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# Administration of Alpha$_{s1}$-Casein Hydrolysate Increases Sleep and Modulates GABA$_A$ Receptor Subunit Expression

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## Abstract

Sleep is the most basic and essential physiological requirement for mental health, and sleep disorders pose potential risks of metabolic and neurodegenerative diseases. Tryptic hydrolysate of $\alpha_{S1}$-casein ($\alpha_{S1}$-CH) has been shown to possess stress relieving and sleep promoting effects. However, the differential effects of $\alpha_{S1}$-CH on electroencephalographic wave patterns and its effects on the protein levels of $\gamma$-aminobutyric acid A (GABA$_A$) receptor subtypes in hypothalamic neurons are not well understood. We found $\alpha_{S1}$-CH (120, 240 mg/kg) increased sleep duration in mice and reduced sleep-wake cycle numbers in rats. While $\alpha_{S1}$-CH (300 mg/kg) increased total sleeping time in rats, it significantly decreased wakefulness. In addition, electroencephalographic theta ($\theta$) power densities were increased whereas alpha ($\alpha$) power densities were decreased by $\alpha_{S1}$-CH (300 mg/kg) during sleep-wake cycles. Furthermore, protein expressions of GABA$_A$ receptor $\beta_1$ subtypes were elevated in rat hypothalamus by $\alpha_{S1}$-CH. These results suggest $\alpha_{S1}$-CH, through GABA$_A$ receptor modulation, might be useful for treating sleep disorders.

**Key Words:** Sleep, $\alpha_{S1}$-CH, Electroencephalogram, GABA$_A$ receptor

## INTRODUCTION

Sleep is the most basic and essential physiological requirement for maintaining health, mental stability, and memory retrieval (Lo *et al*., 2016; Schouten *et al*., 2017). Based on electroencephalogram frequency-band rhythms, that is, delta ($\delta$), theta ($\theta$), alpha ($\alpha$), beta ($\beta$), and gamma ($\gamma$) rhythms, sleep is classified into five stages, namely, non-rapid eye movement (NREM) sleep (stages I to IV) and rapid eye movement (REM) sleep (stage V), which occur in alternating cycles (Doroshenkov *et al*., 2007), although recently an automatic, 6-stage, electroencephalographic sleep classification method was proposed (Diykh *et al*., 2016). While $\delta$ rhythm dominates NREM sleep, $\theta$ rhythm is commonly observed during REM sleep (Doroshenkov *et al*., 2007; Luppi *et al*., 2017). Accordingly, electroencephalography (EEG) can be employed to identify sleep disorders and to aid the predictions of treatment outcomes in various psychiatric diseases (Olbrich *et al*., 2015).

Sleep disorders not only reduce quality of life but also serve as risk factors of dementia (Mishima, 2016) and metabolic diseases, like atherosclerosis (Tobaldini *et al*., 2017), and hence, early intervention is clinically relevant as it potentially mitigates harmful consequences. Recently developed drugs that have been used to treat insomnia, but can have undesirable side effects (Kay-Stacey & Attarian, 2016). Furthermore, reports indicate lotus leaf extract augments hypnosis by binding to $\gamma$-aminobutyric acid A (GABA$_A$) receptor (Tian and Liu, 2015; Yan *et al*., 2015), and that consuming dairy products supports sleep in a better way (Kitano *et al*., 2014). Alpha ($\alpha$)$_{s1}$-casein hydrolysate ($\alpha_{S1}$-CH) is a milk protein with reported chronic stress relieving properties (Guesdon *et al*., 2006; Kim *et al*., 2007). However, although the tryptic hydrolysate of $\alpha_{S1}$-casein appears to improve sleep quality (Dela Peña *et al*., 2016), little data is available on the way it affects pentobarbital-induced sleep in mice or influences EEG band rhythms during stages of sleep. Furthermore, it has not been determined whether $\alpha_{S1}$-CH mediates its hypnotic action in mice via GABA$_A$ receptor in hypothalamus. Therefore, we investigated the effects of

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$\alpha_{S1}$-CH on sleep duration, sleep quality as determined by electroencephalography, and on the protein expression of GABA$_A$ receptor subunits ($\alpha$1, $\beta$1, $\gamma$3) in the rat hypothalamus.

## MATERIALS AND METHODS

### Chemicals

Bovine $\alpha_{S1}$-casein hydrolysate ($\alpha_{S1}$-CH), commercialized as Lactium®, was obtained from Ingredia (Arras, France). Pentobarbital sodium was obtained from Hanlim Pharm (Seoul). Diazepam and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Animals and treatments

Male C57BL/6 mice (28-30 g) and Sprague-Dawley rats (male, 260-280 g) were purchased from the Orient Bio (Seoul, Korea) and allowed free access to water and food. Mice were grouped into six per cage, and maintained in an ambient atmosphere at 23°C under a 12 h diurnal light cycle. Rodents were divided into groups: 6 groups of mice for sleep testing, 3 groups of rats for EEG recording, and 3 groups of rats for western blotting. All behavioral experiments were carried out in a nearby room maintained where under the same environmental conditions. Experiments were conducted according to Animal Care and Use Guidelines of the School of Medicine, Ewha Womans University, Korea.

Mice were given a single dose (30-240 mg/kg, p.o.) of $\alpha_{S1}$-CH or saline 30 min prior to an injection of pentobarbital sodium (42 mg/kg, i.p.) to determine the onset and duration of sleep, as previously described by Ma *et al*. (2009) with slight modification. Time elapsed between disappearance (sleep onset) and reappearance of righting reflex (up to a maximum of 2 h) was defined as sleep duration. Experiments were performed in mouse cages with aspen bedding. Animals that did not sleep within 15 min after pentobarbital injection were excluded. Rats were treated with $\alpha_{S1}$-CH (150 or 300 mg/kg) orally once per day for 3 days before electroencephalography (EEG) or western blotting. EEG recordings were started at 2 hrs after last treatment, and hypothalami were collected at 6 hrs after last treatment for western blotting.

### Electroencephalography

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and a transmitter was implanted for EEG recording via telemetry as previously described (Sanford *et al*., 2006). Briefly, in each case, the body of the transmitter was subcutaneously implanted just posterior to the scapula using three sutures for stabilization. The transmitter electrodes were led subcutaneously to the skull, and their bare ends were placed in contact with the dura through holes in the skull. Electrodes were anchored to the skull with screws and dental cement. All surgical procedures were performed stereotaxically under aseptic conditions.

For telemetric recording of cortical EEG signals, transmitter gain was set at -0.5/+0.5 volts per unit. Raw output signals, which ranged from 0.5 to 0.0 Hz, were processed using a Data Sciences analog converter and routed to an analog-to-digital (AD) converter (Eagle PC30, Data Sciences International, St. Paul, MN, USA), which digitized EEG and activity signals. Subsequently data were transferred to a computer and graphically displayed. An on-line fast Fourier transformation (FFT) program was used to analyze EEG data and generate power density values from 0.0 to 20.0 Hz at a resolution of 0.5 Hz. FFT data were further averaged between 0 to 20 Hz at 10-s intervals. Sleep data and FFT results were saved to hard disk every 10 s for additional off-line analysis. Number of animal movements related to telemetry receiver generated transistor-transistor logic pulses were viewed as measures of activity. Data were gathered on the 1st and 3rd days after $\alpha_{S1}$-CH treatment and percentage power densities were calculated. EEG signals were measured for 6 hrs between 11:00 am 5:00 pm. Each group contained 5-6 rats.

### Determination of sleep behaviors using EEG signals

Times elapsed in wakefulness, NREM sleep, or REM sleep was determined using digitized data using animal sleep analysis software Sleep-Sign 2.1 (Kissei Comtec, Matsumoto, Japan). Briefly, this software identifies wakefulness as high-frequency, low-amplitude EEG, NREM sleep as spikes interspersed with slow waves, and REM sleep as $\delta$-waves (0.75 to 4.0 Hz) with $\theta$-wave activity (5.0 to 9.0 Hz) of peak frequency 7.5 Hz.

### Western blotting

Six hrs after the last administration of $\alpha_{S1}$-CH, rats were decapitated and brains were quickly removed and chilled in ice-cold saline. Coronal sections were obtained using a rodent brain matrix (ASI Instruments, Warren, MI, USA). Hypothalami were dissected out, immediately frozen on dry ice, and stored at -80°C. Frozen tissue samples were homogenized in PRO-PREP protein-extraction solution (Intron Biotechnology Inc., Seongnam, Korea) and centrifuged at 13,000 rpm at 4°C for 20 min. Protein concentrations in supernatants were determined and 40 μg aliquots were subjected to polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene fluoride membranes (Hybond-P; GE Healthcare, Amersham, UK) using a wet transfer system, and membranes were incubated with one of the following primary antibodies: rabbit anti-GABA$_A$ $\alpha$1 polyclonal antibody (diluted 1:2,000 in TBS containing 0.5% Tween 20; Abcam, Cambridge, UK); rabbit anti-GABA$_A$ $\beta$1 polyclonal antibody (diluted 1:2,500 in TBS containing 0.5% Tween 20); rabbit anti-GABA$_A$ $\gamma$3 polyclonal antibody (diluted 1:2,500 in TBS containing 0.5% Tween 20); rabbit anti-glutamic acid decarboxylase (GAD) polyclonal antibody (diluted 1:2,000 in TBS containing 0.5% Tween 20); and $\beta$-actin antibody. Membranes were then washed and incubated with the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (diluted 1:3,000 in TBS containing 0.5% Tween20). Immunoreactive bands were developed using a chemiluminescence detection kit (Roche Diagnostics, Mannheim, Germany) and quantitative analysis was performed by densitometric scanning.

### Statistical analysis

Sleep onset and duration data were analyzed using analysis of variance (ANOVA). The Newman-Keuls test was used to perform intergroup comparisons. Values were expressed as means ± SEM, and statistical significance was accepted for $p$ values <0.05.

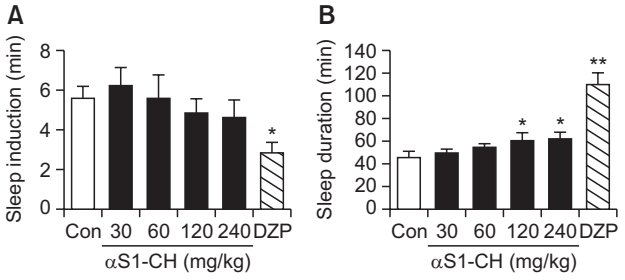


**Fig. 1.** Effects of $\alpha_{s1}$-CH on the onset and duration of sleep in pentobarbital-treated mice. Mice were food-deprived for 12 h prior to being treated with $\alpha_{s1}$-CH (30-240 mg/kg) or diazepam (DZP, 1mg/kg, i.p.). Sleep latency (A) and total sleeping time (B) were recorded for 120 min after injecting pentobarbital (42 mg/kg, i.p). Columns contain mean values and SEMs (n=8-10) as determined by ANOVA. Comparisons were made using the Newman-Keuls test. **p*<0.05, ***p*-values of <0.01 were considered significant as compared with pentobarbital-treated controls.

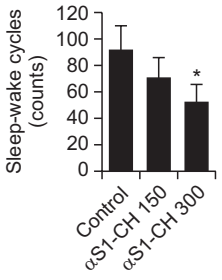


**Fig. 2.** Effects of $\alpha_{s1}$-CH on sleep-wake counts. $\alpha_{s1}$-CH (150, 300 mg/kg) was orally administered to rats once daily for 3 days. Sleep-wake cycles were measured by EEG for 6 hrs and analyzed using Sleep Sign 2.1 software. Values were compared using the Newman-Keuls test. Column contain mean values and SEMs (n=5-6). **p*-values of <0.05 were considered significant as compared with naïve controls.

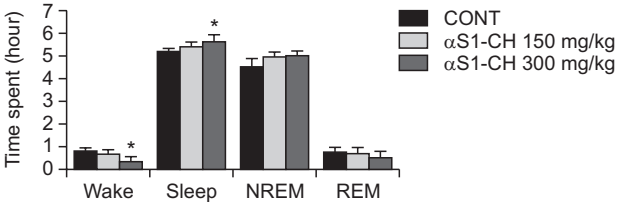


**Fig. 3.** Differential effects of $\alpha_{s1}$-CH on sleeping stages in rats. Wakefulness, total sleep, REM sleep, and NREM sleep were determined after administering $\alpha_{s1}$-CH (150, 300 mg/kg) orally to rats once daily for 3 days. Sleeping was determined using telemetric cortical EEG records and analyzed using Sleep Sign2.1 software. To compare each group versus naïve control, we used the Newman-Keuls test. Columns represent mean values and SEMs (n=5-6). **p*<0.05 compared with naïve controls.

## RESULTS

### Pretreatment with $\alpha_{S1}$-CH prolonged sleep duration in mice

It has been demonstrated $\alpha_{S1}$-casein hydrolysate protects rat from chronic mild stress-induced sleep disorders (Guesdon *et al*., 2006). In our preliminary experiment, $\alpha_{S1}$-CH showed an anxiolytic effect at relatively low doses (25, 50 mg/kg) in mice (data not shown). Therefore, we investigated whether $\alpha_{S1}$-CH improves sleep duration in pentobarbital-treated mice. Mice treated with $\alpha_{S1}$-CH in the dose range 30-240 mg/kg tended to have lower sleep-onset times (Fig. 1A). However, this effect of $\alpha_{S1}$-CH was not significant as compared with pentobarbital controls. Diazepam (1 mg/kg, i.p.) exhibited significantly earlier sleep-onset times than that of control after pentobarbital treatment. One-way ANOVA showed significant differences in sleep induction between control and diazepam group [$F(5,50)=5.0$, $p<0.01$]. In contrast, the duration of sleep significantly elevated when mice were treated with $\alpha_{S1}$-CH at higher doses (120 or 240 mg/kg, p.o.) [$F(5,51)=15.02$, $p<0.01$] (Fig. 1B).

### Rats pretreated with $\alpha_{S1}$-CH had fewer sleep-wake cycles

Sleep-wake cycle disruption has been associated with stress, which suggests that reducing the number of sleep-wake cycles may provide relief from neurodegenerative diseases (Cedernaes *et al*., 2017). Therefore, we investigated whether $\alpha_{S1}$-CH could reduce the number of sleep-wake cycle disruptions in rats. In the preliminary test, administration of $\alpha_{S1}$-CH at doses of 50 or 100 mg/kg did not significant affect sleep/wake cycles or EEG patterns in rats, and thus, the dose of $\alpha_{S1}$-CH was increased to 150 or 300 mg/kg. We found $\alpha_{S1}$-CH at 300 mg/kg significantly reduced the number of sleep-wake cycles by ~50% (Fig. 2). Furthermore, total time awake was reduced by $\alpha_{S1}$-CH pretreatment and total asleep was increased (Fig. 3). Although REM sleep was decreased and NREM sleep was increased after treatment with $\alpha_{S1}$-CH, no significant differences were found between treatment groups (Fig. 3).

### The effect of $\alpha_{S1}$-CH on frequency bands of EEG during sleep-wake cycles

Protein $\alpha_{S1}$-CH (150 or 300 mg/kg, p.o.) was administered to rats once per day for 3 days. Wakefulness, REM sleep and NREM sleep were monitored using the power densities of delta ($\delta$), theta ($\theta$), and alpha ($\alpha$) frequency bands in rats treated without or with $\alpha_{S1}$-CH (150 or 300 mg/kg). Whereas the percentage of $\theta$ power density was significantly increased by treatment with $\alpha_{S1}$-CH (300mg/kg) in sleep-wake cycles, $\delta$ frequency bands showed negligible differences. On the other hand, treatment with $\alpha_{S1}$-CH at 300 mg/kg decreased the percentage of $\alpha$ power density (Fig. 4).

### $\alpha_{S1}$-CH modulated the expression levels of $\beta_1$ and $\gamma^3$ subtypes of $GABA_A$ receptor in the rat hypothalamus

$GABA_A$ receptor subtypes in neuronal tissue have been reported to be targets for insomnia treatment (Luppi *et al*., 2017). We investigated whether the protein expressions of the $GABA_A$ receptor subunits $\alpha_1$, $\beta_1$, and $\gamma^3$ were modulated in the hypothalami of rats treated with $\alpha_{S1}$-CH. Treatment using $\alpha_{S1}$-CH at 150 mg/kg or 300 mg/kg increased the protein expression of $\beta_1$, but the protein expression level of $\alpha_1$ and glutamic acid decarboxylase ($GAD_{65/67}$; catalyzes the formation of GABA in neuronal tissues) were unaltered (Fig. 5). Although the level of $\gamma_3$ tended to be elevated by $\alpha_{S1}$-CH treatment, this was not statistically significant.

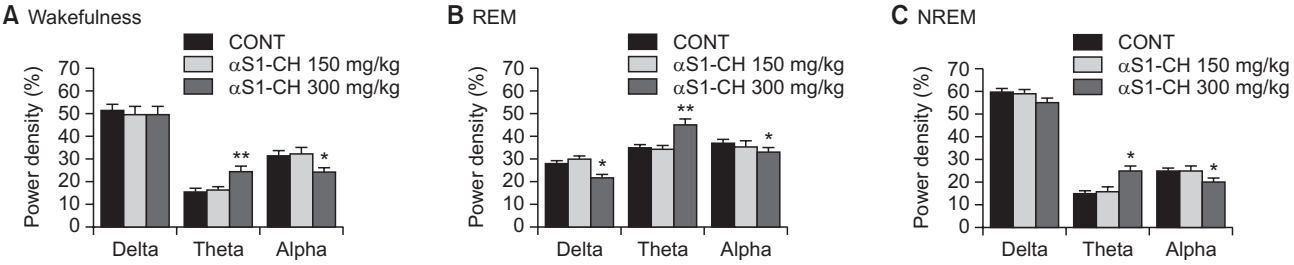


**Fig. 4.** The effects of $\alpha_{s1}$-CH on EEG power densities during wakefulness (A), REM sleep (B) and NREM sleep (C) in rats. $\alpha_{s1}$-CH (150, 300 mg/kg) was orally administrated to rats once daily for 3 days. Power densities were classified as $\delta$-wave, $\theta$-wave, and $\alpha$-wave densities. The Newman-Keuls test was used to compare groups versus the naïve control group. Columns represent mean values and SEMs (n=5-6). *p*-values of <0.05 or <0.01 were considered significant (as indicated by * or **, respectively).

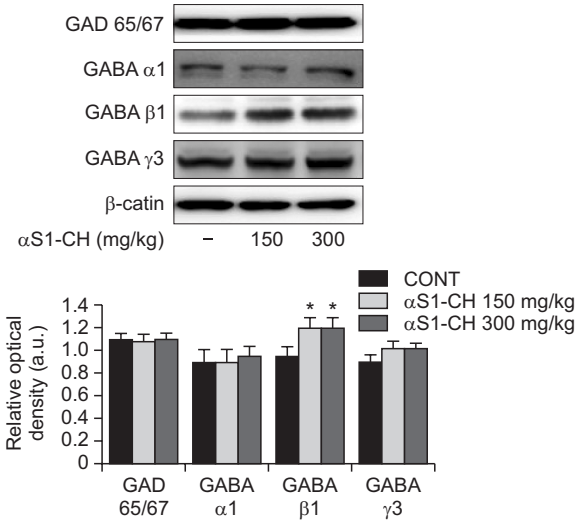


**Fig. 5.** Modulation of glutamic acid decarboxylate (GAD) and GABA$_A$ receptor subunits in the rat hypothalamus by $\alpha_{s1}$-CH (150, 300 mg/kg). After three days of consecutive treatment by $\alpha_{s1}$-CH, hypothalamic tissues were isolated, homogenized, and immunoblotted. Immunoreactive band intensities were measured by densitometry. Protein levels were normalized versus GAPDH. Columns contain mean values and SEMs (n=3). The Newman-Keuls test was used to compare groups versus the naïve control group. **p*-values of < 0.05 were considered significant.

## DISCUSSION

Considering the importance of nutrition-based hypnosis over that of recently developed drugs with undesired side effects, our report on $\alpha_{S1}$-CH has merit with respect to prolonged duration of sleep, and fewer sleep-wake cycles, which suggests a new avenue for developing alternative therapeutic options against the insomnia experienced during stressful conditions. Sleep disorders are associated not only with mental problems (Yu *et al*., 2013) but are also linked to various health conditions, such as, metabolic disease and reduced testosterone levels accompanied by altered sexual behavior (Alvarenga *et al*., 2015). Recently, a disordered protein architecture of receptors was suggested to be related to sleep problems (Tou and Chen, 2014), which implies a complex mechanism underlies insomnia. Several drugs that ameliorate insomnia have been developed, but many are associated with unwanted side effects (Kripke, 2016; Sirdifield *et al*., 2017). Alternative options have been sought, such as, acupuncture (Lee and Lim, 2016) and the use of natural products (Shi *et al*., 2014). Milk contains a wide variety of bioactive peptides, including those in tryptic hydrolysate of $\alpha_{S1}$-casein, which has been reported to modulate the architecture of sleep (Dela Peña *et al*., 2016).

Little is known of EEG band rhythms and membrane receptor expressions in hypothalamic neurons, and EEG parameters are considered essential during sleep examinations and are used to evaluate sleep patterns or problems. Therefore, we investigated the effects of $\alpha_{S1}$-casein in mouse model of pentobarbital-induced sleep .

We found $\alpha_{S1}$-CH (30-240 mg/kg) did not modulate sleep onset, but that at 120 or 240 mg/kg it prolonged sleep duration in mice. Similarly, $\alpha_{S1}$-CH at 300 mg/kg reduced the number of sleep-wake counts nearly by a half in rats. Moreover, total sleeping time was increased but wakefulness was diminished by $\alpha_{S1}$-CH at 300 mg/kg. Together, these findings strongly support previous findings that suggested the tryptic hydrolysate of $\alpha_{S1}$-casein had sleep promoting properties. Pena *et al*. also showed that EEG $\delta$ waves increased in NREM sleep whereas $\alpha$ waves decreased (Dela Peña *et al*., 2016). In the present study, we also found the power density of $\theta$ waves were significantly increased and $\alpha$ densities significantly decreased by $\alpha_{S1}$-CH (300 mg/kg) in rats. It has been known $\delta$ waves are slow waves related to the governance of sleep, and that $\alpha$ waves are high frequency waves related to sedatives and hypnotics (Stahl, 2008). Interestingly, it was reported that $\delta$ rhythm is predominantly seen during NREM sleep in contrast to $\theta$ rhythm, which is usually observed during REM sleep (Luppi *et al*., 2017). In general, in our EEG signals, $\theta$ waves were significantly enhanced during REM sleep, NREM sleep, and wakefulness when rats were treated with $\alpha_{S1}$-CH (300 mg/kg), which indicates higher concentrations of $\alpha_{S1}$-CH influence EEG signals. In contrast to $\theta$ rhythms, $\alpha$ rhythms are present during waking (Doroshenkov *et al*., 2007), and in the present study were found to be decreased by pretreatment with $\alpha_{S1}$-CH at higher concentration (300 mg/kg). This result may seem contradictory given the aforementioned EEG patterns of REM and NREM, and we cannot provide an explanation for this result. However, Rajaratnam *et al*. showed that melatonin administration does not significantly change $\delta$ or $\alpha$ activities in man (Rajaratnam *et al*., 2004), and suggested melatonin facilitates rather than induces sleep. This might also be the case for $\alpha_{S1}$-CH.

Despite controversies regarding the properties, functions,

and subunit arrangements of $GABA_A$ receptors, they have been established to be pentameric ligand-gated channels that negatively mediate neurotransmission in the central nervous system, (Puthenkalam *et al*., 2016; Wongsamitkul *et al*., 2016). Dela Peña *et al*. (2016) suggested $GABA_A$ receptor subunits play a role in mediating $\alpha_{S1}$-CH induced sleeping based on results obtained using bicuculline, a competitive $GABA_A$ receptor antagonist, and that the dose-dependent increase in chloride ion influx induced by $\alpha_{S1}$-CH in cultured human neuroblastoma cells was blocked by bicuculline. In the present study, we found the protein expression of the $\beta_1$ receptor subunit of $GABA_A$ was increased in the hypothalami of rats treated with $\alpha_{S1}$-CH (150, 300 mg/kg), but that $\alpha$1 and $GAD_{65/67}$ protein levels were unchanged. The activation of GAD plays an important role in the GABAergic system because GABA is generated from glutamate by the action of GAD. In the present study, protein levels of $GAD_{65/67}$ were unaltered by $\alpha_{S1}$-CH administration, suggesting $\alpha_{S1}$-CH might not modulate GABA generation. Nevertheless, our report indicates the importance of $\beta_1$ subunits of $GABA_A$ receptor in $\alpha_{S1}$-CH enhanced sleep, though further studies are warranted on receptor subtypes and their arrangements in $GABA_A$ receptor (Mohler *et al*., 2005; Wongsamitkul *et al*., 2016), especially since Liang and Marks (2014) observed the involvement of the $GABA_A$ $\gamma^2$ receptor subunit in REM sleep.

In the present study, we found $\alpha_{S1}$-CH significantly enhanced pentobarbital-induced sleep duration in mice, increased total sleep, and EEG $\theta$ wave during sleep in rats. Given increased protein expressions of $GABA_A$ receptor $\beta_1$ subunits after $\alpha_{S1}$-CH treatment observed in rats, further work is required to explore other $GABA_A$ receptor subtypes and their arrangements to clearly delineate the sleep-enhancing effect of $\alpha_{S1}$-CH. Nonetheless, our findings suggest $\alpha_{S1}$-CH dietary supplementation could be deployed to treat sleep disorders.

## ACKNOWLEDGMENTS

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