

Links between gut dysbiosis and neurotransmitter disturbance in chronic restraint stress-induced depressive behaviours: the role of inflammation

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Abstract

Accumulating evidence has shown that inflammation, the gut microbiota and neurotransmitters are closely associated with the pathophysiology of depression. However, the links between the gut microbiota and neurotransmitter metabolism remain poorly understood. The present study aimed to investigate the neuroinflammatory reactions in chronic restraint stress (CRS)-induced depression and to delineate the potential links between the gut microbiota and neurotransmitter metabolism. C57BL/6 mice were subjected to chronic restraint stress for 5 weeks, followed by behavioural tests (the sucrose preference test, forced swim test, open field test and elevated plus maze) and analysis. The results showed that CRS significantly increased IL-1 β , IL-2, IL-6 and TNF α levels and decreased BDNF expression, accompanied by the activation of I κ B α -p-NF- κ B signalling in the mouse hippocampus. In addition, the neurotransmitter metabolomics results showed that CRS resulted in decreased levels of plasma 5-HT, DA, and NE and their corresponding metabolites, and gut microbiota fecal metabolites with the 16S rRNA gene sequencing indicated that CRS caused marked microbiota dysbiosis in mice, with a significant increase in *Helicobacter*, *Lactobacillus*, and *Oscillibacter* and a decrease in *Parabacteroides*, *Ruminococcus*, and *Prevotella*. Notably, CRS-induced depressive behaviours and the disturbance of neurotransmitter metabolism and microbiota dysbiosis can be substantially restored by dexamethasone (DXMS) administration. Furthermore, a Pearson heatmap focusing on correlations between the microbiota, behaviours and neurotransmitters showed that *Helicobacter*, *Lactobacillus*, and *Oscillibacter* were positively correlated with depressive behaviours but were negatively correlated with neurotransmitter metabolism, and *Parabacteroides* and *Ruminococcus* were negatively correlated with depressive behaviours but were positively correlated with neurotransmitter metabolism.

Taken together, the results suggest that inflammation is involved in microbiota dysbiosis and the disturbance of neurotransmitter metabolism in CRS-induced depressive changes, and the delineation of the potential links between the microbiota and neurotransmitter metabolism will provide novel strategies for depression treatment.

1. Introduction

Depression is a devastating disorder with a lifetime prevalence of more than 10% (Global Burden of Disease Study 2013 Collaborators, 2015; Gulbins et al., 2018). As one of the most common neuropsychiatric diseases, it is mainly characterized by a pervasive low mood, lack of interest, anhedonia and helplessness (Kessler et al., 2003). It is well known that the decreased levels of dopamine (DA), norepinephrine (NE) and serotonin (or 5-hydroxytryptamine, 5-HT) in the central nervous system is the hallmark of the development of depression. Current clinical drugs for depression are also dominated by serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs). However, approximately half of patients fail to achieve sustained remission (Roiser; Elliott; Sahakian, 2012). Accordingly, the neurobiological mechanisms underlying depression still need to be characterized.

Accumulating studies suggest that depressive symptoms are closely associated with the level of proinflammatory cytokines (IL-1 β , IL-6 and TNF α) and polymorphisms in inflammation-related genes (Kenis and Maes, 2002; Miller and Raison, 2016). Clinical research has found that antidepressants, such as SSRIs, can inhibit elevated levels of inflammatory mediators in peripheral blood (Hashioka et al., 2009). In animal experiments, chronic inflammatory molecule administration, such as lipopolysaccharides (LPS), IL-1 β and TNF α , triggers depressive-like responses, and the inhibition of inflammation alleviates depressive-like behaviour in some animal models (Goshen et al., 2008; Jiang et al., 2017). For example, the administration of infliximab (a TNF α inhibitor) decreases depression-like behaviour in a rat model of chronic mild stress (Ertenli et al., 2012). Curcumin, which has anti-inflammatory and antioxidant effects, has been proven to improve depressive symptoms by inhibiting the NF- κ B pathway and upregulating the expression of brain-derived neurotrophic factor (BDNF), which is related to cell proliferation (Lopresti; Hood; Drummond, 2012). The NF- κ B signalling pathway plays an important role in the pathological symptoms of depression, and its activation causes excess expression of a spectrum of proinflammatory cytokines, such as IL-1 β , IL-6 and TNF α (Zhang; Lenardo; Baltimore, 2017). In stressed mice, depressive-like behaviours can be blocked by the administration of the NF- κ B inhibitors JSH or SC (Koo et al., 2010). In addition, in the central nervous system, desipramine reduces LPS-induced increases in TNF α , IL-1 β and p65-NF- κ B levels in rat cortical tissue (O'Sullivan et al., 2009). These results supported the critical roles of inflammation in depression. However, the potential roles of inflammation are still ambiguous, and the potential mechanisms in depression treatment remain to be clarified.

In recent years, studies along the gut-brain axis on mental health have gained considerable attention (Martin et al., 2018; Mayer et al., 2014). The epidemiological and animal studies indicate that gut microbiome diversity and gut microbiota composition are associated with depression (Aslam et al., 2018; Luo et al., 2018). Reports from patients with depression and healthy controls have demonstrated that faecal bacterial α -diversity was increased in depressed patients and the levels of several predominant genera were significantly different between the depression and healthy groups (Jiang et al., 2015). Indeed, recent human and animal studies have suggested that gut microbiota interventions, including probiotics or faecal microbiota transplantation, may be helpful to treat depression (Guida et al., 2018; Li et al., 2018). However, the links between the gut microbiota and the brain remain poorly understood. Intriguingly, intestinal flora can also synthesize 5-HT, DA and NE, and secrete to the blood, which is closely associated with depression treatment (Dinan and Cryan, 2016). Additionally, an early-life stress animal study showed that the gut microbiota is significantly associated with depressive behaviour and inflammatory cytokines (IL-6) in the hippocampus (Doherty et al., 2017). Therefore, this evidence prompted us to infer that inflammation may be involved in neurotransmitter metabolism disorder and microbiota dysbiosis in depression.

In the present work, the chronic restraint stress (CRS) mouse depression model, which is widely used to induce depressive behaviour was exploited (Wang et al., 2017). The depressive-like behaviours, neuroinflammatory reactions, gut microbiota and monoamine neurotransmitter metabolism in the CRS

mouse model of depression were evaluated. Moreover, the associations between depressive behaviour, gut microbiota and neurotransmitter metabolism were also analysed.

2. Materials And Methods

2.1 Animals and treatments

Seven-week-old male C57BL/6J mice, weighing 22–30 g, were purchased from Vital River (Beijing, China). Mice were housed under standard conditions of temperature ($23 \pm 2^\circ\text{C}$), humidity ($55 \pm 10\%$) and light (light/dark cycle of 12/12 h each). All mice were allowed to acclimatize for period of 1 week, with free access to food and water. Then, the mice were randomly divided into 4 groups ($n = 15$): control, control + DXMS, CRS, and CRS + DXMS. While the mice in the control + DXMS and CRS + DXMS groups were intraperitoneally injected with 1 mg/kg DXMS (the First Affiliated Hospital of Nanjing Medical University, Jiangsu, China), the control and CRS groups received the same volume of saline (Bska et al.,2004; Cassol-Jr et al.,2010; Luo et al.,2018). The injection time was one hour before the restriction. For CRS experiments, the mice were restrained in a 50 mL polystyrene tube with 12 evenly spaced vent-holes (0.5 cm diameter each and 1.0 cm apart from each other) 4 h/d (10:00–14:00) for 5 weeks (Khalid et al.,2016). The timeline of CRS is illustrated in Fig. 1A. All animal treatments were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University and in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

2.2 Behavioural tests

2.2.1 Sucrose preference test (SPT)

The SPT is a well-known test for the evaluation of anhedonia, a typical depressive-like behavior in mice (Wang et al.,2017). Before the SPT, mice were housed alone and deprived of water for 12 h. On day 36, two pre-weighed drinking bottles containing 1% sucrose solution or tap water were placed in the drinking hole of each cage. The sides of the two bottles were randomly placed and switched in the middle of the SPT to avoid spatial bias. After 12 h, the bottles were weighed again, and the change in weight of each bottle was considered to be the mouse intake. The preference for sucrose was calculated as the percentage of sucrose solution intake relative to the total liquid intake.

2.2.2 Open field test (OFT) and elevated plus maze (EPM)

The OFT and EPM tests are used to assess the anxiety state existing in depressive-like behaviours (Wang et al.,2017). The day after the SPT, the OFT was conducted in a square area ($40 \text{ cm} \times 40 \text{ cm} \times 30 \text{ cm}$). Mice were placed individually into the centre of the area for 5 min while being monitored with a video camera. During the test, the time spent in the central square ($20 \text{ cm} \times 20 \text{ cm}$) was recorded. After the OFT, the EPM test was also performed to evaluate anxiety-like behaviour. The maze apparatus has two opposite open arms ($50 \times 10 \text{ cm}$) and two opposite closed arms ($50 \times 10 \text{ cm}$) with 10 cm walls, connected by a central platform ($10 \times 10 \text{ cm}$) and elevated 70 cm from the floor. The mice were placed at the centre of the apparatus with their head facing towards an open arm. The time spent in the closed

arms during the 5 min was recorded with a video camera. The apparatus was cleaned with 20% alcohol between trials.

2.2.3 Forced swim test (FST)

The FST is a common measurement for evaluating the behavioural despair of depressive-like behaviours (Wang et al., 2017). On day 38, all groups were subjected to the FST. Mice were placed individually in 2 L beakers containing 1.5 L water (25°C), and the water was changed after each round of testing. A camera recorded the movement of the mice for 6 min, and the immobility time (the time during which the mice not explored or escaped) was recorded during the last 4 min of the 6 min test session. The video was analysed by Tail Suspension Scan (Clever Sys Inc., Reston, VA, United States).

2.3 Western blot analysis

One day after the behavioural tests, the mice were decapitated and then the prefrontal cortex (PFC), hippocampus and striatum were quickly removed and kept in a -80°C freezer. For western blotting, the tissues were homogenized by a tip sonicator with 200 µL/5 mg radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma, St. Louis, MO, USA) containing protease inhibitors and then centrifuged at 12000 rpm for 15 min. The protein concentrations were analysed using the BCA Protein Assay Kit (Thermo Scientific Rockford, IL, USA). The loading buffer was added to the samples of supernatants and heated to 100°C for 5 min. The equal samples (30 µg) were separately loaded onto 12% SDS-PAGE gels and then transferred onto PVDF membranes. After blocking with 5% milk solution, the bolts were incubated at 4°C for 12 h with primary antibodies against IκBα, p65, and IL-1β (1:1000, Cell Signalling Technology, USA); IL-2 and IL-6 (1:1000, Proteintech, China); TNFα (1:3000, Proteintech, China); p-p65 (1:1000, Affinity, China); BDNF (1:3000, Abcam, USA), and β-Actin (1:5000, Santa Cruz Biotechnology, USA). After primary antibody incubation, membranes were washed with TBST (3 cycles for 5 min each) and incubated with secondary antibodies for 2 h. The membrane signals were scanned by enhanced chemiluminescence HRP substrate (Millipore Corporation, Billerica, MA, USA) and quantified using ImageJ software (NIH, USA). β-Actin was used as an internal standard for signal normalization.

2.4 Immunohistochemistry (IHC)

For immunohistochemistry, the mouse brains were collected and rapidly fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin and cut into serial slices 30 µm thick in the coronal plane. After being deparaffinized and rehydrated, sections of brain were subjected to antigen retrieval with sodium citrate buffer, followed by quenching of endogenous peroxidase. To prevent nonspecific staining, the sections were blocked with 5% goat serum blocking solution for 10 min and then incubated with primary antibodies (as described in the western blot analysis section above) overnight at 4°C. The next day, after washing with PBS 3 times, the tissues were incubated with the corresponding secondary antibodies for 10 min at 37°C. Finally, the sections were treated with aminoethyl carbazole and counterstained with Mayer's haematoxylin. After the slices were dried completely, IHC images were scanned by Panoramic Scan. Representative images were captured from the CA1 region of the hippocampus, the PrL region of the cortex and the striatum by Panoramic Viewer software.

2.5 16S rRNA gene sequencing

Before the behavioural tests, fresh faeces of the mice were collected for 16S rRNA gene sequencing on the Illumina MiSeq system. Briefly, DNA extraction was performed using an environmental sample DNA extraction kit (OMEGA). The V3–V4 region of the 16S rRNA gene was amplified using 16S primers by a thermocycler PCR system (Applied Biosystems 2720 Thermal Cycler). The PCR products were purified by using the AxyPrep Mag PCR Clean-Up Kit (AXYGEN) and quantified using Qubit picogreen (Thermo Scientific, Shanghai, China). Equimolar quantities of the products were mixed and paired-end sequenced at 2 x 300 bp on the Illumina MiSeq platform according to the manufacturer's protocols. The resulting raw fastq files were quality filtered using Trimmomatic and assembled by FLASH. The high-quality reads were clustered into operational taxonomic units (OTUs) at the 97% similarity level using UPARSE, and the representative sequence of each OTU was analysed by the RDP Classifier algorithm against Silva123 using a confidence threshold of 0.8. The species abundance and beta-diversity analysis were performed using R, and alpha-diversity analysis was calculated within Mothur.

2.6 UHPLC-MS/MS neurotransmitter analysis

Blood samples obtained by the retro-orbital bleeding assay were clotted, and serum supernatant was separated by centrifugation at 1500 × g for 15 min at 4°C and then kept at -80°C. The content (5-HT, DA, NE) was detected using ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS/MS). The UHPLC separation was conducted on an Acquity BEH-C18 column (100 mm × 2.1 mm, 1.7 μm) (Thermo Fisher Scientific) at 35°C. A total of 5 μl of supernatant was injected for the UHPLC-MS/MS analysis. The mobile phase comprised A and B, where A was 0.1% formic acid water (A) and B was 0.1% formic acid acetonitrile. The linear gradient programme was as follows: 20%-60% B from 0 to 3 min; 60% B from 3 to 6 min; 60%-80% B from 6 to 10 min; 80%-95% B from 10 to 13 min; 95% B from 13 to 17 min; return to the initial conditions (80% A and 20% B) from 17 to 17.1 min; and re-equilibration from 17.1 to 20 min. The UHPLC-MS/MS was run at a flow rate of 0.25 ml/min. The mass spectra were obtained on a Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source in positive mode. The resolution was 17,500, and the scan type was parallel reaction monitoring (PRM). The parameters were as follows: spray voltage 3 kV, S-Lens RF level 50, capillary temperature 350°C, heater temperature 425°C, sheath gas flow 50 arbitrary units (arb), auxiliary gas flow 13 (arb) and sweep gas 3 (arb).

2.7 Data analysis

SPSS statistics 18.0 software was used for statistical analyses. Multiple comparisons were conducted using one-way ANOVA, and Dunnett's multiple comparison procedures were used for post hoc comparisons when the ANOVA showed obvious effects. Pearson correlations were performed between relative microbiota, behaviour and neurotransmitters. $p < 0.05$ was considered statistically significant. GraphPad Prism 5 was used for generating graphs. Data are reported as the mean ± SEM.

3. Results

3.1 Chronic restraint stress induces depressive-like behaviours in mice

To investigate the effects of inflammation on depression, we established a murine model of depressive-like behaviour by CRS, and DXMS, one anti-inflammatory drug commonly used in the clinic, was applied. In the SPT, the sucrose preference index was significantly decreased in CRS mice, an effect that was partially restored by treatment with DXMS (Fig. 1B), suggesting that anti-inflammatory treatment improved anhedonia in depressed mice. For the FST, the immobility time in the CRS mice was significantly increased when compared with the saline group, and similarly, the effect was markedly ameliorated by the anti-inflammatory treatment (Fig. 1C), suggesting that anti-inflammation attenuated the depressed-like behaviour in mice. To confirm these results, the OFT and EPM were also performed to evaluate the anxiety level of mice. Compared with the control group, CRS mice exhibited substantially decreased time in the centre of the OFT and increased time in the closed arms of the EPM, while pretreatment with DXMS in CRS mice remarkably reversed the anxious behaviour (Fig. 1D, E).

3.2 Neuroinflammation and brain-derived neurotrophic factor (BDNF) expression in the hippocampus, PFC and striatum in CRS mice

Having determined the benefits of anti-inflammatory treatment in depressive-like behaviour in CRS mice, we then sought to examine the inflammatory reactions in the mouse brain. The hippocampus, PFC and striatum, which are associated with depression, were selected in the current research. In the hippocampus, western blot analysis showed that the levels of IL-1 β , IL-2 and TNF α were obviously elevated by CRS, while the inflammatory signalling protein p-p65 NF- κ B was significantly increased accompanied by I κ B α degradation. Moreover, BDNF, a neurotrophin, plays a key role in neuronal development and neurogenesis and was significantly decreased in CRS mice (Fig. 2A, B, C). Notably, these effects can be partially attenuated by DXMS treatment. In the PFC, IL-1 β and TNF α expression was significantly increased, though IL-6 and p-p65 showed a slight reduction in CRS mice. Additionally, the I κ B α and BDNF levels were significantly decreased in CRS mice (Fig. 2D, E, F). Similarly, the changes in IL-1 β , IL-6, TNF α , p-p65 and BDNF were also reversed by DXMS. For the striatum, the expression of IL-1 β , IL-6 and TNF α was increased, and the BDNF level was decreased in CRS mice (Fig. 2G, H, I). As speculated, the administration of DXMS substantially retarded CRS-induced IL-1 β upregulation and BDNF downregulation. Taken together, these results confirmed that the neuroinflammation was involved in the depressive-like changes in CRS-induced depression. To further confirm the results of the western blot analysis and to clarify the localization of neuroinflammation, an immunohistochemical assay was performed to examine the expression of IL-1 β , IL-2, IL-6, TNF α and BDNF. Consistent with the western blot results, CRS significantly increased the expression of IL-1 β and TNF α and decreased the BDNF level in the hippocampal CA1 region, the prelimbic cortex (PrL) of the PFC and the striatum (Fig. 3A, B, C). Consistently, the changes in IL-1 β , TNF α and BDNF in the CRS mouse brain were reversed by DXMS administration.

3.3 Effects of chronic restraint stress on monoamine neurotransmitters and their metabolites in serum

The disturbance of neurotransmitters is closely associated with the development of depression. We then sought to investigate CRS-mediated neurotransmitter metabolism and evaluate the potential role of inflammation in driving neurotransmitter disturbance. The serum of the mice was collected and subjected

to UHPLC-MS/MS analysis. The neurochemical data showed that CRS caused a significant decrease in the concentration of 5-HT with a simultaneous reduction of its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) (Fig. 4A). Moreover, NE and its metabolite, 3-methoxy-4-hydroxy-phenylglycol (MHPG), were notably decreased in the CRS group (Fig. 4B). CRS also affected DA metabolism, with decreased levels of DA and its metabolites, 3-methoxytyramine (3-MT) and homovanillic acid (HVA) (Fig. 4C), and the reduction in monoamine neurotransmitters and their corresponding metabolites was reversed by anti-inflammatory treatment.

3.4 Effects of chronic restraint stress on faecal microbiota composition

Since gut microbiota dysbiosis has been found to be closely associated with depression, and this effect is associated with inflammation, we then examined the homeostasis of the gut microbiota after CRS and the effects of anti-inflammatory treatment on gut microbiota. The stacked bar chart shows the relative abundance of various microbes in faecal samples from the four groups at the genus level (Fig. 4A). The relative abundance of *Helicobacter* and *Lactobacillus* was significantly increased in the CRS group, and anti-inflammatory intervention markedly decreased the abundance. Moreover, the abundance levels of *Oscillibacter* and *Pseudoflavonifractor* were obviously increased, while the *Parabacteroides*, *Ruminococcus*, and *Prevotella* levels were decreased in the CRS group when compared with the control group. *Butyricicoccus* was more abundant in the CRS mice than in the CRS + DXMS group, though there were no changes compared with the control mice (Fig. 4B). Additionally, DXMS treated alone did not obviously affect the abundance of microbiota in mice. Furthermore, the alpha- and beta-diversity analysis showed that there were no significant changes in microbiota structure among the four groups (Fig S1).

3.5 Correlations between microbiota, behaviours and neurotransmitters in CRS mice

To explore the potential correlations between the changes in microbiota, salient individual behaviours and neurotransmitter metabolism in CRS mice, a Pearson heatmap focusing on the correlations between microbiota, behaviours and neurotransmitters was performed. The microbiota significantly differed between groups and was highly correlated with the sucrose preference index in the SPT and the immobility time in the FST (Fig. 6). *Oscillibacter* and *Pseudoflavonifractor* were negatively correlated with the sucrose preference index in the SPT, while the *Parabacteroides* displayed a positive association with the sucrose preference index in the SPT. *Helicobacter* and *Pseudoflavonifractor* were positively correlated with the immobility time in the FST, while *Parabacteroides* was negatively correlated with the immobility time in the FST. A significant correlation was also shown between the microbiota and neurotransmitter metabolism (Fig. 6). *Parabacteroides* and *Ruminococcus* were positively correlated with the metabolites of DA (3-MT, HVA) and NE (MHPG), while *Lactobacillus* was negatively correlated with MHPG. Moreover, a negative relationship was found between the abundance of *Helicobacter* and NE and 3-MT. Paradoxically, *Oscillibacter* abundance was positive for DA but negative for HVA.

4. Discussion

It has been demonstrated that the disturbance of the gut-brain axis contributes to the development of depression; however, the potential mechanisms remain poorly understood. In the current study, CRS

successfully induced depressive-like behaviours in mice, accompanied by increased IL-1 β and TNF- α expression and decreased BDNF levels in the hippocampus, PFC and striatum. Moreover, the disturbance of neurotransmitter metabolism and gut microbiota may be associated with inflammation, as anti-inflammatory treatment not only alleviated IL-1 β and TNF α levels and increased BDNF levels in the hippocampus, PFC and striatum in CRS mice but also rebalanced the abundance of gut microbiota and improved monoamine neurotransmitter levels in CRS mice, underscoring a critical role in gut-microbiota-brain modulation in CRS-induced depression.

CRS is widely used in depression animal models in rodents(Wang et al.,2017). In the current work, we found a decreased sucrose preference index in the SPT, increased immobility time in the FST, decreased time in the centre of the OFT and increased time in the closed arms of the EPM in CRS mice. Considerable evidence indicates that CRS induces depression via neuroinflammatory pathways(Miller and Raison,2016; Zhu et al.,2019). Clinical and animal studies have demonstrated neuroinflammation in major depressive disorder (MDD) patients and depression animal models which manifested as the excess accumulation of proinflammatory cytokines and inflammatory signalling activation(Miller and Raison,2016). In the present study, more brain regions associated with depression, including the hippocampus, PFC and striatum, were investigated, showing that IL-1 β and TNF α levels were significantly increased in these brain regions in CRS mice, which was consistent with previous studies showing that stress models induce IL-1 β and TNF α increases in microglia in the brain(Guo et al.,2018; Koo et al.,2010). The NF- κ B complex including p50, p52, Rel A (p65), Rel B, and c-Rel, plays a pivotal role in proinflammatory signal transduction; the activation of p65 NF- κ B contributes to downstream cytokine release, an effect that may be closely associated with the degradation of I κ B α , an inhibitory protein of NF- κ B(Koo et al.,2010; Zha et al.,2014; Zhang; Lenardo; Baltimore,2017). In the present study, CRS significantly increased I κ B α degradation and NF- κ B p65 phosphorylation in the mouse hippocampus. However, in the PFC, the phosphorylation of NF- κ B p65 was slightly decreased, which may be due to the specificity of each specific brain region of the PFC(Yu and Sharp,2012). It should be noted that the anti-inflammatory intervention with DXMS, a first-line immunosuppressive drug used for anti-neuroinflammation in clinical practice(Mai et al.,2009; Meneses et al.,2017; Vizquete et al.,2018), significantly improved CRS-induced depressive-like changes in the SPT, FST, OFT and EPM, partially by suppressing proinflammatory cytokines and antagonizing the NF- κ B pathway, suggesting the involvement of the inflammatory reaction in CRS-induced depression.

BDNF has been shown to be reduced by inflammatory cytokines and NF- κ B pathway activation in stress-induced animal models of depression(Zhao et al.,2019). A spectrum of evidence has shown that BDNF is substantially decreased in mood disorders and plays an essential role in antidepressant treatments(Guo et al.,2018; Pesarico et al.,2016). In our study, CRS caused a marked decrease in BDNF expression in the hippocampus, PFC and striatum, while anti-inflammation obviously retarded CRS-induced BDNF reduction, deciphering the potential roles of inflammation in depression-like behaviour.

The abnormalities in the synthesis and metabolism of monoaminergic neurotransmitters are proposed to be associated with depression(Perez-Caballero et al.,2019). Currently, first-line antidepressants act to

increase the level of monoamine neurotransmitters by inhibiting the reuptake of 5-HT and NE, thereby exerting an antidepressant role (Perez-Caballero et al., 2019). Therefore, the levels of monoamine neurotransmitters and the corresponding metabolites in mouse serum were detected. CRS induced a significant decrease in the concentrations of 5-HT, DA, NE and their metabolites, and these effects were reversed by anti-inflammation. These results strongly support the evidence that the high expression of proinflammatory cytokines (TNF- α , IL-6 and IL-1 β) is related to the disturbance of neurotransmitter metabolism (Miller and Raison, 2016). Moreover, our studies expanded the finding that the downstream metabolites were significantly changed, implying the abnormal activity or expression of some limited-step enzymes that are responsible for 5-HT, DA and NE metabolism (Miller and Raison, 2016). However, further studies on this issue are needed.

Accumulating evidence has revealed that chronic stress disturbs the gut microbial community (Guo et al., 2018, 2019). A recent study in CRS mice has shown that the Shannon index was enhanced in depressed mice and that the proportion of sequences of *Firmicutes* and *Bacteroidetes* was significantly different between depressed and control mice (Guo et al., 2018). In the current work, CRS significantly increased the abundance of *Helicobacter*, one microbe is associated with gastritis, gastric ulcer, and digestive dysfunction (Kim; Lim; Kim, 2018). Although there are reports that digestive dysfunction is sometimes comorbid with several mental illnesses with the highest proportion of depression (McGee; Lu; Disbrow, 2018), the specific mechanism of *Helicobacter* in mediating depression is still very limited. In our study, *Helicobacter* may be involved in mediating depression at least in part, through inflammation (Kim; Lim; Kim, 2018), because our correlation analysis showed that the *Helicobacter* abundance was significantly positively correlated with the immobility time in the FST, and DXMS treatment significantly decreased the abundance of *Helicobacter*. Furthermore, *Helicobacter* was negatively correlated with NE and 3-MT, suggesting that it may also be involved in monoamine neurotransmitter metabolism to mediate depression. It is worth noting that CRS induced an increase in the abundance of *Lactobacillus* and anti-inflammatory intervention reversed this change. Although reports have shown that *Lactobacillus* is a probiotic (Cheng et al., 2018; Guo et al., 2018), some studies have demonstrated that *Lactobacillus* members elicit IL-1 β production and inflammasome activation (Miettinen et al., 2012; Wong et al., 2016). In accordance with the previous work, here we also showed that the *Lactobacillus* abundance is related to inflammation, which might be associated with the diverse roles of *Lactobacillus* in specific diseases (Cheng et al., 2018); however, investigations of these underlying mechanisms are required for further elucidation. A recent study demonstrated that *Parabacteroides distasonis* alleviates obesity and metabolic dysfunctions through the production of succinate and secondary bile acids (Wang et al., 2019). In this study, the abundance of *Parabacteroides* was decreased in the CRS mice and similar effects were observed in male offspring exposed to prenatal stress (Gur et al., 2019). Moreover, we also showed that *Parabacteroides* abundance was negatively correlated with immobility time in FST and positively correlated with 3-MT, HVA and MHPG, which are the metabolites of DA and NE, suggesting that *Parabacteroides* may be involved in DA and NE metabolism. In CRS mice, the abundance of *Ruminococcus*, which is reported to be positively correlated with tryptamine metabolism (Dinan and Cryan, 2016; Jiang et al., 2015), was obviously decreased. The abundance of *Ruminococcus* was

positively correlated with 3-MT, HVA and MHPG in the current work, suggesting that *Ruminococcus* is positively correlated with dopamine metabolism. These results were consistent with previous studies that *Ruminococcus* exerts antidepressant effects and was less abundant in depressed patients and rodents (Cheng et al., 2018). For *Oscillibacter*, its main metabolite is valeric acid, which structurally resembles GABA and binds to the GABA_A receptor. Here, the abundance of *Oscillibacter* in the CRS mice was markedly increased and negatively correlated with the sucrose preference index in the SPT, suggesting that *Oscillibacter* is related to the induction of depressive behavior, which might be ascribed to *Oscillibacter* produced valeric acid (Naseribafrouei et al., 2014). In addition, *Oscillibacter* abundance was positively correlated with DA in this study, which contradicts the increased *Oscillibacter* in depressed mice, which might be due to the compensatory effect of the DA pathway (Wąsik et al., 2013). These results indicated correlations between microbiota, depressive-like behaviours and neurotransmitters, suggesting that microbiota dysbiosis may contribute to depression-like behaviour by affecting DA and NE metabolism.

Available reports have revealed complex reciprocal relationships between the intestinal microbiota and inflammation (Bruce-Keller; Salbaum; Berthoud, 2017), the gut microbiota is associated with systemic host immunity which participates in inflammation (Bruce-Keller; Salbaum; Berthoud, 2017; Dinan and Cryan, 2016). Oral probiotics ameliorate the behavioural deficits in depressed mice via the gut microbiota-inflammation axis, while inflammatory stimuli, such as LPS, induce gut microbiota dysbiosis, highlighting the potential role of inflammation in linking the gut to the brain (Guo et al., 2019; Li et al., 2018). In our work, it was proved that the interaction between microbiota dysbiosis and the neuroinflammatory response was involved in stress-induced depression, and anti-inflammatory treatment obviously improved the intestinal flora and neuroinflammation, which might occur through the gut microbiota-inflammation axis (Fig. 7). Inflammation might play a potential role in the process of gut microbiota DA and NE metabolism. Further research on the underlying mechanisms involving inflammation and the gut microbiota in the gut-brain axis is needed.

In conclusion, CRS contributes to depressive-like behaviour, with a significant reduction in monoamine neurotransmitter metabolism (5-HT, DA, NE, 5-HIAA, 3-MT, HVA, and MHPG) accompanied by gut microbiota dysbiosis. These effects are partially improved by anti-inflammatory treatment, underscoring the critical roles of inflammation in CRS-induced depressive-like changes. Therefore, fully deciphering the potential links between neurotransmitter metabolism, the gut microbiota and behaviour will provide novel therapeutic strategies and targets for depression intervention.

Abbreviations

CRS: chronic restraint stress; PFC: prefrontal cortex; DXMS: dexamethasone; DA: dopamine; NE: norepinephrine; 5-HT: serotonin; BDNF: brain-derived neurotrophic factor; SPT: sucrose preference test; OFT: open field test; EPM: elevated plus maze; FST: forced swim test; PrL: prelimbic cortex; 5-HIAA: 5-

hydroxyindoleacetic acid; MHPG: 3-methoxy-4-hydroxy-phenylglycol; 3-MT: 3-methoxytyramine; HVA: homovanillic acid.

Declarations

Ethics Approval Animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory, and all procedures were approved by the animal ethical and welfare committee of Nanjing Medical University (NJMU).

Consent for publication Approved by all authors.

Data Availability: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest: The authors have no conflicts of interest to declare.

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Figures

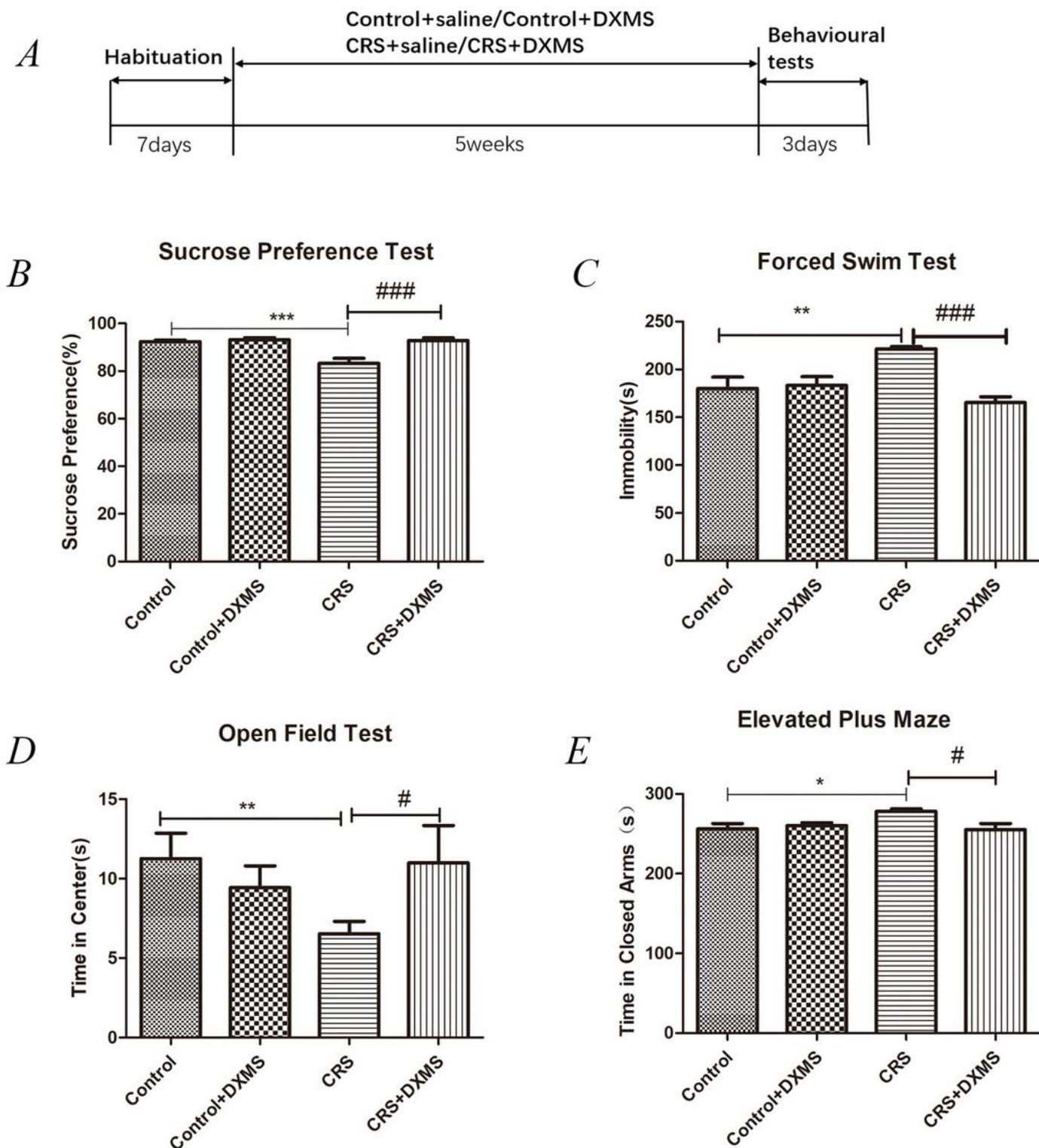


Figure 1

Antidepressant effects of dexamethasone in the chronic restraint stress (CRS) mouse model. (A) Experimental process of CRS modelling or administration. (B) Sucrose preference index in the SPT. (C) Immobility time in the FST. (D) Time in the centre of the OFT. (E) Time in the closed arms of the EPM. Data are presented as the mean \pm SEM, $n = 6-12$ for the behaviour test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control, # $p < 0.05$, ### $p < 0.001$ vs. CRS.

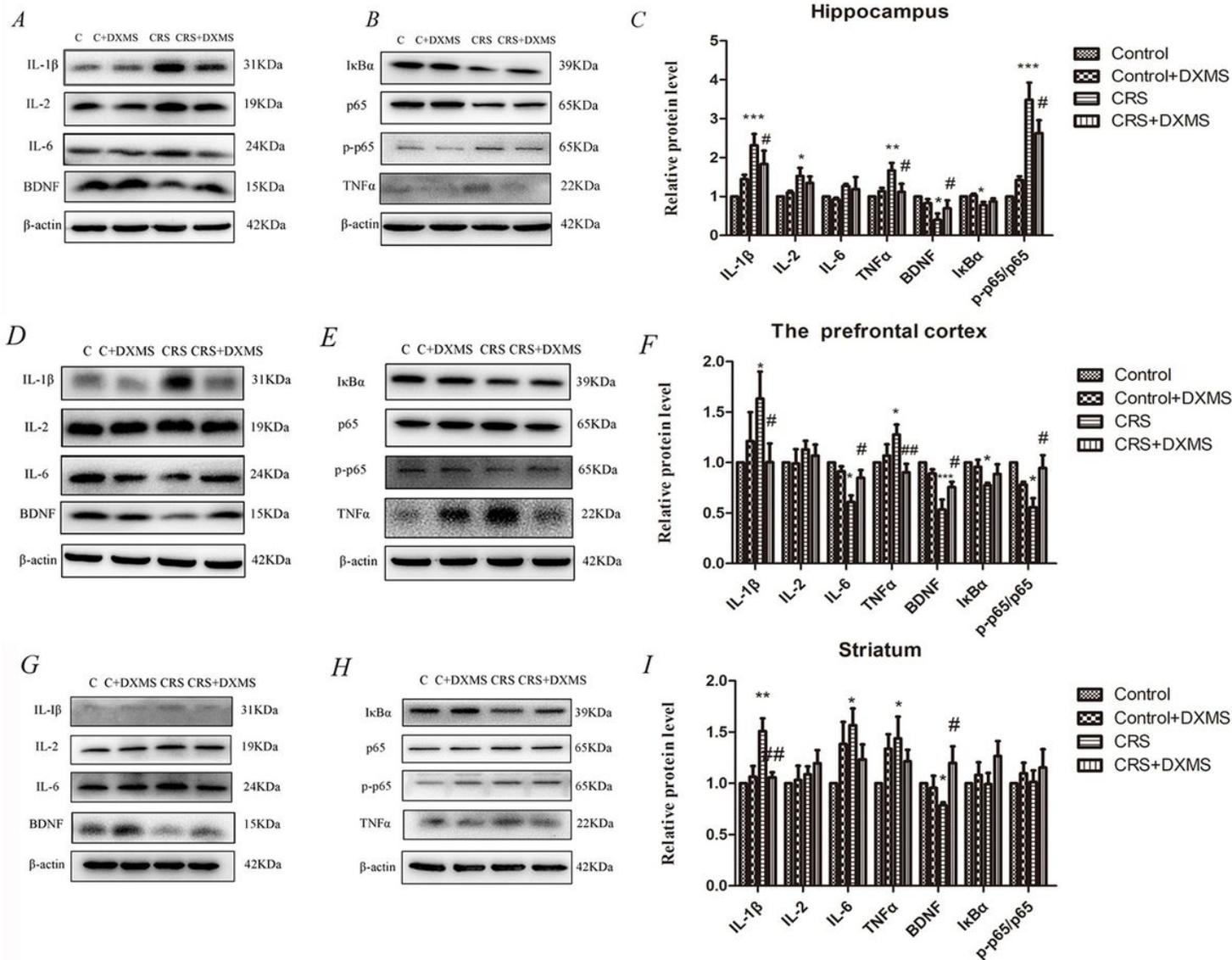


Figure 2

Inflammation mediators and BDNF expression in the hippocampus (A, B, C), PFC (D, E, F) and striatum (G, H, I). Inflammation mediators and BDNF expression were detected by western blot. Data are presented as the mean \pm SEM, n = 4-6. *p < 0.05, **p < 0.01 vs. control, #p < 0.05, ###p < 0.001 vs. CRS.

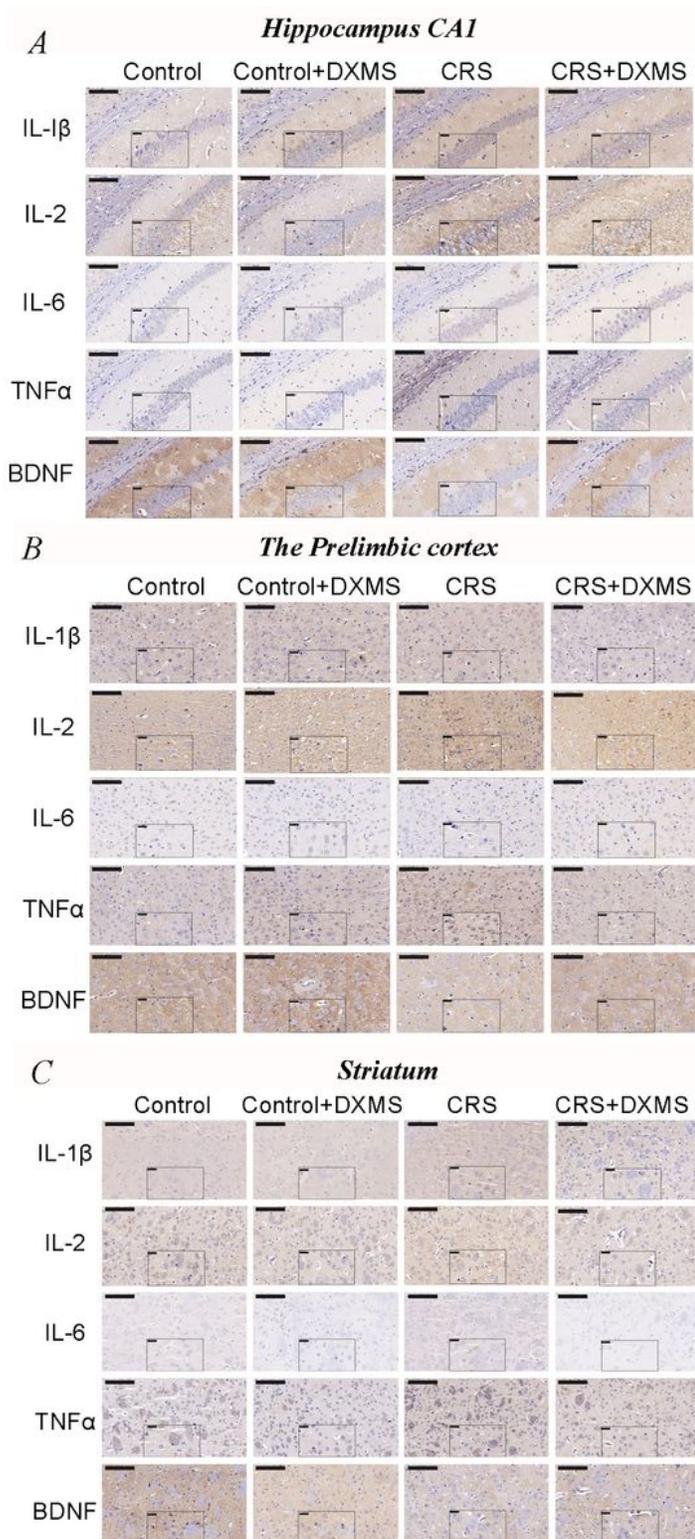


Figure 3

Protein expression of IL-1 β , IL-2, IL-6, TNF α and BDNF in the CA1 region of the hippocampus (A), the prelimbic cortex (B) and the striatum (C) detected by immunohistochemistry. The images are representative of 3-6 mice per group. Scale bars: 200 μ m.

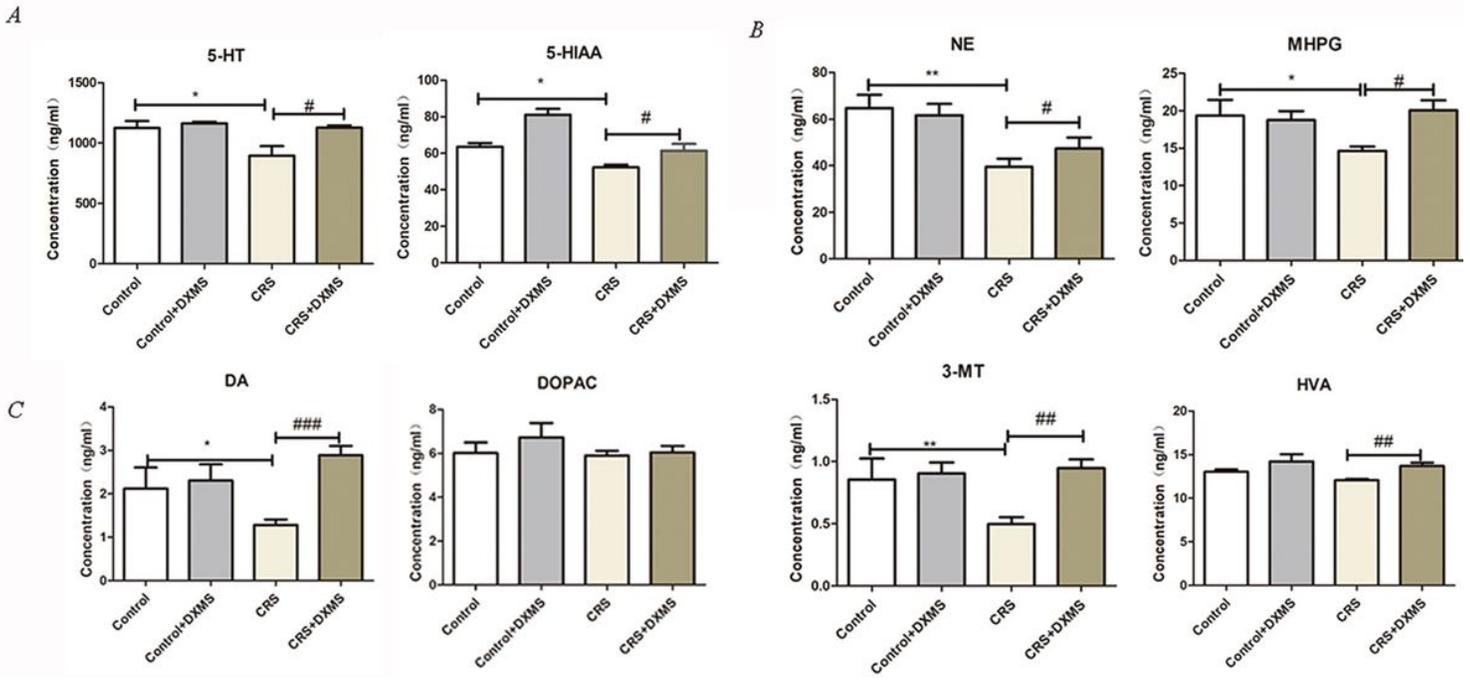


Figure 4

The concentration of monoamine neurotransmitters and their metabolites in serum in the four groups. (A) Concentration of 5-HT and a metabolite of 5-HT (5-HIAA). (B) Concentration of NE and a metabolite of NE (MHPG). (C) Concentration of DA and metabolites of DA (DOPAC, 3-MT, HVA). Data are presented as the mean \pm SEM, $n = 5-8$. * $p < 0.05$, ** $p < 0.01$ vs. control, # $p < 0.05$, ### $p < 0.001$ vs. CRS.

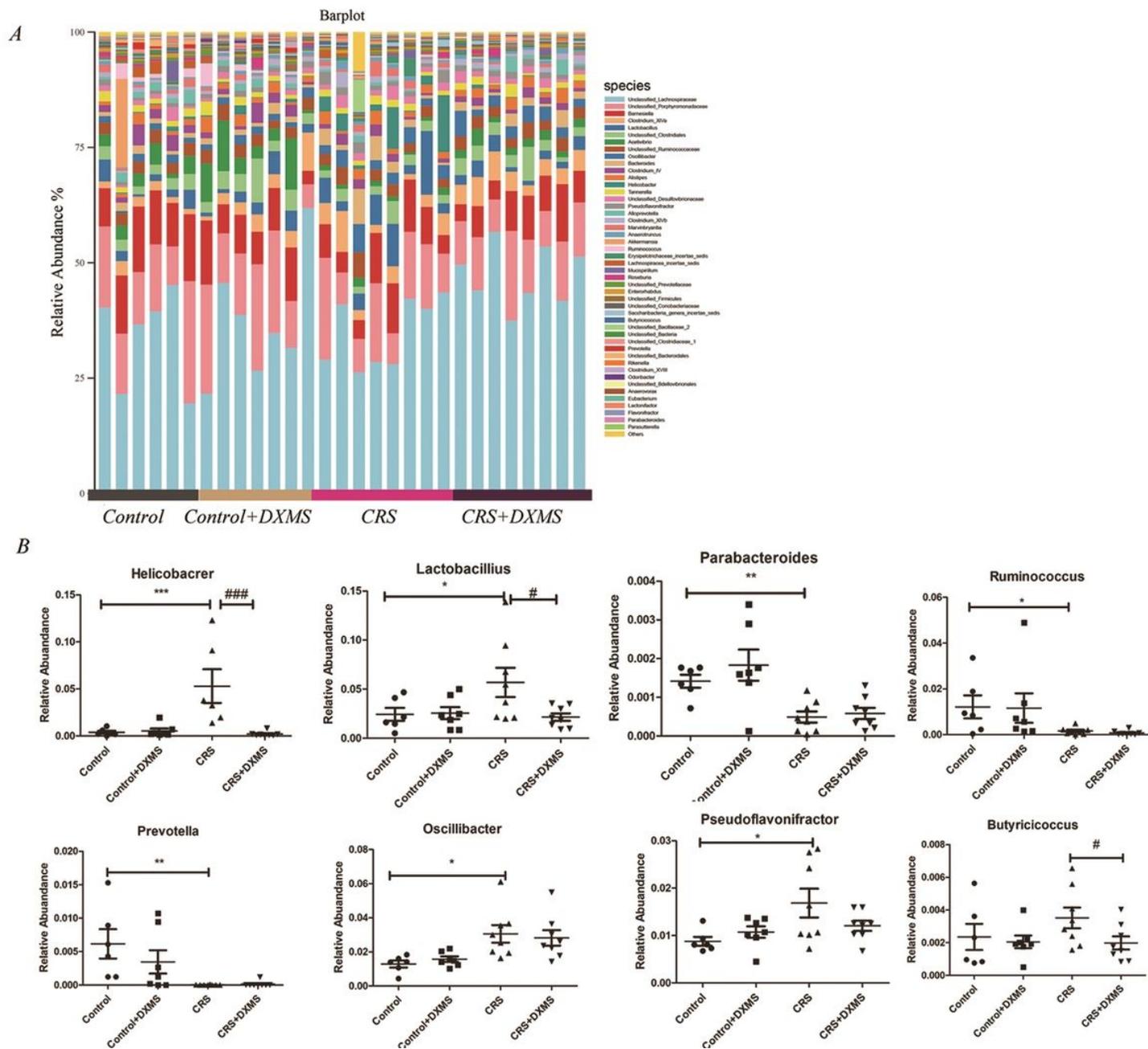


Figure 5

Changes in the microbial relative abundance in faecal samples from the four groups at the genus level. (A) Stacked bar chart of microbial relative abundance. (B) One-way ANOVA at the genus level of *Helicobacter*, *Lactobacillus*, *Parabacteroides*, *Ruminococcus*, *Prevotella*, *Oscillibacter*, *Pseudoflavonifractor* and *Butyricoccus*. Data are presented as the mean \pm SEM, n = 6-8. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control, #p < 0.05, ###p < 0.001 vs. CRS.



Figure 6

Pearson heatmap focusing on correlations between the microbiota and the behaviours and neurotransmitters found to be significantly different in the same mouse. Top x-axis columns: SPT (sucrose preference index in the SPT), FST (immobility time in the FST), OFT (time in the centre of the OFT), EPM (time in the closed arms of the EPM), 5-HT and a metabolite of 5-HT (5-HIAA), NE and a metabolite of NE (MHPG), DA and metabolites of DA (3-MT, HVA), respectively. Right y-axis: gut microbiota names at genus. The scale (right legend) indicates the level of positive (red) or negative (blue) correlation, and asterisks indicate significance (*p < 0.05, **p < 0.01).

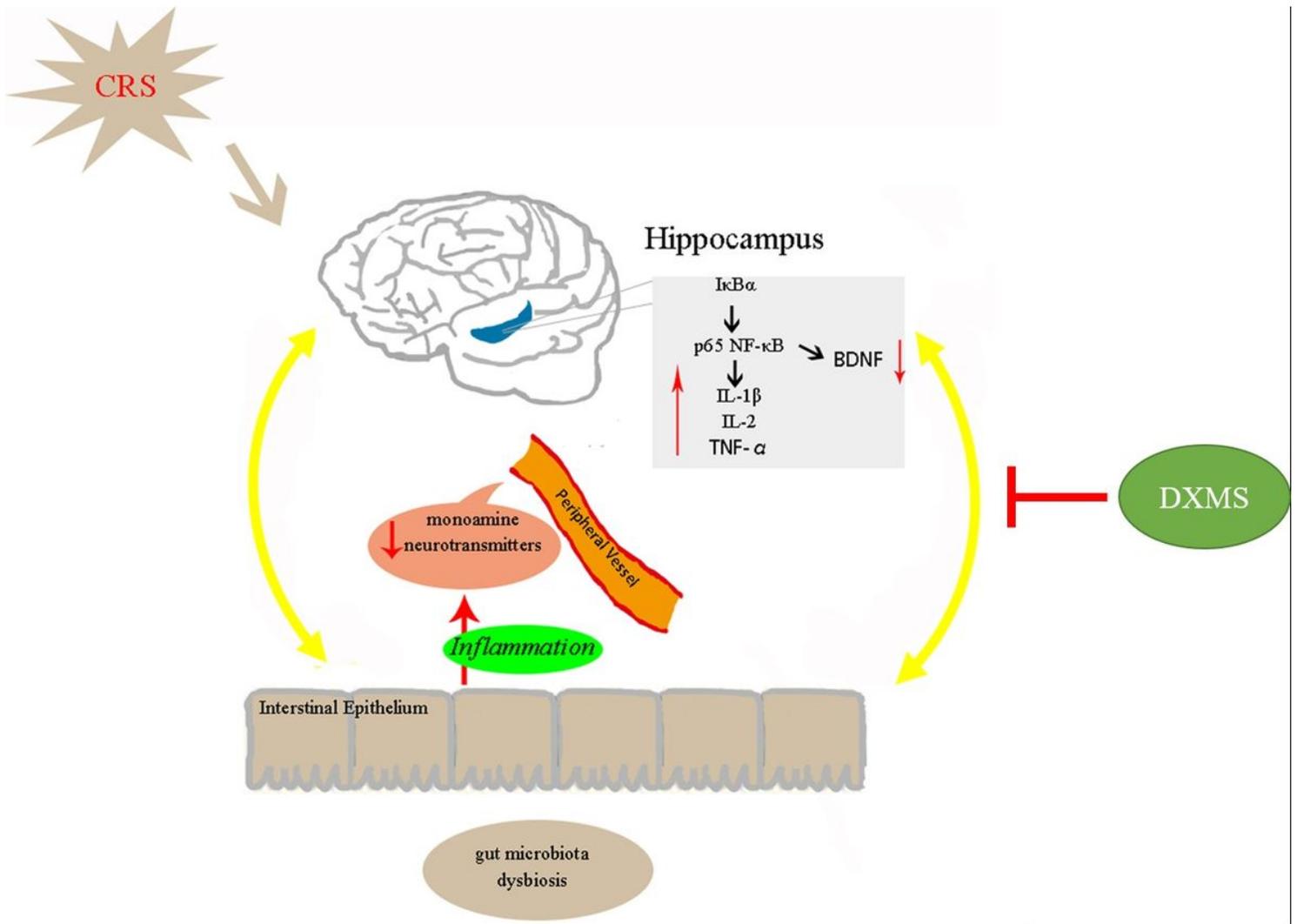


Figure 7

A schematic depicting the role of inflammation in the modulation of gut microbiota and neurotransmitter metabolism in CRS depressive-like mice. CRS contributes to neuroinflammation, neurotransmitter metabolism disturbance and gut microbiota dysbiosis, which are substantially rescued by anti-inflammatory treatment. Additionally, gut microbiota dysbiosis is closely associated with abnormal neurotransmitter metabolism and depressive-like behaviour, providing potential therapeutic strategies for depression.

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