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Ziwuliuzhu Acupuncture Modulates Glu/GABA-Gln Metabolic Loop Abnormalities in Insomniac Rats

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Research Article

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ABSTRACT

Ziwuliuzhu acupuncture is widely considered an effective treatment for insomnia in clinics, but little is known about its possible mechanisms. This study investigated the therapeutic effect of Ziwuliuzhu acupuncture on insomnia and its regulatory mechanism on the Glutamic acid (Glu)/γ-aminobutyric acid (GABA) glutamine (Gln) metabolic loop in a rat model of insomnia. Hematoxylin and eosin staining was used to observe the pathological tissue in the hypothalamus. The levels of the neurotransmitters Glu and GABA in the hypothalamus were determined by high performance liquid chromatography (HPLC) Mass Spectrometry (MS)/MS. Immunohistochemistry (IHC) was used to detect the expression of the GABAA receptor in the hypothalamus. The expression levels of glutamate decarboxylase (GAD65/67) and Glutamine Synthetase (GS) in the hypothalamus were determined by Western blotting. Compared with the model group, the Ziwuliuzhu acupuncture groups showed decreased Glu levels ($p<0.001$) and GABAA receptor expression ($p<0.01$), increased GABA levels ($p<0.05$), and a decreased Glu/GABA ratio. In comparison to the model group, Ziwuliuzhu acupuncture increased the protein expression of GAD65 ($p<0.001$) and GAD67 ($p<0.05$) in the hypothalamus and reduced the expression of GS ($p<0.01$). Glu/GABA-Gln metabolism may be regulated by Ziwuliuzhu acupuncture to produce sedative and hypnotic effects, affecting Glu and GABA synthesis and decomposition, as well as restoring the excitatory/inhibitory balance between Glu and GABA.

Keywords: Ziwuliuzhu acupuncture; Glutamic acid; γ-aminobutyric acid; Glutamic acid/γ-aminobutyric acid–glutamine metabolism loop; Insomnia

INTRODUCTION

Currently, insomnia is one of the world's most serious health problems due to a variety of factors. Clinical medicine typically uses sedatives and hypnotics to combat insomnia, but these drugs can easily lead to dizziness, sleepiness, rebound insomnia, and even drug dependence or addiction. Traditional Chinese medicine is renowned for its effectiveness in treating insomnia. Specifically, acupuncture can enhance local blood circulation, reduce tension and spasm of soft tissues, accelerate tissue repair, and inhibit the production of inflammatory factors. Additionally, acupuncture regulates the functions of the viscera, stabilizes the heart rate, and improves sleep quality. Chinese time medicine, a theory that explains human life phenomena through the lens of time, includes methods such as Ziwuliuzhu acupuncture. This acupuncture method selects points and applies treatment at fixed times, ensuring that human blood correctly circulates through the twelve meridians, maintaining the rhythm of sleep/wake cycles, and thus improving insomnia symptoms. Ziwuliuzhu acupuncture can improve insomnia symptoms; however, the specific mechanism underlying its effects on insomnia remains unclear, and no studies have explored the subject [1].

Presently, benzodiazepines and nonbenzodiazepines are the two most commonly prescribed clinical drugs for insomnia; both are γ-aminobutyric acid A (GABAA) receptor agonists, primarily altering the effects of γ-aminobutyric acid (GABA) and other neurotransmitters. The main inhibitory neurotransmitter in the mammalian central nervous system is GABA and the main excitatory neurotransmitter is Glutamate (Glu). Astrocytes take up Glu released from neurons during synaptic activity and convert it to Glutamine (Gln) *via* Glutamine Synthetase (GS). A glutamate decarboxylase enzyme (GAD65 or GAD67) directly synthesizes GABA from Glu [2]. Animal experiments have shown that the excitatory/inhibitory imbalance between Glu and GABA is closely associated with the occurrence of insomnia. Glu and GABA metabolic conversion in the brain takes place through the Glu/GABA-Gln metabolic loop [3].

The purpose of this experiment was to determine the effect of Ziwuliuzhu acupuncture on the concentrations of Glu and GABA, the expression level of the GABAA Receptor (GABAAR), and the expression levels of GAD65, GAD67, and GS in the hypothalamus of a rat model of insomnia. Additionally, the relationship between the Glu/GABA-Gln metabolic loop and insomnia was investigated to establish an experimental foundation for the development of a clinical treatment for insomnia [4].

MATERIALS AND METHODS

Animals and ethics statement

Male Sprague Dawley (SD) rats (n=40, 4–6 weeks old, SPF grade, weight 220 ± 20 g) were purchased from Beijing Huafukang Biotechnology Co., Ltd. SCXK (Jing; animal certificate number 20210011). Food and water were provided to all animals before they were subjected to random experiments for a week in isolation. The laboratory animal ethics committee of Jinan university (license number SCXK) (Yue) 2017-0174) approved our experimental protocol (IACUC-20211229-05) and provided oversight and direction to all experimenters. All operations were conducted in accordance with the statute on the administration of laboratory animals [5-10].

Model establishment and group creation

Forty adult healthy SPF male SD rats were randomly divided into four groups (n=10 rats each): the control, model, Najia and Nazi groups (the latter two are different methods of Ziwuliuzhu acupuncture). The model, Najia, and Nazi groups received intraperitoneal injections of p-Chlorophenylalanine (pCPA; DL-4-Chlorophenylalanine, C6505, Sigma, Burlington, MA, USA). The pCPA suspension (dissolved in 0.9% sodium chloride solution at 100 mg/ml and a dose of 450 mg/kg) was injected intraperitoneally within one hour after 9:00 a.m. (according to the local time in Guangzhou) for two consecutive days. At 36 hours after injection, the pentobarbital sodium sleep synergy experiment was conducted to evaluate the insomnia model.

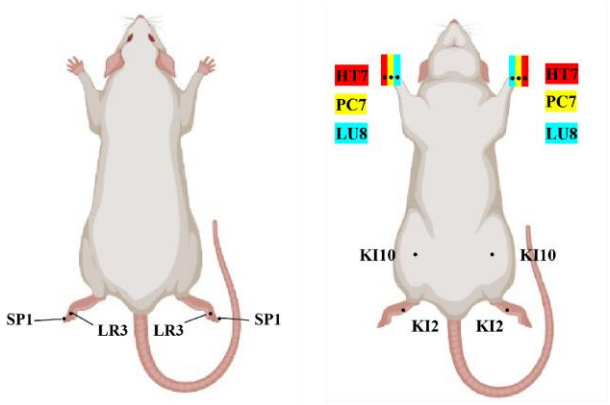
Pentobarbital sodium sleep synergy experiment

At 8:00 p.m. on the night of the last treatment, an intraperitoneal injection of sodium pentobarbital (No: AS5062-5, Merck, Germany) (50 mg/ml, dissolved in 0.9% sodium chloride solution at a dose of 35 mg/kg) was administered to all 40 rats. The righting reflex of rats was then assessed on a flat plate (Figure 7). When the rats were placed on the flat plate in a supine position (abdomens upward), the righting reflex disappeared after more than 60 seconds. The time from the intraperitoneal injection to the disappearance of the righting reflex was recorded as the sleep latency; the rats that turned over more than twice within 60 seconds were regarded as recovering the righting reflex, and the duration from the disappearance of the righting reflex to the recovery of the righting reflex was regarded as the duration of sleep [11-20].

Animal treatment

After the insomnia model was successfully established, treatment was administered to the Najia and Nazi groups. Based on the acupoint pattern of the rats, acupuncture points were selected. Acupoints with the most abundant blood channels were selected for treatment in accordance with the Ziwuliuzhu Najia method. According to the treatment time (2022.3.14-2022.3.20), stimulation was separately applied to the following acupoints: The bilateral Yingu (KI10), bilateral Taichong (LR3), bilateral Daling (PC7), bilateral Yinbai (SP1), bilateral Jingqu (LU8), bilateral Rangu (KI 2), and bilateral Taichong (LR3). According to the Ziwuliuzhu Nazi method, the heart meridian's original bilateral Shenmen (HT7) were selected as acupoints (Figure 1). Rats were anatomically stimulated at acupuncture points that corresponded to human acupoints. For acupuncture treatment, following stereotactic fixation of rats, sterile acupuncture needles (0.18 mm diameter, 15 mm length; Suzhou Huatuo medical instrument Co., Ltd., Suzhou, China) were inserted perpendicularly into the acupuncture points to a depth of 2 mm. The needle was retained at that depth for 15 min, and a reinforcing reducing method was used every five min to turn each needle. Acupuncture was applied at the following times: 1) In the Najia group, after successful modeling, at 9:00 a.m. on 7 consecutive days; and 2) In the Nazi group, after successful modeling, at 11:00 a.m. for 7 consecutive days. Before modeling, the control group was intraperitoneally injected with an equal volume of 0.9% sodium chloride solution and continuously injected for 2 days. The model group and the Najia and Nazi groups were subjected to model establishment. After model establishment, the control and model groups underwent the same period of stereotactic fixation (for 15 min per day) but did not receive acupuncture treatment. During acupuncture, all rats were fixed in place with a stereotactic apparatus.

Figure 1. The prone and supine positions of the rats. Rats were stimulated using specific acupuncture points with anatomical correspondence to human acupoints. SP1 (Yinbai) is located at the medial aspect of the big toe, approximately 0.1 cun posterior to the corner of the nail. LR3 (Taichong) is located in the depression distal to the junction of the 1st and 2nd metatarsal bones. HT7 (Shenmen) is situated in the transverse crease of the wrist of the forepaw, radial to the flexor carpi ulnaris tendon. PC7 (Daling) is on the midpoint of the transverse crease of the wrist between the tendons of the palmaris longus and flexor carpi radialis. LU8 (Jingqu) is located in the depression between the styloid process of the radius and the radial side, 1 cun above the transverse crease of the wrist. KI2 (Rangu) is located on the medial border of the foot, inferior to the tuberosity of the navicular bone, at the junction of the pink and pale skin. KI10 (Yingu) is located on the medial side of the popliteal fossa between the tendons of the semitendinosus and semimembranosus.



Histology

Within 12 hours after the end of the treatment period, we collected samples from the rats and placed them in a stereotactic apparatus. As part of the anesthesia process, all rats were fasted and rested after treatment, and 2% sodium pentobarbital (0.15 mL/100 g) was injected intraperitoneally. To fully expose the abdominal cavity of the rat, the abdominal skin was cut open. During the procedure, blood was collected from the abdominal aorta following blunt dissection, and then rats were decapitated on ice to remove the brain. The hypothalamus was separated from the taken brain tissue and the SCN tissue was stripped under the microscope, placed into an EP tube, and labeled with the group. EP tubes were stored in an ice box. For preservation, the specimen tissues were transferred from the ice box to the -80°C freezer after all brain tissues were collected. Paraformaldehyde (4%, P1110-2, Nocon Bio Company, Guangzhou, China) was used to fix part of the hypothalamus of the rats [21-25].

Indicator detection

Hematoxylin and eosin staining: Hypothalamic tissue fixation was dehydrated with the following parameters: Xylene I (SCRC 10023418, Sinopharm Chemical Reagent Co., Ltd.) for 20 min, Xylene II (SCRC 10023418, Sinopharm Chemical Reagent Co., Ltd.) for 20 min, 100% ethanol I (SCRC 100092683, Sinopharm Chemical Reagent Co., Ltd.) for 5 min, 100% ethanol II (SCRC 100092683, Sinopharm Chemical Reagent Co., Ltd.) for 5 min, and 75% ethanol for 5 min, followed by rinsing with tap water. The sections were stained with hematoxylin solution for three to five min and then rinsed with tap water. After applying hematoxylin differentiation solution, the slices were rinsed with tap water and rinsed again with tap water after bluing. Sections were then stained with eosin dye (DIAPATH Giotto, Italy) for 5 min after being submerged in 85% and 95% ethanol for 5 min each. The sections were dehydrated as follows: Xylene I for

5 min, Xylene II for 5 min, 100% ethanol I for 5 min, and 100% ethanol II for 5 min. Finally, sections were sealed with neutral gum. The sections were examined under a microscope (NIKON ECLIPSE E100, Nikon, Japan), which was also used for picture capture and analysis (DS-U3, Nikon, Japan).

HPLC-MS/MS

The neurotransmitters GABA and Glu were extracted from the rat hypothalamus. For the chromatographic procedure, a Waters Bradas Energy Holdings (BEH) column was used to separate the sample, and water (phase A, containing 0.1% formic acid) and acetonitrile (phase B, containing 0.1% formic acid) were used as elution solvents. The elution gradient of the HPLC (Waters Technology Co., Ltd., USA) system was 1% B for 0~2 min, 2~90% B for 2~4 min, 90% B for 4~4.5 min, 90~1% B for 4.5~5 min, and 1% B for 5~6 min. The flow rate was 0.2 ml/min and the column temperature was 40℃. After the sample was separated by Ultra Performance Liquid Chromatography (UPLC) (Waters Technology Co., Ltd., USA), it entered the MS system for MS/MS analysis and detection. The MS conditions were as follows: detection in positive ion mode by means of spray ionization, Multireaction Modeling (MRM) scanning mode, and the following ion pairs used for quantitative analysis: m/Z 104 → m/Z 45 (γ-aminobutyric acid) and m/Z 148→m/Z 84 (glutamic acid) [26-30].

Immunohistochemistry

The steps of fixation, dehydration, embedding in paraffin, and sectioning were the same as those described above. Hypothalamic sections were washed in distilled water after dewaxing in water. The tissue sections were placed in a container filled with citric acid (pH=6.0, G1202, Servicebio, Wuhan, China) antigen retrieval buffer for antigen retrieval in a microwave oven, placed in phosphate buffered saline (PBS) (pH=7.4, G0002, Servicebio, Wuhan, China) and shaken on a decolorization shaker 3 times for 5 min each. The sections were placed in 3% hydrogen peroxide (Anjie High Tech, Shandong, China) and incubated at room temperature in darkness for 25 min. Bovine Serum Albumin (BSA, 3%) (G5001, Servicebio, Wuhan, China) was added to evenly cover the tissue, and the tissues were sealed for 30 min at room temperature. Tissues were incubated with primary antibodies overnight at 4°C and incubated with secondary antibodies at room temperature for 50 min. Newly prepared DAB color developing solution (G1211, Servicebio, Wuhan, China) was added to the plate after the sections were slightly dried. The color development time was controlled under the microscope. Brownish yellow nuclear counterstaining indicates positive cells. The sections were dehydrated and mounted with SweSuper Clean BioMount Medium. A microscope (E100, Nikon, Japan) was used to visualize the stained tissue and to acquire and analyze images (Nikon DS-U3, Nikon, Japan) [31-35].

Western blotting

On ice, hypothalamic tissue samples were lysed in Radioimmunoprecipitation Assay (RIPA) buffer (beyotime biotechnology, Shanghai, China) containing 1 mM Phenylmethylsulfonyl Fluoride (PMSF) (beyotime biotechnology, Shanghai, China) and 1 M protease and phosphatase inhibitor cocktail. A Bicinchoninic Acid (BCA) protein assay kit (beyotime biotechnology, Shanghai, China) was used to quantify the proteins extracted from hypothalamic tissues of rats. On SDS–PAGE gels (P0012AC, beyotime biotechnology, Shanghai, China), an equal amount of protein (20 mg/lane) was electrophoresed using an electrophoresis apparatus (BIO-RAD, USA). A trans blot turbo transfer system (BIO-RAD, Hercules, CA, USA) was then used to transfer the selected target gel band to Polyvinylidene Fluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). Membranes were blocked in 5% skim milk (FD0080, Fdbio science,

Hangzhou, China) for one hour before they were incubated overnight at 4°C with the following primary antibodies: rabbit anti-glutamine synthetase monoclonal antibody (1:500, GB111177, Service bio, Wuhan, China); rabbit anti-GAD65 monoclonal antibody (1:500, GB11562, Servicebio, Wuhan, China); rabbit anti-GAD67 monoclonal antibody (1:500, GB111397, Service bio, Wuhan, China); and rabbit anti-GAPDH monoclonal antibody (1:500, GB15004, Abbkine, Wuhan, China). Next, TRIS buffered saline (TRIS) and TBS with Tween (TBST) were used to wash the membranes, after which the membranes were incubated with goat anti-rabbit IgG (H+L) secondary antibody (1:10000, #A21020, Abbkine, Wuhan, China) for 2 hours. Finally, the protein bands were analyzed using an Electrochemiluminescence (ECL) system (Bio-Rad laboratories Inc., California, USA). A total of three replicates were performed, and imageJ (NIH, Bethesda, USA) was used to perform the quantitative analysis.

Data analysis

Statistical analysis was performed using IBM SPSS 25.0 software (V13.0, Chicago, IL, USA). The experimental data are presented as the mean ± SD. Groups were compared using one-way Analyses of Variance (ANOVA). Post hoc tests included the LSD test for homogeneous variance, and Tamhane's T2 test was for heterogeneous variance. A p value <0.05 was considered significant. A very significant difference was considered when the p value was <0.01 [36-38].

RESULTS

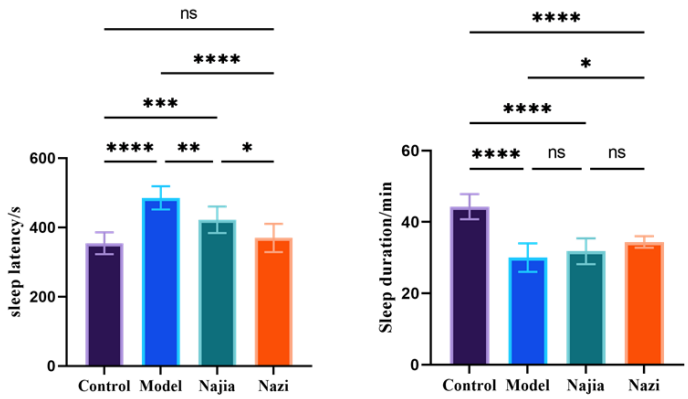
Evaluation of the therapeutic effect of Ziwuliuzhu acupuncture in insomniac rats

Rats in the model group were aggressive, biting and fighting, and behaved in a similar manner to the same-sex control rats. These rats were active during the night and rested during the day. Compared to the model group, the Najia and Nazi groups exhibited decreased daily activity and significant reductions in jumping and fighting. Their activity was reduced, and they became less aggressive. After model establishment, the sleep latency of the model group was significantly increased (P<0.0001), and the sleep duration was significantly decreased (P<0.0001) relative to the control group, indicating that the insomnia model was successfully established. Compared with the model group, the sleep latency of the Nazi group (P<0.0001) and Najia group (P<0.01) was significantly decreased. The sleep duration of the Nazi group was increased compared to the model group (P<0.05), but the difference in sleep duration in the Najia group was not significant (P>0.05). The difference in sleep duration between the Najia group and the Nazi group was not significant (P>0.05). These findings indicate that sleep was improved in rats treated with Ziwuliuzhu acupuncture (Table 1 and Figure 2).

Table 1. Results of the pentobarbital sodium assay (sleep latency and sleep duration) in the control, model, Najia, and Nazi groups. Each group was assessed 7 days after treatment. Values are means ± SDs (n=10).

Group	Sample size	Sleep latency (s)	Sleep duration (min)
Control	10	354.2 ± 31.37	44.3 ± 3.529
Model	10	485.2 ± 33.68	30 ± 4
Najia	10	422.2 ± 38.24	31.8 ± 3.645
Nazi	10	369.5 ± 40.75	34.4 ± 1.647

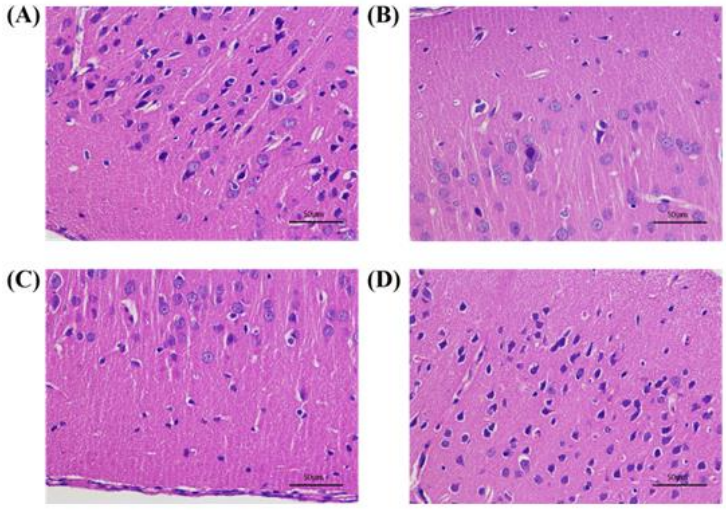
Figure 2. The sleep latency (s; A) and sleep duration (min; B) of the Control, Model, Najia, and Nazi groups after treatment. Values are means ± SDs (n=10). Post hoc LSD test: ****p<0.0001, ***p<0.001 compared to the control group; *p<0.05, **p<0.01 compared to the model group; ns indicates that the difference was not significant.



Effect of Ziwuliuzhu acupuncture on the pathological morphology of the hypothalamus in insomniac rats

In the control group, the hypothalamic neurons were orderly, the cytoplasm was uniform, the nucleolus was clear, and the structure was intact. In contrast, the hypothalamic neurons in the model group were loosely arranged and disordered, irregular in shape, had indistinct layers, had enlarged cell bodies, exhibited nuclear pyknosis, and exhibited process reduction; the hypothalamus also exhibited neuronal loss (Figure 3). Compared with the model group, the neurons in the Najia group and the Nazi group were arranged in a regular manner, with distinct layers, and with a reduction in nuclear pyknosis; the number of neurons also increased.

Figure 3. HE staining was used to assess the severity of inflammation and pathological reactions in the hypothalamic area of rats in each group (HE, 400×) (scale bar=50 μm). (A) Control group; (B) Model group; (C) Najia group; (D) Nazi group.



Effect of Ziwuliuzhu acupuncture on Glu and GABA concentrations in hypothalamic tissue of insomniac rats

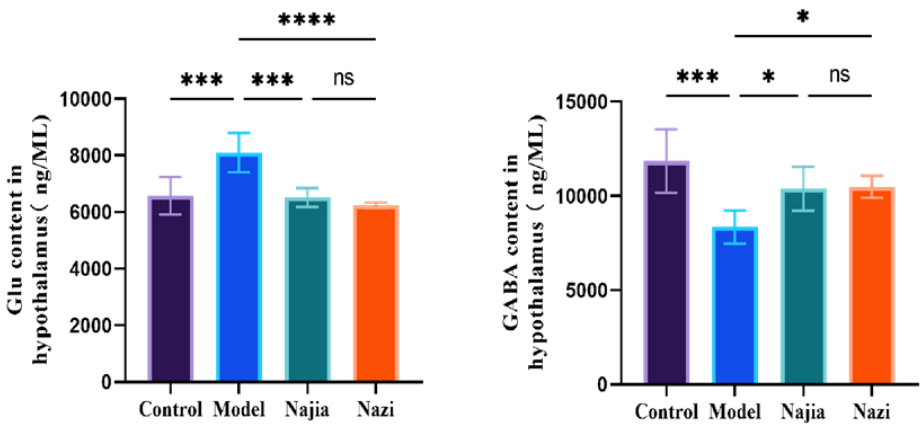
The GABA and Glu concentrations and ratio are provided in Table 2. The model group had significantly lower levels of GABA than the control group (P<0.001). Compared to the model group, the Nazi group and the Najia group had a significant increase in GABA levels (P<0.05). There were no significant differences in GABA levels between the Najia

and Nazi groups (P>0.05). The Glu concentration was significantly higher in the model group than the control group (P<0.001). The Najia group (P<0.001) and the Nazi group (P<0.001) showed significant decreases in Glu concentrations compared to the model group. The Glu concentrations of the Najia and Nazi groups did not differ significantly (P>0.05). A significant increase in the Glu/GABA ratio was observed in the model group compared to the control group, as Glu expression increased and GABA expression decreased. Compared to the model group, the Najia and Nazi groups had lower Glu/GABA ratios (Table 2 and Figure 4).

Table 2. Comparison of hypothalamus levels of Glu, GABA and the Glu/GABA ratio among all groups. Values are means ± SDs (n=6).

Group	Glu (ng/mL)	GABA (ng/mL)	Glu/GABA ratio
Control	6576 ± 664	11851 ± 1681	0.5549 ± 0.0037
Model	8099 ± 695.6	8350 ± 878.6	0.970 ± 0.0078
Najia	6513 ± 335.7	10388 ± 1166	0.6270 ± 0.0064
Nazi	6236 ± 104.9	10489 ± 585.1	0.5945 ± 0.0042

Figure 4. Effect of Ziwuliuzhu acupuncture on the expression of GABA and Glu in the hypothalamus. (A) The expression levels of Glu and (B) GABA in the hypothalamus were assessed with HPLC–MS/MS. Values are means ± SDs (n=6). Post hoc LSD test: ***p<0.001 compared to the control group; ****p<0.0001, ***p<0.001, *p<0.05 compared to the model group; ns indicates that the difference was not significant.



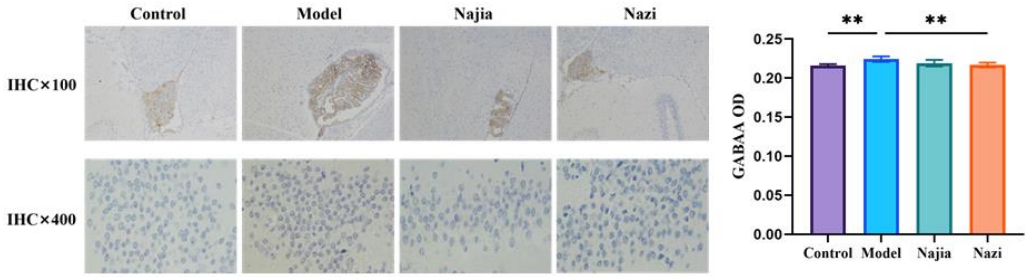
Effect of Ziwuliuzhu acupuncture on GABAAR expression in the hypothalamus of insomniac rats

After Immunohistochemistry (IHC) staining, GABAAR positive cells appeared yellow or brownish yellow and were mainly found in the nucleus, cytoplasm, and intercellular matrix. Light microscopy showed GABAAR expression in the hypothalamus of each group; in the model group, GABAAR expression was more extensive and more intense. Compared to the control group, the model group showed a significant increase in the positive expression of GABAARs and an increase in average Optical Density (OD) values (P<0.01) (Table 3). The Najia and Nazi groups showed lower levels of GABAAR expression in the hypothalamus than the model group, and the OD values decreased on average. The difference in GABAAR expression between the Najia group and the control group was not significant (P>0.05); however, the Nazi group had a significant decrease in GABAAR expression compared to the model group (P<0.01) (Figure 5).

Table 3. The mean OD values of GABAAR in the hypothalamus of rats in each group (IHC, 400×). Values are means ± SDs (n=6).

Group	Sample size	OD values
Control	6	0.2161 ± 0.001671
Model	6	0.2243 ± 0.0035
Najia	6	0.2191 ± 0.004197
Nazi	6	0.2167 ± 0.003023

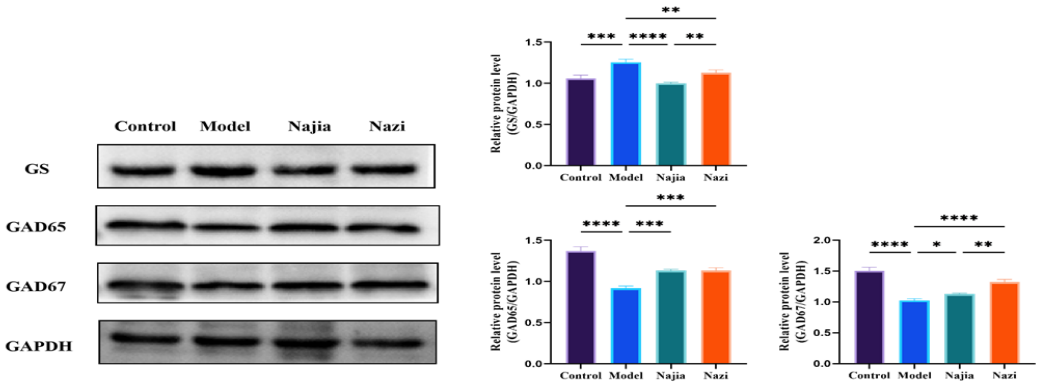
Figure 5. (A) Expression of GABAARs in the hypothalamus of rats in the Control, Model, Najia and Nazi groups (IHC; 100× and 400× magnification); (B) Mean OD values of GABAAR in the hypothalamus of each group. Values are means ± SDs (n=6). Post hoc LSD test: **p<0.01 compared to the control group; **p<0.01 compared to the model group.



Effect of Ziwuliuzhu acupuncture on the protein expression of GAD65, GAD67 and GS in the hypothalamus of insomniac rats

Compared with the control group, the model group had significantly lower levels of GAD65 and GAD67 in the hypothalamus (P<0.0001) and significantly higher levels of GS (P<0.001). The Najia and Nazi groups exhibited a significant increase in GAD65 (P<0.001) and GAD67 (P<0.05) levels compared to the model group. GS levels in the hypothalamus of the Najia group were significantly lower than those in the hypothalamus of the model group and Nazi group (P<0.01). The GS level and GAD67 level were higher in the Nazi group than in the Najia group (P<0.01), but the difference between the Nazi group and the Najia group in GAD65 was not significant (P>0.05) (Figure 6).

Figure 6. Effect of Ziwuliuzhu acupuncture on the expression of hypothalamus related proteins in rats. (A)Western blotting was used to measure the protein expression of GAD65, GAD67, and GS in hypothalamic tissues, and (B) Quantitative analysis of the data was performed. Values are means ± SDs. Post hoc LSD test: ****p<0.0001, ***p<0.001 compared to the control group; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 compared to the model group; **p<0.01 compared to the Najia group.



DISCUSSION

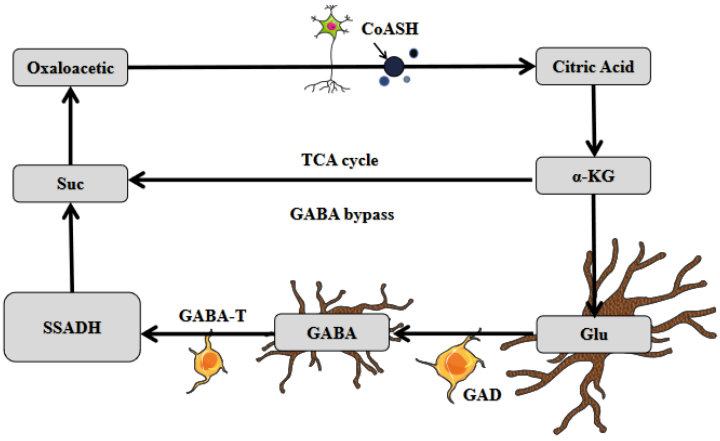
Glu and GABA are important excitatory and inhibitory neurotransmitters, respectively. A balance between Glu and GABA is needed to maintain normal brain function. Numerous studies have demonstrated a close association between insomnia and an excitatory/inhibitory imbalance of Glu and GABA. In the present study, we observed a decrease in GABA expression in the hypothalamus of sleep deprived rats, an increase in GABAAR expression, and an increase in Glu levels and the Glu/GABA ratio. Increased GABAAR expression results in memory loss and decline, inhibition of the expression of relevant biological clock genes, and insomnia. Tetrandrine acts directly on GABAARs, blocking the opening of Cl channels and thereby blocking GABA transmission, reducing inhibitory input. As GABAA expression increases, the balance between GABA and Glu changes. Glu and GABA metabolism in the brain occurs primarily through the Glu/GABA-Gln metabolic loop. A variety of neuropsychiatric disorders may be associated with this metabolic loop according to recent studies. Glu/GABA-Gln metabolism appears to be closely related to insomnia. In the Glu/GABA-Gln metabolic loop, Glu is converted to GABA by GAD65 and GAD67, and Gln is converted to Glu by GS; this loop maintains synaptic transmission of Glu. We found that the protein expression of GAD65 and GAD67 in the hypothalamus of insomniac rats was significantly reduced, hindering the conversion of Glu to GABA and resulting in a decrease GABA levels. When Glu is released or taken up in excessive quantities, it leads to excitotoxicity and dysfunction of glutamatergic neurons, which is associated with a variety of neurological disorders. Astrocytes play an important role in the clearance and transformation of extracellular Glu. GS is a specific enzyme in astrocytes that converts Glu released by neurons into Gln and then transmits Gln back to nearby neurons for reuse. These actions maintain the synaptic transmission of Glu. We found that the protein expression of GS in the hypothalamus of insomniac rats increased, suggesting that more Glu was converted into Gln, thereby maintaining a relatively stable Glu level.

GABAergic neurons are mainly distributed in the hypothalamus, and the discharge of these neurons increases during sleep. According to some experimental studies, GABA concentration increases during Nonrapid Eye Movement (NREM) sleep and decreases during Rapid Eye Movement (REM) sleep. A significant increase in GABA levels was observed in the brains of narcolepsy patients, especially those who did not experience night sleep disturbances, while a decrease in GABA levels was observed in the brains of patients with primary insomnia compared to healthy individuals. To exert its effects, GABA must bind to receptors. GABARs are classified into three types: GABAARs, GABABRs, and GABACRs. The inhibitory effects of GABA are mediated primarily by GABAARs, and it is currently believed that their molecular mechanism involves binding to a specific recognition site on GABAARs to enhance their inhibitory effects. Glu is the most important excitatory neurotransmitter in mammals. Under physiological conditions, glutamatergic neurons regulate sleep and promote wakefulness by stimulating orexin neurons and cholinergic neurons. Glucose and glutamine are two important sources of Glu. However, only Glu synthesized through the "Glu-Gln" cycle acts as a neurotransmitter and plays a role in neural function. To maintain the normal activity of neurons, the synthesized Glu must be cleared in a timely manner after synaptic release. Excessive accumulation of Glu in the brain is excitotoxic to neurons. The "Glu-Gln" cycle in the normal brain is responsible for inactivating Glu and terminating its effect. Additionally, Glu can be decarboxylated to produce GABA; therefore, GABA and Glu work together to maintain sleep wake cycles, and the metabolic balance between these neurotransmitters maintains sleep stability. To date, no study has examined changes in the concentration of Glu and GABA in the brains of insomniac rats. A previous study

indicated that after sleep deprivation, Glu and GABA levels in the hypothalamus increased, decreased, and then returned to normal. Another study found that in the hypothalamus of sleep deprived rats, GABA levels decreased, while Glu levels and the Glu/GABA ratio increased. Thus, insomnia is closely related to Glu and GABA concentrations as well as the Glu/GABA ratio.

In the brain, Glu and GABA metabolism is closely related to the circulation of Tricarboxylic Acids (TCAs). Glu is generated in the TCA cycle by the first reaction of α-Ketoglutarate (α-KG), which is an intermediate metabolite generated by glucose degradation. GAD catalyzes the dehydroxylation of Glu to produce GABA; GABA released by nerve terminals is taken up by astrocytes, and GABA is degraded by GABA-Transaminase (GABA-T) and succinate Semialdehyde Dehydrogenase (SSADH) to produce Succinic acid (Suc). Suc then returns to the TCA cycle to generate Glu (Figure 7). Astrocytes lack GAD and cannot convert Glu to GABA. Instead, Gln converts Glu into Gln under the action of GS, and Gln returns to nerve terminals to form Glu, which is a precursor to GABA. GAD is the primary metabolic enzyme responsible for converting Glu to GABA in the Glu/GABA-Gln metabolic loop, whereas the astrocyte-specific enzyme GS is responsible for converting Glu to Gln, thereby maintaining synaptic transmission of neuronal Glu.

Figure 7. Schematic diagram of the Tricarboxylic Acid (TCA) cycle.



Ziwuliuzhu theory states that the law of time governs circadian rhythms and gene expression in the twelve meridians. Ziwuliuzhu acupuncture is an integral part of Chinese time medicine, a branch of Traditional Chinese Medicine (TCM), and it has been shown to effectively treat insomnia among Chinese patients. In TCM, physicians compared the movement of human blood to the flow of water. In the body, twelve meridians regulate the rhythmic ebb and flow throughout the day. Thus, blood in the meridians may exhibit the most vigorous flow at certain times of day. Under the Najia method, the acupoint with the most vigorous blood is selected for treatment, while under the Nazi method, the acupoint selected for insomnia treatment is the heart meridian (in TCM, an individual with insomnia suffers from a mental illness, and such diseases are rooted in the heart). There is evidence that both Ziwuliuzhu acupuncture methods can reduce sleep latency and increase the duration of sleep as well as alleviate the damage to hypothalamic neurons in insomniac rats. We found that Ziwuliuzhu acupuncture increases GABA levels in the hypothalamus of insomniac rats, reduces GABAAR expression, and reduces Glu levels and the Glu/GABA ratio. Regarding the expression of related proteins, a decrease in GS and an increase in GAD65 and GAD67 protein expression were observed in the

hypothalamus of insomniac rats after Ziwuliuzhu acupuncture treatment. By regulating the protein expression of GAD65, GAD67, and GS in the Glu/GABA-Gln metabolic loop, Ziwuliuzhu acupuncture influences the expression of Glu and GABA as well as the Glu/GABA ratio.

However, this study has some limitations. No further analysis was conducted to determine whether the Najia method or Nazi method differ in the mechanisms underlying insomnia treatment. Since the purpose of this study was to examine the mechanism through which Ziwuliuzhu acupuncture regulates insomnia, no routine acupuncture group was established for comparison. Furthermore, it appears that other neurotransmitters and their respective receptors or transporters may contribute to insomnia in rats. A future study should utilize a larger sample size, establish a control group that receives routine acupuncture treatment, and compare the effects of the Nazi method and the Najia method. To examine and compare the impact of other neural signaling pathways on corresponding indicators, lesions or inhibitors should be used to block the effect of pathways regulated by SCN on downstream neurotransmitters; the resulting changes should be compared to those in healthy rats to further explore the benefits of Ziwuliuzhu acupuncture.

CONCLUSION

In summary, this study examined the effects and mechanisms of Ziwuliuzhu acupuncture on the Glu/GABA-Gln metabolic loop in a rat model of insomnia. Ziwuliuzhu acupuncture increased GABA concentrations and the protein expression of GAD65 and GAD67 in the hypothalamus but decreased Glu concentrations, GS protein expression, and GABAAR expression, thus alleviating the imbalance in the Glu/GABA-Gln metabolic loop. In addition to providing a reliable basis for the treatment of insomnia, these findings also provides a means to promote the popularization and application of Ziwuliuzhu acupuncture in clinical settings.

AUTHOR CONTRIBUTIONS

Initiated and designed the experiments: A.H., D.P., and Y.C.; conducted the experiments: Z.H. and Z.Z.; collected and analyzed the data: P.L. and Y.H.; wrote the manuscript: A.H. and D.P.; obtained the funding: C.Z. and P.Q.; supervised the research: C.Z. and P.Q. All authors read and agreed to the final manuscript.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest. The funders had no role in the design of the study; the collection, analyses, or interpretation of data; the writing of the manuscript; or the decision to publish the results.

ETHICS APPROVAL

This study was performed in line with the principles of the Declaration of Helsinki. All experiments were performed following relevant guidelines and regulations, including the ARRIVE guidelines and approved by the laboratory animal ethics committee of Jinan university, China (SCXK (Yue) 2017-0174).

CONSENT TO PARTICIPATE/PUBLISH

Not applicable.

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