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An integrated targeted metabolomics and network pharmacology approach to exploring the mechanism of ellagic acid against sleep deprivation-induced memory impairment and anxiety

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Abstract

Background: As a neuroprotective agent, ellagic acid (EA) is extremely beneficial. Our previous study found that EA can alleviate sleep deprivation (SD)-induced abnormal behaviors, although the mechanisms underlying this protective effect have not yet been fully elucidated.

Objective: An integrated network pharmacology and targeted metabolomics approach was utilized in this study to investigate the mechanism of EA against SD-induced memory impairment and anxiety.

Methods: Behavioral tests were conducted on mice after 72 h of SD. Hematoxylin and eosin staining and nissl staining were then carried out. Integration of network pharmacology and targeted metabolomics was performed. Eventually, the putative targets were further verified using molecular docking analyses and immunoblotting assays.

Results: The present study findings confirmed that EA ameliorated the behavioral abnormalities induced by SD and prevented histopathological and morphological damage to hippocampal neurons. Through multivariate analysis, clear clustering was obtained among different groups, and potential biomarkers were identified. Four key targets, catechol-*O*-methyltransferase (COMT), cytochrome P450 1B1 (CYP1B1), glutathione S-transferase A2 (GSTA2), and glutathione S-transferase P1 (GSTP1), as well as the related potential metabolites and metabolic pathways, were determined by further integrated analysis. Meanwhile, in-silico studies revealed that EA is well located inside the binding site of CYP1B1 and COMT. The experimental results further demonstrated that EA significantly reduced the increased expression of CYP1B1 and COMT caused by SD.

Conclusion: The findings of this study extended our understanding of the underlying mechanisms by which EA treats SD-induced memory impairment and anxiety, and suggested a novel approach to address the increased health risks associated with sleep loss.

Keywords

Targeted metabolomics, network pharmacology, ellagic acid (EA), memory impairment, anxiety

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Introduction

Sleep is imperative for maintaining healthy body function, both in terms of quality and quantity.[1] Particularly, as an essential component of routine brain function, sleep plays a crucial role in metabolic homeostasis, energy restoration, synapse structure related to learning, and neuronal

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reactivation.[2] Sleep deprivation (SD) is caused by suboptimal work timetables, around-the-clock lifestyles and psychosocial pressure collectively contributing to sleep pathology, and even impairing neurocognitive function.[3] Moreover, the long-term definition of SD is usually found in contemporary society and is involved in the ordinary definition of SD.[4] A varied spectrum of extensional SD patterns has been widely studied in vivo and are connected with brain sensitivity decreasing to hypermetabolism, neurotoxicity, emotional disturbances and systemic immune system damage.[5] Furthermore, the hippocampus is a brain structure especially vulnerable to SD in terms of morphological and functional aspects.[6] Diverse reports argue that 72 h of SD causes cognitive impairment by decreasing cells' proliferation in the hippocampal formation's dentate gyrus (DG) by as much as 50%.[7] In spite of the fact that general cognitive function can be recovered after subsequent sleep, chronic loss of sleep leads to a number of neurobiological alterations that accumulate over time, leading to adverse health consequences.[8] It is therefore desirable to identify natural compounds that could alleviate SD's detrimental effects.

Polyphenols, which are products of plant metabolism, may reduce the risk of age-related neurodegeneration, according to several epidemiological investigations.[9–11] Ellagic acid (EA) is an active component of natural polyphenols that can also be produced in nuts, fruits, and plants. A variety of pharmacological properties are associated with EA, including antioxidant, neuroprotective, and anti-inflammatory activities.[12,13] Our previous research demonstrated that EA could ameliorate anxiety and memory impairment by reducing inflammatory responses and oxidative stress in sleep-deprived mice.[14] The mechanisms and targets of EA against SD-induced memory impairment and anxiety, however, have yet to be fully identified. Prominently, there is considerable evidence that dietary polyphenols undergo extensive metabolism following ingestion, and the constituents acting at the cellular and tissue level will, in most cases, be metabolites instead of native polyphenols.[15]

Metabolomics, mainly including targeted metabolomics and untargeted metabolomics, offers a novel perspective for studying diseases' multifactorial mechanisms and for assessing drug effects from a holistic and comprehensive standpoint.[16] Nevertheless, the terminal variation of treatment and disease could only be reflected by targeted metabolomics.[17] There is a lack of clarity around the endogenous mechanisms that govern metabolite changes, such as how these metabolites are produced, what are their upstream and downstream proteins and pathways, and through which key proteins EA exerts its effects. Network pharmacology combines the ideas of systems biology and multidirectional pharmacology to analyze the mechanism of action of drugs by constructing a complex network among drugs, targets, and diseases.[18] However, the single computational method relies on public databases and lacks experimental verification, which is the biggest drawback.[19] Thus, the integration of network pharmacology and targeted metabolomics can make up for their respective deficiencies[20] and improve knowledge regarding the therapeutic rule of natural compounds against SD-induced cognitive impairment and anxiety.

In this research, targeted metabolomics was utilized to ascertain the impacts of EA on SD and to discover the leading metabolites. Posteriorly, network pharmacology was performed to analyze the proteins and reactions that modulated the metabolites, as well as the key targets that EA acted on. This research will hopefully elucidate the mechanism of EA in treating memory impairment and anxiety induced by SD.

Materials and methods

Chemical and reagents

Deionized water was obtained utilizing the Milli-Q water purification system (EMD Millipore, Bedford, MA, USA). Methanol (chromatographic grade), acetonitrile, and formic acid were acquired from Fisher Scientific (FairLawn, NJ, USA). All other reagents and solvents were of analytical grade. EA (purity > 95%) was supplied by Xi'an Xiaocao Biological Technology Co. Ltd (Xi'an, Shaanxi, China).

Animals and treatments

C57BL/6J mice (weight: 18–22 g) were supplied by the Fourth Military Medical University's animal care facility. All animals were kept under lab routine conditions (temperature: 25°C ± 2°C, humidity: 55% ± 5%, 12 h lighting) and with access to food and water without restriction. After the 7-day adaptation period, mice were randomly divided into three groups: control group, SD group, and SD treated with EA group (eight animals in each group). EA was administered intraperitoneally (50 mg/kg) daily to the mice, and physiological saline (0.9% NaCl, 10 ml/kg, i.p.) was administered at the same time to the control and SD groups. The study protocol was approved by the Ethics Committee of Animal Experimentation of Chengdu University of Traditional Chinese Medicine (No. CDUTCM-2022-35).

As previously described, the SD model was implemented by applying the modified multiple-platform method.[14,21,22] Following 3 days of SD habituation (only 3 h per day, in order to avoid stress, fear, or anxiety that might impair the startle response in mice in an unfamiliar environment), all groups except the control group were consecutively subjected to SD for 72 h, and behavioral tests were then executed. Finally, the mice were sacrificed for metabolism analysis (Supplemental Figure 1). The mice were freely given food and water throughout the continuous 72 h SD period, while the control group mice were kept in cages only.

Evaluation of SD model and EA effect

Behavioral tests were used to measure the effects of EA on SD-induced memory impairment and anxiety. Exploring

behavior was assessed employing the open field test (OFT) and the elevated plus maze test (EPMT). The spatial learning and memory of the mice were evaluated using the Morris water maze test (MWMT).

Hematoxylin and eosin staining

Mice were anesthetized using an intraperitoneal injection of sodium pentobarbital and instantaneously sacrificed. Following transcardial perfusion with phosphate-buffered saline, the mice in each group were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The brains of coronal parts (20 μm) from the hippocampus were slashed on a cryostat, and stained with hematoxylin and eosin (H&E). Of note, the sections were observed to assess the histopathological structure of the hippocampus under light microscopy (Olympus, Japan) after staining.

Nissl staining

Nissl bodies are very abundant among neurons with strong metabolic functions. Under certain conditions, when neurons are damaged, nissl bodies in their cytoplasm may decrease or disappear, resulting in nerve cell necrosis. Nissl staining is a classic method to observe the state of nissl bodies. Therefore, to observe the morphological changes of hippocampal neurons damaged by SD and the protective effect of EA, nissl staining was performed. Coronal parts of the brains were hydrated in gradients of 95%, 85%, and 70% ethanol for 5 min, respectively. After being stained with 0.1% cresyl violet for 10 min, rinsed with distilled water, dehydrated in gradient alcohol, cleared with xylene, and sealed with neutral gum. Then images were selected utilizing an Olympus VS120 Virtual Slide Scanner.

Sample collection and preparation

The blood samples were gathered into pipes without shaking. Centrifugation was then performed at 4°C and 3500 *g* for 10 min, and the plasma was subsequently centrifuged at 10,000 *g* for 5 min at 4°C. The consequent plasma samples were pipetted into pipes and stored at −80°C. The plasma samples were thawed at ambient temperature before analysis. Then, 30 μL of acetonitrile was added to 10 μL of plasma, vortexed for 60 s, and centrifuged for 2 min at 13,200 *g* at 4°C to taken the suspension. The protein precipitation process was repeated three times, then the sample was transferred to an autosampler vial with a 250 μL insert tube for testing.

Chromatographic analysis

The mouse blood samples were quantitatively analyzed with high sensitivity by a tandem quadrupole mass spectrometer (UPLC XEVO TQ-S, Waters, USA). Chromatographic separation was executed on an UPLC RP Column (2.1 mm × 150 mm, 1.8 μm). The analytical column temperature was sustained at 40°C, and the flow rate was 0.6 mL/min. The mobile phase comprised solution A (0.1% formic acid in water) and solution B (acetonitrile and water, 95:5). The gradient elution was optimized as follows: 0 to 0.5 min, 96% to 4% B; 0.5 to 2.5 min, 90% to 10% B; 2.5 to 5 min, 72% to 28% B; 5 to 7 min, 5% to 95% B; and 7 to 9 min, 96% to 4% B. Multiple reaction monitoring analyses were carried out adopting a Xevo TQ-S mass spectrometer. All experiments were executed in positive electrospray ionization mode. The capillary voltage and ion source temperature were kept invariant and set to 2.0 kV and 150°C. The cone gas flow rate, desolvation temperature, and desolvation gas flow were 150 L/h, 600°C, and 1000 bar, respectively. The system was commanded by Masslynx software.

Data analysis and pattern recognition

The ultra performance liquid chromatography-liquid chromatography with tandem mass spectrometry (UPLC-LC-MS/MS) raw data was foremost converted into mzXML data file format and then passed through Skyline. Ultimately, data was imported into SIMCA 14.1 (Umetrics AB, Umea, Sweden) to perform principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA) after data preprocessing. A permutation test (200 iterations) was performed for validating and overfitting the OPLS-DA model. Plasma potential metabolites among the groups were picked following the variable importance for projection (VIP > 1.0) and *P*-value of analysis of variance (ANOVA, $p < 0.05$) in this study. The databases HMDB (http://www.hmdb.ca), METLIN (https://metlin.scripps.edu), KEGG (http://www.kegg.jp), etc., were used to identify the potential metabolites. Then, the potential metabolic pathways were analyzed through applying MetaboAnalyst 4.0.

Network pharmacology construction

To visualize the metabolite–protein–pathway network and to reveal the key metabolites and related proteins, network pharmacology was conducted. First, all of the molecular targets associated with EA were gathered from TCMSP database version 2.3 (http://tcmspw.com/tcmsp.php), the Comparative Toxicogenomics Database (http://ctdbase.org/), and A Bioinformatics Analysis Tool for Molecular Mechanism of Traditional Chinese Medicine (http://bionet.ncpsb.org/batman-tcm/). Second, the therapeutic target genes of SD-induced memory impairment and anxiety were acquired from the Therapeutic Target Database (https://db.idrblab.org/ttd/), the Online Mendelian Inheritance in Man (http://www.omim.org/), GeneCards (https://www.genecards.org/) and a database of gene–disease associations (DisGeNET, http://www.disgenet.org/). Then, the Cytoscape version 3.7.1 was used to constructed a network,

which was considered to be the predicted target of EA against SD-induced memory impairment and anxiety. And a protein–protein interaction (PPI) network was established by STRING 11.0 (https://string-db.org/). Finally, the metabolites–reaction–enzyme–gene network was analyzed by MetScape in Cytoscape to identify the key genes.

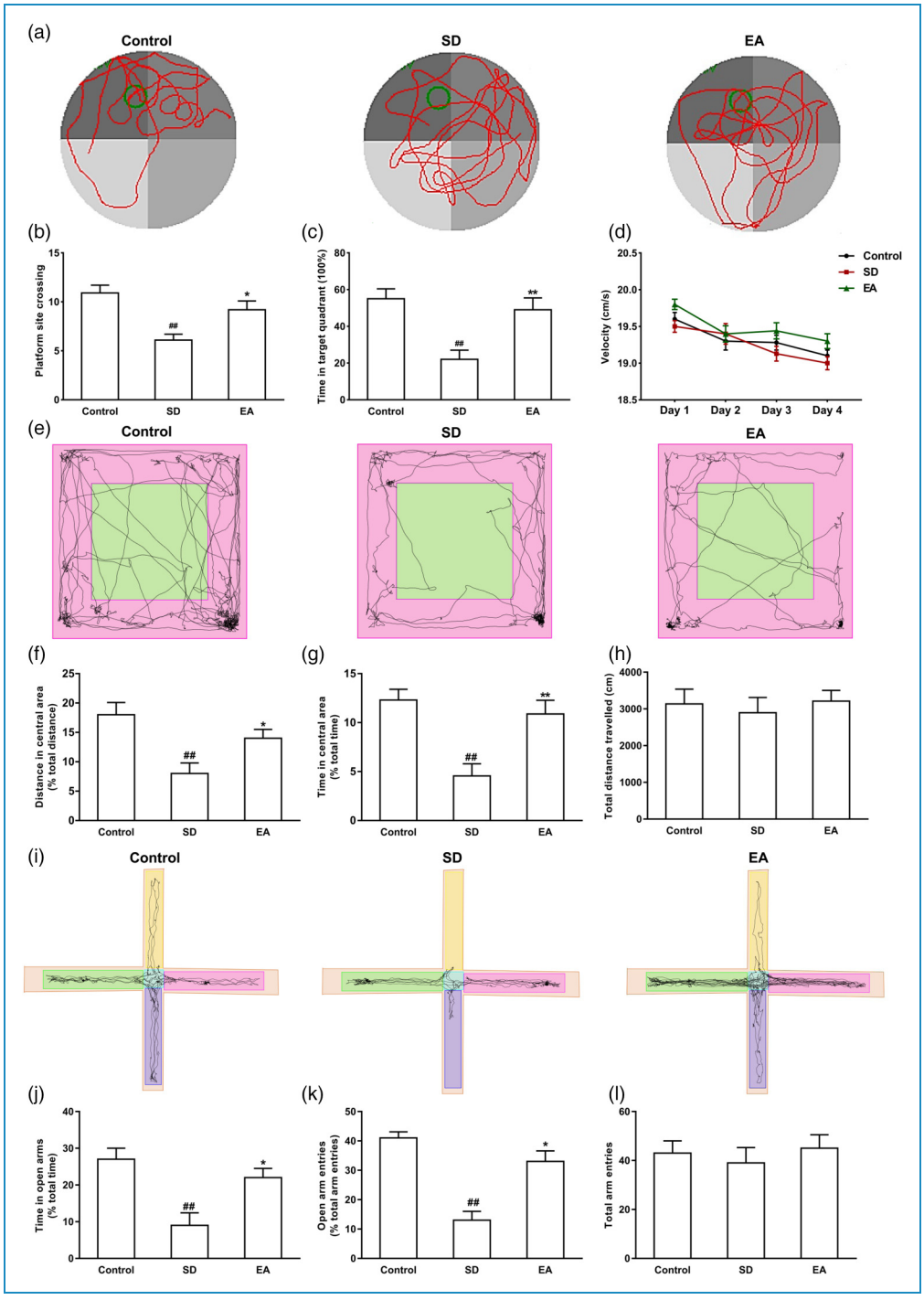

Figure 1. Effect of EA on spatial reference memory and anxiety-like behaviors. (a) Representative swimming tracks in the MWMT during the probe trial. (b) Frequency of crossing the target quadrant during the probe trials. (c) The percentage of time spent in the target quadrant during the probe trial. (d) The swimming velocity of the mice. (e) Sample traces of locomotor activity in the OFT. (f) The total distance traveled and (g) time spent in the center area. (h) The total distance traveled was summarized. (i) Sample traces of locomotor activity in the elevated plus maze test. (j) The time spent in the open arms and (k) entrance into the open arms. (l) The total arm entrances. The results are presented as means ± sd for each group ($n = 8$), $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus SD group.
EA: ellagic acid; MWMT: Morris water maze test; OFT: open field test; SD: sleep deprivation.

Molecular docking

Autodock Vina conducted an in-silico study of the docking of EA with fundamental genes. The three-dimensional model of EA was accessed from PubChem Compound (https://www.ncbi.nlm.nih.gov/pccompound), then converted to PDB file format. The crystal structures of targets were downloaded from the RCSB Web site (http://www.rcsb.org/pdb) in PDB format. By removing water molecules and adding hydrogen atoms, the structures were optimized, and default options for default parameters were selected (non-polar hydrogen combination and $40 \times 40 \times 40$ Grid Box size). The theoretical binding affinities of EA to genes were predicted based on docking scores.

Western blot analysis

The hippocampus was homogenized in an ice-cold RIPA lysis buffer, and the bicinchoninic acid protein assay kits (Jiancheng, Nanjing, China) were used to measure the protein concentration. Then, quantitative protein samples (30 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Subsequently, 5% nonfat milk in 0.1% Tween 20 in Tris-buffered saline was used to block nonspecific binding for 1 h at 37°C. The membrane was incubated with the corresponding primary antibodies (catechol-*O*-methyltransferase (COMT), cytochrome P450 1B1 (CYP1B1), and β-actin) diluted to 1:1000 overnight at 4°C. At room temperature, the membrane was incubated with a secondary antibody (1:5000) for 2 h and then washed three times. Immunoreactive bands were detected by an enhanced chemiluminescence kit and imaged using a Tanon imaging system (Tanon 4200, China).

Statistical analysis

Data analyses were performed using GraphPad Prism 8.0 (GraphPad Prism Software, La Jolla, CA, USA). The results are presented as means ± sd for each group. To assess the differences between the groups, a one-way ANOVA was carried out followed by the Tukey test. Results were considered significant at $p < 0.05$.

Results

EA attenuated memory impairments and anxiety-like behaviors in sleep-deprived mice

The MWMT was developed to evaluate recognition memory and spatial learning (Figure 1(a)). The time in the objective quadrant and the frequency of crossing the platform revealed significant differences between the SD group and the control group, indicating that the sleep-deprived mice could not recollect the objective platform's location ($p < 0.05$, Figure 1(b) and (c)). EA reversed the shorter time in the target quadrant in the SD group, and platform crossing times were also increased with EA ($p < 0.05$, $p < 0.01$, Figure 1(b) and (c)). These results implied that EA could alleviate SD-induced learning and memory deficits. The swimming speed of mice in each group decreased from the second day (the decrease in swimming speed was not obvious on the third day), but there was no significant difference among the groups, which indicated that the mice had complete motor ability without any interference ($p > 0.05$, Figure 1(d)).

In the OFT, the distance moved and the time that was passed at the center location were decreased in sleep-deprived mice, and these results were significantly increased in the EA group ($p < 0.01$, Figure 1(e) to (g)). However, no difference in the travel distance was detected among all three groups, which indicated that the mice had intact locomotor activity without any interference ($p > 0.05$, Figure 1(i) to (k)). Furthermore, the number of entrances into the open arms and the time passed in the open arms were significantly increased in EA ($p < 0.05$, $p < 0.01$, Figure 1(i) to (k)). Similar to the OFT, no distinction in the total entrance to the closed and open arms was detected among all three groups in the EPMT, which indicated that the mice had intact locomotor activity without any interference ($p > 0.05$, Figure 1(l)). All the above results implied that EA could relieve SD-induced anxiety-like behaviors.

EA prevented the histopathological and morphological change of hippocampal neurons in sleep-deprived mice

It is essential for animals to have neurons with a normal function and a definite number. SD induced significant pathological abnormalities with loosely arranged neurons, pyknotic nuclei and loss, or dark color staining in the CA1 regions of the mouse hippocampus (Figure 2(a) to (c)), compared with that in the control group. And EA prevented histopathological changes and significantly increased the number of normal neurons compared to the SD group ($p < 0.01$, Figure 2(d)). Meanwhile, SD caused significant injury to the neuronal structure in the CA3 and DG regions of the mouse hippocampus (Figure 2(e) to (g)). In contrast, most of the EA treatment group mice's neuronal cells were evenly stained light blue and had regularly shaped cell bodies. In the CA3 region, the percentage of injured cells was also significantly decreased by EA ($p < 0.01$, Figure 2(h)). Together, these findings confirmed that EA could restrain morphological damage and histopathological on neurons in the hippocampal regions of mice that are induced by SD.

Metabolic profiling of plasma and multivariate data analysis

Multivariate analysis was used to assess serum metabolic profiles in the control group, the model group, and the

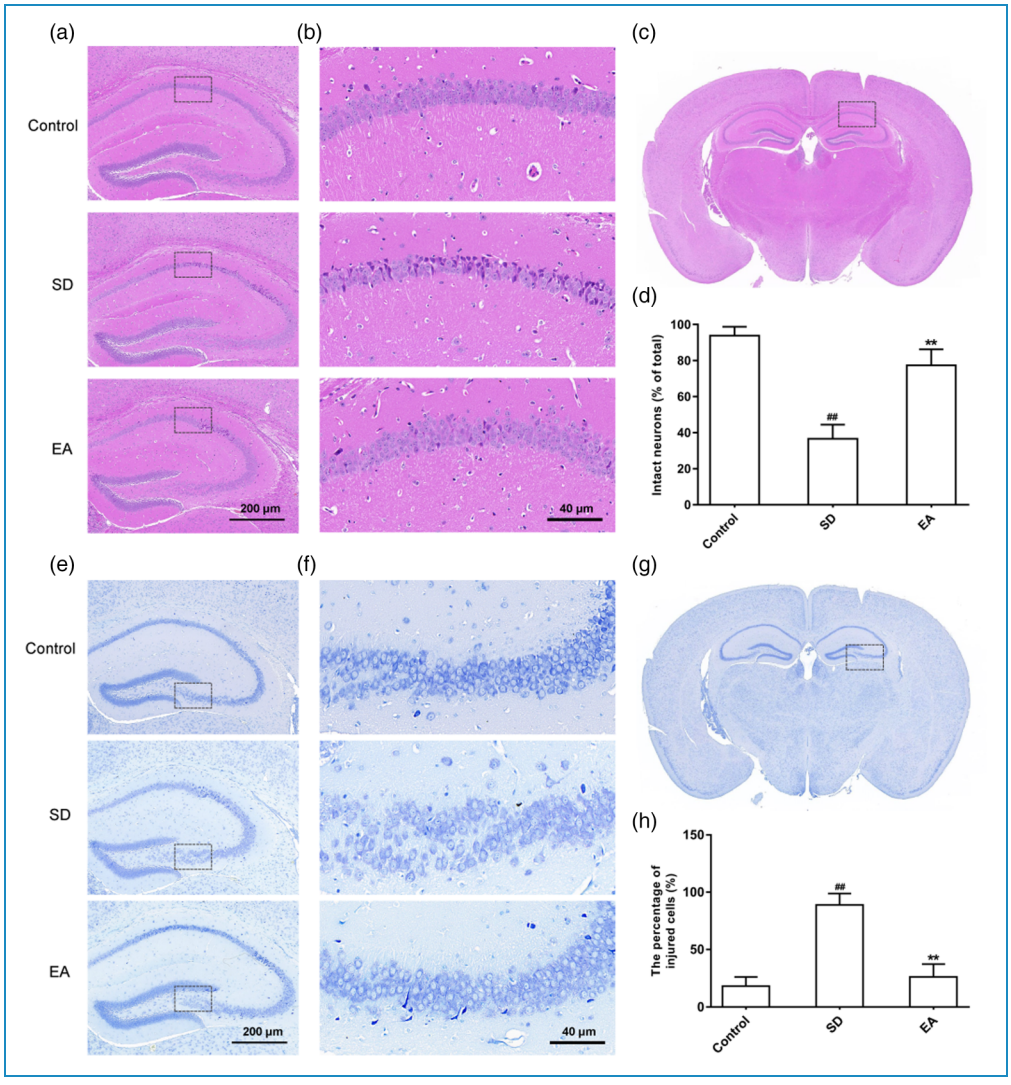

Figure 2. The effect of EA on hippocampal neuronal histopathology and morphology of mice after SD for 72 h. (a) H&E staining of CA1 of the hippocampus. (b) The enlarged regions are within respective rectangular boxes. (c) An illustration of a mouse brain section. The region in the rectangular box was examined using H&E staining. (d) The percentage of intact neurons relative to the total neurons in each group. (e) Nissl staining of the dentate gyrus and CA3 of the hippocampus. (f) The enlarged regions are within respective rectangular boxes. (g) An illustration of a mouse brain section. The region in the rectangular box was stained with nissl staining. (h) The percentage of injured cells in the enlarged CA3 region of the hippocampus. The results are presented as means ± sd for each group (n = 3), $^{\#\#}p < 0.01$ versus control group; $^{**}p < 0.01$ versus SD group.
EA: ellagic acid; SD: sleep deprivation; H&E: hematoxylin and eosin.

EA group. Substantial separations were observed among the control, model, and EA groups in PCA and partial least square discrimination analysis (PLS-DA) score plots (Figure 3(a) to (d)). Additionally, the EA group was much closer to the control group than the SD group, supporting the notion that pretreatment with EA can result in some metabolites returning to their normal levels. A permutation test was used to verify the model to avoid the transition fit of the OPLS-DA mode (Figure 4(a) and (b)). The quantification parameters for this OPLS-DA model ($R^2Y = 0.97$ and $Q^2 = 0.959$) were all positive, which also indicated that the OPLS-DA model was stably established. In addition, 200 times permutation tests showed that the established OPLS-DA model was reliable, and the R^2 and Q^2 values were lower than the original model (Figure 4(c) and (d)). Then the levels of the potential biomarkers were selected based on the OPLS-DA analysis of the S-plots (Figure 4(e) and (f)) and VIP plots (Figure 4(g) and (h)). Potential differential metabolites were identified among the control, SD, and EA groups using a VIP > 1.0 and a p-value < 0.05 in the OPLS-DA models. A total of 10 differential metabolites

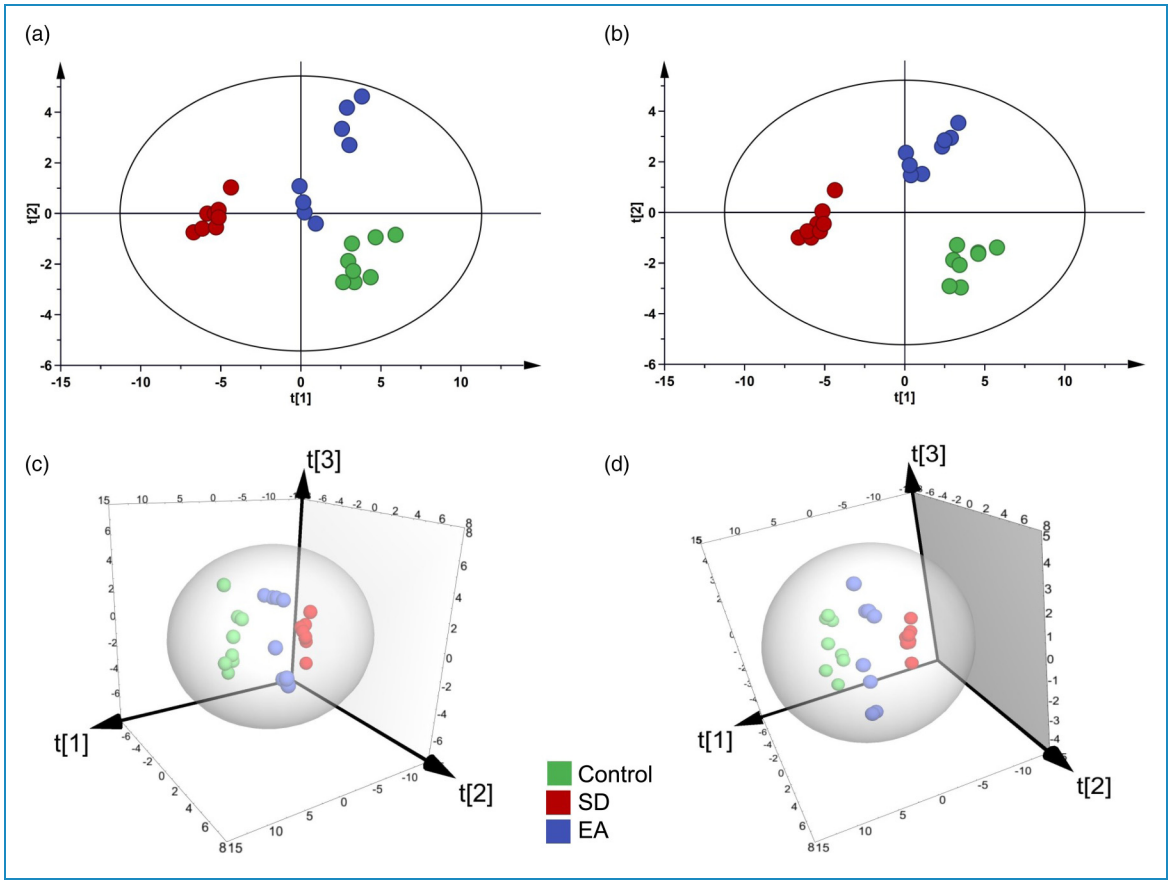

Figure 3. Score plots of plasma in each group based on UPLC-LC-MS/MS. (a) and (b) PCA and PLS-DA 2D score plots of metabolite profiles in the control, SD and EA groups. (c) and (d) PCA and PLS-DA 3D score plots of metabolite profiles in the control, SD and EA groups. Each symbol represents the metabolomics profile of an individual sample. The red circle represents the control group, the red circle represents the SD group, and the blue circle represents the EA group.
EA: ellagic acid; SD: sleep deprivation; PCA: principle component analysis; UPLC-LC-MS/MS: ultra performance liquid chromatography-liquid chromatography with tandem mass spectrometry; PLS-DA: partial least square discrimination analysis; SD: sleep deprivation; 2D: two-dimensional; 3D: three-dimensional.

were identified in the control and SD groups, and 12 in the SD and EA groups.

Identification of metabolites significantly altered

The relations between these differential metabolites were depicted by the heat map of the differential metabolites' hierarchical clustering analysis in varying samples (Figure 5). Furthermore, eight metabolites, including glycine, L-glutamine, taurine, L-alanine, L-citrulline, L-serine, L-glutamic acid, and L-proline, were discovered through searching databases such as HMDB (http://www.hmdb.ca/), KEGG (http://www.kegg.com/), and METLIN (http://metlin.scripps.edu/) (Supplemental Table 1). In contrast to the control group, the levels of glycine, L-glutamine, taurine, L-alanine, L-citrulline, L-serine, and L-proline were significantly reduced ($p < 0.05$, $p < 0.01$), while the L-glutamic acid was significantly increased in the SD group ($p < 0.01$). However, compared with the SD group, the levels of glycine, L-glutamine, taurine, L-alanine, L-citrulline, L-serine, and L-proline were significantly increased ($p < 0.05$, $p < 0.01$), and the L-glutamic acid was significantly reduced in the EA group ($p < 0.01$). Importantly, except taurine, glycine and L-citrulline, the above biomarkers were adjusted to the normal level in the EA group (Figure 6).

Metabolic pathway analysis

On the basis of the potential biomarkers of sleep-deprived mice, metabolic pathways that were most closely connected were discovered utilizing metaboanalyst based on the KEGG database. As stated by the impact value (pathway impact ≥ 0.1), the metabolic pathway analysis revealed that eight pathological processes were associated with EA treatment, including

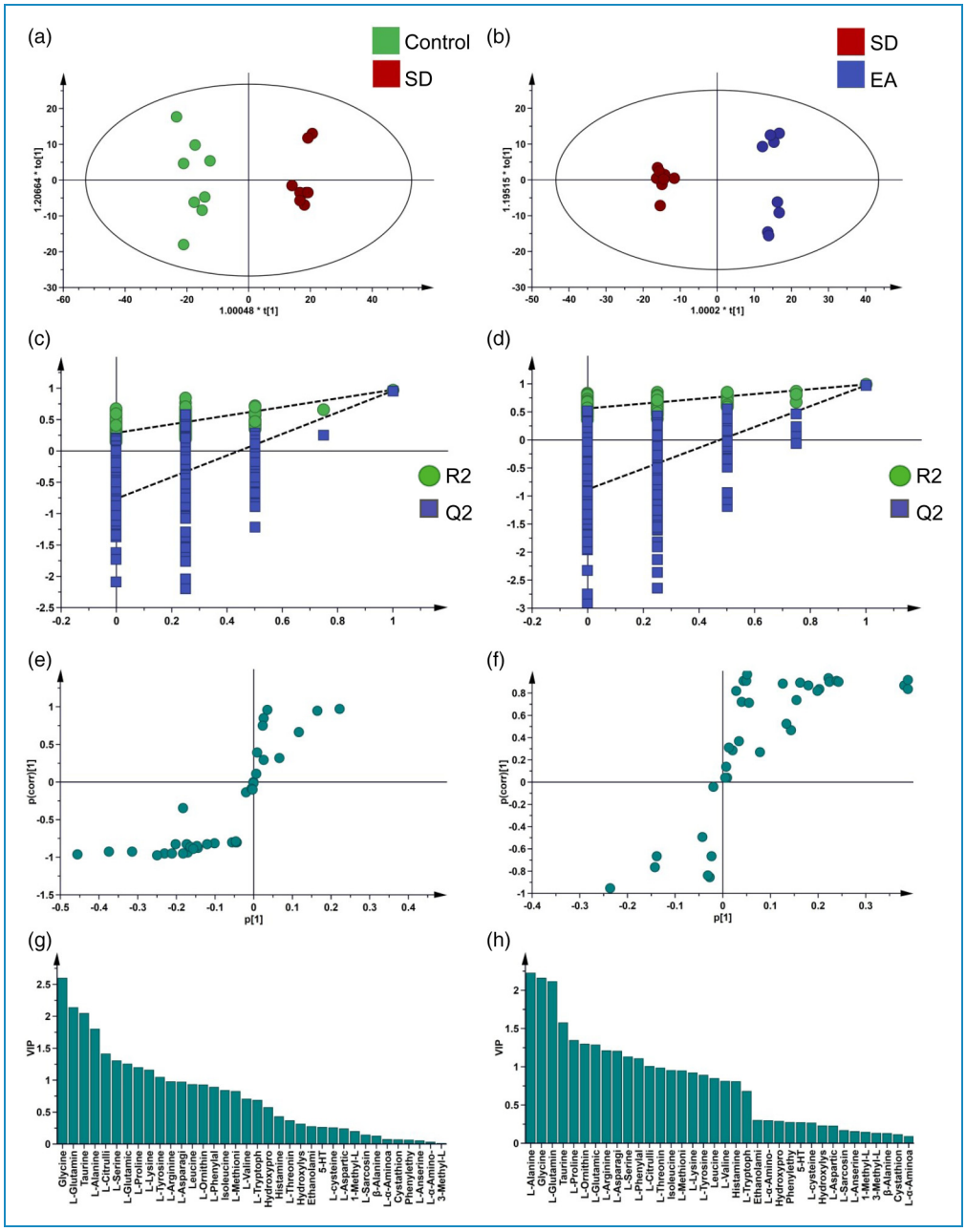

Figure 4. Multivariate statistical analysis of serum metabolites. (a) OPLS-DA score plot for the control and SD groups. (b) OPLS-DA score plot for the SD and EA groups. (c) Permutation test for the validity of the OPLS-DA model for the control and SD groups. (d) Permutation test for the validity of the OPLS-DA model for the SD and EA groups. (e) and (f) The S-plot of OPLS-DA for control group versus SD group and SD group versus EA group. (g) and (h) The VIP value of metabolites in the OPLS-DA for control group versus SD group and SD group versus EA group.

OPLS-DA: orthogonal partial least squares discriminant analysis; SD: sleep deprivation; EA: ellagic acid; VIP: variable importance in the projection.

D-glutamine and D-glutamate metabolism, glycine, serine and threonine metabolism, taurine and hypotaurine metabolism, alanine, aspartate and glutamate metabolism, arginine biosynthesis, nitrogen metabolism, aminoacyl-tRNA biosynthesis and glyoxylate and dicarboxylate metabolism (Figure 7(a)). Synchronically, these substantially changed pathways are related to amino acid modifications, indicating that SD-related metabolic pathways may be highly involved in regulating amino acids (Figure 7(b)). Herein, these metabolic pathways play an integral part in EA's ability to improve SD-induced memory impairment and anxiety.

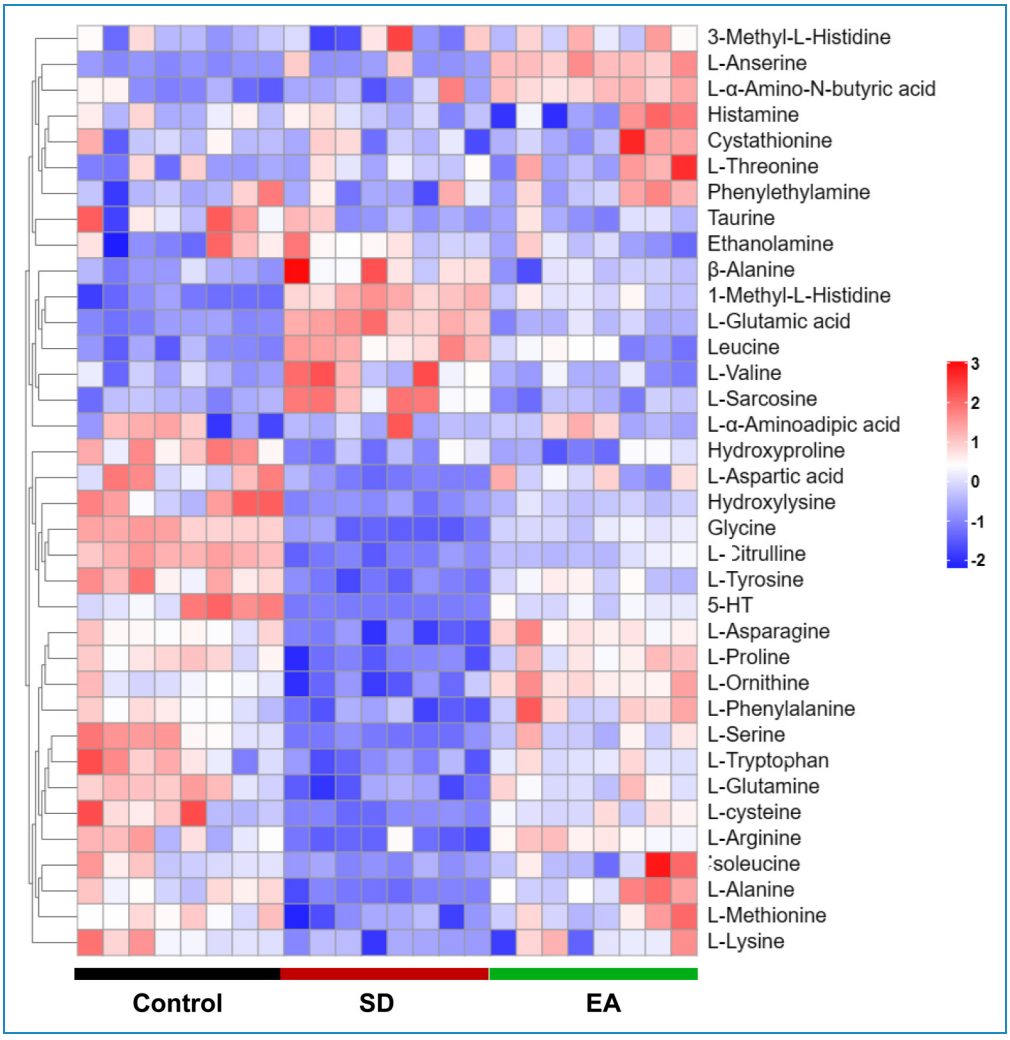

Figure 5. Heat map of the hierarchical clustering analysis of the differential metabolites for the control group versus the SD group and the SD group versus the EA group. Each column represents one serum sample, and each row represents one differential metabolite; the color represents the relative level of the differential metabolite with a gradient from blue (low levels) to red (high levels).
SD: sleep deprivation; EA: ellagic acid.

Key protein network construction and molecular docking analysis

To further explore the mechanisms of EA against memory impairment and anxiety caused by SD, we conducted network pharmacology. A total of 68 EA-related targets (Supplemental Table 2) and 2605 SD-related targets were identified (Supplemental Table 3). Subsequently, as a result of connecting the targets of EA to genes associated with SD, 28 targets were identified, which were considered as potential targets of EA against SD-induced memory impairment and anxiety (Figure 8(a)). All these genes were imported into STRING to obtain PPI relationships. Moreover, 26 genes (the other two genes were disconnected) were considered the key targets that EA against SD-induced memory impairment and anxiety (Figure 8(b)). The metabolites–reaction–enzyme–gene network was constructed based on targeted metabolomics and network pharmacology (Figure 8(c)). By matching the potential targets identified in network pharmacology with the differential metabolites in targeted metabolomics, we found four essential targets, including COMT, CYP1B1, glutathione S-transferase A2 (GSTA2), and glutathione S-transferase P1 (GSTP1), which may play key roles in the therapeutic effect of EA on SD-induced memory impairment and anxiety.

A series of in-silico studies was performed to examine the binding affinity of EA for COMT, CYP1B1, GSTA2, and GSTP1 (Figure 9). The docking scores of EA with fundamental target receptors ranged from −6.2 to −10.7 (Supplemental Table 4). Above all, the molecular docking scores of EA with CYP1B1 and COMT were higher than those with the other two proteins (binding energy < −7 kcal/mol), which indicated that EA was well located inside the interaction site with CYP1B1 and COMT.

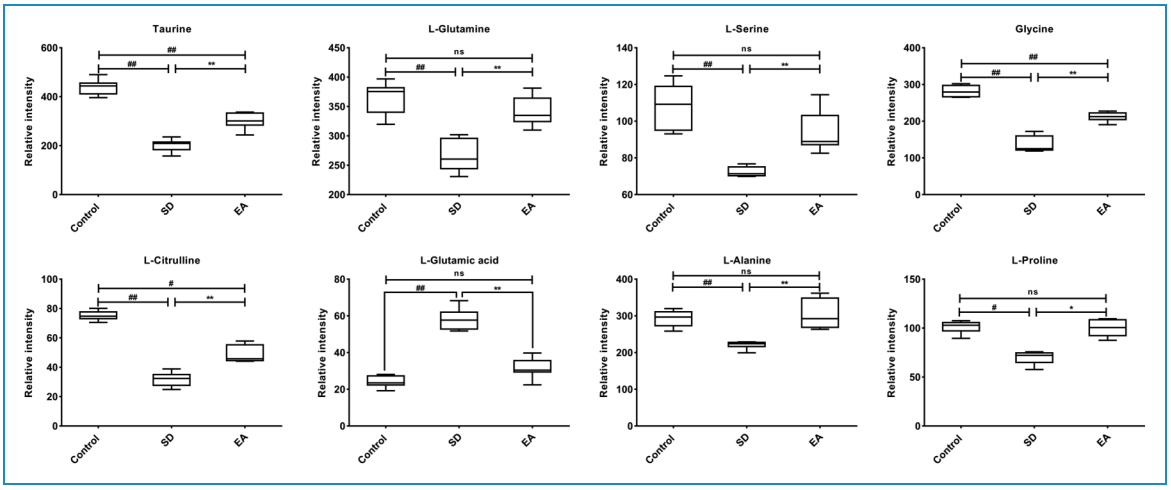

Figure 6. Box plots showed the normalized relative contents of these differential metabolites in different groups. The results are presented as means ± sd for each group ($n = 8$), $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus SD group.

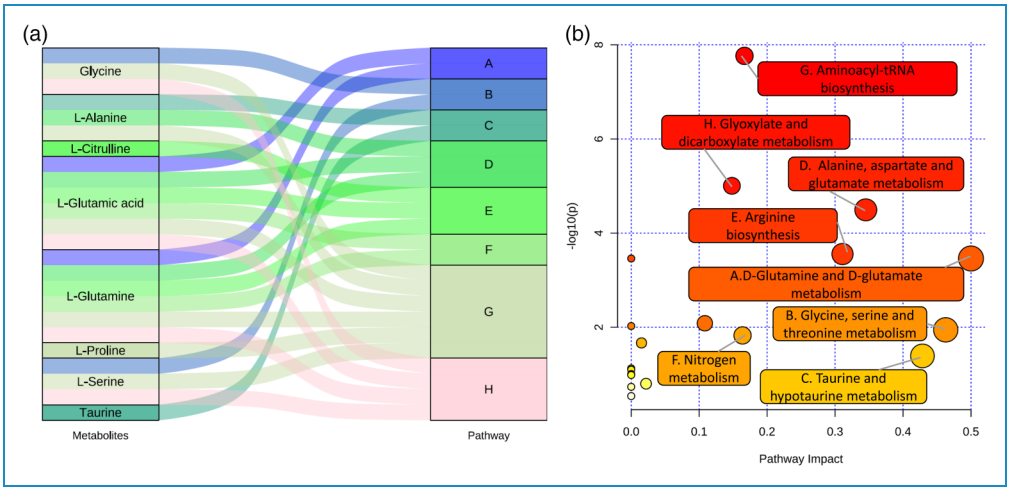

Figure 7. (a) Pathways showing high connections with amino acid metabolism. (b) The topology analysis of the related metabolic pathway.

Validation of hub target proteins

In order to further validate the putative targets for EA against memory impairment and anxiety caused by SD, CYP1B1 and COMT were assayed for validation of the protective mechanism of EA (Figure 10). The western blotting results showed that the expression level of CYP1B1 and COMT was significantly decreased in the SD group compared to the control group ($p < 0.01$). However, EA significantly increased the expression of CYP1B1 and COMT compared to the SD group ($p < 0.05$, $p < 0.01$).

Discussion

SD has been proven to lead to mood-related monoaminergic network fluctuation in the hippocampus and striatum, as well as cognitive impairment and anxiety-like behavior.[23] Furthermore, SD is also known to damage neuronal networks on a physiological, molecular, and synaptic level.[22] Multiple testimony strengthens the opinion that SD is involved in specific metabolome alterations, SD relevant biomarker candidates and protein alterations.[24] Collectively, SD varies across diverse processes of the metabolic network, and intervention with metabolic markers or targets is a crucial approach to ameliorating SD-induced cognitive impairment and anxiety-like behavior.

A dietary-derived phenolic compound with a chemical formula of $C_{14}H_6O_8$, EA is also referred to as a dimeric derivative of gallic acid.[25] Due to EA's poor water solubility and bioavailability, plasma levels of the compound are restricted after consumption of fruits and vegetables (normal plasma concentrations range from 0.1 to 0.4 μmol/L).[26] The research on water-soluble and absorbable EA formulations has, however, been incessantly focused, involving novel formulations (i.e. nanoparticles, liposomes, microemulsions, and polymeric) as well as

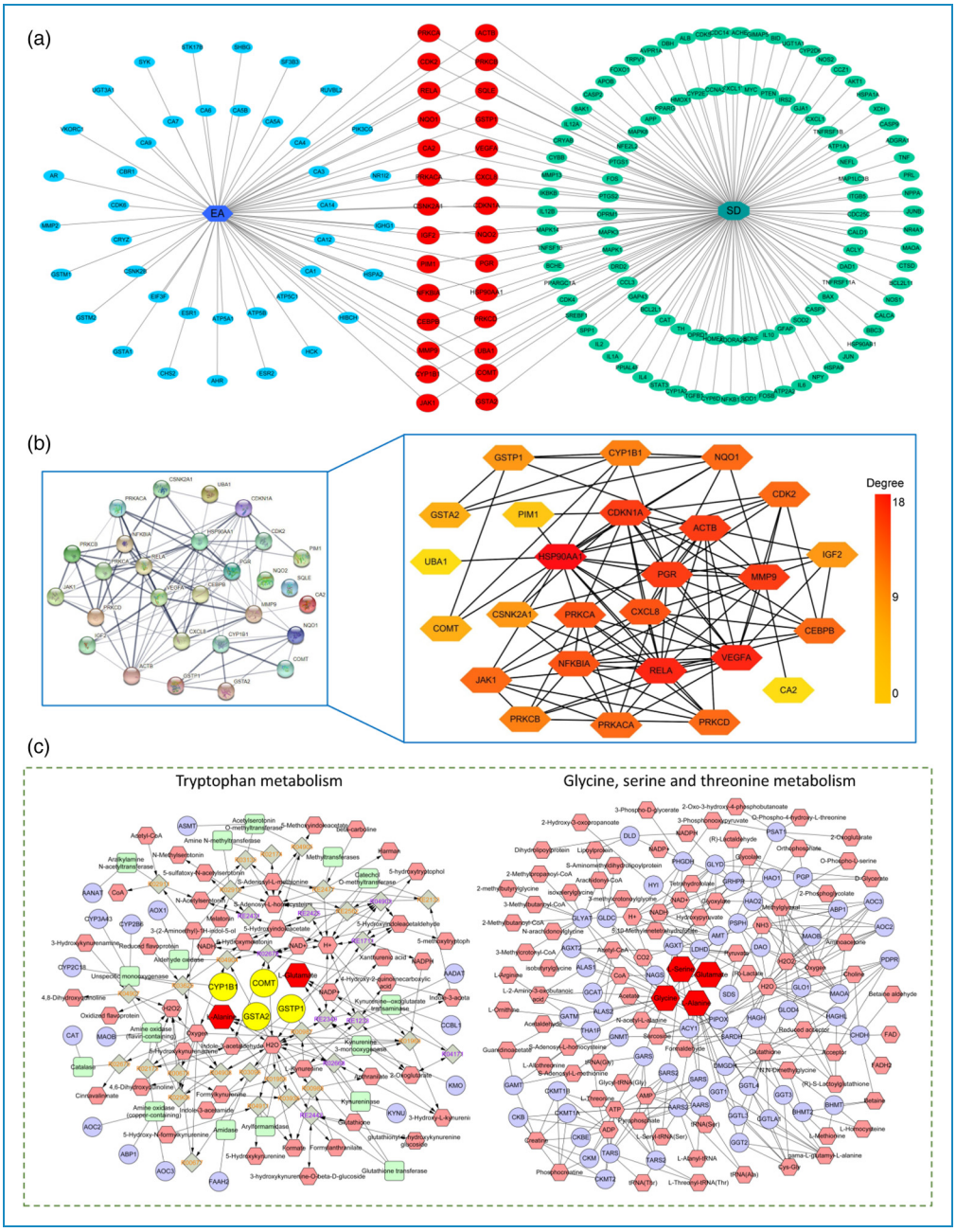

Figure 8. Integrated network pharmacology and targeted metabolomics analysis of EA treating SD-induced memory impairment and anxiety. (a) The network of potential targets of EA against SD-induced memory impairment and anxiety. (b) The PPI network of EA treatment for SD-induced memory impairment and anxiety. Node color reflects its degree. (c) The compound–reaction–enzyme–gene networks of the key metabolites and targets. The red hexagons, green round rectangle and blue circles represent metabolites, reactions and genes, respectively.
EA: ellagic acid; SD: sleep deprivation; PPI: protein–protein interaction.

drug delivery systems (i.e. nanoparticles, liposomes, microemulsions, and polymeric) in order to significantly improve the bioavailability and solubility of EA.[27] Furthermore, EA shows some salient pharmacological properties.[28] Previous reports showed that EA has a neuroprotective effect,[29] and our study also found that EA can improve learning and memory impairments caused by SD. Nevertheless, the interpretation of the action mechanisms of EA remains unclear as a consequence of the complex pathogenesis of SD.

It is noteworthy that in this study, the mechanisms underlying the therapeutic effects of EA on sleep-deprived mice were explored for the first time from the viewpoint of network pharmacology and targeted metabolism. The present research confirmed that EA ameliorated behavioral

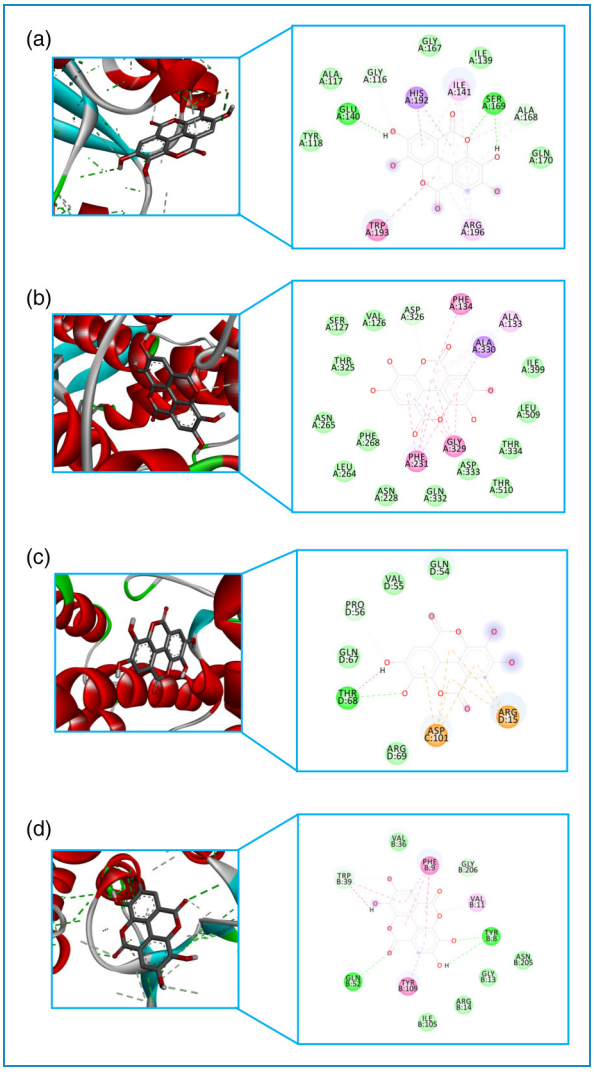

Figure 9. (a) to (d) Structural interactions of EA with COMT, CYP1B1, GSTA2 and GSTP1 receptors in in-silico studies, respectively.
EA: ellagic acid; COMT: catechol-*O*-methyltransferase; CYP1B1: cytochrome P450 1B1; GSTA2: glutathione S-transferase A2; GSTP1: glutathione S-transferase P1.

abnormalities induced by SD, which was in line with our previous study.[14] Meanwhile, EA significantly prevented histopathological and morphological damage to hippocampal neurons in sleep-deprived mice by *H&E* and nissl staining. Consistent with the previous study,[30] the significant metabolic disturbances between SD mice and normal controls were exhibited by the UPLC-LC-MS/MS. Targeted metabolomics was used to detect metabolic differences between SD mice and normal controls and to investigate the metabolic pathways and mechanisms involved in SD. It is interesting to note that the results suggest that EA may possess an important therapeutic function in SD, as it was found to keep sleep-deprived mice near normal levels in the treatment group receiving EA. We found four crucial targets through the combination of network pharmacology and targeted metabolomics, including COMT, CYP1B1, GSTA2, and GSTP1. Particularly, the molecular docking analysis of EA showed that it was well located within the binding site of CYP1B1 and COMT, and the experimental results demonstrated that EA significantly reduced the expression of CYP1B1 and COMT induced by SD.

Amino acids form the basic building blocks of proteins, which in turn constitute the second-largest component of human muscles, cells, and other tissues.[31] As many neurotransmitters are amino acids, variations in amino acid levels in biological fluids have been found to be closely associated with a range of neurological diseases.[32,33] Meanwhile, SD induced the release of neurotransmitters, suggesting that altered amino acid levels are responsible for memory impairment and anxiety.[34] Metabolic analysis indicated that the metabolites of the three groups were similar, but there were some differences. An aggregate of 10 differential metabolites were discovered in the control and SD groups, and 12 in the SD and EA groups. Multivariate analysis showed that eight amino acids, including L-glutamine, glycine, taurine, L-alanine, L-citrulline, L-serine, L-glutamic acid, and L-proline, were potential biomarkers in the established classification model. We also found that the levels of L-glutamine, glycine, taurine, L-alanine,

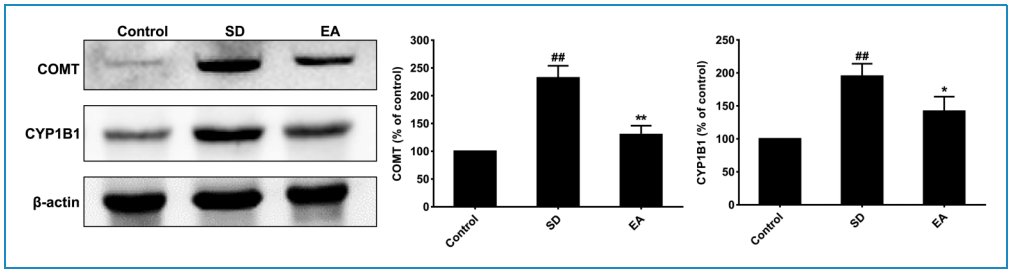

Figure 10. Effects of EA on the expression of CYP1B1 and COMT induced by SD ($n = 5$). $^{\#\#}p < 0.01$ versus control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus SD group.
EA: ellagic acid; CYP1B1: cytochrome P450 1B1; COMT: catechol-*O*-methyltransferase; SD: sleep deprivation.

L-citrulline, L-serine, and L-proline were reduced compared to the control group, while L-glutamic acid was significantly increased in the SD group. Most interestingly, some of the metabolites such as L-glutamine, L-glutamic acid, and taurine have been identified as biomarkers for SD. Nevertheless, compared with the SD group, the levels of glycine, L-glutamine, taurine, L-alanine, L-citrulline, L-serine, and L-proline were significantly increased, and L-glutamic acid was significantly reduced in the EA group. Importantly, except taurine, glycine, and L-citrulline, the other five biomarkers were adjusted to normal level in the EA group. Accordingly, the results can therefore be deduced that EA against SD-induced memory impairment and anxiety may vary with amino acid levels.

Among the eight potential amino acids, L-glutamic acid, being a major excitatory neurotransmitter that can excite all neurons in the central nervous system,[30] was involved in six metabolic pathways, namely, those of D-glutamine and D-glutamate metabolism, alanine, aspartate, and glutamate metabolism, arginine biosynthesis, nitrogen metabolism, aminoacyl-tRNA biosynthesis and glyoxylate and dicarboxylate metabolism. Moreover, excessive amounts of L-glutamic acid can cause excitotoxicity through the *N*-methyl-D-aspartic acid (NMDA) receptor-dependent pathway.[34] A previous study showed a significantly higher increase in glutamic acid levels in the hippocampus after 6 or 12 h of SD compared with non-SD.[35] Interestingly, metabolic analysis revealed that L-glutamine and L-glutamic acid metabolites, involved in the metabolism of D-glutamine and D-glutamate, were highly induced in the SD group, and reduced after the administration of EA. In addition, it has been shown that increasing the level of L-glutamic acid reduces the activity of glutathione peroxidase (GSH-px) and superoxide dismutase, increases malondialdehyde levels, and stimulates reactive oxygen species (ROS) production, while lowering L-glutamic acid levels inhibits oxidative stress injury,[36] which was in line with our prior study that EA exhibited a normalizing effect on oxidative stress parameters levels in SD mice's hippocampus. However, contrary to L-glutamic acid, glycine and other amino acids (including taurine) are inhibitory neurotransmitters, which can improve sleep and memory.[37] Meanwhile, glycine is a potent antioxidant that has the ability to scavenge free radicals. Glycine, L-glutamic acid, and cysteine combined tripeptide, glutathione (GSH), is the main antioxidant in the body. Moreover, GSH is a coenzyme of many enzymes, including GSH-px, that participate in various biological processes to assist in scavenging ROS and preventing oxidative damage to the body.[38]

As part of the energy metabolism pathway, the glycine, serine, and threonine pathway is thought to provide a significant precursor substance to the tricarboxylic acid cycle. Importantly, glycine, as the core amino acid in the pathway, plays an instrumental role in the synthesis of phospholipids and collagen, as well as in the release of energy. Literature studies have shown that glycine levels are dramatically decreased in the serum of major depressive disorder patients.[39] In the same way, glycine was thought to be an inhibitory neurotransmitter capable of combining with NMDA receptor antagonists in order to protect against anxiety and SD-induced cognitive impairment.[40] Aside from being a coagonist of the NMDA receptor, serine is also involved in the synthesis of bioactive lipids such as phosphatidylserine,[41] which promote the release of acetylcholine in the inner leaflet of neural plasma membranes. Besides, tryptophan metabolism is implicated in SD-induced memory impairment and anxiety. The amino acid tryptophan is an indispensable component of protein synthesis as well as the substrate for kynurenine and serotonin. In agreement with earlier reports,[30] a decreased level of L-tryptophan was detected in mice that had been sleep deprived, which may negatively impact brain structure, mood, and cognitive function, whereas EA reversed this abnormality.

The COMT gene is one of the primary enzymes of the metabolic degradation of catecholamines, which has been shown to be closely associated with a variety of mental disorders.[42] COMT catalyzes the transfer of a methyl group from *S*-adenosyl-methionine to a hydroxyl group on a catechol nucleus of major neurotransmitters such as dopamine, epinephrine, and norepinephrine.[43] CYP1B1, a member of the CYP family 1, metabolizes a large number of compounds.[44] CYP family of proteins catalyze many monooxidase reactions, among which mutagenic metabolites and ROS are produced, leading to oxidative stress and cell death.[45] Previous studies have shown that CYP1B1 deficiency protects mice from learning and memory deficits.[46] The results of this study revealed that COMT and CYP1B1 expression in the sleep-deprived mice's hippocampus were significantly up-regulated, while EA treatment significantly decreased these changes, indicating that EA may play a part in improving SD-induced cognitive impairment and anxiety by inhibiting COMT and CYP1B1 genes expression. It remains to be seen if differences in the expression of CYP1B1 and COMT with and without EA treatment are related to metabolic changes. On this basis, more advanced probe technology should be utilized for further verification.

Conclusions

In summary, this study demonstrated the underlying mechanisms of EA's therapeutic effects on sleep-deprived mice for the first time from the perspective of network pharmacology and targeted metabolomics. We identified that EA prevented the histopathological and morphological damage of hippocampal neurons in sleep-deprived mice. By modulating four key targets, as well as relevant metabolites and pathways, EA plays a pivotal role in SD-induced memory impairment and anxiety. Based on molecular docking results, EA

has high affinity for two fundamental targets, and experimental results have confirmed that EA significantly reduces the increased expression of CYP1B1 and COMT caused by SD. Altogether, these findings provide a deeper understanding of the functional relationship between SD and metabolites, as well as the molecular mechanism of EA in improving cognitive impairment and anxiety caused by SD.

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Data availability statement: The original contributions presented in the study are included in the article/Supplemental Material, further inquiries can be directed to the corresponding authors.

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