

Original Research Article

Effect of Songyu Anshen Fang on expression of hypothalamic GABA and GABA(B) receptor proteins in insomniac rats induced by para-chlorophenylalanine

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Abstract

Purpose: To investigate the effects of the Chinese compound, Songyu Anshen Fang (SYF) on levels of GABA and GABA(B) receptor proteins in insomniac rats induced by para-chlorophenylalanine (PCPA).

Methods: All rats were randomly separated into either a control group, insomnia group, or a SYF group (at a dose of 8.5 g/kg or 17 g/kg body weight per day). The rat model of insomnia was induced by intraperitoneal injection of PCPA, and SYF was administered intragastrically in suspension. All experimental groups were treated with a corresponding agent for one week. The levels of glutamic acid (Glu) and γ -aminobutyric acid (GABA) were determined by high performance liquid chromatography (HPLC); mRNA and protein expressions, and GABA(B) receptor levels were detected by real-time polymerase chain reaction (RT-PCR) and western blot.

Results: SYF treatment with 8.5 or 17 g/kg/day decreased the levels of Glu and Glu/GABA ratios in the hypothalamus following abnormal increase by PCPA. Moreover, GABA(B) receptor, mRNA and protein expression decreased by PCPA in hypothalamus were significantly normalized by SYF.

Conclusion: The study indicates that the effects of PCPA-induced insomnia can be alleviated by SYF modulation of neurotransmitter levels and the expression of GABA(B) receptor in the hypothalamus. This suggests that clinical application of SYF to treat insomnia may be feasible.

Keywords: Songyu Anshen Fang, Para-chlorophenylalanine (PCPA), γ -Aminobutyric acid (GABA), GABA(B) receptor, Insomnia

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INTRODUCTION

Insomnia is a widespread health problem and increasing incidence of insomnia, due to an accelerated life pace, renders insomnia research extremely important. Insomnia is attributed at

least in part to the role of neurotransmitters in regulation of sleep and wakefulness. These neurotransmitters include histaminergic, noradrenergic, and cholinergic neurotransmission [1,2]. GABA is the major inhibitory neurotransmitter and plays an important role in

the regulation of sleep [3]. Monoaminergic mechanisms have been reportedly involved in sleep regulation in various mammals [4-6]. Administration of para-chlorophenylalanine (PCPA), a specific inhibitor of serotonin (5-HT) synthesis, decreases the level of 5-HT in several species of mammals, leading to reduced sleep [7-10]. Therefore, in this study, we developed a rat model of insomnia based through the administration of PCPA.

The Chinese compound Songyu Anshen Fang (SYF) exhibits significant clinical therapeutic effects on insomnia. Previous pharmacological studies have additionally shown that SYF can improve cognitive disorders in insomniac rats via the modulation of neurotransmitters 5-HT and DA [11]. There is also evidence that GABA and GABA(B) receptors are involved in the occurrence and development of insomnia. However, the underlying mechanism of SYF's therapeutic effect on insomnia remains unclear. In this study we investigated the effects of SYF on levels of glutamic acid, γ -aminobutyric acid and GABA(B) receptors in the hypothalamus of insomniac rats.

EXPERIMENTAL

Animals

Adult male Sprague-Dawley rats (weighing 230 – 280 g, Shanghai Slaccas Animal Center, Shanghai, China) were selected for these experiments. All the rats were housed individually at a temperature of 22 °C and a humidity of 50 % under controlled illumination (12:12 h dark:light cycle; light on at 06:00 hours). At all times, rats had free access to food and water. The experimental procedures described herein were approved by the Animal Care Committees at the Fujian Academy of Traditional Chinese Medicine (no. FJATCM-IAEC 2016003) and the study followed the Guide for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use [12].

Drugs

DL-p-chlorophenylalanine hydrochloride (PCPA, Sigma) was dissolved in saline with a pH adjusted to 7.2. The insomnia model was established by injection of 300 mg/kg PCPA intraperitoneally (i.p.) at 19:00 h for two consecutive days. The rats in the control group received 0.9 % saline in place of PCPA. Rats in the Songyu Anshen Fang (SYF) group were administered a suspension of SYF (8.5 and 17 g/kg per day) intragastrically at 8:00 h for 7

consecutive days. Rats in the model group and control group received equal amounts of sterile water. Two hours after the last day of SYF administration, the animals were deeply anesthetized by intraperitoneal injection of pentobarbital sodium (65 mg/kg) and the hypothalamus was excised and flash frozen in liquid nitrogen, followed by storage at –80 °C.

Measurement of excitatory amino acids

The levels of GABA and glutamate were detected by high performance liquid chromatography. Seventy (70 μ L) of AccQ:Tag Fluor borate buffer and 20 μ L of AccQ:Tag derivative reagent were added to 10 μ L of sample or standard solution followed by vortexing. The mixture was allowed to react for 10 min at 55 °C. The derivatized sample (10 μ L) was then injected onto a C18 reversed phase column. A Waters HPLC-FLD (model: 2475) fitted with a Waters AccQ:Tag column (150 mm \times 3.9 mm, 4 μ m) was set to an excitation wavelength (Ex) = 250 nm, emission wavelength (Em) = 395 nm, and column temperature of 40 °C. The mobile phase was comprised of 0.1 M sodium acetate buffer (pH 6.8, A) and acetonitrile (B). The flow rate was set to 1.0 ml/min. All solvents were filtered by vacuum through a 0.22 μ m membrane (Millipore) and degassed by sonication for 30 min before use.

Quantitative real-time PCR

Total RNA was extracted by the RNeasy pure tissue kit (Qiagen). Contaminating DNA was removed by DNase (Qiagen) treatment. The absorbance of the product was detected at 260 and 280 nm by a spectrophotometer to determine the concentration and purity, respectively. Total RNA was reverse-transcribed into cDNA using the QuantiTect First strand Synthesis system (Qiagen). cDNA amplification was performed by 7500 fast Sequence Detection System (Applied Biosystems, Foster, CA, USA) and SYBR Premix Ex Taq (Takara). The PCR conditions were: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, at 60 °C for 30 s. All samples were examined in triplicates. Primer Express software was used to design primer sequences, the results of which are presented in Table 1.

Table 1: Primer sequences for real-time PCR

Gene	Forward (5'–3')	Reverse (5'–3')
GAPDH	GTTTGTGATGGG TGTGAAC	TCTTCTGAGTGCCAG TGAT
GABA(B)	GCTTATCCACCA CGACAGCAA	CAGAACTACAGCCAG GCATGAGA

Western blotting

Western blot was used to assess GABA(B) proteins in the hypothalamus quantitatively. Frozen specimens were homogenized in 300 μ L of RIPA buffer containing 50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 1 % NP-40, 0.1 % sodium dodecyl sulfate (SDS), and 1 mM PMSF. Following centrifugation (13,100 g for 10 min, 4 °C), the supernatant was used to estimate the total amount of protein. Samples with equal amounts of protein were loaded onto a 8 % SDS–polyacrylamide gel for electrophoresis

Proteins were transferred to a PVDF membrane. After blocking of nonspecific binding sites with T-TBS containing 3 % bovine serum albumin (2 h at room temperature), membranes were incubated with the primary antibody rabbit anti-GABA(B)1 (1:200; Santa Cruz) at 4 °C overnight. Next, the membrane was incubated with secondary antibody, HRP-conjugated goat anti-rabbit IgG (1:5000; Abcam), for 1 h. After rinsing with a buffer, the immuno-complexes were visualized by chemiluminescence using the ECL kit (Thermo). Protein expression in the hypothalamus was determined by measuring the optical density using an image analysis system (Protein Simple Imaging system, FluorChem M).

Statistical analysis

Experimental data are displayed as mean \pm SEM. Data were processed with SPSS 17.0 statistical analysis software. Statistical significance between groups was examined by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of SYF on levels of Glu and GABA in the hypothalamus of rats

To observe the effects of SYF on levels of Glu and GABA in the hypothalamus of insomniac rats, the levels of Glu and GABA were determined by HPLC. The regression equation correlation coefficient of the determined amino acids is shown in Table 2 and the representative chromatograms of Glu and GABA are presented in Figure 1 A and B. Results showed that after PCPA treatment, the levels of Glu in hypothalamus were remarkably increased ($p < 0.001$), and Glu/GABA ratios increased significantly ($p < 0.05$) compared with the saline group. However, the levels of Glu were significantly decreased ($p < 0.01$, $p < 0.001$) in response to varying doses of SYF for 7 days.

Glu/GABA ratios were also significantly reduced ($p < 0.01$, $p < 0.001$) by SYF treatment when compared with the PCPA group (Figure 2)

Table 2: Regression equation and correlation coefficient of the determined amino acids

Amino acid	Regression equation	R
Glu	$Y = 1.92e + 008X + 4.11e + 005$	0.9999
GABA	$Y = 2.50e + 008X + 2.55e + 006$	0.9991

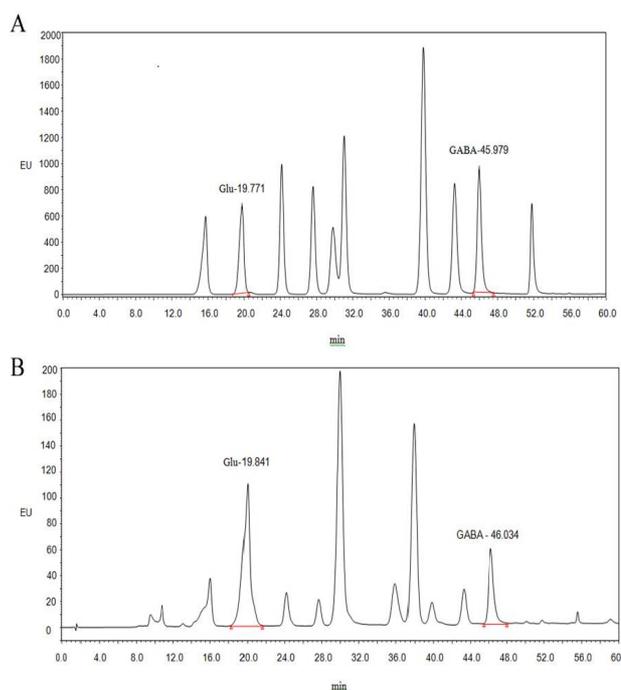


Figure 1: Representative chromatograms of Glu and GABA. A is chromatogram of amino acid solution standards; B is chromatogram of the hypothalamus of insomniac rats

Effect of SYF on mRNA expression of GABA(B)

RT-PCR results showed that after PCPA treatment, GABA(B) mRNA expression was significantly decreased ($p < 0.001$) in rat hypothalamus compared with the saline control group. However, GABA(B) mRNA expression in rat hypothalamus was significantly increased ($p < 0.05$, $p < 0.001$) in response to SYF intervention when compared with the PCPA group (Figure 3).

Effects of SYF on GABA(B) protein expression

Western blot analysis showed that after PCPA treatment, GABA(B) was significantly decreased ($p < 0.001$) in rat hypothalamus when compared with saline control group. On the other hand, GABA(B) expression in rat hypothalamus was significantly increased ($p < 0.05$, $p < 0.001$) after

administration of SYF when compared with PCPA group (Figure 4).

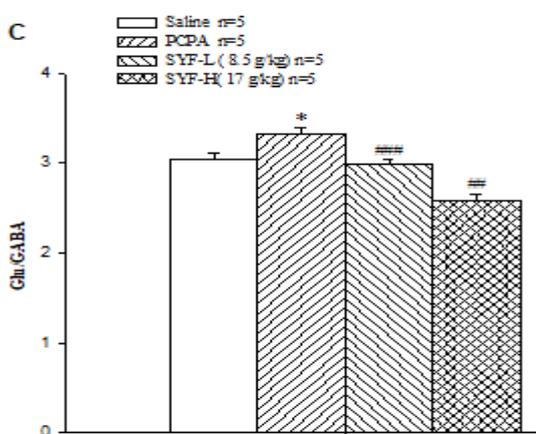
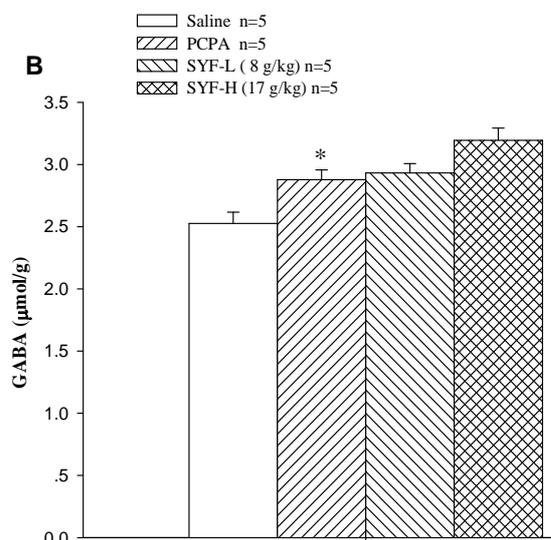
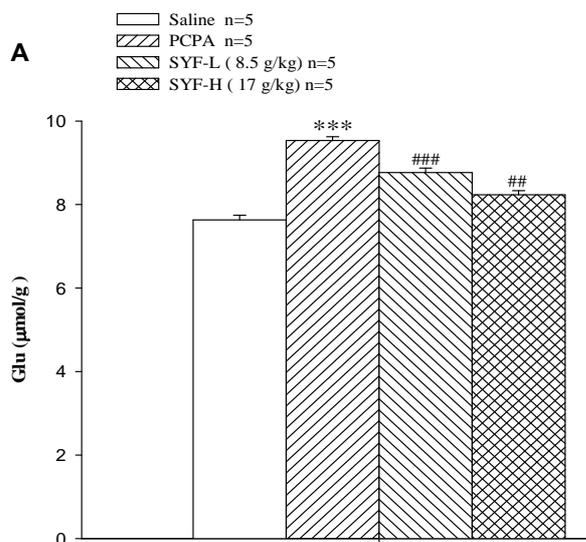


Figure 2: Effect of SYF on Levels of Glu and GABA in the rat hypothalamus; *** $p < 0.001$, * $p < 0.05$ vs Saline group; ### $p < 0.001$, ## $p < 0.01$ vs PCPA group

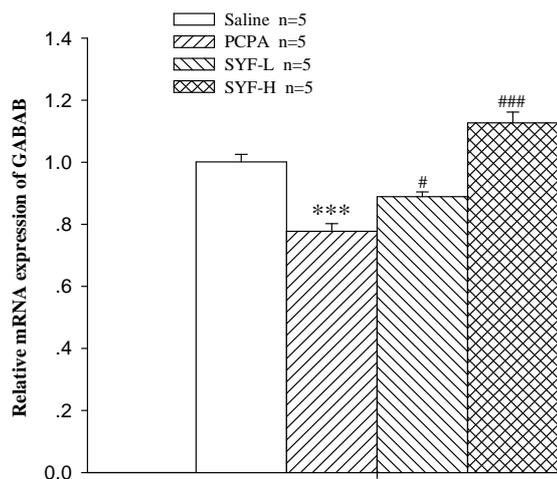


Figure 3: Effect of SYF on the expression level of GABA(B) mRNA in the hypothalamus of insomnia rats induced by PCPA; *** $p < 0.001$ vs Saline group ; ### $p < 0.001$, # $p < 0.05$ vs PCPA group

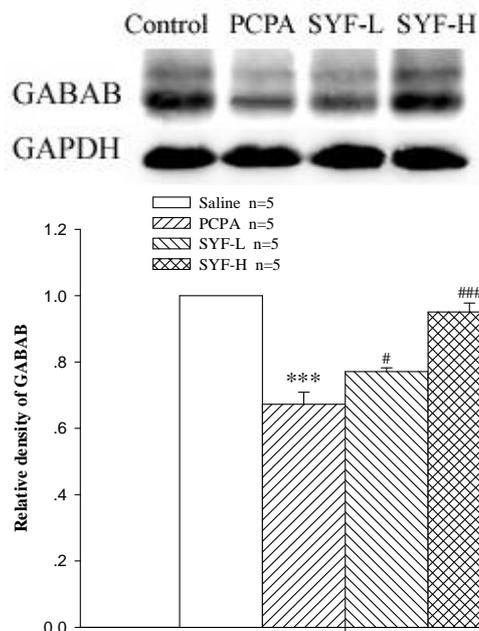


Figure 4: Effect of SYF on the level of GABA(B) in the hypothalamus of insomnia rats induced by PCPA; *** $p < 0.001$ vs Saline group; ### $p < 0.001$, # $p < 0.01$ vs PCPA group

DISCUSSION

As a specific and irreversible inhibitor of the aromatic amino acid hydroxylase tryptophan 5-monoxygenase, PCPA is the rate-limiting enzyme of serotonin (5HT) biosynthesis [7]. Application of PCPA significantly reduces the concentration of 5-HT across several species of mammals, a phenomenon accompanied by a reduction in sleep [7-10].

Brain regions that are involved in the sleep process include the brainstem, hypothalamus, basal forebrain, pontine reticular formation, and locus coeruleus. These structures form the activation-induction system of sleep and receive fiber projections from serotonin-releasing nerves in the dorsal raphe nucleus. Many inhibitory neurotransmitters exist in the sleep-related hypothalamic region [13]. Damage of these brain regions or alterations of the balance of neuronal transmission between the major excitatory neurotransmitter (Glu) and the inhibitory substance (GABA) can directly affect the sleep architecture [14]. Therefore, we chose the hypothalamus as a detection area and GABA and Glu as indicators in our assessment of changes in sleeping patterns in response to PCPA-induced insomnia and SYF intervention.

GABA is the major inhibitory neurotransmitter and has a key role in modulating neuronal activity. GABA acts through ionotropic (GABAA R and GABAC R) and metabotropic (GABAB R) receptors, which produce slow and fast inhibitory signals, respectively. As a heterodimer, GABAB R1 and GABAB R2 play an important role in the fine-tuning of inhibitory synaptic transmissions which have been implicated in muscle relaxation, hippocampal long-term potentiation, slow wave sleep and antinociceptive events.

SYF is a Chinese herbal formula that contains Gansong (the radix of *Nardostachys chinensis Batal*), Yujin (the tuberous root of *Curcuma petiolata*), Meiguihua (the flower of *Rosa rugosa*), Shenglonggu (*Fossilia Osis Mastodi*), Zhenzumu (the powder of *Concha Margaritifera Usta*), Yejiaoteng (*Cauls Polugoni Multiflori*), Danshen (the radix of *Salviae Miltiorrhizae*), Suanzaoren (the kernel of *Semen Ziziphi Spinosa*), and Hehuanpi (the skin of *Cortex Albiziae*). This study investigated the effect of SYF on the expression of hypothalamic GABA and GABA(B) receptor proteins in PCPA-induced insomnia in rats. In the present study, SYF treatment with 8.5 or 17 g/kg/day significantly normalized the expression of GABA(B) mRNA and GABA(B) protein in the hypothalamus of PCPA-treated rats. The results indicate that the effect of SYF on PCPA-treated rats might be associated with the modulation of neurotransmitter levels and the GABA(B) receptor expression in the hypothalamus.

CONCLUSION

The findings of this study demonstrate that PCPA-induced insomnia is alleviated in rats by SYFs. The mechanism may be related to the modulation of neurotransmitter levels and the

expression of GABA(B) receptor in the hypothalamus by SYF. Thus, SYF has potentials for clinical application in the treatment of insomnia, but further studies are required in this regard.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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