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Article

α-Pinene, a major constituent of pine tree oils, enhances non-rapid eye movement sleep in mice through $GABA_A$-benzodiazepine receptors

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Running title: Sleep-enhancing effect of α-pinene through $GABA_A$ receptor

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Abbreviations:

ACSF, artificial cerebrospinal fluid; **BZD,** benzodiazepine; **EEG,** electroencephalogram; **EMG,** electromyogram; **ICR,** Imprinting control region; **i.p.,** intraperitoneally; **NREMS,** rapid eye movement sleep; **p.o.,** oral administration; **REMS,** non-REM sleep; **sIPSCs,** spontaneous inhibitory postsynaptic currents; **Wake,** wakefulness.

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Abstract

α-Pinene is a major monoterpene of the pine tree essential oils. It has been reported that α-pinene shows anxiolytic and hypnotic effects upon inhaled administration. However, hypnotic effect by oral supplementation and the molecular mechanism of α-pinene have not been determined yet. By combining *in vivo* sleep behavior, *ex vivo* electrophysiological recording from brain slices, and *in silico* molecular modeling, we demonstrate that (-)-α-pinene shows sleep enhancing property through a direct binding to $GABA_A$-BZD receptors by acting as a partial modulator at the BZD binding site. The effect of (-)-α-pinene on sleep-wake profiles was evaluated by recording EEG and EMG. The molecular mechanism of (-)-α-pinene was investigated by electrophysiology and molecular docking study. (-)-α-Pinene significantly increased the duration of NREMS and reduced the sleep latency by oral administration without affecting duration of REMS and delta activity. (-)-α-Pinene potentiated the $GABA_A$ receptor-mediated synaptic response by increasing the decay time constant of sIPSCs in hippocampal CA1 pyramidal neurons. These effects of (-)-α-pinene on sleep and inhibitory synaptic response were mimicked by zolpidem, acting as a modulator for $GABA_A$-BZD receptors, and fully antagonized by flumazenil, an antagonist for $GABA_A$-BZD receptor. (-)-α-Pinene was found to bind to aromatic residues of α1 and □2 subunits of $GABA_A$-BZD receptors in the molecular model. We conclude that (-)-α-Pinene enhances the quantity of NREMS without affecting the intensity of NREMS by prolonging GABAergic synaptic transmission, acting as a partial modulator of $GABA_A$-BZD receptors and directly binding to the BZD binding site of $GABA_A$ receptor.

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Introduction

Pine trees of the genus *Pinus* comprise of more than 100-250 species and are widely spread out all over the world (Inoannou et al., 2014; Judzentiene and Kupcinskiene, 2008). It has been one of the most popular plants worldwide due to their medicinal and aromatic properties (Lee et al., 2005; Yang et al., 2010). It has been accepted that these traditional and pharmaceutical uses of pines are due to their essential oils (Hmamouchi et al., 2001). Pine essential oils possess numerous biological activities, such as anti-inflammatory, anti-microbial, analgesic, and anti-stress effects (Suntar et al., 2012; Xie et al., 2015). They are mainly composed of monoterpene such as α- and β-pinene, 3-carene, limonene, and terpinene (Judzentiene and Kupcinskiene, 2008).

α-Pinene (2,6,6,-trimethylbicyclo(3.1.1)-2-hept-2-ene) is the major monoterpene of pine essential oils (Groot and MacDonald, 2002) and a hydrocarbon group of bicyclic terpenes with a strong turpentine odor (Bakkali et al., 2008). It has been widely used as a food flavoring ingredient (Limberger et al., 2007; Rivas et al., 2012), and was approved as a food additive with GRAS (Generally Recognized as Safe) by US FDA (FDA, 2015). In addition, a number of studies have attributed biological properties including anti-microbial (Gomes-Carneiro et al., 2005), hypertensive (Kamal et al., 2003), anti-nociceptive (Him et al., 20008), and anti-inflammatory (Orhan et al., 2006) effects to α-pinene.

Recently, Satou et al. reported that inhalation of α-pinene produces anxiolytic activity in an elevated plus maze test in mice (Satou et al., 2014). They also confirmed the accumulation of α-pinene in the brain. According to Yamaoka et al., inhalation of α-pinene significantly increased rapid-eye movement sleep in rats (Yamaoka et al., 2005). Despite the popular usage of α-pinene as a food ingredient and therapeutic agent, its

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action on sleep and anxiety, the related molecular mechanism, and effect by oral supplementation of α-pinene have not been determined yet. In this study, we investigated the hypnotic effect of orally administrated α-pinene. Using pharmacological tools (e.g. zolpidem and flumazenil), electrophysiology, and molecular modeling, we set out to identify the molecular mechanism of α-pinene.

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Materials and methods

Materials

(-)-α-Pinene (CAS no. 7785-26-4) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Zolpidem (CAS no. 82626-48-0), a $GABA_A$-benzodiazepine (BZD) receptor agonist, was used as a reference hypnotic drug and obtained from Ministry of Food and Drug Safety, Cheongwon-gun, Chungcheongbuk-do, Korea. Flumazenil (CAS no. 78755-81-4), an antagonist of $GABA_A$-BZD receptors, was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Molecular structures and weights of (-)-α-pinene, 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°C, 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.

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Pentobarbital-induced sleep test

The initial screening for hypnotic effect of (-)-α-pinene sleep was done with pentobarbital-induced sleep, as previously described (Cho et al., 2011). 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. (-)-α-Pinene 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 (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 the loss and the recovery of the righting reflex.

Analysis of sleep architecture

Pharmacological treatments

(-)-α-Pinene 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 25, 50, or 100 mg/kg. The positive control zolpidem (10 mg/kg) was administered in the same manner as (-)-α-Pinene. Flumazenil was dissolved in sterile saline and injected intraperitoneally (i.p.) 15 min before (-)-α-Pinene 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.).

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Polygraphic recordings and vigilance state analysis

Under pentobarbital anesthesia (50 mg/kg, i.p.), the C57BL/6N mice were chronically implanted with a head mount (#8201, Pinnacle Technology Inc., Lawrence, KS, USA) installed with electroencephalogram (EEG) and electromyogram (EMG) electrodes for polysomnographic recordings. The front edge of the head mount was placed 3.0 mm anterior to bregma, and four electrode screws for EEG recording were positioned in holes perforated into the skull. Two EMG wire electrodes were inserted into the nuchal muscles. The head mount was fixed to the skull with dental cement. After surgery, mice were allowed to recover in individual cages for 1 week, and habituated to the recording conditions for 3–4 days before the experiment. The EEG and EMG recordings were carried out by means of a slip ring designed so that the movement of the mice was not restricted. EEG and EMG were recorded using the PAL-8200 data acquisition system (Pinnacle Technology Inc., Lawrence, KS, USA). The EEG and EMG signals were amplified (100×), filtered (low-pass filter: 25 Hz EEG and 100 Hz EMG), and stored at a sampling rate of 200 Hz. Sleep states were monitored for a period of 48 h, which comprised baseline and experimental days. Baseline recordings were taken for each animal during 24 h, beginning at 17:00 h. These baseline recordings served as controls for the same animal. The mice were considered asleep showing no EMG signal. The vigilance states were automatically classified by a 10 s epoch as wakefulness (Wake), rapid eye movement sleep (REMS), or non-REM sleep (NREMS) by SleepSign ver. 3.0 (Kissei Comtec, Nagano, Japan). As a final step, defined sleep-wake stages were examined visually and corrected if necessary. The sleep latency was defined as the time from drug administration to the appearance of the first NREMS episode lasting for at least 20 s.

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Bouts of NREMS, REMS and Wake were defined as periods of one or more consecutive epochs (each epoch: 10 s). Each delta power of NREMS in the range of 0.5–4 Hz was first summated and then normalized as a percentage of the corresponding mean delta power of NREMS.

Electrophysiological measurement

Slice preparation

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 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4, 1 $CaCl_2$, 3 $MgCl_2$, 10 glucose at pH 7.4, and was bubbled with 5% CO_2 / 95% O_2. 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.

Recording of sIPSCs

The standard ACSF recording solution was composed of (mM): 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4, 1.5 $CaCl_2$, 1.5 $MgCl_2$ and 10 glucose saturated with 95% O_2–5% CO_2, 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 (2-amino-5-phosphonopentanoic acid; 50μM; Tocris) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; 20μM; Tocris) were added into ACSF. Recordings were

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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

Homology Modeling

Homology modeling was performed with MODELLER in DiscoveryStudio program (Accelrys, CA, USA). The X-ray crystal structure of a human GABAA receptor, the β3 homopentamer (PDB code: 4COF) was employed as template for the most abundant α1β2□2 subtype of GABAA receptor (Miller and Aricescu, 2014). The sequences of the human α1 (P14867), β2 (P47870) and □2 (P18507) were retrieved from the UniProt database (http://www.uniprot.org) and aligned with ClustalW implemented in DiscoveryStudio program. The mismatch residues between α1 and β3 subunits were manually edited to remove gaps following the sequence alignment reported by Miller et al. (Miller and Aricescu, 2014). The sequence identities of α1, β2 and □2 with β3 were 40.6%, 89.8% and 41.4%, and the sequence similarities of them were 62.6%, 95.8% and 63.7%. Procheck validation indicated that 1669 of total 1670 residues of homology model was in allowed region of Ramachandran plot, and only one residue Arg204 of α1 subunit was an outlier. The co-crystalized ligands containing benzamidine, sugars, and ions were removed and the best model of α1β2□2 subtype $GABA_A$ receptor among 50 generated models having various conformations with MODELLER was selected by optimally satisfying spatial restraints derived from the alignment and expressed as probability density functions (pdfs) for the features

restrained, which is calculated from the relationship of Cα-Cα pairs and main-chain dihedral angles between homology model and template as spatial restraints (Sali and Blundell, 1993).

Molecular Docking Study

Molecular Docking was performed using Schrodinger package program (Schrodinger LLC, NY, USA). Homology model of α1β2□2 subtype GABAA receptor was neutralized and energetically minimized with OPLS2005 forcefield using Protein Prep Wizard. Parameters of Protein Prep Wizard were set to default value. Chemical structures of (-)-α-pinene, zolpidem and flumazenil were sketched using ChemDraw program. Ligands were prepared with protonation at pH 7.4 and energy minimization with OPLS2005 forcefield using LigPrep module. Parameters of Ligprep were set to default value. Binding modes of ligands were predicted into benzodiazepine (BZD) site of GABAA receptor using InduceFit docking module. The grid box was automatically set into the centroid region of α1Y209, α1H101, α1Y159, □2F77, □2M130 and □2T142 residues. Glide XP docking algorithm was used for more extensive through torsional refinement and sampling. Other parameters were default. Predicted binding poses of compound were selected with low Gscore for interactions. Gscore is an empirical scoring function that includes interaction energies of hydrogen bond, hydrophobic, van- der Waals in binding site and ligand strain energy.

Data analysis

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All data were expressed as the mean ± SEM (standard error of mean). Statistical analysis was performed with the Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). 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.

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Results

Effects of (-)-α-pinene in the pentobarbital-induced sleep test in ICR mice

In order to investigate whether oral administration (p.o.) of (-)-α-pinene (Fig. 1) produces sedative-hypnotic effects, we first used the pentobarbital-induced sleep test in ICR mice. As expected, a well-known hypnotic drug, zolpidem (Fig. 1; 10 mg/kg, p.o.) significantly ($p < 0.01$) potentiated pentobarbital-induced sleep in mice relative to the control group (Fig. 2). (-)-α-Pinene (12.5, 25, 50, and 100 mg/kg, p.o.) also decreased sleep latency and increased sleep duration in a dose-dependent manner. In particular, administration of 100 mg/kg of (-)-α-pinene was found to prolong sleep duration up to 114.7 ± 8.2 min, to the level similar to that of zolpidem at 10 mg/kg (124.2 ± 4.9 min).

Effects of (-)-α-pinene on sleep architecture in C57BL/6N mice: Sleep latency and amounts of NREMS and REMS

To determine the effects of (-)-α-pinene (p.o.) on normal sleep, we used analysis of sleep architecture based on EEG and EMG recordings (Fig. 3A). Representative waveforms of EEG and EMG upon sleep states were shown in Fig. 3B. EEG power of delta (0.5 – 4 Hz) and theta (6 – 10 Hz) waves were measured by Fast Fourier Transformation (FFT) (Fig. 3C). Fig. 4 presents the effects of (-)-α-pinene (25, 50 and 100 mg/kg) and zolpidem (10 mg/kg) on sleep latency, NREMS, and REMS in C57BL/6N mice. Examples of EEG and EMG signals and corresponding

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hypnograms from a single mouse during the first 3 h were shown in Fig. 4A. Concentration of (-)-α-pinene was chosen from the pentobarbital-induced sleep test.

The values of sleep latency for (-)-α-pinene (100 mg/kg) and the positive control zolpidem (10 mg/kg) were 19.6 ± 3.5 and 8.1 ± 2.0 min, respectively (Fig. 4B). Both of (-)-α-pinene ($p < 0.05$) and zolpidem ($p < 0.01$) produced a significant decrease in sleep latency. The decrease in sleep latency in mice treated with (-)-α-pinene indicates that it accelerates the initiation of NREMS just as zolpidem does. We calculated the amounts of NREMS and REMS during the first 3 h after the administrations of (-)-α-pinene and zolpidem (Fig. 4C). As expected, the positive control zolpidem (10 mg/kg) increased the amount of NREMS by 1.7-fold ($p < 0.01$) compared to that of the vehicle. Administration of (-)-α-pinene (100 mg/kg) was found to significantly increase the NREMS by 1.3-fold ($p < 0.05$). There was no significant difference in the amount of REMS between (-)-α-pinene and zolpidem. Notably, in the pentobarbital-induced sleep test, (-)-α-pinene at 50 mg/kg significantly ($p < 0.01$) decreased sleep latency and increased sleep duration (Fig. 2A and B), whereas in the EEG and EMG experiment, (-)-α-pinene at the same dose did not show any significant difference (Fig. 4B and C).

Time-course change of NREMS, REMS, and Wake

Fig. 5 shows the time course of NREMS, REMS, and Wake for 24 h after the administration of (-)-α-pinene (100 mg/kg) and zolpidem (10 mg/kg). (-)-α-Pinene (100 mg/kg) significantly increased the amount of NREMS during the second and third hours after administration by 1.37- and 1.58-fold relative to vehicle, respectively (Fig. 5A). This enhancement of NREMS was accompanied by a significant decrease in Wake during the same hours (Fig. 5A). The significant increase in NREMS by

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zolpidem (10 mg/kg) lasted for 5 h after administration (Fig. 5B). Unlike zolpidem, (-)-α-pinene did not produce a significant increase in NREMS for the first hour. Remarkably, both (-)-α-pinene and zolpidem did not affect REMS for 24 h. After an initial increase in NREMS for the first 3 h, there was no further significant change in sleep architecture during the subsequent periods (Fig. 5). This result indicates that (-)-α-pinene induced NREMS without causing adverse effects after sleep induction, which is consistent with previous findings (Masaki et al., 2012).

Characteristics of sleep-wake episodes and power density

To better understand the nature of sleep-enhancing effects of (-)-α-pinene, we additionally analyzed the total number and mean duration of NREMS, REMS, and Wake episodes, as well as sleep stage transition and EEG power density (Fig. 6). Both (-)-α-Pinene and zolpidem significantly increased the number of bouts of Wake ((-)-α-pinene: 1.9-fold, $p < 0.05$; zolpidem: 2.1-fold, $p < 0.01$) and NREMS ((-)-α-pinene: 40%, $p < 0.05$; zolpidem: 70%, $p < 0.01$) but not REMS (Fig. 6A). Moreover, both (-)-α-pinene and zolpidem significantly decreased the duration of Wake ((-)-α-pinene: 52%, $p < 0.05$; zolpidem: 56.5%, $p < 0.01$) without affecting that of NREMS or REMS (Fig. 6A). Finally, both (-)-α-pinene and zolpidem significantly increased the number of state transitions from Wake to NREMS and from NREMS to Wake, whereas the number of stage transitions from NREMS to REMS and from REMS to NREMS were not affected. These results indicate that (-)-α-pinene inhibited the maintenance of Wake, consistent with the previous report (Omori et al., 2012).

So far, we observed an enhancement of quantity of sleep by (-)-α-pinene as evidenced by decrease of sleep latency and increase of NREMS time. Next, to evaluate the sleep intensity, we analyzed the EEG power density in mice during

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NREMS and measured the delta activity. We found that (-)-α-pinene did not affect the EEG power density (0-20 Hz) including delta activity (frequency range of 0.5–2.5 Hz) in NREMS compared to vehicle (Fig. 6C), whereas zolpidem significantly decreased the delta activity, indicating a slight loss of sleep intensity by zolpidem (Fig. 6C). These results suggest that, unlike zolpidem, (-)-α-pinene increased the quantity of sleep without compromising the sleep intensity.

Molecular mechanism of (-)-α-pinene

It has been reported that some monoterpenes such as borneol, verbenol, and pinocarveol act as a positive modulator of the $GABA_A$ receptors (Granger et al., 2005; Kessler et al., 2014). For this reason, we first investigated whether the GABAergic system is involved in the hypnotic effects of (-)-α-pinene. To confirm the molecular mechanism of (-)-α-pinene on $GABA_A$-BZD receptors, flumazenil (the antagonist at the $GABA_A$-BZD receptor) was pre-treated 15 min before administration of (-)-α-pinene, and then analyzed the properties of sleep. Firstly, we found that injection of flumazenil (1 mg/kg) alone did not produce significant changes in the sleep architecture (Fig. 7A), and concluded that flumazenil is not an inhibitor of sleep. Secondly, the hypnotic effect of zolpidem, the well-known $GABA_A$-BZD receptor agonist, was fully antagonized by flumazenil (Fig. 7A). Finally, flumazenil also completely inhibited the hypnotic effect of (-)-α-pinene (Fig. 7A). The time-course plot of duration of each sleep stage showed that in the presence of flumazenil (-)-α-pinene failed to alter the sleep architecture during 24 h (Fig. 7B). These results suggest that the sleep-enhancing effects of (-)-α-pinene could be due to its modulation of $GABA_A$ receptor by acting at the BZD site.

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To test the effect of (-)-α-pinene on $GABA_A$-BZD receptors at GABAergic synapses, we performed whole-cell path-clamp recordings of IPSCs from hippocampal CA1 pyramidal neurons. These neurons are known to contain α1 and α2 subunits of $GABA_A$ receptors, which are the known targets of BZD drugs (Somogyi et al., 1996; Wisden et al., 1992). We found that zolpidem (10 μM) significantly enhanced the decay time constant of sIPSCs in dose-dependent manner (Fig. 8B and C) without affecting the amplitude and frequency of sIPSCs (Fig. 8D and E), consistent with previous reports (Perrais and Ropert, 1999; Woo et al., 2014). We found that (-)-α-pinene (10 μM) also enhanced the decay time constant of sIPSCs in dose dependent manner (EC_{50}: 4.9nM, Fig. 8G and H), without affecting the amplitude and frequency of sIPSCs (Fig.8I and J). This enhancement of decay time constant by (-)-α-pinene was fully inhibited by flumazenil (1 μM) (Fig. 8G). These results suggest that (-)-α-pinene prolongs the GABAergic synaptic transmission effectively by modulating the $GABA_A$ BZD receptor.

Binding mode of (-)-α-pinene in BZD binding site of $GABA_A$ receptor

To predict the binding modes of zolpidem, flumazenil and (-)-α-pinene at the BZD binding site, the human α1β2□2 subtype of $GABA_A$ receptor was generated based on the X-ray crystal structure of β3 homopentamer $GABA_A$ receptor (PDB: 4COF; Fig. 9A, Supplemental Fig. 1) (Miller and Aricescu, 2014). Each compound was docked into the BZD binding site between α1 and □2 subunits in extracellular domain of $GABA_A$ receptor using InduceFit docking method of Maestro Program. As shown in Fig. 9B, C, and Supplemental Fig. 2, 3, the binding mode of zolpidem and flumazenil commonly showed that 1) α1Tyr209 is engaged in π-π interaction with phenyl rings, and 2) amine groups of loop C α1Ser205 is forming a hydrogen bond of carbonyl group

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of compounds. This is consistent with the fact that α1Tyr209 is crucial for high binding affinity of diazepam, zolpidem and flumazenil as shown by mutagenesis studies (Amin et al., 1997; Buhr et al., 1997). In addition, zolpidem participates three π-π interactions with loop D □2Phe77, loop A α1His101, and loop D □2Ala79, while flumazenil has hydrophobic contacts with □2Tyr58, □2Phe77 and □2Met130. This is consistent with experimental data that mutations of □2Phe77 and α1His101 lead to loss of the affinity for zolpidem, whereas the same mutations lead to only slight change in the affinity for flumazenil (Buhr et al., 1997; Wieland et al., 1992). The binding energy of (-)-α-pinene with Gscore of -6.57 kcal/mol was lower than zolpidem and flumazenil with Gscore of -9.22 and -8.78 kcal/mol, respectively. These results predict that (-)-α-pinene probably show lower binding activities than zolpidem and flumazenil. The α-pinene makes strong hydrophobic interactions with aromatic residues of α1Phe99, α1Tyr159, α1Tyr209, and □2Phe77 (Fig. 9D and Supplemental Fig. 4). These results indicate that (-)-α-pinene may positively modulate the biological function of $GABA_A$ receptor by directly binding at the BZD binding site

Discussion

We demonstrated that (-)-α-pinene enhances NREMS by prolonging the decay time constant of GABAergic synaptic transmission by directly acting at BZD binding site of $GABA_A$ receptor. Until now, it has been reported that inhalation of (-)-α-pinene produces sedative and anxiolytic effects. To our best knowledge, this is the first study to investigate the effects of oral administration of (-)-α-pinene on sleep and its hypnotic mechanism. We report that the effect of (-)-α-pinene in sleep was specific in NREMS rather than REMS by oral administration in mice (Fig. 4). Contrary to our findings, it has been reported that there was an increase of REMS by inhaled (+)-α-

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pinene in rat (Satou et al., 2014). This discrepancy might be due to different administration method, concentration, enantiomer type, or species.

It has been reported that sleep quantity, as indicated by increased duration of NREMS, is enhanced by various hypnotics including natural compounds as well as well-known drugs such as diazepam and zolpidem (Chen et al., 2012; Cho et al., 2011; Masaki et al., 2012). In addition to an increase in duration of NREMS, zolpidem can increase sleep quantity by changing the sleep architecture such as changes in bout number and sleep state transition (Chen et al., 2012; Qu et al., 2012). We found that, just like zolpidem, (-)-α-pinene not only enhanced the duration of NREMS (Fig. 4), but also decreased the Wake bout (Fig. 6) and increased the number of transition in Wake to NREMS and NREMS to Wake (Fig. 6). Based on these results, we conclude that (-)-α-pinene acts as a hypnotic by enhancing sleep quantity.

When it comes to "good sleep," it is important to consider sleep quality in addition to sleep quantity. Although it is difficult to define sleep quality in rodents, it has been widely accepted that delta (0.5-4 Hz) activity could be a good indicator of the quality or intensity of NREMS (Chen et al., 2012; Tobler et al., 2001). In human and rodent, it has been reported that diazepam and zolpidem increase the sleep quantity in NREMS, but paradoxically reduce the delta activity (Feinberg et al., 2000; Tobler et al., 2001). In our study, zolpidem produced a typical decrease in delta activity as expected. Interestingly, (-)-α-pinene did not alter delta activity (Fig. 6C), consistent with other natural compounds that show hypnotic effects (Cho et al., 2014; Yoon et al., 2014). Therefore, (-)-α-pinene shows an advantage over zolpidem or diazepam in that it preserves intensity of NREMS.

A bicyclic monoterpene borneol, which is found in essential oils for analgesia and anesthesia in traditional Chinese medicine, acts as a positive modulator at $GABA_A$ receptors (Granger et al., 2005). It has been reported that the other monoterpenes verbenol and pinocarveol potentiated the GABA action of the $GABA_A$

receptors (Kessler et al., 2014). These reports suggest that hypnotic effect of (-)-α-pinene may involve GABAergic mechanism. Therefore, in order to demonstrate the action mechanism of (-)-α-pinene, the effect of flumazenil on the hypnotic effect of (-)-α-pinene was tested. Flumazenil is a well-known antagonist of the $GABA_A$-BZD receptors, and inhibits the effects of BZD agonists such as zolpidem and diazepam by competitively blocking their binding (Chen et al., 2012; Johnston, 2005). Hypnotic effects of zolpidem and (-)-α-pinene were completely blocked by flumazenil (Fig. 7). These findings support that the hypnotic effects of (-)-α-pinene should be attributed to the positive allosteric modulation of $GABA_A$ receptors at the BZD-binding site, via a mechanism similar to that of zolpidem.

BZD agonists such as diazepam and zolpidem are known to enhance GABAergic inhibitory signaling by prolonging the decay time constant of $GABA_A$ receptors in various brain regions including thalamus, hippocampus, and neocortex (Bacci et al., 2003; Perrais and Ropert, 1999; Woo et al., 2014). In our recent study, we reported that the single compound, isoliquiritigenin, derived from flavonoids shows the same effects of prolonging the decay time constant of $GABA_A$ receptor-mediated IPSCs, acting as a modulator of $GABA_A$-BZD receptors, and thus enhancing the quantity of NREMS (Cho et al., 2011; Woo et al., 2014). In the current study, we found the same effects of (-)-α-pinene. Based on these findings, we can predict based on the ability to prolong the decay time constant of IPSCs whether a certain drug or chemical can act as a potential modulator acting at the BZD site of $GABA_A$-BZD receptors and possibly enhance the quantity of NREMS. This assay can serve as a simple screening method for finding potential hypnotics mimicking benzodiazepines.

The molecular modeling results demonstrate that (-)-α-pinene directly binds to the BZD binding site of $GABA_A$. To investigate the binding mode of (-)-α-pinene at the BZD binding site of $GABA_A$, we generated a homology model of α1β2□2 $GABA_A$ receptor, which is most abundant in physiological system, based on the X-ray crystal

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structure of β3 homopentamer $GABA_A$ receptor (Miller and Aricescu, 2014) because the β3 homopentamer lacks the BZD binding site. We conducted molecular docking study of (-)-α-pinene including zolpidem and flumazenil at the BZD binding site of putative model of α1β2□2 GABAA receptor. The binding modes of zolpidem and flumazenil at the BZD binding site between α1 and □2 subunits were in well agreement with experimental study of mutagenesis. It has been reported that the residue of α1Tyr209 is essential for binding of ligands at BZD binding site, whereas the residues of α1His101 and □2Phe77 are needed for binding of zolpidem, but not for flumazenil (Buhr et al., 1997; Wieland et al., 1992). The (-)-α-pinene was well accommodated into the binding pocket and shared the similar hydrophobic interactions as flumazenil and zolpidem, with key residues of α1Phe99, α1Tyr159, α1Tyr209 of and □2Phe77 at the BZD binding site.

From the molecular modeling, we obtained binding energy for each ligand and its rank order: (-)-α-pinene (-6.57 kcal/mol) < flumazenil (-8.78 kcal/mol) < zolpidem (-9.22 kcal/mol). Based on this rank order, we can make various predictions on physiological effects of each ligand. First of all, it is possible that the deficiency of both π-π interaction of (-)-α-pinene with α1Tyr209 and hydrogen bond of (-)-α-pinene with α1Ser205 might have resulted in lower binding energy, lower potency, and lower efficacy than those of zolpidem and flumazenil. For example, zolpidem at 1μM enhanced the sIPSC decay by about 35%, whereas (-)-α-pinene enhanced by about 20% at the same concentration, indicating a lower potency of (-)-α-pinene than zolpidem. The efficacy of (-)-α-pinene, which at 1μM already showed saturation at 30% of decay, appears to be lower than zopidem, which has not been saturated even at 10μM (about 60%), suggesting that (-)-α-pinene might act as a partial modulator at the BZD binding site of $GABA_A$ receptor. These rank orders of potency and efficacy are in line with the rank order of binding energy for (-)-α-pinene and zolpidem. In contrast, (-)-α-pinene

showed higher affinity (EC50: 4.9 nM) than zolpidem in sIPSC decay, which did not correlate well with the rank order of binding energy.

In summary, when we compare the effectiveness between zolpidem and (-)-α-pinene based on electrophysiology, molecular modeling, and sleep behavior, the sleep quantity was positively correlated with efficacy, potency, and binding energy, whereas the sleep intensity was not (Table 1). Furthermore, the sleep intensity was preserved when (-)-α-pinene acted as a partial modulator. Although (-)-α-pinene showed lower binding energy and efficacy than zolpidem, the effectiveness of (-)-α-pinene for sleep seems to be better than zolpidem when we consider both sleep quantity and sleep intensity. Based on these results, we propose that various parameters obtained from electrophysiology and molecular modeling (e.g. affinity, efficacy, potency, and binding energy) could be a good maker for screening of novel drugs for sleep.

In conclusion, by combining sleep behavior analysis, electrophysiology, and molecular modeling, we demonstrate the feasibility of finding a sleep modulator. We report that (-)-α-pinene can be a useful hypnotic by its potent action at the BZD site of $GABA_A$ receptors as well as by its easy accessibility. In addition to hypnotic role of (-)-α-pinene, it has been shown to display a variety of beneficial properties such as anxiolytic, anti-inflammatory, antioxidant. Most importantly, we can obtain these beneficial effects of (-)-α-pinene from our daily life (e.g. strolling in woods or inhaling essential oil). Through our demonstration on the hypnotic effect of (-)-α-pinene by oral administration, we propose that (-)-α-pinene could be a good therapeutic agent for treating sleeping disorder or anxiety.

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Authorship contributions

Participated in research design: Yang, Woo, Lee, and Cho.

Conducted sleep experiments: Yang, Um, and Yoon.

Conducted electrophysiological experiments: Woo.

Contributed molecular modeling: Pae, Cho, and Park.

Wrote or contributed to the writing of the manuscript: Yang, Um, Woo, Pae, Cho, Lee, and Cho.

Supervised the study: Lee and Cho.

The authors have declared no conflicts of interest.

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Footnotes

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Figure legends

Figure 1. Chemical structures and molecular weight (MW) of (-)-α-pinene, zolpidem and flumazenil.

Figure 2. Effects of the administration of (-)-α-pinene alone and with flumazenil on sleep latency and sleep duration in pentobarbital-treated (45 mg/kg, i.p.) ICR mice. The CON (5% tween 80–saline, 10 ml/kg), zolpidem, and (-)-α-pinene were administered orally (p.o.) to mice 45 min before injection (i.p.) of pentobarbital. Flumazenil was intraperitoneally injected (i.p.) 15 min before oral administration of CON, zolpidem, and (-)-α-pinene. Each value the mean ± SEM calculated from 10 mice. *p < 0.05, **p < 0.01, significant compared to the CON (Dunnett's test). ##p < 0.01, significant between the flumazenil treatment and non-flumazenil treatment (unpaired Student's *t*-test). Abbreviations: CON, control group; NS, not significant.

Figure 3. Typical electroencephalogram (EEG) and electromyogram (EMG) waveforms and fast Fourier transform (FFT) spectrum of C57BL/6N mouse in states of wakefulness (Wake), rapid eye movement sleep (REMS) and non-REMS (NREMS).

Figure 4. Sleep-wake profiles in C57BL/6N mice after oral administration (p.o.) of (-)-α-pinene or zolpidem. (A) Typical signals of EEG and EMG recordings and the corresponding hypnograms in a mouse treated with vehicle, (-)-α-pinene, or zolpidem. (B) Effects of (-)-α-pinene and zolpidem on sleep latency. (C) Amount of NREMS and REMS during the 3 h period after administration of vehicle, (-)-α-pinene, and zolpidem. Open and filled bars indicate the baseline day (vehicle administration) and experimental day ((-)-α-pinene or zolpidem administration), respectively. Each value

represents the mean ± SEM of 8 mice in each group. *p < 0.05, **p < 0.01, significantly different from their vehicle (unpaired Student's *t*-test). Abbreviations: EEG, electroencephalogram; EMG, electromyogram; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.

Figure 5. Time-course changes in NREMS, REMS, and Wake in C57BL/6N mice after oral administration (p.o.) of (-)-α-pinene (A) and zolpidem (B) during the 24 h. Open and filled circles indicate the baseline day (vehicle administration) and experimental day ((-)-α-pinene or zolpidem administration), respectively. Each circle represents the hourly mean ± SEM amount of each stage (n = 8). *p < 0.05, **p < 0.01, significantly different from their vehicle (unpaired Student's *t*-test). The horizontal filled and open bars on the X-axis (clock time) indicate the 12 h dark and 12 h light periods, respectively. Vehicle, (-)-α-pinene and zolpidem were administrated at 17:00. Abbreviations: NREMS, non-rapid eye movement sleep; p.o., oral administration; REMS, rapid eye movement sleep; Wake, wakefulness.

Figure 6. Characteristics of sleep–wake episodes in C57BL/6N mice after oral administration (p.o.) of (-)-α-pinene (A) and zolpidem (B). (A) Total number and mean duration of NREMS, REMS, and Wake bouts for 3 h after the administration of (-)-α-pinene and zolpidem. (B) Sleep–wake stage transitions during the 3 h period after the administration of (-)-α-pinene and zolpidem. Open and filled bars indicate the baseline day (vehicle administration) and experimental day ((-)-α-pinene or zolpidem administration), respectively. Each value represents the mean ± SEM of 8 mice in each group. (C) EEG power density curves of NREMS caused by (-)-α-pinene and zolpidem. Delta activity, an index of sleep intensity, is shown in the inset histogram. The bar (▬) represents the range of the delta wave (0.5□4 Hz). *p < 0.05, **p < 0.01, significantly different from their vehicle (unpaired Student's *t*-test). Abbreviations: NREMS (or N),

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non-rapid eye movement sleep; REMS (or R), rapid eye movement sleep; Wake (or W), wakefulness.

Figure 7. Effect of flumazenil treatment on (-)-α-pinene and zolpidem induced sleep of C57BL/6N mice. (A) Amount of NREMS, REMS, and Wake for 3 h after pretreatment with flumazenil (1 mg/kg, i.p. at 16:45 h) and oral administration (p.o.) of (-)-α-pinene (100 mg/kg, p.o. at 17:00 h), zolpidem (10 mg/kg, p.o. at 17:00 h) and each vehicle in mice. (B) Time-course changes in NREMS, REMS, and Wake after administration of vehicle, flumazenil, and (-)-α-pinene. The horizontal filled and open bars on the X-axis (Clock time) indicate the 12 h dark and 12 h light periods, respectively. Open and filled bars (or circles) indicate the baseline day (vehicle administration) and experimental day ((-)-α-pinene and zolpidem administration). Date represents the mean ± SEM of 8 mice in each group. *$p < 0.05$, significantly different from vehicle (unpaired Student's *t*-test). Abbreviations: NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.

Figure 8. (A) Representative traces of sIPSC before (CON) and after treatment of zolpidem (10μM). (B) Averaged sIPSCs after normalization by peak (left). Decay was fitted to one-exponential functions. Summary bar graph of sIPSC decay value before and after treatment of zolpidem (right). Data are represented as mean ± SEM. **$p<0.01$, Students' tailed *t*-test. C-E. Summary graphs of sIPSC decay tau value (C), amplitude (D), and frequency (E) after normalization by control response. (F) Representative traces of sIPSC before (CON) and after treatment of (-)-α-pinene (10μM). (G) Averaged sIPSCs after normalization by peak (left). Summary bar graph of sIPSC decay value before and after treatment of (-)-α-pinene and flumazenil (1μM, right). *$p<0.05$, one-way ANOVA test. (H-J). Summary graphs of sIPSC decay tau

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value (H), amplitude (I), and frequency (J) after normalization by control response. Decay response was fitted using sigmoidal logistic 4 parameters.

Figure 9. Putative binding modes in BZD binding site of $GABA_A$ receptor. Top and side views of $GABA_A$ receptor homology model (A), the binding pose of zolpidem (B), flumazenil (C) and (-)-α-pinene (D) obtained by InduceFit docking method implemented in Schrodinger program. The PDB files of putative binding modes are in Supplementary data figures 1-4. Representation shows the α1-subunit (blue), β2-subunit (Green) and □2-subunit (red). Ligand and key residues are shown with stick. Hydrogen bond interactions are depicted by green dotted line and hydrophobic phobic interactions are indicated by purple dotted line. Square denotes the BZD binding site between α1-subunit and □2-subunit in the human $GABA_A$ receptor. ECD: extracellular domain, TMD: transmembrane domain.

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Table

	Zolpidem	(-)-α-pinene
Electrophysiology		
Affinity	Low	High
Efficacy	High	Low
Potency (at 1μM)	High	Low
Molecular modeling		
Binding energy	High	Low
Sleep effect		
Quantity	Increase	Increase
Intensity	Decrease	No effect

Table 1. Comparison of effectiveness between zolpidem and (-)-α-pinene in electrophysiology, modeling and sleep behavior.

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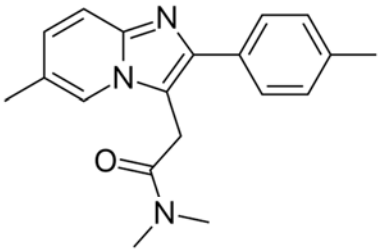
Figure 1

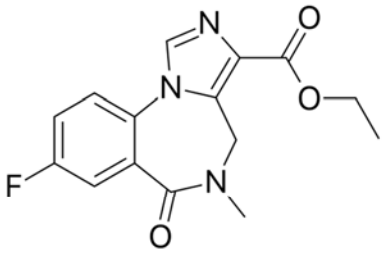
(-)-α-Pinene

$C_{10}H_{16}$ (MW: 136.23)



Zolpidem

$C_{23}H_{27}N_3O_7$ (MW: 457.48)



Flumazenil

$C_{15}H_{14}FN_3O_3$ (MW: 303.29)

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Figure 2

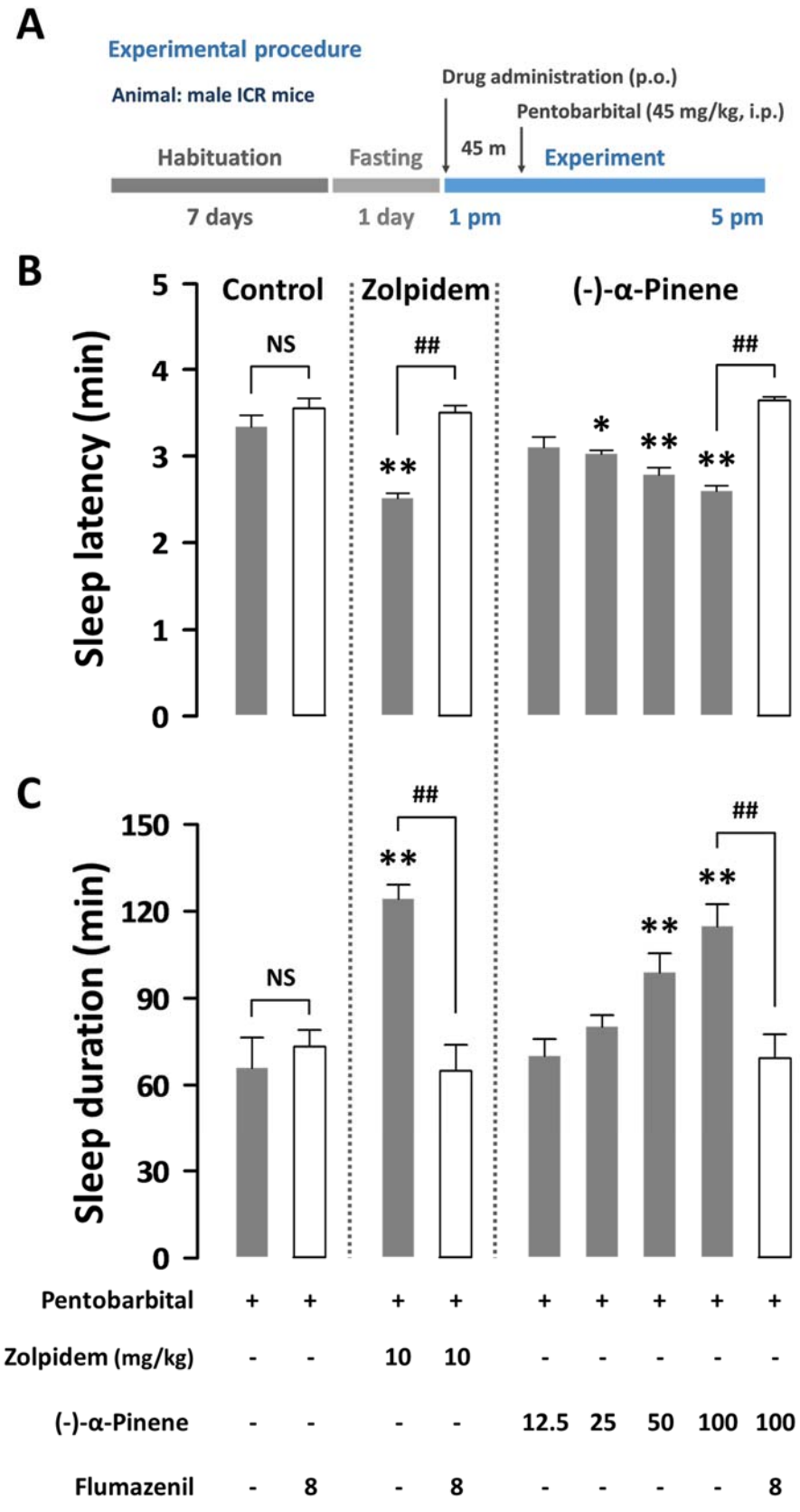

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Figure 3

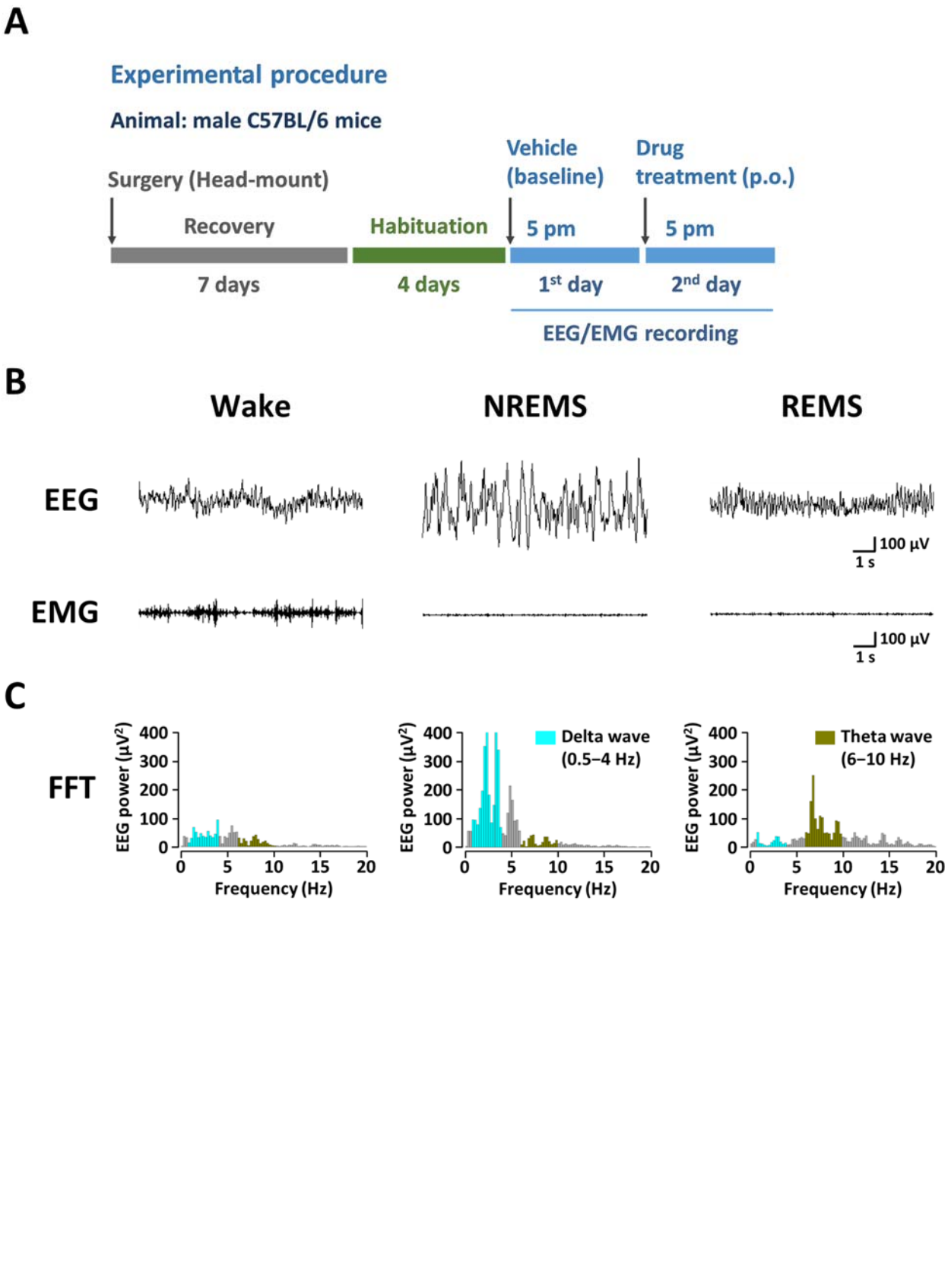

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Figure 4

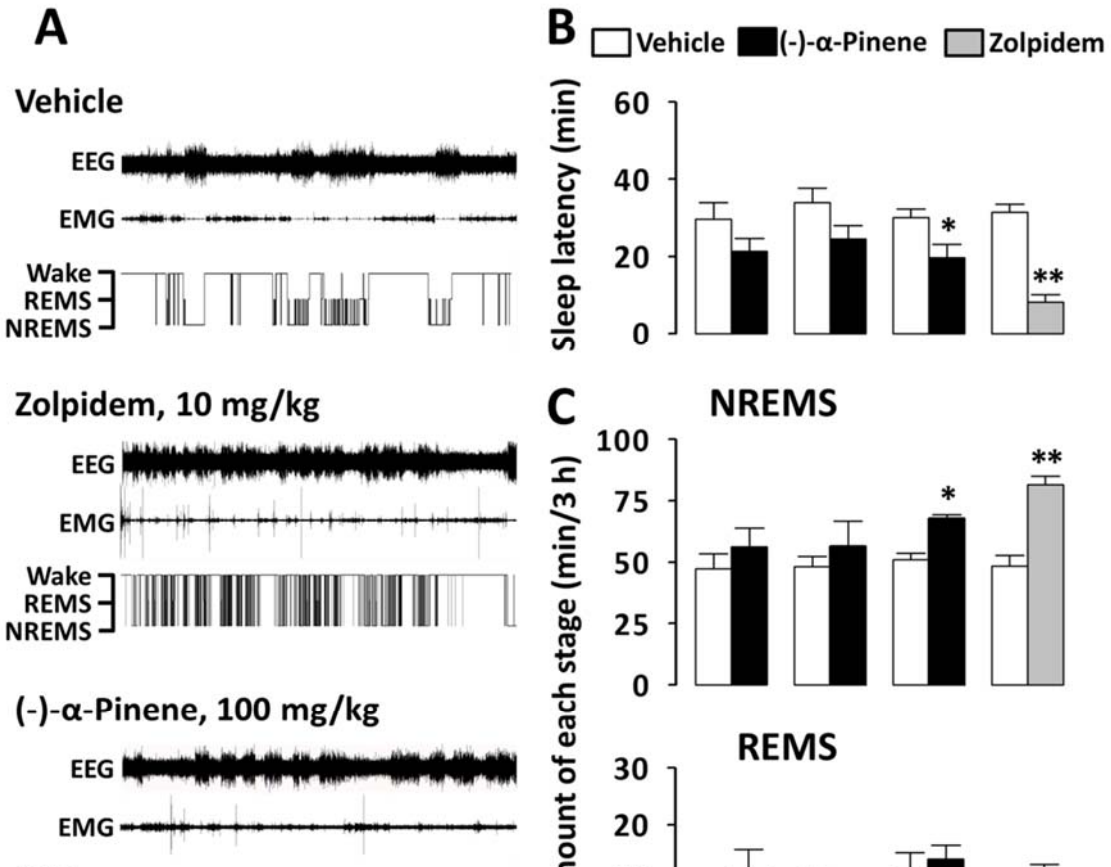

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Figure 5

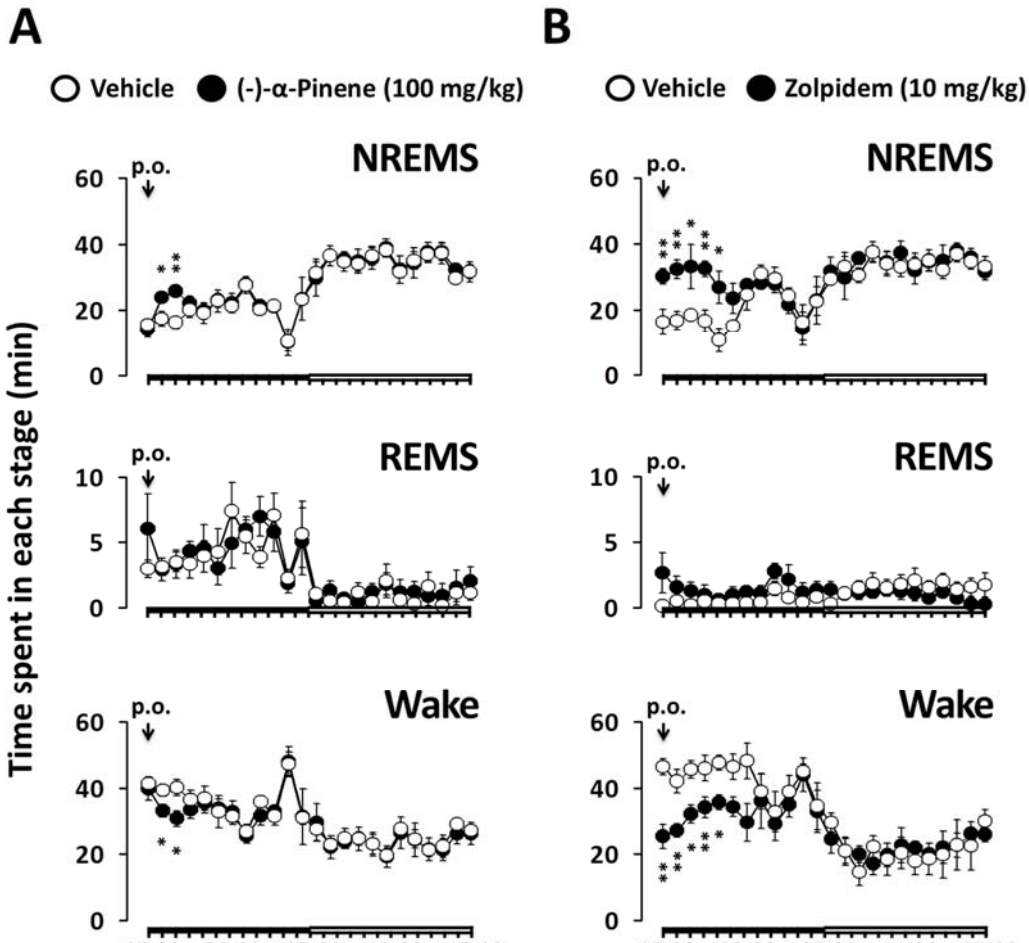

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Figure 6

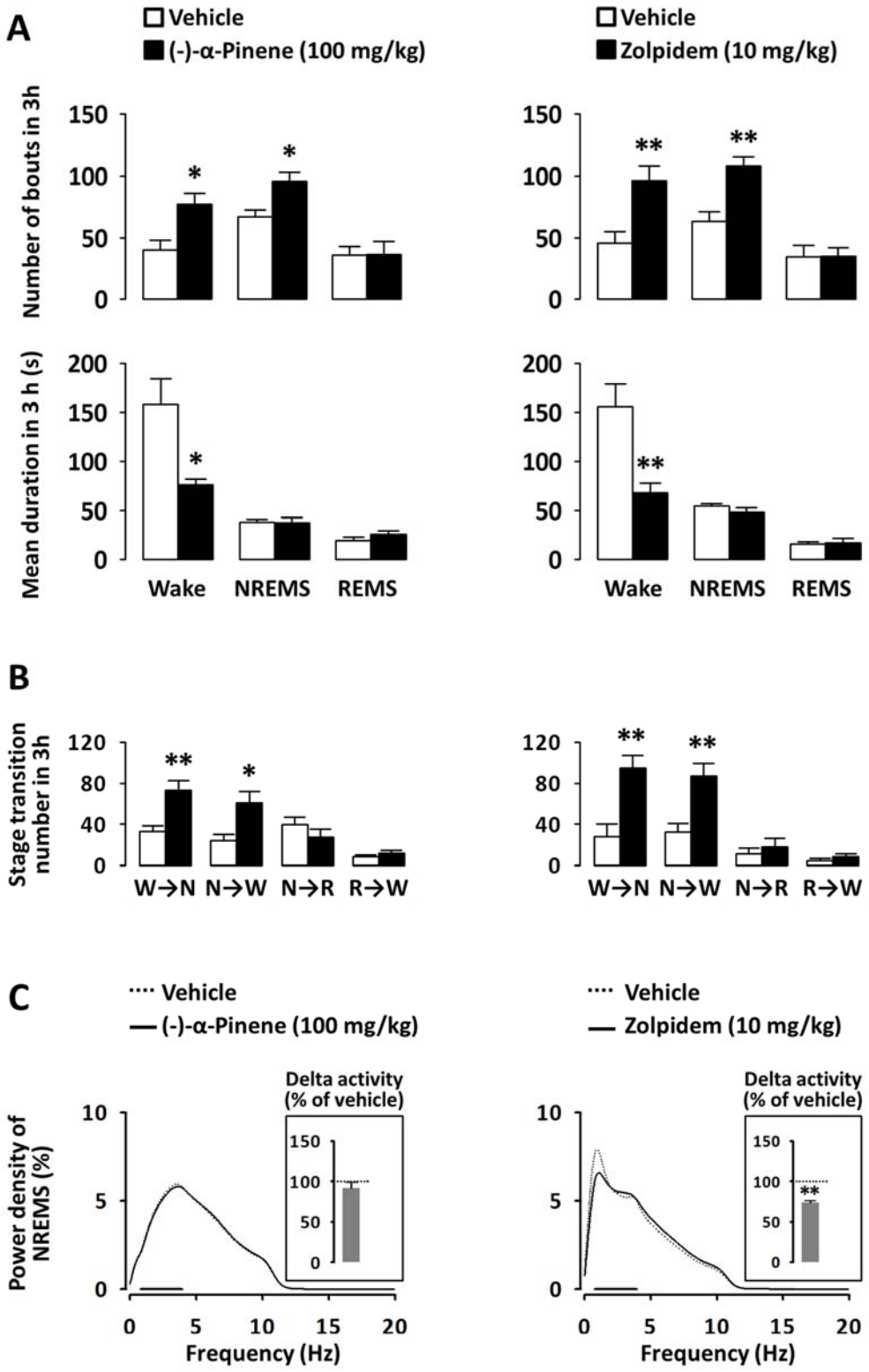

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Figure 7

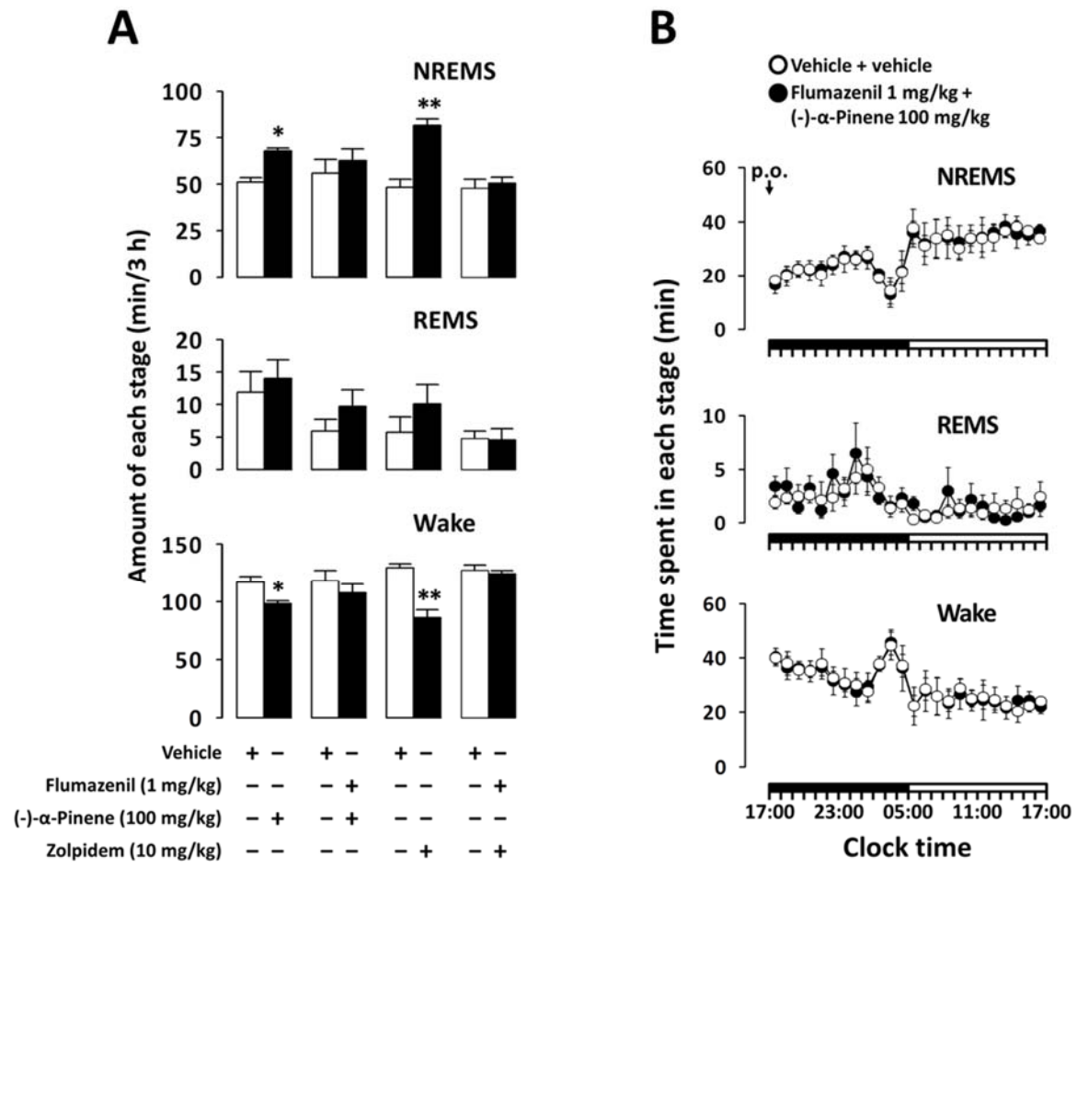

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Figure 8

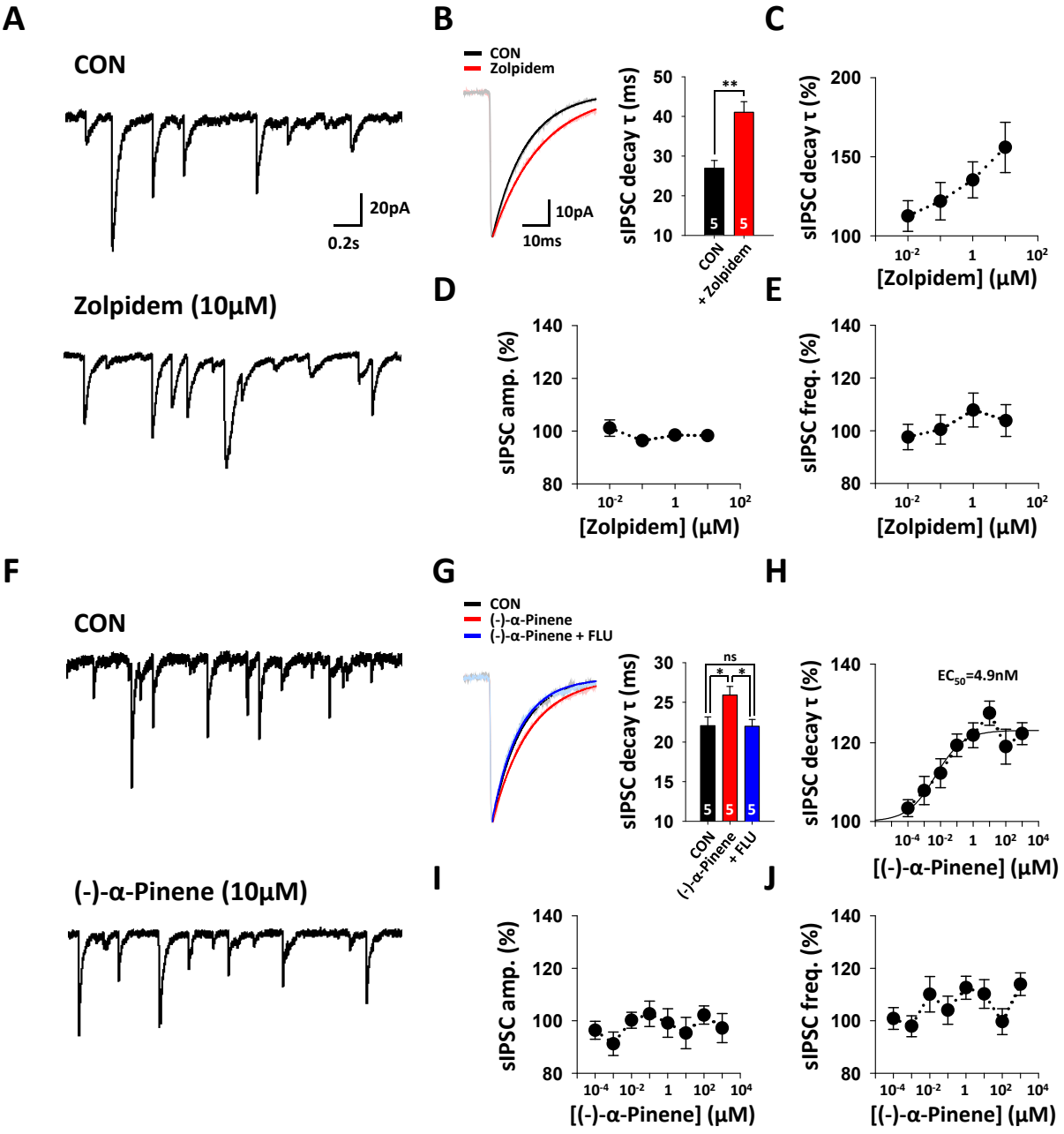

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Figure 9