Effects of chronic stress on intestinal amino acid pathways

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1. Introduction

Major depressive disorder (MDD) is a debilitating mental disorder with a high prevalence and severe impacts on quality of life. However, the pathophysiological mechanisms underlying MDD remain poorly understood. Here, we used high-performance liquid chromatography with ultraviolet detection-based targeted metabolomics to identify amino acid changes in the small intestine, in a rat model of chronic unpredictable mild stress (CUMS). Pearson’s correlation analysis was conducted to investigate the correlations between amino acid changes and behavioral outcomes. Western blot analysis was employed to verify intestinal amino acid transport function. Moreover, we performed an integrated analysis of related differential amino acids in the hippocampus, peripheral blood mononuclear cells (PBMCs), urine and cerebellum identified in our previous studies using the CUMS rat model to further our understanding of amino acid metabolism in depression. Decreased concentrations of glutamine and glycine and upregulation of aspartic acid were found in CUMS model rats. These changes were significantly correlated with depressive-like behaviors. Western blot analysis revealed that CUMS rats exhibited a reduction in the expression levels of amino acid transporters ASC2 and B0AT1, as well as an increase in LAT1 expression. Impaired transport of glycine and glutamine into the small intestine may contribute to a central deficiency. The current findings suggest that the glycine and glutamine uptake systems may be potential therapeutic targets for depression. The integrated analysis strategy used in the current study may provide new insight into the cellular and molecular mechanisms underlying the gut-brain axis, and help to elucidate the pathophysiological changes in central and peripheral systems in depression.
neurotransmitters (NE, 5-HT, DA) [7], as well as regulating mood and depressive-like behavioral performance [8,9], and are closely bound up with systemic energy levels [10]. Using clinical and animal models, previous studies in our laboratory revealed several amino acids as potential biomarkers of depression [11–13]. However, current understanding of the roles played by these biomarkers in MDD is limited.

Recent studies suggest that MDD constitutes a systemic disease, with more widespread effects than impaired brain function alone [14], and the peripheral inflammatory response and metabolic disorders hypothesis of MDD is becoming more widely accepted [15,16]. Moreover, patients with clinical depression are reported to be more likely to develop intense somatic symptoms, including fatigue or loss of energy, considerable weight change, and digestive problems [17]. Thus, exploring peripheral systems might provide innovative insight into the pathogenesis of MDD and help to elucidate the intimate relationship between the central and peripheral systems. The small intestine is the primary site of amino acid absorption [18], and may play a key role in the amino acid imbalance in depression. Importantly, diseases of the small intestine such as IBS and functional gastrointestinal disorders, have been found to exhibit high comorbidity with depression [19,20]. The small intestine is a major component of the gastrointestinal tract, which communicates bidirectionally with the brain through the gut-brain axis (GBA), linking the emotional and cognitive centers of the brain with intestinal functions [21]. Recent studies have emphasized the critical role of the GBA in depression, via its ability to modulate the immune response, gastrointestinal hormone levels and microbial activity [22,23]. However, the impact of the small intestine on metabolism remains unclear.

In the present study, we aimed to gain a better understanding of systemic amino acid metabolism in depression by investigating amino acid metabolism and transport dysfunction in the small intestine. First, depressive-like behaviors in rats were induced via exposure to chronic unpredictable mild stress (CUMS), which is considered to provide an effective model for mimicking the behavioral and physiological symptoms of depression [24]. To detect changes in amino acids, targeted metabolomics approach - high-performance liquid chromatography (HPLC) combined with ultraviolet (UV) detection was applied [25]. The correlation between these changes and behavioral phenotypes related to CUMS exposure was analyzed using Pearson's correlation analysis. Meanwhile, to characterize the changes in intestinal function associated with amino acids, three neutral amino acid transporters (ASCT2, LAT1, B0AT1) involved in the uptake of glutamine and glycine in the small intestine [26] were examined using western blot assays. To extend overall understanding of systemic amino acid metabolism in depression, we integrated related differential amino acids identified from the rat hippocampus, peripheral blood mononuclear cells (PBMCs), urine and cerebellum under CUMS conditions in our previous research [15,27–29]. The current findings provide new insight into the pathophysiology of depression by linking amino acid metabolic changes in the central and peripheral systems, and identifying new potential drug targets for the treatment of depression.

2. Methods and materials

2.1. Animals

This study was given consent by the Ethics Committee of Chongqing Medical University (reference number 2017013), and all procedures were in accordance with the National Institutes of Health Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals). Healthy adult male Sprague-Dawley rats (initial weight: 230–280 g; age: 2–3 months) were obtained from the animal facility at Chongqing Medical University (Chongqing, China). Before random grouping, all animals were maintained under standard laboratory conditions (12-h light/dark cycle, lights on at 8:00 AM; 22 ± 1 °C ambient temperature; 52 ± 2% relative humidity; with unrestricted access to food and water) for a week to acclimatize to the environment. The CUMS protocol was conducted as previously reported (Fig. 1A) [30]. Special care was taken to minimize the number and suffering of animals.

2.2. CUMS paradigm

To reduce the heterogeneity in the experimental rats, rats with sucrose preferences of lower than 80% in the sucrose preference test (SPT)
were eliminated from subsequent experiment [31]. The remaining 40 rats were then randomly divided into a CUMS group (n = 20) and a control group (CON; n = 20). The CUMS procedure was conducted as described in our previous study [11]. The animals were exposed to mild stressors for a period of 4 weeks, as shown in Fig. 1B. In the CUMS group, the same stressor was not given for two consecutive days, and all stressors were applied in a separate procedure room. CON rats were left undisturbed in their home cages, except for cage cleaning, which was carried out periodically [32].

2.3. Behavioral assessment

2.3.1. SPT and body weight

The SPT was conducted before and after the CUMS procedure. Prior to the SPT, rats were habituated to 1% sucrose solution for 48 h. On the testing day, water and food were removed from the cage for 6 h. The rats were then provided with pre-weighed identical bottles of 1% sucrose and water. All fluid consumption was recorded by weighing the two bottles before and 1 h after the start of the test. Sucrose preference was defined as (sucrose consumption/total consumption) × 100%. Body weight was measured before the CUMS exposure and weekly throughout the CUMS period.

2.3.2. Open field test (OFT)

For the OFT, the rat was placed in the center zone of an open field apparatus (100 × 100 × 40 cm) in a soundproof room, and was allowed to move freely for exploration. All behaviors were recorded for a period of 5 min and 30 s, with the first 30 s used for adaptation. Between each test, the box was cleaned with alcohol and dried to eliminate olfactory cues from the previous rat. Total and central distances traveled, central activity (i.e., [central distance/total distance] × 100%), and number of rears (defined as an upright posture maintained on the hind paws) were calculated.

2.3.3. Forced swim test (FST)

In the FST, rats were individually placed into cylinders (50 cm high, 20 cm in diameter) filled with water (23 ± 2°C) to a depth of 30 cm for a 15-min training session, then gently dried and returned to their home cages. They were placed in the cylinders again 24 h later, and the 5-min FST was conducted. The total immobility time in the FST was recorded as an index of depressive-like behaviors. All sessions in the FST and OFT were recorded with an automated video tracking system (EthoVision XT 13, Noldus, Virginia, United States).

2.4. Sample collection

Animals were euthanized with 10% chloral hydrate (100 g/0.4 ml) by intraperitoneal injection, and the distal small intestine was immediately dissected out because of its high expression of amino acid transporters compared with other parts of the small intestine [33]. Feces were gently separated away using tweezers. The tissues were then rinsed in cold saline solution, and stored at −80°C until HPLC analysis or protein extraction.

2.5. Preparation of standard solutions

Stock solutions of 20 amino acids and Norleucine (IS) were acquired by dissolving each standard mixture (18 amino acids AAS18, L-glutamine 76,523, L-asparagine 51,363, N-Lorleucine N8513, Sigma-Aldrich, Germany) in 0.1 M HCl solution, then labeled and stored at −20°C. The working standard solutions were prepared by diluting the stock solutions with water to the desired concentrations of 250 nmol/ml before use, TDPA (0.2%, w/v) was generally added as an antioxidant.

2.6. Dabyslation of standard amino acids and intestinal samples

Dabsylation was performed as previously reported [34,35], with minor modification. In brief, frozen intestine samples (n = 7 for each group) were slowly defrosted and transferred to Eppendorf tubes, weighed, homogenized with a Tissue Lyser (Jingxin Co., Ltd., Shanghai, China) at 70 Hz for 2 min, and resuspended in 500 μl 0.01 M HCl containing 0.2% TDPA and internal standard (250 nM). After deproteinization by mixing with 40% (w/v) trichloroacetic acid, the mixture was kept in an ice bath for 10 min and centrifuged at 10,000 g for 15 min. The supernatants, containing amino acids, were transferred and filtered through a 0.45 μm pore size Millipore filter, then dehydrated in vacuo. The dehydrated samples and amino acid standards were redissolved in 50 μl reaction buffer (0.15 mM NaHCO3, pH 8.6). A 5-μl aliquot of standard amino acids was blended with 45 μl reaction buffer, and all samples and standard amino acids were mixed with 50 μl 12.4 M dabsyl chloride reagent (40 mg dabsyl chloride in 10 ml of acetone), heated at 70°C, and vortexed at 1400 rpm for 15 min with a thermal shaker (Thermo Fisher Scientific). The reaction was stopped by incubating in an ice bath for 5 min. Then, 100 μl dilution buffer (50 ml acetonitrile, 25 ml ethanol and 25 ml mobile phase A) was added to the solution and centrifuged at 15,000 g for 5 min. The supernatant was transferred to a 250 μl insert (polypropylene; Agilent Technologies, USA) and subjected to HPLC analysis.

2.7. HPLC gradient system for determination of amino acids

In addition to reacting with amino acids, dabsyl chloride also reacts with water to produce sodium dimethylaminoazobenzene-4-sulfonate, which is detected as a large early peak in the HPLC trace. The determination of dabsylated amino acids from intestinal samples was carried out on an Agilent 1260 HPLC system with a UV detector at 436 nm. A reverse-phase column (Hypersil GOLD Thermo, 250 mm × 4 mm i.d., 5 μm particle size) coupled with a C18 cartridge (12 mm × 8 mm i.d., 5 μm) was used. The column temperature was set at 50°C. The flow rate and injection volume were 1.0 ml/min and 20 μl, respectively. Mobile phase A (pH = 6.55) consisted of 9 mM KH2PO4, 4% DMF, 0.1% TEA and 0.055% phosphoric acid. Mobile phase B consisted of 80/20 acetonitrile (Merck KGaA, Darmstadt, Germany)/ultrapure water. Both mobile phases were passed through a 0.22-μm membrane filter prior to use. The gradient elution profile is shown in Table 1.

2.8. Western blotting

Frozen small intestinal tissue samples were obtained from both the CUMS group (n = 6) and the CON group (n = 6). Each sample was homogenized in RIPA Lysis Buffer (Beyotime, Beijing, China), containing 0.1% proteases inhibitors, 1% phosphatase inhibitors and 1% PMSF, on ice. The homogenates were then sonicated (70 Hz, pulse for 18 s, rest for 20 s; 3×) and centrifuged at 14,000 g at 4°C for 5 min. The protein concentrations were quantified using a BCA protein assay kit (Beyotime). Solubilized proteins were resolved by electrophoresis on 10% SDS-PAGE gels, then transferred onto polyvinylidene difluoride membranes (Merck, Germany) and subjected to immunoblot analysis with a rabbit anti-ASCT2 polyclonal antibody (ab84903, Abcam; 1:1000), rabbit anti-B0AT1 (ab180516, Abcam; 1:500), mouse

Table 1

<table>
<thead>
<tr>
<th>Gradient elution profile of the HPLC.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
</tr>
<tr>
<td>Solvent B(%)</td>
</tr>
<tr>
<td>Solvent A(%)</td>
</tr>
</tbody>
</table>

Solvent B: 80/20 acetonitrile; solvent A: 9 mM KH2PO4, 4% DMF, 0.1% TEA and 0.055% phosphoric acid (pH 6.55).
monoclonal anti-LAT1 (sc-374,232, Santa Cruz, China; 1:1000) or a rabbit monoclonal β-actin antibody (generous gift from Bioes, China) overnight at 4 °C. All membranes were washed and incubated with their respective horseradish peroxidase-coupled secondary antibody (Bio-Rad). The signal intensities of the immunoreactive bands, detected with an ECL kit (Millipore), were quantified using Quantity One software (Bio-Rad). The proteins were normalized to β-actin, and the control value was set to 1.

2.9. Statistical analysis

Data are presented as mean ± standard error. Repeated measures analysis of variance (ANOVA) was used to analyze body weight data. Unless otherwise stated, Student's t-tests were used to analyze significant differences between the two groups for the OFT, SPT, FST, amino acid concentrations and western blot analysis. All tests were two-tailed. Pearson's correlation analysis was used to evaluate the association between the behavioral phenotype and the altered amino acid concentration. The significance threshold for all analyses was set at p < .05. All analyses were performed using SPSS Statistics 21.0 (IBM, Armonk, NY, USA). The plots were produced with GraphPad Prism 6.0 (La Jolla, China), Cluster 3.0 (Tokyo) and Venny 2.1.0 (bioinfogp.cnb.csic.es/tools/venny/).

3. Results

3.1. A 4-week CUMS protocol successfully induced depressive-like behavior

Before the CUMS protocol, no significant differences were found between the two groups in body weight or sucrose preference (Figs. 2A and B). After 4 weeks of exposure to the stressors, rats in the CUMS group gained significantly less body weight than control rats (p < .001, repeated measures ANOVA). Body weight was significantly lower in the CUMS group than in the CON group over the last 3 weeks of the CUMS exposure protocol (week 2, p < .05; week 3, p < .001; week 4, p < .001; Fig. 2A). The SPT is considered to be a reliable test for the evaluation of chronic stress-related anhedonia, a core symptom of depression [36]. At the end of the 4-week CUMS protocol, rats in the CUMS group displayed impaired sucrose preference compared with baseline period (p < .001; Fig. 2B) and sucrose preference was markedly reduced in the CUMS group compared with the CON group (p < .05; Fig. 2B). The FST serves for assessing depression-related despair-like behaviors [37,38]. Compared with the CON group, immobility time in the FST was significantly elevated in the CUMS group (p < .01; Fig. 2C). Additionally, in the OFT, rats in the CUMS group showed decreased exploratory behavior, with significantly reduced central distance (p < .05; Fig. 2E) and central activity (p < .01; Fig. 2F). However, there was no significant difference in the total distance (Fig. 2D) or the number of rearings (Fig. 2G) between the two groups.

3.2. Altered intestinal Asp, Gln, Gly concentration among 20 amino acids

Previous validation studies have confirmed that the HPLC method exhibits good performance in terms of linearity, accuracy, repeatability and stability [35,39]. The chromatograms for the CON and CUMS groups and the standard curve are presented in Fig. 3. The chromatograms displayed strong signals as well as a large peak capacity and good reproducibility in the retention times. Identification of the 20 amino acids and the dabsyl chloride reagent were performed as previously reported [35]. For the determination of the concentration of the respective amino acids, the ratio of the peak areas of the amino acid and the corresponding internal standard was used. Table 2 lists the concentrations of the 20 amino acids in the small intestinal tissue. Fig. 4A shows a heat map of the fold-changes in the amino acids. The analysis revealed a number of differential amino acids between the control and CUMS groups. Glutamine (p < .05; Fig. 4B) and glycine (p < .05; Fig. 4C) concentrations were reduced, while aspartic acid concentration was increased (p < .05; Fig. 4D) in the CUMS group compared with the control group.

3.3. Analysis of the correlation between differential amino acids and behavioral phenotypes

In the present study, the correlations of glutamine, glycine and aspartic acid levels with behavioral outcomes (body weight, total distance, central distance, central activity, rearing times, sucrose preference, immobility time) were analyzed. Of all the results, a significant negative correlation was observed between aspartic acid concentration and body weight in the fourth week (r = −0.65, p = .011; Fig. 5A), and glycine levels were positively associated with sucrose preference parameters (r = 0.53, p = .047; Fig. 5B). In addition, glutamine levels exhibited a moderate correlation with immobility time (r = −0.53, p = .049; Fig. 5C) and sucrose preference (r = 0.54, p = .045; Fig. 5D). No other significant correlations emerged.

3.4. Dysfunction of three amino acid transporters was uncovered by western blot analysis

Three candidate proteins participating in the uptake of glutamine and glycine in the small intestine (ASCT2, B0AT1 and LAT1) were subjected to western blot analysis for validation (Fig. 6A). The expression levels of ASCT2 (p < .01; Fig. 6B) were significantly decreased in the CUMS group compared with the CON group. In contrast, LAT1 expression was increased (p < .001; Fig. 6C), while B0AT1 expression was reduced (p < .05; Fig. 6D) in the CUMS group. Details of the protein bands are included in Supplemental Fig. 1.

3.5. Integrated analysis for amino acid changes induced by CUMS

Previous studies in our laboratory revealed numerous metabolite changes in CUMS model rats using non-targeted profiling, including a set of differential metabolites in the hippocampus, cerebellum, urine and PBMCs involved in amino acid metabolism. A Venn diagram of all the significant metabolites in the various tissues in the CUMS rat is shown in Fig. 7A. An overview of changes in glycine (GLY), glutamine (GLN) and aspartate (ASP) in the hippocampus, cerebellum, PBMCs, urine and small intestine in the CUMS rat model is shown in Fig. 7B.

4. Discussion

MDD is increasingly recognized as a systemic disease that frequently occurs with various metabolic disorders [40]. Although substantial research effort has focused on the investigation of amino acid metabolism under depressed conditions, previous studies have typically focused on the central nervous system and circulatory system rather than the small intestine. In the enterocytic apical membrane of the small intestine, an initial step of amino acid intake takes place, likewise, it acts as the main source of amino acids in the body [41]. Herein, we successfully induced a depressive phenotype in rats by exposure to a 4-week CUMS protocol. Afterward, a targeted metabolomics approach was used for the first time to identify CUMS-induced amino acid changes in the small intestine. Rats in the CUMS group exhibited significantly decreased levels of neutral amino acids glutamine and glycine, and increased levels of aspartic acid. Subsequently, western blot analysis was carried out to disclose the impaired biological processes of glutamine and glycine transport in the small intestine by examining the expression levels of corresponding transporters.

4.1. Reduced transport of glutamine and glycine in the small intestine

Two Na+-dependent neutral amino acid transporters, B0AT1
SLC6A19 and ASCT2 (SLC1A5), undertake glutamine absorption from the diet across the apical membrane of intestinal epithelial cells [26]. B0AT1 belongs to the B0 system, which accepts a broad variety of neutral amino acids, with the highest affinity for glutamine [42]. B0AT1 plays a critical role in glutamine uptake, as shown by B0AT1-deficient mice, in which Na+-dependent uptake of glutamine in the small intestine is completely abolished. These mice also display a decrease in glucose uptake of approximately 50%, and a reduction in body weight [43]. Thus, the decrease in B0AT1 expression might underlie the reduction in body weight during the 4-week CUMS procedure. ASCT2, an important Na+-dependent transporter of the ASC system that is mainly localized in the intestine [44], mediates the obligatory exchange of amino acids such as glutamine [45]. ASCT2 is a member of the solute carrier 1 (SLC1) family. Interestingly, the other members of this family are intimately related to depression. The SLC1 proteins, including excitatory amino acid transporters EAAT4 (SLC1A6), EAAT3 (SLC1A1), EAAT2 (SLC1A2) EAAT1 (SLC1A3) [46,47]. LAT1 (SLC7A5) is a Na+- and pH-independent transmembrane transporter in the SLC7 family that belongs to the L system [48]. LAT1 imports large and neutral essential amino acids in exchange for intracellular amino acids (e.g., glutamine) on the cell membrane surface [49]. Here, we found increased expression of LAT1 in the CUMS group compared with controls. This increase might diminish net glutamine uptake via the small intestine. Interestingly, LAT1 expression is highly upregulated in many tumor cell lines and cancers [50]. Therefore, the elevated expression of LAT1 in depressed animals might increase their risk of cancer. The transport pathway for glutamine is shown in Fig. 8A.

The majority of glycine appears to be transported by the B0 system and hPAT1 in brush border epithelial cells in the intestinal mucosa [51]. Moreover, ASCT2 mediates glycine influx across apical membranes in the rat jejunum [52]. ASCT2 has substrate specificity, transporting small neutral amino acids with Km values of 20 μM. Glycine is transported with the highest Km value (300–500 μM) [53]. This process is depicted in Fig. 8B.

Taken together, our findings suggest that alterations in the expression of transporters might cause a reduction in the absorption of glutamine and glycine in the CUMS rat model.

4.2. Systemic amino acid changes induced by CUMS

Glutamine, the most abundant free amino acid in the body, is a conditionally essential nutrient under stressful conditions [54]. Patients with stress-related major depression typically exhibit reduced brain glutamine levels [40]. In the CUMS rat model, a lack of glutamine was simultaneously detected in the hippocampus and cerebellum, in line with the results of another study reporting that glutamine levels are downregulated in both the rat hippocampus and cerebral cortex after CUMS exposure [34]. This deficiency in the brain has been reported to increase despair-like behaviors and anhedonia [9], and these behaviors are also associated with reduced intestinal glutamine levels as well through correlation analysis. Furthermore, as it easily crosses the blood-brain barrier, it is not surprising that glutamine is frequently used as a
biomarker of depression [55], as supported by urine metabolism data in the CUMS model. Here, we found decreased glutamine caused by absorption disorder in the small intestine. The small intestine is the major absorption site for glutamine, providing the vast majority of this amino acid to the body [56]. Therefore, the markedly reduced transport of glutamine in the small intestine might contribute to the circulating glutamine deficiency to regulate depressive-like behavior in the CUMS model. Importantly, however, a glutamine-enriched diet is reported to directly ameliorate the uptake of glutamine in the rat small intestine [57]. Meanwhile, this oral glutamine intervention pattern has been reported to lead to mood improvement and [58] and alleviation of anxiety-related behavior [59]. The current finding of decreased absorption of glutamine in the small intestine appears to support the possibility that a glutamine-enriched diet could be an effective treatment for depression.

Glycine is an important inhibitory neurotransmitter in the brain and functions as an NMDAR co-agonist [60]. In addition, decreased glycine levels were found in the hippocampus under CUMS by another group [61]. Here, we observed reduced glycine content in both the PBMCs and cerebellum, and an increased loss of the amino acid via the urine in the CUMS model, likely caused by intestinal dysfunction. The currently prevailing view is that mammals may fail to adequately synthesize glycine under certain conditions [62]. Thus, exogenous supplementation could potentially satisfy this need, and oral glycine has been reported to increase serum glycine, as well as ameliorating cognitive and depressive symptoms [8,63]. However, despite its substantial protective effect [64], there is evidence that excessive glycine intake can lead to toxicity [65]. Therefore, identifying a glycine transport pathway disorder in the intestine and describing the relationships between intestinal glycine and depressive behavior could reveal diversified and safe approaches for the treatment of this symptom.

Increased levels of aspartic acid have also been found in the small intestine using HPLC [66]. In the current study, we did not find a change in aspartate transport in the small intestine. In the central

Fig. 3. HPLC chromatogram of (A) control rats and (B) CUMS rats. (C) Standard curve (250 nmol/l) peaks: (1) aspartic acid, (2) glutamic acid, (3) DAPS-CL, (4) asparagine, (5) glutamine, (6) serine, (7) threonine, (8) glycine, (9) arginine, (10) alanine, (11) proline, (12) valine, (13) methionine, (14) isoleucine, (15) leucine, (16) NorLeu, (17) phenylalanine, (18) ammonia, (19) lysine, (20) histidine, (21) tyrosine.
Table 2
The concentrations of the 20 intestinal amino acids in the CON and CUMS groups.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Peaks</th>
<th>Retention time (min)</th>
<th>CON(ng/mg)</th>
<th>CUMS(ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>1</td>
<td>27.92</td>
<td>76.178 ± 44.1347</td>
<td>131.5736 ± 45.1296</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2</td>
<td>28.821</td>
<td>149.6524 ± 117.5978</td>
<td>115.5661 ± 48.8441</td>
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<tr>
<td>DABS-Cl</td>
<td>3</td>
<td>37.117</td>
<td>419.9245 ± 197.4087</td>
<td>407.0917 ± 84.5289</td>
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<tr>
<td>Asparagine</td>
<td>4</td>
<td>39.753</td>
<td>190.0276 ± 49.2903</td>
<td>133.1381 ± 25.2770</td>
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<tr>
<td>Glutaminea</td>
<td>5</td>
<td>40.338</td>
<td>721.6484 ± 246.2514</td>
<td>525.4560 ± 111.4971</td>
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<tr>
<td>Serine</td>
<td>6</td>
<td>41.796</td>
<td>547.0401 ± 189.0790</td>
<td>456.1394 ± 112.1896</td>
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<td>Threonine</td>
<td>7</td>
<td>43.004</td>
<td>388.0096 ± 97.7157</td>
<td>274.2105 ± 57.7117</td>
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<tr>
<td>Glycine</td>
<td>8</td>
<td>44.151</td>
<td>2376.6764 ± 1071.9974</td>
<td>2322.9480 ± 534.9061</td>
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<tr>
<td>Arginine</td>
<td>9</td>
<td>45.754</td>
<td>905.6265 ± 339.2173</td>
<td>856.8156 ± 232.2708</td>
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<tr>
<td>Alanine</td>
<td>10</td>
<td>47.982</td>
<td>4608.5908 ± 1513.8826</td>
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<tr>
<td>Proline</td>
<td>11</td>
<td>53.105</td>
<td>761.2319 ± 202.2400</td>
<td>611.7856 ± 98.1757</td>
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<tr>
<td>Valine</td>
<td>12</td>
<td>55.253</td>
<td>268.4017 ± 108.7400</td>
<td>211.7385 ± 46.7570</td>
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<tr>
<td>Methionine</td>
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<td>59.668</td>
<td>511.5241 ± 138.0210</td>
<td>417.1333 ± 67.8860</td>
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<tr>
<td>Isoleucine</td>
<td>14</td>
<td>63.306</td>
<td>741.3980 ± 120.5776</td>
<td>956.6248 ± 282.2273</td>
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<tr>
<td>Leucine</td>
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<td>64.263</td>
<td>1358.7845 ± 416.9936</td>
<td>1183.4732 ± 146.7748</td>
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<tr>
<td>Norleucine (IS)</td>
<td>16</td>
<td>64.905</td>
<td>499.0420 ± 215.6406</td>
<td>444.2102 ± 136.8661</td>
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<tr>
<td>Phenylalanine</td>
<td>17</td>
<td>66.036</td>
<td>797.2727 ± 212.1280</td>
<td>624.3178 ± 103.4970</td>
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<tr>
<td>Ammonia</td>
<td>18</td>
<td>79.979</td>
<td>163.0843 ± 91.5155</td>
<td>166.5961 ± 85.7381</td>
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<td>Histidine</td>
<td>19</td>
<td>80.711</td>
<td>40.7241 ± 15.7827</td>
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<td>Tyrosine</td>
<td>20</td>
<td>84.053</td>
<td>62.5686 ± 37.0554</td>
<td>57.8573 ± 24.0056</td>
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<td>Glycinea</td>
<td>21</td>
<td>40.338</td>
<td>2376.6764 ± 1071.9974</td>
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CON: control; CUMS: chronic unpredictable mild stress; DABS-Cl: dabsyl chloride; IS: internal standard. The concentrations of the amino acids are expressed as mean ± SEM (n = 7).

*p < .05.

Fig. 4. Concentrations of amino acids in the small intestine measured by HPLC. (A) Hierarchical clustering was performed using the amino acids in both groups based on their fold changes. (B, C, D) The concentrations of glutamine and glycine in the CUMS group (n = 7) were downregulated, while the concentration of aspartic acid was upregulated compared with the CON (n = 7) group.
nervous system, aspartate usually functions as a regulator of neural plasticity by specifically activating the NMDA receptor in its acetylated form, N-acetyl-L-aspartic acid, which is the second most abundant amino acid in the brain [67]. This process is generally considered to exert antidepressant effects [68]. In the enteric nervous system, which has been described as “the brain within the gut” [69], Nagahama et al. reported that aspartate could function as a neuromodulator by detection of Asp-immunoreactive neurons in the small intestine [70]. Aspartate also regulates intestinal function via the NMDAR [71]. In CUMS-exposed rats, reduced concentrations of N-acetyl-L-aspartic acid were found in the hippocampus, and increased levels were found in the small intestine. We speculate that the resultant changes in aspartate signaling might contribute to the intestinal and central symptoms in depression.

4.3. Amino acids involved in the GBA

The GBA has been increasingly linked to mood and cognition with gut activity in MDD. The existence of reciprocal interactions is unclear, but represents an intriguing possibility [22]. Changes in the brain (often triggered by stress) can influence immune activation, entero-endocrine signaling, epithelial cell functions, as well as intestinal permeability and barrier function. Conversely, intestinal components, such as cytokines, short-chain fatty acids, nutrients and microflora, may affect central processes, such as neurotransmission and behavior [72]. Moreover, glutamine, glycine and aspartate play a critical role in maintaining gut

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Fig. 5. Correlation between differential amino acids (CON, n = 7; CUMS, n = 7) and corresponding behavioral results (CON, n = 7; CUMS, n = 7) (A) aspartic acid and body weight (B) glycine and sucrose preference (C) glutamine and immobility time (D) glutamine and sucrose preference.

Fig. 6. Western blot validation of selected proteins involved in the absorption of amino acids. (A) protein bands (30 μg in each line) for ASCT, B0AT1 and LAT1 in the CUMS (n = 6) and CON (n = 6) groups were analyzed using Quantity One software. (B, D) The expression of ASCT2 and B0AT1 were significantly decreased in the CUMS rats compared with controls. (C) Expression levels of LAT1 were significantly increased in CUMS rats compared with controls. The expression levels are normalized to β-actin. *p < .05, **p < .01, ***p < .001.
health and preventing intestinal diseases [73]. These amino acids regulate inflammation by controlling NF-κB signaling and proinflammatory cytokine secretion, the growth and viability of cells maintained in culture, and apoptosis [74]. These metabolites might modulate other GBA signaling pathways, and exacerbate mental and physical symptoms in depression.

5. Conclusion

Taken together with our previous findings, the current results suggest that the decreased absorption of glycine and glutamine in the small intestine may contribute to their systemic deficiency in depressive rats. Furthermore, this findings provide insight into the cellular and molecular mechanisms underlying the GBA. Therefore, in addition to microbiota, cytokines and gastrointestinal hormones, which have already been reported to link the GBA with MDD, amino acid disorder might also play a pivotal role in the pathophysiology of the disorder. Importantly, the current results indicate novel therapeutic targets for the treatment of depression, and lays the foundation for future studies of this debilitating mental disorder.

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Conflicts of interest

The authors declare no conflict of interest in the submission of this manuscript.

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