Electronic Supplementary Information

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

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1. Untargeted LC-MS/MS-based metabolomics

1.1 Sample processing

The serum samples of each group were thawed at 4 ℃, 100 μL of serum was taken out and put into a 2 ml EP tube, 200 μL of 0.1% acetonitrile formate (V/V) was added, vortex for 2 min, centrifuged at 13000 rpm for 15 min at 4 ℃, and the supernatant was taken for sample analysis. In addition, we took 10 μL of each serum sample from the above four groups and mixed them. The mixed sample was prepared according to the sample preparation method above, and we get the quality control (QC) samples at the same time.

1.2 Liquid phase conditions

The separation was performed on Waters ACQUITY UPLC HSS T3 column (2.1 × 100mm, 1.7 μm). The mobile phases conclude A (water, containing 0.1% formic acid) and B (acetonitrile, containing 0.1% formic acid), and the mobile phase gradient is as follows: 0 ~ 2 min, 2% B; 2 ~ 3 min, 2 ~ 35% B; 3 ~ 13 min, 35 ~ 68% B; 13 ~ 17 min, 68 ~ 70% B; 17 ~ 22 min, 70 ~ 98% B; 22 ~ 25 min, 98% B; 25 ~ 25.5 min, 98 ~ 2% B; 25.5 ~ 28 min, 2% B. The injection volume was 5 μl. The flow rate was 0.2 ml/min, and the column temperature was 40 °C [20].

1.3 Mass spectrometry conditions

The ESI electrospray ionization method, positive and negative ion switching acquisition mode, spray voltage positive 3.5 kV, negative 2.5 kV. Capillary temperature 320 ℃, heater temperature 300 ℃, sheath gas volume flow 35 ARB, auxiliary gas volume flow 10 ARB. The scanning mode is full scan / dd-ms2, the acquisition range is m / Z 100 ~ 1500, and the positive and negative ions switch the acquisition mode. Resolution: MS full scan 35 000 FWHM, MS / MS 17 500 FWHM, collision energy: 12.5, 25, 37.5 eV

1.4 LC-MS Data analysis

The raw LC-MS data files were imported into compound discoverer 3.0 software (Thermo Fisher, USA) so that we can obtain the peak data of matching and alignment. The parameters were set as follows: mass range 100-1500, mass deviation 5×10^{-6}; retention time deviation 0.05 min; SNR threshold 1.5. The peak data including retention time (RT), molecular formula, accurate relative molecular weight, and peak area information are imported into Excel. Then we used peak area normalization, and finally, the normalized peak area data were imported into Simca-p V14.1 (umetrics, Sweden) for multivariate

statistical analyses, such as PLS-DA and OPLS-DA. At a subsequent time, VIP > 1 in the s-plot and P < 0.05 in the independent sample t-test were used to select the most significant difference variables as the differential biomarkers. Through the HMDB database and the second-order fragment ions, the differential biomarkers were distinguished and identified. The pathway enrichment analysis was carried out by Metaboanalyst 3.0, and the related metabolic pathways involved in the differential metabolites were analyzed by KEGG online database and related literature reports.

The following figure is the typical total ion chromatograms (TICs) obtained from the ESI positive and ESI negative.

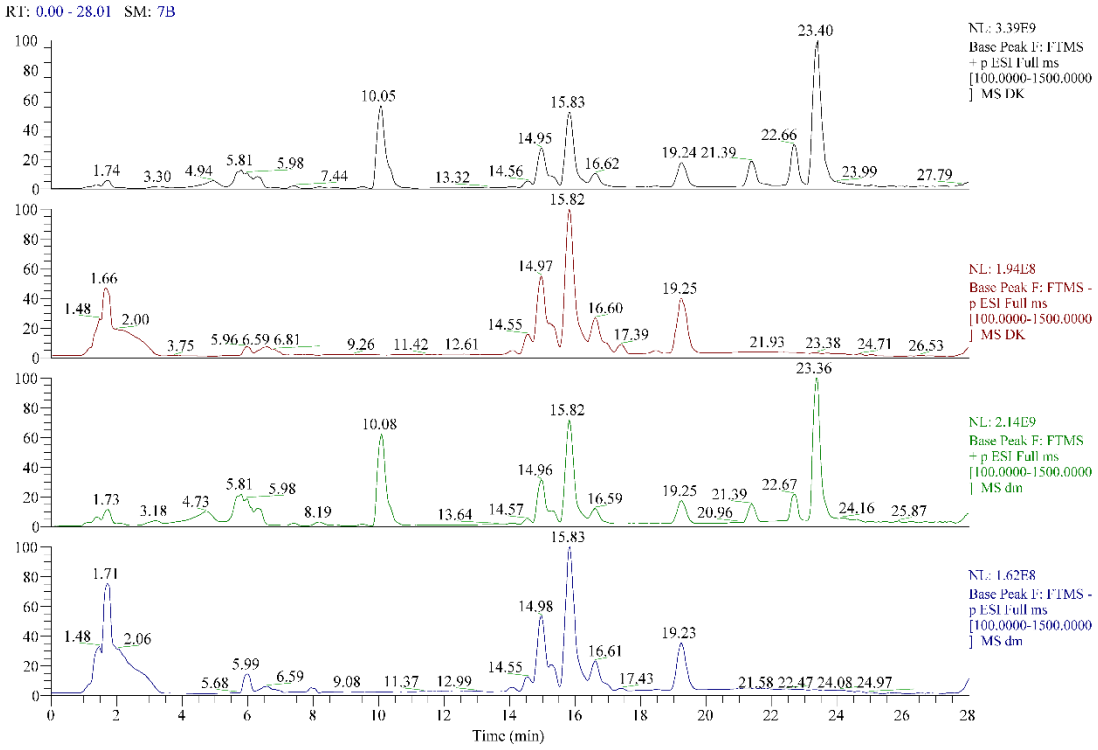

Fig. S1 The typical total ion chromatograms (TICs). (A) The TIC of the control group obtained from the ESI positive. (B) The TIC of the control group obtained from the ESI negative. (C) The TIC of the model group obtained from the ESI positive. (D) The TIC of the model group obtained from the ESI negative.

There are 10 ions extracted from basic peak intensity chromatography of six QC samples for method validation. The RSD of the 10 ions was 5.18 %~15.70 % (Table. S1)

Supplementary Table 1 The stability of LC-MS/MS method using the QC sample (n=6).

No.	RT (min)	m/z	Mean of Relative peak area	SD of Relative peak area	RSD (%)

1	1.58	283.931	167954.6	23480.92	13.98%
2	1.63	367.865	171895.1	19209.64	11.18%
3	1.76	357.835	181183.2	17060.93	9.42%
4	1.77	601.715	149997.6	17700.41	11.80%
5	2.16	280.047	116519.7	17980.21	15.43%
6	5.98	234.042	9319.9	1180.91	12.67%
7	14.58	650.317	157185.6	8137.58	5.18%
8	15.28	725.314	115418.5	6679.65	5.79%
9	15.46	1156.672	160518.3	25202.51	15.70%
10	17.07	720.320	133533.6	9602.09	7.19%

A total of 24 significantly different metabolites were found, the details of differential metabolites in Table S2.

No.	Metabolites	Formula	m/z	RT(min)	VIP	*P*	S-plot	Tread	Scan mode
1	Spermidine	C7 H19 N3	145.1581	1.141	2.0662	0.011*	-0.5039	↓	+
2	Choline	C5 H13 N O	103.1001	1.36	2.2485	0.033*	-0.3208	↓	+
3	Arginine	C6 H14 N4 O2	174.1118	1.375	1.4427	0.045*	0.04269	↓	+
4	Creatine	C4 H9 N3 O2	131.0697	1.432	3.5654	0.007**	-0.1836	↓	+
5	Valine	C5 H11 N O2	117.0792	1.692	2.7610	0.008**	-0.2020	↓	+
6	Acetyl-L-carnitine	C9 H17 N O4	203.116	1.701	4.8862	0.014*	0.3172	↑	+
7	Citric acid	C6 H8 O7	192.0269	1.704	2.0686	0.010*	0.5355	↓	-
8	Hypoxanthine	C5 H4 N4 O	136.0386	1.704	1.3168	0.046*	-0.05244	↑	-
9	Methionine	C5 H11 N O2	149.0512	1.704	1.2744	0.017*	0.3355	↓	+

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10	Xanthine	C5 H4 N4 O2	152.0333	1.704	3.6083	0.005*	-0.2098	↑	-
11	Leucine	C6 H13 N O2	131.0948	1.705	4.0623	0.031*	-0.2681	↓	+
12	Betaine	C5 H11 N O2	117.0793	1.937	3.3577	0.010*	-0.4055	↓	+
13	Uric acid	C5 H4 N4 O3	168.0282	2.261	1.4411	0.024*	0.1766	↓	-
14	Phenylalanine	C9 H11 N O2	165.0792	5.646	4.5457	0.014*	-0.2348	↓	+
15	PEG-4	C8 H18 O5	194.1157	5.75	1.4070	0.029*	0.1028	↓	+
16	Trans-3-Indoleacrylic acid	C11 H9 N O2	187.0635	5.757	4.3739	0.001*	-0.2092	↑	+
17	Tryptophan	C11 H12 N2 O2	204.0901	5.902	2.7782	0.023*	0.5806	↓	-
18	Indole	C8 H7 N	117.0581	5.902	1.1568	0.048*	-0.2397	↓	+
19	Palmitoylcarnitine	C23 H45 N O4	399.335	15.229	1.3430	0.003*	0.5391	↓	+
20	Platelet-activating factor	C26 H54 N O7 P	523.3646	19.241	1.4435	0.030*	-0.5514	↓	+
21	Linoleamide	C18 H33 N O	279.2565	21.377	12.956	0.012*	-0.5937	↑	+
22	Oleamide	C18 H35 N O	281.2721	22.077	12.4575	0.039*	0.3632	↑	+
23	Retinyl acetate	C22 H32 O2	328.2404	23.149	2.0800	0.044*	0.3942	↓	+
24	Arachidonic acid	C20 H32 O2	304.2403	23.468	1.2380	0.001**	-0.4120	↓	+

2. Targeted LC-MS/MS-based metabolomics

LC-MS analysis was performed using a 1290 Infinity binary pumps Liquid Chromatography System (Agilent Technologies) equipped with a Waters ACQUITY UPLC BEH C18 Column (2.1 mm×100 mm, 1.7 μm) with a 3200 Q Trap (AB Sciex) mass spectrometer. The separation mobile phases were a water/formic acid (0.1%) mixture (solvent A) and acetonitrile (solvent B). The elution gradient program for the samples is shown in Table S3. Optimal multiple-reaction monitoring (MRM) transitions were further identified for the analyses of individual AAs (Table S4). Data acquisition and analysis were all performed with Analyst 1.6.3 software (AB SCIEX).

2.1 Sample processing

The serum samples of each group were thawed at 4 ℃, 100 μL of serum was taken out and put into a 2 mL EP tube, add 20 μL internal standard solution and blank matrix, and 200 μL of 0.1% acetonitrile formate (V/V) was added, vortex for 2 min, centrifuged at 13000 rpm for 15 min at 4 ℃, and the supernatant was taken for sample analysis. In addition, we took 10 μL of each serum sample and mixed them. The mixed sample was prepared according to the sample preparation method above, and we get the quality control (QC) samples at the same time.

The hippocampal samples of each group were weighed about 30 mg, and added 10 times the 0.1% acetonitrile formate amount (V/V) of 4 °C, and centrifuged at 13000 rpm for 15 min at 4 ℃ after tissue homogenation. Take 250 μL supernatant in EP tube, add 20 μL internal standard solution and blank matrix. Fully mixed and placed in a freeze centrifugal dryer to blow dry. Finally, 125 μL initial flow phase are used to dissolve the residue, and centrifuged at 13000 rpm for 15 min at 4 ℃ after fully mixed full. the supernatant was taken for sample analysis.

2.2 Liquid phase conditions

The separation was performed on Waters ACQUITY UPLC BEH C18 column (2.1 × 100mm, 1.7 μm). The mobile phases conclude A (water, containing 0.1% formic acid) and B (acetonitrile), and the mobile phase gradient is shown in Table S2. The injection volume was 3 μL. The flow rate was 0.2 ml/min, and the column temperature was 30 °C [20].

Supplementary Table 3 Gradient elution conditions

Time	solvents A (%) 0.1% formic acid + H_2O	solvents B (%) acetonitrile
0	100	0
0.5	99	1
1	98	2
2	95	5
5	90	10
7	40	60

9	100	0
9.5	100	0

2.3 Mass spectrometry conditions

The system was operated at room temperature. The detection was performed after electrospray ionisation in Positive ion mode. Dwell time was kept at 100 ms. The source temperature was maintained at 500 °C and the spray voltage was set at 5500 V. Ion source gas 1, ion source gas 2 and curtain gas were set at 45, 50 and 40, respectively. The MS-parameters were determined and optimised by post-column infusion of neat solutions of the compounds with a syringe pump. Two ions were monitored for each molecule.

Supplementary Table 4 Transition ions and their corresponding deprotonation and collision energies

Compound	Retention time (min)	MRM	DP (V)	CE (V)
Leu	1.85	132.1→86.2	32.00	14.00
Ile	1.57	132.1→86.3	27.50	13.00
Val	1.52	118.3→72.4	23.00	16.00
Trp	8.43	205.3→149.1	31.00	15.00
DHBA	1.48	149 0→121.1	49.00	22.00

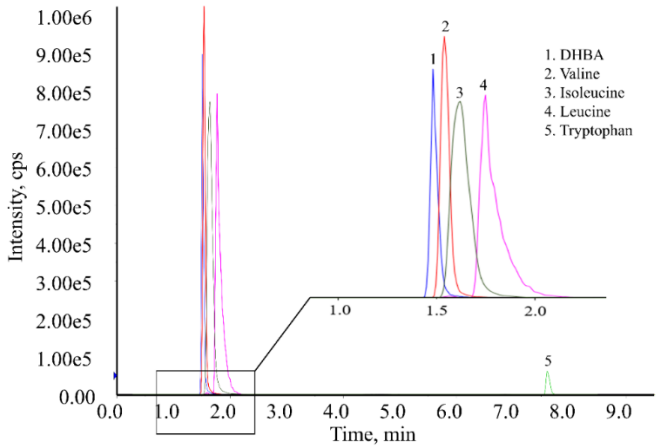

Fig. S2 The chromatographic separation of the selected BCAAs and tryptophan is shown in the chromatogram.

Methodological validation

Preparation of standard solution: 5.0 mg of Leu, Ile, Val, Trp and DHBA were accurately weighed in 5 ml volumetric flasks respectively. Then add 0.1% formic acid water to dissolve them, prepare a stock solution with a certain mass concentration, and prepare a mixed standard solution with a corresponding gradient. Then they would be stored in a refrigerator at 4 ℃ for standby.

The methodological validation mainly evaluated the linearity, precision, repeatability and stability (including the intraday and inter-day stability of samples and standard solution).

The results are as follows: the linearity of the regression equation meets the requirements of $r \geq 0.9900$. The standard curve range of BCAAs and tryptophan in the serum and hippocampus is shown in Table S5-6.

The precision, repeatability and stability results of BCAAs and tryptophan are shown in Table S7-S9 respectively. The RSD values of peak area ratio are less than 10% instability, indicating that the method has good repeatability, and its RSD values of precision and repeatability peak area ratio also meet the requirements of analytical method verification in biological samples.

Supplementary Table 5 Linear equation of BCAAs and tryptophan in seurm

Amino acid	Regression equation	Determination coefficient（r^2）	Linear range （μg/mL）	LLOQ （μg /mL）
Leu	y = 57.408x + 0.3897	0.9988	0.1037-10.3700	0.1037
Ile	y = 86.644x + 0.565	0.9986	0.1037-5.1850	0.1037
Val	y = 55.537x + 0.2277	0.9994	0.0800-3.250	0.0800
Trp	y = 2.1866x + 0.0512	0.9903	0.0837-4.1840	0.0837

Supplementary Table 6 Linear equation of BCAAs and tryptophan in hippocampus

Amino acid	Regression equation	Determination coefficient（r^2）	Linear range （ng/mL）	LLOQ（ng/ mL）

Leu	y = 73.785x + 0.1209	0.9931	10-1555.5	10
Ile	y = 102.12x + 0.1224	0.9930	10-518.5	10
Val	y = 60.329x + 0.013	0.9990	0-1220.4	0
Trp	y = 2.7422x + 0.0037	0.9982	8-1255.2	8

Supplementary Table 7 RSD value of precision detection peak area of BCAAs and tryptophan

Amino acid	precision（RSD %）	
	intraday	inter-day
Leu	4.04	7.91
Ile	4.95	6.79
Val	4.77	6.37
Trp	4.53	8.12

Supplementary Table 8 RSD value of repetitive detection peak area of BCAAs and tryptophan

Amino acid	stability（RSD %）		
	Standard	serum	hippocampus
Leu	7.42	1.26	8.55
Ile	9.27	6.58	8.75
Val	9.60	1.99	2.20
Trp	5.24	2.09	6.93

Supplementary Table 9 RSD value of peak area affecting the stability of BCAAs and tryptophan

Amino acid	stability（RSD %）					
	Standard		serum		hippocampus	
	intraday	inter-day	intraday	inter-day	intraday	inter-day
Leu	10.45	12.46	2.01	3.16	0.66	4.32

Ile	12.57	8.80	7.38	9.57	3.03	7.78
Val	12.41	11.36	3.08	6.32	7.02	4.09
Trp	7.23	7.75	2.80	5.59	10.77	10.14

3. Determine Oxidative Stress Index

To assess the oxidative stress status of hippocampal tissue, we further determined both SOD and MDA by ELISA. Compared with C group, the levels of both SOD and MDA showed significantly increasing in hippocampus of M group. The levels of both SOD and MDA increasing mean that the increasing of oxidative stress in hippocampal tissues.

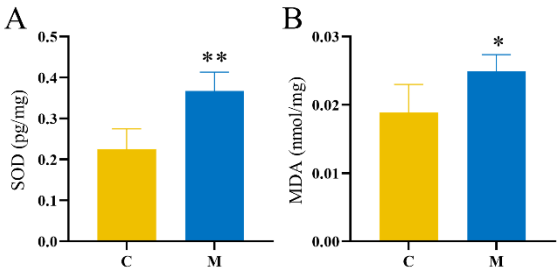

Fig. S3 The levels of SOD (A) and MDA (B) in hippocampus. *p < 0.05, **P < 0.01, compared with the C group (n=6)