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RESEARCH ARTICLE

Hypnotic Effect of GABA from Rice Germ and/or Tryptophan in a Mouse Model of Pentothal-induced Sleep

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Abstract γ-Aminobutyric acid (GABA), a primary inhibitory neurotransmitter, and tryptophan (Trp), a substrate for melatonin, are found in functional foods and exert hypnotic effects. The hypnotic effects of 3 doses of GABA and a combined-preparation of GABA and Trp (GABA+Trp) were investigated in mice. Hypnotic activity was evaluated using pentothal-induced sleep time testing. Treatments included low, middle, and high doses of GABA and GABA+Trp. Low doses of GABA (low-GABA) and low-GABA+Trp reduced sleep latency and significantly (p<0.05) prolonged the sleep time induced by pentothal, compared with controls, although the melatonin concentration in the serum was not affected. On the other hand, the adenosine A_1 receptor (AA1R) immunoreactivity in the suprachiasmatic nucleus of the hypothalamus was significantly (p<0.05) increased after administration of low-GABA and/or low-GABA+Trp, compared to controls. Low doses of GABA and/or Trp cause hypnotic effects that may be related to AA1R activation.

Keywords: γ-aminobutyric acid, tryptophan, melatonin, adenosine receptor, suprachiasmatic nuclei

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Introduction

γ-Aminobutyric acid (GABA), a primary inhibitory neurotransmitter, plays an important role in regulating neuronal excitability in the mammalian central nervous system (1-4). Lower amounts of GABA are found in grains and rice germ (5). Recently, interest in functional foods containing GABA has increased because GABA exerts diverse physiological functions, such as relaxation, anti-anxiety, mood-improving, and hypotensive effects (6-8).

Tryptophan (Trp) is an essential amino acid that can be converted, via metabolic pathways, to serotonin (a neurotransmitter) and melatonin (an end product of Trp metabolism), both of which are thought to be related to a stable mood and sleep (9-13). Dietary Trp can enhance the rate of synthesis and the function of serotonin and melatonin in the brain, and L-Trp has been widely used for effective treatment of acute insomnia and depression (14,15). In addition, melatonin is involved in the sleep-promoting actions of the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (16).

Insomnia is a common sleep disorder. Approximately 10-15% of the adult population suffers from chronic insomnia, and an additional 25-35% suffer transient or occasional insomnia (17). Natural sleep aids are widely used as alternatives to prescription drugs to improve the sleep quality and to avoid side-effects, including impaired cognitive function, tolerance, and dependence (18,19).

Herbal drugs as natural sleep aids have been gradually accepted worldwide due to safety and efficiency (20). More effective substitutes for prescription drugs with fewer side effects are needed. In this study, therefore, GABA was investigated and a combined-preparation of GABA and Trp showed a hypnotic effect as a functional natural sleep aid using pentothal-induced sleeping time testing in mice.

Materials and Methods

Experimental animals Adult male ICR mice weighing 25-30 g were used. Animals were obtained from the Experimental Animal Center of Hallym University, Chuncheon, Korea, and were housed in a conventional state under adequate temperature (23ºC) and humidity (60%) controls with a 12 h light/12 h dark cycle with light onset at 07:00 a.m. Mice were provided with free access to water and food. To ensure adaptation to a new environment, mice were kept in the departmental holding room for 1 week before experiments. Procedures involving animals and care conformed to university guidelines in compliance with current international laws and policies following the NIH Guide for the Care and Use of Laboratory Animals, NIH publication No. 85-23, 1985, revised 1996.

Materials Rice gem ferment extract powder was provided by Biovan Co., Ltd. (Chuncheon, Korea) in March of 2013. The content of GABA was about 15% (w/w). L-Trp was purchased from Sigma-Aldrich (T4196; Sigma-Aldrich Korea Ltd., Yongin, Korea).

Treatments with GABA and GABA+Trp Animals were divided into the 7 groups (n=12 in each group) of 1) a vehicle-treated group (vehicle-group); GABA treated groups of 2) low-GABA (22 mg/kg), 3) middle-GABA, (44 mg/kg), and 4) high-GABA (66 mg/kg); GABA+Trp treated groups of 5) low-GABA+Trp, (22+8 mg/kg), 6) middle-GABA+Trp (44+16 mg/kg), and 7) high-GABA+Trp (66+25 mg/kg). Each test sample was dissolved in saline (Junsei Chemical Co., Ltd., Tokyo, Japan) before use and orally administered using a feeding needle once a day between 1:00 p.m. to 2:00 p.m.

Pentothal-induced sleeping time testing All measurements were performed under blind conditions by 2 observers for each experiment in order to ensure objectivity. All experiments were conducted from 1:00 p.m. to 5:00 p.m. Animals were fasted for 24 h prior to experiments. A total of 30 min after oral administration of test samples, a 40 mg/kg hypnotic dose of pentothal (JW Pharm. Co., Ltd., Seoul, Korea) was administered with intraperitoneal injection to each mouse. Animals were observed during the latent period, defined as the time between pentothal administration to loss of the righting reflex, and the duration of sleeping time, defined as the time between the loss and recovery of the righting reflex. Animals that failed to fall asleep within 15 min after pentothal administration were excluded from experiments (21,22).

Assay of melatonin in the serum After pentothal-induced sleeping time testing, animals (n=12 in each group) were anesthetized with intraperitoneal injection of 1 g/kg urethane (Sigma-Aldrich Co., St. Louis, MO, USA), and blood was collected via cardiac puncture. The ELISA procedure was used for quantitative measurement of melatonin in the serum using a commercial kit from Immuno Biological Laboratories (IBL, Hamburg, Germany) with modification. Briefly, this assay is based on the competition principle and microtiter plate separation. First, each sample was passed through a C18 reversed phase column (IBL), extracted using methanol, evaporated to dryness, and reconstituted using distilled water. Then, each sample was added to a well coated with the antibody goat anti-rabbit Ig of a microtiter plate (Sigma-Aldrich Co.). An unknown amount of antigen present in the sample and a fixed amount of enzyme-labeled antigen competed for the binding sites of the antibodies coating the wells. Then, 50 μL of melatonin biotin and 50 μL of rabbit-antiserum were added into each well, shaken carefully, sealed with adhesive foil and incubated overnight (14-20 h) at 2-8ºC. After washing three times with 250 μL diluted washing buffer, 150 μL of anti-biotin conjugated alkaline phosphatase was added into each well and incubated 2 h at room temperature. The reaction was developed using *p*-nitrophenyl phosphate (Thermo Scientific, Rockford, IL, USA) and optical densities were determined at 405 nm in an automatic microplate reader. Melatonin standards were used to construct a calibration curve against which unknown samples were compared. The sensitivity of the melatonin assay was 3.0 pg/mL.

Immunohistochemistry After blood collection, animals (n=7 in each group) were perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4; Sigma-Aldrich Co.) followed by 4% paraformaldehyde (Samchun Chemicals, Pyeongtaek, Korea) in 0.1 M PBS (pH 7.4; Sigma-Aldrich Co). The brain was removed and brain tissue was post-fixed using the same solution for 6 h. Brain tissue was cryoprotected using infiltration with 30% sucrose (Junsei Chemical Co., Ltd.) overnight. Brain tissues were then frozen and sectioned at 30 μm using a cryostat (Leica, Wetzlar, Germany). Consecutive sections were collected in 6-well plates containing 0.1 M PBS (pH 7.4; Sigma-Aldrich Co).

To examine GABA and/or Trp-related changes in the

adenosine A1 receptor (AA1R) immunoreactivity in the mouse SCN, immunohistochemical staining was performed according to a previous report (23). Rabbit anti-AA1R (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a primary antibody. A negative control test was carried out using pre-immune serum (Vector, Burlingame, CA, USA) instead of the primary antibody in order to establish the specificity of immunostaining. The negative control resulted in the absence of immunoreactivity in all structures.

A total of 7 sections per animal were used to quantitatively analyze AA1R immunoreactivity, which was graded. Digital SCN images were captured using an AxioM1 light microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Axiocam digital camera (Carl Zeiss) connected to a PC monitor. Semi-quantification of the immunostaining intensity of AA1R immunoreactivity was evaluated using digital image analysis software (Optimas 6.5; CyberMetrics, Scottsdale, AZ, USA). The mean intensity of AA1R immunostaining in each immunoreactive structure was evaluated on the basis of a optical density (OD), which was obtained after the transformation of the mean gray level using the formula: OD=log (256/mean gray level). The OD of background was taken from areas adjacent to the measured area. After the background density was substrated, a ratio of the optical density of image file was calibrated as % (relative optical density, ROD) using Adobe Photoshop version 8.0 and then analyzed using NIH Image 1.59 software (NIH, Bethesda, MD, USA).

Western blot analysis To confirm changes in AA1R levels in the mouse SCN, animals (n=5 in each group) were used for Western blot analysis after blood collection. In brief, tissues were homogenized in 50 mM PBS (pH 7.4; Sigma-Aldrich Co.) containing EGTA (pH 8.0; Santa Cruz Biotechnology), 0.2% NP-40 (Sigma-Aldrich Co.), 10 mM EDTA (pH 8.0, Sigma-Aldrich Co.), 15 mM sodium pyrophosphate (Santa Cruz Biotechnology), 100 mM β-glycerophosphate (Santa Cruz Biotechnology), 50 mM NaF (Santa Cruz Biotechnology), 150 mM NaCl (Santa Cruz Biotechnology), 2 mM sodium orthovanadate (Santa Cruz Biotechnology), 1 mM PMSF (Santa Cruz Biotechnology), and 1 mM DTT (Santa Cruz Biotechnology). After centrifugation at 16,000×g in a microcentrifuge for 5 min, the protein level in the supernatants was determined using a Micro BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). Aliquots containing 20 μg of total protein were boiled in a loading buffer containing 150 mM Tris (pH 6.8; Santa Cruz Biotechnology), 3 mM DTT (Santa Cruz Biotechnology), 6% SDS (Santa Cruz Biotechnology), 0.3% bromophenol blue (Santa Cruz Biotechnology), and 30% glycerol (Junsei Chemical Co., Ltd.). Aliquots were then loaded onto polyacrylamide gel. After electrophoresis (Bio-Rad, Hercules, CA, USA), gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY, USA) and incubated with 5% non-fat dry milk (Santa Cruz Biotechnology) in PBS (Sigma-Aldrich Co.) containing 0.1% Tween 20 (Santa Cruz Biotechnology), followed by incubation with each primary antibody and peroxidase-conjugated donkey anti-rabbit IgG (Sigma-Aldrich Co.) using an ECL kit (Pierce Chemical). The result of Western blot analysis was scanned, and densitometric analysis for the quantification of the bands was done using Scion Image software (Scion Corp., Frederick, MD, USA), which was used to count relative optical density (ROD): A ratio of the ROD was calibrated as %, with vehicle-treated group designated as 100 %.

Statistical analysis Data were expressed as a mean± standard error of the mean (SEM). Data were evaluated using a one-way analysis of variance (ANOVA) with SPSS software (IBM Corporation, Armonk, NY), and differences between means were analyzed using Duncan's multiple range test. Statistical significance was considered at $p<0.05$.

Results and Discussion

Effects of GABA and/or Trp on sleep latency and sleeping time In the vehicle-treated group, the sleep latency was 3.8±0.4 min. Administration of GABA decreased the latency of sleep in all the groups (3.2±0.2 min in the low-GABA-treated group, 3.4±0.3 min in the middle-GABA-treated group, and 3.0±0.2 min in the high-GABA-treated group); however, there were no significant ($p>0.05$) differences between the latency of sleep for all the treatment groups and the vehicle-treated group (Fig. 1). In the vehicle-treated group, the sleeping time was 70.1±9.5 min. The duration of sleep time in the low-GABA-treated group was 107.9±8.8 min, and this group showed a significant ($p<0.05$) hypnotic activity, compared with the vehicle-treated group. However, the sleeping times in the middle-GABA and high-GABA-treated groups were 81.0±9.2 min and 78.5±11.0 min, respectively, with no significant ($p>0.05$) differences, compared with the vehicle-treated group (Fig. 1).

The administration of low-GABA+Trp significantly ($p<0.05$) decreased the sleep latency (2.7±0.3 min), compared with the vehicle-treated group. However, administration of middle and high-GABA+Trp did not significantly ($p>0.05$) affect sleep latency (3.5±0.4 and 3.6±0.4 min, respectively) (Fig. 2), compared with the vehicle-treated group. In addition, administration of low-GABA+Trp showed a significant ($p<0.05$) increase in the sleeping time (125.6±8.7 min), compared with the vehicle-treated group. However,

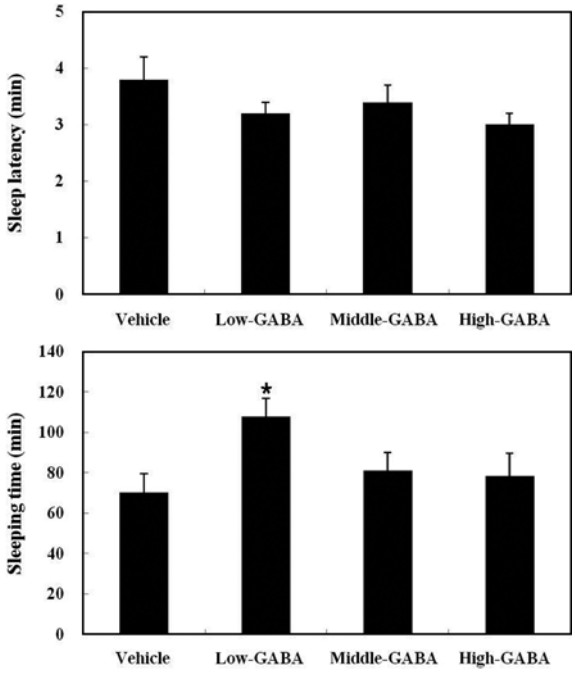

Fig. 1. Effects of GABA on sleep latency and sleeping time in pentothal-treated mice (*n*=7 per group; **p*<0.05 versus the vehicle-treated group). Data are presented as means±SEM.

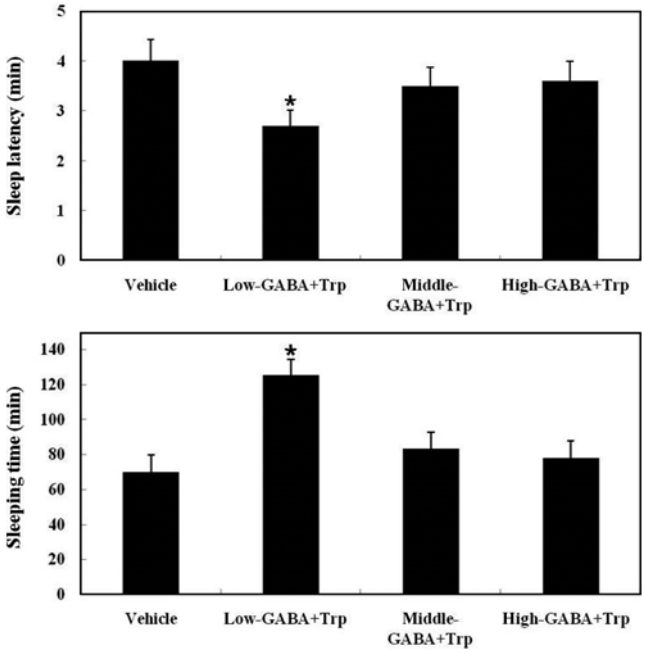

Fig. 2. Effects of combined-preparations of GABA and Trp on sleep latency and sleeping time in pentothal-treated mice (*n*=7 per group; **p*<0.05 versus the vehicle-treated group). Data are presented as means±SEM.

administration of middle and high-GABA+Trp did not significantly ($p>0.05$) change the sleeping time, compared with the vehicle-treated group (83.5±9.0 and 77.9±10.0 min, respectively) (Fig. 2).

It has been reported that activation of $GABA_A$, $GABA_B$, and $GABA_C$ receptors mediates the inhibitory process as a major mechanism for sleep (24). Most conventional pharmacological treatments for insomnia are $GABA_A$ receptor agonists, such as benzodiazepines/non-benzodiazepines (25,26). Low-GABA may exert hypnotic effects by hyperpolarization of the CNS via GABA receptors. On the other hand, the pentothal-induced sleeping latency and sleeping time were not affected in this study by administration of middle and high-GABA, or by combined preparations of middle and high-GABA+Trp. Although the effects of Trp alone were not examined in this study, middle and high-Trp treatments probably did not affect the pentothal-induced sleeping latency and sleeping time because the pentothal-induced sleeping latency and sleeping time were not affected by administrations of middle and high-GABA doses, or by the combined preparations of middle and high-GABA+Trp.

Melatonin concentration in the serum The level of melatonin was 77.8±4.9 pg/mL in the serum of the vehicle-treated group. Administration of GABA increased the melatonin concentration in the serum in all the groups (98.9±5.8 pg/mL in the low-GABA-treated group, 93.3±8.2 pg/mL in the middle-GABA-treated group, and 94.5±5.6 pg/mL in the high-GABA-treated group); however, there were no statistically significant ($p>0.05$) differences in the melatonin concentrations in the serum between all GABA administered groups and the vehicle-treated group. (Fig. 3). A similar profile of melatonin concentration was observed in all the GABA+Trp-treated groups. The levels of melatonin in the low, middle, and high-GABA+Trp-treated groups were slightly increased, compared with the vehicle-treated group; however, there was no statistically significant ($p>0.05$) differences in the melatonin concentrations in the serum between all GABA+Trp-treated groups and the vehicle-treated group (100.4±8.1, 95.2±7.2, and 96.1±6.2 pg/mL, respectively) (Fig. 3).

These results were consistent with a previous study (15) where it was reported that although Trp intake enhanced the rate of melatonin synthesis, Trp intake did not drastically increase melatonin levels in the brain because the Trp content is balanced out by other amino acids, proportionally reducing the amount of Trp entry into the brain (27).

AA1R immunoreactivity and protein levels AA1R-immunoreactive cells were easily observed in the SCN in the vehicle-treated group, and AA1R immunoreactivity was identified in the cytoplasm of the SCN (Fig. 4A). In the low-GABA-treated group, the expression pattern of AA1R immunoreactivity was similar to the pattern in the

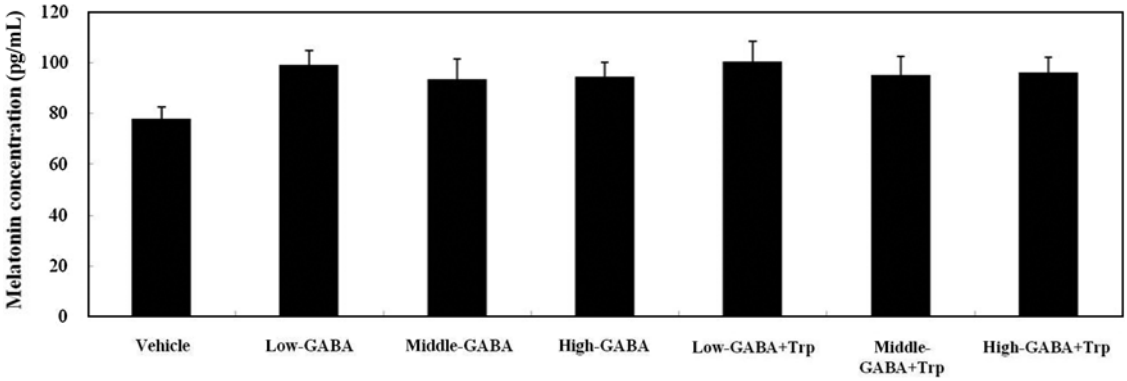

Fig. 3. Comparison of the melatonin concentration in the serum of mice after different oral administration treatments with GABA and/or Trp (*n*=12 per group). Data are presented as means±SEM.

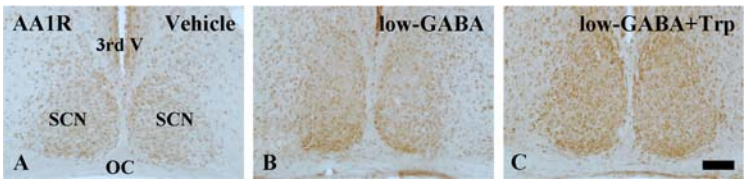

Fig. 4. AA1R immunohistochemistry in the SCN of the hypothalamus in the vehicle-(A), low-GABA-(B), and low-GABA+Trp-(C) treated groups. AA1R immunoreactivity was increased in the low-GABA and low-GABA+Trp-treated groups. 3rd V, 3rd ventricle; SCN, suprachiasmatic nuclei; OC, optic chiasm; scale bar, 200 μm

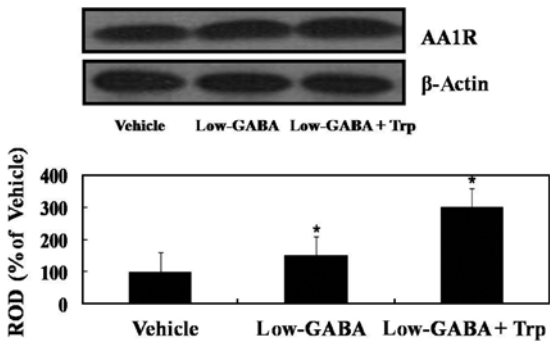

Fig. 5. Western blot analysis of AA1R levels in the SCN of hypothalamus. The relative optical density (ROD) of immunoblot bands is shown as percent values (*n*=5 per group; **p*<0.05 versus the vehicle-treated group). Data are presented as means±SEM.

vehicle-treated group, and the immunoreactivity and the protein level were significantly ($p<0.05$) increased in the SCN, compared with the vehicle-treated group (Fig. 4B, 5). Similarly, AA1A immunoreactivity and the protein level in the low-GABA+Trp-treated group were significantly ($p<0.05$) increased, compared with the vehicle-treated group and with the low-GABA-treated group (Fig. 4C, 5).

Adenosine shows inhibitory effects due to hyperpolarization of neurons via AA1R and is considered to be an important endogenous and homeostatic sleep factor (28,29). It was reported that adenosine exerted sleep-promoting effects in the hypothalamus by inhibiting hypocretin/orexin neurons through AA1R (30). In addition, it was reported that AA1R interacted with other types of G-protein coupled receptors, such as the $GABA_B$ receptor, and that supra-additive synergistic interactions occurred at sub-saturating concentrations of AA1R and $GABA_B$ agonists (31,32). Based on these results, the hypnotic effects of low-GABA and/or Trp might be related to actions on AA1A, which might be highly interactive with the GABA receptor in the SCN of the hypothalamus. A low dose of Trp might exert a synergistic effect.

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Disclosure The authors declare no conflict of interest.

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