Integrative Proteomics-Metabolomics Strategy for Pathological Mechanism of Vascular Depression Mouse Model

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1. Metabolomics & Proteomics Analysis

2. Pathway Integrated Analysis

3. Biomarker Validation

graphic abstract

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Integrative Proteomics-Metabolomics Strategy for Pathological Mechanism of Vascular Depression Mouse Model

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Key words: Proteomics; Metabolomics; Vascular depression; LC-MS

Abbreviations

VD Vascular depression
GCI Global cerebral ischemia
PSD Post-stroke depression
iTRAQ Isobaric Tags for Relative and Absolute Quantitation
SPF Specific pathogen Free
OFT Open-field test
TST Tail suspension test
FST Forced swimming test
UPLC-Q-TOF-MS Ultra-performance liquid chromatography-quadruple-time of flight tandem mass spectrometry
<table>
<thead>
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<th>Term</th>
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<tbody>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>PCA</td>
<td>Principal component analysis</td>
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<tr>
<td>PLS-DA</td>
<td>Partial least squares-discriminate analysis</td>
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<td>IPA</td>
<td>Ingenuity pathway analysis</td>
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<tr>
<td>OEA</td>
<td>N-oleoyl ethanolamine</td>
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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
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<tr>
<td>BCA</td>
<td>Bicinchonininc acid</td>
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<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gelelectrophoresis</td>
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<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
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<td>TIC</td>
<td>Total ion chromatography</td>
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<td>EIC</td>
<td>Extracted ion chromatography</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartic acid receptor</td>
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<td>AMPA</td>
<td>N-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid</td>
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<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
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<td>GO</td>
<td>Gene ontology</td>
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<td>VIP</td>
<td>Variable importance</td>
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<td>MRM</td>
<td>Multiple reaction monitor</td>
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<td>LPT</td>
<td>Long Term Potentiation</td>
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<tr>
<td>NAA</td>
<td>N-acetyl-L-aspartic acid</td>
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<td>NAAG</td>
<td>N-acetyl-L-aspartic acid-L-glutamic acid</td>
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<tr>
<td>NAALADase</td>
<td>N-acetylated alpha-linked-acidic enzyme</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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Abstract

Vascular depression (VD), a subtype of depression, is caused by vascular diseases or cerebrovascular risk factors. Recently, the proportion of VD patients has increased significantly, which severely affects their quality of life. However, the current pathogenesis of VD has not yet been fully understood, and the basic research is not adequate. In this study, based on the combination of LC-MS-based proteomics and metabolomics, we aimed to establish a protein metabolism regulatory network in a murine VD model to elucidate a more comprehensive impact of VD on organisms.

We detected 44 metabolites and 304 proteins with different levels in the hippocampus samples from VD mice using a combination of metabolomic and proteomics analyses with an isobaric tags for relative and absolute quantification (iTRAQ) method. We constructed a protein-to-metabolic regulatory network by correlating and integrating the differential metabolites and proteins using ingenuity pathway analysis. Then we quantitatively validated the levels of the bimolecules shown in the bioinformatics analysis using LC-MS/MS and Western blotting. Validation results suggested changes in the regulation of neuroplasticity, transport of neurotransmitters, neuronal cell proliferation and apoptosis, and disorders of amino acids, lipids and energy metabolism. These proteins and metabolites involved in these dis-regulated pathways will provide a more targeted and credible direction to study the mechanism of VD. Therefore, this paper presents an approach and strategy that was applied in integrative proteomics and metabolomics for research and screening potential targets and biomarkers of VD, which could be more precise and credible in a field lacking adequate basic research.

Introduction

Depression is a common mental health problem in modern society, and the symptoms are mainly manifested as sadness and loss of interest or fun in surrounding activities. Patients often experience guilt, low self-esteem, low sleep quality, loss of appetite, fatigue, and reduction in attention, and sometimes commit suicide. Vascular depression (VD) is a subtype of depression, first proposed by Alexopoulos et al. VD is defined as depression caused by vascular disease or cerebrovascular risk factors. Statistics have shown that
up to 33% of cerebral ischemia patients caused by myocardial infarction will develop depression. The proportion of patients with VD in the total population of patients with depression is increasing, and studies have found that patients with VD accounted for 1/5 of adult depression patients. In addition, VD accounts for 50% of patients with severe depression. Recent clinical studies have shown that the elderly are more prone to VD, resulting in not only an increase in cognitive dysfunction, but also a serious negative impact on the prognosis. At the same time, VD will increase the risk of cerebrovascular abnormalities and even stroke. Population aging, a fast pace of life, and increasing stress have resulted in a high occurrence of cerebrovascular diseases, which also leads to an increased incidence of VD; meanwhile, VD is usually interrelated with high blood pressure disease; therefore, the treatment strategy for VD is more complicated than that of other types of depression. Currently, the vast majority of studies on depression primarily focus on severe depression, and the diagnosis of VD and the corresponding treatment strategy are undefined because of difficulties in the clarification of the relevant mechanisms. Therefore, it is imperative to conduct studies with greater precision and credibility that would benefit future studies on VD, which is a clinically common disease.

Systems biology, which elucidates the physiological state of an organism by integrating biological information at different levels, such as various interactions between genetic elements and proteins, and metabolic and regulatory pathways, is very suitable for establishing early directional contours for a follow-up study. In the post-genome era, system biology focuses on "functional interpretation", and proteomics and metabolomics are its main research approaches. To have a comprehensive and in-depth understanding of complex biological activities of life, proteomic studies are very useful. With the development of liquid chromatography-mass spectrometry technology, modern proteomics techniques, such as quantitative proteomics using iTRAQ markers, have greatly improved the detection abilities and reproducibility, and it has been widely used in a variety of studies on the molecular markers and mechanisms of diseases. Additionally, the metabolites produced during life activities directly and accurately reflect the pathophysiology of organisms. Metabolomics studies all the endogenous metabolites in organisms or cells and reveals their changes and is considered to be an extension and supplementation of genomics and proteomics.
combination of proteomics and metabolomics\textsuperscript{19,21} can provide direct evidence and a clearer explanation for the changes in organisms.

Therefore, this study used bilateral ligation of the common carotid artery to induce global cerebral ischemia (GCI) to build a model of VD in mice\textsuperscript{22,23}. Then, by employing proteomics and metabolomics analyses based on LC-MS, we conducted integrative studies on mouse hippocampus tissue to establish an overall regulatory protein-metabolite network related to VD, and we validated the levels of the molecules involved in the pathways revealed by our bioinformatics study. This whole experimental flow chart is shown in Fig. 1. The results suggest a number of biological pathways associated with VD, which provide a more targeted and more reliable research direction for future study of the mechanisms of VD. Moreover, these biomolecules could be potential therapeutic targets or biomarkers for VD.

Experimental procedures

Chemicals and reagents-Acetonitrile (LC/MS grade) and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Formic acid, niacinamide, L-aspartic acid, hypoxanthine, L-methionine, xanthine, L-phenylalanine, L-arginine, tryptophan, inosine, oleoylethanolamide and other standard chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies were purchased from Abcam Corporation (Abcam, UK). An iTRAQ kit was purchased from AB SCIEX (AB SCIEX, Foster City, CA, USA) and other reagents (analytic quality) were commercially available.

Animal model, behavior studies, and sample collection

Global cerebral ischemic (GCI) Depression Model Building- A total of 57 SPF grade ICR mice (18-20g) were purchased from Shanghai Super-B&K Laboratory Animal Corp. Ltd. and maintained at 20-23°C and a humidity of 40-60%, with a 12h light/dark cycle. Animals had free access to food and water. After one week of adaption to the environment, all mice were randomly divided into two groups: 26 in the sham group and 31 in the GCI model group. We established a model of VD based on a previous model of ischemia-reperfusion injury\textsuperscript{23}. The sham group was treated the same way except the blood supply was blocked. After the operation, the
mouse’s normal body temperature was maintained using an electric heater until the mice could adjust their body temperature. The mice were kept for 3 days to heal the wounds before participating in the behavior tests. The numbers of surviving animals in both the final sham group and the GCI group were 26.

**Behavior tests**- The weight of each mouse was recorded at the same time every two days. An open-field test (OFT) 24, tail suspension test (TST) 25, and forced swimming test (FST) 26 were conducted as described in the previous literature. The results of OFT were evaluated by the total number of horizontal passes and the number of vertical standing times. The TST and FST were examined by the time the animals became immobile. Then, we used SPSS software to analyze the results of the behavioral experiments using independent sample T tests.

**Sample collection and immunofluorescence**- All animals were given anesthesia and immediately decapitated after the behavioral experiments. The hippocampus was dissected out on ice. Among the whole sample of each group, the hippocampus of 6 mice was used for proteomics analysis and Western blot analysis, then 6 were used for metabolomics analysis and 10 for metabolites targeted validation. These samples were quickly frozen in liquid nitrogen and stored at -80 °C for further study. The last 4 samples were fixed in 4% paraformaldehyde overnight for immunofluorescence and then incubated with 30% glucose solution for cryopreservation. The consecutive frozen sections (20 µm) of the hippocampus along the coronal plane were incubated with goat anti-DCX antibodies (Santa Cruz,1: 500) and rabbit polyclonal GFAP (Abcam,1: 1000) overnight at 4 °C. After washing with PBS, the sections were incubated with the corresponding secondary antibody Alexa Fluor594 donkey anti-goat IgG (Abcam,1 : 500) and Alexa Fluor 488 goat anti-rabbit IgG (Abcam,1: 500) and then incubated at 37 °C for 2 h in the dark. The images were observed and acquired under immunofluorescence microscopy.

**Metabolomics analysis**- The metabolomics study was based on previously reported methods 27. The specific experimental procedure is attached in a Supplementary file A. To verify the accuracy of the identities of metabolites whose levels were different between the two groups, we first confirmed the exact molecular weights of these metabolites using an extracted ion flow chromatograph (EIC) and then compared the exact molecular weights with the common online databases, such as the Human Metabolome Database.
We also compared the retention times of some metabolites and their fragments in MS/MS with their standards and fragments under the same conditions and compared MS/MS fragments of other metabolite molecules with the MS/MS fragments in the database above to correctly identify these metabolites. The relative levels of metabolites between the groups are shown in a heat map analysis based on the MetaboAnalyst platform.

**Proteomic Analysis** - The quantitative analysis of iTRAQ proteomics was based on a previous report. Half of each hippocampus of the mice in each group was randomly taken and every 3 half-hippocampus were randomly pooled into one sample in each group. Each group undergo the same pre-process and labeled with 113,114 tags for sham group and 115,116 tag for model group. Samples were analyzed by the eksigent nano LC-UltraTM system tandem TripleTOF™5600 mass spectrometer (AB SCIEX, Foster City, CA, USA). Using an auto sampler, 4 μl of sample was loaded onto a Nano LC trap column (ChromXP C18_CL-3 μm,120A,350 μm *0.5mm) and eluted with solvent A (2% acetonitrile with 0.1% (v/v) formic acid) at 3 μL/min for 15 min and separated on the analyzed column(0.075×150mm, 3μm, 120A) with a linear gradient of solvent A and solvent B (98% acetonitrile with 0.1% (v/v) formic acid) at a flow rate of 300 nL/min over 120 min: 0-0.1 min, 5-10% B; 0.1-80 min, 10-23% B; 80-105 min, 23-35% B; 105-106 min, 35-80% B; 106-110 min, 80% B; 110-110.5 min, 80-5% B; 110.5-120 min, 5% B. Mass spectrometry condition is as follow: using ESI ion source (SilicaTipTM FS360-20-10-N-20-C12,Tip: 10±1 μm, USA.) in positive condition; and ion data in m/z range of 350-1250Da was selectively acquired. Curtain gas: 30psi; atomized gas: 15psi; ion atomization voltage: 2.3kv; ESI temperature: 150°C; accumulative scan time in high resolution scan mode is 250ms; MS/MS spectra of at most 40 precursor are acquired in every cycle time and the charge of fragmentors are +2~+5. iTRAQ adjust rolling collision energy was used during the whole process. Tandem mass spectrometry data were analyzed using an AB Sciex MS data converter (version 1.3) followed by using Mascot (Matrix Science, London, UK; version 2.5.1). The mouse total protein database was downloaded from Uniprot (http://www.uniprot.org/). Peptides and proteins were identified using Scaffold (version Scaffold_4.6.1, Proteome Software Inc., Portland, OR). At the FDR <1%, proteins with at least two identified unique peptides
were considered credible with probability > 99%. The quantitative analysis of peptides and proteins was primarily carried out using Scaffold Q+ (version Scaffold_4.6.1, Proteome Software Inc., Portland, OR) software. First, the sample peaks were iteratively normalized. In this experiment, we used the Mann-Whitney Test to compare the differences between two groups. After the correction for multiple hypothesis test, we found that proteins whose P value less than 0.00833 were much more credible. Finally, we determined the numbers of the proteins whose statistically significant fold changes between groups were above ±1.3. These proteins with differential levels between the groups were then analyzed by Gene Ontology (GO) (http://www.geneontology.org/) for differential protein enrichment at the levels of both the “biological process” and “cellular component”.

**Bioinformatics Analysis of Metabolites and Proteins** - To investigate the mechanism of VD, we performed metabolomics profile analysis and proteomics analysis of differential metabolites by using QIAGEN’s Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) which evaluate the results mainly by two parameters (p value and z-score), p value is based on Right-Tailed Fisher’s Exact Test algorithm, it shows whether the association between a set of meaningful molecules in your experiment and the known process / pathway / transcription comes from random matching , it neither takes into account the effects of molecules nor fold change between molecules in the data set. While z-score evaluate the effect of molecular changes on biological processes, in general, z-score > 2 suggests that the corresponding molecules / functions are significantly activated, Z < -2 thinks that the corresponding molecules / functions are significantly inhibited.

**Validations of the differently expressed proteins and metabolites** - To demonstrate the reliability of the differences in the levels of metabolites between the groups obtained by mass spectrometry, we quantitatively validated 10 metabolites. The specific experimental procedure is attached in the **Supplementary file B. Table 1** shows the optimized MRM parameters for each target metabolite. The standard curves were generated using the standards diluted in 80% ACN. In addition, 100ng of L-chlorophenylalanine was added as an internal standard in other standards except (N-oleylethanolamine, OEA) and samples. Student’s t test was used to analyze the significant differences between the two groups. Among them, the levels of all metabolites were normalized with the internal controls and each sample mass.
Moreover, to verify the accuracy of the results obtained by mass spectrometry in proteomics analysis, we examined some proteins with different levels in the two groups by Western blotting. First, the protein samples (n=3, the rest parts of every two mice was mixed into one sample, six mice randomly divided into three samples.) were prepared the same way as for the proteomics experiment described above. Nine proteins were identified by Western blotting, namely, Camk2a, Ppp1r1a, Prkcb, Gria1, Grin2b, Gria2, Prkcg, Gnaq, and Slc17a7, and the corresponding antibodies (ab92332, ab40877, ab195039, ab109450, ab183942, ab133477, ab108961, ab199533, and ab180188) were from Abcam (Abcam, UK). The experimental procedures were as reported previously. The signals were visualized with Odyssey® Imager (LI-COR, Lincoln, NE, USA), gray scanning analysis were carried out after normalized by internal reference GAPDH and use independent sample T-test to analyze the difference with two groups.

Results

Behavioral analysis- Deceleration of activity, loss of appetite and weight loss seem to be the manifestations of somatic symptoms of depression in mice. The motions of mice slow down. The mouse body weight change curve is shown in Fig. 2A. Within one week of adaptive feeding, there was no difference in body weight between the two groups. However, after the ischemic procedures, the difference was significantly increased with prolongation. At the time point of tissue collection, the body weights of the experimental group were significantly lower than those of the sham group (p<0.001). The OFT was mainly used to measure the spatial exploration behavior of rodents. The results are shown in Fig. 2B. Compared with the sham group, the number of horizontal squares and vertical squares in the experimental group were significantly reduced (p <0.05). The results of TST and FST are shown in Fig. 2C, 2D. The immobility times of the experimental group in these two experiments were significantly higher than that in the sham operation group (p<0.001, p<0.05).

Immunofluorescence Microscopy- The results of immunofluorescence staining of GFAP and DCX in the hippocampus of mice are shown in Fig. 3. GFAP-positive cells were identified as spider-like cells with green fluorescence in the cytoplasm at the excitation wavelength of 488 nm. In the model group, the fluorescence intensity of GFAP was enhanced, the cell size increased, and the protrusion became thinner and elongated. At
the excitation wavelength of 594nm, cytoplasmic red fluorescent DCX positive cells were neonatal neurons: spindle-shaped and with a morphology of migrating cells. These neonatal neurons were located in the hippocampal dentate gyrus area along the edge. These results showed that the number of DCS-positive neonatal neurons in the experimental group was significantly reduced.

[Fig. 3]

Metabolomics analysis- The samples from two groups were analyzed with UPLC-Q-TOF/MS. In this study, we detected a total of 1625 variables, among which 1134 were in positive ion mode and 491 were in negative ion mode. Then these variables were and analyzed with SIMCA software (version 11.0, Umetrics, Umea, Sweden) for multivariate statistical analysis respectively. First, under the unsupervised condition, the PCA score chart (Fig. 4A and 4B) between the two groups was analyzed in the positive and negative ion modes and showed that there were obvious differences between the two groups. We also performed PLS-DA and the two groups were also separated very well under supervised conditions in positive and negative ion modes, as shown in Fig. 4C, 4D. The PLS-DA model was mainly used to detect the fitness of the model. As shown in Figs. 4E and 4F, the model did not appear to over fit. These results suggest that the hippocampus in the model of VD had changed significantly at the metabolic levels compared with the sham operation group. To screen the metabolites with different levels between the groups, we obtain S-VIP diagrams in the positive and negative ion modes using the PLS-DA data model, as shown in Fig. 4G, 4H. The S-VIP diagrams show that the greater the difference between the two groups is, the greater are the differences between the groups. Metabolites were considered to have different levels in the groups when the VIP(variable importance, a value which is show the contribution of the variable to the difference between the groups) value was greater than 1 and considered as a candidate differential metabolite. There were 281 variables changed between two groups and 118 were identified. Finally, by comparing with the standards and their fragments in MS/MS, 44 metabolites were identified, in which 20 were unregulated and 24 were down-regulated with FDR<1% (Supplementary Table S1). The heat map shown in Supplementary fig. S1 shows the differences in the levels of these metabolites between the two groups.

[Fig. 4]
Proteomics Analysis- To explore the changes in protein levels caused by VD, we used isotope-labeled relative and absolute quantification (iTRAQ). In this experiment, we identified 123,204 spectra in the hippocampus samples of the two groups and identified 3690 proteins (Supplementary Table S2). Compared with the sham group, 38 proteins were unregulated (fold change≥1.3, p <0.05) and 162 proteins were down-regulated (fold change≤0.77, p <0.05) with FDR<1%. The results of the enrichment analysis using Gene Ontology (GO) (using both the “cellular component” and the “biological process”) are shown in Supplementary fig.S2. Most proteins identified from the “cellular component” analysis were related to neurons, cell projections, synapses, vesicles, cells and organelles. Biological process enrichment analysis showed that VD was associated with synaptic transmission regulation, neuronal growth, synaptic shrinkage regulation, transport of amino acids, cell endocytosis and other functional regulation of the nervous system regulation process.

IPA analysis- In this study, we analyzed all significantly different metabolites and proteins with IPA analysis. We obtained information regarding “pathway”, “a disease and function”, and “a network” from these metabolites and proteins for better explorations on the internal changes associated with VD. The results of IPA analysis showed that 24 credible signal pathways (| z score |>2,P<0.05) (fig.5A), indicating that the mouse VD caused changes in multiple signal pathways, such as synaptic long term potentiation (p = 4.27E-07), Rac signaling (p = 2.09E-07), calcium signaling (p = 0.024), and pathways associated with immune responses. In addition, fig.5B and C represent significant involvements of these metabolites and proteins in diseases and functions, respectively, such as related to the regulation of neural functions, neurotransmission (p=5.74E-14), transport of L-amino acids (p=2.34E-06), exocytosis (p=7.7E-06), and neonatal death (p=3.99E-04). Moreover, there were some annotations related to the regulation of neurological diseases and behavior, such as seizures (p = 2.43E-06), memory (p = 5.08E-04), and spatial learning (p = 1.18E-04). On the basis of all differential proteins, we categorized the enriched diseases and functions in Table 2. These differentially expressed proteins were involved in cell-to-cell signaling and interaction, nervous system development and function, behavior and neurological diseases, amino acid metabolism, cellular function and maintenance, cell death and survival, lipid metabolism and nucleic acid metabolism. Additionally, to understand their overall biological effects, studying the interactions between these proteins and metabolites is important. Therefore, by using IPA analysis, we
conducted network analysis on these proteins and metabolites, as shown in fig. 6.

**Target validations of the metabolites and proteins**—To verify the changes in biomarkers associated with each pathway and function, we performed targeted MRM validation for some metabolites. The MRM target analysis of metabolites was quantitatively determined by the linear regression equation of peak area and concentration using their respective standard solution, then every metabolites concentration in each sample was calculated by peak area and linear regression equation. The reliability of the test method and the stability of the instrument were evaluated by the precisions of low, medium and high concentrations of the respective standards. We measured a total of 10 metabolites, as shown in Supplementary fig.S3, including nicotinamide, aspartic acid, hypoxanthine, methionine, xanthine, phenylalanine, arginine, tryptophan, inosine and oleylethanolamine. The respective correlation coefficient R and the low, medium and high concentrations of the precision are shown in Table1. After normalized by each sample mass, we compared the concentration of each metabolite between two groups using Student’s t test. The results show that the levels of nicotinamide, hypoxanthine, methionine, xanthine, phenylalanine, arginine and oleoethanolamine in the model group were exactly lower than those in the sham group ($\text{FC(M/C)}<1, P<0.05$). There was no significant difference in the levels of aspartic acid and inosine, but the changes in these metabolites between the two groups were consistent with that in the metabolomics study above.

Western blotting were performed to validate nine proteins, as shown in fig.7. The figure shows that the levels of the nine proteins in the model group were significantly lower than the sham group. These results were consistent with the results of proteomic analysis. These validations confirmed the reliability of metabolomics and proteomics, both at the metabolic level and the protein level, but also indicated that the vascular depressive model causes multiple signaling pathways and functional changes.

**Discussion**

About the mouse model—At present, cerebrovascular disease and depression are often linked together,
especially for the elderly, and depression resulting from vascular risk factors; i.e., VD, is very common. In this paper, we used a mouse VD model in which the bilateral carotid arteries are temporarily blocked. It has been reported that blocking bilateral carotid arteries is directly related to the damage in fragile brain cells such as hippocampus cells, whereas the hippocampus is thought to be involved in the pathophysiological mechanisms of various mood disorders such as depression. Blocking the bilateral common carotid arteries induces cerebral ischemia, resulting in decreased learning and memory in mice that show depressive symptoms. Therefore, this study established a VD model by temporary blocking bilateral common carotid arteries that effectively caused ischemia-induced damage to the hippocampus and symptoms of depression. Currently the evaluation of the vascular depressive model is through assessing changes in body weights and behaviors of the animals. Our results showed that after the surgery, the body weights in the experimental group were lower than those in the sham operation group, and this difference became greater as time went by. The numbers of horizontal and vertical movements in OFT, and the immobility time in TST and FST differed significantly between the two groups, indicating the same trend as reported in the literature. The OFT has been primarily used to evaluate independent behaviors and the ability to explore the new environment of the experimental animals. In FST and TST, if the animals stop active struggling and float in the water or stay still in an inverted position, the animals have been in a mood of disappointment/despair.

**Molecular pathways integration**- Using a combination of metabolomics and proteomics coupled with of IPA software, we found a series of changes in the levels of biomolecules in the hippocampus in mice with VD, and constructed a corresponding pathway diagram (Fig. 8). Vascular risk factors such as ischemia result in direct and indirect abnormal changes in the levels and functions of the protein and metabolites involved in their respective signaling pathways. These changes could be the major reasons accounting for depressive symptoms in mice, including passiveness and depression. The specific mechanisms are as follow:

![Fig. 8](image-url)

**Changes in transport and function of excitatory neurotransmitters**- As a central excitatory neurotransmitter, glutamate participates in information transfer in the central nervous system. Although glutamatergic neurons in some pathological conditions, such as Parkinson's disease, will over-release glutamate, leading to increased
extracellular glutamate levels, and excitotoxicity, studies have reported that the hippocampus from chronic unpredictable depression exhibited significant decreases in the levels of a variety of neurotransmitters, including glutamate. A recent meta-analysis also showed that, compared with the normal group, glutamate levels indeed were significantly reduced in the depression group\textsuperscript{41}. The reason for this is multifaceted, including neurotransmitter endocytosis and efflux, which cannot be ignored. Neurotransmitters are transported to vesicles through transporter proteins, and we found that the levels of two transporter proteins, SLC17A6 and SLC17A7, showed downward trends in the experimental group, indicating that the experimental group had abnormalities in the beginning of the transportation of neurotransmitters. Subsequently, decreases in the levels of SYT1, SYT7 and RIM proteins were observed in the experimental group, among which SYT1 and SYT7 are synaptic binding proteins, and RIM is a synaptic membrane exocytosis protein. All these proteins are involved in the vesicle initialization and spontaneous and induced release of neurotransmitters\textsuperscript{42,43}. After the release of the neurotransmitter, vesicle inward fusions are induced by endocytosis-related protein (AP-2, clathrin) to form hollow vesicles. Meanwhile, after H\textsuperscript{+} acidification and the effects of ATP6v0a1, the vesicles continue to transport neurotransmitters, constituting a complete vesicle transport cycle\textsuperscript{44}. The levels of some key proteins in every step of this cycle were down-regulated in the experimental group, thereby affecting the efficiency of the entire endocytic efflux process and ultimately affecting the delivery of neurotransmitters. On the other hand, reduction in the glutamate receptor protein level is also a factor that cannot be ignored in the occurrence of depressive symptoms. The excitatory effect of glutamate is mainly mediated by activation of AMPA receptors and NMDA receptors; AMPA receptors mainly regulate fast excitatory synaptic transmission, NMDA receptors mainly regulate the specific form of synaptic shrink ability and neuropsychiatric disorders\textsuperscript{45-47}.

More evidence has shown that NMDA receptors have a pivotal role in the transmission of glutamate neurotransmitters in the pathophysiological mechanisms of depression\textsuperscript{48-50}. Reductions in glutamate receptor transporter levels and function lead to decreased glutamate uptake and decreased excitatory effects\textsuperscript{51}. The slowdown in synaptic conduction in depression is due in part to the decrease in the number of glutamate receptors on the synapse\textsuperscript{52}. Therefore, we speculate that the reduction in excitatory neurotransmitter content
and the down regulation of glutamate receptor protein leads to the weakening of the neural excitatory effect, which is an important manifestation of the occurrence of VD.

**Regulation of neural plasticity** - AMPAR and NMDAR are ionotropic receptors, both of which are directly involved in the regulation of synaptic plasticity during the transmission of excitatory neurotransmitters. Among them, the most representative form of synaptic plasticity regulation of the central nervous system is Long Term Potentiation (LTP), which is often associated with mental illnesses such as depression and learning associated with learning and memory. Downstream of the neurotransmitter receptor, multiple proteins in the posterior synaptic dense region interact with each other and maintain the normal physiological function of synapses. CaMKII, an enzyme primarily in the brain, belonging to the calcium-activated serine/threonine protein kinase family, is a key protein downstream of the NMDA receptor signaling pathway, and a core protein in the calcium ion signaling pathway is also involved in the synthesis and release of glutamate, the regulation of ion channels, and the regulation of synaptic retractions. Moreover, CaMKII has a long-lasting enzymatic activity for LTP regulation and is therefore considered to be a molecular switch. The presence of the Ca signaling pathway is an integral part of the normal function of synaptic maintenance in the hippocampus. The transfer of neurotransmitter to the postsynaptic membrane is accompanied by the influx of Ca$^{2+}$ into the membrane through NMDAR, which in turn causes the activation of CaMKII A and the whole signaling pathway. Down-regulations in the levels of a large number of proteins in this pathway in VD inevitably cause serious effects on the normal physiological function of the hippocampus.

In addition to being closely related to synaptic retraction regulation, CaMKII A is closely related to the regulation of cell proliferation, learning and memory, and nerve excitability regulation. PKC is also an important protein in synaptic plasticity regulation, especially the PKC gamma subtype PRKCG (sometimes referred to as PRKCC), which is associated with synaptic plasticity and memory in the hippocampus. Reduced PRKCC expression leads to depressive behavior, and this study also found that VD also showed down-regulation at the protein level of PRKCC, while the levels of multiple proteins in the postsynaptic area associated with long-term potentiation were changed, indicating that passiveness, low mood and other
depression-like symptoms after the global cerebral ischemia are inseparable from nerve plasticity regulation disorders.

**Energy Metabolism**- The process of neurotransmitter transfer and vesicle circulation is an energy-consuming process that requires the mitochondrial energy supply. There is evidence that showed the mitochondria are involved in the regulation of neuronal apoptosis after brain damage. Abnormal functions of mitochondria lead to oxidative stress of mitochondria and the production of reactive oxygen species, resulting in inflammatory responses and neuronal apoptosis, and this phenomenon is primarily manifested in the levels of multiple enzymes involved in the regulation of mitochondrial function. Among them, Ndufs7 and Nduf11 are mainly involved in complex I of the oxidative phosphorylation pathway to provide hydrogen ions. ATP6v0a1 and ATPase are involved in ATP synthesis in complex V of mitochondria. Atpif1 is an ATPase inhibitor, and its increase will significantly inhibit ATPase activity. **On the other hand, energy related metabolites include L-glutamate, citrate, L-aspartate, arginine also decreased in model group. Therefore, when the VD occurs, the changes of these enzymes(Ndufs7, Nduf11, ATP6v0a1, ATPase, Atpif1, ACly, Slc8A2) together with metabolites** lead to mitochondrial oxidative phosphorylation disorder and thereby affect the synthesis of ATP and cause the entire energy metabolism dysfunction.

**Amino Acid Metabolism**- Some amino acids and proteins participate together in homeostasis regulation and, in the central nervous system(CNS), glutamic acid and aspartic acid are most important for maintaining nervous system homeostasis. Under normal circumstances, the postsynaptic membrane stimulated by the excess glutamate neurotransmitter sends feedback signals to the presynaptic membrane to reduce the release of glutamate. The excess glutamate and N-acetyl-L-aspartic acid (NAA) form N-acetyl-L-aspartic acid-L-glutamic acid (NAAG). NAAG is present in the presynaptic membrane and is a major short-term source of glutamate in the synapse. When the receptor protein is down-regulated and the feedback regulation is reduced, NAAG is cleaved into NAA and glutamate by the N-acetylated alpha-linked-acidic enzyme (NAALADase). NAAG, as an endogenous peptide in the mammalian hippocampus, possesses an anti-NMDA receptor effect, and plays a role in blocking the neurotransmitter effect. In this study, the level of glutamate in the VD group decreased and the level of NAAG increased. We speculate that the up-regulation of NAAG exacerbated the conduction and
effect of excitatory neurotransmitters and, due to the occurrence of the disease, negative feedback regulatory
effects were likely not functional. There is a relatively definite view that amino acid metabolism is intertwined,
for example, with some amino acid metabolisms that may contribute to the synthesis of some non-essential
amino acids. 

Arginine, as one of the essential amino acids in mammals, plays an important role in a number of
physiological processes, such as DNA and protein synthesis, removal of reactive oxygen species (ROS),
inhibition of autophagy, cell proliferation, and lipid metabolism. In addition, arginine is also associated
with the regulation of immune function. In the amino acids with altered levels in this study, the level of
arginine in the disease group was 0.85 times that in the sham operation group, undoubtedly affecting the
pathways with the arginine involvement. As shown in fig. 7, among the affected pathways and functions, those
related to individual death of brain cells and the occurrence of neurodegenerative diseases were up-regulated,
and those related to cell proliferation and survival, immune regulation-associated B cell receptor signaling,
nucleotide synthesis, and lipid metabolism were inhibited. These changes are closely associated with the
reduction of arginine level VD.

Conclusion

In this study, we used the method of global cerebral ischemia and reperfusion to establish a murine model
of VD. Integrative proteomics and metabolomics analysis based on the LC-MS platform were used to interpret
the mechanisms of this model for the first time through whole regulatory networks of protein metabolism in the
hippocampus of mice. The results showed that the changes in biomolecules in the hippocampus were mainly
related to the internal imbalance of neurotransmitter transmission in this disease state. On the one hand, the
down-regulations of the levels of excitatory neurotransmitters and their downstream proteins cause a series of
disorders of signaling pathways and neural functions, such as reduced neural plasticity, abnormal Ca signaling
pathways, neuronal cell proliferation and apoptosis imbalance, and all these are closely related to the
occurrence of depression. On the other hand, in the state of VD, we found neurotransmitter endocytosis and
intake compromised abnormalities in the metabolism of amino acids, lipids, and energy, and other functional
disorders and abnormalities.
Acknowledgements

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**Figure Legends**

**Fig. 1** Experimental flow chart.

**Fig. 2** Evaluation of behavior test. (A) Body weight change curve of mice in two groups. (B) Performance of mice in
open-field test. (C, D) Immobility duration induced by GCI in the tail suspension test and force swimming test respectively. Results are expressed as mean ± SD. (*P<0.05 vs. Sham, (**)P<0.01 vs. Sham, (***)P<0.001 vs. Sham.

**Fig. 3** Immunofluorescence staining of GFAP and DCX in the hippocampus of mice. (A, B) immunofluorescence staining of GFAP in Sham group and Model group, respectively. (C, D) Immunofluorescence staining of DCX in Sham group and Model group, respectively.

**Fig. 4** Plots of multivariate statistical analysis of the experimental group in ESI positive and negative ion mode. (A, B) PCA score plot of the sham and model group in ESI positive and negative ion mode respectively; (C, D) PLS-DA score plot of the sham and model group in ESI positive and negative ion mode respectively; (E, F) Permutation test plot of the sham and model group in ESI positive and negative ion mode respectively; (G, H) Scatter coupled with variable importance plot of the sham and model group in ESI positive and negative ion mode respectively.

**Fig. 5** Pathways and Function characterization of all the differentially expressed proteins and metabolites. (A) The creditable canonical pathways. (B, C) Diseases and function annotations. Orange, significantly increase; Blue, significantly decrease. **LTP**: Long term potentiation, **EAJ**: Epithelial Adherens Junctions, **LTD**: Long Term Depression, **AN**: Actin Nucleation, **NPS**: Neuropathic Pain Signaling, **CRH**: Corticotropin Releasing Hormone, **LE**: Leukocyte Extravasation Signaling, **BC**: brain cells, **AA**: amino acid, **ST**: synaptic transmission, **CNS**: central nervous system, **CP**: cellular protrusions, **PMP**: plasma membrane projections.

**Fig. 6** Network analysis of differentially expressed proteins and metabolites altered in VD model group. A-C: The top ranked networks enriched based on part of changed products. Red, significantly increase; Green, significantly decrease.

**Fig. 7** Western blot validations of 9 proteins. (A) The images of western blot. (B, C) Quantification of 9 proteins compared with GAPDH level. Values are means ±SD of three independent experiments. Student’s t test, *p<0.05, **p<0.01, ***p<0.001 compared to sham group.

**Fig. 8** Molecular profiling showing the proposed mechanisms underlying global cerebral ischemia(GCI) induced vascular depression (VD). Box: protein; Circle: metabolite; Blue: Decreased in model group compared with shame group; Red:
Increased in model group compared with shame group:  Grey: Undetected in this experiment.

**Fig. S1** Heat map based on the relative levels of potential marker metabolites in hippocampus of mouse in VD.

**Fig. S2** Gene Ontology(GO) analysis of different expressed proteins in vascular depression in mouse model.(A) The 40 top-ranked cellular component annotations. (B) The 40 top-ranked Biological Process annotations.

**Fig. S3** Extracted ion Chromatogram(EIC) from10 metabolites MRM analysis. **A-I:** EIC of niacinamide, L-aspartic acid, hypoxanthine, L-methionine, xanthine, L-phenylalanine, L-arginine, L-Tryptophan and inosine respectively. **J:** EIC of IS from metabolites MRM analysis. **K:** EIC of oleylethanolamide. **Upper window:** EIC from each standard, **Middle window:** EIC from each sham group, **Lower window:** EIC from each model group.
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FC: fold change; M: model; S: sham; PRE: precursor; PRO: product ion; CE: collision energy; LC: low concentration; MC: medium concentration; HC: high concentration

Table 2 Diseases or Functions Annotation associated with all significantly changed proteins

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Figure 1

**Discovery**

- Sham
- Model

- Metabolomics Analysis (6 samples)
- iTRAQ Proteomics Analysis (6 samples, one half)

**Validation**

- Sham
- Model

- Targeted Metabolomics (10 samples)
- Western Blot (6 samples, the rest)

**Ingenuity Pathway Analysis**
Figure 3
Figure 4
Figure 6

A

B

C

- Chemical/metabolist
- Transmembrane Regulator
- Cytokine
- Transporter
- Chemical/metabolist
- Enzyme
- Ion Channel
- Complex/Group
- Other

- Phosphatase
- Kinase
- Inhibits and acts on
- Inhibition, ubiquitination
- Translocation
- Activation
- Direct interaction
- Indirect interaction
Figure 8
Figure S3