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Revealing the role of leucine in improving the social avoidance behavior of depression through a combination of untargeted and targeted metabolomics†

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Leucine is a common nutritional supplement, and recently, research concerned with the improvement role of leucine in neuropsychiatric disorders has been increasingly emphasized. However, it is unclear what role leucine plays in depression. In this study, the chronic social defeat stress (CSDS) model was used to simulate depression associated with social avoidance in humans. CSDS mice display a depressive state and social avoidance behavior. Untargeted serum metabolomics and pathway analysis indicated that abnormal amino acid metabolism may be the key to abnormal behavior in CSDS mice. Among these metabolites, leucine shows a specific and significant positive correlation with social interaction rate. Targeted metabolomics determine the decreased level of leucine and related metabolites in the serum and hippocampus of CSDS mice. Moreover, immunohistochemical results also indicate an increasing expression of IDO1 in hippocampal tissues in CSDS mice, and neurons may be damaged. Subsequently, leucine was administered to investigate its influence on CSDS mice, and the results revealed that leucine had a good effect on depressive states and social avoidance behaviors. Taken together, we aim to identify the important role of leucine as a functional food supplement to improve depression and social avoidance behavior through the above findings.

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1. Introduction

Depression is a widespread psychiatric disorder that substantially impairs the social function of afflicted individuals. At present, an estimated 350 million individuals worldwide are grappling with this condition.[1,2] Social interaction is the inherent requirement of the nature of animals, and it is also an essential activity of human society. Inevitably, some people endure recurring negative events and show a depressive state with social avoidance as the core during social interaction. Under the guidance of the holistic medical model, social stress is identified as one of the critical contributing factors in the onset of depressive disorders in humans.[3,4] However, the mechanism underlying the depression associated with social function disordered is still unclear. Current treatments for depression are mostly carried out after the clinical symptoms of patients are relieved. Based on the STAR*D study conducted in the USA, post systemic antidepressant treatment, merely 47% of patients witnessed a reduction of more than half from their baseline QIDS-SR16 score. This suggests that the desired therapeutic effect remains unattained for over half of the depressed patients, leaving them with residual symptoms after primary antidepressant treatment.[5] However, the ultimate objective should be not only symptom relief but also the restoration of social function and quality of life in depressed individuals.[6] Therefore, it makes sense to search for several endogenous metabolites that could potentially serve as functional foods.

The chronic social defeated stress model (CSDS) model is used for the following research. It innovatively simulates a depression state *via* physiological and psychological social stress. The experimental animals would be exposed to different dominant rodents and repeatedly suffer from double pressure for a period. Eventually, the experimental animals showed signs of depression and anxiety, such as obedience, surrender, depression, loss of fun, and social avoidance.[7]

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Utilizing the stress principle of the CSDS model, it can be used to study the mechanism of social avoidance and assess the effect of antidepressants on social function improvement.

Currently, the research on the mechanism of the CSDS model mainly focuses on the differential proteins that undergo changes in the brain. The research has identified LRP6 and NYP as pivotal proteins in resilient and susceptible mice, as opposed to control mice, using iTRAQ coupled with LC-MS/MS proteomics.[8] Another research has highlighted the role of TGR5 in CA3 PyNs in depression in CSDS mice.[9] Untargeted metabolomics, a subset of systems biology, is an effective tool for elucidating drug action mechanisms and disease pathogenesis.[10–12] At the same time, serum samples can encapsulate the overall level of metabolism in the body using LC-MS/MS.[13] Consequently, LC-MS/MS untargeted metabolomics methods were used to investigate the change of metabolic profile in experimental animals exposed to CSDS stress in this study. In addition, targeted metabolomics is chosen to detect the concentration and content of a range of target metabolites and to validate the potential differential metabolic pathways found in untargeted metabolomics. The combination of untargeted and targeted metabolomics can provide a reliable basis for the study of functional small molecular substances.

Based on the result of untargeted and targeted metabolomics, it emerged that abnormal amino acid metabolism might be the key to abnormal behavior in CSDS mice. Leucine, isoleucine, and valine collectively constitute Branched-Chain Amino Acids (BCAAs), which have the ability to flow into the brain. These three amino acids are essential amino acids and are known for their abilities to bolster protein synthesis and maintain normal physiological function in humans.[14] Among these, leucine exhibits greater activity and acts as an energy supply substrate for neurons and astrocytes.[15] Besides, leucine competes with aromatic amino acids (like tryptophan) to enter the brain *via* the large neutral amino acid transporter 1 (LAT1) in the blood–brain barrier (BBB).[16] Several clinical studies have reported significant reductions in serum leucine levels in patients with depression, positing it as a potential biomarker.[17,18] Lower levels of leucine can cause dysplasia in the brain.[19] Then, we undertook exogenous leucine supplementation experiments to corroborate these findings.

This study aimed at investigating the alterations in the metabolic profile of CSDS mice and demonstrating the role of leucine in improving the social avoidance behavior of CSDS-induced mice. Initially, we copied the CSDS models and made a preliminary judgment based on the results of behavioral tests. Subsequently, the study on changes in metabolic phenotype in mice was conducted with untargeted metabolomics utilizing LC-MS/MS, and we conducted a correlation analysis on metabolites and behaviors to discuss the protentional interrelationships. It was focused on the modifications in the content of BCAAs and tryptophan in the peripheral serum and central hippocampus employing targeted metabolomics. Further, we evaluated the level of LAT-1 and the oxidative stress status in the hippocampus. Finally, the exogenous supplementation of leucine in mice was proved to improve social avoidance behavior. This study hopes to offer new insights into the role of leucine in improving social avoidance behavior.

2. Materials and methods

The main materials and procedures are summarized here, and the other details are described in ESI.†

2.1. Animal experiment design

In two experiments, a total of 48 SPF-grade male C57BL/6N (almost 5–8 weeks of age, 18–22 g) and 31 SPF-grade male CD-1 mice (almost 5–8 months of age, 35–40 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The animals were given time to adapt to new environmental conditions and were provided with the standard temperature (23 ± 1.5 °C), and relative humidity (45 ± 15%). They were maintained on a 12-hour light and 12-hour dark cycle with a free diet and water. After the adaptation period, they were divided into different groups.

All animal experiments were carried out strictly following the NIH Guidelines for Care and Use of Laboratory Animals (U.S.A.) and the Prevention of Cruelty to Animals Act (1986) of China. The experiments also obtained approval from the Committee of Scientific Research at Shanxi University (CSRSX), and the approved number was SXULL2020009.

2.2. Experimental procedures and sample collection

CSDS stress was used to establish a depression model with social avoidance, and some adjustments were made based on that model (Fig. 1A).[20,21] The mice of the C group were separated by a plexiglass plate without any stimulation, and the contact objects on the opposite side of the partition were changed daily. The mice of the M group were introduced into the cage of CD-1, which was a large, aggressive, and dominant male rodent. C57BL/6N was attacked and defeated for 5–10 min when they were subjected to social pressure from a physiological perspective. Then, C57BL/6N and the CD-1 were separated by the plexiglass plate for the following 24 h. The plexiglass plate was transparent with several holes so that they could feel the existence of each other from the vision and smell when they were under psychological pressure. The psychological stress and physiological stress were repeated for 14 days, and C57BL/6N mice were challenged by different CD-1 every day. The summary of the procedure is shown in the following figure (Fig. 1C).

In experiment 1, twenty C57BL/6N were randomly divided into two groups, the control group (C) and the CSDS model group (M).

In experiment 2 of leucine supplementation, 28 mice were randomly divided into 4 groups: the control group (CON), CSDS model group (MOD), CSDS + paroxetine hydrochloride (PCM), and CSDS + leucine (LEU). Mice in the PCM group were treated with paroxetine hydrochloride (7.85 mg kg^{-1}) once a day for 14 consecutive days. Mice in the LEU group were orally

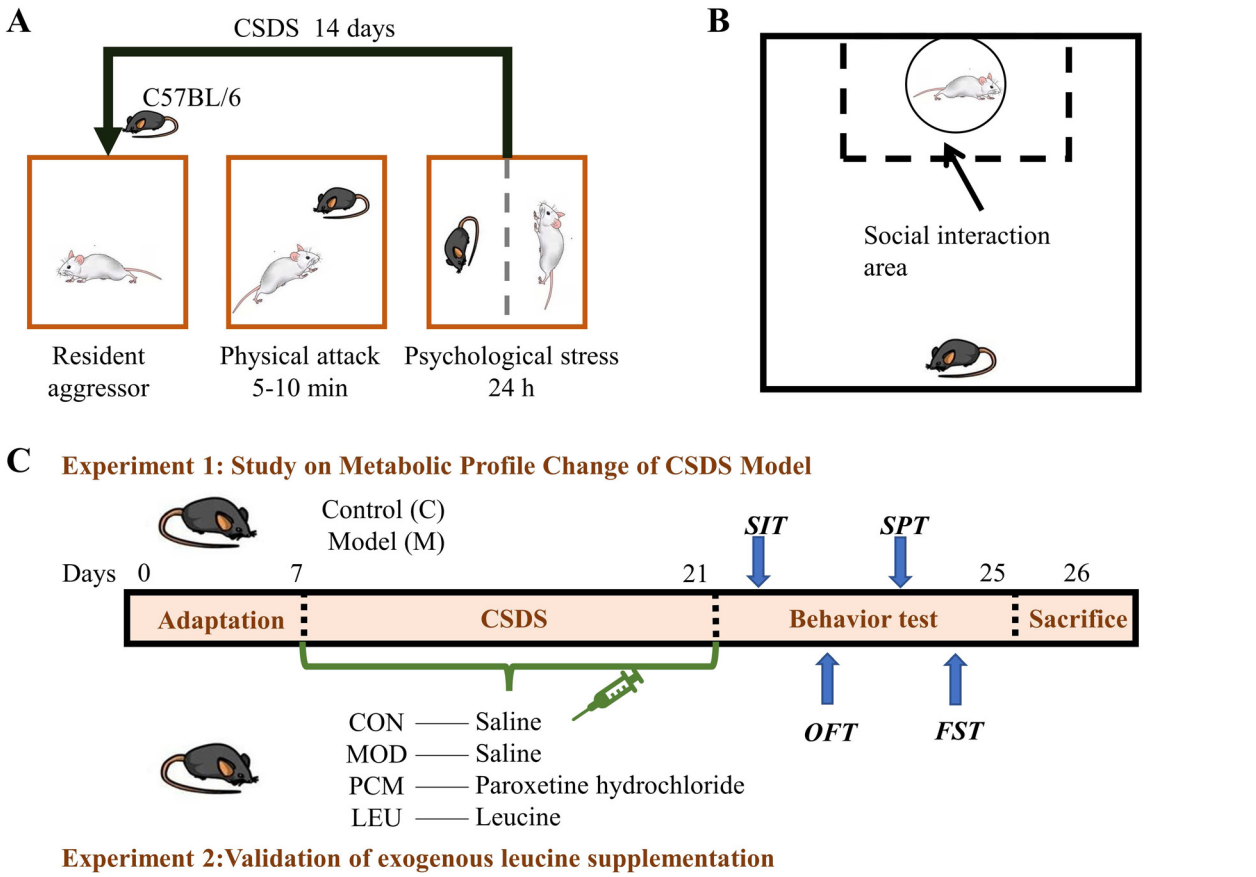

Fig. 1 The CSDS experimental procedures. (A) The specific schematic diagram of the CSDS model is summarized. C57BL/6N was attacked and defeated for 5–10 min, so when they were subjected to social pressure from a physiological perspective. Then, the experimental animals C57BL/6N and the rodent CD-1 were separated by the plexiglass plate for the following 24 h, but they could feel each other's existence from the vision and smell. (B) The specific schematic diagram of the social interaction test is summarized. The time would be recorded which C57BL/6N spent in the social interaction area with or without CD-1. (C) The C57BL/6N mice suffered CSDS for consecutive 14 days. On day 22, SIT was used to evaluate social avoidance. On day 23, the OFT was used to assess depression-like behavior and ability to activity. On day 24, the SPT was used to assess anhedonia. On day 25, the FST was used to assess the state of despair.

supplemented with leucine (40 mg kg^{-1}). Mice in the CON group and MOD group were given an equal volume of saline (Fig. 1C).

The blood of mice was collected from the retro-orbital plexus in a tube after anesthetization and was centrifuged for 10 min at 3500 rpm at 4 °C to obtain serum samples. The hippocampus tissues were obtained on ice and frozen with liquid nitrogen. All samples were stored at −80 °C until analysis. In addition, the right hippocampal tissues of four mice from each group were collected and fixed in 4% paraformaldehyde for subsequent immunohistochemistry.

2.3. Behavioral tests

All behaviors tested were examined under quiet environments and dim light.

2.3.1. The social interaction test (SIT) was performed to assess social avoidance. The social interaction experiment was performed in a social test box (50 cm × 50 cm × 25 cm) according to the literature, and the experimental mice were tested within 24 h after the end of CSDS stress.[22] In the first stage, a C57BL/6N was placed in the social test box with an empty transparent acrylic cage with holes (10 cm diameter, 10 cm height) for 150 s (Fig. 1B). While in the second stage, a strange CD1 mouse was in the acrylic cage, and then the C57BL/6N was placed into the box for 150 s again. During the experiment, the time of the mouse in the social interaction area (10 cm × 15 cm) was recorded. The social interaction ratio (SIR) is the time of the second stage divided by the time for the first stage in the interaction area.

2.3.2. The sucrose preference test (SPT) was able to assess anhedonia. This method was modified based on the original method and the result of sucrose preference rate (SPR) was obtained.[23] The training method involves placing two similar bottles (one bottle of sucrose solution (1%, w/v) and another one of pure water) in the cage for 48 h and exchanging positions every 12 h avoiding the remembering of position. In the test, mice freely obtained liquids, sucrose solution on the left and pure water on the right. In 12 h, the consumption of two

bottles was measured, and the SPR was calculated by the consumption of sucrose solution (g)/(the consumption of sucrose solution [g] + the consumption of pure water [g]) × 100%.

2.3.3. The open field test (OFT) was used to explain depression-like behavior and activity ability. The methods of the test were performed as previously described.[24] The open field box is an acrylic box painted black (50 cm × 50 cm × 40 cm), and the floor is divided into 25 squares on average. Each mouse was placed in the apparatus for 5 min, and the number of crossings and rearing was measured in the last 4 min.

2.3.4. The forced swim test (FST) was used to assess the state of despair. In the FST, the apparatus is a cylindrical barrel (diameter: 10 cm and height: 25 cm). It is filled with constant temperature water of 23 ± 2 °C with a height of 15 cm. In the test, each mouse was adapted for 2 min, and the total immobility time was accumulated plus in the last 4 min[25]

2.4. Reagents and instruments

Acetonitrile and formic acid (Thermo Fisher, USA). Ultrapure water (Millipore, USA). Glucose (Tianjin Bodi Chemical Co., Ltd); glutamate (A600221), leucine (A604076), tryptophan (A601911), isoleucine (A600914), and valine (A602796) (Biotechnology Co., Ltd).

Thermo Scientific Q Exactive and relative Xcalibur workstation (Thermo Fisher Scientific Company, USA); 1290 Infinity binary pumps Liquid Chromatography System (Agilent Technologies, USA) with 3200 Q Trap (AB Sciex, USA) mass spectrometer; electronic analytical balance BSA210S (Nanjing Leibu Science and Technology Industry Co., Ltd); ultrasonic instrument (Kunshan Ultrasonic Instrument Co., Ltd).

2.5. Untargeted and targeted LC-MS/MS-based metabolomics methods

The untargeted metabolomics profile was carried out using simultaneous scanning of positive and negative ion scan modes to measure the serum samples of CSDS mice. A targeted quantitative study of BCAAs and tryptophan was carried out using multiple reaction monitoring (MRM) model after the preparation of serum and hippocampal tissue samples. The sample preparation methods, liquid phase and mass spectrometry conditions, and LC-MS data analysis methods are described in the ESI.†

2.6. Immunofluorescence analytics

The sample is fixed by 4% polyformaldehyde, and the fixed state is good, strictly under the pathology test SOP procedure for trimming, dehydration, burial, slicing, dyeing, and sealing the final microscope examination of the qualified sample.

2.7. ELISA assay

The levels of superoxide dismutase (SOD) activity and malondialdehyde (MDA) in the hippocampus measured by ELISA kits according to the manufacturer's instructions (Andy gene, Beijing, China).

2.8. Statistical analysis

All data are presented as means ± SD. Statistical analysis was performed using SPSS 20.0 and GraphPad Prism 8. The statistical significance of the two-group comparisons was analyzed using the Student's *t*-test and the Mann–Whitney test. The statistical significance of multi-group comparisons was used for one-way analysis of variance (ANOVA) and the Kruskal–Wallis test. The Spearman rank correlation analysis was employed for correlation analysis performed by the genescloud tools (**https://www.genescloud.cn**). GraphPad Prism 8 was used for chart drawing.

3. Results

3.1. CSDS-induced depressed mice showed social avoidance and depression-like appearances

Prior to CSDS experiments, the body weight of the two groups is similar without significant difference. The C57BL/6N mice in the M group showed a series of depression-like appearances after the stress, like less weight gain and lack of pleasure (Fig. 2A and B). During the open field test, the M group showed a significant extension in the time stayed in the center, while the number of crossings and rearings significantly decreased (Fig. 2D–F). In the forced swimming test, the immobility time of the M group showed a significant increase, indicating a state of desperation in the mice (Fig. 2G). In addition to the above results, mice in the M group also showed social avoidance behavior, and the social interaction ratio was significantly lower than that of the C group in the social interaction test (Fig. 2C). The above results illustrated that the mice receiving CSDS pressures showed a series of social avoidance and depression-like behavior. Then, we investigated the potential changes in the serum metabolic profile of CSDS mice.

3.2. Explore possible pathogenesis of depressive and social avoidance behavior *via* LC-MS metabolism

The metabolic profile in the serum of CSDS was analyzed using UPLC-MS/MS in both positive and negative modes to identify significantly differential metabolites. The typical total ion chromatograms (TICs) obtained from both ESI positive and negative are shown in Fig. S1.†

The results showed that the metabolites changed in the serum between the C group and the M group. The score scatter plot of PLS-DA demonstrates remarkable distinctions (Fig. 3A). The permutation testing showed that the model was not overfitting (Fig. 3B). To estimate the significance of Q^2 and R^2 for single response models, the number of permutations is 200, and the values of R^2 and Q^2 were 0.998 and 0.704, respectively. Meanwhile, there are 10 ions extracted from the basic peak intensity chromatography of six QC samples for method validation. The RSD of the 10 ions was 5.18%–15.70% (Table S1†). The OPLS-DA and S-plot (Fig. 3C and D) were used to identify these significantly differential metabolites, and filtering criteria are as follows: *P*(corr) value that was either less than −0.58 or greater than 0.58, VIP values greater than 1.0 and *p*-values less than 0.05. Metabolites were identified according

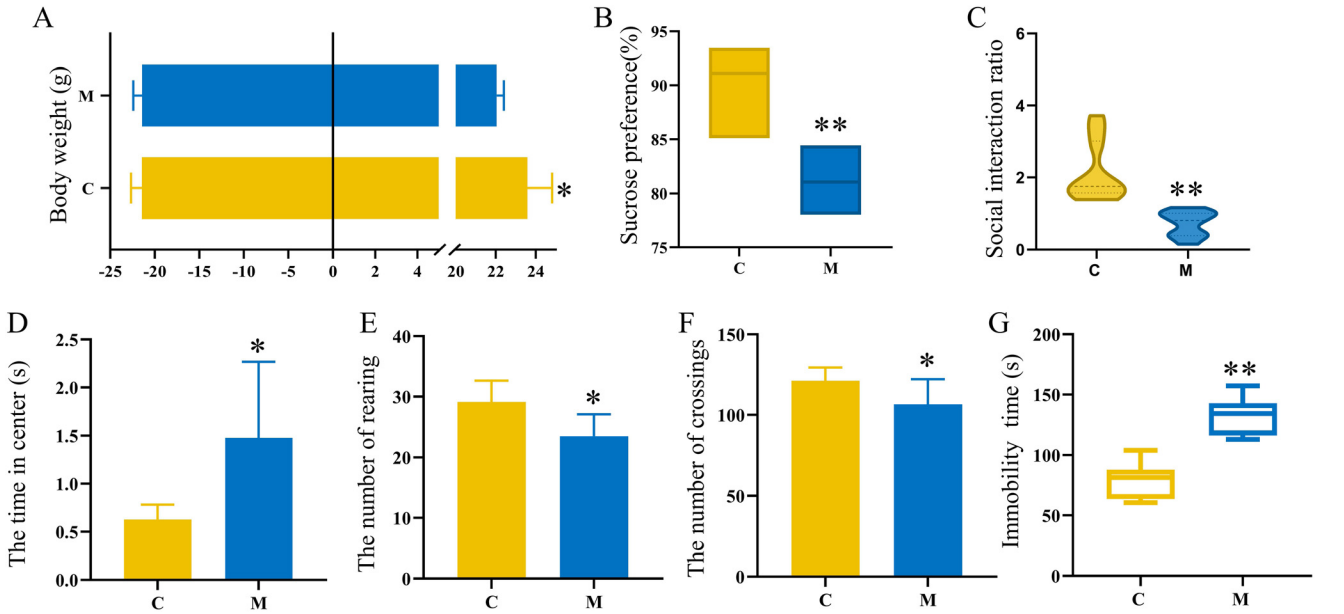

Fig. 2 The behavior test results of C57BL/6N mice after CSDS. (A) The body weight of mice on the 7th and 21st day. (B) Sucrose preference. (C) Social interaction rate. (D) The time in center in OFT. (E) The number of crossings in OFT. (F) The number of rearing in OFT. (G) Immobility time in FST. *P < 0.05, **P < 0.01 compared with C group (n = 10).

to m/z values, retention time, molecular formula, MS/MS fragments, and online databases, including HMDB (**https://www.hmdb.ca**), Massbank (**https://www.massbank.jp**), KEGG (**https://www.kegg.jp**), and PubChem (**https://pubchem.ncbi.nlm.nih.gov/**). A total of 24 significantly different metabolites were found (Table S2†). Compared with the C group, 6 differential metabolites (acetyl-L-carnitine, hypoxanthine, xanthine, *trans*-3-indoleacrylic acid, linoleamide, and oleamide) were significantly increased, and 18 differential metabolites (spermidine, choline, L-arginine, creatine, L-valine, citric acid, methionine, L-leucine, betaine, uric acid, L-phenylalanine, PEG-4, L-tryptophan, indole, palmitoylcarnitine, platelet-activating factor, retinyl acetate, arachidonic acid) were significantly decreased in the M group.

3.3. BCAAs and tryptophan metabolism might be associated with social avoidance behavior of depression

To investigate the potential mechanism of depressive and social avoidance behavior, the differential metabolites were analyzed using MetaboAnalyst 5.0 website.[26] The pathway library of Mus musculus in the KEGG database was selected to support the result (Fig. 4A). The −log (P) of the Y-axis represents the significance, and the greater value of −log (P) represents the more significant difference. The larger Impact represents the node hit, and the node hit means how much the ratio of metabolic belongs to the pathway. When the KEGG database was used to support the analyses, the top ten pathways included aminoacyl-tRNA biosynthesis (1), valine, leucine, and isoleucine biosynthesis (2), glycine, serine, and threonine metabolism (3), arginine and proline metabolism (4), phenylalanine, tyrosine, and tryptophan biosynthesis (5), purine metabolism (6), valine, leucine, and isoleucine degradation (7), phenylalanine metabolism (8), arginine biosynthesis (9), pantothenate and CoA biosynthesis (10). As we can see, the same pathways were concentrated on amino metabolism and fatty acid metabolism. The enrichment analysis was used to support the results above (Fig. 4B). The top 10 pathways were enriched in the front. The result also showed that amino metabolism and fatty acid metabolism were important in the potential mechanism. As shown in Fig. 4C, amino acid metabolism accounts for a large proportion, such as tryptophan degradation, BCAAs degradation, and methionine. There were several other metabolic pathways, including purine metabolism, the TCA cycle, and the urea cycle. Therefore, we further study the content changes of BCAAs and tryptophan.

3.4. Searching the critical metabolites related to social avoidance behavior by correlation analysis

The Spearman correlation analysis was conducted to identify the critical metabolites associated with social avoidance and depression-like appearances (Fig. 5). Among the six behavioral indicators, SIR was considered the best indicator to assess social avoidance behavior. As a result, 16 metabolites (spermidine, creatine, valine, citric acid, xanthine, leucine, betaine, uric acid, phenylalanine, PEG-4, palmitoylcarnitine, platelet-activating factor, linoleamide, oleamide, retinyl acetate, arachidonic acid) showed significant correlation with SIR. Among these metabolites, leucine shows a specific and extremely significant positive correlation with SIR. Although other 15 metabolites are related to SIR, they also correlate with other behavioral indicators. Based on this result, we further discuss the important role of leucine in social avoidance in CSDS mice.

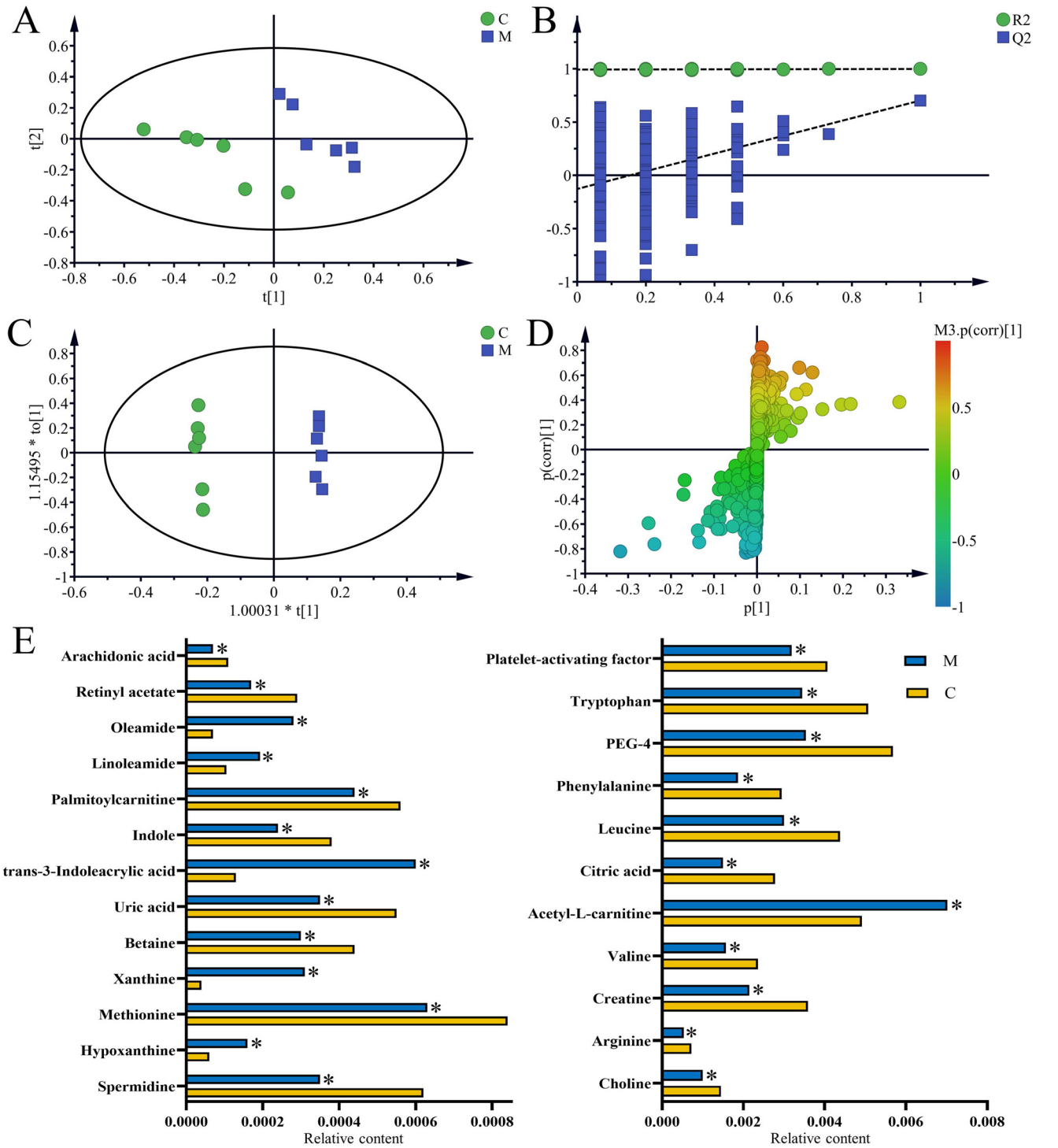

Fig. 3 Multivariate data analysis from UPLC-MS/MS of the C group and the M group. (A) PLS-DA score plots from the C group and the M group. (B) PLS-DA model validation diagram, (C) OPLS-DA score plots from the C group and the M group. (D) S-plot of OPLS-DA. (E) The relative content of significantly different metabolites *$P < 0.05$, compared with the C group ($n = 6$).

3.5. Abnormal balance of BCAAs and tryptophan induced by CSDS might lead to hippocampus inflammation increase in CSDS mice

Changes in the metabolite content of the BCAAs and tryptophan in CSDS-induced depressed mice were detected through the LC-MS/MS in the MRM mode. The LC-MS base-peak chromatograms of various metabolites are shown in Fig. S2.† The optimized MS parameters for the detection of the selected BCAAs and tryptophan are summarized in Table S4.† The methodological validation results are shown in Tables S5–S9.† Compared with the C group, the contents of leucine in the M

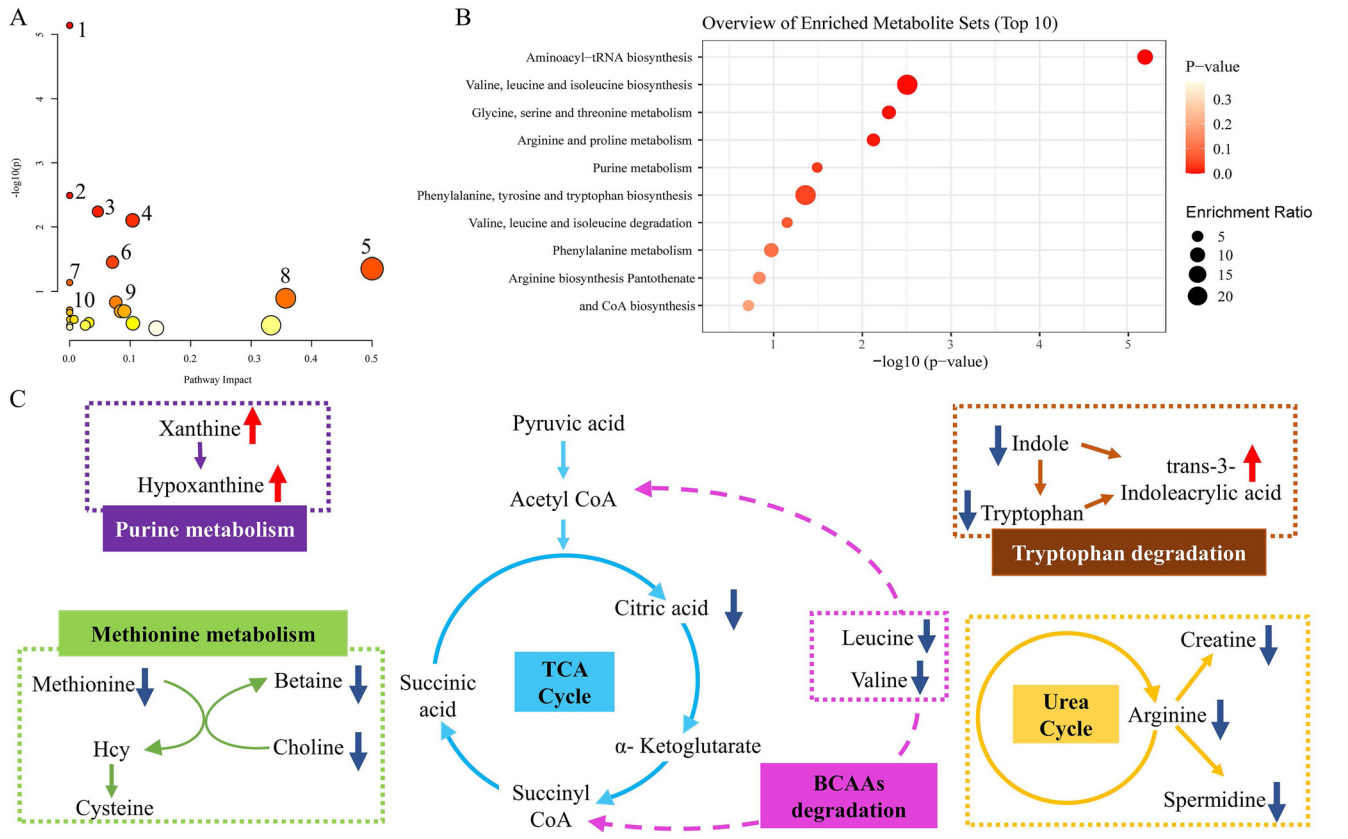

Fig. 4 The metabolic pathway analysis of CSDS. (A) The metabolic pathway analysis is based on the KEGG database. Aminoacyl-tRNA biosynthesis (1), valine, leucine, and isoleucine biosynthesis (2), glycine, serine, and threonine metabolism (3), arginine and proline metabolism (4), phenylalanine, tyrosine, and tryptophan biosynthesis (5), purine metabolism (6), valine, leucine, and isoleucine degradation (7), phenylalanine metabolism (8), arginine biosynthesis (9), pantothenate and CoA biosynthesis (10). (B) The metabolic enrichment analysis based on the KEGG database. (C) The summary of metabolic pathways. The red arrows indicated the increase in metabolite level in the model group, and the blue arrows indicated the decrease in metabolite level in the model group.

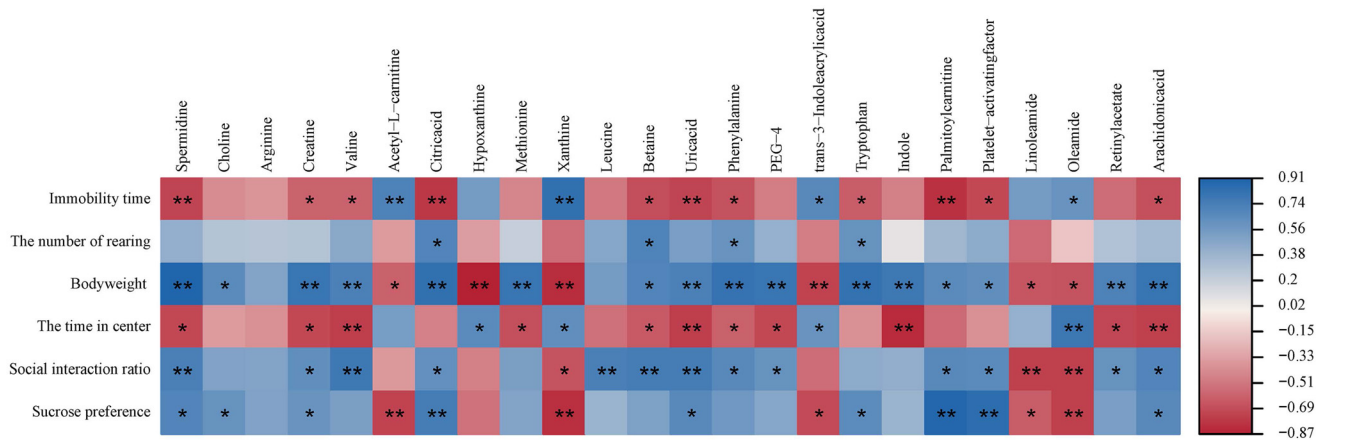

Fig. 5 The correlation analysis between endogenous metabolites and behavior indicators related to depression according to the Spearman correlation coefficient. The blue color indicated that $|r|$ was a positive value and red indicated that $|r|$ was a negative value. The darker the color, the larger the $|r|$ value. $^{*}P < 0.05$, $^{**}P < 0.01$ and $|r| > 0.6$.

group are significantly decreased in the serum (Fig. 6G), and show a decreasing trend in the hippocampus (Fig. 6B). Compared with the C group, the contents of tryptophan in the M group are significantly decreased in the serum and hippocampus (Fig. 6A and F). The above results indicate that the balance of BCAAs and tryptophan was disordered in CSDS

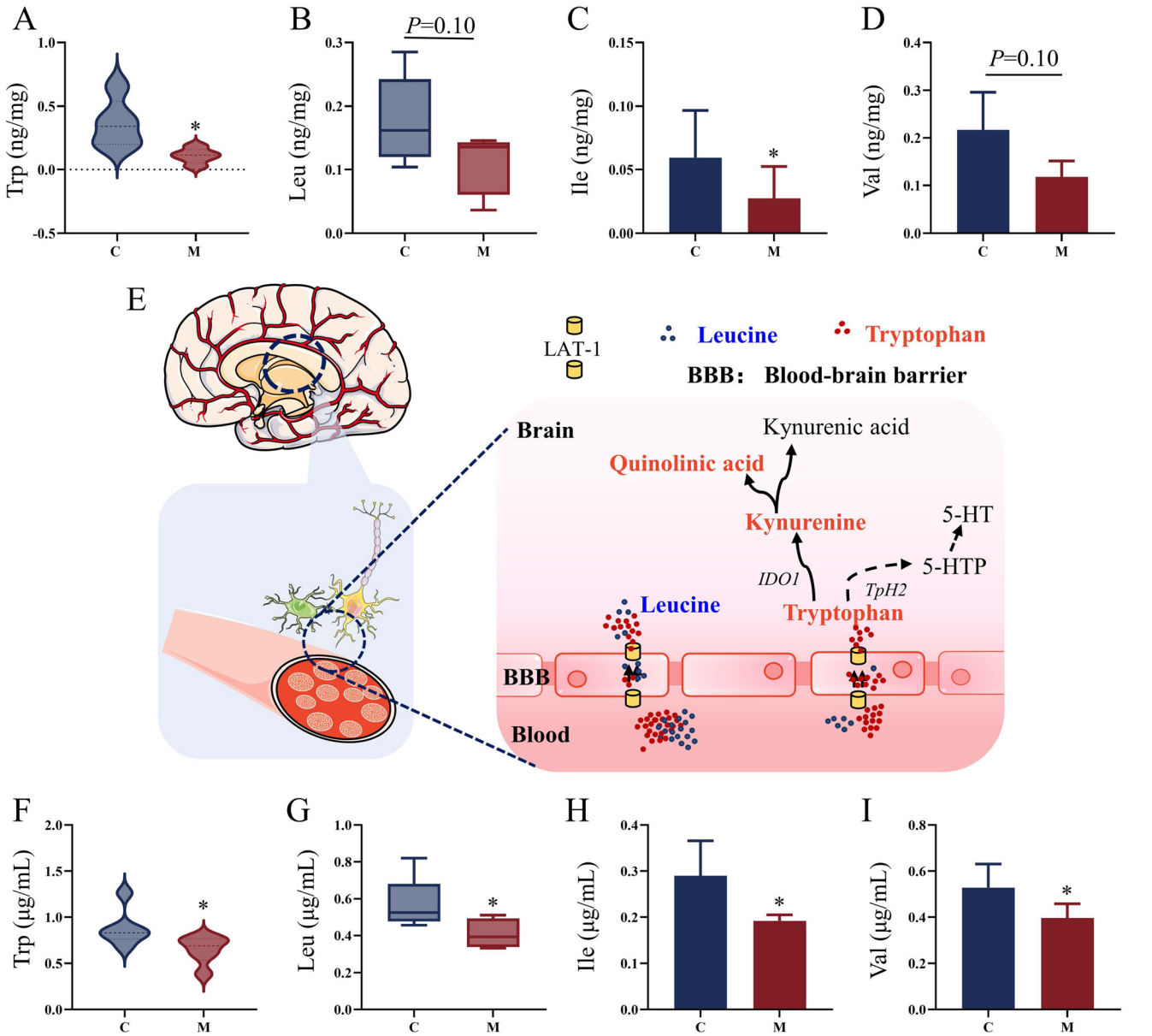

Fig. 6 The content of BCAAs and tryptophan in serum and hippocampal tissues, and the schematics shows the competitive entry of tryptophan and leucine into the brain through the BBB that would affect neuronal. (A–D) Changes in the above metabolites in serum. (E) The competitive entry of tryptophan and leucine into the brain through the BBB would affect neuronal. Lower levels of leucine lead to more tryptophan entering the brain tissue, and tryptophan may metabolize kynurenine and quinolinic acid to further affect neurons. (F–I) Changes in the above metabolites in hippocampal tissues. *$P < 0.05$, compared with the C group ($n = 6$).

mice. Therefore, we next want to explore whether the imbalance of BCAAs and tryptophan is related to the transporter LAT-1 on the BBB, and whether this imbalance will cause changes in the level of oxidative stress in the brain (Fig. 6E).

As shown in Fig. 7, the coding protein SCL7A5 of transporter LAT-1 did not change significantly between the two groups, but the expression of IDO in the brain tissue of the M group increased significantly. In addition, we determined SOD and MDA in mice hippocampus and found that both SOD and MDA levels increased significantly (Fig. S3†). It shows that CSDS does not affect transporter expression but increases oxidative stress levels in brain tissue. Combined with the results of the above quantitative analysis, it was found that leucine significantly decreased in serum and showed an obvious decreasing trend in hippocampal tissue of CSDS-induced mice, but the expression of transporter LAT-1 was not affected. The decrease in leucine content resulted in a significant decrease in serum tryptophan levels. This decrease in leucine content caused tryptophan to have a greater chance of crossing the blood-brain barrier, leading to an increase in tryptophan levels in the brain. CSDS stress increases the expression of IDO in hippocampal tissue, promotes metabolic pathways of trypto-

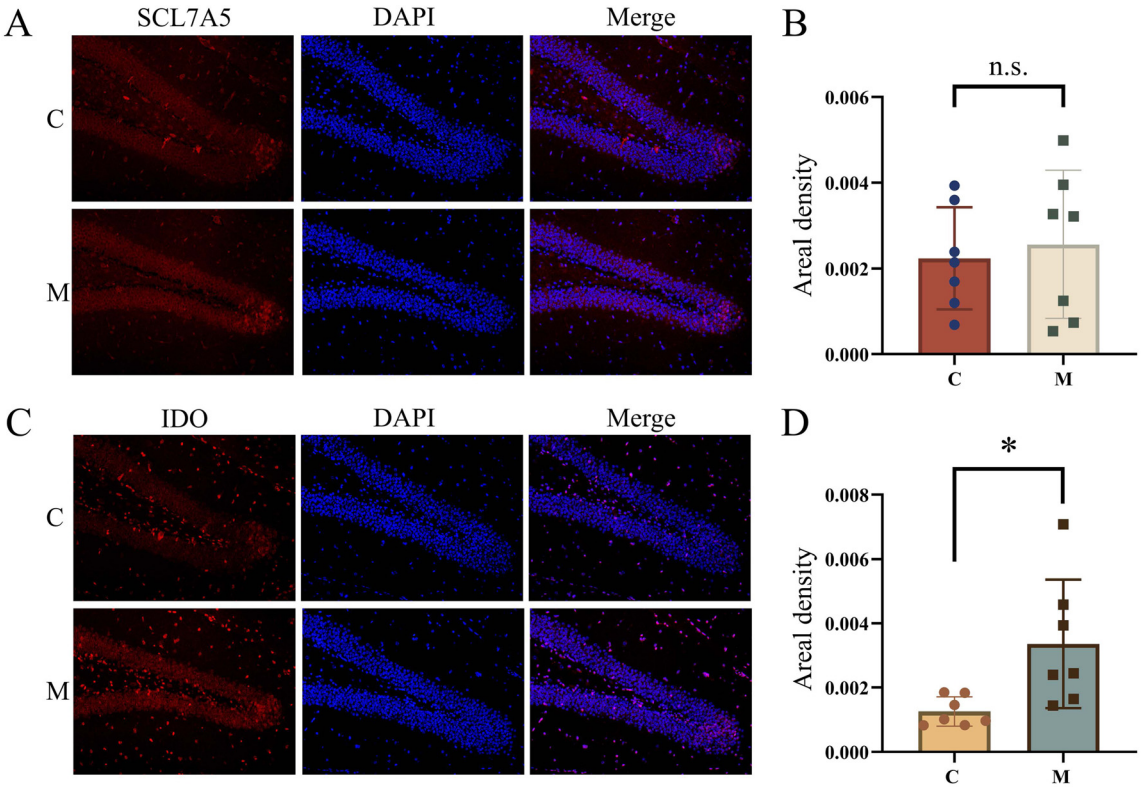

Fig. 7 The level of LAT-1 transporters and IDO in the hippocampus. (A and B) Distribution and expression of LAT-1 (C and D) Distribution and expression of IDO. *P < 0.05, compared with the C group.

phan to kynurenine, increases oxidative stress levels in hippocampal tissue, and may further affect the number of neurons. Therefore, supplementing leucine to reduce tryptophan in the brain may be a new strategy to slow the onset and development of depression.

3.6. Exogenous leucine supplementation improved social avoidance and depressive behavior

In experiment 2, the CSDS model was used to validate exogenous leucine supplementation to further evaluate its role in social avoidance and depressive behavior. The paroxetine hydrochloride was selected as a positive control medicine. At the end of the adaptation period, the body weight was without significant difference in the four groups. After the CSDS stress, the leucine could reverse the slow weight gain caused by CSDS, which is slightly lower than positive medicine without significant differences (Fig. 8A). In addition, leucine improved the social avoidance behavior of mice, and the proportion of time spent in the social zone was significantly higher than that of the MOD group in the social interaction test (Fig. 8B). At the same time, the proportion of the occurrence of social avoidance behavior in the LEU group was slightly higher than the rate of the PCM group (Fig. 8C). Meanwhile, the decrease in interest and mobility induced by CSDS can also be improved to some extent by leucine (Fig. 8D–G). Based on the above results, it is indicated that exogenous leucine supplementation could regulate depressive-like behavior in mice to some extent and improve social avoidance behavior in mice. On a previous basis, leucine may be an important small molecule compound for the process of depression and social avoidance behavior.

4. Discussion

In line with our current findings, the CSDS model and other depression animal models consistently display behavioral parallels with clinical depression patients. These include weight loss, lack of pleasure, decreased activity, and increased despair. Importantly, the CSDS model additionally exhibits a state of social avoidance, which is a real state of many depression patients in life. As CSDS model simulates primary stressors that human beings endure during social interactions, and it is commonly used for studying the pathogenesis of depression. The unity of multiple behavioral indexes can increase the credibility of the model.[27] So, we adopt the sucrose preference rate, the total number of crossing and rearing in the open field test, and the social interaction rate to evaluate the ameliorative effect of exogenous leucine. Consequently, the CSDS model was employed in this study to identify crucial metabolites linked with abnormal behavior and verify the function of leucine.

Small molecule metabolites, as downstream components of proteins and genes, provide terminal information.[28] The metabolites in the serum can reflect the changes in the overall

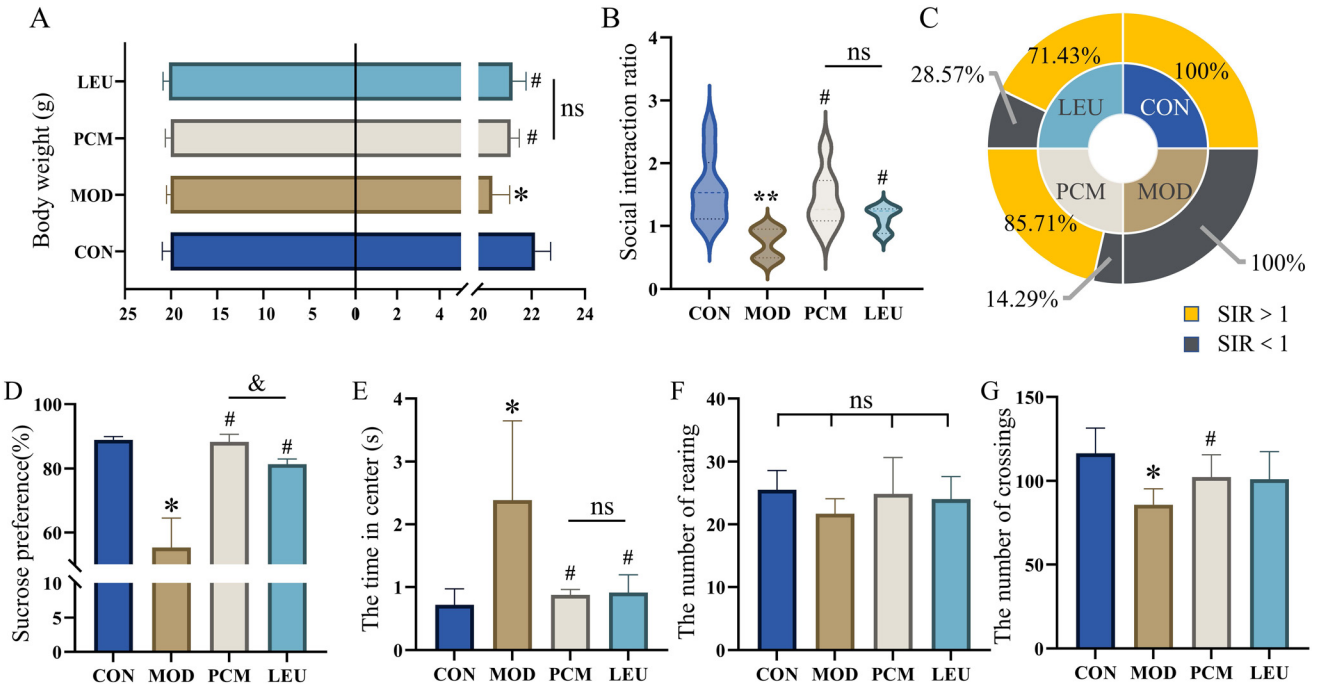

Fig. 8 The behavior test results of exogenous leucine supplementation to CSDS mice. (A) The body weight of mice on the 7th and 21st day. (B) Social interaction rate. (C) The percentage of SIR score in mice. (D) Sucrose preference. The time in center in OFT. (E) The number of crossings in OFT. (F) The number of rearing in OFT. (G) Immobility time in FST. All data were expressed as mean ± SD, (n = 10). *P < 0.05, **P < 0.01 compared with C group.

metabolic profile in experimental animals. Through untargeted metabolomics technology, there are 24 differential metabolites between the control mice and the depressive mice. More interestingly, the majority of these metabolites are belonging to amino acids, such as leucine, valine, arginine, *etc*. Meanwhile, there are potential links between these amino acids and the development of depression.[17,29,30] Valine, leucine, and isoleucine were shown a decrease in the depressive-like model group, which was consistent with the situation of patients with clinical depression.[31,32] Therefore, we speculated that the degradation pathway might be the key mechanism in CSDS-induced mice.

BCAAs and tryptophan are competitively inhibiting relationships, and they would cross the BBB and affect the corresponding brain functions.[33] The activity of leucine is highest in BCAAs, and we found that the content of leucine was decreased in the peripheral and central nervous systems. At higher concentrations, leucine triggers the mTOR signaling cascade in hypothalamic neurons.[34] Several studies have shown that mTOR expression is decreased in depressive patients and model animals.[35] The above information is lateral evidence of a potential relationship between leucine and depression. So that more tryptophan would translate through LAT-1 into the brain when leucine decreases in the body. Increased expression of IDO in CDSD mice also indicates that the tryptophan in the hippocampus is more metabolized into kynurenine when it enters the brain. A recent study demonstrated that the leucine administration combined with LAT-1 hindered the transport of kynurenine into the brain, thereby preventing the development of depression-like behavior in response to LPS.[16] Triggered by inflammatory factors, the kynurenine pathway activates and disrupts the balance between its neuroprotective and neurotoxic branches.[36] More metabolism of tryptophan to kynurenine also reduces its metabolism to 5-HT, further facilitating depression onset.[37] At the same time, the increasing levels of SOD and MDA also indicated that oxidative stress had occurred in the hippocampus.[38]

The role of critical metabolites can be directly validated by exogenous supplementation. Therefore, we chose the antidepressant paroxetine hydrochloride, known for treating social disorders. By comparison, we can more effectively evaluate the role of leucine. Our findings showed that the depressive and social avoidance behavior of mice could be improved by supplementing leucine to a certain extent. Although the effect of leucine was lower than that of positive control medicine, there is no significant difference between the two groups. This is most likely because leucine is further metabolized by oral administration as an endogenous metabolite. Paroxetine, as a mature drug, can quickly reach the target organ to play a role. Through this experiment, we initially explored the role of leucine. Thus, in subsequent experiments, leucine can be supplemented at varying doses or its proportions with isoleucine and valine to ascertain the optimal supplemental measures. It should also be set up with LAT-1 inhibitors to reflect the key role of leucine from another perspective. Meanwhile, we do

not know the metabolic differences in other organs. Subsequent research can employ other test methods, such as GC-MS or NMR.[39–41] At the same time, other research subjects such as the hippocampus, liver, feces, and intestinal contents should also be tested.[42]

In summary, our study verifies the positive effect of leucine on depressive state and social avoidance behavior. At the same time, it provides new evidence for the important role of leucine in neuropsychiatric disorders.

5. Conclusion

This study, which utilized a combination of untargeted and targeted metabolomics, found a significant correlation between decreased levels of leucine in CSDS mice and social avoidance behavior. Further experiments involving exogenous supplementation confirmed that leucine has a positive impact on social avoidance behavior. These findings suggest that the leucine metabolic pathway may be a significant pathological mechanism of depression, and leucine supplementation may be a new way to improve social avoidance behavior in depression.

Author contributions

Junsheng Tian and Xuemei Qin: funding acquisition and resources. Junsheng Tian: conceptualization, data curation, and writing – review & editing. Qi Wang: investigation, methodology, formal analysis, visualization, and writing – original draft. Huan Xiang and Zhenning Wu: visualization and software. All authors read and approved the final version of this manuscript.

Conflicts of interest

There are no conflicts to declare.

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