Glycyrrhizin extracted from licorice alleviates insomnia by regulating signaling pathways of γ-aminobutyric acid (GABA) and serotonin in a mice model

Keywords
insomnia, mice, GABA, signaling, glycyrrhizin, licorice

Abstract

Introduction
Licorice could exhibit beneficial effect in the management of insomnia by regulating key modulators such as GABA and serotonin, we therefore aimed to study the potentail hypnotic effect of licorice extract, glycyrrhizin (GCZ), on insomnia and the underlying mechanisms.

Material and methods
Mice were randomly grouped as a Control group, a GCZ (low) group, a GCZ (high) group and a DZP group with 8 mice in each group. Sleep latency, sleep duration and locomotor activities were recorded. ELISA assays were performed to evaluate the level of GABA, glutamate (Glu) and GABA/Glu ratio in mice tissues. Real-time PCR and IHC were performed to study the expression of GABAA-R.

Results
Oral GCZ treatment at 300mg/kg and 100mg/kg dose-dependently shortened the sleep latency and increased the sleep duration of mice. Locomotor activity was also inhibited by GCZ, while GABA binding affinity was higher in mice treated with GCZ. Moreover, GCZ treatment in mice also increased the GABA level and GALA/Glu ratio in mice plasma, brain, hippocampus and cerebrospinal fluid. Moreover, the level of GABAA receptor (GABAA-R) was also increased by GCZ treatment in a dose-dependent manner.

Conclusions
Our study validated that the glycyrrhizin, content of licorice, could alleviate insomnia by upregulating the level of GABA and its receptor.

Glycyrrhizin extracted from licorice alleviates insomnia by regulating signaling pathways of γ-aminobutyric acid (GABA) and serotonin in a mice model

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Abstract

Background: Licorice could exhibit beneficial effect in the management of insomnia by regulating key modulators such as GABA and serotonin, we therefore aimed to study the potentail hypnotic effect of licorice extract, glycyrrhizin (GCZ), on insomnia and the underlying mechanisms. **Method**: Mice were randomly grouped as a Control group, a GCZ (low) group, a GCZ (high) group and a DZP group with 8 mice in each group. Sleep latency, sleep duration and locomotor activities were recorded. ELISA assays were performed to evaluate the level of GABA, glutamate (Glu) and GABA/Glu ratio in mice tissues. Real-time PCR and IHC were performed to study the expression of $GABA_A$-R. **Result**: Oral GCZ treatment at 300mg/kg and 100mg/kg dose-dependently shortened the sleep latency and increased the sleep duration of mice. Locomotor activity was also inhibited by GCZ, while GABA binding affinity was higher in mice treated with GCZ. Moreover, GCZ treatment in mice also increased the GABA level and GALA/Glu ratio in mice plasma, brain, hippocampus and cerebrospinal fluid. Moreover, the level of $GABA_A$ receptor ($GABA_A$-R) was also increased by GCZ treatment in a dose-dependent manner. **Conclusion:** Our study validated that the glycyrrhizin, content of licorice, could alleviate insomnia by upregulating the level of GABA and its receptor.

Keywords: glycyrrhizin, licorice, insomnia, mice, GABA, signaling

Running title: Glycyrrhizin alleviates insomnia in mice

Introduction

As a fundamental physiological process which is crucial to one's health, sleep has been a frequently discussed topic worldwide. Lack of sleep often results in dissatisfactory cognitive performance, substandard learning ability, weakened immune system and even diseases including hypertension and diabetes [1]. According to a previous meta-analysis on the prevalence of insomnia in China, nearly 1 our of 7 Chinese people suffered from insomnia, regardless of genders or generations [2]. Compared with the prevalence of insomnia in Western countries such as USA, which is 27.1%, the prevalence of insomnia is China is evidently lower [3]. However, when compared with Asian countries such as Singapore, which is 15.3%, similarity was spotted [4].

For patients seeking for professional help, cognitive-behavioral therapy (CBT) and pharmacotherapy are often recommended [5]. However, some reports suggested that the sleep restriction of CBT for insomnia may impair the some patients' consciousness, and result in temporary daytime drowsiness and fatigue [6]. Meanwhile, when applied at potent doses, compounds targeting GABA, a crucial inhibitory neurotransmitter in the central nervous system (CNS), could evidently enhance sleep quality [7]. However, the safety and efficacy of the alternative pharmacotherapies using γ-aminobutyric acid (GABA)-A receptor agonists such as benzodiazepines (BZD) were also challenged when being applied for long-term usage over 6 months. Side effects such as rebound insomnia, nocturnal confusion and unsteadiness were reported, especially in elderly people [8]. Therefore, in consideration of the above mentioned side effects, many patients turn to herbal medicines for help, which may offer an exceptional safety and efficiency profile with much less side effects reported compared with pharmacotherapies [9, 10].

Moreover, flavonoids derived from various plants have been reported to modulate GABA-A receptor at the CNS level. And Glycyrrhiza glabra (G. glabra), also known as licorice, has been reported to act as an modulator of GABA-A receptor [11]. By effective modulation of GABA-A receptors, licorice could promote the sedative and anti-anxiety effects precipitated by GABA [12]. In their study which investigated licorice ethanol extract, Cho et al. reported that G. glabra increased the frequency of non-rapid eye movement (NREM) sleep in mice induced by pentobarbital [13]. And NREM sleep, also known as quiescent sleep, is reported to be associated with reduced incidence of insomnia with both low breathing rate and low blood pressure

observed during NREM sleep [14]. Therefore, G. glabra is shown to exhibit hypnotic effects on pentobarbital-induced sleep in mice models [13].

Serotonin, also known as 5-hydroxytryptamine (5-HT), has been shown to be associated with insomnia and depression [15]. By functioning in the CNS, 5-HT mainly promote wakefulness and inhibit rapid eye movement (REM) sleep, although the underlying mechanism was not thoroughly investigated [15, 16]. Previous evidence mostly signifies that the influence of the antagonist or the inverse agonist of 5-HT(2A) receptor on hypnotic could be considered as a legitimate treatment for patients suffering from insomnia [17, 18]. Moreover, in post-traumatic stress disorder (PTSD) rat models, it was found that glycyrrhizin (GCZ) could help to recover the circadian rhythm changes and diurnal fluctuations of serotonin in the amygdala [19]. And it was also reported that an excessive dosage of GCZ could restore the dysregulated serotonin and ameliorate the upregulated GABA, which recovered brain neuronal damage of rats pharmacologically depleted of norepinephrine [20].

Since licorice has been reported to benefit the management of insomnia by regulating key modulators of insomnia including GABA and serotonin [11, 12, 13, 16, 17, 18, 19, 20], we therefore hypothesized that licorice extract, GCZ, could alleviate insomnia by interacting with GABA and serotonin. Accordingly, we established mice modes to study the effect of GCZ on the insomnia-related parameters in the plasma, brain, hippocampus and CSF tissues in mice.

Materials and Methods

Animals

A total of 32 mice weighing between 18g to 22g were housed for 7 days with free access to food and water. The temperature was controlled at 24±2 ◦C and the humidity was controlled at 55±10 %. The animal facility utilized a 12 h light/dark cycle with the light period being 7:00 a.m. to 7:00 p.m. After accommodating the animals to the facility's environment, the mice were randomly divided into 4 groups: a control, a GCZ (low) group, a GCZ (high) group and a diazepam (DZP) group. For mice in the GCZ groups, the mice were subjected to different doses of GCZ, i.e., 100 mg/kg in the GCZ (low) group and 300 mg/kg for the GCZ (high) group. For mice in the DZP group, the mice were subjected to the oral administration with 2 mg/kg GCZ which was a common reference hypnotic drug. After the oral treatment, the mice were subjected to sleep

test and locomotor activity test. After finishing these physical test, the mice were sacrificed and plasma, brain, hippocampus and CSF samples were collected from each mice for subsequent analysis. The oral administration was performed by using a sonde needle. All animal experiments were performed in line with the Guide for the Care and Use of Laboratory Animal and were approved by the institution's animal ethics committee (Approval ID: SHZY-2019-33VXM-02).

Sleep test

After the oral administration of GCZ or DZP, the sleep latency and duration were observed and recorded by two observers blind to the treatment methods of each mice group. The absence of righting reflex for more than 60 seconds was considered as asleep. Righting reflex is defined as the ability of right itself for more than 10 seconds after being placed on its back. Sleep latency was defined as the time between oral administration and the onset of sleep, and sleep duration was defined as the time between asleep and awake (success of righting reflex).

Locomotor activity test

The mice were individually placed in a paper box, which is 60 cm in length, 50 cm in width and 40 cm in height in a dark environment. Each mouse was accommodated to the environment for 5 min before its moving distance was tracked and recorded by an Animal Trajectory Tracking System (Noldus Information Technology Co., Ltd, Beijing).

Assay of $GABA_A$-BZD receptor binding

The receptor membrane was prepared from the cerebral cortex of male SD rats weighing between 220g to 265g, and the binding of $GABA_A$-BZD receptor was evaluated according to a previously published method [21]. Briefly, the cerebral cortex was homogenized, and the membrane suspension was subjected to a 15-min centrifugation at 27000 xg before the pellet was washed by a 10 min centrifugation at 27000 xg. Subsequently, to remove endogenous GABA, the washed pellet was homogenized before being resuspended for the binding assay. Subsequently, 180 µl of membrane suspension, 10 µL of test solution and 10 µL of [3H] flumazenil were mixed and incubated on ice for 40 min. The binding reaction was then terminated to remove the unbound [3H] flumazenil. The filter-bound radioactivity was determined by conventional liquid scintillation counting using a liquid scintillation counter. The total binding was evaluated by utilization of binding buffer and DZP.

Enzyme-linked immunosorbent assay (ELISA) assay of GABA and Glu level in mice

ELISA assays were performed to evaluate the levels of GABA and Glu in the plasma, brain, hippocampus and CSF tissues in the mice groups. The samples were processed in an RIPA lysis buffer and centrifugated at 1070 xg for 10 min to remove the cell debris. ELISA kits of GABA and Glu (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China) were then used and the measurement was performed according to the instructions provided by the kit manufacture.

IHC assay of $GABA_A$-R in brain tissues

The levels of $GABA_A$-R in the brain tissues collected from mice were measured by IHC assay. The brain tissues collected were fixed in a 10% formalin solution with a 7.4 pH value. Subsequently, the brain tissue were paraffin embedded, sliced into 2 mm thick sections and de-paraffined using xylene. After that, the brain sections were blocked with 0.3% hydrogen peroxide for 20 min, incubated with 0.01 m/L citrate with a 6.0 pH value, blocked with 10% FBS, stained with primary antibodies against $GABA_A$-R (Abcam, Cambridge, MA) overnight, and incubated with horseradish peroxidase conjugated secondary antibodies (Abcam, Cambridge, MA) for 1h. Finally, the sections were counter-stained with a DAB substrate (Sigma-Aldrich, St. Louis, MO) before being observed and assessed under an Olympus light microscope (Olympus, Tokyo, Japan).

Real-time PCR quantification of GABAR mRNA

Total RNA from brain samples collected from the mice groups was isolated by utilizing a Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The extracted RNA was then subjected to reverse transcription to cDNA utilizing a Taqman Reverse Transcription assay kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Then, real-time PCR analysis was performed to evaluate the expression level of $GABA_A$-R mRNA on a PRISM 7500 real time PCR machine (Applied Biosystems, Foster City, CA). The primers used for $GABA_A$-R mRNA were: Forward primer: 5'-ACCACGACATGGAGTACAC-3'; Reverse primer: 5'-CCATTGGGCCTTTAAATTTCAG-3'. The $2^{-\Delta\Delta Ct}$ approach was utilized for the calculation of $GABA_A$-R mRNA expression relative to β-actin, the primers of which were: .

Statistical analysis

All experiment data were analyzed using SPSS (v 19.0,). Differences between the groups were tested and analyzed with one-way ANOVA and Tukey's test (post-hoc test). Data were expressed as means ± standard deviation. The P value less than 0.05 was deemed as statistical significant.

Results

Sleep latency and duration were influenced by GZC

The oral administration of GCZ significantly decreased the sleep latency (Fig.1A) and increased the sleep duration (Fig.1B) in a dose-dependent manner, i.e., GCZ at a dose of 300 mg/kg exhibited stronger hypnotic effect in mice compared with GCZ at a dose of 100 mg/kg. However, compared with GCZ at a high dose of 300 mg/kg, the reference hypnotic drug DZP most significantly improved the sleep quality in mice (Fig.1).

GZC influenced the locomotion distance and GABA affinity in mice

Furthermore, by observing the locomotion distance of each mice group, we could also evaluate the hypnotic effect of GZC. As shown in Figure 2A, the total distance moved is shortest in the DZP group, and longest in the control group. And GZC significantly decreased the total distance moved in the box, with GZC treatment at high dosage exhibiting the stronger reductive effect. Therefore, these results indicate that, like DZP, GZC could also exert hypnotic effect in mice in a dose-dependent manner. Subsequently, the GABA affinity in the mice groups were also studied. As shown in Figure 2B, over 90 % of [^{3}H] flumazenil binding of GCZ was shown at the concentration of 10 mg/mL, while DZP at the same concentration displayed higher percentage of binding [^{3}H] flumazenil binding.

Level of GABA, Glu and GABA/Glu ratio in the mice models

Subsequently, the level of GABA, Glu and the GABA/Glu ratio were studied in the plasma, brain, hippocampus and CSF samples in mice models. As shown in Figure 3, the level of plasma GABA (Fig.3A) was increased while the level of Glu (Fig.3B) was decreased in mice treated with GCZ in a dose-dependent manner. In comparison, the DZP group exhibited the highest level of GABA (Fig.3A) and lowest level of Glu (Fig.3B). Moreover, as shown in Figure 4C, the GABA/Glu ratio was highest in the DZP group and lowest in the control group, and GCZ at a higher dosage also exhibited higher GABA/Glu ratio than GCZ at a lower dosage. Similarly, the level of GABA, Glu,

and GABA/Glu ratio all showed the same tendency among the mice groups in the brain (Fig.4A-C), hippocampus (Fig.4D-F), and CSF samples (Fig.5).

Level of $GABA_A$-R in mice brain

We also studied the level of $GABA_A$-R in mice brain. As shown in Figure 6A, by performing IHC assays, we found that the level $GABA_A$-R was increased in mice treated with GCZ in a dose-dependent manner, although the level of $GABA_A$-R was highest in the DZP group. And real-time PCR analysis also showed higher level of $GABA_A$-R mRNA in mice of the GCZ group, with GCZ treatment at a higher dosage inducing higher level of $GABA_A$-R mRNA (Fig.6B).

Discussion

As reported by previous investigations, the safety and efficacy profiles of multiple approved insomnia therapies were questioned, and some hypnotics are even abandoned due to the negative effects caused or unexpected drug abuse [22]. However, many natural herb treatments provide an excellent safety and efficacy profile for the management of insomnia, which made herbal medicines become a popular choice among patients suffering from insomnia [6, 7, 8]. Many recent investigations also demonstrated that the number of patients taking complementary herbal medicines is increasing, due to the dissatisfaction or concern of adverse effects of pharmacological remedies [10, 23]. Among these herbal medicines, some could be dated back to centuries before, and are still used in many insomnia treatment products nowadays. For example, valerian and chamomile are quite popular among insomnia patients due to their good safety profile and efficacy [10, 24, 25]. In this study, we chose licorice, a widely studied herb, to study the potential therapeutic effect of its extract glycyrrhizin in the management of insomnia. Extracts from licorice have been shown to exhibit therapeutic effects in many diseases. For example, glabridin from licorice has been reported to antagonize vascular inflammation and promote vascular remodeling of the left anterior descending coronary artery in diabetic rats [26]. Moreover, even the de-glycyrrhizinated extract from licorice could antagonize renal tubular epithelial-mesenchymal transition [27]. In this study, we studied the effect of GCZ, and found that GCZ treatment shortened the sleep latency of mice and increased the sleep duration of mice in a dose-dependent manner. Moreover, the locomotor activity was also inhibited by GCZ treatment.

It is also noteworthy that natural herbs are commonly combinations of serval complex constituents, which makes it difficult to identify the active components and their mechanism [28]. There are four clinically approved effective pharmacological therapies for insomnia: the regulation of melatonin receptor agonism, the regulation of histamine 1 receptor antagonism, the regulation of hypocretin/orexin antagonism and the regulation of $GABA_A$-R [1, 29]. And Vereczki et al. reported in their study that the ratio of GABAergic neurons in basal amygdala is 22% while the ratio of GABAergic neurons in lateral amygdala is 16%, while over 20% brain neurons are estimated as GABAergic neurons [30]. And three different type of GABA receptors, $GABA_A$-R, $GABA_B$-R and $GABA_c$-R are reported in the mechanism of sleep [31]. As a matter of fact, many studies which reported the potential mechanism of licorice have identified the altered neurotransmission of GABA as a key factor which participated in the presences of these hypnotic and sedative effects [11, 12, 13]. Therefore, in this study, we also studied the effect of GCZ on the expression of GABA and its receptors. Accordingly, we found that GCZ not only exhibited high GABA binding affinity, but also dose-dependently increased the GABA level as well as the $GABA_A$-R level.

Glutamate is a major excitatory neurotransmitter which distributed widely in the CNS. Glu can influence the cognitive functions including learning and memory abilities, and the high level of Glu was associated with higher levels of pathological incidence such as neurotoxicity [32, 33]. Although Glu is highly expressed in brain tissues, only a small fraction of Glu functions as neurotransmitters, and the level of Glu is associated with the metabolic level, which enables the researchers to identify the nerve function as excitatory or inhibitory by evaluating the Glu level alone [34]. However, the level of GABA is produced by the decarboxylation of Glu, thus making the ratio of GABA/Glu as an effective parameter to refer to when the CNS functions is evaluated [35, 36, 37]. In our current study, by studying the level of GABA and Glu, we found that GCZ increased the ratio of GABA/Glu in a dose-dependent manner, which indicated the inhibitory effect of GCZ on CNS.

The demand for safer remedy options for insomnia is increasing. Although the results of this study identified hypnotic effect in glycyrrhizin, there is a long way to go before it can be prescribed in clinical practices. Our conclusion is limited since the safety profile of licorice extract

glycyrrhizin remains to be investigated. In the previous report which investigated the safety profile of licorice in treating cardiovascular disorders, licorice was reported to induce hypokalemia [38], cardiac arrest [39] or even coronary artery spasm [40]. Therefore, the prescription of licorice for the treatment of insomnia still requires enormous investigations to screen out the active components and study their molecular mechanisms to exert the hypnotic and sedative effect.

Conclusions

In conclusion, our study validated that GCZ exerts sedative and hypnotic effect in mice, which is one of the active components of licorice responsible for its ability to alleviate insomnia. And the GCZ-induced sedation and hypnosis may be caused by regulating the level of GABA, GABA/Glu ratio and its receptor $GABA_A$-R in a mice model.

Declarations

Ethics approval and consent to participate

All animal experiments were performed in line with the Guide for the Care and Use of Laboratory Animal and were approved by the animal ethics committee of Shanghai Municipal Hospital of Traditional Chinese Medicine.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

Qing Deng designed this study, performed the experiments, collected the data, processed the data and composed the manuscript.

Qing He performed the experiments, collected the data, processed the data and approved the final manuscript.

Nana Li performed the experiments and approved the final manuscript.

Jian Xu collected the literatures, collected the data, processed the data, visualized the data and improved the manuscript.

Figure legend

Figure 1

A: Sleep latency of mice was shortened by GCZ treatment in a dose-dependent manner.

B: Sleep duration was longer in mice treated with GCZ in a dose-dependent manner.

Figure 2

A: The total distance moved which indicated locomotor activity was reduced by the GCZ treatment in a dose-dependent manner.

B: The [^{3}H] flumazenil binding which indicated GABA binding affinity was higher in mice treated with GCZ in a dose-dependent manner.

Figure 3

A: Level of plasma GABA was increased by GCZ treatment in a dose-dependent manner.

B: Level of plasma Glu was suppressed by GCZ treatment in a dose-dependent manner.

C: The plasma GABA/Glu ratio was increased by GCZ treatment in a dose-dependent manner.

Figure 4

A: GABA level in mice brain was up-regulated by GCZ treatment in a dose-dependent manner.

B: Glu level in mice brain was down-regulated by GCZ treatment in a dose-dependent manner.

C: The ratio of GABA/Glu in mice brain was elevated by GCZ treatment in a dose-dependent manner.

D: Level of hippocampal GABA was increased by GCZ treatment in a dose-dependent manner.

E: Level of hippocampal Glu was suppressed by GCZ treatment in a dose-dependent manner.

F: The hippocampal GABA/Glu ratio was increased by GCZ treatment in a dose-dependent manner.

Figure 5

A: GABA level in CSF samples was promoted by GCZ treatment in a dose-dependent manner.

B: Glu level in CSF samples was inhibited by GCZ treatment in a dose-dependent manner.

C: The ratio of GABA/Glu in CSF samples was raised by GCZ treatment in a dose-dependent manner.

Figure 6

A: IHC assay upon the $GABA_A$-R showed that $GABA_A$-R expression was increased by GCZ in a dose-dependent manner.

B: Real-time PRC analysis upon the $GABA_A$-R mRNA indicated significant up-regulation by GCZ treatment in a dose-dependent manner.

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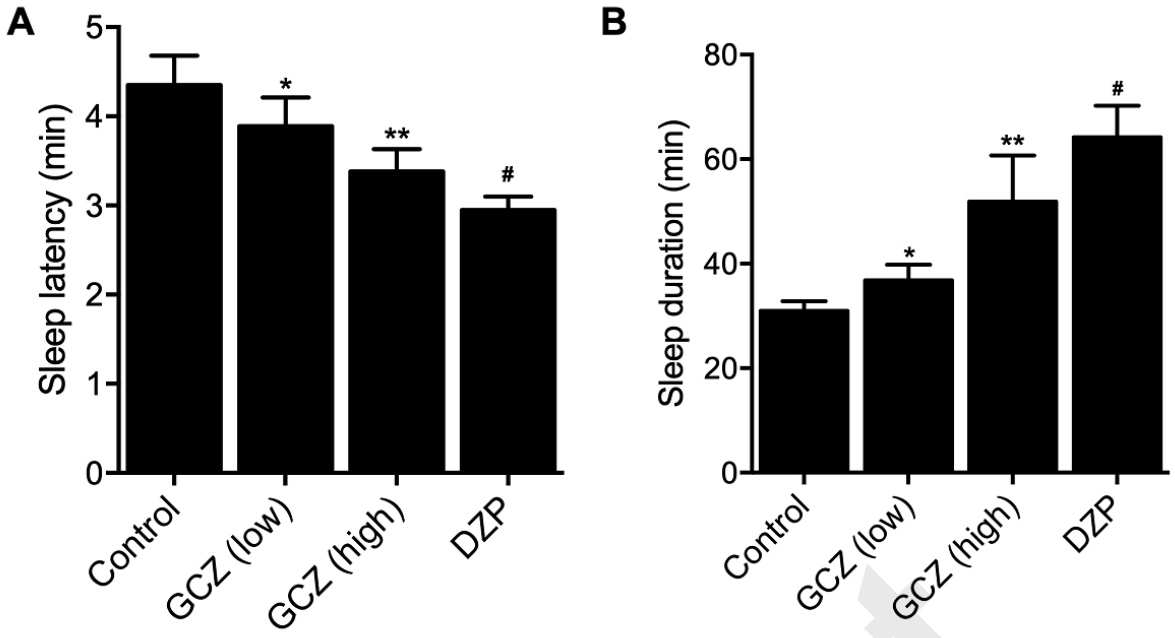

Figure 1
A: Sleep latency of mice was shortened by GCZ treatment in a dose-dependent manner.
B: Sleep duration was longer in mice treated with GCZ in a dose-dependent manner.

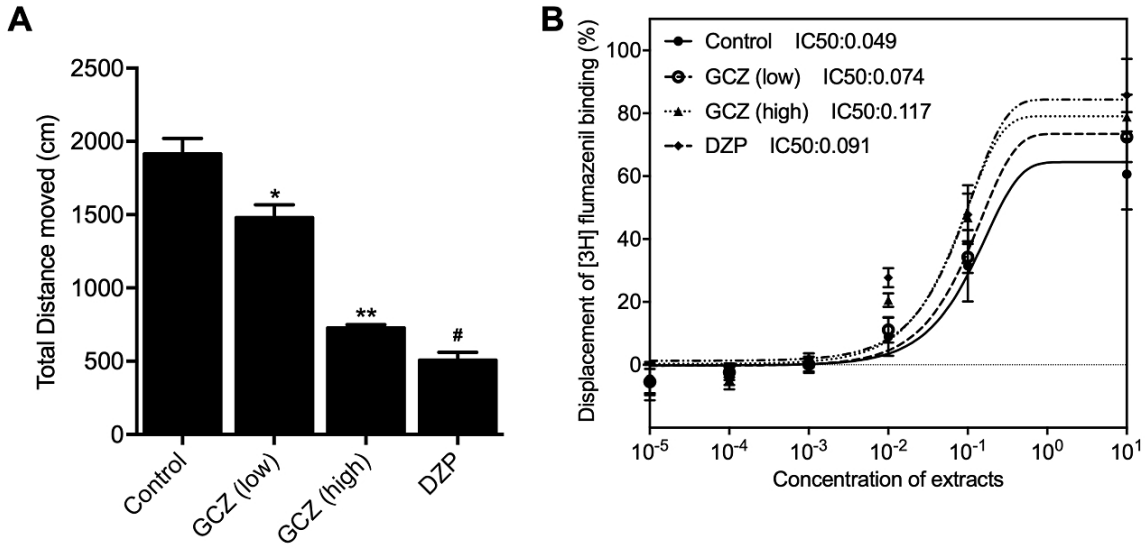

Figure 2

A: The total distance moved which indicated locomotor activity was reduced by the GCZ treatment in a dose-dependent manner.

B: The [3H] flumazenil binding which indicated GABA binding affinity was higher in mice treated with GCZ in a dose-dependent manner.

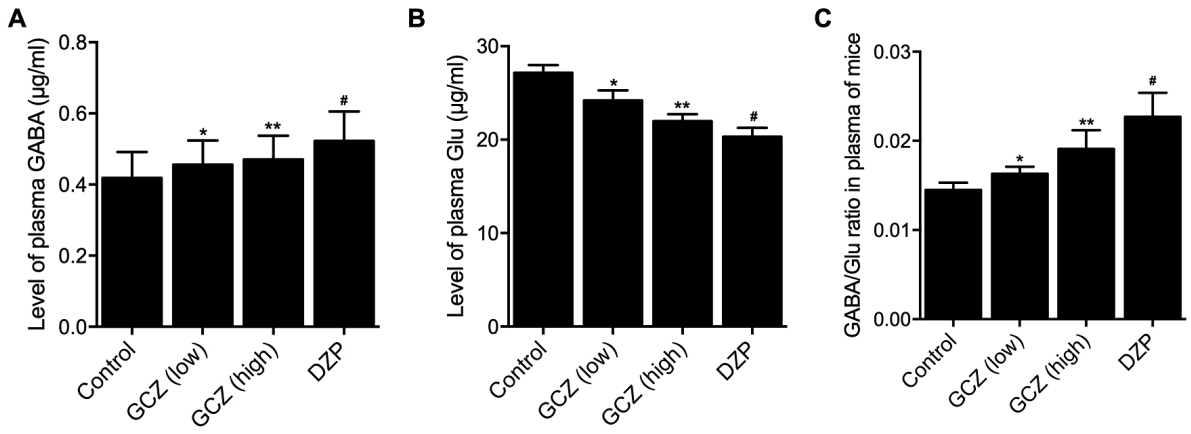

Figure 3
A: Level of plasma GABA was increased by GCZ treatment in a dose-dependent manner.
B: Level of plasma Glu was suppressed by GCZ treatment in a dose-dependent manner.
C: The plasma GABA/Glu ratio was increased by GCZ treatment in a dose-dependent manner.

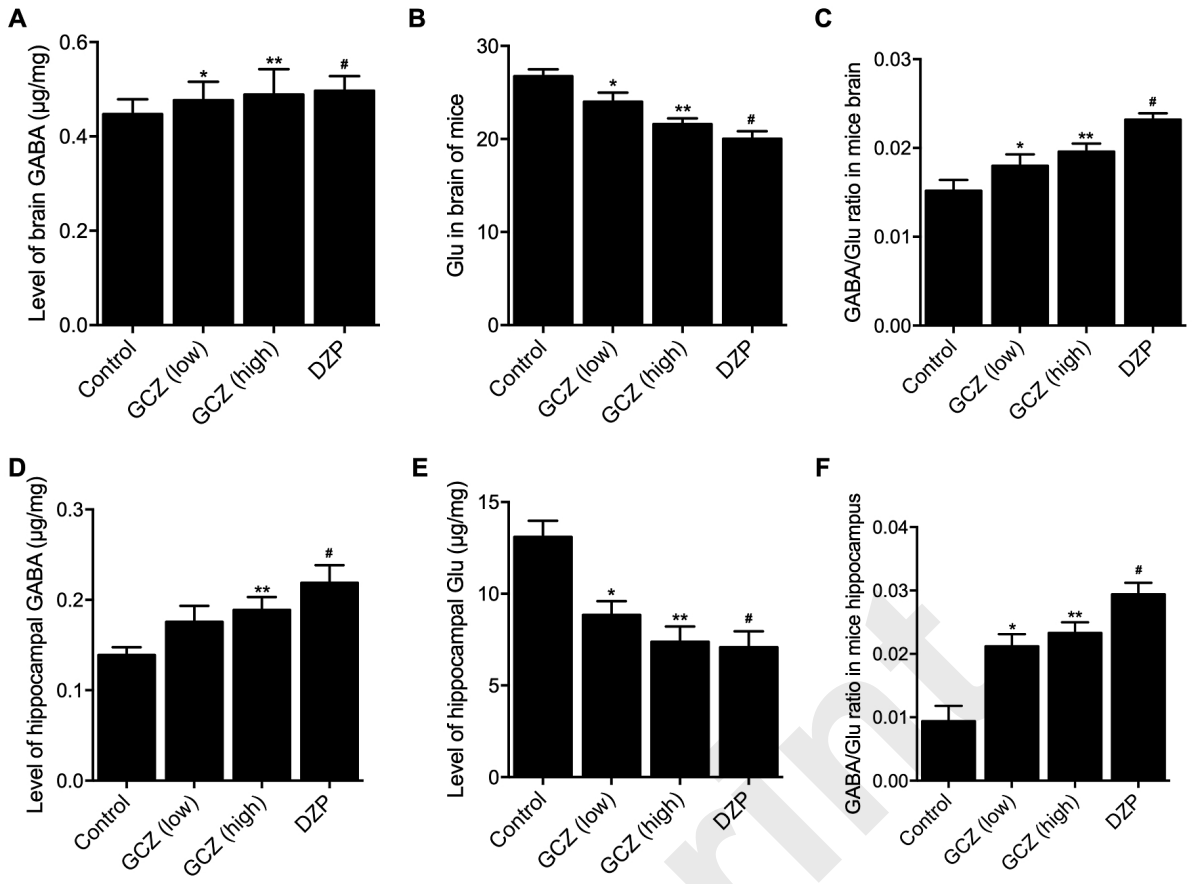

Figure 4

A: GABA level in mice brain was up-regulated by GCZ treatment in a dose-dependent manner.

B: Glu level in mice brain was down-regulated by GCZ treatment in a dose-dependent manner.

C: The ratio of GABA/Glu in mice brain was elevated by GCZ treatment in a dose-dependent manner.

D: Level of hippocampal GABA was increased by GCZ treatment in a dose-dependent manner.

E: Level of hippocampal Glu was suppressed by GCZ treatment in a dose-dependent manner.

F: The hippocampal GABA/Glu ratio was increased by GCZ treatment in a dose-dependent manner.

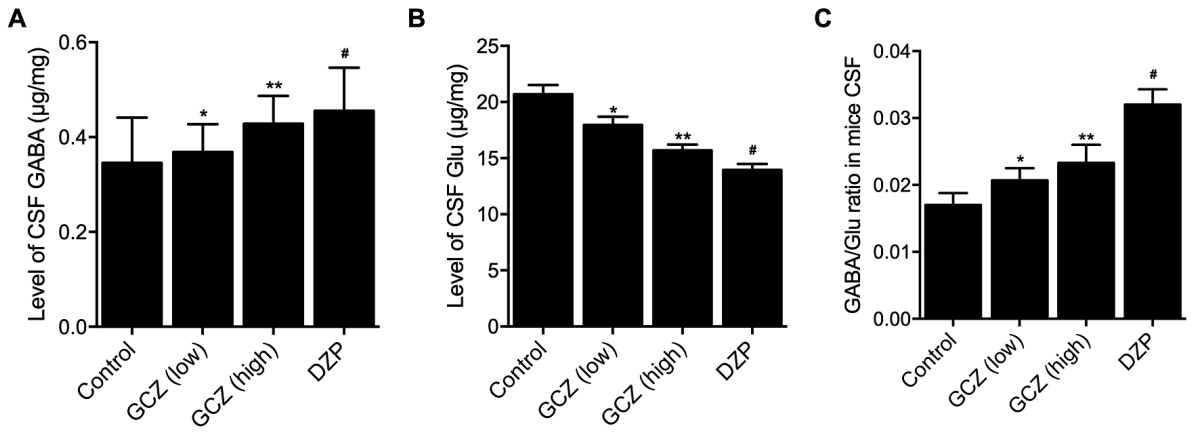

Figure 5
A: GABA level in CSF samples was promoted by GCZ treatment in a dose-dependent manner.
B: Glu level in CSF samples was inhibited by GCZ treatment in a dose-dependent manner.
C: The ratio of GABA/Glu in CSF samples was raised by GCZ treatment in a dose-dependent manner.

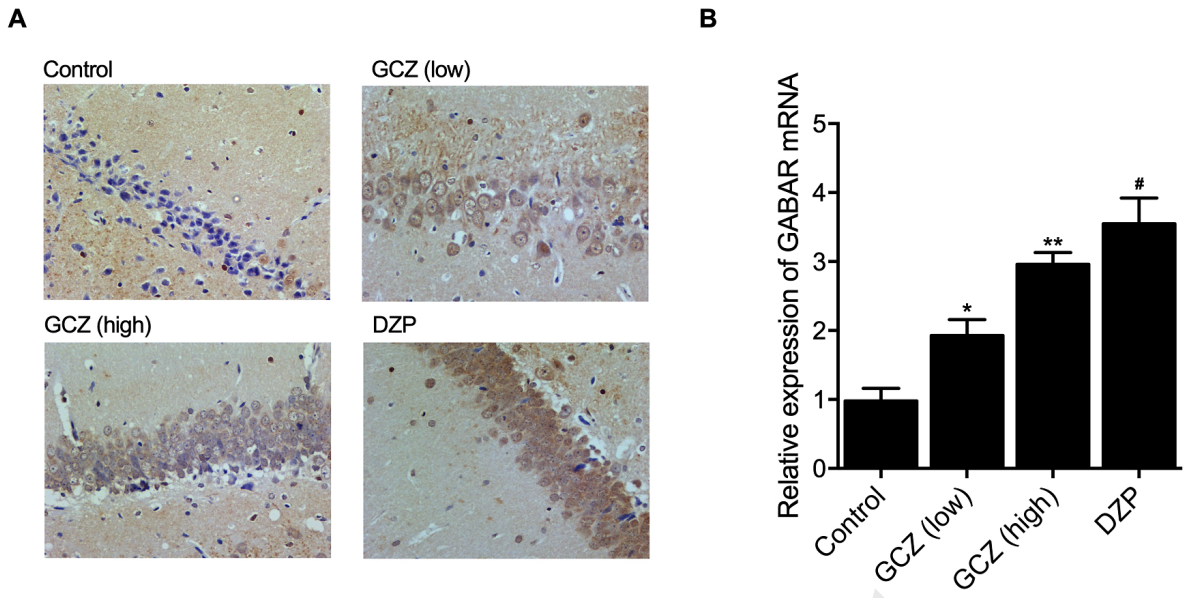

Figure 6
A: IHC assay upon the GABAA-R showed that GABAA-R expression was increased by GCZ in a dose-dependent manner.
B: Real-time PRC analysis upon the GABAA-R mRNA indicated significant up-regulation by GCZ treatment in a dose-dependent manner.