

Schisandrin B exerts hypnotic effects in PCPA-treated rats by increasing hypothalamic 5-HT and γ -aminobutyric acid levels

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Abstract. Schisandrin B (SchB) is one of the primary active components of *Schisandra chinensis* (Turcz.) Baill., a traditional Chinese herb that has been used to treat insomnia for hundreds of years. Our previous studies revealed that SchB exerts sedative and hypnotic effects, increasing the content of γ -aminobutyric acid (GABA) and the expression of its receptors in the brain tissues of rats. 5-hydroxytryptamine (5-HT) is another important neurotransmitter involved in sleep regulation, although, to the best of our knowledge, there are no reports of its association with SchB. Therefore, the present study aimed to determine whether the hypnotic effect of SchB was partly due to alterations in the expression of 5-HT. The results indicated that SchB reduced sleep latency and increased sleep duration in parachlorophenylalanine (PCPA)-induced rats with insomnia by increasing 5-HT and 5-hydroxyindoleacetic acid, and upregulating the expression of the 5-HT receptor 1A in the hypothalamus. SchB also increased the ratio of GABA to glutamic acid and the activity of glutamic acid decarboxylase, decreased the activity of GABA transaminase, and upregulated the expression of GABA_A receptor $\alpha 1$ and GABA_A receptor $\gamma 2$ in the rat hypothalamus. These results suggested that SchB improved PCPA-induced insomnia in rats, and its effects may be associated with the regulation of GABA and 5-HT levels in the hypothalamus.

Introduction

All species maintain a 24-h solar cycle of rest and activity, and disrupting this cycle affects adaptation and homeostasis (1). Similar to fish that do not sense the water except if they are placed in a dry environment, the quotidian normality of sleep is not perceived until it is disrupted (1). For example, 30% of the adult population complain of transient insomnia, and 10% experience chronic insomnia that disrupts daytime function (2). Patients with chronic insomnia experience low work productivity and a high number of absenteeism incidents, accidents and hospitalizations, resulting in treatment costs of 60 billion dollars annually (3). Schisandra, a Traditional Chinese Medicine, is the dried ripe fruit of *Schisandra chinensis* (Turcz.) Baill. (Magnoliaceae), and has been used to treat insomnia for hundreds of years (4,5). Schisandrin B (SchB) is a monomeric compound of Schisandra lignans (Fig. 1). According to a previous study in rats, SchB exerts sedative and hypnotic effects, which are associated with the upregulation of the inhibitory neurotransmitter γ -aminobutyric acid (GABA) and both the mRNA and protein expression of its two receptors, GABA_A receptor $\alpha 1$ (GABA_A R $\alpha 1$) and GABA_A receptor $\gamma 2$ (GABA_A R $\gamma 2$) (6). However, reports have demonstrated that as well as GABA, 5-hydroxytryptamine (5-HT), another central neurotransmitter, also plays an important role in sleep regulation (7,8). The decarboxylation of 5-hydroxytryptophan, a 5-HT precursor, is a key step during the synthesis of 5-HT, and the tryptophan hydrogenase enzyme is essential for this step (9). Parachlorophenylalanine (PCPA) can antagonize tryptophan hydrogenase and inhibit the synthesis of 5-HT, resulting in insomnia with less slow wave sleep and circadian rhythm disturbances (10,11). Therefore, in the present study, a PCPA-induced insomnia rat model was used to determine whether SchB exerted its sedative and hypnotic effects by regulating 5-HT. The study aimed to provide experimental evidence for further development of SchB as a sleep-promoting supplement and medicine.

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Materials and methods

Chemicals and reagents. SchB (Chengdu Pukang Biotechnology Co., Ltd.), the crude powder of *Schisandra*

sphenanthera Rehd. et Wils, was added to 10X the volume of ethanol and extracted three times for 1 h each. The combined filtrate was then concentrated to a small volume, and the sample was mixed with diatomite and extracted three times with n-hexane-ethyl acetate at a 10:1 ratio; the filtrate was then concentrated once more. The sample was mixed with silica gel for gradient elution with petroleum ether-ethyl acetate (1:0.5:1), and the sample fraction containing SchB was collected and concentrated until dry, and then dissolved in anhydrous ethanol at 4–10°C until crystallization. After repeated recrystallization with anhydrous ethanol (2–3 times), the purity of SchB reached >98%. PCPA was purchased from Sigma-Aldrich (Merck KGaA), and rat GABA, glutamate (Glu), glutamic acid decarboxylase (GAD), γ -aminobutyric acid transaminase (GABA-T), 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) ELISA kits were purchased from Zhongsheng Beikong Biotechnology Co., Ltd. (<http://www.zhongsheng.com.cn/>). GABA_A R α 1 (1:1,000; cat. no. ab252430) and GABA_A R γ 2 (1:1,000; cat. no. ab87328) were acquired from Abcam. 5-hydroxytryptamine receptor 1A (HTR1A; 1:1,000; cat. no. A2801), β -actin (1:100,000; cat. no. AC026) and HRP goat anti-rabbit IgG (H+L) (1:5,000; cat. no. AS014) were acquired from ABclonal Biotech Co., Ltd. All antibodies were diluted with TBS with containing 0.1% Tween-20 (TBST).

Experimental animals. A total of 52 eight week-old male Wistar rats, weighing 200 \pm 10 g, were provided by Changchun Yis Experimental Animal Technology Co., Ltd., license no. SCXK (Jilin) 2016-0003. The animals were maintained in separate cages in a quiet environment at 18–22°C and 50–60% humidity with a 12-h light/dark cycle and under specific pathogen-free conditions, with free access to food and water, and were acclimated to the laboratory environment for 7 days before experimentation.

Animal grouping and treatment. For reproduction of the insomnia model, rats were maintained for 1 week and then intraperitoneally administered with PCPA (300 mg/kg) once a day for 3 days. The disappearance of the circadian rhythm and continuous animal activity during the day indicated successful model establishment.

For sleep behavioral experiments, 24 male Wistar rats were randomly divided into the blank control (CON), model (PCPA), SchB, and PCPA + SchB groups (6 rats per group). Rats in the CON and PCPA groups were administered equal volumes of distilled water, and those in the SchB and PCPA + SchB groups received 3.5 mg/kg SchB once a day for 7 days. In our previous research, we observed the sedative and hypnotic effects of SchB in mice at an effective dose of 5 mg/kg (6). Based on this, we calculated the equivalent dose of 3.5 mg/kg for rats, which was effective in the preliminary experiment. So, 3.5 mg/kg SchB was selected as the appropriate dose in the present study. Following the sleep behavioral experiments, the rats were anesthetized with ether, as it is easy to evaporate using a simple device, rarely causes animal death due to overdose, and has no effects on the experimental data. The status of anesthesia was judged by falling, relaxed limbs and loss of the skin pain reflex. The presence of a heartbeat and breathing confirmed that the rats were alive. The rats were sacrificed by exsanguination from

the abdominal aorta under ether anesthesia for 10 min (12). The animal experiments were approved by the Institutional Animal Care and Use Committee of Beihua University (approval no. 20190301).

A total of 28 male Wistar rats (grouped and treated as aforementioned; 7 rats per group) were used for the detection of biomarkers associated with sleep regulation. The rats were sacrificed by exsanguination from the abdominal aorta after 10 min of ether anesthesia. The hypothalamus was then removed from the brain tissue, homogenized, and separated to harvest the supernatant. The levels of 5-HT, 5-HIAA, GABA and Glu, as well as the activities of GAD and GABA-T in the rat hypothalamus were determined by ELISA. Protein expression of HTR1A, GABA_A R α 1 and GABA_A R γ 2 was detected by western blotting.

Sleep experiments for the synergistic effects of SchB and pentobarbital sodium. On the 7th day, 40 min after the last administration of SchB, the rats in all four groups were intraperitoneally treated with a subthreshold dose of pentobarbital sodium (28 mg/kg), and the number of sleeping rats was observed. After another two days, the rats received a threshold dose of pentobarbital sodium (35 mg/kg) to distinguish sleep latency and duration. At 30 min post-pentobarbital sodium administration, the number of sleeping rats (determined by the disappearance of the righting reflex for >1 min), the sleep latency (the time from the start of administration to the start of sleep) and the sleep duration were observed.

Detection of 5-HT, 5-HIAA, GABA and Glu levels, and GAD and GABA-T activity in the rat hypothalamus. On the 7th day, 40 min after the last administration of SchB, the rats were anesthetized with ether and sacrificed by exsanguination from the abdominal aorta. The hypothalamus was rapidly isolated on ice, homogenized and separated to generate the supernatant. 5-HT, 5-HIAA, GABA and Glu levels, as well as the activities of GAD and GABA-T were detected by ELISA kits (5-HT, cat. no. 201506; 5-HIAA, cat. no. 201506; GABA, cat. no. 201512; Glu, cat. no. 201512; GAD, cat. no. 201512; and GABA-T, cat. no. 201512) from Zhongsheng Beikong Biotechnology Co., Ltd. (<http://www.zhongsheng.com.cn/>), according to the manufacturer's instructions.

Western blot detection of HTR1A, GABA_A R α 1 and GABA_A R γ 2 protein expression in the rat hypothalamus. The hypothalamus of each rat was immersed in lysis buffer (protein lysate; Beijing Solarbio Science & Technology Co., Ltd.) on ice for 1 h, and the supernatant was separated by centrifugation at 16,000 \times g and 4°C for 10 min. Protein concentration was determined using the BCA method, and the target proteins (60 μ g per lane) were separated via SDS-PAGE on a 10% gel, and subsequently transferred to PVDF membranes for 2 h. The membranes were rinsed with Tris buffer for 5 min, and then blocked with blocking buffer (TBST buffer containing 5% skimmed milk powder) for 1 h at room temperature. The blocking buffer was discarded, and the corresponding primary antibodies were added and incubated at 4°C overnight. The membranes were washed with TBST five times for 5 min each time, and incubated for 2 h with the corresponding secondary antibodies. The membranes were washed with TBST five

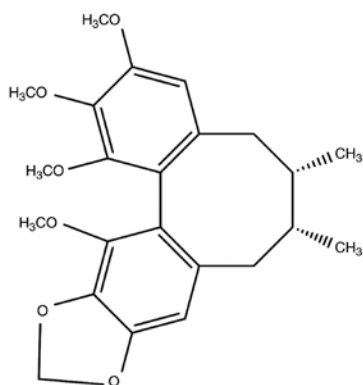


Figure 1. Chemical structure of Schisandrin B.

times (5 min each) and were visualized using the enhanced electrochemiluminescence method. ImageJ (version 1.51j8; National Institutes of Health) was used to perform the western blotting densitometric analysis.

Statistical analysis. SPSS 20.0 software (IBM Corp.) was used for all statistical analyses. All quantitative data are expressed as the mean \pm SD, the mean value is taken from each rat in each group treated with or without SchB. The data between the two groups were compared using one-way ANOVA (followed by Tukey's test). Each assay was performed in three experimental repeats. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of SchB in combination with a subthreshold dose of pentobarbital sodium on the number of sleeping rats. To evaluate the hypnotic effects of SchB, a sleep experiment was performed on rats treated with a subthreshold dose of pentobarbital sodium. As shown in Fig. 2, the number of sleeping rats in the SchB group was significantly increased compared with that in the CON group ($P < 0.01$), indicating the hypnotic role of SchB. Compared with the PCPA group, the number of sleeping rats in the PCPA + SchB group was significantly higher ($P < 0.01$), suggesting that SchB and pentobarbital sodium exerted a synergistic effect to induce sleep in rats.

Effects of SchB in combination with a threshold dose of pentobarbital sodium on sleep regulation in rats. The effects of SchB in combination with a threshold dose of pentobarbital sodium on sleep latency and duration were observed in rats. The results indicate that compared with the CON group, the sleep latency time of the SchB group was significantly shorter, and the sleep duration was significantly prolonged ($P < 0.01$). Compared with the PCPA group, sleep latency was reduced and sleep duration was significantly prolonged in the PCPA + SchB group ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 3). In our previous study, SchB alone could not produce significant hypnotic effects. However, following the addition of a subthreshold (28 mg/kg) or threshold (35 mg/kg) dose of pentobarbital sodium, SchB showed a significant synergistic effect with pentobarbital sodium in comparison with control groups (6).

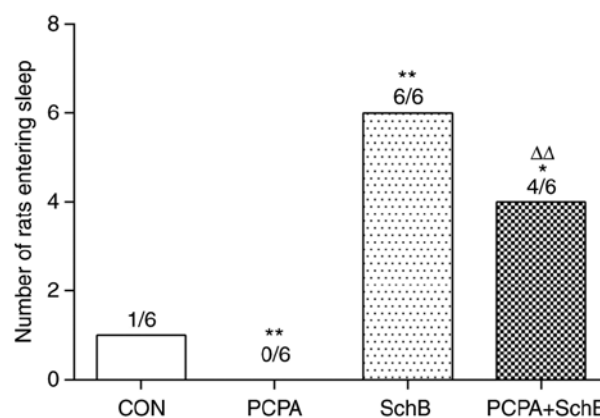


Figure 2. Effect of SchB in combination with a subthreshold dose of pentobarbital sodium on the number of sleeping rats ($n=6$). * $P < 0.05$, ** $P < 0.01$ vs. CON group; $\Delta\Delta P < 0.01$ vs. PCPA group. SchB, Schisandrin B; CON, control; PCPA, parachlorophenylalanine.

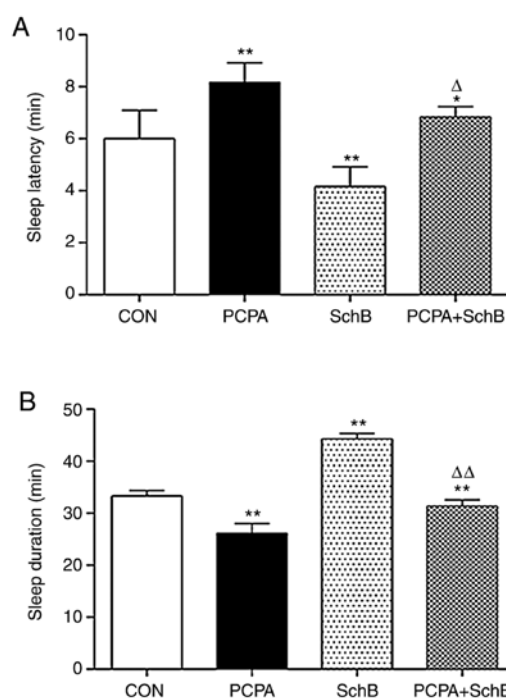


Figure 3. Effect of SchB in combination with a threshold dose of pentobarbital sodium on the sleep latency and sleep duration in rats. (A) Effects of SchB in combination with a threshold dose of pentobarbital sodium on sleep latency in rats. (B) Effects of SchB in combination with a threshold dose of pentobarbital sodium on sleep duration in rats. Data are presented as the mean \pm SD ($n=6$). * $P < 0.05$, ** $P < 0.01$ vs. CON group; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ vs. PCPA group. SchB, Schisandrin B; CON, control; PCPA, parachlorophenylalanine.

Effects of SchB on the levels of 5-HT and 5-HIAA in the hypothalamus. 5-HT is an important central neurotransmitter in sleep regulation, of which 5-HIAA is a primary metabolite (13). Compared with the CON group, the levels of 5-HT and 5-HIAA in the hypothalami of rats in the SchB group were significantly increased ($P < 0.05$ and $P < 0.01$, respectively). Compared with the PCPA group, the levels of 5-HT and 5-HIAA in the hypothalami of rats in the PCPA + SchB group were also significantly increased ($P < 0.05$), suggesting that SchB exerted a certain therapeutic effect on PCPA-induced insomnia (Fig. 4).

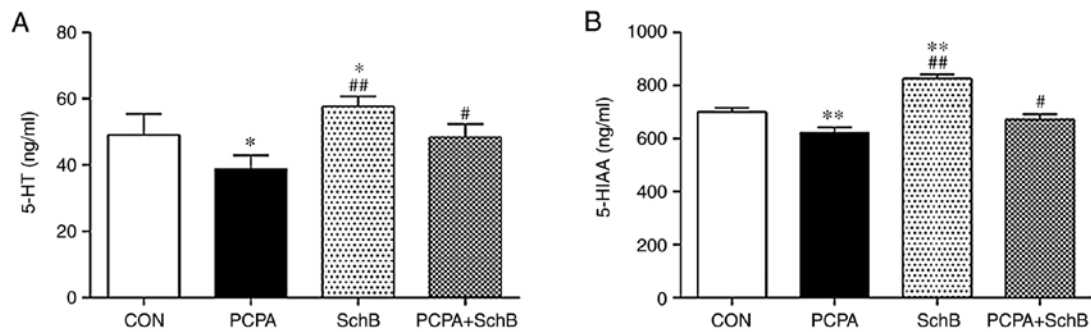


Figure 4. Effect of SchB on the levels of 5-HT and 5-HIAA in the hypothalami of rats. (A) Effects of SchB on the levels of 5-HT in hypothalami of rats. (B) Effects of SchB on the levels of 5-HIAA in the hypothalami of rats. Data are presented as the mean \pm SD (n=4). *P<0.05, **P<0.01 vs. CON group; #P<0.05, ##P<0.01 vs. PCPA group. SchB, Schisandrin B; CON, control; PCPA, parachlorophenylalanine; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid.

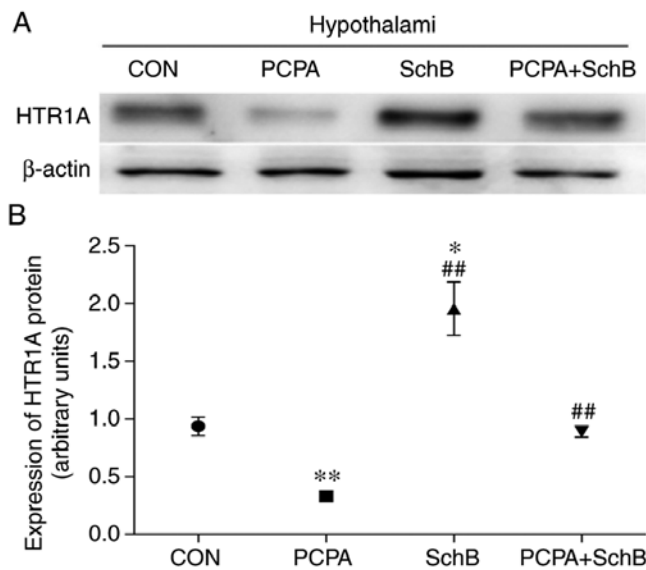


Figure 5. Effect of SchB on the expression of HTR1A protein in the hypothalami of model rats. (A) Representative western blotting images demonstrating HTR1A protein expression in the hypothalami of rats in each group. (B) The expression of HTR1A protein in the hypothalami of rats in each group. Data are presented as the mean \pm SD (n=3). *P<0.05, **P<0.01 vs. CON group; ##P<0.01 vs. PCPA group. SchB, Schisandrin B; CON, control; PCPA, parachlorophenylalanine; HTR1A, 5-hydroxytryptamine receptor 1A.

Effects of SchB on HTR1A protein expression in the hypothalamus. As shown in Fig. 5, HTR1A protein expression in the hypothalami of rats in the SchB group was significantly higher than that in the CON group (P<0.05), and the content of HTR1A in the hypothalami of rats in the PCPA + SchB group was significantly higher than that in the PCPA group (P<0.01). These findings suggested that SchB played a sedative and hypnotic role by regulating the expression of HTR1A in the rat hypothalamus.

Effects of SchB on the levels of GABA and Glu, and the activity of GAD and GABA-T in the rat hypothalamus. GABA is the primary inhibitory neurotransmitter in the central nervous system of mammals (14). GAD converts Glu to GABA (15) and GABA-T is the hydrolytic enzyme of GABA (16). Therefore, the activities of GAD and GABA-T were detected in the

present study. Compared with the CON group, the expression levels of GABA in the hypothalami of rats in the SchB group were increased, the expression of Glu was decreased, and the GABA/Glu ratio was significantly increased. Furthermore, GAD activity was increased, whereas the activity of GABA-T significantly decreased. Compared with the PCPA group, the expression levels of GABA were increased and those of Glu were decreased; the ratio of GABA/Glu was increased, GAD activity was increased and GABA-T activity was significantly decreased in the PCPA + SchB group (P<0.05 or P<0.01) (Fig. 6).

Effects of SchB on GABA_A R α 1 and GABA_A R γ 2 protein expression in the rat hypothalamus. The results demonstrated that the expression levels of GABA_A R α 1 and GABA_A R γ 2 protein in the hypothalami of rats in the SchB group were significantly higher than those in the CON group, and that GABA_A R α 1 and GABA_A R γ 2 protein expression in the hypothalami of the PCPA + SchB group were significantly higher than in the PCPA group (P<0.05 or P<0.01) (Fig. 7). Collectively, these data indicated that SchB exerted its sedative and hypnotic effects by regulating the expression of GABA_A R α 1 and GABA_A R γ 2 in the rat hypothalamus.

Discussion

As early as 1969, Jouvet (17) and Koella (18) proposed the theory that 5-HT induces sleep. Later research indicated that low levels of 5-HT in the brain resulted in insomnia, which could be reversed by the recovery of 5-HT levels in the brain (19,20). PCPA has the capacity to selectively inhibit tryptophan activity, which blocks the synthesis of 5-HT (21,22). However, once PCPA is metabolized, the synthesis of 5-HT can be restored without substantial neuronal damage (23), and thus is recognized as a suitable tool for establishing animal models of insomnia. In the present study, PCPA predictably decreased the number of sleeping rats, prolonged the latency of sleep and decreased sleep duration. Conversely, SchB administration increased the number of sleeping rats, reduced sleep latency and increased sleep duration in rats treated with PCPA, indicating that SchB may improve insomnia resulting from low 5-HT levels.

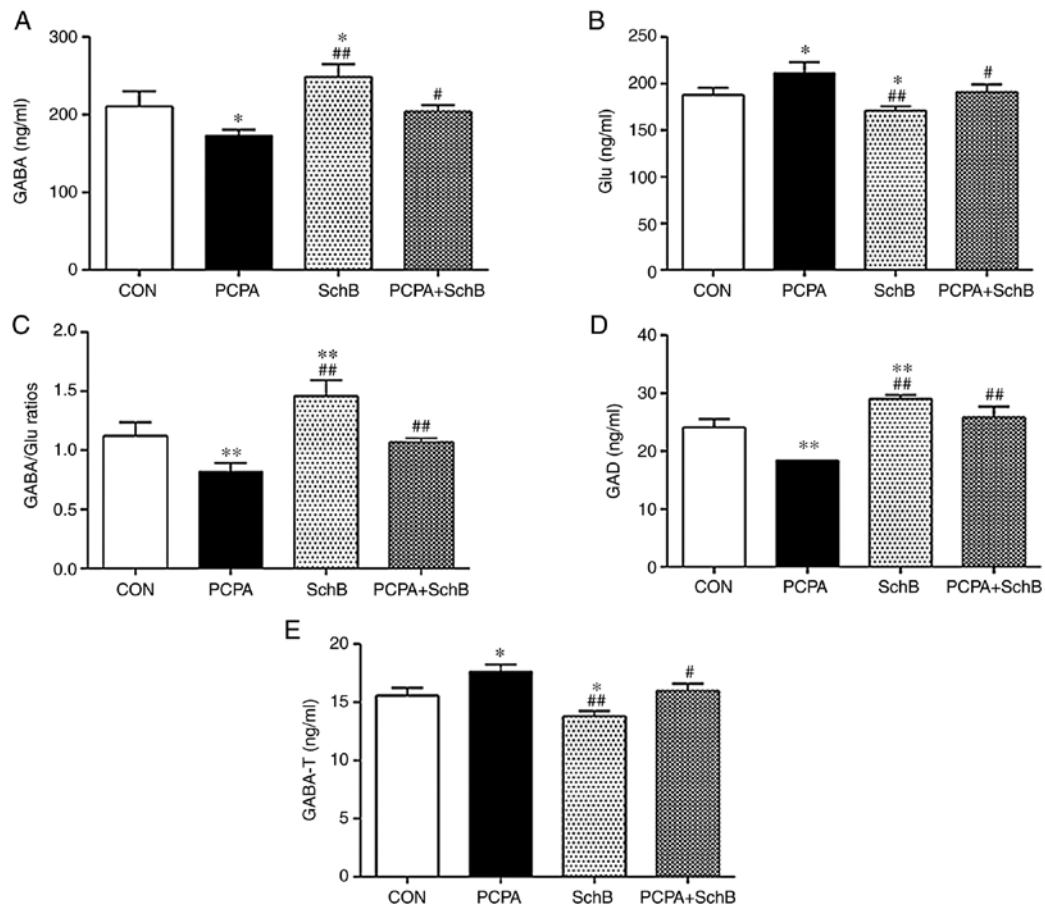


Figure 6. Effect of SchB on GABA and Glu expression in the hypothalami of rats. (A) Effect of SchB on GABA expression in the hypothalami of rats. (B) Effect of SchB on Glu expression in the hypothalami of rats. (C) Effect of SchB on the GABA/Glu ratio in the hypothalami of rats. (D) Effect of SchB on GAD activity in the hypothalami of rats. (E) Effect of SchB on GABA-T activity in the hypothalami of rats. Data are presented as the mean \pm SD (n=4). *P<0.05, **P<0.01 vs. CON group; #P<0.05, ##P<0.01 vs. PCPA group. SchB, Schisandrin B; CON, control; PCPA, parachlorophenylalanine; GABA, γ -aminobutyric acid; Glu, glutamate; GAD, glutamic acid decarboxylase; GABA-T, γ -aminobutyric acid transaminase.

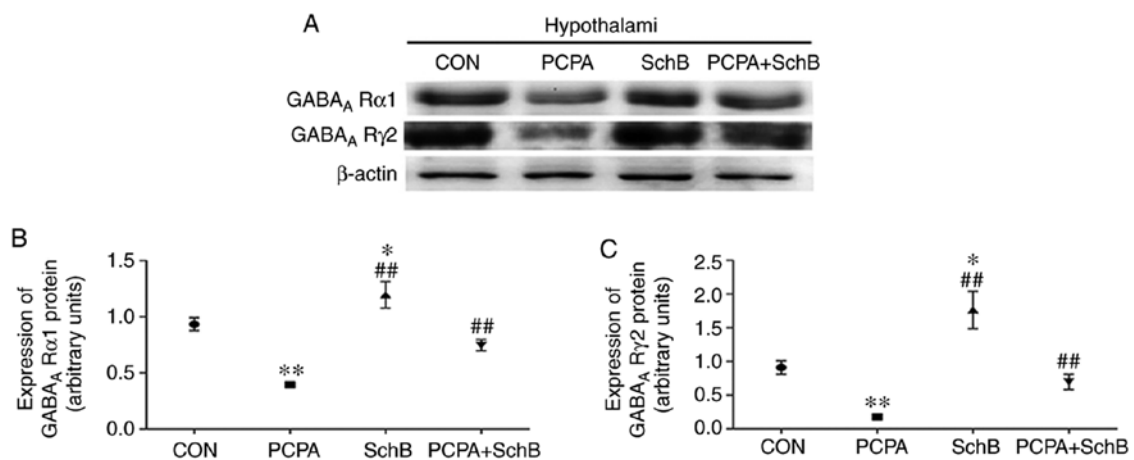


Figure 7. Effect of SchB on the expression of GABA_A R α 1 and GABA_A R γ 2 protein in the hypothalami of model rats. (A) Representative western blotting images demonstrating GABA_A R α 1 and GABA_A R γ 2 protein expression in the hypothalami of rats in each group. (B) The expression of GABA_A R α 1 protein in the hypothalami of rats in each group. (C) The expression of GABA_A R γ 2 protein in the hypothalami of rats in each group. Data are presented as the mean \pm SD (n=3). *P<0.05, **P<0.01 vs. CON group; ##P<0.01 vs. PCPA group. SchB, Schisandrin B; CON, control; PCPA, parachlorophenylalanine; GABA_A R α 1, GABA_A receptor α 1; GABA_A R γ 2, GABA_A receptor γ 2.

5-HT neurons affect the higher hypothalamic center through ascending projections, and 5-HT and its receptors in the hypothalamus are important components in the regulation

of sleep and arousal (24,25). 5-HIAA is a metabolite of 5-HT. 5-HT is deaminated and converted into 5-hydroxyindole acetaldehyde by monoamine oxidase, which is itself catalyzed

by aldehyde dehydrogenase into 5-HIAA; thus, the levels of 5-HIAA in the brain reflect the activity of the 5-HT-nergic nervous system (13,26).

5-HIAA is associated with analgesia and sleep. Previous studies have revealed that in animal models of insomnia or sleep deprivation, 5-HIAA levels decrease with those of 5-HT (27,28). The HTR1A gene encodes the 5-HT_{1A} receptor, the latter of which binds to the free form of 5-HT in the synaptic space, thus mediating 5-HT function, increasing its nervous system activity and promoting sleep (29). HTR1A is the most abundantly expressed 5-HT receptor in the mammalian brain. Among various biochemical reactions involving the 15 receptor subtypes of 5-HT (and the associated receptor family), 5-HT dysfunction is the most closely associated with HTR1A (30-32). The results of the present study demonstrated that the expression levels of 5-HT and 5-HIAA in the hypothalamus, and protein expression in the hypothalami of rats with PCPA-induced insomnia, were significantly decreased, but subsequently increased by SchB, suggesting that the hypnotic effect of SchB may be associated with 5-HT.

GABA and Glu are the primary neurotransmitters in the central nervous system of mammals (14,33,34). When the levels of Glu are increased, neurons are activated, promoting arousal and awakening of the body. When the level of GABA is increased, neuronal excitation is inhibited and arousal is inactivated, thus promoting sleep (35,36). The GABA/Glu ratio maintains the balance between the inhibition and excitation of nerve cells, and therefore, is often used to study and evaluate the excitatory and inhibitory state of central nervous system function. Specific binding of GABA and its receptor instigates post-synaptic membrane chloride influx, resulting in membrane hyperpolarization and the inhibition of neuronal activity. This inhibition can be specifically blocked by GABA receptor inhibitors (37). GABA_A α 1 and GABA_A γ 2 are two important subunits of the GABA receptor. In our previous study, SchB was found to increase the levels of GABA, decrease the levels of Glu, increase the ratio of GABA/Glu, and increase the expression of GABA_A α 1 and GABA_A γ 2 in the hypothalami of rats (4). In the present study, similar changes in these indicators were observed in the hypothalamic tissues of rats treated with both SchB and PCPA, further confirming that the hypnotic effect of SchB is associated with an increase in GABA and a decrease of Glu.

GAD is a rate-limiting enzyme in the synthesis of GABA from Glu (38), and under GAD catalysis, the levels of GABA and Glu are increased and decreased, respectively (15,39). GABA-T hydrolyses GABA. Mature GABA is first deaminated by GABA-T to form succinic semialdehyde (SSA); SSA is then converted to succinic acid, (catalyzed by succinate semialdehyde dehydrogenase), and the end product (succinic acid) then enters the tricarboxylic acid cycle to form Glu once again (16,40-42). It is therefore evident that the activities of GAD and GABA-T can directly influence the levels of GABA and Glu. The present results revealed that SchB significantly increased GAD activity and decreased GABA-T activity in rats, with or without PCPA treatment, suggesting that SchB altered the levels of GABA and Glu via GAD and GABA-T.

In conclusion, the present study demonstrated that SchB was able to improve PCPA-induced insomnia in rats, which may be partly associated with its ability to elevate the levels of 5-HT

and GABA in the hypothalamus. The present study provided experimental evidence showing the potential of Schisandra and SchB to be further developed into sleep-promoting health supplements and treatments for insomnia.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JLL and JGC conceived and designed the study. MYW, JHS, CMW, NL and SJ performed the animal experiments. MYW and HL performed the data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Institutional Animal Care and Use Committee of Beihua University (Jilin, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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