity.**

***Conclusions:*** **This study demonstrates that BDNF may mediate ELS-induced anxiety and visceral hypersensitivity through hippocampal TrkB-CRF signaling, providing a mechanistic basis for targeting BDNF in stress-related IBS.**

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**KEYWORDS**

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## INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder arising from bidirectional dysregulation of the brain-gut axis, involving aberrant neuroendocrine-immune communication [1,2]. Early-life stress (ELS) is a critical risk factor that disrupts this axis, promoting the development of visceral hypersensitivity and psychiatric comorbidities such as anxiety in IBS [3,4]. Clinically, this comorbidity is highly significant, as anxiety can intensify abdominal pain and discomfort in affected individuals [5,6]. Nevertheless, the mechanisms through which brain-gut dysfunction contributes to IBS-like visceral hypersensitivity remain elusive.

As a pivotal brain-gut neuropeptide, brain-derived neurotrophic factor (BDNF) is abundantly expressed throughout the central and gastrointestinal systems, where it plays a central role in regulating brain-gut axis signaling [7]. The limbic system, encompassing the hippocampus, amygdala, and cingulate cortex, plays a central role in emotional regulation. Neuroimaging studies have revealed structural and functional abnormalities in these regions in patients with anxiety disorders [8], and molecular alterations have been similarly observed in rats exhibiting anxiety-like behaviors induced by environmental enrichment [9]. Within the limbic system, overexpression of BDNF in the amygdala and hippocampus facilitates anxiety-related responses [10]. Corticotropin-releasing factor (CRF), a hypothalamic neuropeptide, serves as a principal mediator of stress responses and has been implicated in the pathogenesis of visceral hypersensitivity in IBS [11]. Previous studies have demonstrated that intracerebroventricular administration of CRF induces stress-related visceral hypersensitivity, an effect that can be abolished by CRF receptor antagonists [12]. BDNF enhances CRF release in the central nervous system by activating tyrosine receptor kinase B (TrkB) receptors located on CRF neurons [13]. This mechanistic interaction suggests that the BDNF-CRF signaling axis may underlie ELS-induced visceral hypersensitivity in IBS.

The maternal separation (MS) paradigm provides a robust model of early-life stress that recapitulates key IBS-like features-including visceral hypersensitivity and anxiety-like behaviors-through disruption of the brain-gut axis [4]. Despite these advances, the contribution of the BDNF-CRF axis to MS-induced visceral hypersensitivity remains poorly understood. In the present study, we investigated the role of BDNF in mediating ELS-induced anxiety and visceral hypersensitivity in rats exposed to maternal separation.

## MATERIALS AND METHODS

### Maternal separation model

Six pregnant Sprague-Dawley (SD) rats, acquired at gestational days 13 - 14 (with parturition occurring around days 21 - 22), were sourced from SPF Biotechnology Co. Ltd (Beijing). Prior to giving birth, all rats were housed individually in standard Makrolon cages, which were lined with wood shavings, in the level 2 animal facility at Shandong University Laboratory Animal Center. The rats were provided aseptic food and water ad libitum, and maintained under controlled environmental conditions: lighting from 7:00 AM to 7:00 PM, temperature kept at approximately 22°C, and humidity maintained near 45%. Each litter typically consisted of 8 - 12 pups, from which 5 - 6 male pups were selected per litter, resulting in a total of 31 male pups for the experiment. Female pups were reserved for other studies within our laboratory. The selected male pups (n = 31) were randomly assigned: 18 underwent maternal separation (MS), while the remaining 13 formed the control group.

MS procedures were conducted as previously described [14]. The entire litter of male pups was separated from their dams for 180 minutes daily. This separation regimen was carried out over an 11-day period, spanning postnatal days 2 to 12. Separations were consistently performed from 9:00 AM to 12:00 PM. The pups were housed in plastic cages placed on heating pads that maintained a temperature range of 30 - 33°C, in a dedicated separate room. After separation, the pups were returned to their original cages. In the control group, no special treatment was administered to the male pups from postnatal days 2 through 12. All rats were weaned on day 22 after birth. No other procedures were performed on the rats in either group until they reached 70 days post-birth.

Four rats from the MS group received an intracerebroventricular (ICV) injection of K252a, forming the MS+K252a group (n = 4). Therefore, the control group consisted of 13 rats, the MS group had 14 rats, and the MS+K252a group included 4 rats. Six to seven control rats and seven to eight MS rats were used for the open-field test (OFT), visceral sensitivity assessments, enzyme-linked immunosorbent assay (ELISA), and PCR tests (the number of rats in each test is detailed in the Results section). The same rats from each group were used for all of the tests. The remaining six rats from both the control and MS groups were subjected to immunohistochemistry. Four rats from the MS+K252a group were used for visceral sensitivity assessment, ELISA, and immunohistochemistry. Rats were anesthetized with 4% isoflurane for colorectal distension (CRD) and ICV procedures. At the conclusion of the experiment, rats were humanely euthanized by intraperitoneal injection of pentobarbital sodium (150 mg/kg), while surviving animals were retained for subsequent breeding. All experimental procedures adhered to the ARRIVE guidelines and received prior approval from both the Shandong University Laboratory Animal Center and the Ethics Committee on Animal Experiment of Shandong University Qilu Hospital (Animal Experiment Approval Certificate Number: Dwll-2020-043).

**Efficiency experiment**

***Open-Field Test (OFT)***

On day 71 post-birth, rats from both groups underwent the OFT between 9:00 AM and 12:00 PM to assess anxiety-like behavior, as previously described [15]. The test was conducted in a black square enclosure (100 × 100 × 40 cm), dimly lit by a 40-lux white light source, and monitored by an overhead digital camera. One hour prior to testing, all rats were placed in an adjacent room for acclimatization. Each rat was then placed in the center of the open field and given 10 minutes for free exploration. The rat's movement was recorded by the digital camera, which was connected to a radio recorder and a computer equipped with a smart tracking system from Spain [16]. Anxiety-related indices were automatically recorded, including: the percentage of time spent in the central zone, the frequency of center crossings, the number of rearings (rising on hind legs), which correlates negatively with anxiety, and the amount of grooming (self-cleaning), which correlates positively with anxiety.

***Visceral sensitivity assessment***

Five days after the OFT, all rats were fasted for 16 hours before undergoing colorectal distension (CRD). First, rats were lightly sedated with 4% isoflurane (temporary inhaled anesthesia). A latex balloon catheter (Ultracover 8F; International Medical Products, Zutphen, The Netherlands) was gently inserted into the colon, positioned 5 cm from the anus, secured to the tail, and connected to a syringe. Rats were then placed in a plexiglass restraint box (20 × 6 × 8 cm), allowing only forward and backward movement. After a 30-minute recovery period, CRD was performed. The balloon was inflated with increasing volumes of 37℃ water (0.4 mL, 0.6 mL, 0.8 mL, 1.0 mL, and 1.2 mL). Each distension lasted 20 seconds, followed by a 4-minute relaxation interval, and was repeated three times per volume. Two blinded experimenters evaluated the visceral pain response using the Abdominal Withdrawal Reflex (AWR) scoring system:

0: No observable behavioral response to CRD.
1: Brief head movement followed by immobility.
2: Contraction of abdominal muscles.
3: Lifting of the abdomen off the platform.
4: Body arching and lifting of the pelvic structures.

**Molecular biology experiment**

***TrkB antagonist K252a ICV***

Rats were anesthetized with 4% isoflurane and securely positioned in a stereotaxic apparatus for surgery. K252a (1 μg, Sigma, USA), in a volume of 2.5 μL, was carefully injected into the bilateral ventricles at the following coordinates relative to the bregma: anterior/posterior -1.4 mm, medial/lateral 1.8 mm, and dorsal/ventral -3.0 mm. The injection was delivered at a controlled rate of 0.5 μL/min using a micropump [17]. To ensure complete injection, the cannula was left in place for one additional minute before removal. Three hours after the K252a ICV injection, rats underwent colorectal distension (CRD).

***Sampling***

One hour after CRD, rats were euthanized via intraperitoneal injection of pentobarbital sodium (150 mg/kg). For half of the rats, rapid decapitation was performed, blood was collected from the carotid arteries, and the brains were quickly removed. Coronal brain sections (1 mm thick) were prepared using a rat brain slicer (Braintree Scientific, USA). The cingulate gyrus, amygdala, and hippocampus were then dissected, flash-frozen in liquid nitrogen, and stored at -80℃ for subsequent ELISA and PCR analysis. For the remaining rats, hearts were exposed, and 300 mL of normal saline (0.9%) was perfused via the ascending aorta, followed by 300 mL of 4% paraformaldehyde. Brains were then removed and post-fixed in 4% paraformaldehyde for more than 24 hours at 4℃ for immunohistochemical analysis.

***Immunohistochemistry***

The tissues were embedded in paraffin and 6 μm coronal sections were cut. Regions of the cingulate gyrus, amygdala, and hippocampus were dissected according to the rat brain atlas by Paxinos and Watson [18]. After antigen retrieval, sections were rinsed with 0.01 M PBS and incubated in 3% hydrogen peroxide for 20 minutes. Sections were then incubated with a blocking buffer (goat serum, SP9001 from Zhongshan, Beijing, China) for 30 minutes. Primary antibodies (BDNF (ab108319) at a dilution of 1/50; CRF (ab272391) at a dilution of 1/500, both from Abcam, USA) were incubated with sections for 48 hours at 4℃. Secondary antibody incubation (diluted 1/200, from Zhongshan Gold Bridge, China) occurred for 30 minutes at 37℃. Sections were washed and incubated with peroxidase-labeled streptavidin working solution for 30 minutes at 37℃. Visualization was achieved using 3,3’-diaminobenzidine tetrahydrochloride. Images were captured using a light microscope (Olympus Bx51). Immunostaining was quantified using Image Pro-Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). Positive immunostaining was detected using a grey-shade threshold and expressed as the percentage (% area) of the scanned field.

***Real-time quantitative PCR***

BDNF expression was assessed via quantitative real-time PCR. After decapitation, the cingulate gyrus, amygdala, and hippocampus were dissected from each group. Total RNA was extracted using TRIzol reagent (TOYOBO, Japan) and then 1 μg of RNA was reverse transcribed into cDNA using the ReverTra Ace® qPCR RT kit (TOYOBO, Japan). PCR amplification was performed on a StepOne Plus system (Thermo ABI, Singapore) in 10 μL reactions containing 1 μL cDNA, 5 μL SYBR Green (TOYOBO, Japan), 0.4 μM primers, and distilled water. BDNF primers (designed by the Neurobiology Lab of Shandong University) were as follows:

Forward: 5′-TAA ATG AAG TTT ATA CAG TAC AGT GGT TCT ACA-3′

Reverse: 5′-AGT TGT GCG CAA ATG ACT GTT T-3′ [19]

PCR reactions were performed in duplicates. β-actin mRNA was used as the internal control for normalization with the $2^{-\Delta\Delta Ct}$ method; the primers were:

Forward: 5′-CAA CTT GAT GTA TGA AGG CTT TGG T-3′

Reverse: 5′-ACT TTT ATT GGT CTC AAG TCA GTG TAC AG-3′.

***ELISA for BDNF and CRF***

Brain tissue from the cingulate gyrus, amygdala, and hippocampus was homogenized in a lysis buffer containing 137 mM NaCl, 20 mM Tris (pH 8.0), 1% NP40, 10% glycerol, 1 mM PMSF, 10 mg/mL aprotinin, 1 mg/mL leupeptin, and 0.5 mM sodium vanadate. After centrifugation at 13,000 rpm for 15 minutes at 4°C, the supernatants were collected for protein quantification and subsequent ELISA analysis. Specific ELISA kits (BDNF: KeYingMei, Beijing, China; CRH: Anoric Biotechnology Co., Tianjin, China) were used to precisely quantify BDNF and CRF levels, following the manufacturer's instructions. The detection range for the assay was 0 to 1,000 pg/mL. All samples were tested in duplicates. The resulting BDNF and CRF concentrations were normalized to total protein content.

**Statistical analysis**

Data are expressed as mean ± standard deviation or median (interquartile range). Statistical analyses were performed using SPSS software (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY, USA). Photomicrographic differences were analyzed employing the Mann-Whitney U test and independent *t*-tests. Independent *t*-tests were utilized to compare differences between two groups for OFT, ELISA, and qPCR results. Differences in AWR scores between the control vs. MS group and the MS vs. MS+K252a group were also assessed using independent *t*-tests. Spearman's rank correlation analysis was conducted to evaluate the correlations between BDNF levels with anxiety and visceral hypersensitivity. Statistical significance was defined as a two-tailed $p < 0.05$.

## RESULTS

**ELS-induced anxiety and visceral hypersensitivity in MS rats**

MS rats (n = 7) displayed significantly increased anxiety-like behaviors compared to control rats (n = 6). Rats in the MS group spent significantly less time in the central zone than the control group (control: 3.87 ± 2.82% vs. MS: 0.80 ± 0.71%, t = -2.80, df = 11, p = 0.02; Figure 1a). MS rats also crossed the center zone fewer times (control: 12.33 ± 7.31 vs. MS: 3.71 ± 3.86, t = -2.72, df = 11, p = 0.02; Figure 1b) and exhibited fewer rearings (control: 40.67 ± 21.47 vs. MS: 11.71 ± 5.59, t = -3.21, df = 5.58, p = 0.02; Figure 1c). Moreover, MS rats performed more grooming behaviors (control: 17.67 ± 7.23 vs. MS: 33.57 ± 15.11, t = 2.35, df = 11, p = 0.04; Figure 1d). Compared to the control group, MS rats exhibited higher AWR scores (0.4 mL: control: 0.03 ± 0.07 vs. MS: 0.33 ± 0.21, t = -3.56, df = 7.36, p = 0.009; 0.6 mL: control: 0.50 ± 0.3 vs. MS: 1.00 ± 0.37, t = -2.63, df = 11, p = 0.02; 0.8 mL: control: 1.25 ± 0.44 vs. MS: 2.05 ± 0.78, t = -2.21, df = 11, p = 0.049; 1.0 mL: control: 2.33 ± 0.46 vs. MS: 3.00 ± 0.54, t = -2.36, df = 11, p = 0.04; 1.2 mL: control: 3.03 ± 0.25 vs. MS: 3.74 ± 0.21, t = -5.61, df = 11, p = 0.00; Figure 1e). Spearman's rank correlation analysis revealed a significant negative correlation between AWR scores and the percentage of time spent in the central zone in the MS group (r = -0.79, p = 0.04; n = 7; Figure 1f).

**BDNF concentration increased in the hippocampus of MS rats**

BDNF was abundantly expressed in the hippocampus, cingulate gyrus, and amygdala (Figure 2). Quantitative analysis showed that hippocampal BDNF expression was significantly elevated in MS rats compared to control rats (MS: median 18.5, interquartile range 15.1 - 24.3 vs. control: 11.7, interquartile range 8.6 - 14; p = 0.01; n = 6 per group; Figure 2a and 2b). However, no significant differences in BDNF expression were found in the cingulate gyrus and amygdala between MS and control rats (cingulate gyrus: median 7.1, interquartile range 5.5 - 10.6 in MS rats vs. median 8.1, interquartile range 9.7 - 15.6 in control rats, p =1.0; amygdala: median 5.2, interquartile range 2.1 - 6.8 in MS rats vs. median 4, interquartile range 3 - 7.7 in control rats, p = 0.87; n = 6 per group; Figure 2c - 2f).

The PCR results demonstrated that the mRNA levels of BDNF were significantly higher in the hippocampus of MS rats compared to control rats (control: 1.00 ± 0.22 vs. MS: 3.25 ± 0.79, t = -2.74, df = 8.04, p = 0.03; Figure 3a). However, no significant differences in BDNF mRNA expression were found in the cingulate gyrus (control: 1.00 ± 0.32 vs. MS: 1.13 ± 0.20, t = -0.34, df = 13, p = 0.74; Figure 3b) and amygdala (control: 1.00 ± 0.65 vs. MS: 1.03 ± 0.36, t = -0.05, df = 13, p = 0.97; Figure 3c) between MS and control rats (n = 7 in the control group, n = 8 in the MS group).

Hippocampal BDNF levels were significantly higher in MS rats than in control rats (control: 17.96 ± 5.38 pg/mg vs. MS: 41.08 ± 23.50 pg/mg, t = -2.53, df = 6.73, p = 0.04; Figure 3d). However, no significant changes in BDNF levels were observed in the cingulate gyrus (control: 37.24 ± 18.1 pg/mg vs. MS: 36.43 ± 16.57 pg/mg, t = 1.04, df = 11, p = 0.32; Figure 3e) or amygdala (control: 32.83 ± 9.60 pg/mg vs. MS: 38.97 ± 16.97 pg/mg, t = -1.26, df = 11, p = 0.24; Figure 3f; n = 6 in the control group, n = 7 in the MS group), which was consistent with the immunohistochemical results. Additionally, serum BDNF levels in MS rats were comparable to those in control rats (control: 802.98 ± 82.14 pg/mL vs. MS: 892.02 ± 136.55 pg/mL, t = -1.45, df = 10, p = 0.18; n = 6 in the control group, n = 7 in the MS group).

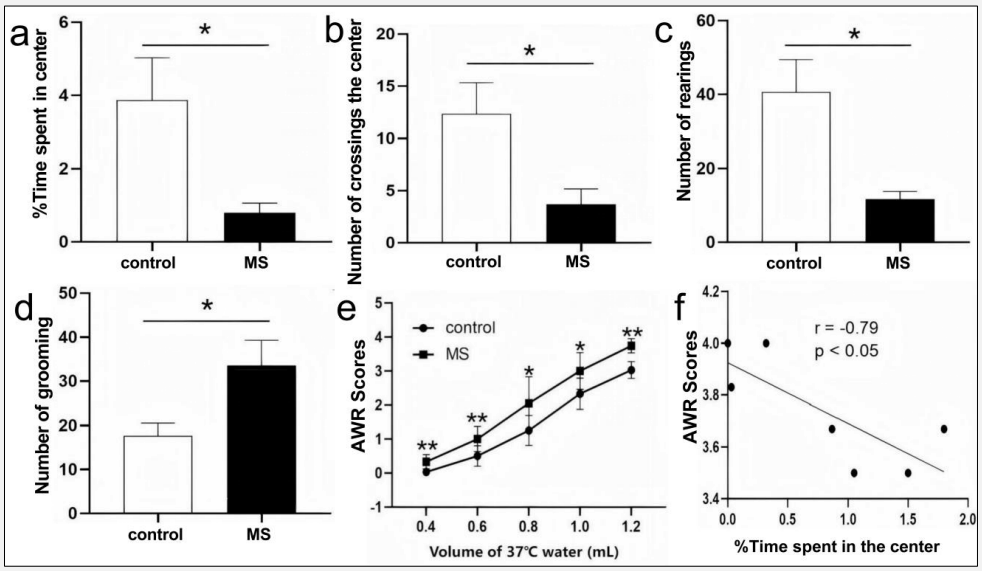


**Figure 1. Compared to control rats, MS rats exhibited significantly heightened anxiety-like behaviors.**

**a) MS rats demonstrated a markedly decreased percentage of time spent in the center relative to controls. b, c) MS rats displayed fewer crossings past the center b) and significantly less rearings c). d) Conversely, MS rats showed a significantly larger number of grooming events. * $p < 0.05$. e) MS rats also registered substantially higher abdominal withdrawal reflex (AWR) scores at every tested volume of 37°C water (0.4, 0.6, 0.8, 1.0, 1.2 mL) compared to controls. * $p < 0.05$, ** $p < 0.01$. f) Spearman's rank correlation analysis demonstrated a significant negative correlation between AWR scores and the percentage of time spent in the center zone exclusively in the MS group. N = 6 in the control group and n = 7 in the MS group.**

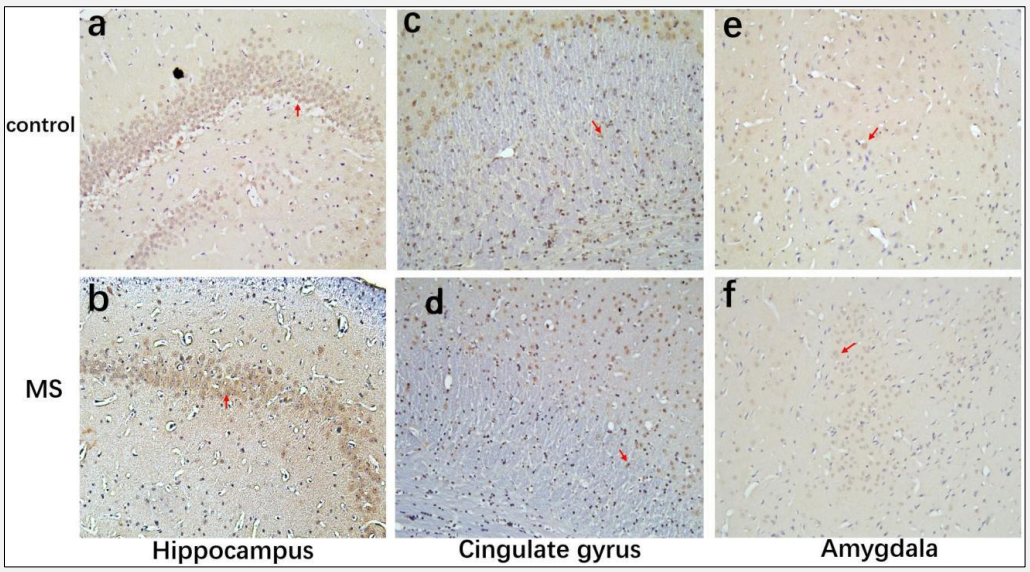


**Figure 2. Immunohistochemistry visualized BDNF expression (brown staining).**

**Representative photomicrographs reveal BDNF-immunoreactive cells within the hippocampus (control: a; MS: b), the cingulate gyrus (control: c; MS: d), and the amygdala (control: e; MS: f). Arrows mark typical cells exhibiting positive BDNF immunoreactivity. Magnification, × 20. n = 6 per group.**

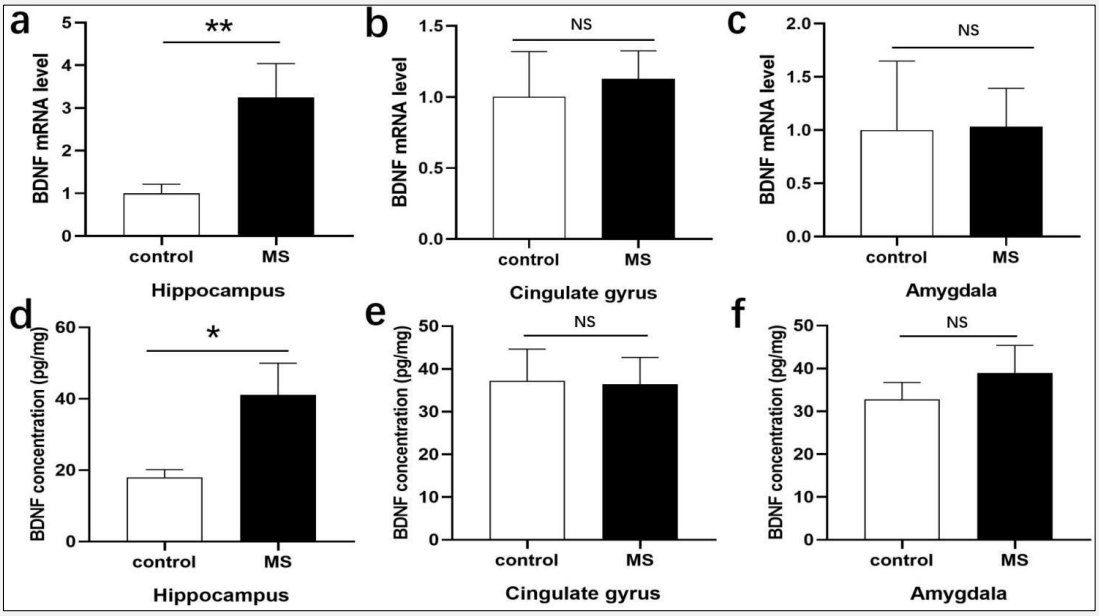


**Figure 3. The mRNA level and protein concentration of BDNF was detected by RT-PCR and ELISA.**

**a) In the hippocampus, BDNF mRNA expression revealed a significant increase in the MS group compared to controls. b and c) Conversely, within the cingulate gyrus b) and amygdala c), BDNF mRNA levels remained unchanged in the MS group relative to controls. ** $p < 0.01$. NS, not significant. n = 7 control, n = 8 MS. d) Hippocampal BDNF protein concentration demonstrated a significant elevation in the MS group versus controls. e and f) However, BDNF protein concentrations in the cingulate gyrus e) and amygdala f) showed no alteration in the MS group compared to controls. * $p < 0.05$. NS, not significant. n = 6 control, n = 7 MS.**

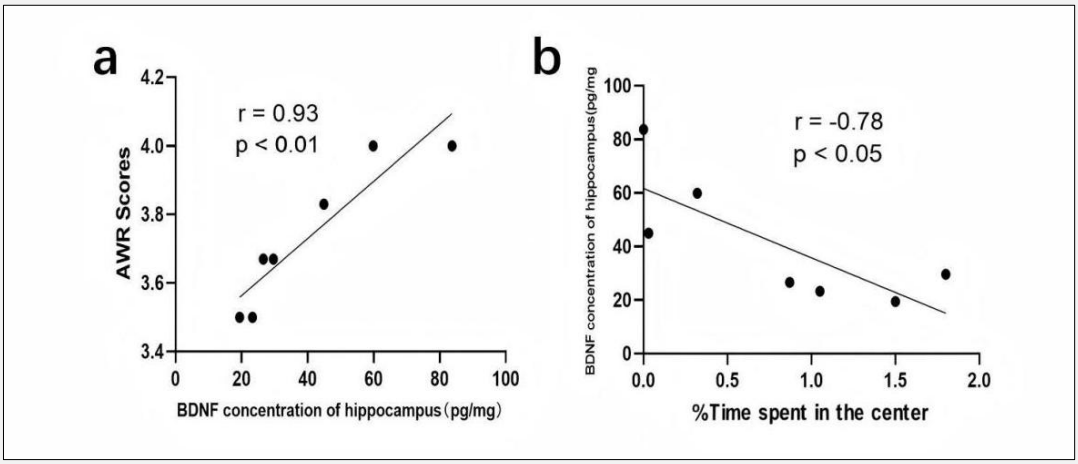


**Figure 4. Spearman's rank correlation test.**

**a) BDNF concentration in the hippocampus was positively correlated with the AWR score in MS group. b) BDNF concentration in the hippocampus was negatively correlated with the percentage of time spent in the center in MS group. n = 7.**

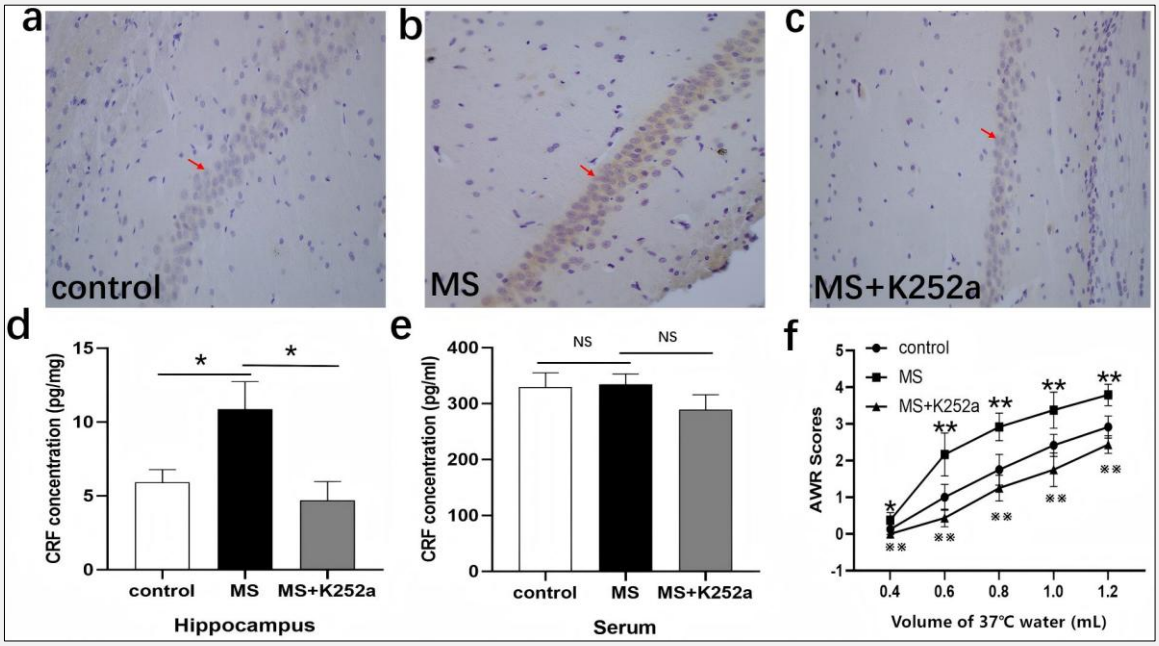


**Figure 5. The expression and concentration of CRF was detected by immunohistochemistry and ELISA.**

**CRF-immunoreactive cells were shown in the hippocampus of control group a), MS group b), and MS+K252a group c). Typical cells with positive CRF immunoreactivity are indicated by arrows. Magnification × 40. d) in the hippocampus, the concentration of CRF was significantly increased in MS group compared to control group; However, the concentration of CRF was significantly decreased in MS+K252a group compared to MS group. e) in the serum, the concentration of CRF was not changed in MS group compared to control group and MS group compared to MS+K252a group. f) compared to control rats, MS rats showed higher AWR scores to each volume of 37°C water (0.4, 0.6, 0.8, 1.0, 1.2 mL); however, compared to MS rats, MS+K252a rats showed lower AWR scores to each volume of 37°C water (0.4, 0.6, 0.8, 1.0, 1.2 mL). * p < 0.05, ** p < 0.01. NS, not significant. n = 6 in the control and MS groups and n = 4 in the MS+K252a group.**

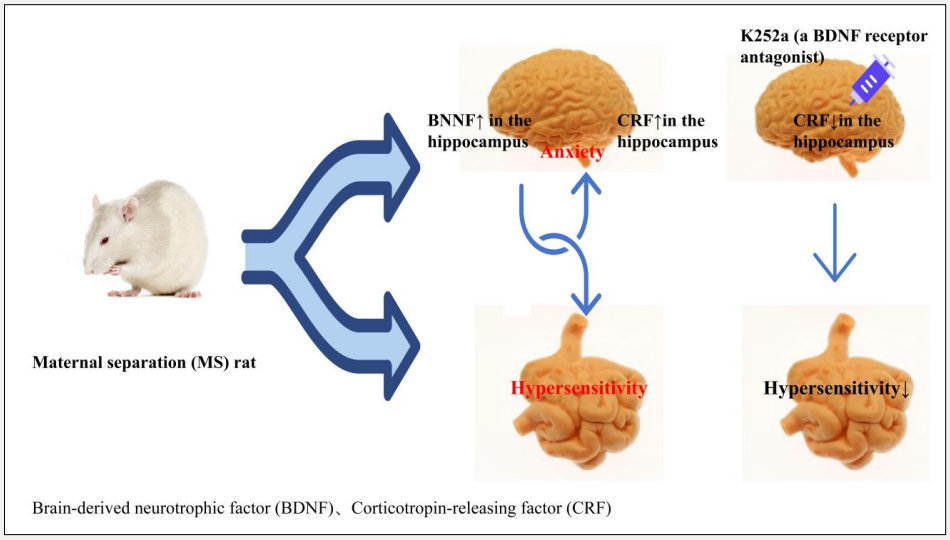


**Figure 6. BDNF/TrkB-CRF signaling drives early-life stress-induced anxiety and visceral hypersensitivity.**

**Hippocampal BDNF Correlated with Anxiety and Visceral Hypersensitivity in MS Rats**

Correlation analysis revealed a significant positive correlation between hippocampal BDNF levels and the AWR score ($r = 0.93$, $p = 0.003$; $n = 7$; Figure 4a). In contrast, hippocampal BDNF levels showed a significant negative correlation with the percentage of time spent in the central zone ($r = -0.78$, $p = 0.04$; $n = 7$; Figure 4b).

**K252a decreased CRF expression and alleviated visceral hypersensitivity**

CRF-like immunoreactivity was abundantly distributed throughout the hippocampus. Quantitative analysis revealed that CRF expression in the hippocampus was significantly elevated in MS rats compared to control rats (MS: median 19.3, interquartile range 16 - 26.4 vs. control: 10.5, interquartile range 7.7 - 14.1; $p = 0.02$; Figure 5a and 5b; $n = 6$ per group). ELISA results showed that the concentration of CRF in the hippocampus was higher in MS rats than in control rats (control: 5.93 ± 2.09 pg/mg vs. MS: 10.88 ± 4.54 pg/mg, $t = -2.43$, $df = 10$, $p = 0.04$; Figure 5d; $n = 6$ per group).

Following the ICV injection of K252a (a BDNF receptor antagonist) in MS rats, CRF expression in the hippocampus was reduced compared to the MS group (MS group: median 19.3, interquartile range 16 - 26.4 vs. MS+K252a group: 12.9, interquartile range 10.1 - 15.1; $p = 0.02$; Figure 5b and 5c; MS group $n = 6$, MS+K252a group $n = 4$). The CRF concentration in the hippocampus of the MS+K252a group was also lower than in the MS group (MS: 10.88 ± 4.54 pg/mg vs. MS+K252a: 4.69 ± 2.56 pg/mg, $t = 2.45$, $df = 8$, $p = 0.04$; Figure 5d; MS group $n = 6$, MS+K252a group $n = 4$). The AWR scores for each volume of 37°C water (0.4, 0.6, 0.8, 1.0, 1.2 mL) were significantly lower in the MS+K252a group compared to the MS group (0.4 mL: MS: 0.38 ± 0.21 vs. MS+K252a: 0.00 ± 0.00, $t = 4.39$, $df = 5$, $p = 0.007$; 0.6 ml: MS: 2.17 ± 0.58 vs. MS+K252a: 0.44 ± 0.24, $t = 6.48$, $df = 7.09$, $p = 0.00$; 0.8 mL: MS: 2.92 ± 0.38 vs. MS+K252a: 1.25 ± 0.35, $t = 7.02$, $df = 8$, $p = 0.00$; 1.0 mL: MS: 3.38 ± 0.49 vs. MS+K252a: 1.75 ± 0.46, $t = 5.24$, $df = 8$, $p = 0.001$; 1.2 mL: MS: 3.79 ± 0.29 vs. MS+K252a: 2.44 ± 0.24, $t = 7.67$, $df = 8$, $p = 0.00$; Figure 5f; MS group $n = 6$, MS+K252a group $n = 4$).

However, no significant difference in serum CRF concentrations was found among control rats, MS rats, and MS+K252a rats (control: 329.62 ± 62.61 pg/mL vs. MS: 334.92 ± 44.61 pg/mL, $t = -0.17$, $df = 10$, $p = 0.87$; MS: 334.92 ± 44.61 pg/mL vs. MS+K252a: 289.03 ± 53.33 pg/mL, $t = 1.48$, $df = 8$, $p = 0.18$; Figure 5e; $n = 6$ in the control and MS groups, $n = 4$ in the MS+K252a group).

## DISCUSSION

This study demonstrates that early life stress (ELS) induces upregulation of hippocampal BDNF, which activates TrkB receptors and enhances the expression of corticotropin-releasing factor (CRF), a key mediator of anxiety and visceral hypersensitivity in IBS [20]. These findings highlight the central role of BDNF in the pathogenesis of ELS-induced anxiety and visceral hypersensitivity. The study also suggests that BDNF functions as a brain-gut peptide that modulates the dysregulation of the bidirectional brain-gut axis in IBS.

Visceral hypersensitivity is a core pathophysiological feature of IBS, often co-occurring with psychiatric comorbidities, particularly anxiety disorders. Clinical evidence confirms that anxiety exacerbates both central and peripheral visceral sensitivity in IBS patients [21], strongly implicating brain-gut axis dysfunction in the pathogenesis of IBS. ELS triggers long-lasting changes in this axis, making it a valuable model for exploring the underlying mechanisms of IBS [14]. MS rats, a well-established model of early life stress, display characteristic anxiety and visceral hypersensitivity symptoms, making them an excellent model for investigating brain-gut dysfunction in IBS [22]. Previous studies have found that anxiety levels in rats increase after 180 minutes (HMS180) of MS, and visceral sensitivity to colorectal distension (CRD) is elevated in separated animals [23]. In this study, we found that MS rats spent significantly less time in the central zone, crossed the center less frequently, and exhibited fewer rearings, while showing notably increased grooming behavior compared to control rats. These behavioral changes collectively suggest increased anxiety in the MS group. Additionally, MS rats showed heightened visceral sensitivity to CRD, further supporting the presence of visceral hypersensitivity. Spearman's rank correlation analysis revealed a positive correlation between anxiety and visceral hypersensitivity in MS rats, suggesting that anxiety exacerbates visceral hypersensitivity. In line with several previous studies, we preferentially used male rats in this study, as female rats are influenced by hormonal fluctuations [14,24]. A recent study indicated that female rats exhibit less anxiety-like behavior, so female rats were intentionally excluded to avoid potential bias [25].

BDNF is highly expressed in both the central nervous system and the gastrointestinal tract. This neurotrophic factor has a profound impact on the development of anxiety and visceral hypersensitivity. Research shows that overexpression of BDNF in the amygdala and hippocampus leads to heightened anxiety, particularly in the hippocampus [25]. Additionally, a recent study showed that withdrawal from an obesogenic diet increased hippocampal BDNF levels, which mediated anxiety-like behaviors. The increase in BDNF likely occurred in its mature forms (14 kD monomer and 28 kD dimer) [26]. However, previous studies have reported reduced hippocampal BDNF expression in adulthood following

early life stress (ELS), possibly because these animals did not exhibit anxiety. Overexpression of BDNF in the intestinal tract has been positively correlated with the severity of abdominal pain, underscoring BDNF's role in promoting visceral hypersensitivity in IBS patients [27]. However, research on how BDNF regulates ELS-induced anxiety and visceral hypersensitivity remains limited [28]. We used functional magnetic resonance imaging to show that hippocampal activity was enhanced in both IBS patients with anxiety and control groups when subjected to maximal rectal stimulation. These studies suggest that the hippocampus may serve as the center for regulating anxiety-induced visceral hypersensitivity. The hippocampus plays a critical role in mediating anxiety-exacerbated pain. In this study, BDNF concentrations were measured in the cingulate gyrus, amygdala, and hippocampus, revealing a significant increase in BDNF protein and mRNA levels specifically in the hippocampus of MS rats, while levels in the cingulate gyrus and amygdala remained largely unchanged. Additionally, a strong negative correlation was found between the percentage of time MS rats spent in the central zone and hippocampal BDNF expression, indicating that heightened anxiety in MS rats corresponds directly to elevated BDNF expression in the hippocampus. A strong positive correlation was observed between hippocampal BDNF expression and the AWR score. These results suggest that ELS-induced anxiety and visceral hypersensitivity are closely associated with increased BDNF expression in the hippocampus.

Serum BDNF acts as a biomarker for anxiety severity and is correlated with the symptom burden of IBS, reflecting its involvement in brain-gut axis dysregulation [29]. However, in MS rats, serum BDNF levels did not show a significant difference from controls, suggesting that central BDNF may not readily cross the blood-brain barrier (BBB) into systemic circulation. CRF is a key regulator of emotional and psychological stress responses [11]. It is widely distributed throughout both central and peripheral nervous systems and easily crosses the blood-brain barrier. Barna et al. found increased CRF mRNA expression in the central nervous system of MS rats, suggesting that CRF may contribute to the onset of anxiety and visceral hypersensitivity in this model [30]. This study further demonstrates elevated CRF expression in the hippocampus of MS rats, indicating that CRF is involved in the brain-gut dysfunction underlying early-life stress-induced visceral hypersensitivity. TrkB, the high-affinity receptor for BDNF, is activated by BDNF, which binds to TrkB receptors on CRF neurons, triggering CRF release [31]. Additionally, BDNF is highly expressed in the hippocampus, a key region for integrating stress responses and processing visceral pain [31]. The hippocampus, where the bilateral ventricle is located, is the primary site for intracerebroventricular (ICV) injection [17]. This study found that following ICV injection of the TrkB antagonist K252a in MS rats, CRF expression in the hippocampus and the AWR scores decreased, alleviating visceral hypersensitivity. These findings suggest that the overexpression of CRF in the hippocampus was induced by increased BDNF in this region. Moreover, the visceral hypersensitivity in MS rats was relieved after K252a ICV administration. These results suggest that CRF mediates BDNF's regulatory role in the brain-gut dysfunction underlying early-life stress-induced visceral hypersensitivity in MS rats. However, to confirm this hypothesis, further investigation into the expression of the BDNF receptor TrkB in MS rats and the effect of K252a on anxiety is necessary. Thus, this represents a limitation of the current study.

No significant change in serum CRF concentrations was observed in this study, suggesting that CRF does not cross the blood-brain barrier into the bloodstream. Previous studies have shown that CRF in the spinal dorsal root ganglia (DRG) contributes to visceral hypersensitivity, indicating a potential role for CRF in neural pathways [32].

## CONCLUSION

This study demonstrates that hippocampal BDNF/TrkB-CRF signaling drives early-life stress-induced anxiety and visceral hypersensitivity. Our research establishes a foundational theoretical framework for understanding the pathogenesis of ELS-induced IBS-like anxiety and visceral hypersensitivity, while also revealing new insights into potential therapeutic targets for IBS.

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The authors declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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