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ORIGINAL RESEARCH

ATAS-EMIT Activates the 5-HT/AC/cAMP Pathway to Ameliorate Insomnia in PCPA Insomniac Rats

Liangxian Liu[1], Zheng Zuo[2], Zuhong Wang[1], Li Li[1], Yongjiang Fang[1], Chunyan Guo[1], Xuejiao Pan[1], Xiao Xiao[1]

[1]Department of Acupuncture, Kunming Municipal Hospital of Traditional Chinese Medicine, Kunming, Yunnan, People's Republic of China; [2]Second Clinical Medical College, Yunnan University of Traditional Chinese Medicine, Kunming, Yunnan, People's Republic of China

Correspondence: Li Li, Department of Acupuncture, Kunming Municipal Hospital of Traditional Chinese Medicine, 25 Dongfeng Dong Lu, Kunming, Yunnan, 663100, People's Republic of China, Email LiLi200201@sina.com

Background: Insomnia may lead to or be co-morbid with mental disorders. However, available treatments have significant side effects. Time-acupoints-space acupuncture, eight methods of intelligent turtle (ATAS-EMIT), as a low-hazard insomnia treatment, has been focused on.

Methods: Thirty-six rats were randomly divided into 4 groups: normal (Control), insomnia (Model), ATAS-EMIT treatment (Treatment), and sham acupuncture treatment (Sham). Behavioral experiments were used to assess the treatment of ATAS-EMIT for insomnia. ELISA, Western blot, and immunofluorescence staining were used to detect the expression of 5-HT, AC, cAMP, $5HT_{1A}$ receptor ($5HT_{1AR}$), and $5HT_{2A}$ receptor ($5HT_{2AR}$) in rat hippocampus. Transcriptome sequencing was used to explore more therapeutic mechanisms.

Results: ATAS-EMIT treatment significantly reduced anxiety-like behaviors and activities in PCPA insomniac rats in the open field test. Additionally, ATAS-EMIT significantly shortened the sleep latency period in insomnia-prone rats while prolonging both sleep duration and hanging rest time. These behavioral studies suggest that ATAS-EMIT is a treatment for insomnia. The insomnia-related indicators showed that ATAS-EMIT significantly activated the 5-HT/AC/cAMP pathway, promoted the expression of the $5HT_{1AR}$, and inhibited the expression of the $5HT_{2AR}$ for therapeutic purposes. Subsequent transcriptome sequencing revealed that ATAS-EMIT-treated DEGs were enriched for multiple insomnia-related functions such as phototransduction, stimulus-response, and tryptophan metabolism. Six key genes, *Cngb1, Cabp4, Sag, Tyr, Trpm1*, and *Adipoq*, were screened and validated.

Conclusion: ATAS-EMIT significantly improved insomnia symptoms in PCPA insomniac rats, and activation of the 5-HT/AC/cAMP pathway was involved. Various mechanisms, such as phototransduction, tryptophan metabolism, and reduction of stimulation, contributed to the therapeutic effects of ATAS-EMIT.

Keywords: insomnia, acupuncture, 5-hydroxytryptamine, 5ht1/2a receptor, transcriptome sequencing, mental disorders

Introduction

Insomnia is often used as a term to describe disturbed sleep. People with insomnia often exhibit hypervigilance during the day and difficulty falling asleep and maintaining sleep at night.[1,2] About 30% of the sample studies from different countries were plagued by symptoms such as difficulty in starting or maintaining sleep and waking up too early.[3] Moreover, insomnia is highly prevalent in older adults and women, with 66.7% of 468 women observed to have insomnia in a population-based study of sleep disorders.[4] Sleep deprivation caused by insomnia is a key factor in reduced anti-inflammatory responses and cognitive function.[5,6] The consequences of insomnia are not only sleep deprivation, but may also cause damage to brain plasticity, mood, and immunity, which contributes to mental disorders.[7–10] It is estimated that 40% of people with insomnia also suffer from a mental disorder.[11] Currently, sedative antidepressants or atypical antipsychotics are commonly used to treat insomnia symptoms in patients with mental disorders, but they are not suitable for the treatment of insomnia.[12] Among the drugs used to treat insomnia, benzodiazepines (BZ)/BZ receptor agonists (BZRA) have been approved. However, long-term BZ/BZRA therapy can lead to the development of adverse

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events such as tolerance and dependence. Long-term use of these drugs beyond 3–4 weeks is not allowed.[12,13] The need to find a long-term, effective treatment for insomnia is urgent.

In recent years, acupuncture has gained great appeal for insomnia patients as a treatment that improves sleep quality, fatigue, and mood symptoms with low risks.[14] A randomized controlled trial showed acupuncture improves sleep quality in insomnia patients.[15] Another animal study found that acupuncture on Baihui, Sanyinjiao, and Shenmen acupoints could regulate immunity and improve sleep disorders by regulating intestinal bacteria.[16] Time-acupoints-space acupuncture (ATAS) is a system of acupoint selection and acupuncture methods established by Dr. Miansheng Zhu of the Department of Traditional Chinese Medicine at the Leonardo da Vinci School of Medicine in Paris, France, who has been in clinical practice for more than 30 years.[17] Current research has demonstrated that ATAS is effective in the treatment of a variety of diseases. For instance, researchers have found that ATAS is effective in treating persistent chronic cough in patients with lupus nephropathy.[18] Besides, ATAS has shown improvement in both chemotherapy-induced fatigue and quality of life aspects in breast cancer patients.[19,20] The eight methods of intelligent turtle (EMIT) is one of the four systems of ATAS. The ATAS-EMIT acupuncture method differs from traditional acupuncture by combining astronomy with the patient's acupoints. The acupoints, mainly on the chest, abdomen, face, and head, are divided into nine palaces. The acupuncture is performed in sequence according to the numbering of the palaces.[19] Current studies have found EMIT to be therapeutically effective in the treatment of chronic superficial gastritis.[21] Moreover, it showed effective modulation in a guinea pig model of kidney yang deficiency.[22] Notably, our clinical research has found that ATAS-EMIT effectively improves insomnia symptoms in breast cancer patients throughout the chemotherapy process.[23] Moreover, clinical study results for insomnia indicate that the ATAS-EMIT treatment group achieved an overall response rate of 92.5%, significantly higher than that of the control group.[24] However, the positive therapeutic effect of ATAS-EMIT on insomnia and its mechanism are unclear.

Insomnia is a contributing factor to mental disorders, mainly depression is also a typical symptom of mental disorders. Can early and adequate treatment of insomnia prevent depression? Researchers have found that strategies for treating insomnia have shown depression-preventive properties.[25] Notably, 5-Hydroxytryptamine (5-HT) was found to play an important role in both depression and insomnia. Previous studies have found that 5-HT mediates multiple gene interactions in the AC-cAMP pathway that are associated with the progression of depression susceptibility.[26–29] Meanwhile, 5-HT plays an important role in promoting arousal and inhibiting rapid eye movement sleep.[30] Additionally, p-chlorophenylalanine (PCPA), a 5-HT inhibitor, is commonly used to establish animal models of insomnia. Similarly, this study employed PCPA, a 5-HT synthesis inhibitor, to generate rat models of insomnia. This approach avoids confounding effects from multi-target interference, thereby enhancing the credibility of our findings.[31] These suggest that it is practicable to study the insomnia treatment mechanism of ATAS-EMIT from 5-HT.

The purpose of this study was to treat insomnia using ATAS-EMIT. We analyzed the behavioral and insomnia-related indices in the hippocampal tissues of a PCPA-induced insomnia rat model to investigate the therapeutic effect of ATAS-EMIT on insomnia. Transcriptome sequencing and analysis contributed to our investigation of the mechanism of insomnia treatment by ATAS-EMIT. This study is the first to combine ATAS-EMIT and transcriptomics analysis to elucidate systemic mechanisms for treating insomnia. Our results will find a low-risk and high-efficiency treatment for insomnia. And provide theoretical support for ATSA-EMIT treatment of insomnia.

Methods

Insomnia Model

This study was approved by the Ethical Review Committee for Animal Experiments of Yunnan University of Traditional Chinese Medicine (R-062021LH031) and was performed in strict compliance with the ARRIVE guidelines. Random sequences were generated using the statistical software SPSS 23.0. Thirty-six SPF-grade male SD rats (250±15 g) were randomly divided (n=9: three for tissue sectioning; three for hippocampal extraction for basic research; and three for hippocampal extraction for transcriptomic analysis) into a normal group (Control), model group (Model), acupuncture treatment group (Treatment), and sham acupuncture group (Sham). Rats were obtained from the Laboratory Animal Center of Kunming Medical University (License No. SCXK(Dian)K2020-0004). The construction of the insomnia model

followed the methods of the previous researchers.[32] Briefly, PCPA (7424–00-2, Sigma, USA) was configured as an injection solution at a concentration of 45 mg/mL using weakly alkaline saline (pH 7–8). PCPA was injected intraperitoneally at a dose of l mL/100 g once a day at 8:30–9:00 a.m. for 2 consecutive days to induce the insomnia model. The control group replaced the PCPA injection with the same volume of weakly alkaline saline. Thirty hours after the 2nd intraperitoneal injection of PCPA, behavioral changes and neurological symptoms of rats were observed and recorded. The model replication was successful if it showed the disappearance of diurnal alternation, strong response to external sound, light, and other natural stimuli, fast feeding, drinking, hyperactivity, increased stools with white color, and the difference between the observation and that of the control group was obvious. All rats in this study were housed in a room-temperature environment with free access to food and water. The housing conditions maintained a 12-hour light/12-hour dark photoperiod. Behavioral identification was performed at the end of the treatment. Subsequently, the rats were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (120 mg/kg) and subsequently euthanized by cervical dislocation.

ATAS-EMIT Acupuncture Intervention

Rats with successful modeling were selected, and the treatment was carried out on the day after successful modeling, once a day for a total of 6 times. The ATAS-EMIT method was used to treat the ATAS-EMIT rats, in which a 0.18×13 mm needle was inserted into the corresponding depths of the acupoints, and the needle was left in each acupoint for 30 seconds and then discharged. The total duration of the treatment was maintained at 7 min. The Sham group simulated acupuncture by needling the acupoints at the lower edge of both ribs of the rats. The Control and Model groups were given the same time and intensity of scratching stimulation as the Treatment group to exclude interference.

ATAS-EMIT acupuncture point selection referring to an acupoint atlas for rats:[33] 1) Time points: first acupuncture bilateral zhaohai acupoints (SI8, 6 mm, operator and rat face to face to apply needles, left to right), then acupuncture lower back nine palaces, and finally acupuncture limbs abdominal nine palaces (Figure 1). 2) Spatial acupoints: consist of 2 sets of nine palace acupoints: a) lower back; b) limbs and abdomen. The nine palaces start from point number 2. Bilateral xinyu (BL15, 6 mm), piyu (BL20, 6 mm), shenyu (BL23, 6 mm), quchi (LI11, 4 mm), neiguan (PC6, 1 mm), sanyinjiao (SP6, 5 mm), and single points of dazhui (GV14, 5 mm), jizhong (DU6, 4 mm), baihui (GV20, anterior and posterior oblique s tabbing 2 mm), danzhong (CV17,1.5mm straight stabbing), zhongwan (CV12, 2 mm), guanyuan (CV4, 2 mm).

Behavioral Assessment

Open Field Test

A 100 cm x 100 cm x 40 cm open field test chamber is used. The bottom of the chamber was divided into 25 compartments. The room was kept quiet and dark during the test. The animals were placed in the center of the chamber

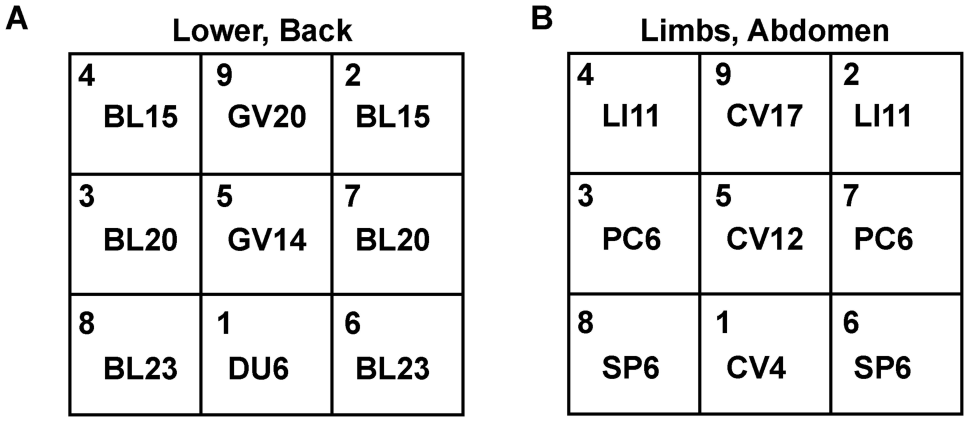

Figure 1 Acupuncture points for ATAS-EMIT. (**A**): ATAS-EMIT's nine acupuncture points for the lower and back. (**B**): ATAS-EMIT's nine acupuncture points for the limbs and abdomen.

and acclimatized for 2–5 min. The distance traveled and the number of times the animals stood up were recorded and analyzed within 10 min. At the end of the test, each rat was cleaned of its feces, and the chamber was wiped with an alcohol swab to remove any residual odor.

Barbiturate Synergistic Sleep Experiment

The optimal dose of sodium pentobarbital was determined to be 45 mg/kg by pre-testing. The rats were injected intraperitoneally with 45 mg/kg of 1% pentobarbital sodium solution, and the disappearance of the reflex of turning over for more than 1 min was regarded as a sign of sleep. Sleep latency and sleep duration were recorded. Sleep latency was defined as the time from the start of sodium pentobarbital injection to the time when the reflex disappeared for more than 1 min, and sleep duration was defined as the time from the time when the reflex disappeared for more than 1 min to the time when the reflex returned.

Tail Suspension Test

The height between the tip of the rat's nose and the ground was maintained at approximately 20 cm, and the rat was placed in a head-inverted position. After 2 min of suspension, the prohibited time was recorded for 4 min.

ELISA

The levels of 5-HT, AC and cAMP in the hippocampal tissue of PCPA insomniac rats were detected according to the instructions of Serotonin 5-Hydroxytryptamine (5-HT) ELISA Kit (E-EL-0033, Elabscience, China), Rat Adenylate Cyclase (AC) ELISA Kit (JL21346, Mlbio, China) and Cyclic Adenosine Monophosphate (cAMP) ELISA Kit (E-EL-0056, Elabscience, China).

Western Blot

RIPA lysate (400 μL, P0013B, Biotronik) was used to extract proteins from 100 mg of tissue. Proteins were quantified and homogenized, and each sample was subjected to SDS-PAGE electrophoresis at an amount of 60 μg. The membrane was transfected and then incubated with 5 mL of primary antibody at 4°C overnight. Primary antibodies used were: rabbit anti-mouse anti-β-actin monoclonal antibody (1:4000, TA-09, Zhongsui Jinqiao, China); rabbit anti-$5HT_{1A}$ Receptor ($5HT_{1AR}$) (1:2000, bs-1124R, Bioss, China) and rabbit anti-$5HT_{2A}$ Receptor ($5HT_{2AR}$) (1:2000, bs-1056R, Bioss, China). China). After rewarming, the excess primary antibody was discarded and incubated with the secondary antibody at room temperature for 2 h. The secondary antibodies used were: mouse anti-Goat Anti Mouse IgG-HRP (1:4000, M21001L, Abmart, China) and Goat Anti Rabbit IgG-HRP (1:4000, M21002L, Abmart, China). Subsequently, chemiluminescence and the 5200Multi chemiluminescence instrument (Tanon, China) were used for color development observation.

Immunofluorescence (IF)

Rat hippocampal tissue was prepared into 5 μm thick sections and then deparaffinized. Subsequently, the sections were antigenically repaired and occluded using 5% sheep serum for 1 h. Then, primary antibodies rabbit anti-$5HT_{1AR}$ (1:80, bs-1124R, BIOSS) and rabbit anti-$5HT_{2AR}$ (1:100, bs-1056R, Bioss, China) diluted in 2% sheep serum were incubated with the antibody at 4°C overnight. After Sheep anti-Affinity purified antibody 488 labeled goat Anti-rabbit IgG (H+L) (1:1000, 5230–0385, KPL, USA) was added for labeling. The slices were sealed with an anti-quenching sealer (containing DAPI, 50 μL) and then scanned into slices under a fluorescence microscope.

Transcriptome Sequencing and Functional Enrichment Analysis

Hippocampal tissue samples were analyzed by transcriptome sequencing. Each set of differential genes (DEGs) was subjected to Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) bioinformatics analysis, and protein-protein interaction network analysis (PPI). Transcriptome sequencing was performed by Oebiotech Shanghai. Hisat2 was used for sequence comparison of clean reads.[34] Htseq-count software was used to obtain the number of reads compared to protein-coding genes.[35] The FPKM method was used to exclude errors due to differences in protein-coding gene lengths and sequencing volumes. The DESeq2 software was used to process each sample gene and screen for DEGs

based on a multiplicity of differences of 1.5 and significance of difference test ($p < 0.05$).[36,37] The DEGs were subjected to GO[38] and KEGG[39] enrichment analyses and calculated by the hypergeometric distribution test. $p < 0.05$ was considered significant. Protein-Protein Interaction Networks (PPI) were used to obtain gene interrelationships and to map interactions based on the annotation of species information in the STRING database.

RT-qPCR

Total RNA was extracted using TRIzol (15596026, Lifetech, China). Subsequently, RNA was converted to cDNA following the guidelines of the manufacturer of FastKing RT cDNA (With gDNase) kit (KR116, Tiangen, China). The cDNA was extracted using Taq Pro Universal SYBR qPCR Master Mix (Q712-02, Vazyme, China) was used to extract cDNA and amplify the target gene for real-time quantification. The primers used were as follows, and the primer directions were all 5'-3': β-actin(R)-F: CTGGAGAAGAGCTATGAG; β-actin(R)-R: GATGGAATTGAATGTAGTTTC; Cngb1(R)-F: AACTTCCAAGACTCAAGAC; Cngb1(R)-R: TAGCACCTTCTCCAGATT; Cabp4(R)-F: CTGGATGAGATGTTGAGAG; Cabp4(R)-R: TCAGCCTGTAGATAGCAT; Sag(R)-F: GACCAATAACACGGAGAA; Sag(R)-R: CGAGTAGAGAACCACATT; TyR(R)-F: TAAGGACATCAACATCTA; TyR(R)-R: CATAATAGCAAGAACAGT; Trpm1 (R)-F: AGACCTCAACACCTACAA; Trpm1(R)-R: CTAAGACCTCGGACAAGT; Adipoq(R)-F: ATGTATCACTCAGCATTC; Adipoq(R)-R: CTGTTGGTTGTAGAAGAT.

Statistical Analysis

Each set of data had at least 3 parallel. Researchers conducting behavioral assessments, molecular analyses, and transcriptomic sequencing were unaware of the group assignments. Statistical probability was assessed using SPSS 23.0. The Shapiro–Wilk test was used to confirm normality, and the Brown-Forsythe test was employed to verify homogeneity of variance. After satisfying these assumptions, a one-way ANOVA was conducted, followed by Tukey's post hoc test with Bonferroni correction. The $p< 0.05$ was considered significant. GraphPad Prism 9.5 and Origin 2021 for visualization.

Results

Therapeutic Effect of ATAS-EMIT on PCPA Insomnia Rat Model

Acupuncture treatment of insomniac rats, followed by open field test characterization, revealed improved anxiety in ATAS-EMIT-treated rats. Significant reduction in voluntary activity, as evidenced by a significant reduction in total distance moved and number of times standing up (Figure 2A–C). Moreover, the results of the barbiturate synergistic sleep experiments also revealed a significant reduction in sleep latency and a significant increase in sleep duration in ATAS-EMIT-treated rats (Figure 2D–F). Furthermore, the tail suspension test also revealed a significant increase in the resting time of rats in the ATAS-EMIT treatment group (Figure 2G and H). Besides, the insomniac rats in the sham acupuncture group (Sham) performed consistently with the Model group, and no improvement in insomnia symptoms was found. These results indicate that ATAS-EMIT is effective in treating insomnia and demonstrate that the therapeutic effect is not a placebo effect.

Effects of ATAS-EMIT Treatment on Hippocampal Organization in Insomniac Rats

Hippocampal tissues of PCPA-induced insomnia rats were isolated and evaluated for the levels of 5-HT, adenylate cyclase (AC), and cyclic adenosine monophosphate (cAMP). 5-HT is a key central neurotransmitter for sleep onset and maintenance.[30] AC is a key enzyme that catalyzes the production of cAMP from ATP and is closely associated with the development of insomnia.[29] Our findings revealed that PCPA induced a significant decrease in the expression of 5-HT, AC, and cAMP in the hippocampal tissue of insomniac rats. Notably, these phenomena were significantly reversed after ATAS-EMIT treatment (Figure 3A–C). Moreover, the 5-HT_{1A} receptor and 5-HT_{2A} receptor are the major subtypes of the 5-HT receptor subfamily regulating sleep-wakefulness.[40,41] In this study, Western blot (Figure 3D and E) and IF (Figure 3F–H) assays for 5-HT_{1AR} and 5-HT_{2AR} revealed that insomnia led to a significant decrease in the expression of 5-HT1_{AR} and a significant increase in the expression of 5-HT2_{AR} in the hippocampal tissues of rats. Notably, ATAS-

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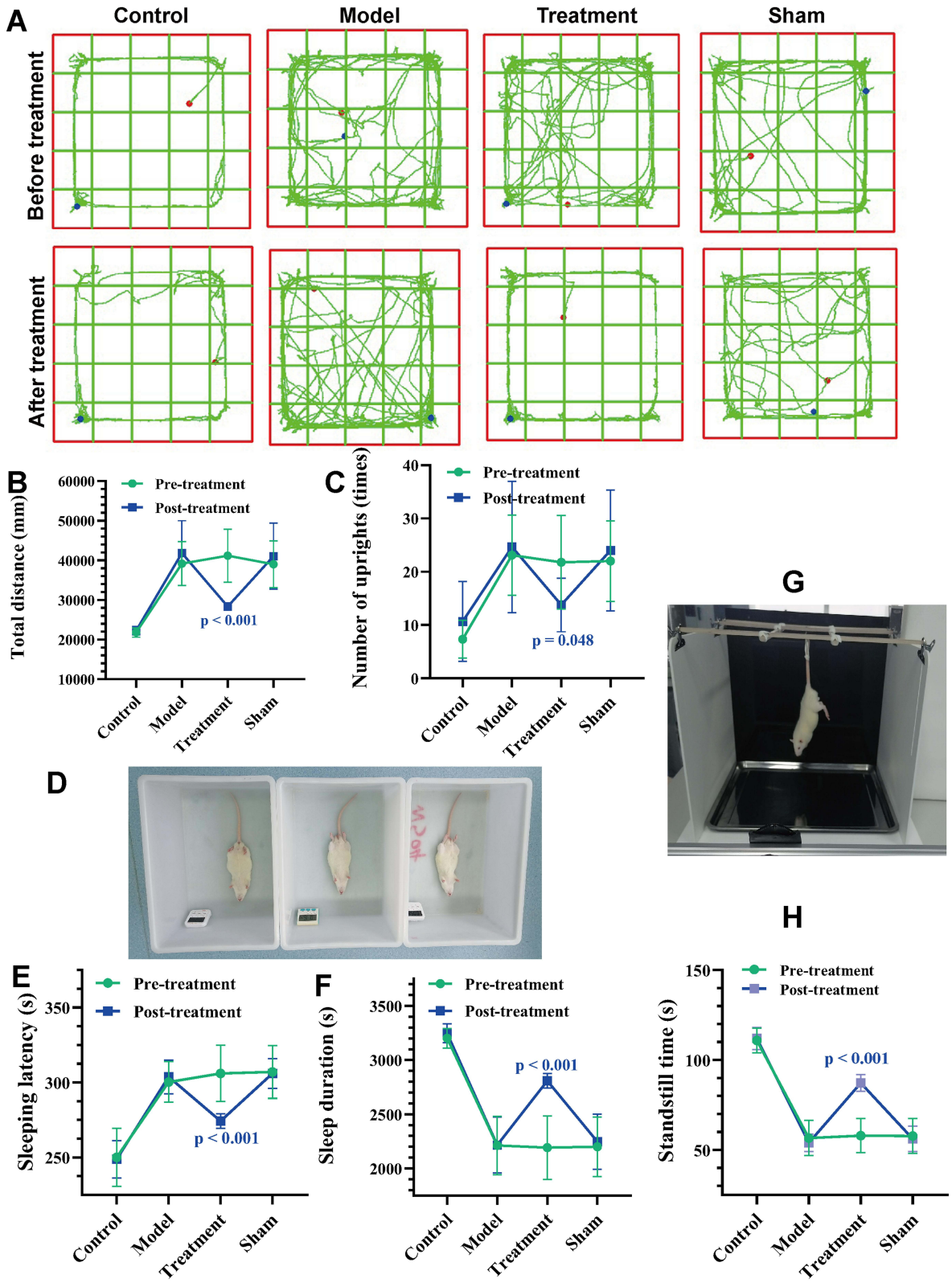

Figure 2 ATAS-EMIT treatment effectively improves insomnia behavior in PCPA-induced insomnia rats. (**A**): Trajectory diagrams of open field tests. (**B** and **C**): Comparison of total distance (**B**) and number of uprights (**C**) before and after treatment. (**D**–**F**): The barbiturate synergistic sleep experiment (**D**) was used to compare sleep latency (**E**) and sleep duration (**F**) before and after treatment. (**G** and **H**): The tail suspension test (**G**) was used to compare the standstill time of rats before and after treatment. Each set of data had at least 3 replicates. $p < 0.05$ was considered a significant difference.

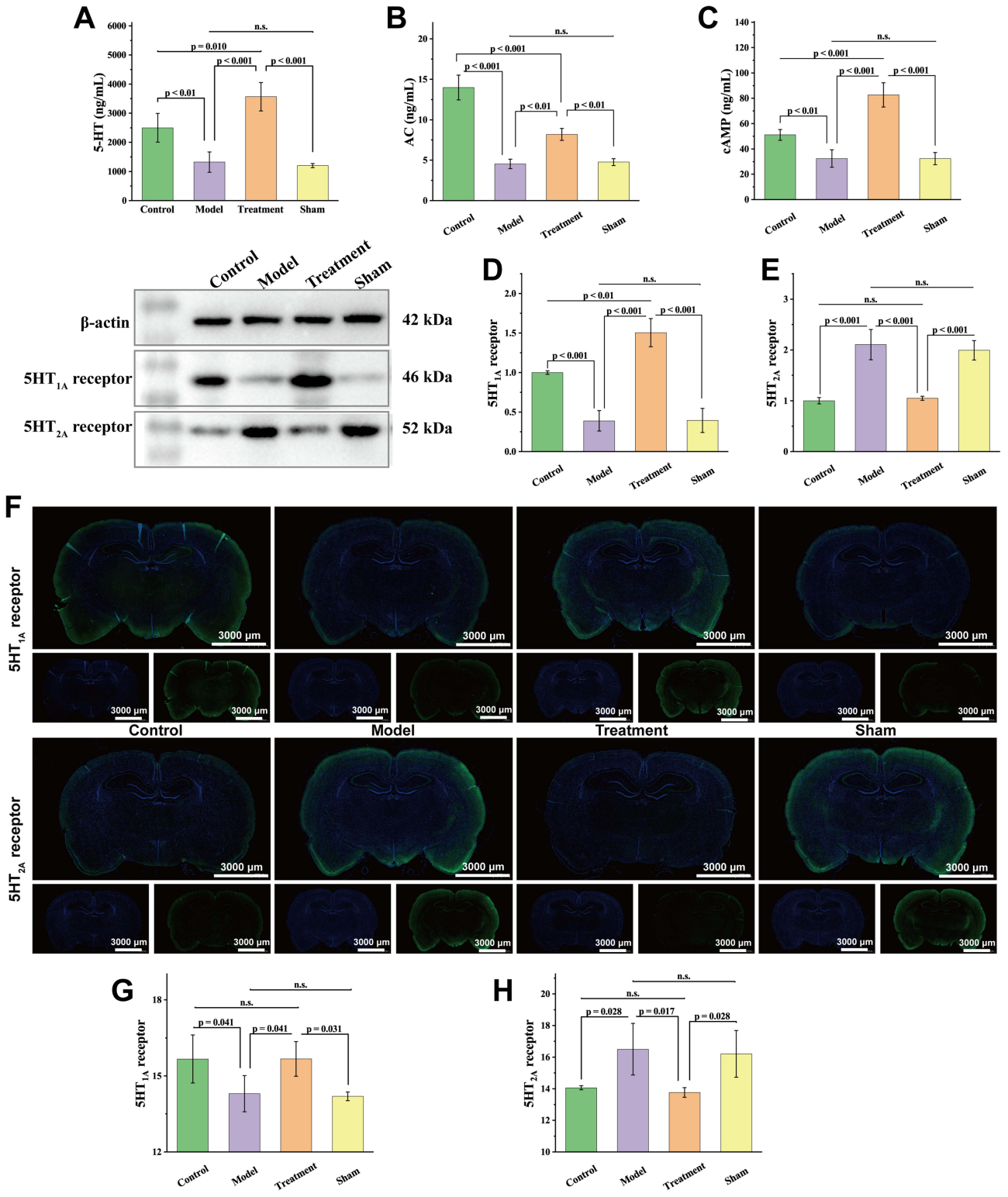

Figure 3 ATAS-EMIT exerts therapeutic effects on insomnia through activation of the 5-HT/AC/cAMP pathway. (**A**–**C**): ELISA was used to assess the effects of ATAS-EMIT on the expression of 5-HT (**A**), AC (**B**), and cAMP (**C**) in hippocampal tissues of insomniac rats. (**D** and **E**): Western blot was used to assess the effects of ATAS-EMIT treatment on the expression of $5HT_{1A}$ (**D**) and $5HT_{2A}$ (**E**) receptors in hippocampal tissues of insomniac rats. (**F**–**H**): Immunofluorescence (**F**) was used to assess the effect of ATAS-EMIT treatment on $5HT_{1A}$ (**G**) and $5HT_{2A}$ (**H**) receptor expression in hippocampal tissues of insomniac rats. Each set of data had at least 3 replicates. ns indicates that the difference between the comparison groups is not statistically significant; $p < 0.05$ was considered a significant difference.

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EMIT treatment effectively promoted the expression of 5-HT_{1AR} and restored 5-HT_{2AR} content to normal levels. These results suggest that ATAS-EMIT can effectively regulate the expression of sleep-related factors in the hippocampus of insomniac rats to treat insomnia.

Transcriptome Sequencing and Functional Enrichment Analysis of Hippocampal Tissue

Reference transcriptome sequencing of hippocampal tissues from rats in the Control, Model, and Treatment groups yielded a total of 61.41 G of clean data, with a Q30 base distribution ranging from 96.8 to 97.02% and an average GC content of 52.60% (Figure 4A). DEGs were screened at $p < 0.05$ and the absolute value of log2 FC was greater than 1. Model vs Control had a total of 271 DEGs (153 downward and 118 upward) (Figure 4B). Treatment vs Control had 204 DEGs (145 downward and 59 upward) (Figure 4C). Model vs Treatment had a total of 235 DEGs (92 downward and 143 upward) (Figure 4D). These DEGs were analyzed for GO functional enrichment. Insomniac rats are upregulated relative to control rats in the biological processes such as visual perception (0007601), response to stimulus (0050896), phototransduction (0007602), detection of light stimulus involved in visual perception (0050908), retinal cone cell development (0046549), and photoreceptor cell outer segment organization (0035845). Furthermore, insomniac rats are upregulated photoreceptor outer and inner segment (0001750 and 0001917), interphotoreceptor matrix (0033165), cone photoreceptor outer segment (0120199), heterotrimeric G-protein complex (0005834), intracellular cyclic nucleotide-activated cation channel complex (0017071), and extracellular space (0005615) in cellular component. Moreover, cGMP binding (0030553), opsin binding (0002046), intracellular cGMP-activated cation channel activity (0005223), and spectrin binding (0030507) in molecular function are upregulated (Figure 4E). These results suggest that PCPA-induced insomnia is closely related to the processes of visual perception of light and phototransduction. It is noteworthy that all these GO terms mentioned above showed down-regulation after ATAS-EMIT treatment (Figure 4F). Additionally, DEGs of Treatment vs Control were enriched on GO terms such as down-regulated monoamine transport (0015844), serotonin uptake (0051610), dopaminergic synapse (0098691), and neuron projection (0043005) (Figure 4G). These findings suggest that ATAS-EMIT treatment is effective in treating insomnia through gene expression alteration. In addition, KEGG functional enrichment analysis revealed that the phototransduction (rno04744), serotonergic synapse (rno04726), tryptophan metabolism (rno00380), and GABAergic synapse (rno04727) related pathways were up-regulated in insomnia rats. Meanwhile, the viral protein interaction with cytokine and cytokine receptor (rno04061) pathways was down-regulated (Figure 5A). Notably, like the GO functional enrichment analysis, ATAS-EMIT treatment was effective in reversing these changes (Figure 5B). In addition, ATAS-EMIT can down-regulate pathways such as cocaine addiction (rno05030), amphetamine addiction (rno05031), alcoholism (rno05034), and parkinson disease (rno05012), suggesting that ATAS-EMIT not only treats insomnia but also has a variety of beneficial effects (Figure 5C).

Screening and Validation of Hub Genes in ATAS-EMIT for the Treatment of Insomnia

To screen for hub genes, DEGs were analyzed by PPI. We first counted the DEGs in the Model and Treatment groups and performed protein interaction analysis on DEGs that did not produce intersections (Figure 6A and B). Subsequently, DEGs that produced intersections between the two groups were analyzed for protein interactions (Figure 6C). Finally, the genes *Cngb1, Cabp4, Sag, Tyr, Trpm1*, and *Adipoq* were selected as hub genes. RT-qPCR experiments revealed that the expression of *Cngb1, Cabp4, Sag, Tyr*, and *Trpm1* was elevated in the insomnia model group, and was further elevated in the treatment group (Figure 6D–H). The mRNA expression of *Adipoq* was elevated in the insomnia model group and significantly decreased in the treatment group relative to the model group (Figure 6I).

Analysis of Hub Gene Interactions with 5-HT Proteins

The interactions between hub genes and MAOA (a gene controlling 5-HT degradation), HTR1A, and HTR2A (genes of 5HT1/2A receptor) were analyzed by using the String database. The results of the analysis revealed that these genes have an interaction relationship (Figure 7A). Subsequent functional enrichment analyses revealed that they were enriched on stimulus-related GO terms such as phototransduction, external stimulation, photostimulation, and abiotic stimulation (Figure 7B). Besides, they were enriched in neuron projection term in cellular component (Figure 7C). KEGG enrichment analysis revealed that they were enriched in pathways such as serotonergic synapse, phototransduction, and tyrosine

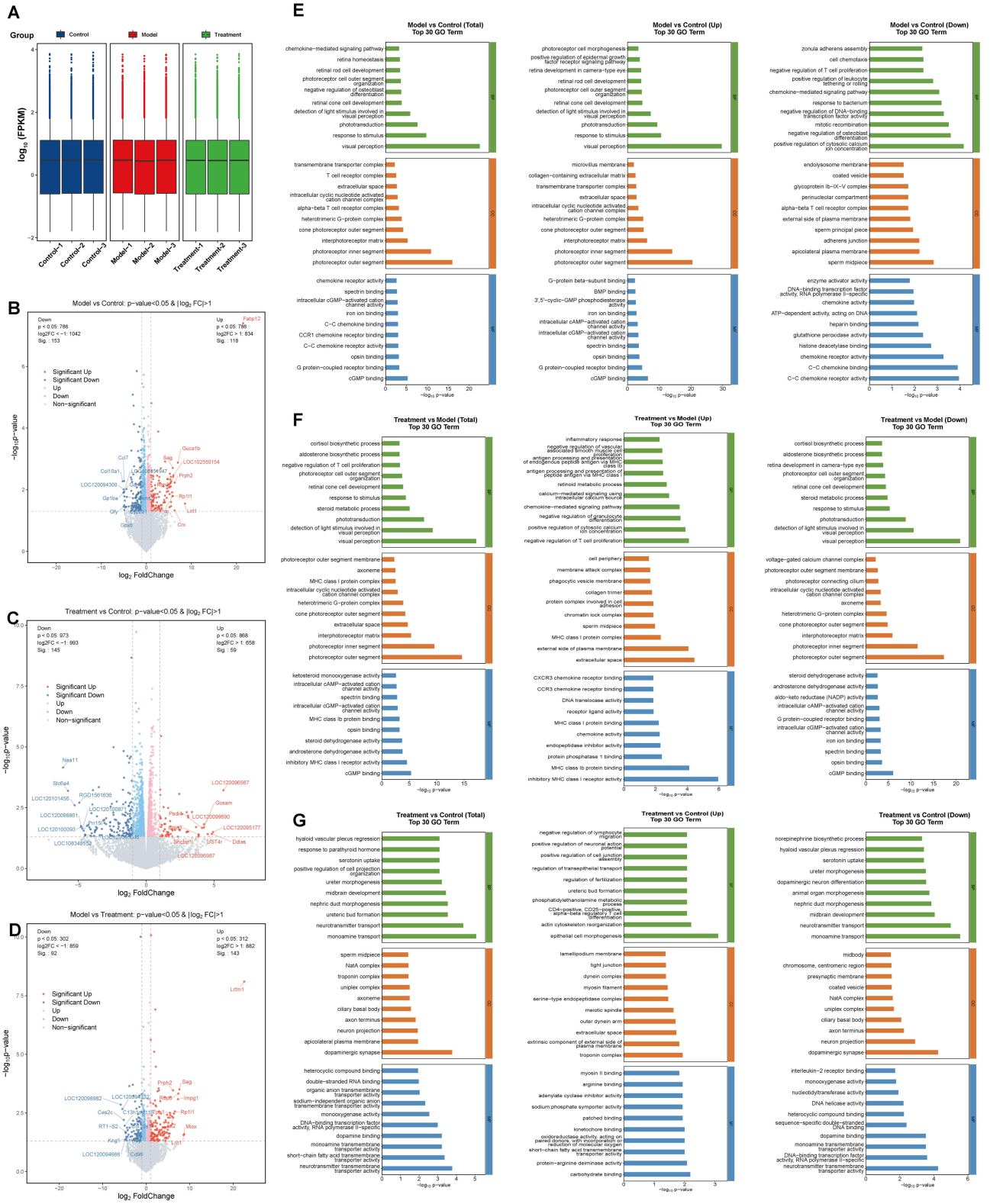

Figure 4 Transcriptome sequencing was used to explore more potential mechanisms of ATAS-EMIT for insomnia. (**A**): Box-whisker plot of FPKM values for each sample gene. (**B–D**): DEGs in Model vs Control group (**B**), Treatment vs Control group (**C**), and Model vs Treatment group (**D**); Genes with up-regulated expression (red) and genes with down-regulated expression (blue). (**E–G**): GO functional enrichment of DEGs in Model vs Control group (**E**), Treatment vs Model group (**F**), and Treatment vs Control group (**G**). $p < 0.05$ was considered a significant difference.

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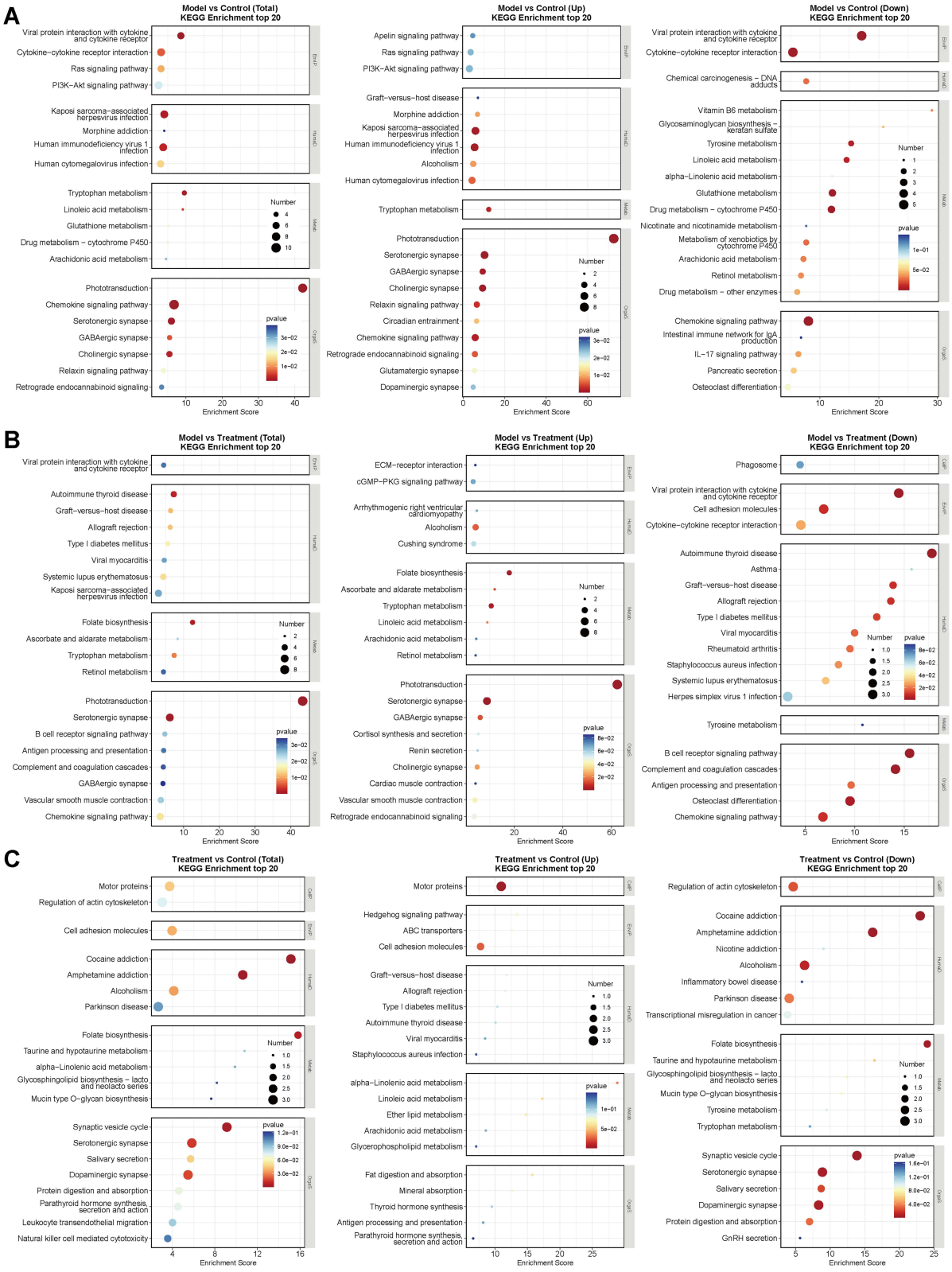

Figure 5 KEGG functional enrichment analysis of DEGs. (**A**–**C**): Model vs Control group (**A**), Model vs Treatment group (**B**), and Treatment vs Control group (**C**) KEGG functional enrichment analysis of DEGs. $p < 0.05$ was considered a significant difference.

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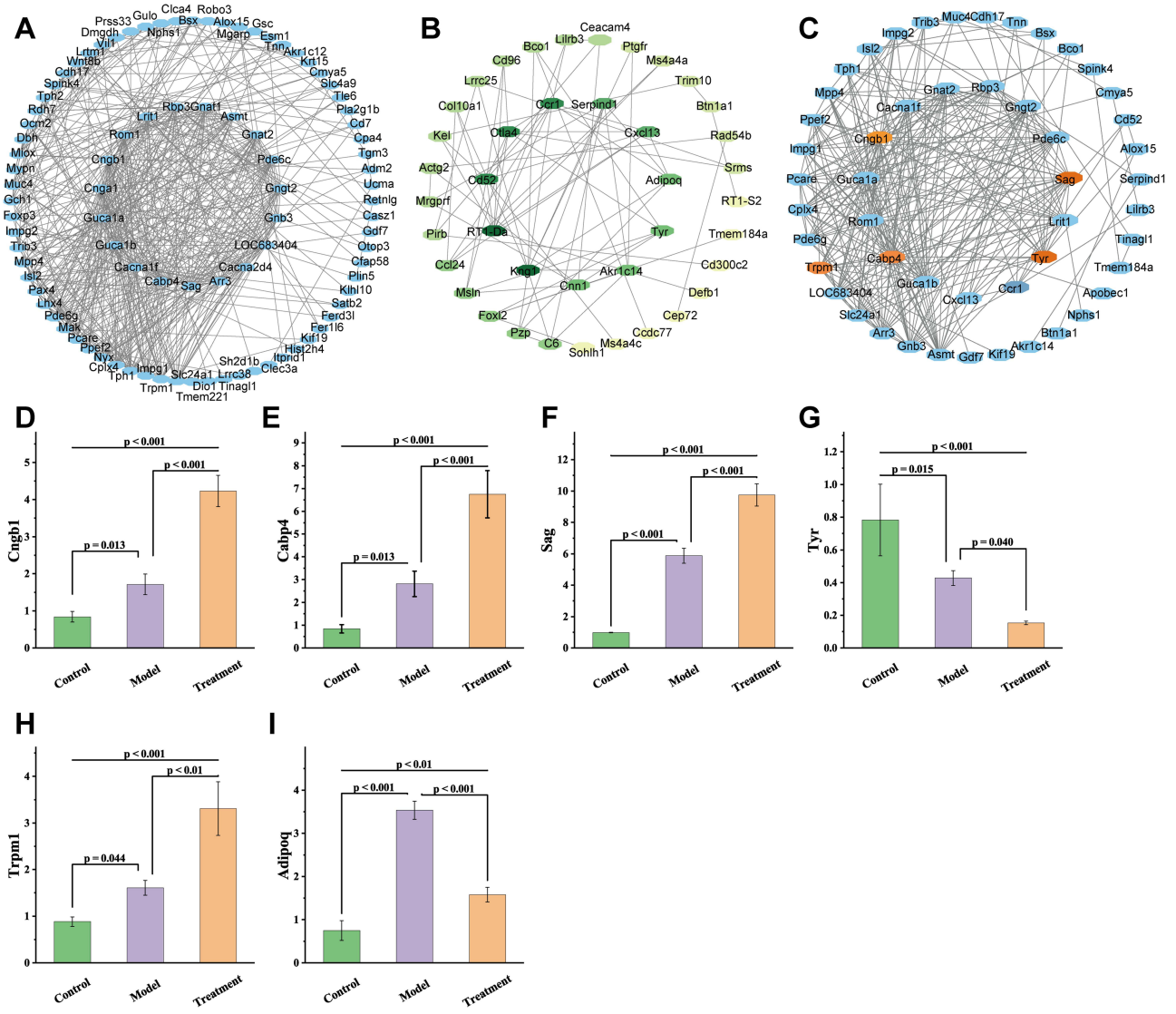

Figure 6 Screening and validation of hub genes. (**A** and **B**): PPI analysis of DEGs with up-regulated (**A**) and down-regulated (**B**) expression, which did not produce intersections in the Model vs Treatment group. (**C**) PPI analysis of DEGs that produced intersections in the Model vs Treatment group. (**D–I**): RT-qPCR was used to validate the mRNA expression levels of hub genes, *Cngb1* (**D**), *Cabp4* (**E**), *Sag* (**F**), *Tyr* (**G**), *Trpm1* (**H**), and *Adipoq* (**I**), β-actin as the internal control gene. Each set of data had at least 3 replicates. $p < 0.05$ was considered a significant difference.

metabolism (Figure 7D). Diseases-gene association enrichment analysis revealed that they were enriched for mental disorders such as obsessive-compulsive disorder, psychotic depression, and congenital stationary night blindness (Figure 7E). These findings suggest that ATAS-EMIT may improve insomnia by reducing neural projections from external stimuli.

Discussion

Insomnia itself is not only a disease that affects people's normal life, but may also lead to a variety of mental disorders or co-morbidities, of which depression is the most common.[42] Current treatment options for insomnia are limited and prone to negative consequences, such as alcohol or drug abuse, and hypnotherapy does not address the root cause of the problem.[43,44] It is interesting to note that several clinical studies have shown that conventional acupuncture has significant efficacy in the treatment of both secondary and primary insomnia, and the establishment of a sham acupuncture group revealed that this treatment effect is not a placebo effect.[45–48] Meanwhile, there is no shortage of such findings in animal studies.[49,50] These studies suggest that acupuncture may be a worthwhile treatment option for

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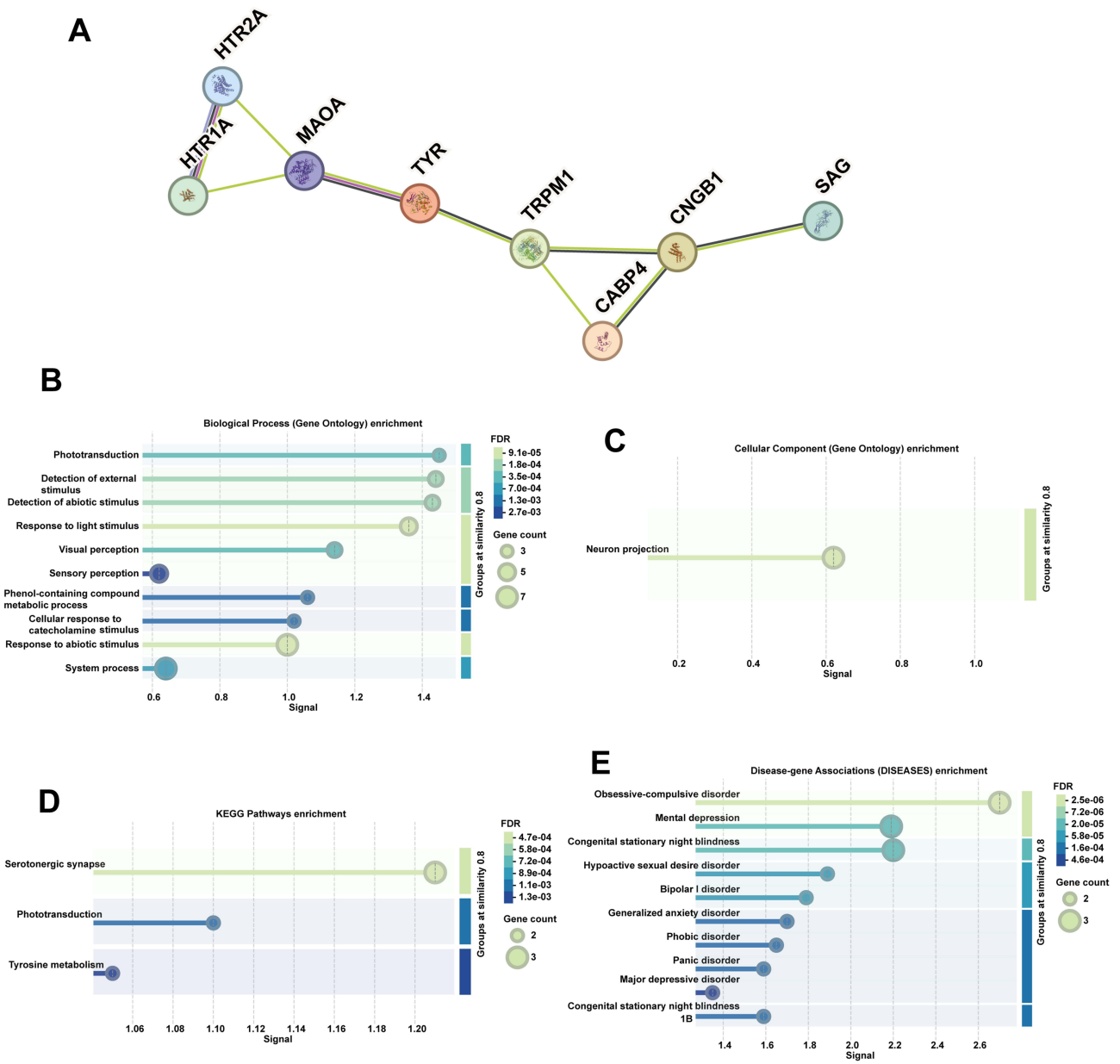

Figure 7 PPI analysis of hub genes and 5-HT-related proteins. (**A**) Interaction relationship between hub genes and 5-HT-related proteins. (**B** and **C**) GO enrichment analysis of this interaction in biological process (**B**) and cellular component (**C**). (**D**) KEGG pathway enrichment analysis of this interaction chain. (**E**) Disease gene association enrichment analysis of this interaction chain. $p < 0.05$ was considered.

insomnia with unrestricted cycles of use, low toxicity, low dependence, and high efficacy. In our animal study, we also observed a significant improvement in insomnia performance in rats with PCPA-induced insomnia because of ATAS-EMIT treatment. Specifically, there was a reduction in anxiety-like behaviors, total distance traveled, and number of uprights, sleep latency, and a prolongation of sleep duration and suspended resting time. This is similar to previous studies that found electroacupuncture in *cymba concha* prolonged sleep duration and reduced anxiety-like behaviors in open field tests.[51] Differently, electroacupuncture treatment in the *cymba concha* did not observe a reduction in motor activity in the open field test, whereas ATAS-EMIT treatment in our study was able to significantly reduce the total distance traveled and the number of times of uprightness in the open field test in insomniac rats, suggesting that ATAS-EMIT may be a more efficacious acupuncture treatment.

Although the efficacy of ATAS-EMIT in the treatment of insomnia is remarkable. However, it is necessary for scientific evidence to elucidate the underlying mechanisms. In this study, we examined insomnia-related indices and found that ATAS-EMIT significantly reversed the reduction of 5-HT, AC, and cAMP expression in the hippocampus caused by insomnia. Besides, the decrease in the expression of 5-HT in the hippocampus of rats with PCPA-induced insomnia has been observed in several studies, and the increase in its expression content suggests that the treatment has a potential therapeutic effect on insomnia.[52,53] Furthermore, researchers have found that AC inhibition causes a decrease in cAMP, which is an important second messenger closely related to mental disorders,[54,55] which is similar to what we have found. Furthermore, analysis of the two subtypes of 5-HT revealed that ATAS-EMIT significantly up-regulated the expression of 5HT1A receptor ($5HT_{1AR}$) and decreased the expression of 5HT2A receptor ($5HT_{2AR}$) in the hippocampus of insomniac rats, which is in line with the trend of the previous studies on the saponin of *Liriope spicata* Lour, and that this saponin was effective as a sedative and hypnotic agent in PCPA-induced insomniac mice.[56] These studies suggest that ATAS-EMIT can promote the $5HT_{1AR}$, reduce $5HT_{2AR}$ expression, and activate the 5-HT/AC/cAMP pathway to achieve the therapeutic purpose of PCPA-induced insomnia.

In our animal studies, the mechanism of ATAS-EMIT treatment for insomnia has primarily focused on 5-HT, particularly the 5-HT/AC/cAMP pathway. However, whether additional pathways are involved warrants further investigation. Transcriptome sequencing not only identifies DEGs to guide relevant clinical drug development but also uncovers additional pathways to direct future research. GO functional enrichment analysis of DEGs revealed increased visual perception, phototransduction, and sensitivity to stimuli in PCPA insomniac rats. Moreover, the DEGs were predominantly enriched in cellular components such as photoreceptors. Furthermore, previous studies have also found that insomnia is associated with dim light and circadian photoreceptors.[57] Remarkably, the ATAS-EMIT treatment group was able to downregulate the genes involved in these functions. It is suggested that ATAS-EMIT can regulate circadian rhythms by modulating photoreception to treat insomnia. In addition, ATAS-EMIT treatment down-regulated monoamine transport, serotonin uptake, dopaminergic synapse, and neuron projection, which were found to be associated with the regulation of sleep and wakefulness.[58–60] Moreover, KEGG functional enrichment analysis revealed that ATAS-EMIT could also down-regulate genes of cocaine addiction, amphetamine addiction, alcoholism, and parkinson disease. These findings suggest that the beneficial effects of ATAS-EMIT go far beyond the treatment of insomnia. PPI analysis screened and validated six hub genes, *Cngb1, Cabp4, Sag, Tyr, Trpm1*, and *Adipoq*. It is noteworthy that the expression of all genes except *Adipoq* was elevated in the model group and the treatment group. *Cngb1, Cabp4, Sag*, and *Trpm1* are genes associated with photoreceptors and phototransduction. *Cngb1* encodes the β-subunit of cyclic nucleotide-gated ion channels in rod photoreceptors, participating in photoreceptor regulation.[61,62] *Cabp4* is a photoreceptor-specific protein critical for the development and maintenance of photoreceptor synapses.[63] *Sag* is present in photoreceptor cells and participates in rod phototransduction regulation by desensitizing rhodopsin.[64,65] *Trpm1* is essential for the pupillary light reflex and the contraction of the iris in response to light-mechanical stimuli.[66] Additionally, studies have revealed that casein-derived peptides promote sleep, with Tyr and Pro playing key roles in this process.[67,68] The increased expression in the model group may be attributed to the compensatory regulation of the down-regulation of gene expression due to insomnia. Interaction analysis of hub genes and *MAOA*, a gene controlling 5-HT degradation,[69,70] as well as the genes for 5HT1/2A receptor, *HTR1A*, and *HTR2A*, using the String database, revealed that these genes can interact with each other. Moreover, functional enrichment analysis revealed that it is enriched in neuron projection and may play a role in phototransduction, stimulation, and tyrosine metabolism. Inhibition of tyrosine metabolism was found to be associated with the treatment of insomnia,[71] which is also consistent with our KEGG enrichment analysis finding that ATAS-EMIT treatment downregulated genes enriched in the tyrosine metabolism pathway. Furthermore, disease enrichment analyses found them to be associated with psychiatric disorders such as obsessive-compulsive disorder, psychotic depression, and congenital stationary night blindness. These findings suggest that ATAS-EMIT may improve insomnia by reducing external stimuli and modulating serotonin and may play a role in a variety of psychiatric disorders.

In summary, our results found that ATAS-EMIT is an effective treatment for insomnia and may have better results than other acupuncture treatments. The 5-HT/AC/cAMP pathway activation is involved. Processes such as photoreception, transduction, and sensitivity to stimuli, as well as several factors such as tyrosine and dopamine, were modulated to jointly contribute to the insomnia treatment effect of ATAS-EMIT. It is worth mentioning that the results of transcriptome

sequencing require further experiments for validation and refinement. This study utilized only male rats and lacked long-term follow-up to assess the durability of effects. Interpretation of clinical treatment samples is also necessary. Furthermore, comparing the efficacy of ATAS-EMIT with traditional acupuncture methods for treating insomnia would be beneficial for the research and development of ATAS-EMIT. These research limitations will guide our next steps to provide a clearer outlook for ATAS-EMIT. Despite these limitations, the current study provides valuable insights into the treatment of insomnia with ATAS-EMIT.

Conclusion

ATAS-EMIT is an effective treatment for insomnia. In the PCPA-induced insomnia model, behavioral observations revealed that ATAS-EMIT significantly improved insomnia symptoms. The specific manifestations were a reduction of anxiety-like behavior, sleep latency, and an increase in sleep duration. Further mechanistic exploration revealed the involvement of activation of the 5-HT/AC/cAMP pathway. Moreover, processes such as photoreception, transduction, and sensitivity to stimuli, and several factors such as tyrosine and dopamine, jointly contribute to the insomnia treatment effect of ATAS-EMIT. In short, this study provides an effective and low-harm treatment for insomnia and provides theoretical support for the use of ATAS-EMIT in the treatment of insomnia.

Data Sharing Statement

Data will be made available by the corresponding author on request.

Ethical Approval Statement

This study was approved by the Ethical Review Committee for Animal Experiments of Yunnan University of Traditional Chinese Medicine (R-062021LH031) and was performed in strict compliance with the ARRIVE guidelines.

Author Contributions

LL - data curation, investigation, formal analysis, project administration, writing - original draft, writing -review & editing; **ZZ** - data curation, validation, methodology, writing - original draft; **ZW** - formal analysis, funding acquisition, writing - original draft; **Li L** - data curation, project administration, conceptualization, writing -review & editing; **YF** - investigation, methodology, writing - original draft; **CG** - investigation, software, visualization, writing - original draft; **XP** - funding acquisition, visualization, writing - original draft; **XX** - formal analysis, resources, writing - original draft. All authors gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

None of the authors has any conflicts of interest to declare.

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