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Untargeted metabolomics reveals the mechanism by which walnut enzyme hydrolysate mediates sleep improvement in a mouse model of PCPA-induced insomnia

Ziqi Zhang^a, Rui Fan^a, Siting Li^a, Yingmin Jia^a, Junxia Xia^b, Bing Qi^b, Zhou Chen^{a,*}, Aijin Ma^{a, *}^a School of Food and Health, Beijing Technology and Business University, Beijing, 100048, China.^b Hebei Key Laboratory of Walnut Nutritional Function and Processing Technology, Hengshui, 053000, China

ABSTRACT: Walnut, one of the four major tree nuts, has shown promise as a natural sleep aid. However, the majority of these studies have been limited to phenotypic observations and partial target validation, with a systematic elucidation of the underlying mechanisms at the metabolomic level still lacking. This study examined the sleep-promoting effects of walnut enzymatic hydrolysate (WEL) in a p-chlorophenylalanine (PCPA)-induced insomnia mouse model using untargeted metabolomics. Our results demonstrated that WEL significantly increased total sleep time in insomniac mice, with the high-dose WEL group (WEL-H) showing 2.09 times longer sleep duration than the Model group. Furthermore, WEL-H significantly increased the key inhibitory neurotransmitters 5-HT and GABA in insomniac mice by 23.98% and 15.17%, respectively, while significantly decreasing the excitatory neurotransmitter DA by 22.36%. Metabolite set enrichment analysis revealed that the *neuroactive ligand-receptor* interaction pathway, particularly through *GABAergic* signaling, mediated this sleep improvement. These findings were supported by corresponding changes in the expression of key *GABAergic* neurotransmission proteins. In summary, WEL demonstrates significant sleep-improving effects and shows great potential as a novel natural food ingredient for sleep enhancement.

Keywords: Walnut; Enzymatic hydrolysate; Sleep; Metabolomics

1. Introduction

Insomnia affects nearly one-third of the global population^[1], impairing daily functioning and increasing the risk of comorbid disorders. The utility of available pharmacological treatments is limited by their side effects, including excessive daytime sleepiness, cognitive impairment, and drug intolerance, which often preclude long-term administration^[2]. In contrast, bioactive food compounds have emerged as promising alternatives, offering both sleep-enhancing benefits and a favorable safety profile^[3-4]. These natural components improve sleep quality by extending total sleep duration and reducing sleep latency, positioning dietary interventions as a viable strategy for insomnia management.

Recent studies highlight the sleep-promoting potential of natural products such as milk^[5-6] and *Ziziphi Spinosae Semen*^[7-8], along with their bioactive constituents (e.g., peptides and saponins). These compounds

*Corresponding author
zhouCh2017@btbu.edu.cn; aijinma206@sina.com

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effectively improve sleep quality by modulating GABAergic and serotonergic (5-HT) systems while concurrently regulating gut microbiota composition. This dual mechanism significantly prolongs sleep duration, reduces sleep latency, and mitigates sleep deprivation-induced mitochondrial dysfunction. Such findings underscore the therapeutic potential of natural products in sleep regulation.

Walnuts, rich in high-quality proteins and unsaturated fatty acids, are a particularly promising candidate^[9-10]. Their protein fraction contains substantial glutamate (a direct precursor of GABA-the primary inhibitory neurotransmitter) and arginine, which generates nitric oxide (NO), a known sleep regulator^[11]. In addition, Walnuts have a millennia-long history of use as both a health food and in folk medicine. A growing body of evidence from *in vitro*, *in vivo*, and clinical studies demonstrates that they possess multiple bioactivities^[12]. Such as antioxidant, anti-inflammatory, antihypertensive, and neuroprotective effects^[13]. Recent evidence indicates that walnut-derived peptides not only improve sleep but also counteract cognitive deficits caused by sleep deprivation^[4].

Plant-based protein products are increasingly popular due to their natural, nutritious, and eco-friendly profile. Walnuts, in particular, are a valuable ingredient for these products because of their high-quality protein and bioactive compounds^[14]. Nevertheless, their application is limited by two main factors: their high oil content and a predominant insoluble glutelin fraction (70.11%)^[15]. The high oil content leads to poor water solubility, phase separation, and susceptibility to oxidative rancidity, while the high glutelin content further compromises functionality. To address these issues, enzymatic hydrolysis presents a promising solution^[16]. Studies have shown that enzymatic hydrolysis treatment increased the solubility of walnut glutelin by 1.33-fold, water-holding capacity by 0.23-fold, emulsifying activity by 0.32-fold, and emulsion stability by 0.75-fold^[17]. This process offers advantages such as mild reaction conditions, high efficiency, and controllability^[18]. Building upon these functional characteristics, we developed an efficient preparation method for walnut enzymatic hydrolysate (WEL). The process integrates simultaneous degreasing and enzymatic hydrolysis, with subsequent spray-drying, enabling systematic assessment of WEL's sleep-regulating effects.

However, the mechanisms by which walnut enzymatic hydrolysate improves sleep are not yet fully understood, particularly at the metabolomic level. Despite these advances, the mechanisms underlying walnut's sleep-enhancing effects remain unclear. This study employs untargeted metabolomics to identify potential sleep-related biomarkers in WEL, and systematically elucidate its mechanistic pathways. This study addresses this gap by employing untargeted metabolomics to comprehensively map metabolic alterations induced by WEL in a PCPA-induced insomnia mouse model, with a focused investigation into the GABAergic pathway. Our findings provide critical insights that could support WEL's development as a novel therapeutic for insomnia and related disorders.

2. Materials and methods

2.1. Chemicals and reagents

Amylase derived from *Bacillus licheniformis* (2000 U/g, food grade) and trypsin (20000 U/g, food grade) were obtained from Wanbang Chemical Technology Co., Ltd. (Zhengzhou, China). Sodium pentobarbital and p-chlorophenylalanine (PCPA) were purchased from Sigma-Aldrich (Shanghai, China). Tween-80 was acquired from Solarbio Technology Co., Ltd. (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for mouse γ -aminobutyric acid (GABA), dopamine (DA), and 5-hydroxytryptamine (5-HT) were obtained from Jianglai Biotechnology Co., Ltd. (Shanghai, China). GAD65 and GABA-T antibodies were purchased from ABclonal Technology Co., Ltd. (Beijing, China).

2.2 Preparation of Walnut Enzymatic Hydrolysate (WEL)

The walnut enzymatic hydrolysate (WEL) was prepared using an aqueous enzymatic method to concurrently generate bioactive peptides and remove lipids^[19]. Protease and amylase were selected to hydrolyze proteins and residual starch, facilitating oil separation. The optimal conditions (hydrolysis time, enzyme dosages, solid-to-liquid ratio) were determined through single-factor and orthogonal array designs, maximizing both peptide yield and oil removal efficiency. This optimized process yielded WEL with significantly reduced lipid content and increased peptide content. The detailed preparation procedure is as follows:

Walnut kernels were boiled in a 0.2% (w/v) NaOH solution for 10 min to remove the seed coat, followed by pulverization using an herbal medicine grinder at 10,000 r/min for 90 s. The resulting powder was mixed with deionized water at a solid-to-liquid ratio of 1:4 (w/v) and homogenized using a colloid mill for 1 min. Enzymatic hydrolysis was then performed at 50 °C for 4.5 h with the addition of trypsin (1000 U/g substrate) and amylase (40 U/g substrate). After enzyme inactivation, the mixture was centrifuged at 12,000 r/min for 15 min to remove the upper lipid layer. The remaining aqueous phase was homogenized under high pressure (20 MPa, 5-10 min) and subsequently spray-dried at an inlet temperature of 180 °C, with a feed flow rate of 15% and an air flow rate of 100%.

2.3 Compositional Analysis of Walnut Enzymatic Hydrolysate

2.3.1 Protein Content Determination

The protein content of walnut enzymatic hydrolysate was determined according to the Chinese National Food Safety Standard GB 5009.5-2016 ("Determination of protein in foods") using the Kjeldahl method.

2.3.2 Lipid Content Determination

The fat content in walnut enzymatic hydrolysate was measured following the Chinese National Food Safety Standard GB 5009.6-2016 ("Determination of fat in foods") through Soxhlet extraction.

2.3.3 Moisture Content Determination

Moisture content of the walnut enzymatic hydrolysate was analyzed in accordance with the Chinese National Food Safety Standard GB 5009.3-2016 ("Determination of moisture in foods") using the oven drying method.

2.3.4 Carbohydrate Content Determination

The carbohydrate content in walnut enzymatic hydrolysate was determined using the phenol-sulfuric acid method.

2.4 Experimental Animals

Male ICR mice of specific pathogen-free (SPF) grade, weighing 23-28 g, were used in this study and purchased from the Department of Laboratory Animal Science, Peking University Health Science Center (Animal Certificate No.: 110332241100036328). The animals were housed under controlled environmental conditions: temperature maintained at $(22 \pm 2) ^\circ\text{C}$, relative humidity at $(40 \pm 10) \%$, and a 12-hour light/dark cycle. Food and water were provided ad libitum.

Following a one-week acclimatization period, the mice were randomly divided into five groups ($n = 8$ per group). All animals were maintained in the animal facility of Beijing Huayuan Times Technology Co., Ltd. (Animal Use License No. SYXK(Jing)2021-0040). The mice were fed with CO60 sterilized maintenance diet and provided with autoclaved purified water. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Beijing Huayuan Times Technology Co., Ltd. (Ethical Approval No. HYS2024-8).

2.5 Animal Grouping and Experimental Procedures

Following a 7-day acclimatization period, forty mice were randomly allocated into five groups ($n=8$ per group). The groups were designated as follows: Control group, Model group, Low-dose group (WEL-L), Medium-dose group (WEL-M), and High-dose group (WEL-H).

The insomnia animal model was established by referring to previously reported methods with appropriate modifications^[20-21]. With the exception of the Control group, all other groups received daily intraperitoneal injections of PCPA at a dose of 400 mg/kg for three consecutive days. The Control group was administered an equivalent volume of 5% Tween-80 aqueous solution via intraperitoneal injection. Successful model establishment was confirmed by the observation of circadian rhythm disruption and a declining body weight trend in PCPA-treated mice, exhibiting significant differences compared to the Control group.

The dosage was modified based on the protocol reported by Yi et al^[22]. Based on a daily intake of 5 g of walnut enzymatic hydrolysate for a 60 kg adult, the corresponding doses for mice were calculated according to body weight and surface area, resulting in 1000 mg/kg (medium-dose group), with additional groups of 2000 mg/kg (high-dose group) and 500 mg/kg (low-dose group) to establish a concentration gradient for investigation. All treatments were administered via oral gavage between 8:00-9:00 AM for 9 consecutive days with the following regimens:

Control and Model groups: equivalent volumes of distilled water

WEL-L group: 500 mg/kg WEL in distilled water

WEL-M group: 1000 mg/kg WEL in distilled water

WEL-H group: 2000 mg/kg WEL in distilled water

2.6 Pentobarbital Sodium-Induced Sleep Potentiation Test

Thirty minutes after the final administration on day 8, all mice received an intraperitoneal injection of pentobarbital sodium (60 mg/kg) for the sleep potentiation test. Sleep duration was quantified by monitoring the loss and recovery of righting reflex according to the following parameters: (1) Time of pentobarbital sodium injection; (2) Sleep onset time: defined as loss of the righting reflex within 60 seconds; (3) Sleep termination time: defined as recovery of the righting reflex within 30 seconds.

The total sleep duration was calculated as the time period between persistent loss and subsequent recovery of righting reflex. To minimize observer bias, all behavioral tests were conducted under double-blind conditions. All observations were conducted under standardized environmental conditions to minimize external disturbances.

2.7 Sample Collection

Two hours post-administration is a commonly used time point for neurotransmitters and metabolites to reach peak or steady-state levels^[23]. Sample collection was performed 2 hours after the final administration on day 9. Blood samples were obtained via orbital puncture and allowed to clot at room temperature for 20 minutes. Serum was separated by centrifugation at 3,000 r/min for 20 minutes, immediately flash-frozen in liquid nitrogen, and stored at -80 °C for subsequent biochemical analyses.

Following blood collection, whole brains were rapidly dissected, flash-frozen in liquid nitrogen, and maintained at -80 °C for future biochemical determinations. All tissue processing was completed within 2 minutes of sacrifice to ensure sample integrity.

2.8 Histological Examination

Brain tissues were fixed in 4% paraformaldehyde solution. Tissues were subsequently removed from the fixative and trimmed to the required dimensions. Following dehydration, embedding, sectioning, and staining, the tissue blocks were examined microscopically. Quantitative analysis was performed on images at 100× magnification using Image J software, and statistical methods were employed to analyze the pathological features of the damaged brain tissue^[24]. Randomly selected fixed regions were used to count all morphologically intact neurons. The criteria for morphological integrity were defined as: nuclei being round or oval in shape, clearly stained, with visible nucleoli, and relatively intact cytoplasm and cell boundaries. Cells exhibiting nuclear pyknosis, karyorrhexis, or indistinct cellular contours were excluded.

2.9 Determination of Serum 5-HT Levels

Frozen serum samples stored at -80 °C were thawed on ice and processed according to the manufacturer's protocol provided with the 5-HT ELISA kit. All procedures were performed under chilled conditions to maintain analyte stability.

2.10 Analysis of GABA and DA Levels in Brain Tissue

Brain tissue specimens preserved at -80 °C were thawed on ice and homogenized in ice-cold PBS (1:9, w/v) using pre-chilled grinding beads in a cryogenic homogenizer. The homogenization protocol consisted of two 60-second cycles at 60 Hz, followed by centrifugation at $5,000 \times g$ for 10 minutes at 4 °C to obtain the supernatant. Subsequent quantification of GABA and DA levels was performed strictly according to the manufacturer's instructions for the respective ELISA kits.

2.11 Untargeted Metabolomics Analysis of Mouse Brain Tissue

2.11.1 Metabolite Extraction

Following thawing at 4 °C, tissue samples were homogenized in pre-chilled methanol/acetonitrile/water solution (2:2:1, v/v/v) using vortex mixing and low-temperature ultrasonication for 30 min. After incubation at -20 °C for 10 min, samples were centrifuged at 14,000 g (4 °C) for 20 min. The supernatant was vacuum-dried and reconstituted in 100 μ L acetonitrile/water (1:1, v/v) for mass spectrometry analysis. The solution was vortexed, centrifuged again at 14,000 g (4 °C) for 15 min, and the final supernatant was collected for LC-MS analysis.

2.11.2 LC-MS Analysis

(1) Chromatographic Conditions:

Separation was performed on an Agilent 1290 Infinity LC UHPLC system equipped with a HILIC column (25 °C). The mobile phase consisted of (A) water containing 25 mmol/L ammonium acetate and 25 mmol/L ammonium hydroxide, and (B) acetonitrile. The gradient elution program was as follows: 0-0.5 min, 95% B; 0.5-7 min, 95-65% B; 7-8 min, 65-40% B; 8-9 min, 40% B; 9-9.1 min, 40-95% B; 9.1-12 min, 95% B. The flow rate was maintained at 0.5 mL/min with an injection volume of 2 μ L. All samples were kept at 4 °C in the autosampler.

(2) Q-TOF Mass Spectrometry Conditions:

Mass spectrometric analysis was conducted using an AB Triple TOF 6600 system coupled to the UHPLC system. Both positive and negative electrospray ionization (ESI) modes were employed with the following parameters: Gas1: 60 psi, Gas2: 60 psi, curtain gas: 30 psi, ion source temperature: 600 °C, ion spray voltage: ± 5500 V. Full scan MS data were acquired from m/z 60-1000 (accumulation time: 0.20 s/spectrum), while MS/MS spectra (m/z 25-1000) were collected in data-dependent acquisition (DDA) mode with an accumulation time of 0.05 s/spectrum. The dynamic exclusion window was set to 4 Da with top 10 precursors selected for fragmentation (collision energy: (35 ± 15) eV, declustering potential: ± 60 V).

2.12 Western Blot

Total proteins were extracted from brain tissues using ice-cold RIPA lysis buffer (Biorigin, Beijing, China) supplemented with 1 mmol/L PMSF and protease inhibitor cocktail. Following centrifugation at 12,000 r/min for 10 min at 4 °C, protein concentrations were determined using a BCA protein assay kit (Biorigin, Beijing, China). Equal amounts of protein (45 µg) were separated by SDS-PAGE and subsequently transferred onto PVDF membranes. The membranes were sequentially incubated with primary antibodies specific to target proteins, followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were visualized using an ECL detection kit on an ImageQuant LAS 500 imaging system (Cytiva, USA) and quantified using ImageJ software (National Institutes of Health, USA).

2.13 Statistical Analysis

All data were analyzed using IBM SPSS Statistics 26.0, Microsoft Excel 2019, and GraphPad Prism 10.1.2 software. Intergroup differences were evaluated by one-way ANOVA followed by appropriate post-hoc tests. Results are presented as mean ± SEM, with $P < 0.05$ considered statistically significant.

3. Results and discussion

3.1. Compositional Analysis of WEL

The walnut enzymatic hydrolysate (WEL) produced using the aforementioned protocol was quantitatively analyzed for its major components. The protein content was 46.89 g/100 g, the lipid content was 30.52 g/100 g, the moisture content was 3.16 g/100 g and the carbohydrate content was 6.18 g/100 g (Fig. 1A).

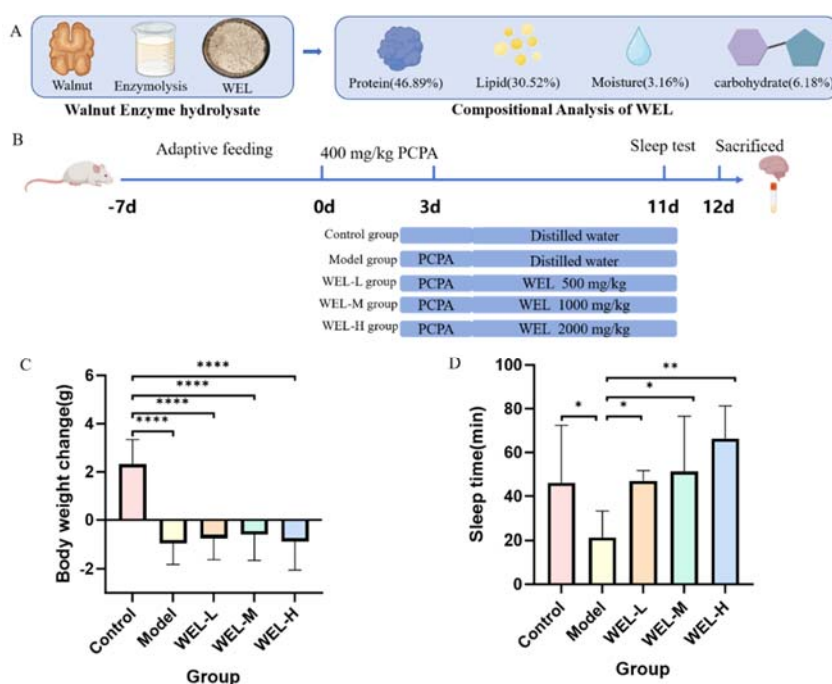


Fig. 1. Effect of WEL on sleep and body weight in mice. (A) Schematic timeline of the animal experimental procedure; (B) Preparation and composition of WEL; (C) Effect of PCPA on body weight changes in mice; (D) Effect of WEL on sleep time in PCPA-modelled mice. Data are presented as mean ± SEM, $n = 3/\text{group}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, versus the control group.

According to previous studies, the measured lipid content in walnut is 67.17%, and the protein content is 18.45%. Therefore, compared to walnuts, walnut enzymatic hydrolysate exhibits significantly reduced oil content and substantially increased protein content. This compositional reconstruction may enhance its potential sleep-improving bioactivity through multiple mechanisms. The bioactive peptides generated during enzymatic hydrolysis may act on the central nervous system via the gut-brain axis, modulating the GABAergic system to subsequently induce sleep.

3.2 Effects of WEL on Behavioral Parameters in PCPA-induced Insomnia Mice

The animal experimental procedure is illustrated in the Fig.1B below. Following PCPA administration, model mice exhibited characteristic behavioral alterations including reduced food intake, partially closed eyes with a slightly haggard appearance and occasional aggressive interactions. As shown in Fig. 1C, a significant difference in body weight change was observed between Model and Control groups ($P < 0.001$). Control mice maintained normal weight gain with a weight change of (2.31 ± 1.03) g, whereas model animals exhibited continuous weight loss with a change of $-(0.94 \pm 0.88)$ g, confirming successful model establishment^[25].

Sleep duration analysis (Fig. 1D) revealed that compared to the Control group, Model mice displayed significantly reduced sleep time ($P < 0.05$), validating the insomnia model. Notably, WEL administration dose-dependently reversed this effect, with all WEL-treated groups showing significantly prolonged sleep duration versus Model ($P < 0.05$). The high-dose WEL group (WEL-H) demonstrated particularly robust effects, increasing sleep time by 2.09-fold compared to Model. These findings demonstrate that WEL effectively ameliorates insomnia by dose-dependently extending sleep duration.

3.3 Effects of WEL on brain cell morphology in PCPA-treated Mice

Fig. 2A demonstrates the pathological findings of brain tissues across all groups. The Control group exhibited abundant hypothalamic neurons with intact morphology, uniform distribution, and well-organized alignment. In contrast, the Model group showed severely deformed hypothalamic cells, loosely arranged with numerous fragmented small cells and irregular pyramidal-shaped cells. Notably, the WEL-treated group demonstrated neatly arranged neuronal cells. Clearly visible nuclei, uniform intercellular matrix, and orderly neuronal arrangement were observed.

Quantitative analysis of H&E staining (Fig. 2B) revealed that the PCPA model induced significant hypothalamic neuronal damage. Compared with the control group, the model group showed a significant reduction in hypothalamic neuron density (275 ± 14.4 of model group cells/mm² vs 841.7 ± 65.1 of control group cells/mm², $P < 0.0001$), along with a significant increase in the mean cross sectional area of neurons (62.8 ± 1.8 of model group μm^2 vs 56.0 ± 0.4 of control group μm^2 , $P < 0.05$), indicating neuronal loss and cellular edema (Fig. 2C). WEL H intervention significantly reversed these pathological alterations: compared with the model group, the WEL H group restored neuron density to 783.3 ± 46.4 of treatment group cells/mm² ($P < 0.0001$), and the mean neuronal area also returned to near normal levels (56.74 ± 0.62 of treatment group μm^2 , $P < 0.05$ vs model group).

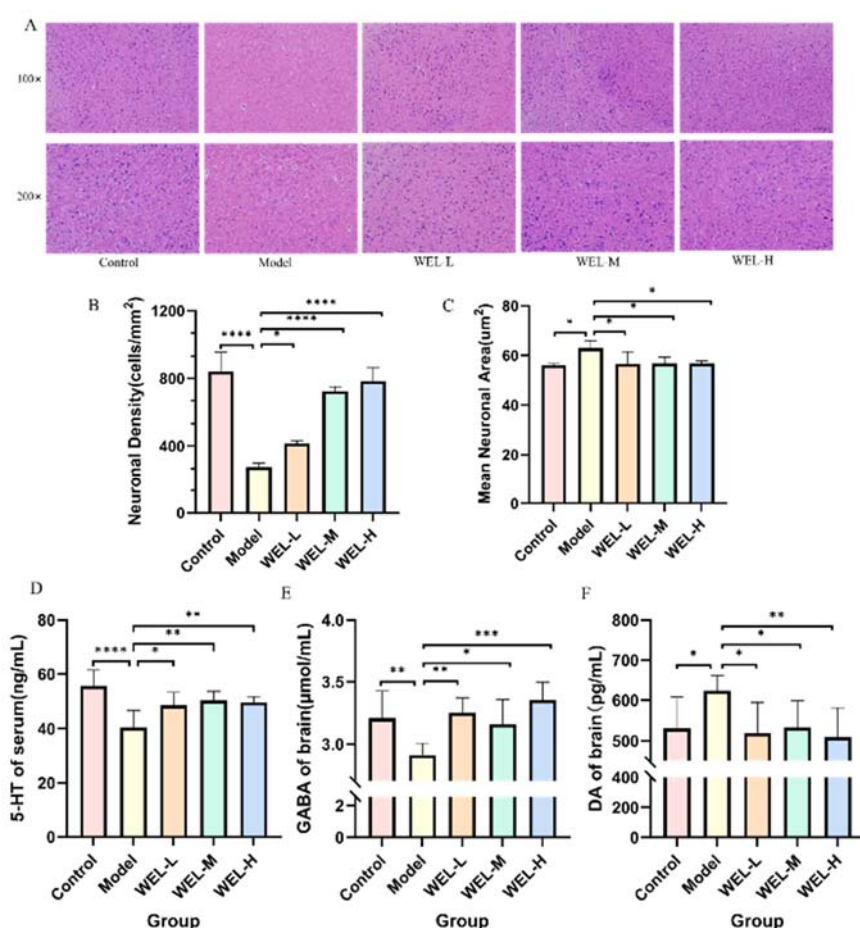


Fig. 2. Effect of WEL on histopathological analysis and neurotransmitter levels in mice. (A) Representative image of brain H&E staining (100 $\times$ and 200 $\times$ magnification, scale bar 100 and 50 μ m); (B) Effect of WEL on Neuronal Cell Count in PCPA Modeled Mice; (C) Effect of WEL on Mean Neuronal Area in PCPA Modeled Mice; (D) Effect of WEL on serum 5-HT levels in PCPA modelled mice; (E) Effect of WEL on brain GABA levels in PCPA modelled mice; (F) Effect of WEL on brain DA levels in PCPA modelled mice. Data are presented as mean $\pm$ SEM, $n = 3/\text{group}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, versus the control group.

3.4 Effects of WEL on Serum 5-HT Levels in PCPA-treated Mice

As shown in Fig. 2D, serum 5-HT levels were significantly reduced in Model mice compared to Controls ($P < 0.001$), and WEL administration significantly restored serum 5-HT concentrations in a dose-dependent manner ($P < 0.01$ vs Model). The WEL-H showed the most pronounced effect, increasing 5-HT levels by 23.98% compared to Model, though remaining slightly below Control levels ($P > 0.05$).

PCPA inhibits 5-HT synthesis by suppressing tryptophan hydroxylation, thereby reducing 5-HT levels in both cerebral and peripheral systems, ultimately inducing insomnia and anxiety^[26]. This neurotransmitter depletion in both central and peripheral systems contributes to the observed insomnia and anxiety-like behaviors, further validating successful model establishment. These results demonstrate WEL's capacity to partially reverse PCPA-induced 5-HT depletion, suggesting its potential mechanism in sleep improvement.

3.5 Effects of WEL on GABA and DA Levels in Brain Tissue of PCPA-treated Mice

Fig. 2E illustrates the impact of WEL on GABA content in brain tissue. Compared to Control, Model mice exhibited significantly reduced GABA levels ($P < 0.01$). WEL administration dose-dependently restored

GABA concentrations ($P < 0.05$ vs Model), with the high-dose group (WEL-H) demonstrating the most pronounced effect (15.17% increase vs Model, $P < 0.001$).

As shown in Fig. 2F, DA levels were significantly elevated in Model mice versus Controls ($P < 0.05$). WEL treatment effectively normalized DA concentrations ($P < 0.05$ vs Model), particularly in the WEL-H group which showed a 22.36% reduction compared to Model ($P < 0.01$).

GABA, the primary inhibitory neurotransmitter in the CNS, plays a crucial role in sleep initiation and maintenance^[27]. Conversely, DA acts as an excitatory neurotransmitter, with its release in the nucleus accumbens influencing REM sleep duration^[28]. The statistically significant ($P < 0.05$) modulation of both neurotransmitters by WEL suggests its dual mechanism in sleep regulation: enhancing GABAergic inhibition while attenuating dopaminergic excitation, thereby restoring neurochemical balance in insomnia mice.

3.6 Effects of WEL on Brain Metabolome in PCPA-treated Mice

3.6.1 Multivariate Statistical Analysis of Metabolites

Untargeted metabolomics was employed to compare metabolic profiles among Model, Control, and WEL-H groups, investigating potential mechanisms underlying WEL-mediated sleep improvement. Principal component analysis (PCA) of UPLC-MS/MS data revealed distinct metabolic clustering patterns (Fig.3A-B), demonstrating significant metabolic alterations in WEL-H treated mice compared to both Control and Model groups ($P < 0.05$) in both positive and negative ionization modes. The clear separation in PCA score plots indicates substantial metabolic reprogramming induced by WEL supplementation. Further analysis revealed that the key metabolites driving this inter-group separation are primarily concentrated in several major categories related to neurotransmitter metabolism and energy metabolism.

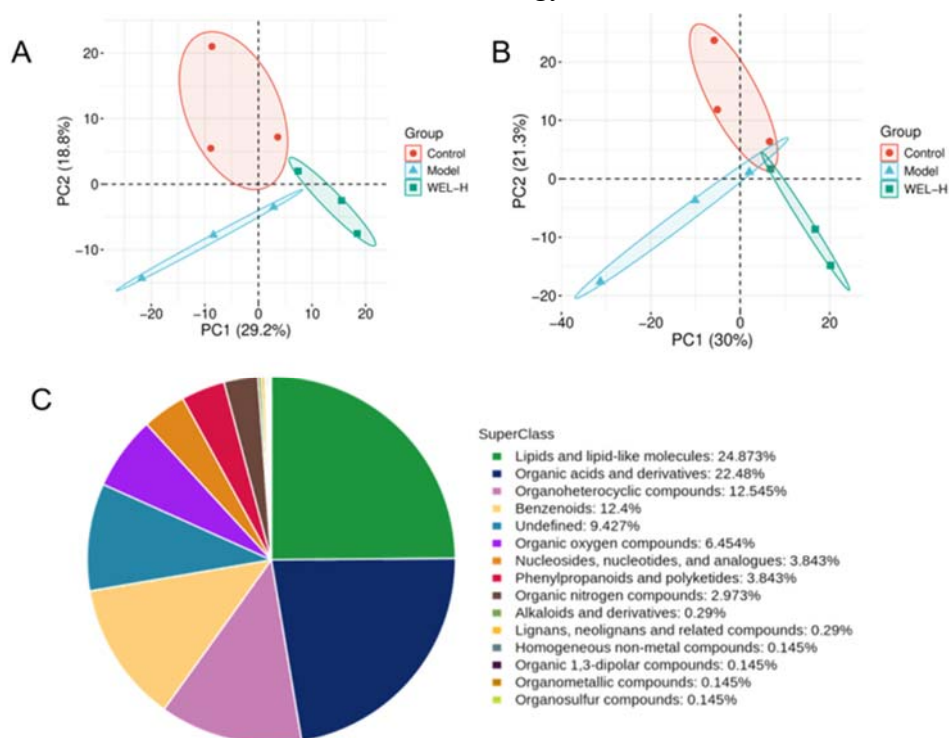


Fig. 3. Comprehensive analysis of components of WEL. (A) PCA in pos mode; (B) PCA in neg mode; (C) Metabolite composition analysis (top 15).

UHPLC and Q-TOF analysis identified 1,379 differentially expressed metabolites, which were categorized into 14 classes (Fig. 3C): lipids (343, 24.87%), organic acids (310, 22.48%), organoheterocyclic compounds (173, 12.55%), benzenoids (171, 12.40%), organic oxygen compounds (89, 6.45%), nucleotides (53, 3.84%), phenylpropanoids and polyketides (53, 3.84%), organic nitrogen compounds (41, 2.97%), alkaloids (4, 0.29%), lignans (4, 0.29%), homogeneous non-metal compounds (2, 0.15%), 1,3-dipolar organic compounds (2, 0.15%), organometallics (2, 0.15%), and organic sulfur compounds (2, 0.15%), with 130 (9.43%) unclassified compounds.

3.6.2 Screening and Analysis of Differential Metabolites

Differential metabolites were identified using fold change ($FC > 1.0$) and t-test ($P < 0.05$) criteria. Comparative analysis revealed 121 significantly altered metabolites between Model and Control groups (22 upregulated, 99 downregulated, Fig. 4A-B), while 164 metabolites were differentially expressed between WEL-H and Model groups (105 upregulated, 59 downregulated, Fig. 4C-D). Venn diagram analysis (Fig. 4E) demonstrated the overlap of differential metabolites across experimental groups.

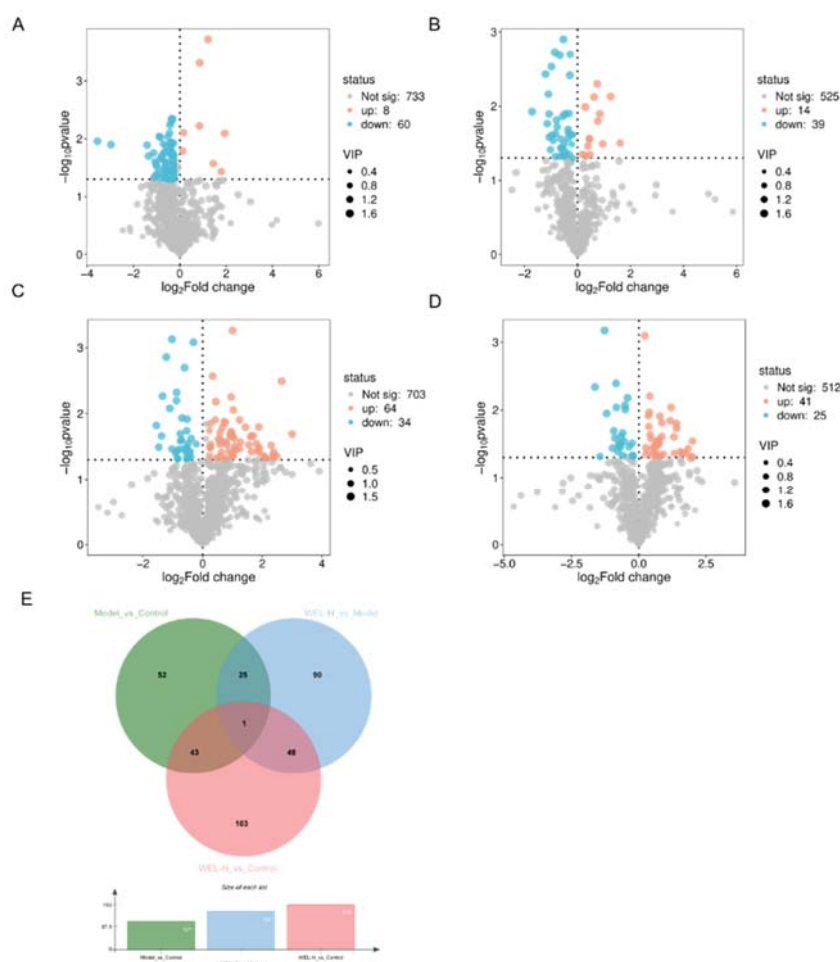


Fig. 4. Multivariate statistical analysis of differential metabolites. (A) Differential metabolite volcano map in Model vs Control pos mode; (B) Differential metabolite volcano map in Model vs Control neg mode; (C) Differential metabolite volcano map in WEL-H vs Model pos mode; (D) Differential metabolite volcano map in WEL-H vs Model neg mode (Proteins significantly upregulated are indicated in red ($P < 0.05$, fold change > 1), while significantly downregulated proteins are shown in blue ($P < 0.05$, fold change < -1)); (E) Venn diagram of identified metabolites in three groups (Control, Model and WEL-H).

The differential metabolite analysis revealed that WEL-H intervention significantly modulated several key metabolic hubs intimately connected to the regulation of sleep-wake circuitry. Key sleep-related metabolites including GABA, 3-hydroxybutyrate, allantoin, DL-serine, magnolol, and AICAR were identified (Fig. 5). Notably, WEL-H treatment significantly increased GABA levels versus Model ($P < 0.05$, Fig. 5A). As the primary inhibitory neurotransmitter, GABA mediates sleep through GABAAR, GABABR, and GABACR^[29]. This finding strongly suggests an enhancement of the central GABAergic inhibitory tone, a cornerstone mechanism for sleep promotion. WEL-H also elevated 3-hydroxybutyrate ($P < 0.05$, Fig. 5B), a metabolite reportedly deficient in individuals with sleep disorders^[30], and a key indicator of ketone body metabolism. Concurrently, WEL-H reduced allantoin ($P < 0.05$, Fig. 5C), which is elevated in REM sleep behavior disorder patients^[31]. Since allantoin is an oxidative product of urate, its reduction indicates a potential mitigation of sleep disorder-associated oxidative stress, thereby creating a more favorable microenvironment for neural function.

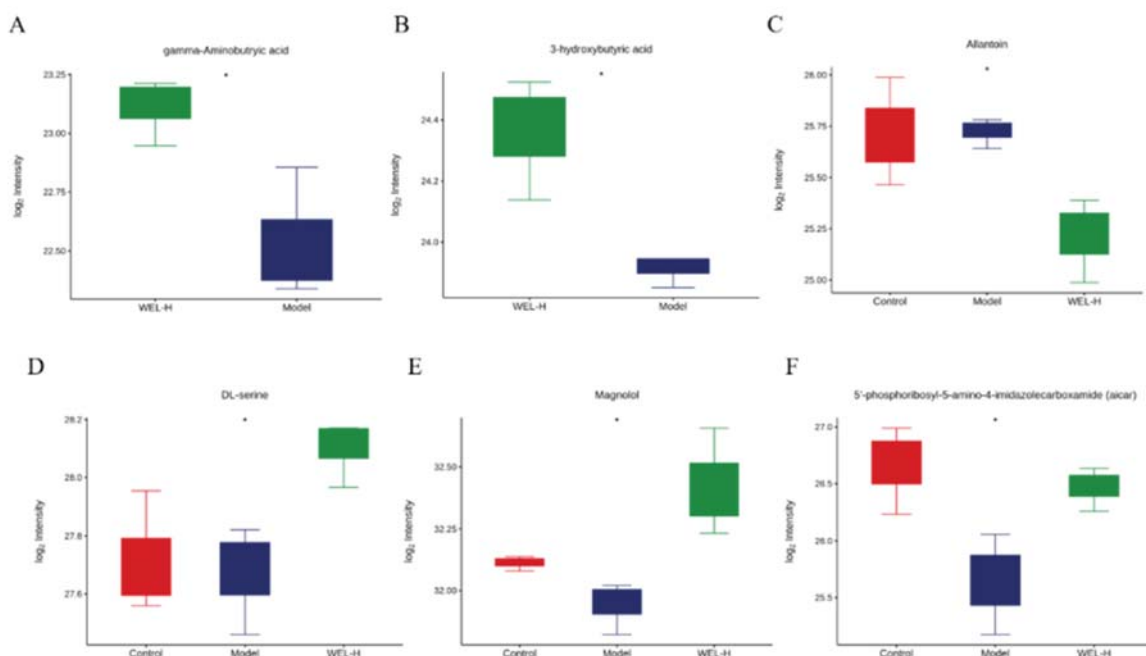


Fig. 5. Key sleep-related metabolites including GABA(A), 3-hydroxybutyrate(B), allantoin(C), DL-serine(D), honokiol(E), and AICAR(F). Data are presented as mean $\pm$ SEM, $n = 3/\text{group}$. * $P < 0.05$, versus the control group.

DL-serine levels were significantly increased in WEL-H treated mice ($P < 0.05$, Fig. 5D). D-serine acts as an NMDA receptor co-agonist influencing sleep-wake cycles^[32], while L-serine serves as a glycine precursor that promotes NREM sleep through its sedative effects^[33]. Chronic L-serine supplementation may alleviate circadian rhythm delays^[34]. Thus, the rise in DL-serine points to a dual modulation of both glutamatergic (via NMDA receptor) and glycinergic pathways, fine-tuning neuronal excitability and sleep architecture. The WEL-H group showed elevated levels of magnolol ($P < 0.05$, Fig. 5E), a compound with demonstrated sedative and hypnotic effects via GABAergic enhancement and 5-HT modulation^[35-36]. Animal studies confirm magnolol's ability to increase both NREM and REM sleep through GABAAR. Its presence further corroborates the activation of the GABAergic pathway identified above. Additionally, increased AICAR levels ($P < 0.05$, Fig. 5F) suggested AMPK pathway activation, which regulates sleep depth and homeostasis^[37]. AICAR, as an AMP mimetic, directly activates AMPK. This links sleep regulation to cellular

energy sensing, suggesting that WEL-H may improve sleep by simulating a state of energy replenishment and activating downstream sleep-promoting signals. These metabolic alterations collectively suggest potential mechanisms for WEL-mediated sleep improvement.

3.6.3 Metabolic Pathway Enrichment Analysis of Differential Metabolites

Metabolite Set Enrichment Analysis (MSEA) was performed to elucidate potential metabolic pathways through which WEL modulates sleep in PCPA-induced insomnia mice. Fig.6 presents the MSEA results of metabolites between different groups.

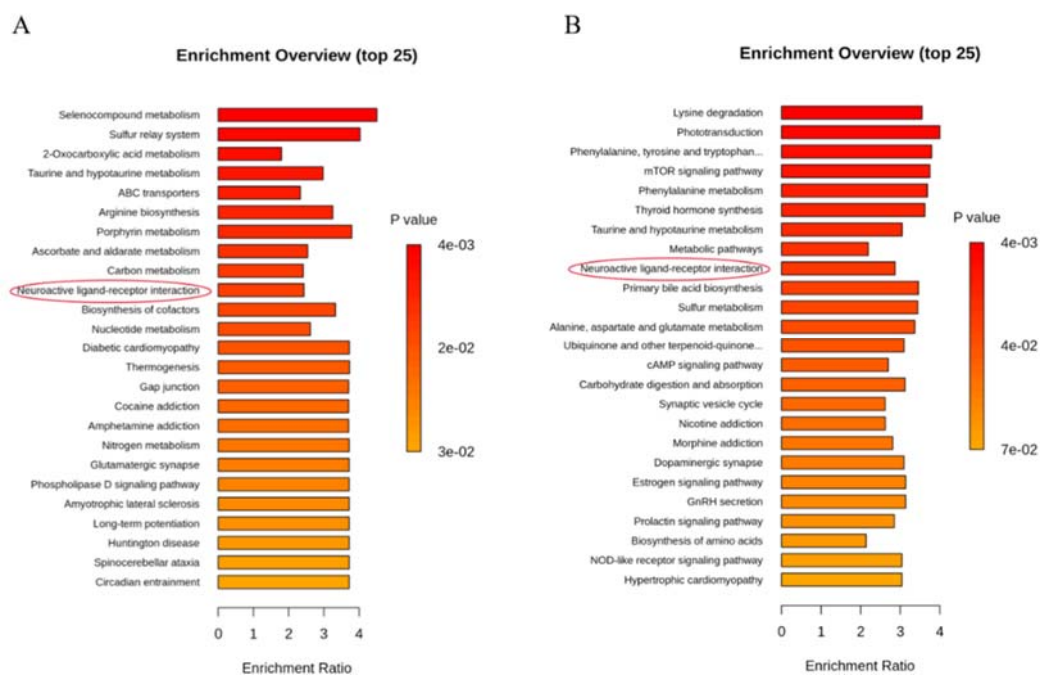


Fig. 6. Metabolites (positive and negative ion combined) enrichment pathway analysis of different groups: Control vs Model (A), WEL-H vs Model (B).

In the Model vs Control comparison, key metabolites were significantly enriched in five sleep-related pathways ($P < 0.05$): Neuroactive ligand-receptor interaction, Cocaine addiction, Glutamatergic synapse, Phospholipase D signaling pathway, Circadian entrainment; The WEL-H vs Model comparison revealed enrichment in six sleep-associated pathways ($P < 0.05$): Neuroactive ligand-receptor interaction, cAMP signaling pathway, Synaptic vesicle cycle, Nicotine addiction, Morphine addiction, Dopaminergic synapse.

Glutamate is the primary excitatory neurotransmitter in the brain, participating in the regulation of adenosine and sleep pressure through glutamatergic synapses^[38]. The phospholipase D (PLD) signaling pathway plays a crucial role in cellular signal transduction, particularly in stress responses, neural functions, and cellular metabolism. The circadian rhythm is an approximately 24-hour cyclical physiological process in organisms, regulated by the master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Sleep is considered to be jointly regulated by the sleep-wake homeostasis system and the circadian system^[39]. Circadian rhythm synchronization facilitates sleep. Studies by Ji et al. suggested that cAMP exerts antidepressant and sleep-promoting effects through the cAMP/PKA-CREB-BDNF signaling cascade by modulating BDNF-induced phosphorylation of TrkB^[40].

Notably, both comparisons showed significant enrichment in the neuroactive ligand-receptor interaction pathway ($P < 0.01$), suggesting its central role in sleep-related metabolic dysregulation and WEL intervention. Integrated analysis of differential metabolites revealed that GABA, as the primary inhibitory neurotransmitter in CNS, directly regulates GABAergic signaling to modulate neuronal excitability and sleep homeostasis^[41]. GABAergic neurons regulate the release of 5-HT by activating GABAAR, thereby indirectly influencing the inhibitory effect of 5-HT and promoting sleep production. Magnolol has been shown to enhance the activity of GABAAR, and supplements containing magnolol will be effective anxiolytics, sedatives, and anticonvulsants^[42]. Other key metabolites including 3-Hydroxybutyrate, allantoin and DL-serine may synergistically interact with GABAergic pathways to maintain neurotransmitter balance and sleep regulation. These findings collectively indicate that GABAergic pathway modulation likely represents the principal mechanism underlying WEL-mediated sleep improvement.

3.6.4 Validation of GABAergic Pathway Through Protein Expression Analysis

To verify whether WEL improves sleep behavior via the GABAergic pathway, we examined the protein expression levels of key GABAergic components. Western blot analysis revealed significant reductions in GAD65 (GABA-synthesizing enzymes) in Model mice compared to Controls ($P < 0.001$), while WEL treatment increased the expression level by 36.7% (Fig. 7A); The expression of GABA-T (a key enzyme for GABA degradation) was significantly increased in model mice ($P < 0.05$), while WEL treatment reduced this expression level by 40.1% (Fig. 7B).

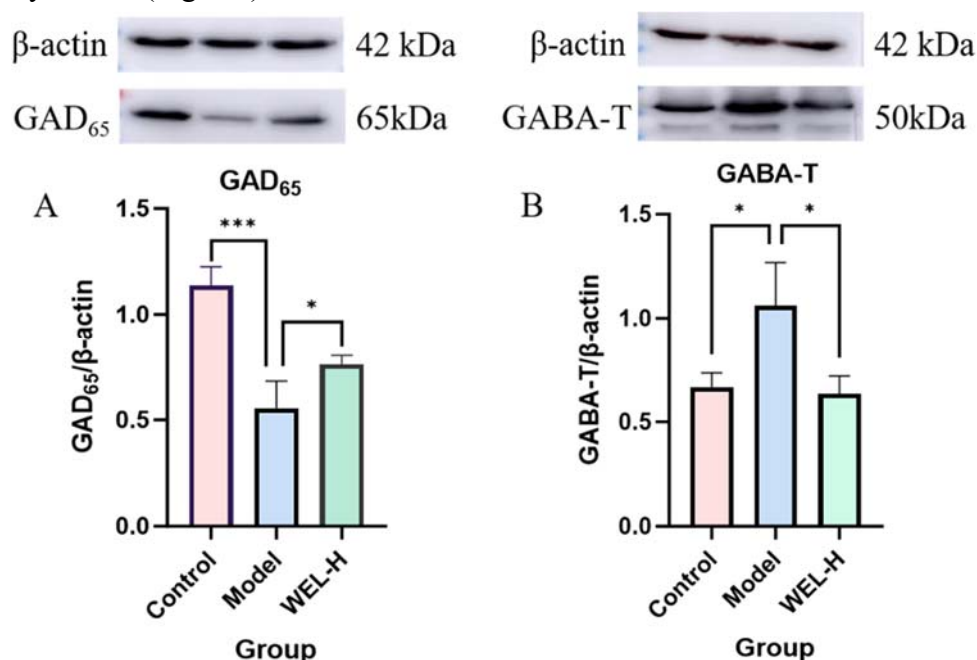


Fig. 7. Effects of WEL on protein expression in mouse brain tissue: GAD65(A); GABA-T(B). Data are presented as mean $\pm$ SEM, $n = 3/\text{group}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus the control group.

These integrated results demonstrate that WEL-H specifically activates the GABAergic pathway to ameliorate sleep disturbances. In the PCPA-induced insomnia model, the downregulation of the rate-limiting GABA synthase (GAD65) and upregulation of the GABA-degrading enzyme (GABA-T) are key molecular impairments that disrupt GABA homeostasis. WEL intervention significantly reversed this suppression,

restoring both protein abundance expression of these key factors. This targeted modulation of key enzymatic regulators provides a direct molecular explanation for our metabolomics findings. It establishes a clear causal link: the restoration of GABA-synthesizing and -degrading enzyme expression is the mechanistic driver behind the elevated brain GABA levels observed in the metabolomics data. Furthermore, this coordinated change at both the protein and metabolite levels robustly validates the enrichment seen in neuroactive ligand-receptor interaction pathways from our earlier analysis. This mechanistic evidence strongly supports WEL's potential as a natural sleep-enhancing agent targeting GABAergic neurotransmission.

4. Discussion

The regulation of sleep and wakefulness relies on highly complex neurotransmitter networks and metabolic homeostasis^[43]. With the accelerated pace of modern life, sleep disorders have become a significant public health concern, whose pathogenesis is closely associated with imbalances in the central nervous excitation-inhibition system^[44]. Although existing pharmacological treatments can provide short-term symptomatic relief, their long-term application is limited by side effects such as drug dependence and cognitive impairment. In this context, functional components derived from natural foods have emerged as a new research direction in sleep medicine due to their multi-target and high-safety profiles^[45].

Walnuts are rich in glutamate (a GABA precursor) and arginine (a nitric oxide synthesis precursor). The enzymatic hydrolysis process employed in this study likely liberates neuroactive small peptides or modified amino acid derivatives from walnut proteins, which may constitute the key bioactive components responsible for the observed cross-system regulation of neurotransmitters^[46]. Numerous studies have demonstrated the sleep-enhancing effects of walnuts and their oligopeptides. Zerón-Rugério et al.^[47] demonstrated that daily consumption of 40 grams of walnuts increased urinary 6-sulfatoxymelatonin (6-SMT) levels, reduced sleep latency, and improved overall sleep quality. Yeh et al.^[48] conducted a randomized double-blind placebo-controlled clinical trial, and the final results demonstrated that walnut oligopeptides effectively improved memory, cognitive function, and sleep quality. Compared to traditional sedative drugs, this multi-target intervention strategy derived from a food matrix offers inherent safety advantages.

This study systematically elucidated the potential mechanism by which walnut enzymatic hydrolysate (WEL) improves sleep through behavioral evaluation, untargeted metabolomics, and molecular biology validation. Utilizing a PCPA-induced insomnia mouse model, the study demonstrated that WEL dose-dependently prolonged pentobarbital sodium-induced sleep duration, with the high-dose WEL group (WEL-H) showing 2.09 times longer sleep duration than the Model group. A benchmark comparison with previously reported sleep-promoting botanical ingredients (including lettuce^[49], hibiscus flower extract^[50], and zucchini extract^[51]) revealed that walnut enzymatic hydrolysate (WEL) demonstrates superior efficacy in prolonging sleep duration in insomnia mouse models. Concurrently, WEL treatment significantly elevated levels of serotonin (5-HT) and γ -aminobutyric acid (GABA), while reducing the content of the excitatory neurotransmitter dopamine (DA) compared to the control group. Studies have shown that the serotonin (5-HT) and γ -aminobutyric acid (GABA) systems are core neurotransmitter pathways regulating the sleep-wake

cycle. Specifically, GABA—the primary inhibitory neurotransmitter in the central nervous system—is closely associated with sleep disorders such as insomnia when dysfunctional, while dopamine (DA) is considered a wake-promoting agent in sleep-wake regulation^[52]. These results provide direct evidence for the sleep-improving effect of WEL from the perspective of neurotransmitter balance.

Metabolomics analysis further expanded the understanding of WEL's mechanism of action. Untargeted metabolic profiling identified 1379 metabolites, with GABA, 3-hydroxybutyric acid, DL-serine, magnolol, and AICAR being identified as key differential metabolites. As a core inhibitory neurotransmitter, elevated GABA levels directly correlate with GABA receptor activation; 3-hydroxybutyric acid, an endogenous neuroactive substance, synergistically enhances GABAergic signaling transmission. DL-serine (a glycine precursor) influences the sleep-wake cycle by modulating glutamatergic neurotransmission^[53], while magnolol^[54] and AICAR^[37] involve in the regulation of sleep depth and sleep homeostasis by enhancing GABAergic transmission and activating AMPK, respectively, collectively forming a multi-target regulatory network. This multi-target, multi-system regulatory profile may confer significant therapeutic advantages over first-line pharmacological agents such as benzodiazepines and Z-drugs, which primarily exert a potent but narrowly focused enhancement of GABAAR function. Drug-related adverse effects frequently stem from the excessive and isolated inhibition of specific neural circuits. The food-derived, multi-component nature of WEL-H suggests a gentler, “systems-level” intervention.

Pathway enrichment analysis revealed that WEL intervention significantly affected the "neuroactive ligand-receptor interaction" pathway (enriched in both Model vs Control and WEL-H vs Model comparisons), with the GABAergic pathway identified as the core mechanism (Fig.8). This finding was validated at the molecular level by Western blot: WEL significantly upregulated the protein expression of the GABA-synthesizing rate-limiting enzyme (GAD65) and downregulated the protein expression of the key GABA-degrading enzyme (GABA-T), comprehensively elucidating the pivotal role of the GABAergic system in sleep improvement from functional to expression levels. GABA is synthesized from glutamate under the catalysis of glutamate decarboxylase (GAD), which acts as the rate-limiting enzyme in this process^[55]. Our findings demonstrate that WEL upregulates the expression of GAD65, thereby facilitating GABA synthesis. Concurrently, WEL downregulated GABA transaminase (GABA-T), the primary catabolic enzyme, indicating a reduction in GABA degradation. This dual-regulatory mechanism—promoting synthesis while inhibiting degradation—effectively elevated synaptic GABA levels, thereby potentiating the inhibitory signaling of the GABAergic system^[56]. It is important to note the limitations of this study. Given the established role of the gut-brain axis in sleep regulation, future work should investigate the impact of WEL on gut-derived metabolites. and integrate multi-omics approaches, such as transcriptomics and proteomics, This would involve conducting 16S rRNA gene sequencing and metagenomic analysis on fecal samples to determine whether WEL modulates the composition and function of the gut microbiota, and subsequently integrating these findings with multi-omics approaches (such as transcriptomics and proteomics) to establish a causal link between microbial shifts and central mechanisms, thereby providing a more comprehensive

understanding of its sleep-improving action. to provide a more comprehensive understanding of its mechanisms.

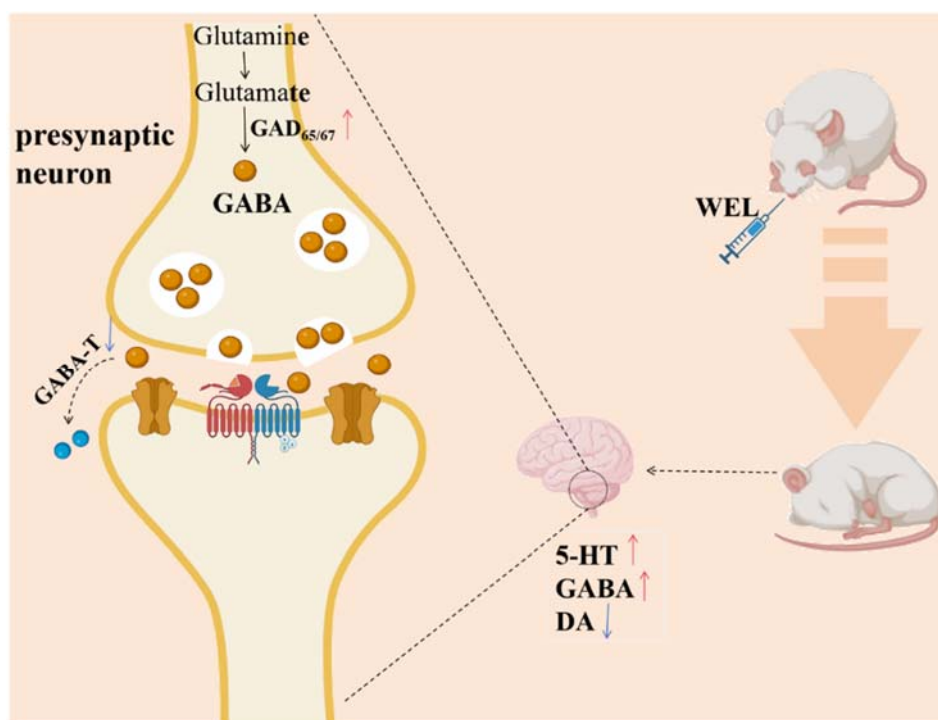


Fig. 8. Mechanism by which the proposed walnut enzyme hydrolysate (WEL) alleviates sleep in insomnia mouse models.

In summary, this study is the first to reveal the potential sleep-improving mechanism of walnut enzymatic hydrolysate at the metabolic level, providing a valid theoretical foundation for its future application and understanding in sleep improvement. Furthermore, these potential biomarkers and metabolic pathways were validated through measurements of relevant protein expression. This study not only deepens the understanding of walnut bioactivity, but also provides crucial theoretical support and application prospects for developing safe and effective natural sleep-enhancing products.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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