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4-Hydroxybenzaldehyde, One of Constituents from Gastrodiae Rhizoma Augments Pentobarbital-induced Sleeping Behaviors and Non-rapid Eye Movement (NREM) Sleep in Rodents

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Abstract – In the previous experiments, we reported that ethanol extract of Gastrodiae Rhizoma, the dried tuber of *Gastrodia Elata*Blume (Orchidaceae) increased pentobarbital-induced sleeping behaviors. These experiments were undertaken to know whether 4-hydroxybenzaldehyde (4-HBD), is one of the major compounds of Gastrodiae Rhizoma increases pentobarbital-induced sleeping behaviors and changes sleep architectures *via* activating $GABA_A$-ergic systems in rodents. 4-HBD decreased locomotor activity in mice. 4-HBD increased total sleep time, and decreased of sleep onset by pentobarbital (28 mg/kg and 40 mg/kg). 4-HBD showed synergistic effects with muscimol (a $GABA_A$ receptor agonist), shortening sleep onset and enhancing sleep time on pentobarbital-induced sleeping behaviors. On the other hand, 4-HBD (200 mg/kg, p.o.) itself significantly inhibited the counts of sleep-wake cycles, and prolonged total sleep time and non-rapid eye movement (NREM) in rats. Moreover, 4-HBD increased intracellular Cl^- levels in the primary cultured cerebellar cells. The protein levels of glutamic acid decarboxylase (GAD) and $GABA_A$ receptors subunits were over-expressed by 4-HBD. Consequently, these results demonstrate that 4-HBD increased NREM sleep as well as sleeping behaviors *via* the activation of $GABA_A$-ergic systems in rodents.

Keywords – 4-Hydroxybenzaldehyde (4-HBD), Pentobarbital, Intracellular chloride, Glutamic acid decarboxylase (GAD), $GABA_A$ receptors subunits, Electroencephalogram (EEG)

Introduction

Insomnia is a common clinical condition characterized by difficulty in sleep onset or maintaining sleep.[1] The current pharmacological treatments for insomnia patients are primarily benzodiazepines and non-benzodiazepines by activating $GABA_A$ receptors and $GABA_A$-ergic systems. Recently, many kinds of phytomedicines have been introduced and focused on insomnia and anxiety because of modulating $GABA_A$-ergic systems.[2] Gastrodiae Rhizomahas traditionally been used to treat headaches, myocardial infarction, atherosclerosis, hyperlipidemia, hypertension, dementia, gout and epilepsy.[3] Phytochemical studies of this plant have revealed the presence of several phenolic compounds, including 4-hydroxybenzyl alcohol, 4-hyroxy-benzaldehyde (4-HBD), vanillin, vanillyl acohol, β-sitosterol and gastrodin.[4] 4-HBD is a major active constituent of Gastrodiae Rhizoma, which is an effective anxiolytic agent via $GABA_A$-ergic systems in the central nervous systems.[5] In addition, 4-HBD inhibits GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase in $GABA_A$-ergic systems.[6] In addition, N^6-(3-methoxyl-4-hydroxybenzyl) adenine riboside, which was originally isolated from *Gastrodia elata*increased GABA levels, activating GAD.[7]

Many pharmacological evidences suggest that traditional Chinese medicine interacts with $GABA_A$ receptors. This receptor, which functions as a chloride ion channel, is activated by the inhibitory neurotransmitter GABA. Thus, $GABA_A$-ergic drugs have induced sedative-hypnotic effects in animals and humans.[8] $GABA_A$ receptors complex has specific binding sites: GABA, barbiturates, benzodiazepines, ethanol, steroids and picrotoxin. Basic subunits are composed to α (1~6), β (1~3) and γ (1~3).[9] These binding sites are triggering chloride channel open with resulting membrane hyperpolarization.[10] Base on previous results, it should be very interested in whether 4-HBD, a major

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component of Gastrodiae Rhizoma also augments pentobarbital-induced sleeping behaviors and modulates sleep architectures in the rodents. The possible mechanisms were involved *via* the $GABA_A$-ergic systems.

Experimental

Reagents and chemicals – 4-HBD, n-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) and cytosine β-D-arabinofuranoside were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Muscimol, pentobarbital and diazepam were used following company respectively: Tocris (Cookson, Avonmouth, UK or Ellisville, MO, USA), Hanlim Pharm. Co., Ltd. (Seoul, Korea) and Samjin Pharm. (Seoul, Korea). Fetal bovine serum (FBS) and DMEM were obtained from GIBCO (Grand Island, NY, USA). The specific rabbit polyclonal antibodies against $GABA_A$ receptors subunits, $GAD_{65/67}$ and the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase were obtained from Abcam Inc. (Cambridge, UK). Chemiluminescent HRP substrate was purchased from Millipore Co. (Billerica, MA, USA).

Animals – The animals used for experiments were 4-weeks old ICR male mice and 8-weeks old male Sprague Dawley rats weighing 20 - 25 g and 300 - 310 g respectively (purchased from Samtako, Osan, Korea). All rodents were housed in acrylic cages (45 × 60 × 23 cm), and were kept at least 1 week for acclimation time. The room condition was maintained at 22 ± 2 ºC, relative humidity (50 - 52%), and a 12-h light/dark cycle with fed *ad libitum*. This experiment was performed in accordance with the Animal Care and Use Guidelines of Chungbuk National University, Korea.

Locomotor activity measurement – The spontaneous locomotor activity was measured by a tilting-type ambulometer (AMB-10, O'Hara, Tokyo, Japan) for 1 h.[11]. Diazepam (2 mg/kg, p.o.) and 4-HBD (50, 100 and 200 mg/kg, p.o.) dissolved in distilled water were administered 30 min and 60 min prior to the experiment, respectively.

Pentobarbital-induced sleeping behaviors measurement – All mice were fasted for a day, and all experiments were carried out between 1:00 and 5:00. Pentobarbital was diluted in 0.9% physiological saline. 4-HBD (50, 100 and 200 mg/kg) and muscimol (0.2 mg/kg) were orally administered before 60 min and 15 min, respectively, and then pentobarbital (42 mg/kg) was injected intraperitoneally. After treatment of pentobarbital, mice were moved to another cage. The sleep latency was recorded as elapse time after pentobarbital injection. The sleep was recorded as the time between the elapse and the righting of animals. The mice that failed sleep within 15 min were excluded from the experiments.[12]

EEG telemetry transmitter implantation and data collection – After administration of pentobarbital (50 mg/kg, i.p.), rats were placed on a pad in the stereotaxic apparatus under aseptic conditions. Transmitters (Data Sciences International TA11CTA-F40, MN, USA) were implanted under the skin after the scalp incision. In detail, the skull periosteum was removed, and then drilled the two holes for insert of electric lines. (A: 2.0 [Bregma], L: 1.5; P: 7.0 [Bregma], L: 1.5 contra-lateral). The lines of transmitter were connected skull and subcutaneously. Dental cement was used to fix the electric lines to the skull. The incision parts were sew up by a silk 4 - 0 suture. Antibiotic were given to all rats after surgery (5 million unit potassium penicillin-G Injection, Kunwha, KOREA). After the implantation of transmitter, rats were given recovery time for a week. 4-HBD (200 mg/kg, p.o) was administered to rats. All signals were transmitted by AD converter (Eagle PC30, USA), and stored in the computer. Results of recording graphically shown were also possible to express by the computer. The FFT analysis generated power density values to 0~20.0 Hz with a resolution of 0.5 Hz. FFT data was also issued a mean in the range of 0~20.0 Hz for every 10 sec. EEG data in all rats were recorded from 11:00 am to 5:00 pm.[13]

Data analysis – Sleep cycle was saved visually by Sleep-Sign 2.1software (KISSEI Comtec Co Ltd, Matsumoto, Japan). Data were classified into wakefulness, non-rapid eye movement (NREM) and rapid eye movement (REM) for every 10 sec.[14] Wakefulness states and NREM states were found in high frequency and slow wave respectively.-wave (0.75~4.0 Hz) and -wave (5.0~9.0 Hz, peak at 7.5 Hz) increased in low waves of EEG during REM sleep. Counts of wakefulness, NREM, REM and total sleep time (NREM+REM) were recorded to each rat for 6 h. The EEG power was set up 0.5~20.0 Hz in 0.5 Hz bins. Sleep architecture was evaluated in the three waves range of, and (8.0~13.0 Hz).[15] Data was calculated as relative value by the Microsoft Excel.

Cell culture – Primary cultures of cerebellar enriched in granule cells were experimented by 7 - 8 days rats.[16] Cerebellar granule cells were seeded amounts of 1.0×10^5 cells in per 96-well dishes coated with poly-L-lysine (50 μg/mL; Sigma, St. Louis, MO, USA). DMEM used for cell cultures contained 10% fetal bovine serum, glutamine (2.0 mM), gentamicin (100 μg/mL), antibiotic-antimycotic solution (10 μg/mL; Sigma) and potassium chloride (25 mM). Cells were incubated at 37 ºC in a humidified atmosphere of 5% CO_2/95% air.After 16 h for cell cultures,

96-well plates were added into cytosine arabinofuranoside (final concentration 10 μM; Sigma) for inhibit of non-neuronal cells growth.

Measurement of intracellular Cl^- influx – MQAE (a sensitive fluorescent substance for Cl^-) was used for measurement of Cl^- influx of cerebellum cells of rats, and it was based on the method of West and Molloy.[17] After treatment of MQAE for overnight, the cells were washed three times in the buffer (pH 7.4) which contained 2.4 mM HPO_4^{2-}, 0.6 mM $H_2PO_4^-$, 10 mM HEPES, 10 mM D-glucose and 1 mM $MgSO_4$. The fluorescence data was measured according to excitation wavelength 320 nm and emission wavelength 460 nm by Elisa Reader (SpectraMax M2e Multi-Mode, PA, USA).[18] The data was calculated by F/F_0 on the basis of the ratio of Cl^- data. F is the fluorescence of each sample, and F_0 is the fluorescence without Cl^- ions.

Western blottings – Protein samples were extracted from the cell cultures of the rat hypothalamus. 4-HBD (final concentration, 10 and 100 g/ml) was dissolve in 0.01% DMSO. The control sample was treated in the same solvent as that used in the 4-HBD treatment. Cell cultures were replaced with fresh medium periodically. After treatment of pentobarbital or 4-HBD, cells were harvested, and treated with cold lysis buffer [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100, 1 mM PMSF, 10 μl/mL aprotinin, 1 mM NaF and 2 mM sodium ortho-vanadate]. Extracts were recovered supernatant after centrifugation at 13,000 × g at 4 ºC for 20 minutes. Protein concentration was measured using the Bradford protein analysis and stored at –20 ºC.[19] The same amounts of protein put in 10% SDS-polyacrylamide gel, and then was loaded the electrophoresis. The protein was transferred to PVDF membranes (Hybond-P, GE Healthcare, Amersham, UK) using semidry transfer system. The blots were blocked for 1 h at room temperature with 5.0% (w/v) BSA [applied to all primary antibodies except for glyceraldehyde 3-phosphate dehydrogenase (GAPDH)], and 5.0% (w/v) skim milk (only applied to GAPDH) in tris-buffered saline solution (TBS) containing 0.1% Tween-20. Both specific rabbit polyclonal antibodies against $GABA_A$ receptors subunits and rabbit anti-$GAD_{65/67}$ polyclonal antibody at the appropriate dilution in TBST and 5.0% BSA (1:2,500 for all the primary antibodies used) were incubated for overnight at 4 ºC. After washing with TBST, the blots were treated 1: 3,000 dilution of a secondary antibody at room temperature for 4 hours (goat anti-rabbit, IgG). A chemical for detecting the secondary antibody was performed by using the light emitting substrate is the ECL system (Roche Diagnostics, Mannheim, Germany).

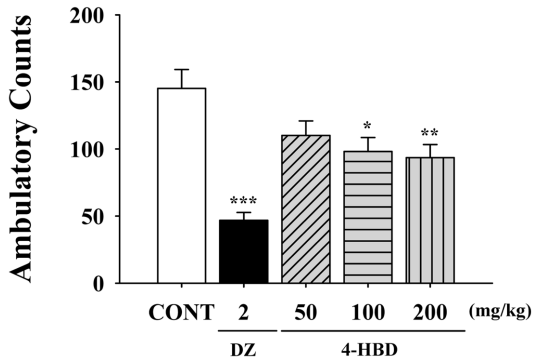

Fig. 1. Effects of 4-HBD on locomotor activity test. Ambulation activity was measured for 1 h, 30 min after oral administration of diazepam and 1 h after the administration of 4-HBD. Each column represents the mean with S.E.M (n = 10). The significance of the effects of the compounds was assessed using analysis of variance (ANOVA) followed by Holm-sidak test. *P < 0.05, **P < 0.01, ***P < 0.001, compared to the naïve control.

Statistical analysis – All statistical analyses were calculated using the Sigma Stat software (SPSS Inc., Chicago, IL, USA).The average for experiments data was calculated as mean ± S.E.M. Statistical significances were compared with control group by Holm-sidak test. All experiments were repeated at least three times for obtain comparable results. $P < 0.05$ was judged as the value of the significance.

Results

Effects of 4-HBD on locomotor activity in mice – 4-HBD (100 and 200 mg/kg) significantly decreased locomotor activity. Diazepam 2 mg/kg, as a positive control also decreased locomotor activity (Fig. 1) From these preliminary experiments, we suggest that 4-HBD might be sedative.

Effects of 4-HBD on pentobarbital-induced sleeping behaviors in mice – 4-HBD (50, 100 and 200 mg/kg) significantly reduced sleep latency time. 4-HBD (200 mg/kg, i.p) and muscimol (0.2 mg/kg, i.p) significantly enhanced pentobarbital (42 mg/kg, i.p)-induced sleeping time in mice (Fig. 2). We suggest that 4HBD could reduce sleep latency, and increase total sleep. Similar results were obtained by diazepam.

Effects of 4-HBD on sleep onset by sub-hypnotic dosage of pentobarbital in mice – 4-HBD (100 and 200 mg/kg) increased the number of sleeping mice, and total sleep time in sub-hypnotic dose (28 mg/kg) of pentobarbital. Muscimol (a GABA agonist), as a positive control also the number of sleeping mice and prolonged

Table 1. Effects of 4-HBD on sleep onset of mice treated by sub-hypnotic dose of pentobarbital (28 mg/kg, i.p).

Group	Dose (mg/kg)	No. falling asleep/total	Sleep time (min)
Control	0	6/12	23.8 ± 1.6
Muscimol	0.2	11/12***	51.3 ± 5.9***
4-HBD	50	7/12	24.1 ± 4.5
	100	8/12***	37.7 ± 2.1***
	200	10/14***	39.8 ± 5.8*

Each value represents the mean ± S.E.M. (n = 12 - 14). *$p < 0.05$ and ***$p < 0.005$ compared to control (Chi-square test and ANOVA followed by Holm-sidak test).

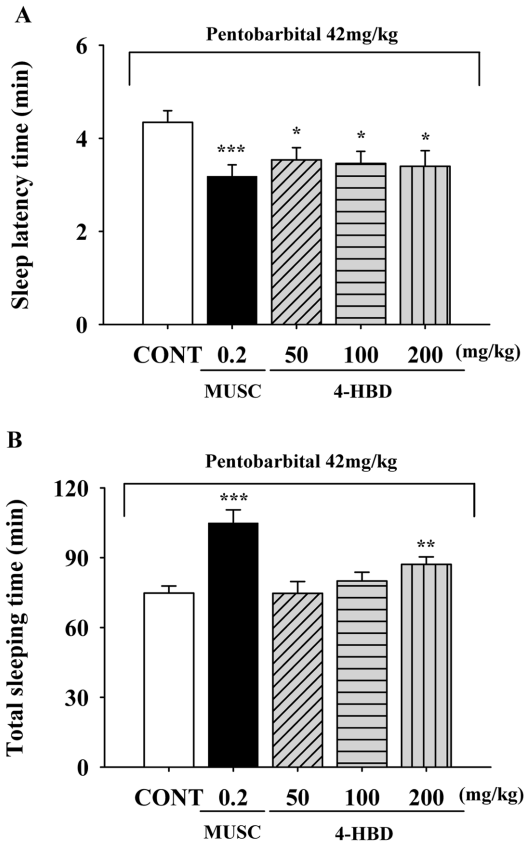

Fig. 2. Effects of 4-HBD on onset and duration of sleep in pentobarbital-treated mice. Mice were food deprived for 24 h prior to the experiment. Pentobarbital (42 mg/kg, i.p) was injected to mice following injection of muscimol or 4-HBD. The sleep latency time (A) and total sleeping time (B) were recorded. Each column represents the mean with S.E.M (n = 12 - 14). The significance of the effects of the compounds was assessed using analysis of variance (ANOVA). Where there was significant variability, the individual values were compared using Holm-sidak test. $^{*}P < 0.05$, $^{*}P < 0.01$, $^{***}P < 0.005$, compared with that of the control.

the sleep time (Table 1). We suggest that 4-HBD would interact with $GABA_A$ receptors.

Effects of 4-HBD on sleep-wake cycles – 4-HBD (200 mg/kg) significantly reduced the sleep-wake cycles (Fig. 3). It means that wakefulness can be reduced by 4HBD.

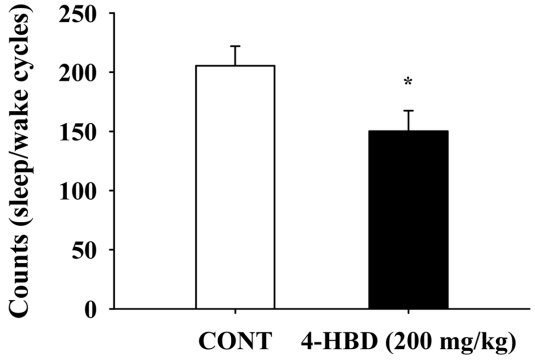

Fig. 3. Effects of 4-HBD on counts of sleep-wake cycles. Where there was significant variability, the individual values were calculated as mean with S.E.M (n = 8) were compared using Holm-sidak test. $^{*}P < 0.05$, compared with that of the control.

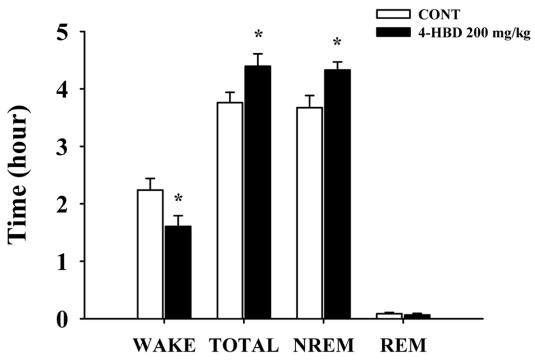

Fig. 4. Effects of 4-HBD on rat sleep architecture. The data represent the mean with S.E.M (n = 8) of time spent, which separated the wakefulness/sleep (NREM and REM sleep) state. The significance of the effects of the compounds was assessed using analysis of variance (ANOVA). Where there was significant variability, the individual values were compared using Holm-sidak test. $^{*}P < 0.05$, compared with that of the naïve control.

Effects of 4-HBD on sleep architectures – After EEG analysis, we found that 4-HBD (200 mg/kg) significantly prolonged total sleep time and especially NREM (slow wave sleep) sleep. 4HBD also decreased wakefulness (Fig. 4).

Effects of 4-HBD on EEG power density – 4-HBD increaseddelta waves in REM sleep (Fig. 5B). However,

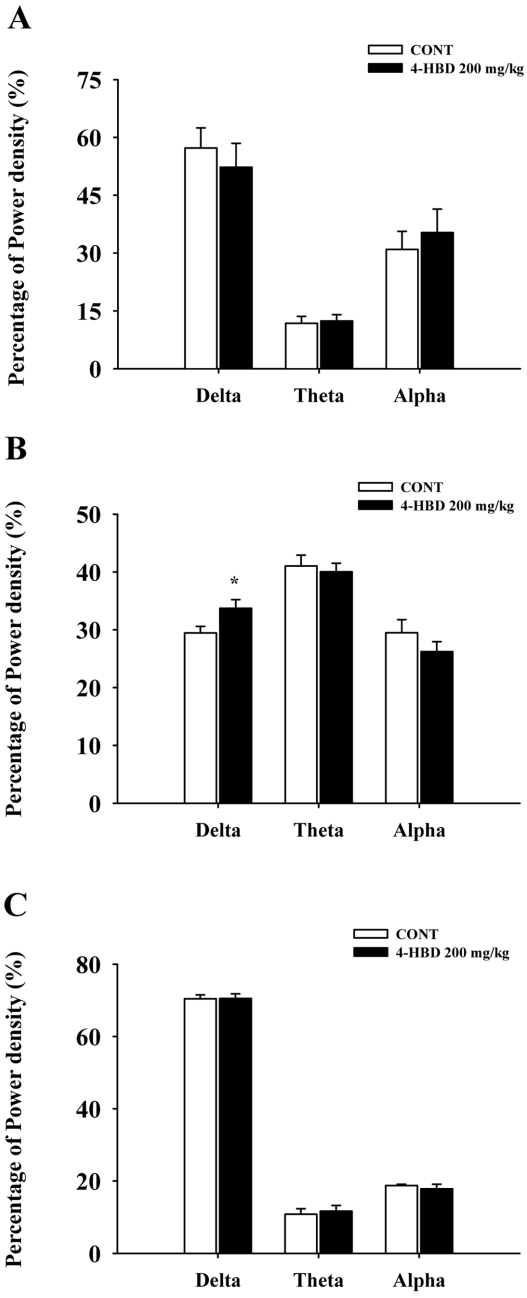

Fig. 5. Effects of 4-HBD on EEG power densities of wakefulness (A), REM sleep (B) and NREM sleep (C). Power densities were departmentalized into δ-wave, θ-wave and α-wave. Each wave represents the mean with S.E.M (n = 8). The significance of the effects of the compounds was assessed using analysis of variance (ANOVA). Where there was significant variability, the individual values were compared using Holm-sidak test. $^{*}P < 0.05$, compared with that of the naïve control.

waves in NREM sleep and wakefulness showed no changes by 4-HBD (Fig. 5A, 5C). So, 4-HBD increases slow waves sleep during REM sleep.

Effects of 4-HBD on intracellular Cl^- influx in primary cultured cerebellar granule cells – 4-HBD (1.0 μM) increased intracellular Cl^- influx, resulting hyperpolarization of neuronal cell membrane. Pentobarbital (10 μM) also significantly increased intracellular Cl^- influx in primary cultured granule cells (Fig. 6).

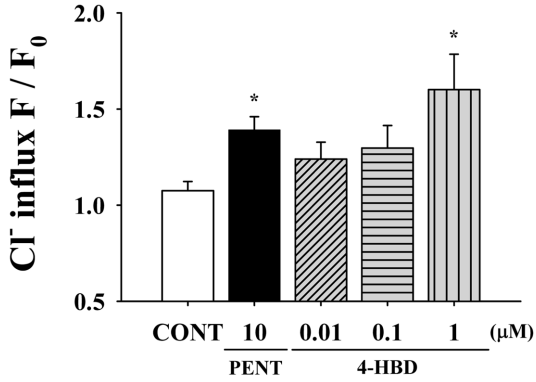

Fig. 6. Effects of 4-HBD on Cl^- influx in primary cultured cerebellar granule cells. After the culture of cerebellar granule cells for 8 days, the cells were incubated with MQAE overnight, and then 4-HBD (0.01, 0.1 and 1 μM) and pentobarbital (PENT 10 μM) were added 1 h prior to measurement. Each column represents the mean with S.E.M (n = 3). The significance of the effects of the compounds was assessed using analysis of variance (ANOVA). Where there was significant variability, the individual values were compared using Holm-sidak test. $^{*}P < 0.05$, compared with that of the control.

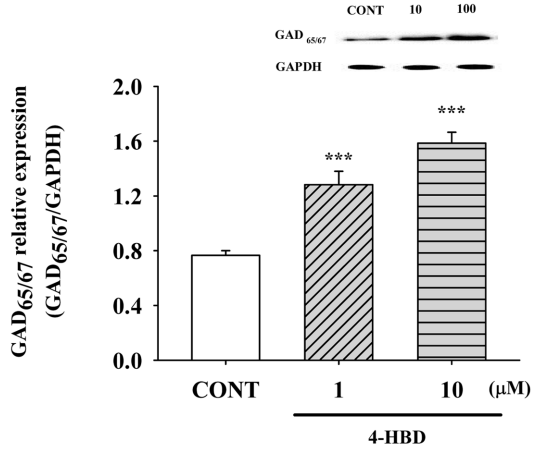

Fig. 7. Effects of 4-HBD on the expression of GAD. Immunoblots of lysed hypothalamic neuronal cells which were treated for 1 h following 4-HBD are shown. GAPDH levels were need for the normalization of the protein expression. Each column represents the mean with S.E.M (n = 3). The significance of the effects of the compounds was assessed using analysis of variance (ANOVA). Where there was significant variability, the individual values were compared using Holm-sidak test. $^{***}P < 0.005$, compared with that of the control.

Effects of 4-HBD on expression of $GAD_{65/67}$ – The expression of $GAD_{65/67}$ was induced by 4-HBD (1.0 and 10 μM) in the primary hypothalamic neuron cells of rats (Fig. 7). We suggest that 4-HBD activates $GAD_{65/67}$.

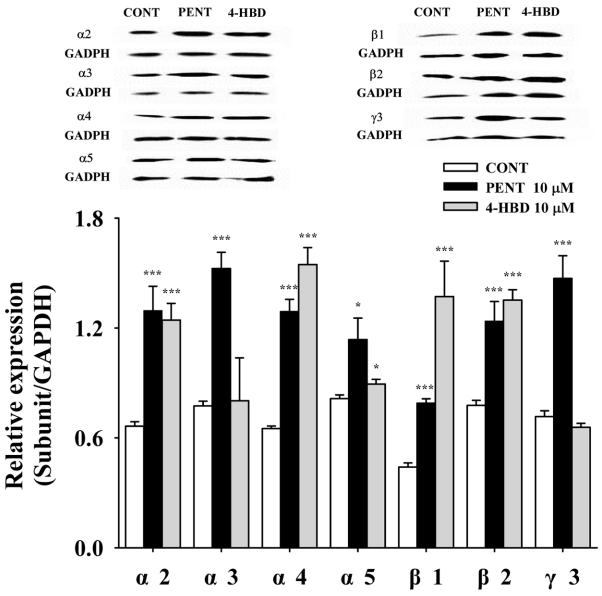

Fig. 8. Effects of 4-HBD on expression of $GABA_A$ receptors subunits. Immunoblots of lysed hypothalamic neuronal cells which were treated for 1 h following 4-HBD are shown. GAPDH levels were need for the normalization of the protein expression. Each column represents the mean with S.E.M (n = 3). The significance of the effects of the compounds was assessed using analysis of variance (ANOVA). Where there was significant variability, the individual values were compared using Holm-sidak test. $^{*}P < 0.05$, $^{***}P < 0.005$, compared with that of the control.

Effects of 4-HBD on expression of $GABA_A$ receptors subunits – From these experiments, the activations of $GABA_A$ receptors subtypes were measured by western blotting. All subtypes of $GABA_A$ receptors except γ subtype were overexpressed by 4-HBD (10 μM). Pento-barbital, as a positive control also showed similar patterns (Fig. 8).

Discussion

The sedative/hypnotic effects of Gastrodiae Rhizoma have been shown in human and animal models.[20] Recent study has reported that N(6)-(3-methoxyl-4-hydroxybenzyl) adenine from Gastrodiae Rhizoma increases $GABA_A$-ergic systems, such as activating GAD enzyme, increasing GABA levels and overexpressing GABA receptors.[7,21] So, it was demonstrated that Gastrodiae Rhizoma would be useful for the treatment of insomnia.[21] It has been evidenced which 4-HBD, an active compound of Gastrodiae Rhizomais neuroprotective.[21] Based on previous studies, we focused on the hypnotic effect with 4-HBD, as an ultimate goal of the experiment. We first found that the locomotor activity was inhibited by 4-HBD. 4-HBD also increased the number of sleep animals and total sleep in sub-hypnotic (28 mg/kg) pentobarbital-induced sleeping behaviors, and shortened sleep onset and increased total sleep in hypnotic (42 mg/kg) pentobarbital-induced sleep. Moreover, 4-HBD decreased sleep/wake cycles by the measurement of EEG. 4-HBD also potentiated pentobar-bital-induced sleep behaviors in combination with muscimol (a GABA agonist). Muscimol and other $GABA_A$ agonists cause potentiation of Cl^- influx when administered with pentobarbital or other $GABA_A$ agonists. We presumed that 4-HBD enhances the hypnotic effects via $GABA_A$-ergic systems in the CNS. Furthermore, the sleep architectures by EEG after oral administration of 4-HBD were analyzed in rats. 4-HBD increased total sleep and NREM sleep. Especially, delta waves in REM sleep were enhanced. The previous research demonstrated that prolongation of REM and NREM sleep as well as special increase of slow waves in NREM plays important roles in the treatment of insomnia and sleep. It has been well known that $GABA_A$ receptors agonists, such as barbiturates and benzodiazepine decrease wakefulness, and increase REM and NREM.[22] Slow waves of NREM plays an important role in sleep quality.[23] δ-Waveswere associated in REM sleep.

To define $GABA_A$-ergic mechanisms, intracellular chloride influx, and GAD and $GABA_A$ receptors activations were measured in the primary cultured cells. Intracellular influx of Cl^- was increased by 4-HBD, causing hyper-polarization of the neuronal membrane and reducing neuronal firing.[24] In addition, GAD is the enzyme for decarboxylase, and plays an important role for GABA synthesis from glutamate in the $GABA_A$-ergic systems.[25] High expression of $GAD_{65/67}$ protein levels by 4-HBD was found in the hypothalamic cell of rats. The expressions $GABA_A$ receptors subunits in primary cultured hypothalamic neuronal cells were also measured. All subunits except γ3, α and β sub-units of $GABA_A$ receptors were overex-pressed. The most abundant $GABA_A$ receptors submits composition, $\alpha_1\beta_2\gamma_2$ is present in most brain regions, and these submits are related to the hypnotic/sedative effects of $GABA_A$ receptors. 4-HBD induced of α- and β-subunitsprotein over-expressions of $GABA_A$ receptors. However, pentobarbital a positive control, induced all the αβγ subtypes in these experiments. From these experiments, 4-HBD enhanced the sedative-hypnotic effect in pento-barbital-induced sleep. 4-HBD, itself decreased sleep-wake cycles, and increased NREM sleep. 4-HBD activated $GABA_A$ receptors chloride channel complex, increasing intracellular chloride, activating GAD and GABAA receptors subtypes. It is concluded that 4-HBD, one of component of Gastrodiae Rhizoma would be useful for

the treatment of insomnia.

Acknowledgements

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