Alterations in splenic function and gene expression in mice with depressive-like behavior induced by exposure to corticosterone

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Abstract. Depressed patients present with increased cortisol levels and attenuated immune responses. However, little is known about the association between depression and the spleen, as this is the largest peripheral immune organ. In this study, we examined alterations in splenic function and gene expression in mice with depressive-like behavior, well as the expression of certain proteins in related pathways. A mouse model of depression was established with the use of corticosterone. Splenic function and histopathology were assessed using Wright and H&E staining. The Agilent Whole Mouse Genome Oligo Microarray containing >41,174 transcript probes was used to measure the levels of gene-expression in the spleens from control and model mice, and the levels of certain proteins associated with depression were measured by western blot analysis in the brain and spleen separately. We found that splenic function and immunity in the mice with depressive-like behavior were markedly impaired. A total of 53 genes exhibited a differential response in the mice with depressive-like behavior, 11 of which were more notable, including collagen, type VI, α5 (*Col6a5*), immunoglobulin superfamily, member 11 (*Igsf11*), D site albumin promoter binding protein (*Dbp*), tachykinin 2 (*Tac2*) and γ-aminobutyric acid B receptor 2 (*Gabbr2*). Pathway analysis revealed that the amino acid biosynthesis and the clock gene pathways were more meaningful among these genes. The levels of GABBR2, DBP and substance P (SP; encoded by the *Tac2* gene) related proteins in the brain were markedly down-regulated, and similar results were observed in the spleen. The anti-depressant, fluoxetine, reversed the changes in the levels of these proteins. The findings of our study regarding changes occurring in the spleen during depression may indirectly elucidate and shed light into the pathogenesis of depression and depressive-like behavior.

Introduction

As the economy rapidly grows in the developing world, the incidence of depression is poised to increase. WHO statistics have indicated that depression will be the leading cause of disease burden worldwide by the year 2030 (1). Even with the armamentarium of pharmacotherapy and psychotherapy, caring for patients with depression remains a challenge due to the lack of universal responses to anti-depressive therapies and frequent recurrences of depressive episodes (2,3).

The currently used anti-depressants mostly target brain neurotransmitters, including 5-hydroxytryptamine (5-HT), norepinephrine [NE also known as noradrenaline (NA)] and dopamine (DA). Following the consumption of antidepressants, it usually takes several weeks for patients to exhibit any improvement (4). In addition, in a great number of patients, anti-depressants are unable to achieve favorable effects. Thus, fully elucidating the mechanisms responsible for the pathogenesis of depression is of great significance and may lead to the development of novel therapeutic methods.

Depression is thought to develop as a result of genetic and environmental changes, and biological factors (5). Although the specific molecular and cellular mechanisms are unclear as regards these factors, an association between chronic stress and glucocorticoid levels has attracted attention (6). Clinical studies have indicated that patients with psychiatric disorders, such as major depression, develop symptoms of chronic stress and mount attenuated immune responses (7). Kiank *et al* (8) found that glucocorticoid levels increase significantly and induce a negative immune response in mice and humans.

It has been reported that chronic stress induces structural alterations in splenic lymphoid tissue, which are relevant to the changes in corticosterone levels in rats (9). However, there is

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no direct evidence of alterations in splenic function and gene expression associated with depression. Thus, in this study, we aimed to examine the histopathological characteristics of spleen tissue, functional changes and differentially expressed genes in mice with depressive-like behavior. We also examined the levels of certain related proteins in the main pathways assessed in the brain and the spleen separately.

Materials and methods

Animals. Adult Balb/c mice (6-7 weeks old, weighing 18-22 g) were purchased from the Experimental Animal Centre of Hualan Bioengineering Co., Xinxiang, China (quality certificate no. 0005496). The mice were bred under aseptic conditions, and allowed to acclimatize for 1 week in an environment with a controlled temperature (25-27˚C), constant humidity (50-60%) and 12 h light/dark cycle, and were given free access to food and water. The animal protocols for this study were approved by the Xinxiang Medical University Institutional Animal Care and Use Committee according to the Guidelines on the Humane Treatment of Laboratory Animals published by the Ministry of Science and Technology of the People's Republic of China in 2006.

Animal grouping and establishment of mouse model of depression. Female mice were used for all the experiments according to the literature (10,11). A total of 72 mice were randomly divided into 2 groups: 22 mice served as the normal control group, and another 50 mice were used to establish the model of depression by an intraperitoneal injection of corticosterone [Sigma-Aldrich, Munich, Germany; purity >98.5% determined by high performance liquid chromatography (HPLC) assay (data not shown)] suspension at 20 mg/kg as previously described by Ago *et al* (12). The mice in the normal control group were administered normal saline at the same volume. All mice were injected once a day for 21 days. The success rate of the depression model was approximately 74%. After excluding all the demised animals (1 mouse died in the contorl group due to unknown reasons) and those that failed to exhibit depression-like behaviors, 21 mice remained in the normal control group, and 37 mice in the depression model group. A total of 9 animals in each group were used for the microarray analysis of spleen and the measurement of neurotransmitters in the brain, and 4 animals in each group were used for the histopathological examination. The remaining animals were divided into 3 groups as follows: the normal control group (n=8), the depression model group (n=12) and the fluoxetine group (n=12), of which 8 mice in each group were used for western blot analysis. The mice in the fluoxetine group were administered fluoxetine by gavage for 15 days, and the mice of normal control group and depression model group were given 0.9% saline solution at the same time.

Open field test. The mice were individually housed in a cage measuring 55x55x40 cm^3 without a cover. The bottom of the cage was divided into 11x11 cm^2 squares and marked with white lines. The entire interior walls and the bottom floor were painted with black color. A canon XF300 video camera (Canon China Co., Beijing, China) was installed on the ceiling at the center of the room. The movements of the mice were recorded using the video camera. The locomotive activities based on the distance of movement and the numbers of standing-up were measured on the 21st day after the beginning of the experiment. Specifically, the mouse was placed at the bottom center of the cage and allowed to run freely for the first 5 min in order to allow the animal to adapt to the new environment. Subsequently, the activities of the mouse in the next 5 min were recorded using the video camera. These measurements were performed at around 10 a.m. after the final administration of normal saline (normal control group) or corticosterone (depression model group). The activities of the mouse were analyzed using points allocated to all the movements. Walking across one small square or 10 cm in distance was allocated 1 point, as previously described (13,14). The lifting of both front limbs or touching the walls of the cage was also allocated 1 point. Mobile scores were calculated based on all the points recorded.

Sucrose preference test (SPT). The sucrose preference test was performed as previously described by Bhatt *et al* with slight modifications (15). The mice were trained before the experiment. Each mouse was housed in a single cage with two bottles of sterile sucrose water, each bottle containing 100 ml solution. Within the next 24 h, one bottle of sterile sucrose water was replaced with a bottle of sterile distilled water, and the position of the left bottle and right bottle was switched. Sucrose and water consumption within the last 1 h of a 24-h period was measured, which was regarded as the baseline values. The sucrose preference rate was calculated using the following formula: sucrose consumption/sucrose consumption + water consumption. On the 21st day of the injection, sucrose and water consumption were measured within 1 h, and the sucrose preference rate was then calculated.

Histopathological examination. The brains and spleen tissue of the mice from both groups were examined using H&E staining as previously described (16). Four animals from each group were sacrificed by decapitation, and the brains and spleens were removed. All specimens were fixed for 24 h by 4% paraformaldehyde phosphate buffer solution (pH 7.4). After the specimens were regularly dehydrated and embedded in paraffin, a 5 mm portion of the forebrain and the spleen tissue was cut into coronal sections. The paraffin-embedded sections (5-μm-thick) were placed on glass slides and stained with hematoxylin for 5-10 min and eosin for 2-3 min after routine dewaxing. The image-analysis software of HMIAS-2000 system (Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) was used for the histological examination of the specimens.

Measurement of neurotransmitters. The NA, adrenaline (Ad), DA, tryptophan (Try) and 5-HT levels in the mouse brains were determined using the HPLC fluorescence method (Varian Prostar 210; Varian, Palo Alto, CA, USA) as previously described (17). Nine mice from each group (control group and model group) were sacrificed by decapitation, and the brains were removed. The prefrontal cortex was weighed and dissected over ice. The brain area sample was placed in a homogenizer, and 0.1 M perchlorate solution that contains 0.004 M sodium metabisulfite was added. The brain tissue to the perchlorate solution ratio was 1:3. The homogenate of each sample were centrifuged for 30 min (8,183 x g) at -4˚C, the supernatants

were filtered by 0.45 μm hydrophilic membranes and transferred to a centrifuge tube and stored at 4˚C. The reagents of NA, Ad, DA, Try and 5-HT (Sigma-Aldrich Co.) were used for the standard solution. All the other chemicals used in this study were of HPLC grade. Measurements were made by injecting 20 μl of each sample into the Varian Prostar 210 series HPLC system with a fluorescence detector (excitation wavelength, 280 nm; emission wavelength, 360 nm), which employed a Varian C18 column (4.x250 mm, 5 μm; velocity, 1 ml/min; column temperature, 20˚C). The range of the concentration of NA, Ad, Try or 5-HT in the standard liquid was 0.0625-1.0 μg/ml and that of DA was 0.125-2.0 μg/ml. The mobile phase was composed of 30% methanol (Concord Technology Co., Tianjin, China), 4% ultrapure water and 66% phosphate buffer, which consisted of 0.02 M KaH_2PO_4 and 0.005 M heptyl sulfonic acid sodium (both from Daily Chemical Co., Shanghai, China) (pH .3). The flow rate was maintained at 1.0 ml/min.

Evaluation of splenic function. Retro-orbital blood collection was conducted before sacrificing the animals by decapitation. A drop of blood was placed on the slide and spread into a thin layer. Cellular morphology was observed by Wright staining, as previously described (18). The red blood cells, Howell-Jolly bodies (HJB) and various types of white blood cells (WBC) were observed using a light microscope (Olympus 009083; Olympus, Tokyo, Japan) and the numbers of the cells were counted with x100 oil lens. Five fields were randomly selected from the upper left, lower left, upper right, lower right and the middle areas of the blood smear and photographed using a HMIAS-2000 high-resolution color medical image analysis system (Qingping Imaging Co., Tongji Medical College, Shanghai, China). HJB and hemosiderosis were observed and analyzed, as previously described (19).

Splenic gene determination and analysis. The spleens of 9 mice from each group were removed immediately after sacrifice. After the spleen tissue was processed by nitrogen and TRIzol reagent (Life Technologies, Carlsbad, CA, USA), the samples were stored in a -80˚C freezer until analysis. Following the manufacturer's instructions, total RNA from the spleen of each mouse was extracted using TRIzol reagent. The equal amounts of RNA from 3 mice in the same group were mixed to yield a testing sample. Totally, 3 mixed testing samples from 9 mice were used for the examination of gene chip. Total RNA was amplified and labeled using the Low Input Quick Amp Labeling kit, One-Color (Agilent Technologies, Santa Clara, CA, USA). The labeled cRNA were purified using the RNeasy mini kit (Qiagen GmBH, Hilden, Germany).

The samples were hybridized to the Agilent Whole Mouse Genome Oligo Microarray (4x44K, including 41,174 genes and transcript) by Gene Expression Hybridization kit (both from Agilent Technologies), stained, washed and scanned according to the standard Agilent oligonucleotide microarray hybridization protocol. The computer data files to be used in data analysis were generated with the GeneSpring software 11.0 (Agilent Technologies) using the statistical algorithm provided. All chip samples were scanned and data were read using the same instrument and following the same protocol. The above process was performed by Shanghai Biotechnology Corp. (Shanghai, China). Data quality assessment was then performed following the guidelines outlined in the Agilent Data Analysis Fundamentals Manual. All quality control results met the criteria recommended by Agilent Technologies.

Western blot analysis. The spleens and prefrontal cortices of 8 mice from each group were removed after sacrifice by cervical dislocation, and all the tissues were kept at -40˚C. The protein concentration of each sample was determined using a bicinchoninic acid assay kit using BSA as standard (Pierce, Rockford, IL, USA). Equal amounts of proteins from each sample (40 μg) were separated by dodecyl sulfate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in 0.01 M phosphate-buffered saline (PBS) at room temperature for 1 h and then incubated overnight at 4˚C with the primary antibodies (Abcam, Cambridge, MA, USA), including anti-D site albumin promoter binding protein (DBP; 1:1,000), anti-γ-aminobutyric acid B receptor, 2 (GABBR2; 1:1,000) and anti-substance P (SP; 1:200) antibodies. The secondary antibody (IRDye 800-conjugated goat anti-rabbit secondary antibodies) was added at 1:2,000 for 1 h at room temperature. Mouse anti-β-actin antibody (1:2,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as internal reference to confirm that the same amount of each type of proteins was loaded. The bands were photographed by a fully automatic chemo-luminescence image analysis system (Tanon-450; Tanon Science and Technology Co., Ltd.. Shanghai, China). The gray-scale of each band was detected using ImageJ image analysis software. The quantities of each western blot product were normalized by dividing the average gray level of the signal by that of the corresponding β-actin, and observed as the semi-quantitative value of the target fragments.

Statistical analysis. The data analysis of the behavioral tests (3 variables), monoamine depletion (5 variables) and blood tests (6 variables) in the mice were performed using an independent-samples t-test. One-way analysis of variance (ANOVA) with post hoc tests, followed by the least significant difference (LSD) test was performed to compare the protein levels of DBP, GABBR2 and SP. The results of quantitative analysis were summarized as the means ± SEM and analyzed using SPSS 17.0 statistical software. A value of $P<0.05$ was set to define statistical significance. All P-values are two-tailed. Gene chip data were analyzed using the SAS system (SBC analysis system).

Results

Changes in animal behavior. The open field test was used to explore the locomotor activity of the mice, as previously described (20). As a test for the degree of anhedonia, sucrose preference rate was determined based on the sucrose consumption. The results revealed that there was no distinctive behavioral difference between the 2 groups before the experiment ($P>0.05$; Fig. 1A). The mobile scores, the number of rearings (standing on hind limbs) and the sucrose intake of the mice in the depression model group were significantly decreased compared with the normal control group (mobile scores, $t=2.342$, $P=0.030$; number of rearing, $t=4.814$, $P=0.000$;

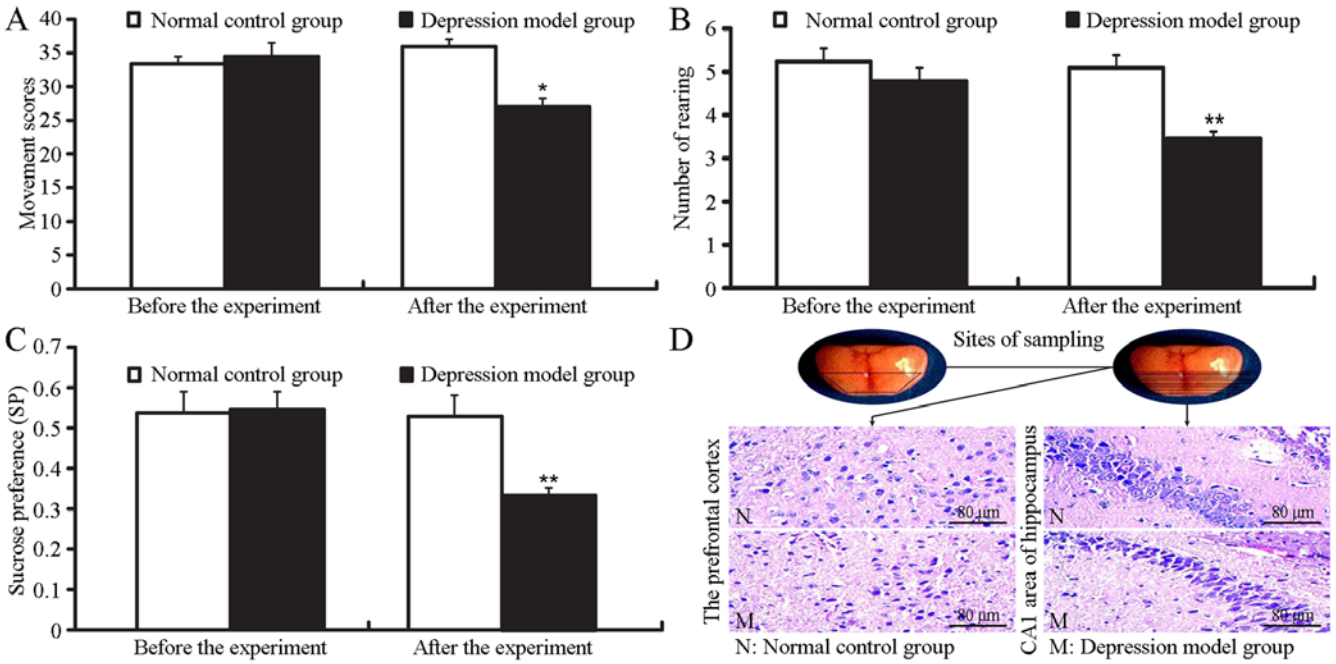

Figure 1. Changes in behavior and histopathological changes in the prefrontal cortex and hippocampus in mice in the depression model group (means ± SEM). (A) Mobile scores; (B) number of rearing; (C) sucrose consumption. There was no distinctive difference between the two groups in the behaviors before the experiment (P>0.05). The mobile scores, number of rearing and sucrose intake of mice in the depression model group were significantly decreased compared with normal control group (P<0.05 or P<0.01); normal control group (n=21); depression model group (n=37); *P<0.05 or **P<0.01 compared with normal control group. (D) H&E staining (x200 magnification, n=4). In the normal control group, the cellular structures are compact with regular arrangement and a clear hierarchy in the prefrontal cortex and hippocampal CA1. In the depression model group, the morphology of the brain cells was changed from an ellipse or round shape to a spindle, strip or irregular shape. The cellular borders appeared fuzzy, and brain structures were disordered. Although the cellular arrangement of hippocampus CA1 region remained largely normal, the morphology of nerve cells changed significantly, and some nerve cells are absent in the depression model group.

sucrose intake, t=3.829, P=0.004) (Fig. 1A-C). The indicators of depression were strongly associated with the length of corticosterone injections. The model of depression was successfully established on the 21st day.

Histopathological changes. The histopathology of the cerebral cortex and hippocampus was evaluated with H&E staining (Fig. 1D). In the normal control group, the morphology of the neurons and glial cells in the prefrontal cortex and hippocampal CA1 region was normal. The cellular structures were compact with regular arrangement and a clear hierarchy. The nucleoli were distinctly visible without any anomalies. In the mice in the depression model group, the brain cells exhibited an altered morphology and the cells presented a spindle-like, or irregular shape. The cellular borders appeared fuzzy, and the brain structures were disordered. A large number of neurons had lost structural integrity with prominent karyopyknosis and the disintegration of nucleoli in the prefrontal cortex. Although the cellular arrangement of the hippocampus CA1 region remained largely normal, the morphology of the nerve cells had altered significantly, and some nerve cells were absent in the mice in the depression model group.

Measurement of monoamine. Brain neurotransmitters assessed by HPLC fluorescence method were used to further confirm the reliability of the depression model from the biochemical point of view. The HPLC chromatogram of a cocktail of DA, NA, Ad, Try and 5-HT standard is presented in Fig. 2A. The peak of each neurotransmitter in the mixed standard solution was well separated and no other significant peaks were present. A series of standard solutions was prepared with a concentration gradient and injected into HPLC. The value of peak areas and concentrations with reference to the each neurotransmitter standard was used to carry out linear regression analysis and create standard curves. As a result, the regression equation and correlation co-efficient of each neurotransmitter is shown in Table I. The results indicated that the peak area had a significant linear correlation with the content level of NE, Ad, DA, Try or 5-HT. The contents of each neurotransmitter in the brains of the mice were then respectively calculated using the regression equations. The chromatogram of a sample from one of the experimental groups is shown in Fig. 2B and C. The peak shape of each neurotransmitter was well delineated, and there was no overlap, double peak and bad tail phenomenon. There were two unknown, well-separated peaks between the peak of Ad and DA, which were worthy of note. All the neurotransmitters in the prefrontal cortex of the mice in the depression model group yielded a lower value and peak size than those of the control group (levels of neurotransmitters: NA, t=2.606, P=0.040; Ad, t=2.930, P=0.026; DA, t=4.931, P=0.003; Try, t=6.530, P=0.001; 5-HT, t=2.819, P=0.030; and peak size: NA, t=2.672, P=0.037; Ad, t=3.544, P=0.012; DA, t=7.442, P=0.000; Try, t=3.608, P=0.011; 5-HT, t=2.612, P=0.040) (Fig. 2D and E).

Changes in splenic function and pathology. The changes in splenic function in the mice in the depression model group was one of our main areas of focus. The numbers of HJB and WBC in the blood correlate with splenic function (21). HJB, WBC, neutrophils and lymphocytes were counted using a light microscope following Wright staining. In the control group,

Table I. The linear regression equation, linear range and the correlation co-efficient of 5 neurotransmitters were measured using the HPLC fluorescence method.

Neurotransmitters	Linear equation	Linear range (μg/ml)	Correlation co-efficient (r)
NA	$y=5.29\cdot10^5x+6.19\cdot10^3$	0.0625-1.0	0.999815
Ad	$y=7.34\cdot10^5x+8.66\cdot10^3$	0.0625-1.0	0.998305
DA	$y=9.32\cdot10^5x+1.61\cdot10^4$	0.125-2.0	0.999789
Try	$y=8.12\cdot10^6x-3.00\cdot10^5$	0.0625-1.0	0.996419
5-HT	$y=1.35\cdot10^7x-1.15\cdot10^6$	0.0625-1.0	0.995741

HPLC, high performance liquid chromatography; NA, noradrenaline; Ad, adrenaline; DA, dopamine; Try, tryptophan; 5-HT, 5-hydroxytryptamine.

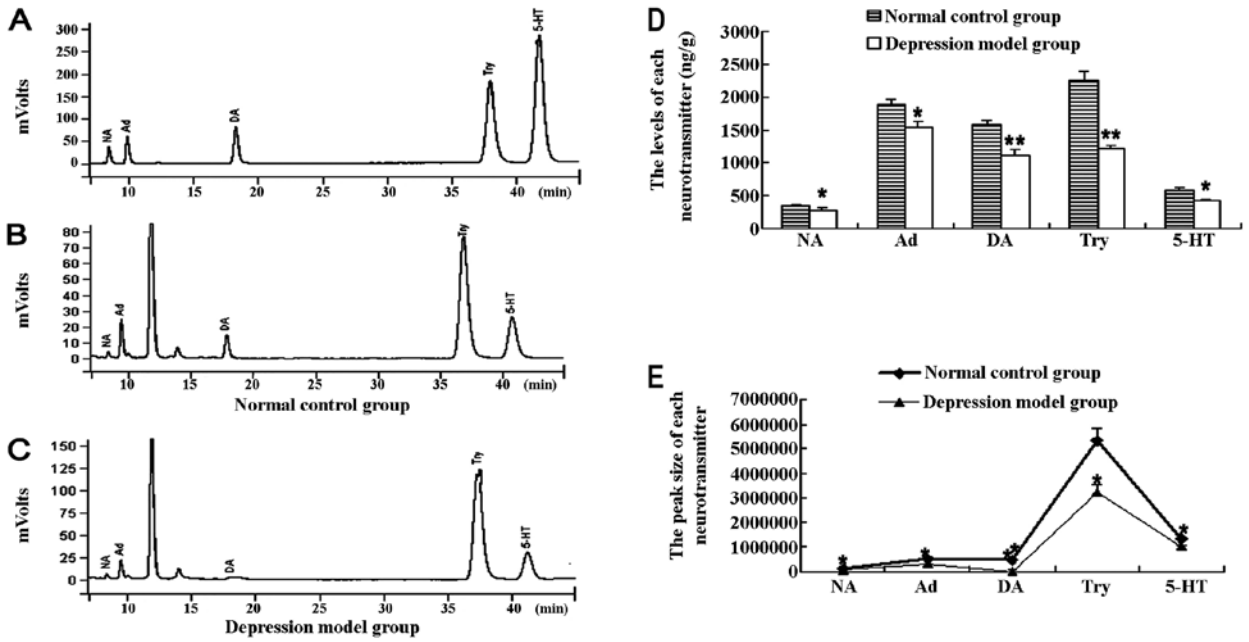

Figure 2. The content and the peak area of neurotransmitters in the prefrontal cortex (n=6). (A) High performance liquid chromatography (HPLC) chromatogram of the standard substance in noradrenaline (NA) (1 μg/ml), adrenaline (Ad) (1 μg/ml), dopamine (DA) (2 μg/ml), tryptophan (Try) (1 μg/ml) and 5-hydroxytryptamine (5-HT) (1 μg/ml). The retention time of NA, Ad, DA, Try and 5-HT is ~7, 9, 18, 37 and 41 min, respectively. (B and C) HPLC chromatograms of neurotransmitters in the brain samples. The retention time of NA, Ad, DA, Try and 5-HT in the sample is the same as each standard substance. (D and E) Contents (ng/g) and peak area of each neurotransmitter in the sample. Summary data are expressed as the means ± SEM. As indicated, the content and the peak size of each neurotransmitter in the prefrontal cortex of depressed mice were lower than those of control group ($P<0.05$ or $P<0.01$); $^*P<0.05$ or $^{**}P<0.01$ compared with normal control group.

cellular morphology was normal without obvious HJB. In the mice in the depression model group, cellular size and staining were mildly abnormal and HJB were clear and obvious (where the virtual arrow is pointing) (Fig. 3A). The histopathology was observed using H&E staining of the splenic sections in both groups (Fig. 3B). In the control group, the white pulp was stained dark-blue, and was round or ellipse-shaped. Cellular structures were dense with numerous lymphocytes and lymphoid nodules. In the mice in the depression model group, the cellular structures of white pulp became sparse, lymphocytes and lymphoid nodules were diminished, and hemosiderin was clearly evident in the splenic tissue. As shown in Fig. 3C and D, the counts of HJB and the lymphocyte ratio in the depression model group increased greatly compared with the normal control group (counts of HJB: t=-3.273, P=0.017; lymphocyte ratio: t=2.755, P=0.033). However, the number of neutrophils, WBC and lymphocytes and the ratio of neutrophils was significantly decreased compared with the normal control group (number of neutrophils: t=3.545, P=0.012; WBC, t=2.575, P=0.042; lymphocytes: t=2.696, P=0.036; ratio of neutrophils: t=3.658, P=0.011).

Differential expression of genes. The differential expression of genes in the spleen was another area of focus in this study. We selected differentially expressed genes whose value ($P<0.05$) and the gene expression levels were increased >2-fold or decreased >2-fold when comparing the depression model group with the normal control group. The results indicated that there were 53 differentially expressed mRNAs in the spleen when comparing the depression model group with the normal control group, including miRNA containing gene (*Mirg*), collagen, type VI, α5 (*Col6a5*), immunoglobulin super-

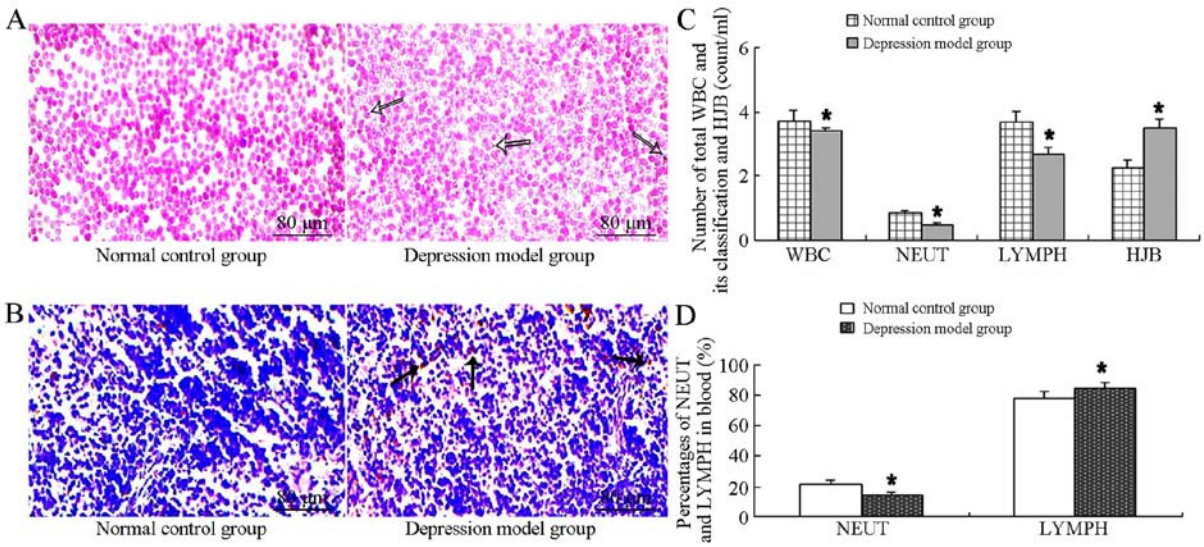

Figure 3. Wright staining of blood smears and H&E staining of spleen (x100 magnification) as well as the indicative changes of blood in mice (means ± SEM). The arrow in (A) points to Howell-Jolly bodies (HJB), and the arrow in (B) points to hemosiderosis. (A) Wright staining showing obvious HJB in the blood cells of mice in depression model group (where the virtual arrow is pointing). (B) H&E staining showing the decreased white pulp (dark-blue, round or ellipse-shaped), the elevated red pulp and hemosiderin in splenic tissue (black arrow) of depression model group. (C) The number of total white blood cells (WBC) and its classification and HJB. (D) The percentage of neutrophils (NEUT) and lymphocytes (LYMPH). Summary data in (C and D) show that the counts of HJB and lymphocyte ratio in depression group were significantly increased compared with the normal control group (P<0.05). However, the numbers of neutrophils and lymphocytes and the ratio of neutrophil were significantly reduced compared with the normal control group (P<0.05). H&E staining, n=4. Wright staining: in the normal control group, n=21; in depressed model group, n=37. *P<0.05 compared with normal control group.

family, member 11 (*Igsf11*), LIM domain binding 3 (*Ldb3*), cytochrome P450 family 2 subfamily E member 1 (*Cyp2e1*), major urinary protein 4 (*Mup4*), cytochrome P450, family 2, subfamily c, polypeptide 37 (*Cyp2c37*), indolethylamine N-methyltransferase (*Inmt*), Dbp, carboxylesterase 1D (*Ces1d*), RNA binding motif protein, X-linked-like 2 (*Rbmxl2*), carbonic anhydrase 3 (*Car3*), cytochrome P450 family 2 subfamily F member 1 (*Cyp2f2*), aryl hydrocarbon receptor nuclear translocator, class E basic helix-loop-helix protein 2 (*Arnt2*), cilia and flagella associated protein 57 (*Cfap57*, also known as *Wdr65*), Smith-Magenis syndrome chromosome region, candidate 8 (*Smcr8*), aquaporin 11 (*Aqp11*), ERCC excision repair 6 like, spindle assembly checkpoint helicase (*Ercc6l*), *1190001L17Rik*, solute carrier family 51, alpha subunit (*Slc51a*), denticleless E3 ubiquitin protein ligase homolog (Dtl), solute carrier family 25 member 21 (*Slc25a21*), breast and ovarian cancer susceptibility protein 1 (*Brca1*), *C530008M17Rik*, N-acetylglutamate synthase (*Nags*), *1700063H04Rik*, erythroferrone (*Erfe*, also known as *Fam132b*), SSX family member 2 interacting protein (*Ssx2ip*), protein disulfide isomerase family A member 2 (*Pdia2*), pyruvate kinase, liver and RBC (*Pklr*), von Willebrand factor C and EGF domain-containing protein (*Vwce*), hyperpolarization activated cyclic nucleotide gated potassium channel 3 (*Hcn3*), Gabbr2, solute carrier family 5 member 2 (*Slc5a2*), coiled-coil domain containing 74A (*Ccdc74a*), transmembrane protein 56 (*Tmem56*), serine dehydratase like (*Sdsl*), acyl-CoA synthetase long-chain family member 6 (*Acsl6*), Threonyl-TRNA synthetase like 2 (*Tarsl2*), *AK129341*, tachykinin 2 (Tac2), interleukin 1 rReceptor like 1 (*Il1rl1*), Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 4 (*Cited4*), receptor accessory protein 6 (*Reep6*), ectonucleotide pyrophosphatase/phosphodiesterase family member 3 (*Enpp3*), asparagine synthetase (*Asns*), SEC14 like lipid binding 2 (*Sec14l2*) and 5 genes of unknown function (Fig. 4). There were 14 upregulated mRNAs and 39 downregulated mRNAs among these genes.

The sieving captured 53 differentially expressed genes annotated to the system of 'GO-pathway'. A GO enrichment analysis of differentially expressed mRNAs was performed to identify GOs with a higher confidence. Enrichment provides a significant means of measuring function. The corresponding function is more specific with the enrichment increasing, which helps us to identify GOs with more concrete functional description in the experiment (22). After this process, the results revealed that the amino acid synthesis and the clock gene were more significantly affected in two pathways associated with depression among these genes. According to microarray analysis and the literature data (23,24), we also found 11 of 55 differentially expressed genes in the spleen, which can influence immune responses, inflammation, metabolism, collagen formation, cell factor, protein transport and nerve myelin formation, were closely linked to depression (Table II).

Expression levels of GABBR2, DBP and SP related proteins. The DBP, GABBR2 and SP proteins in the spleen and brain were further detected. As shown in Fig. 5A, the protein expression levels of GABBR2, DBP and SP in the brain were significantly decreased in the model group compared to those in the control group (GABBR2, t=6.61, P=0.000; SP, t=6.045, P=0.001; DBP, t=6.956, P=0.000). GABBR2 proteins were not detected in the spleen (Fig. 5B), and the alterations in the DBP and SP protein levels in the spleen were coincident with those in the brain (DBP, t=7.496, P=0.00; SP, t=3.651, P=0.006). Moreover, following treatment with fluoxetine,

Table II. The differently expressed genes in spleen associated with depression (n=9).

No.	GenBank no.	Gene name	Gene symbol	Fold change	Regulation	P-value
1	NM_001167923	Collagen, type VI, α5	*Col6a5*	10.022	Up	0.027
2	NM_170599	Immunoglobulin superfamily, member 11	*Igsf11*	2.140	Up	0.039
3	NM_016974	D site albumin promoter binding protein	*Dbp*	2.280	Up	0.015
4	NM_144520	SEC14-like 2 (*S. cerevisiae*)	*Sec14l2*	2.304	Down	0.034
5	NM_010743	Interleukin 1 receptor-like 1	*Il1rl1*	2.632	Down	0.009
6	NM_013631	Pyruvate kinase liver and red blood cell	*Pklr*	2.577	Down	0.015
7	NM_001033599	Acyl-CoA synthetase long-chain family member 6	*Acsl6*	2.13	Down	0.020
8	D14423	Tachykinin 2	*Tac2*	2.19	Down	0.038
9	NM_001081070	Protein disulfide isomerase associated 2	*Pdia2*	2.985	Down	0.039
10	NM_134005	Ectonucleotide pyrophos-phatase/phosphodiesterase 3	*Enpp3*	2.445	Down	0.043
11	NM_001081141	γ-aminobutyric acid (GABA) B receptor, 2	*Gabbr2*	3.717	Down	0.040

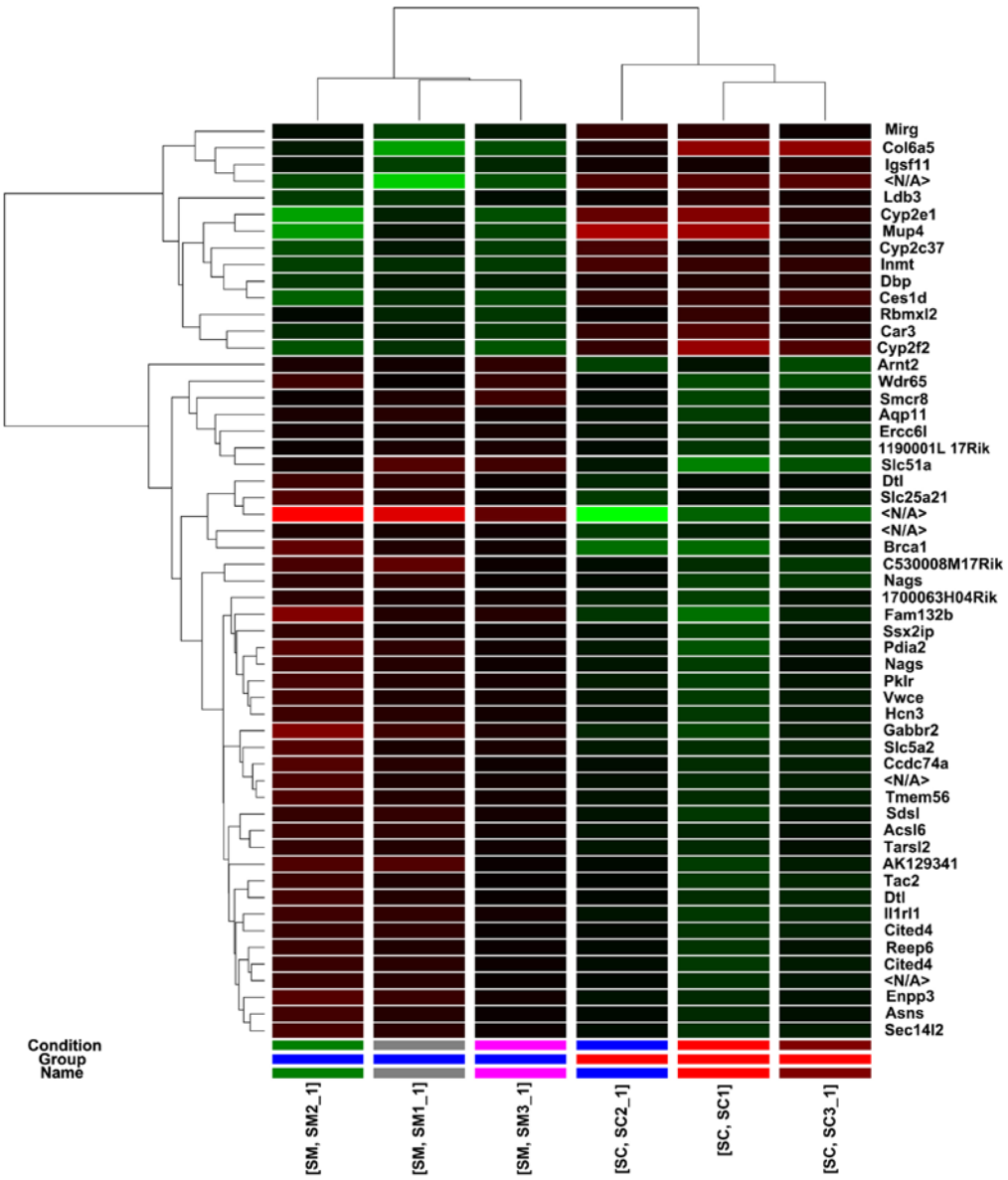

Figure 4. The dendrogram of differentially expressed genes in the spleens of mice (n=9). The 3 columns on the left represent the depression model group and the 3 columns on the right represent the normal control group. Green color indicates the upregulation of gene expression and red color indicates the downregulation of gene expression. The differentially expressed genes are shown on the right side of the image. The results revealed that 53 differentially expressed mRNAs in the spleen were found with comparisons between the depression model group and normal control group, which consisted of 14 upregulated mRNAs and 39 downregulated mRNAs.

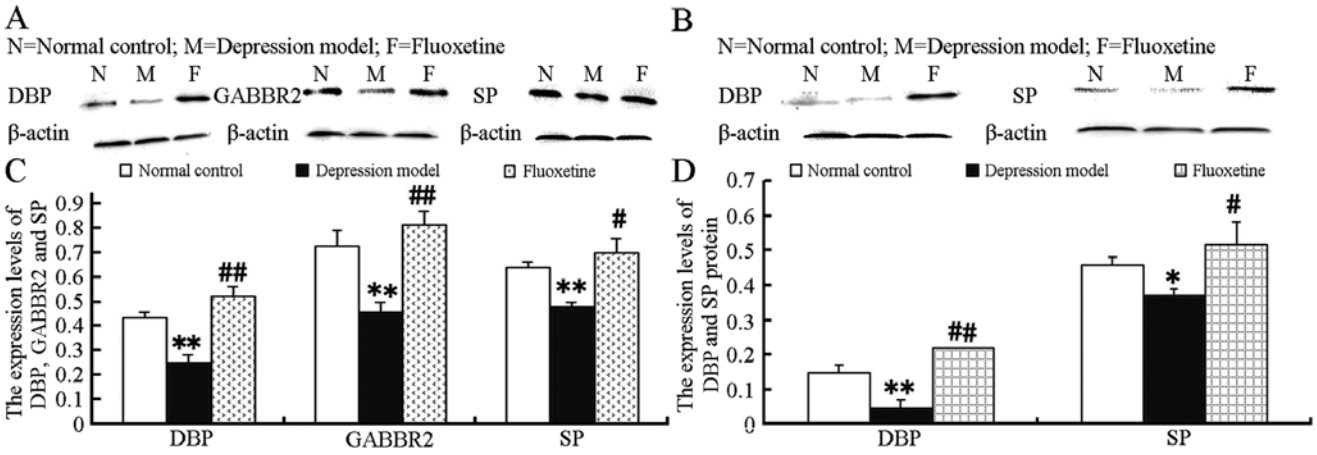

Figure 5. The expression of D site albumin promoter binding protein (DBP), γ-aminobutyric acid B receptor 2 (GABBR2) and SP proteins in the brain and spleen. Shown are representative western blots of proteins (upper panel) and summarized bar graphs (lower panel) (n=8, means ± SEM). (A and C) The expression levels of GABBR2 and SP proteins in the prefrontal cortex of the model group were significantly decreased compared to control group (P<0.01), while DBP expression significantly increased (P<0.01). Fluoxetine treatment significantly reversed the changes in DBP, GABBR2 and SP protein expression in the brains of mice in the depression model group (P<0.05 or P<0.01). (B and D) Similarly, the changes in the expression of DBP and SP proteins occurred in the spleen, which was coincident with the changes in the brain (P<0.01). Fluoxetine also reversed these changes. *P<0.05 or **P<0.01 depression model group compared with normal control group; $^{\#}$P<0.05 or $^{\#\#}$P<0.01 fluoxetine group compared with depression model group.

the protein expression levels of DBP, SP or GABBR2 in the prefrontal cortex and the spleen were markedly enhanced in the mice in the depression model group (in prefrontal cortex: DBP, t=9.02, P=0.000; SP, t=3.587, P=0.007; GABBR2, t=4.843, P=0.001; and in spleen: DBP, t=19.81, P=0.000; SP, t=3.574, P=0.007).

Discussion

There is accumulating evidence to support a close connection between stress and depression (25,26). Glucocorticoids are characteristic mediators in stress responses, and the detrimental effects of chronic stress on health have been proposed to be attributed to their immunosuppressive properties (27). Thus, in this study, we examined the histopathology of the spleen, the splenic function and gene expression in mice with depressive-like behavior, as well as the expression of certain proteins in the related pathways. To the best of our knowledge, we demonstrate for the first time that splenic function in mice with depressive-like behavior was markedly impaired and we also found some differentially expressed genes in the spleen. In addition, the levels of proteins in related pathways were also altered in the mice with depressive-like behavior.

The present study had five main findings: first, depression induces significant damage to the morphology and function of the spleen in mice. Second, 53 genes exhibited a differential response in the spleen of the mice in the depression model group, 11 of which were more notable. Third, the amino acid biosynthesis and the clock gene pathways were more markedly affected in the spleen. Fourth, the related proteins in the pathways were downregulated in the depressed mice. Fifth, fluoxetine reversed the changes in GABBR2, DBP and SP protein levels in the brain and spleen. The results obtained in the spleen confirmed our hypothesis.

Counting the number of erythrocytes with HJB in blood smear under a light microscope is a commonly used method to assess splenic function (28). When the spleen is absent or insufficient, the number of HJB containing erythrocytes is increased (29). In the present study, the mice in the depression model group exhibited higher counts of HJB, which indicated that splenic function was compromised.

The numbers of neutrophils, WBC and lymphocytes and the ratio of neutrophils were significantly decreased, while the ratio of lymphocytes was elevated greatly in the depression model group compared to the normal control group. These are highly consistent with the clinical data that approximately 80% of clinical patients exhibit a low neutrophil ratio and a high lymphocytes ratio (unpublished data). The changes in lymphatic structure and the increase in hemosiderosis suggested that the splenic function and immune response declined (30). At the same time, this study found the neurons and glial cells in the frontal cortex and hippocampus were deformed or damaged, and the depressive behaviors of the mice correlated well with low levels of NE, Ad, DA, Try and 5-HT in the brain. These results are consistent with those of previous studies on brain tissue in depression (31,32). This indicates that the function of the spleen may play an important role in the pathogenesis of depression.

Changes in gene expression are well documented in depression and schizophrenia, and may contribute to the pathologic phenotypes associated with these disorders (33). Although the onset of depression cannot be attributed to a single gene, multiple small genetic effects along with environmental factors have been shown to trigger major depressive episodes (34,35). In this study, when analyzing gene expression profiles in the spleen, we found 53 differentially expressed mRNAs in the mice in the depression model group, and two signaling pathways were more affected. A total of 11 of 53 differentially expressed genes in the spleen wre closely associated with depression, of which many differentially expressed genes were also screened in other experiments as differentially expressed genes in the brain (21). Based on these results, we can infer that the changes in the levels of genes in the spleen may also be one of the pathological mechanisms of depression.

Certain studies have reported that the decreased protein levels of GABBR2 or elevated SP protein levels in the brain are associated with depression (36,37), and DBP-knockout mice exhibit bipolar disorder (38). We found that although there was no GABBR2 protein expression in the spleen (39), the expression of the other two proteins in the spleen was generally consistent with that in the brain: compared to the control group, the protein expression of DBP and SP was significantly decreased. The results of GABBR2 and SP protein expression in the frontal cortex, and SP in the spleen were coincident with those of microarray analysis. Of note, although the *Dbp* gene in the spleen was upregulated, its protein level was downregulated in the brains and spleens of the mice in the depression model group. This result is basically consistent with that of a previous stutdy (38); the effects of depression were reversed by fluoxetine.

In addition, we also noted the discordant modulation of SP in depression. Despite that vast majority of published studies indicates that serum and cerebrospinal fluid SP concentrations in depressed patients are significantly higher than in healthy subjects (40,41), elevated SP levels have also been reported in patients with major depressive disorder under anti-depressant therapy (42), which are in line with our results of reduced SP protein expression in mice with depressive-like behavior, and the same reversed effect of fluoxetine. Indirectly, this reflects a close association between depression and changes in splenic gene expression.

As demonstrated in the present study, the administration of corticosterone in mice affected behavioral and neurobiological changes that mirror some of the core symptoms and neurobiological changes associated with major depression. However, the association between spleen and depression has not gained enough attention. The results of this study suggest that the improvement of splenic function and splenic gene expression may help patients with depression; however, it cannot be determined whether splenectomy or splenic injuries can lead to depression. A cause-and-effect relationship between the changes in the spleen and depression is not yet clear, and further research is required. In conclusion, although preliminary, this study sheds light on splenic changes in animal models of depression and indicate a strong connection between depression and changes in splenic function, pathomorphology and multiple genes, which may lay the ground work for future studies on the pathogenesis in depression.

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