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Original Article

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3-Carene, a Phytoncide from Pine Tree Has a Sleep-enhancing Effect by Targeting the $GABA_A$-benzodiazepine Receptors

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3-Carene, a bicyclic monoterpene, is one of the major components of the pine tree essential oils. It has been reported that, in addition to its known properties as a phytoncide, 3-carene has anti-inflammatory, antimicrobial, and anxiolytic effects. We have previously demonstrated that α-pinene, the major component of pine tree, has a hypnotic effect through $GABA_A$-benzodiazepine (BZD) receptors. However, a hypnotic effect of 3-carene has not been studied yet. Here, we report that oral administration of 3-carene increases the sleep duration and reduces sleep latency in pentobarbital-induced sleep test. 3-Carene potentiates the $GABA_A$ receptor-mediated synaptic responses by prolonging the decay time constant of inhibitory synaptic responses. These enhancing effects of 3-carene are reproduced by zolpidem, a modulator for $GABA_A$-BZD receptor, and fully inhibited by flumazenil, an antagonist for $GABA_A$-BZD receptor. The molecular docking of 3-carene to the BZD site of $GABA_A$ protein structure, suggests that 3-carene binds to the BZD site of α1 and Υ2 subunits of $GABA_A$-BZD receptor. These results indicate that, similar to α-pinene, 3-carene shows a sleep-enhancing effect by acting as a positive modulator for $GABA_A$-BZD receptor.

Key words: 3-carene, Sleep, $GABA_A$-BZD receptor, Phytoncide

INTRODUCTION

Sleep is defined as a naturally recurring state of mind and body, characterized by altered consciousness [1], and is divided into two types: non-rapid eye movement (non-REM or NREM) and rapid eye movement (REM) sleep. Sleep is observed in non-human animals including mammals, birds, reptiles, amphibians, fish, even in insects [2-5] and is required for living animals to live and maintain a normal life. It has been growing numbers of sleep disorders: One fourth of the general population in America have experienced insomnia, one of the most common sleep disorders [6]. Although there have been various ways to treat insomnia such as behavioral therapy, psychotherapy, and light therapy, the most common one is pharmacological treatments using hypnotic drugs targeting $GABA_A$-BZD receptors such as diazepam, zolpidem [7]. However, it has been reported that those drugs have many side effects including cognitive impairment, tolerance, headache, nausea, and rebound insomnia [8, 9]. Therefore, there has been growing need for developing new hypnotic drugs derived from natural products with less side effects.

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Pines are conifer in the genus *Pinus* of the family *Pinaceae* and are widely distributed in the forest throughout the world [10, 11]. Pines have been widely used for their therapeutic and pharmacological properties of the volatile essential oils in the Eastern and Western World [12-14]. Nowadays, there has been growing interest in pine essential oils, also known as phytoncide, for their beneficial effects such as enhancing immune systems and relieving stress [15, 16]. Pine essential oils are mainly consisted of monoterpene such as α- and β-pinene, 3-carene, limonene, and terpinene [11]. These compounds have various beneficial effects, such as anti-inflammatory, anti-microbial, anti-fungal, and anti-stress [17, 18].

3-Carene (3,7,7-Trimethylbicyclo[4.1.0]hept-3-ene) is a bicyclic monoterpene consisting of fused cyclohexene and cyclopropane rings and has a sweet and pungent odor [19]. It has been used as a raw material in perfumes, cosmetics, flavors and terpene resins, having various therapeutic properties including anti-inflammatory, anti-fungal, and sedative effects [20, 21]. 3-Carene is the second abundant monoterpene of pine essential oils after α-pinene. Recently we have reported a hypnotic effect of α-pinene as a positive modulator of $GABA_A$-BZD receptors [22]. Although the beneficial effects and biological properties of 3-carene as a phytoncide of the pine tree have been established, the potential effect of 3-carene on sleep has not been tested yet. In this study, we investigated the hypnotic effect of 3-carene in mice by oral administration method. We also identified the molecular mechanism of 3-carene through the various experimental approaches including electrophysiology, pharmacological tools, and molecular modeling. Through our previous and current study, we suggest that phytoncides from pine trees including α-pinene and 3-carene commonly have sleep-enhancing effect as a positive modulator of $GABA_A$-BZD receptors.

MATERIALS AND METHODS

Materials

3-Carene (CAS no. 13466-78-9) was purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). Zolpidem (Ministry of Food and Drug Safety, Cheongwon-gun, Chungcheongbuk-do, Korea), a $GABA_A$-benzodiazepine (BZD) receptor agonist, was used as a reference hypnotic drug. Flumazenil, an antagonist of $GABA_A$-BZD receptors, was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Molecular structures and weights of 3-carene, zolpidem, and flumazenil are shown in Fig. 1. All other chemicals and reagents were of the highest grade available.

Animals

All procedures involving animals were conducted in accordance with the animal care and use guidelines of the Korea Food Research Institutional Animal Care and Use Committee (permission number: KFRI-M-12027). Imprinting control region (ICR; male, 18~22 g) and C57BL/6N (male 27~30 g) mice were purchased from Koatech Animal Inc. (Pyeongtaek, Korea). The animals were housed in an insulated, sound-proof recording room maintained at an ambient temperature of 23±0.5℃, with a constant relative humidity (55±2%) on an automatically controlled 12 h light/12 h dark cycle (lights off at 17:00). They had free access to food and water. All efforts were made to minimize animal suffering and to use only the number of animals required for the production of reliable scientific data.

Pentobarbital-induced sleep test

The initial screening for hypnotic effect of 3-carene sleep was done with pentobarbital-induced sleep [23]. Experiment was performed between 13:00 and 17:00 h, and the ICR mice were fasted for 24 h before the experiment to minimize the drowsiness induced by food. 3-Carene and zolpidem were administered orally (p.o.) to the ICR mice (n=10) 45 min before the pentobarbital injection (45 mg/kg, i.p.). After the injection intraperitoneally (i.p.) of pentobarbital, mice were placed in individual cages and observed for measurements of sleep latency and duration. The observers were blinded to the individual treatments. The mice were considered asleep if stayed immobile and lost its righting reflex when positioned on its back. The sleep latency was defined as the elapsed time from pentobarbital injection to onset of righting reflex loss. The sleep duration was defined as the difference in time between

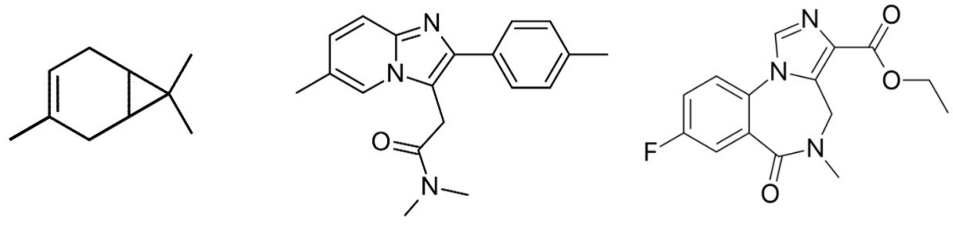

Fig. 1. Chemical structures and molecular weight (MW) of 3-carene, zolpidem and flumazenil.

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the loss and the recovery of the righting reflex.

Pharmacological treatments

3-Carene was dissolved in sterile saline containing 5% tween 80 immediately before use, and administered orally (p.o.) to the C57BL/6N mice (each group, n=8) at 17:00 h on the experimental day at a dose of 12.5, 25, 50, or 100 mg/kg. The positive control zolpidem (10 mg/kg) was administered in the same manner as 3-carene. Flumazenil was dissolved in sterile saline and injected intraperitoneally (i.p.) 15 min before 3-carene or zolpidem administration. For baseline data, mice were injected with the vehicle (saline containing 5% tween 80) at 16:45 h (i.p.) and 17:00 h (p.o.).

Electrophysiological measurement

Adult mice (7~9 weeks) were deeply anaesthetized until cessation of breathing and subsequently decapitated. The brain was rapidly removed and submerged in an ice-cold oxygenated artificial cerebrospinal fluid (ACSF) composed of (in mM) 130 NaCl, 24 NaHCO3, 3.5 KCl, 1.25 NaH2PO4, 1 CaCl2, 3 MgCl2, 10 glucose at pH 7.4, and was bubbled with 5% CO2/95% O2. Transverse mouse brain slices (300 μm) containing hippocampus were acutely prepared with a vibratome (Linear slicer, DSK, Japan), and incubated in a chamber with oxygenated ACSF at room temperature for 1 h before use.

The standard ACSF recording solution was composed of (mM): 130 NaCl, 24 NaHCO3, 3.5 KCl, 1.25 NaH2PO4, 1.5 CaCl2, 1.5 MgCl2 and 10 glucose saturated with 95% O2–5% CO2, at pH 7.4. The internal solution was composed of (mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP, 10 QX-314. To block the spontaneous EPSC, APV (50 μM; Tocris) and CNQX (20 μM; Tocris) were added into ACSF. Recordings were obtained using Axopatch 200A (Axon instruments, CA, USA) and filtered at 2 kHz. In case of sIPSC recording, recordings were digitized at 10 kHz, and analyzed using pCLAMP 10 (Molecular devices, CA, USA) and Mini Analysis Program (Synaptosoft, NJ, USA). The sIPSCs were automatically detected. All experimental procedures described were performed in accordance with the institutional guidelines of Korea Institute of Science and Technology (KIST, Seoul, Korea).

Molecular modeling

In this study, we used previously published model for the most abundant α1β2γ2 subtype of $GABA_A$ receptor [22]. 3D coordinates of 3-carene was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/compound/26049#section=Top). Schrodinger 2015 (Schrodinger LLC, New York, NY) package was used to perform docking study of 3-carene. Ligand structure was imported to the Maestro and prepared using default LigPrep setting at 7.4 pH. The putative binding site for docking study was considered as benzodiazepine (BZD) site on $GABA_A$, and the grid box was assigned to the center of α1Y237, α1H129, α1Y187, γ2F116, γ2M169 and γ2T181 residues. We utilized Glide standard precision (SP) docking algorithm to dock 3-carene inside receptor, and fifty binding poses were generated and ranked according to the more negative Glide Gscore value. Gscore function comprises of terms for H-bond, hydrophobic, van-der-Waals interaction energy, and ligand strain energy. All the figures were rendered using Discovery Studio Client 2017 R2 package. 3-Carene was map on the already developed pharmacophore using Discovery Studio to know whether it is an agonist, partial agonist or an antagonist. Ligands were taken from the literature to develop pharmacophore [24].

Data analysis

All data were expressed as the mean±SEM. Statistical analysis was performed with the SigmaPlot 13.0 (Systat Software Inc.). For multiple comparisons, data were analyzed using one-way ANOVA followed by Dunnett's test. Comparisons between two-group data were analyzed by the unpaired Student's t-test. The significance level was set at $p<0.05$ for all statistical tests.

RESULTS

Effects of 3-carene in the pentobarbital-induced sleep test

To test whether oral administration (p.o.) of 3-carene (Fig. 1) produces a sleep-enhancing effect, we performed the pentobarbital-induced sleep test in ICR mice (Fig. 2A). As expected, zolpidem (10 mg/kg, p.o.), a well-known hypnotic drug and positive modulator of $GABA_A$-BZD receptors [25, 26], significantly decreased the sleep latency and increased the sleep duration (sleep latency: 2.53±0.08 min; sleep duration: 132.1±15.48, Fig. 2B and 2C). We found that 3-carene (100 mg/kg, p.o.) also showed a similar effect in sleep latency and duration in a dose-dependent manner (sleep latency: 2.62±0.05 min; sleep duration: 122.2±8.74, Fig. 2B and 2C) as the zolpidem.

Molecular mechanism of sleep-enhancing effect of 3-carene

Recently we have reported that α-pinene, a monoterpene from pine essential oil, acts as a positive modulator of $GABA_A$-BZD receptor, similarly to other monoterpenes such as borneol, verbenol, and pinocarveol [22, 27, 28]. To confirm 3-carene functions like these monoterpene, we treated flumazenil, an antagonist of $GABA_A$-BZD receptor, 15 min before administration of 3-carene, and then measured the sleep behavior. Flumazenil (1 mg/kg) inhibited the hypnotic effect of zolpidem without affecting the sleep latency

and duration (Fig. 3B and 3C). The hypnotic effect of 3-carene was fully antagonized by flumazenil, suggesting that 3-carene acts as a positive modulator of $GABA_A$-BZD receptor, just like zolpidem.

To confirm that 3-carene is a genuine positive modulator of $GABA_A$-BZD receptor, we performed the conventional slice whole-cell patch clamp experiments. We measured the inhibitory synaptic responses from hippocampal CA1 neurons that express $GABA_A$ receptors containing α1 and α2 subunits, the well-known targets for BZD drugs such as diazepam and zolpidem [29, 30]. We found that 3-carene prolonged the decay time constant of spontaneous inhibitory postsynaptic currents (sIPSCs) in dose-dependent manner (EC_{50}=48.8 nM, Fig. 4B and 4D). This enhancing effect in decay time by 3-carene was fully inhibited by flumazenil (1 μM, Fig. 4B and 4C). There was no significant difference in frequency and amplitude of sIPSCs (Fig. 4E and 4F), consistent with previous reports [22, 31, 32]. These data suggest that 3-carene enhances

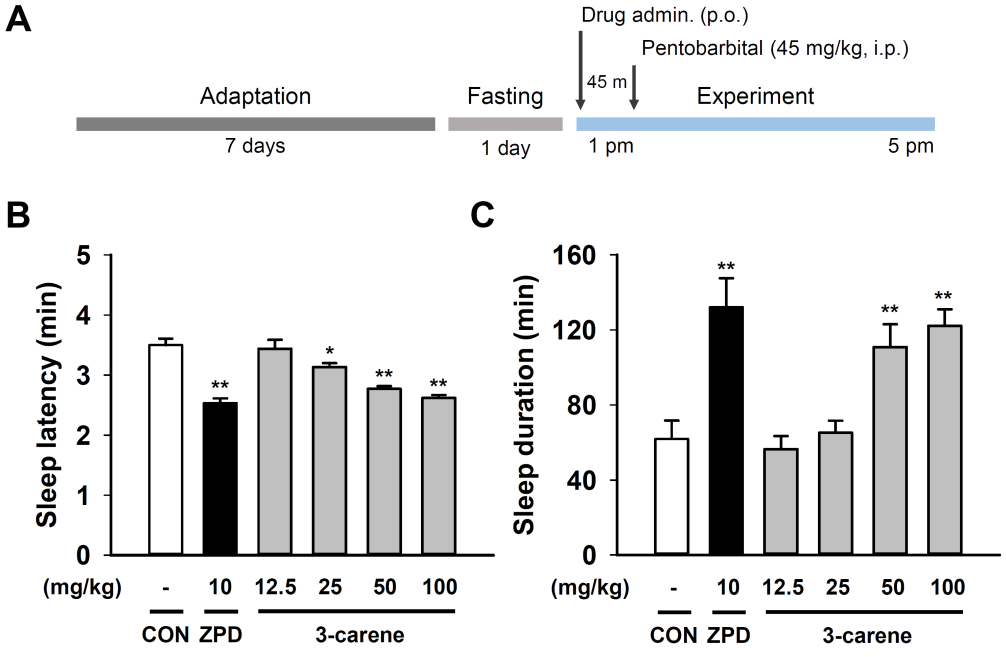

Fig. 2. (A) Experimental procedure for sleep test. Effects of 3-carene on sleep latency (B) and sleep duration (C) in mice induced by hypnotic dose (45 mg/kg, i.p.) of pentobarbital. Each column represents mean±SEM (n=10). *p<0.05, **p<0.01, significant as compared to the control group (One-way ANOVA, Dunnett's test). Con, control group; ZPD, zolpidem group.

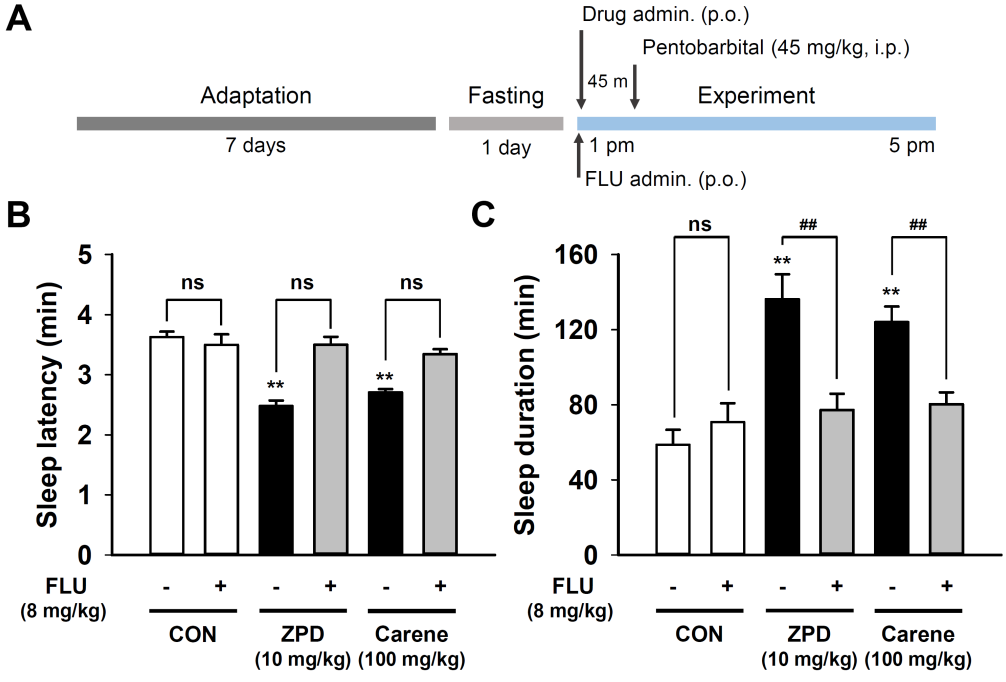

Fig. 3. (A) Experimental procedure for sleep test. Effects of FLU on the changes in sleep latency (B) and sleep duration (C) in mice treated with ZPD and 3-carene. FLU (8 mg/kg, i.p.) was administered 10 min before oral administration. Each column represents mean±SEM (n=10). **p<0.01, significant as compared to the control group (One-way ANOVA, Dunnett's test). ##p<0.01, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). CON, control group; FLU, flumazenil; NS, not significant; ZPD, zolpidem group.

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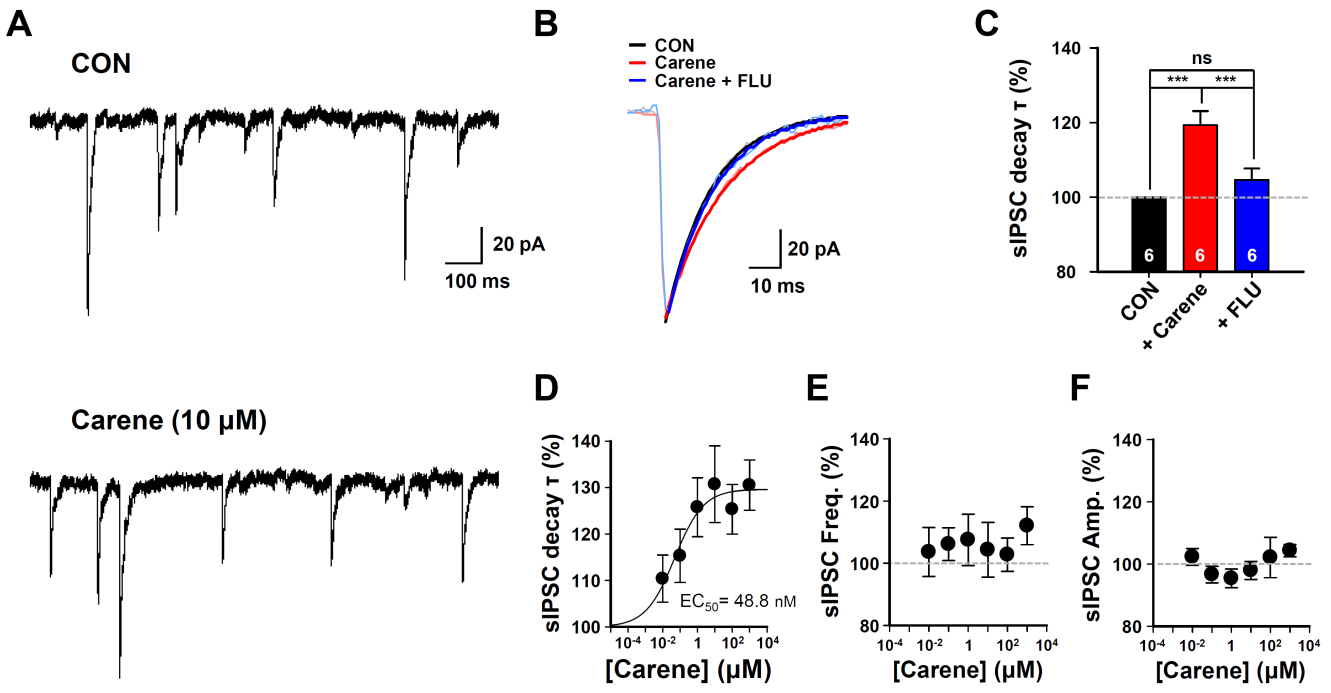

Fig. 4. (A) Representative traces of sIPSC before (CON) and after treatment of 3-carene (10 μM). (B) Averaged sIPSCs after normalization by peak. (C) Summary bar graph of sIPSC decay value before and after treatment of 3-carene and flumazenil (1 μM, right). ***p<0.001, significant as compared to the control group (One-way ANOVA, Dunnett's test). (D~F). Summary graphs of sIPSC decay tau value (D), frequency (E), and amplitude (F) after normalization by control response. Decay response was fitted using sigmoidal logistic 4 parameters.

the GABAergic synaptic responses by targeting the $GABA_A$-BZD receptor and prolonging the decay time of sIPSCs.

Docking model of 3-carene in BZD binding site of $GABA_A$ receptor

Based on the data showing 3-carene acting as a positive modulator of $GABA_A$-BZD receptor, we predicted the binding modes of 3-carene at the BZD binding site in $GABA_A$ receptor through the docking and pharmacophore mapping. We found that 3-carene binds at BZD site of α1 and γ2 subunits of $GABA_A$ receptor with -5.39 kcal/mol glide Gscore energy (Fig. 5A and 5B). Docked pose reveals that ligand docked in the deep crevice and formed van der Waals contact with Ser232, Ser233, Thr234 of α1-subunit, and Met168 and Thr181 from γ2-subunit. Phe116 and Tyr97 forms pi-pi stacking and pi-sigma interactions with the cyclic ring, and 7-methyl, respectively (Fig. 5B). This observation coincides with the previous report about the importance of these residues in ligand interaction identified by modeling and mutagenesis [22, 33-35]. Also, 3-methyl forms alkyl-alkyl interactions with Ala118 and Leu179 of α1- and γ2-subunits, respectively (Fig. 5C). To identify the pharmacological class of 3-carene, we mapped 3-carene over developed pharmacophore models of agonist, partial agonist, and antagonist. We observed that 3-carene only mapped over an antagonist pharmacophore generated by using ZK-93426, APQ2Cl, and Ro13-0699 (Fig. 5D). In fact, 3-carene mapped over three hydrophobic features but missed two hydrogen bond acceptor function. These results indicate that 3-carene positively modulates the biological function of $GABA_A$ receptor by directly binding at the BZD binding site.

DISCUSSION

In this study, we have demonstrated that 3-carene has a sleep-enhancing effect by prolonging the decay time of GABAergic synaptic response as a positive modulator for $GABA_A$-BZD receptor. Although it has been reported that 3-carene has been used as raw material in perfumes, cosmetics, flavors, and terpene resins with various beneficial effects such as anti-inflammatory, anti-fungal, and sedative effects [20, 21], any hypnotic effect of 3-carene has not been tested. This is the first study to identify the effect of orally administered 3-carene on sleep via the pentobarbital-induced sleep test and its molecular mechanism.

Nowadays there has been growing interest in forest bathing, defined as making contact with and taking in the atmosphere of the forest, for its various therapeutic effects such as enhancing immune system function, ameliorating respiratory and cardiovascular sys-

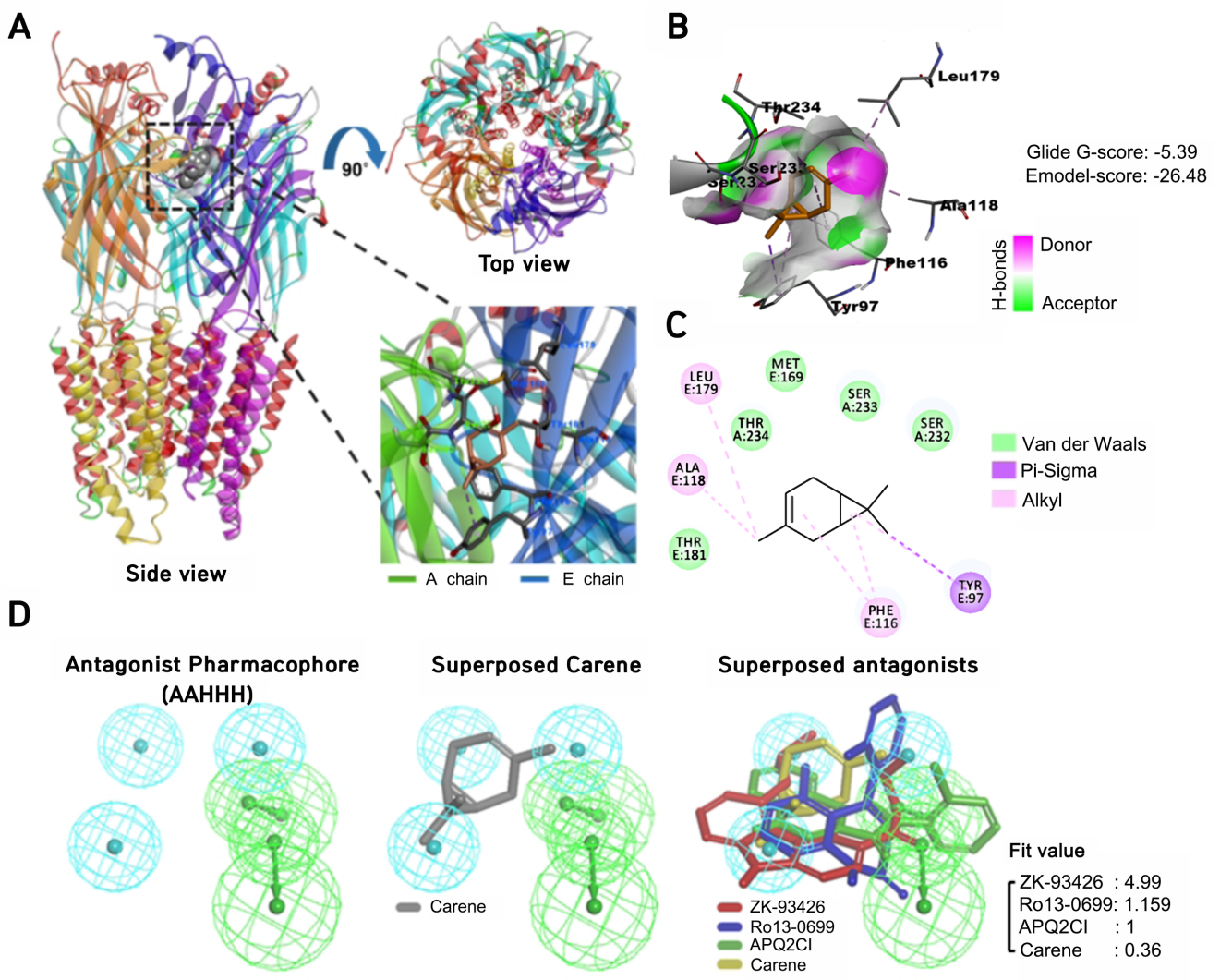

Fig. 5. (A) $GABA_AR$ shown by different chain colors. 3-carene (gray sphere) docked inside chain A and chain B (left). (B) Closer view of docked model of 3-carene, interacting residues was shown by lines and 3-carene by stick model (upper). 2D-interaction plot of 3-carene and surrounding residues of $GABA_A$ receptor. (C) Various interactions were represented by distinct color and dashed lines. α1- and γ2-subunit of $GABA_A$ receptor is shown by A and E chains, respectively. Protein shown by transparent hydrogen bond donor and acceptor surface. Pink and Violet showed Pi-alkyl and pi-sigma interactions. (D) 3-carene mapped over antagonist pharmacophore. Hydrophobic and hydrogen bond acceptor features shown in cyan and green mesh sphere (left, middle). Hydrophobic and hydrogen-bond acceptor features were depicted by cyan and green mesh spheres. ZK-93426, Ro13-0699, APQ2Cl and 3-carene were shown by the red, blue, green and yellow sticks, respectively (right).

tem, relieving stress and anxiety [36, 37]. It has been accepted that the various beneficial effects of the forest are due to plant-derived products or phytoncide, defined as natural volatile compounds derived from trees and plants [37, 38]. The ancient Chinese and Korean medicinal literatures have described the therapeutic effects of pine tree seeds in digestive function, stress, and sleep and usage of pine tree seeds in symptoms or diseases relating to stress and sleep [39, 40]. Based on these lines of ancient evidence, we demonstrate the hypnotic effect of phytoncide from pine tree and its molecular mechanism through the combination of experimental methods including sleep behavior analysis, electrophysiology, and molecular modeling.

We have previously reported that certain purified compounds from rice, marine plant, and pine tree all enhance the $GABA_A$ receptor-mediated synaptic response by prolonging the decay time constant of inhibitory synaptic response by targeting $GABA_A$-BZD receptor [22, 23, 32, 41]. We have shown that these compounds have hypnotic effects with enhanced quantity of non-rapid eye movement sleep (NREMS) [22, 23, 32, 41]. In the current study, we found the same effects of 3-carene in IPSCs and sleep. Based on these findings, we can make a generalization that if a certain drug or compound enhances the decay time constant

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of IPSCs, we can safely predict that it could have a hypnotic effect as a positive modulator of $GABA_A$-BZD receptor. Therefore, we propose that this electrophysiological assay as a simple screening method for finding a potential sleep-enhancing drug.

In this study, we tested the sleep-enhancing effect of 3-carene through pentobarbital-induced sleep test. This method serves as a screening tool for finding potential hypnotics and determining the proper concentration of drugs for further experiments such as electroencephalography (EEG) and electromyography (EMG) to examine the effect of 3-carene in sleep stages such as REM and non-REM. Therefore, without detailed sleep stage analysis with EEG and EMG, there might be a limitation to conclude that 3-carene has an effect on sleep-wake behavior and sleep architecture based only on pentobarbital-induced sleep test. However, in our previous report on α-pinene [22], we performed all the critical experiments including the EEG/EMG and detailed sleep stage analysis for α-pinene. In the same previous report, we have established that combination of pentobarbital-induced sleep test, IPSC recordings, and molecular docking are sufficient to ensure a positive effect on sleep stages. Therefore, in the current study with 3-carene, we did not perform the detailed sleep stage analysis using EEG and EMG, which takes a lot of effort and time. Instead, we believe that the pentobarbital-induced sleep test, IPSC recordings, and molecular docking are sufficient to ensure a positive effect of 3-carene on sleep. Nevertheless, to elucidate the effect of 3-carene in sleep stages and architecture, further experiments of EEG and EMG with 3-carene are needed in the future.

Here we have performed the IPSC recordings from hippocampal CA1 neurons. The rationale for performing the IPSC recordings in hippocampus is that although hippocampus is not the sleep control center, it is possible to test the general action of 3-carene in hippocampal CA1 neurons expressing $GABA_A$ receptors containing α1 and α2 subunits. These $GABA_A$ subunits are the known molecular targets for BZD drugs such as diazepam and zolpidem [29, 30]. Hippocampal CA1 pyramidal neurons also express $GABA_A$ receptors containing α1 and α2 subunits [29], just as the ventrolateral preoptic nucleus in the hypothalamus of the actual sleep center [42, 43]. The effect of BZD drugs has been tested in neurons from various brain regions including thalamus, hippocampus, and neocortex [13, 31, 44]. In our previous studies, we have tested the effect of various natural compounds on the IPSC decay kinetics in hippocampal CA1 neurons and found that the compounds also have hypnotic effects [22, 32]. Therefore, testing the effect of candidate compounds in the hippocampus CA1 is a convenient and simple way for pre-screening for potential hypnotic drugs.

In summary, we have demonstrated that 3-carene as a phytoncide from pine tree has the sleep-enhancing effect by targeting $GABA_A$-BZD receptor by combining sleep behavior analysis, electrophysiology, and molecular modeling. In addition to the hypnotic effect of 3-carene, it is known to have other therapeutic effects such as anti-inflammatory, anti-oxidant, and anti-stress. We can easily obtain these beneficial effects of 3-carene from forest bathing, inhalation of pine essential oils, and oral supplements. We propose 3-carene as an excellent therapeutic agent for patients having sleep disorders or anxiety.

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