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Posted Date: 10 June 2024

doi: 10.20944/preprints202406.0562.v1

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## Article

# GABALAGEN Alleviates Stress-Induced Sleep Disorder in Rats

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**Abstract:** (1) Background: Gamma-aminobutyric acid (GABA) is one of the inhibitory neurotransmitters with beneficial effects including sedative property. However, despite various clinical trials, scientific evidence regarding the impact of orally ingested GABA, whether natural or synthesized through biological pathways, on sleep is not clear.; (2) Methods: The present study work evaluated GABA-A binding affinity of GABALAGEN(GBL), a low-molecular-weight collagen infused with GABA, through receptor binding assays. The sedative effects of GBL were investigated through electroencephalography (EEG) analysis in the electro foot shock (EFS) stress-induced sleep animal model and then we examined the expression orexin, GABA<sub>A</sub> receptor in the brain region using immunohistochemistry.; (3) Results: We found that on the binding assay, GBL displayed high affinity to GABA<sub>A</sub> receptor (IC<sub>50</sub> value, 31.5µg/mL). Also, after treatment of GBL, percent of total time of REM and NREM were significantly and dose-dependently increased compared to the Con group. Consistent with behavioral results, the GBL-treated groups showed that the expression of GABA<sub>A</sub> receptor immune-positive cells in the VLPO were markedly and dose-dependently increased compared to the Con group. Also, the GBL-treated groups showed that the expression of orexin immune-reactive cells in the LH were significantly decreased compared to the Con group.; (4) Conclusions: In conclusion, GBL showed efficacy and potential to be used as an anti-stress therapy to treat sleep deprivation through antagonism of GABA receptors and consequent inhibition of orexin activity.

**Keywords:** GABALAGEN; electroencephalography (EEG); Sleep; Orexin; Gamma-aminobutyric acid (GABA)

## 1. Introduction

Sleep is an essential aspect of human life. worldwide, a significant number of people suffer from sleep disorders, with insomnia being the most commonly reported sleep disturbance [1–4]. Sleep disorder is characterized by difficulties in initiating or maintaining sleep, non-restorative sleep, or poor-quality sleep [5,6]. There has been much research into the effects of stress on sleep in rodents [7]. The primary stressors used in previous studies were immobilization stress and mild electric shock, with few reports using other methods [8–10]. These sleep disturbances are a prominent feature, and potentially even a hallmark, of post-traumatic stress disorder (PTSD) [7].

Orexin is a neuropeptide whose main function is regulation of the sleep-wake cycle. Orexin regulates a wide variety of important bodily functions, such as eating behavior, stress response, reward processing, mood, and cognition [11–14]. Previous studies reported daytime sleepiness, narcolepsy, rapid eye movement (REM) sleep abnormalities, and in some cases an association between orexin deficiency narcolepsy [11–14].

The classes of drugs commonly used for the treatment of sleep disorder include  $\gamma$ -Aminobutyric acid type A (GABA<sub>A</sub>)-benzodiazepine (BZD) receptor agonists, selective serotonin reuptake

inhibitors, melatonin receptor agonists, antidepressants, and antihistamines [15–17]. GABA is an amino acid that occurs naturally in the human body, and it is the primary inhibitory neurotransmitter in the central nervous system of mammals. GABA plays a crucial role in maintaining mental health. It is involved in various physiological processes such as increasing brain protein synthesis, elevating growth hormone concentration, lowering high blood pressure, regulating diabetes, and facilitating diuresis. GABA has a calming effect and is potentially useful in treating insomnia and depression [16–18].

GABALAGEN (GBL) is a type of collagen with a low molecular weight that contains GABA, which has the same structure as collagen molecules. Through a fermentation process involving specialized *Lactobacillus* strains (*L. Brevis*-BJ20 and *plantarum*-BJ21), GBL is converted into a peptide form that is easily absorbed by the body. This process also produces GABA, which is specialized for *Lactobacillus* strains. GBL is known for its positive effects on skin health, stress relief and promoting sleep. However, research on the impact of GBL on sleep is limited despite various research, and there is currently no conclusive understanding of the mechanisms for treating or improving sleep and related disorders.

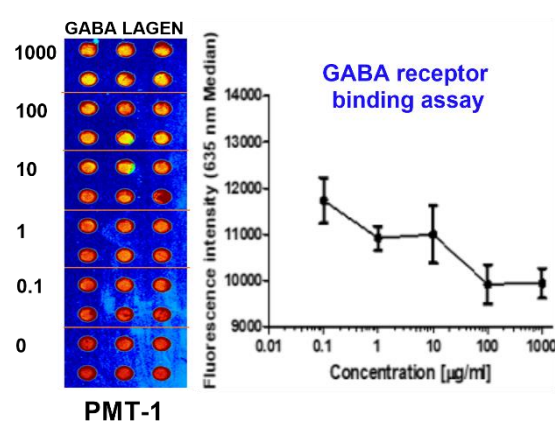
The present study work evaluated the effects of GABALAGEN(GBL), a low-molecular-weight collagen infused with GABA, through receptor binding assays. The sedative effects of GBL were investigated through electroencephalography (EEG) analysis in the electro foot shock (EFS) stress-induced sleep animal model and then we examined the expression orexin, GABA<sub>A</sub> receptor in the brain region using immunohistochemistry.

## 2. Results

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

### 2.1. GABA<sub>A</sub> and 5-HT<sub>2c</sub> Receptor Binding Assay

As shown in Figure 1, the binding activities of GBL to the GABA<sub>A</sub> receptor, indicative of its role as a GABA<sub>A</sub> receptor agonist, were substantiated. The IC<sub>50</sub> value for GBL in this context was measured at 31.5 µg/mL (Figure 1).

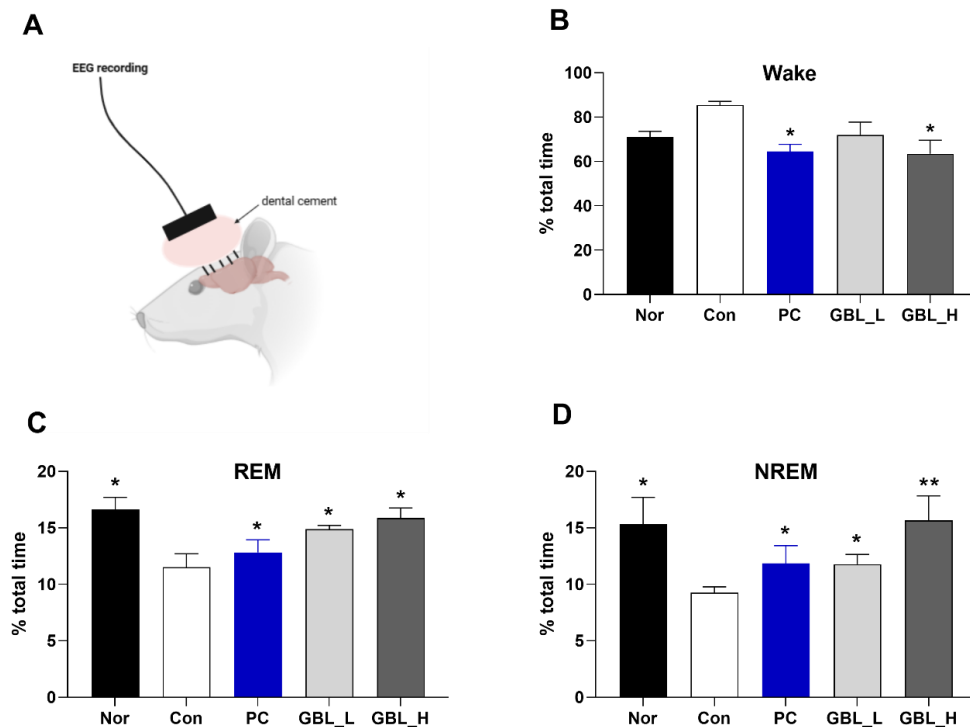


**Figure 1.** The binding activities of GBL to the GABA<sub>A</sub> receptor.

### 2.2. Effect of GBL on EEG Sleep Architecture and Profile

The effect of GBL on EEG sleep architecture and profile was investigated. There was a trend increase in wake time of Con group ( $P > 0.05$ , Figure 2A), however the GBL\_H-treated group showed slightly increased wake time compared to the Con group ( $P < 0.05$ ). Also, the Con group was markedly decrease in REM ( $P < 0.05$ , Figure. 2B), and NREM ( $P < 0.05$ , Figure. 2C), compared to that of the Nor group. However, after treatment of GBL, percent of total time of REM and NREM

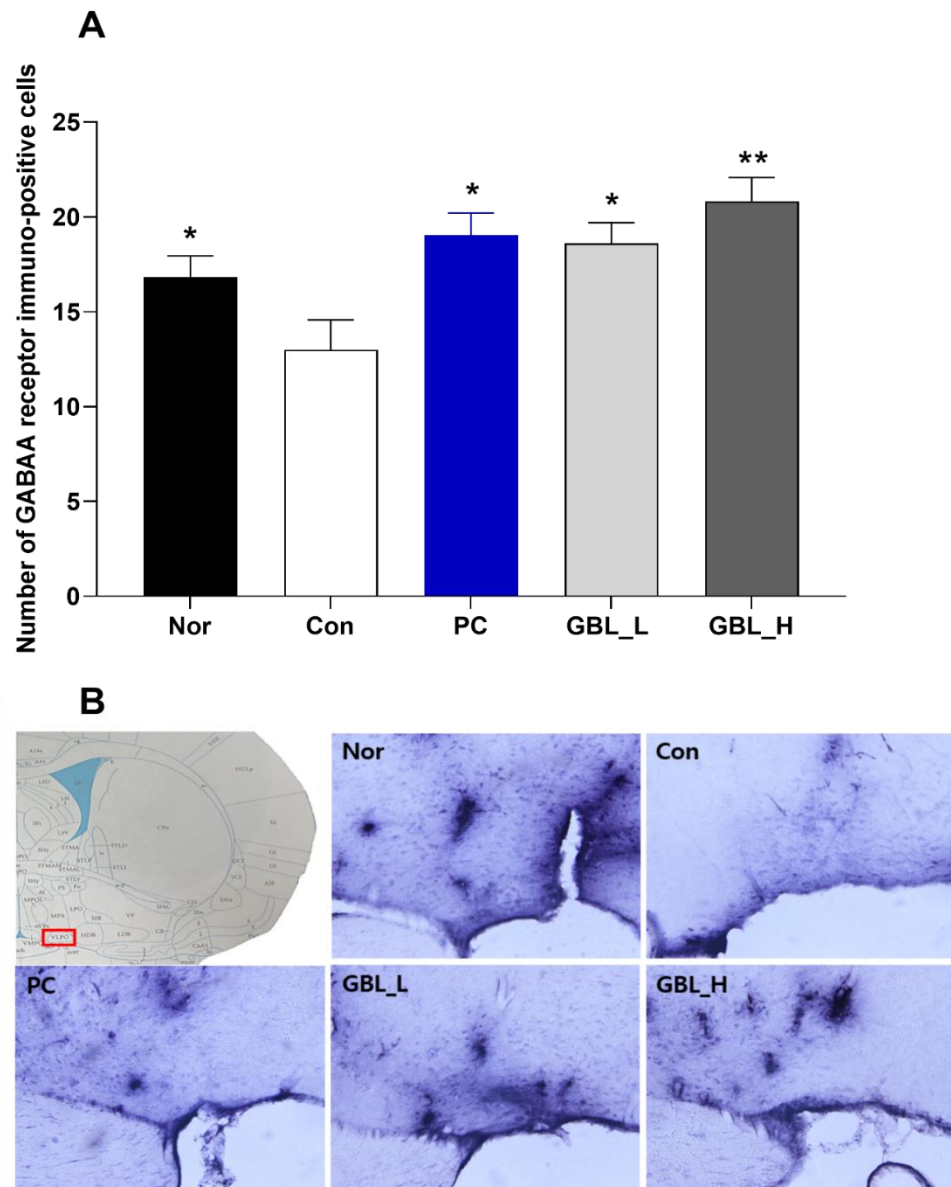
significantly and dose-dependently increased compared to the Con group (REM:  $P < 0.05$ , Figure 2B; NREM:  $P < 0.05$ , Figure 2C).



**Figure 2.** Effect of GBL on EEG sleep architecture and profile. Changes in the percentage of wake (B), REM(C), and MREM(D). The values are presented as means  $\pm$  S.E.M. \*  $P < 0.05$ , \*\* $P < 0.01$  vs Con group.

### 2.3. Effect of GBL on the Number of GABA<sub>A</sub> Receptor Positive Cells in the Ventrolateral Preoptic Nucleus (VLPO)

We measured GABA<sub>A</sub> receptors immunoreactivity in the VLPO (Figure 3(A) and (B)). The numbers of GABA<sub>A</sub> receptor immuno-positive cells were significantly decreased in the Con group ( $P < 0.05$ , Figure 3(A) and (B)). However, the GBL-treated groups showed that the expression of GABA<sub>A</sub> receptor immune-positive cells in the VLPO were markedly and dose-dependently increased compared to the Con group ( $P < 0.05$ ).

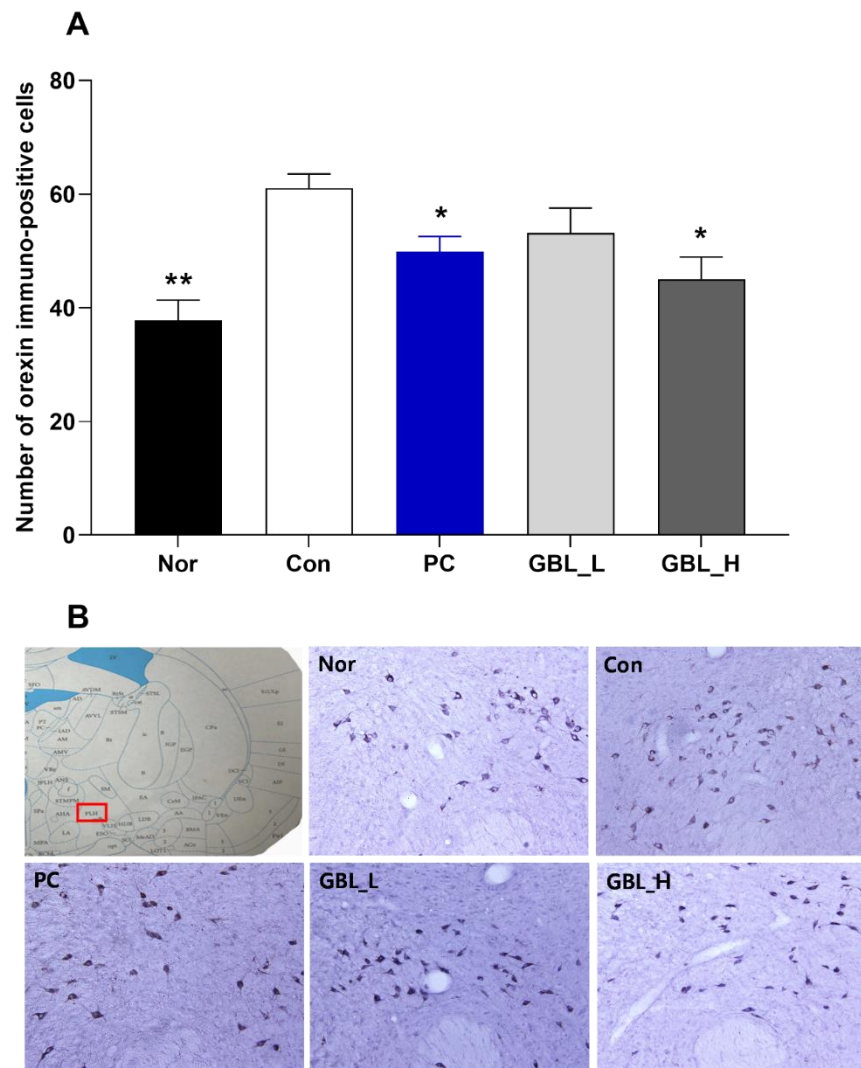


**Figure 3. Effect of GBL on the number of GABA<sub>A</sub> receptor positive cells in the ventrolateral preoptic nucleus (VLPO).** The values are presented as means  $\pm$  S.E.M. \*  $P < 0.05$ , \*\* $P < 0.01$  vs Con group.

#### 2.4. Effect of GBL on the Number of Orexin Positive Cells in the Lateral Hypothalamus (LH)

We measured orexin immunoreactivity in the LH (Figure 4(A) and (B)). The numbers of orexin immuno-positive cells were significantly increased in the Con group ( $P < 0.01$ , Figure 4(A) and (B)). However, the GBL-treated groups showed that the expression of orexin immune-reactive cells in the LH were significantly decreased compared to the Con group ( $P < 0.05$ ).





**Figure 4. Effect of GBL on the number of orexin positive cells in the lateral preoptic nucleus (LH).**

The values are presented as means  $\pm$  S.E.M. \*  $P < 0.05$ , \*\* $P < 0.01$  vs Con group.

### 3. Discussion

Present study found that in the binding assay, GBL displayed binding affinity to GABA<sub>A</sub> receptor (IC<sub>50</sub> value, 31.5 $\mu$ g/mL) and 5-HT<sub>2c</sub> receptor (IC<sub>50</sub> value, 7.14 $\mu$ g/mL). Also, after treatment of GBL, percent of total time of REM and NREM were significantly and dose-dependently increased compared to the Con group. Consistent with behavioral results, the GBL-treated groups showed that the expression of GABA<sub>A</sub> receptor immune-positive cells in the VLPO were markedly and dose-dependently increased compared to the Con group. Also, the GBL-treated groups showed that the expression of orexin immune-reactive cells in the LH were significantly decreased compared to the Con group. GBL showed efficacy and potential to be used as an anti-stress therapy to treat sleep deprivation through antagonism of GABA receptors and consequent inhibition of orexin activity.

Stress can have a significant, long-lasting negative impact on health including changes in behavior and sleep [8–10]. Sleep reactivity tends to cause sleep disturbances during environmental disturbances, pharmacological issues, or stressful life events [20]. As a result, individuals with highly responsive sleep systems are prone to insomnia disorders after stressors, pose a risk of psychopathology, and potentially hinder their recovery from traumatic stress [21–27]. Therefore, there is tremendous value in improving sleep responsiveness, creating a robust sleep system for stress exposure, and ultimately preventing insomnia and subsequent consequences.

GABALAGEN (GBL) is a type of collagen with a low molecular weight that contains GABA, which has the same structure as collagen molecules. GBL is known for its positive effects on skin health, stress relief and promoting sleep. We measured GABA<sub>A</sub> receptors immunoreactivity in the VLPO. The numbers of GABA<sub>A</sub> receptor immuno-positive cells were significantly decreased in the Con group. However, the GBL-treated groups showed that the expression of GABA<sub>A</sub> receptor immune-positive cells in the VLPO were markedly and dose-dependently increased compared to the Con group. Previous studies proved that many herbal medicines have been proposed to enhance GABAergic signaling, many through interactions with the GABA<sub>A</sub> receptor [28–31].

Orexin neurons are characterized by wakefulness-promoting activity, reaching peak firing rates during wakefulness [32–34], and exhibiting the highest extracellular levels of orexin during wakefulness [35]. The LH coordinates sleep-promoting activities, and disruptions in orexin function may upset the delicate balance between sleep and wakefulness, thereby contributing to a spectrum of sleep-related problems [36–38]. This emphasizes the importance of investigating the role of the LH and orexin in comprehending and addressing sleep-related disturbances [33,38]. We found that GBL decreased expression of Orexin positive cells in the LH region. GBL may decrease Orexin in the LH, leading to a reduction in arousal and potentially enhancing the quality of sleep.

Taken together, these data suggest that earthing mats may be helpful in stress management via the regulation of GABAergic mechanisms. In light of such limitations, our finding provides preliminary evidence of the safety of GBL and their potential to improve sleep quality in an animal model.

#### 4. Materials and Methods

##### *Preparation of GBL*

GBL was acquired from Marine Bioprocess Co., Ltd. (Busan, Republic of Korea). GBL production required two consecutive fermentations via *Lactobacillus brevis* BJ20 (accession No. KCTC 11377BP) and *Lactobacillus Plantarum* BJ21 (accession No. KCTC 18911P). Seed media composed of 1% yeast extract (Choheung, Ansan, Korea), 0.5% glucose (Choheung, Ansan, Korea), and 98.5% water was sterilized for 15 min at 121 °C before being inoculated with 0.02% BJ20 and 0.02% *Lactobacillus Plantarum* BJ21 and cultured for 24 h at 37 °C separately. For the first fermentation, 10% (v/v) of the *Lactobacillus brevis* BJ20 cultured seed media was fermented in a fermentation medium (yeast extract 2%, glucose 0.28%, collagen 29% [Geltech Co., Ltd., Busan, Republic of Korea], L-glutamic acid 5.5% [Samin chemical, Siheung, Korea], water 63.22%) at 37 °C for 24 hr. Then, 10% (v/v) of BJ21 cultured seed medium was added and fermented at 37 °C for a further 24 hr. The fermentation medium was sterilized and spray-dried to prepare GBL powder samples.

##### *GABA Receptor Binding Assay*

The GABA receptor was sourced from rat brain tissue. The Protein Chip was obtained from Proteogen, Cy5-labeled muscimol were acquired from Peptron. The stock buffer for the GABA receptors comprised 50 mM Tris-HCL (pH 7.4), 0.5 mM EDTA, 10 mM MgCl<sub>2</sub>, and 10% sucrose. The GABA receptor-binding assay buffer comprised 50 mM Tris-HCL, 10 mM MgCl, 1 mM EDTA, and 0.1% bovine serum albumin (BSA) at pH 7.4. The receptor (50 µg/mL) was immobilized on the Protein Chip, serving as a substrate to capture protein, for 16 h at 4 °C. After double washing in 0.05% phosphate-buffered saline containing 0.2% Triton X-100 (PBST) for 10 min and drying with Nitrogen gas (N<sub>2</sub>), the Protein Chip underwent a 1-hour blocking step at room temperature using 3% BSA. Following three washes with PBST and drying, Cy5-labeled muscimol (500 µM, with 30% glycerol in PBS as a buffer) and GBL (using 30% glycerol in PBS as a buffer) were applied to the Protein Chip and incubated for 1 h at 37 °C. Subsequently, the Protein Chip was rinsed with PBST and DW and dried under a stream of N<sub>2</sub> gas. GBL, dissolved in ethanol and diluted to the desired concentration using PBS, covered a concentration range from 1,000 µM to 15.625 µg/mL. Muscimol were used as negative controls. GBL was used to evaluate the sedative

### *Animals*

Adult male Sprague-Dawley rats were obtained from Samtako (Osansi, Gyeonggi-do, Republic of Korea). These animals were accommodated in a climate-controlled environment with temperatures maintained between 20 °C to 25 °C and humidity levels set at 45% to 65%, following a 12-hour light and 12-hour dark cycle (lights on at 8 a.m.). Food and water were provided ad libitum throughout the study. The laboratory animals were handled and treated in compliance with the guidelines outlined by the Ministry of Food and Drug Safety (MFDS) National Institute of Toxicological Research, per the standards for laboratory animal care and usage (Approval No. KHUASP[SE]-14-051).

### *Surgery*

The subjects were divided into five groups: normal (Nor; n = 7), control (con; n = 8), low-dose GBL-treated (GBL\_L; n = 8), high-dose GBL-treated (GBL\_H; n = 7), and diazepam-treated (DZP; n = 7). Electroencephalogram (EEG) electrodes were surgically implanted to facilitate polygraphic recordings, following the guidelines delineated in the Paxinos and Watson stereotaxic atlas [19]. Surgical anesthesia was induced with intraperitoneal pentobarbital (40 mg/kg). Subsequently, the rats were subjected to chronic implantation of a head mount. The transmitter body was subcutaneously placed off the midline, posterior to the scapula, secured to the skin, and stabilized using three sutures. Skull-mounted electrodes were secured with screws and dental cement. All surgical interventions were performed using a stereotaxic methodology in an aseptic environment. Postoperatively, each rat was allowed a 7-day recovery period in separate transparent enclosures. After electroencephalogram (EEG) operation, the animals received electro foot shock (EFS) once a day for 5 days. EFS was administered in accordance with the following conditions at random: frequency = 10 times in 3 s; intensity = 3 mA; duration = 5 min.

### *Methodology of EEG Recording*

Following the recovery phase, the rats were acclimated to the recording settings before testing. GBL\_L, GBL\_H, and DZP were prepared in 0.9% saline (GBL\_L concentration = 100 mg/kg, GBL\_H concentration = 250 mg/kg, DZP concentration = 10 mg/kg) and orally administered for five consecutive days before the EEG recording commenced. The oral administration of saline, GBL\_L, GBL\_H, and DZP was performed 10 minutes before the EEG recording sessions. The recordings were initiated at 8:00 p.m., capturing 12 h of EEG recordings and activity in all subjects. The cortical EEG signals were amplified (x 100), filtered through a low-pass filter at 100 Hz, digitized at a sampling rate of 200 Hz, and recorded using a PAL-8200 data acquisition system from Pinnacle Technology Inc. The recordings were performed at a chart speed of 25 mm/s.

The SleepSign Ver. 3 software (Kissei Comtec, Nagano, Japan) automatically classified sleep-wake states into three categories: wakefulness (Wake), rapid eye movement (REM) sleep, and NREM sleep. Sleep latency was defined as the duration from the administration of the sample to the onset of the initial uninterrupted NREM sleep episode, lasting a minimum of 2 min and not interrupted by more than six 4-second epochs that were not scored as NREM sleep.

### *Immunohistochemistry*

After transcranial perfusion, the rat brains were removed with 4% formaldehyde solution (Sigma-Aldrich Co.), post-fixed in the same fixative for 24 h, and placed in phosphate-buffered saline containing 20% sucrose for 72 h. Serial 30 µm-thick coronal sections were cut using a cryostat microtome (CM1850UV; Leica Microsystems Inc., Wetzlar, Germany) and stored at -20 °C; they were histochemically processed as free-floating sections. Sections were washed three times with PBST. Primary rabbit polyclonal antibodies against orexin (Abcam, Cambridge, MA, USA) were diluted to 1:800. Antibody targeting the GABA<sub>A</sub> receptor (Abcam, Cambridge, MA, USA) was diluted to 1:800. The sections underwent a 12-hour incubation at 4 °C with constant agitation. After rinsing with PBST, a 2-hour incubation at room temperature was performed using a biotinylated goat anti-rabbit



antibody (Vector Laboratories, Inc., Burlingame, CA, USA) diluted to 1:200 in PBST with 2% v/v normal goat serum. Subsequently, the sections were exposed to an avidin-biotin-peroxidase complex reagent (Vector Laboratories) for 2 h at room temperature. After further rinsing with PBST, the tissues were developed using a DAB substrate kit (Vector Laboratories). The final steps included mounting the sections on slides, air-drying, and covering them for microscopic observation.

#### *Statistical Analysis*

All statistical analyses were conducted using SPSS (IBM® SPSS® Statistics Ver. 23 Chicago, IL, USA). For multiple comparisons, behavioral data were analyzed using one-way analysis of variance (ANOVA). Tukey's post-hoc test was used to identify significant differences among groups. The level of significance was set at  $P < 0.05$ .

## 5. Conclusions

In summary, GBL showed efficacy and potential to be used as an anti-stress therapy to treat sleep deprivation through antagonism of GABA receptors and consequent inhibition of orexin activity.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: title; Table S1: title; Video S1: title.

**Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, I.S.; Data curation, H.P., and W.J.; Writing, H.P., and I.S.; Formal analysis, W.J. and H.P.; Investigation, I.S."

**Funding:** Please add: "This research was funded by the National Foundation of Korea, grant number NRF-2021R1A2C1093825"

**Institutional Review Board Statement:** "The animal study protocol was approved by the Institutional Review Board of Kyung Hee University (protocol code KHUASP[SE]-14-051)." for studies involving animals.

**Informed Consent Statement** "Not applicable."

**Data Availability Statement:** We encourage all authors of articles published in MDPI journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required. Suggested Data Availability Statements are available in section "MDPI Research Data Policies" at <https://www.mdpi.com/ethics>.

**Acknowledgments:** In this section, you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

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## Appendix A

The appendix is an optional section that can contain details and data supplemental to the main text—for example, explanations of experimental details that would disrupt the flow of the main text but nonetheless remain crucial to understanding and reproducing the research shown; figures of replicates for experiments of which representative data is shown in the main text can be added here if brief, or as Supplementary data. Mathematical proofs of results not central to the paper can be added as an appendix.

## Appendix B

All appendix sections must be cited in the main text. In the appendices, Figures, Tables, etc. should be labeled starting with “A” —e.g., Figure A1, Figure A2, etc.

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