Neuroscience Letters 722 (2020) 134832

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Intraperitoneal injection of ginkgolide B, a major active compound of *Ginkgo biloba*, dose-dependently increases the amount of wake and decreases non-rapid eye movement sleep in C57BL/6 mice

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ARTICLE INFO

Keywords:
Mouse
Ginkgolides
Bilobalide
Wakefulness
NREM sleep

ABSTRACT

The terpene lactones of *Ginkgo biloba* extract, namely ginkgolides (A, B, and C) and bilobalide, possess antioxidant, anti-inflammatory, and neuroprotective effects. They are widely prescribed for the treatment of cerebral dysfunctions and neurological impairments. In addition, they demonstrate antagonistic action at the gamma-aminobutyric acid type A and glycine receptors, which are members of the ligand-gated ion channel superfamily. In the present study, the effects of ginkgolides (A, B, and C) and bilobalide on sleep in C57BL/6 mice were investigated. Ginkgolide B was found to dose-dependently increase the amount of wake and decrease that of non-rapid eye movement sleep without changes in the electroencephalography power density of each sleep/wake stage, core body temperature and locomotor activity for the first 6 h after intraperitoneal injection. Of note, the amount of wake after injection of 5 mg/kg of ginkgolide B showed a significant increase (14.9 %) compared with that of vehicle ($P = 0.005$). In contrast, there were no significant differences in the amount of sleep, core body temperature, and locomotor activity in the mice injected with ginkgolide A and C. Bilobalide briefly induced a decrease in locomotor activity but did not exert significant effects on the amounts of sleep and wake. The modes of action of the wake-enhancing effects of ginkgolide B are unknown. However, it may act through the antagonism of gamma-aminobutyric acid type A and glycine receptors because it is established that these inhibitory amino acids mediate sleep and sleep-related physiology. It is of interest to further evaluate the stimulant and awaking actions of ginkgolide B on the central nervous system in clinical and basic research studies.

1. Introduction

Sleepiness is one of the most disabling symptoms for presenteeism at the work place [1,2]. In present society, a large number of individuals tend to resist or ignore the natural key sign that they need more sleep. Sleep deprivation and chronic sleep loss (i.e., sleep debt) may be harmful to the health and productivity of individuals, leading to headache, fatigue, hypertension, obesity, risk of type 2 diabetes, immune system impairment, depression, and cognitive impairment [3]. Having enough sleep is the only the solution to resolve sleep loss. However, many individuals use natural stimulants of the central nervous system (CNS), such as caffeine, for refreshment and to overcome sleepiness during daytime [4,5]. Constituents of naturally occurring non-stimulant thermogenic plants, such as capsaicin, *p*-synephrine (bitter orange extract), and chlorogenic acid (green coffee bean extract) have also been used for these purposes [6–8].

Ginkgo biloba, commonly known as ginkgo, is widely used in traditional medicine and as a source of food [9]. The *Ginkgo biloba* extract EGb 761, which is produced from the leaves of the ginkgo tree, is also extensively prescribed as a pharmacological product or dietary supplement for cerebrovascular and neurodegenerative diseases, owing to its antioxidant, anti-inflammatory, and neuroprotective effects [10–12]. In addition, a previous study showed that EGb 761 alleviated anxiety symptoms in patients with generalized anxiety disorder and adjustment disorder without sedation as opposed to benzodiazepine [13]. EGb 761 contains 22.0 %–27.0 % flavonoid glycosides and 5.4 %–6.6 % terpene lactones [14]. Among the terpene lactones, ginkgolides and bilobalide comprises 2.8 %–3.4 % and 2.6 %–3.2 %, respectively. The structures of

Abbreviations: $GABA_A$, gamma-aminobutyric acid type A; ZT, *Zeitgeber* Time

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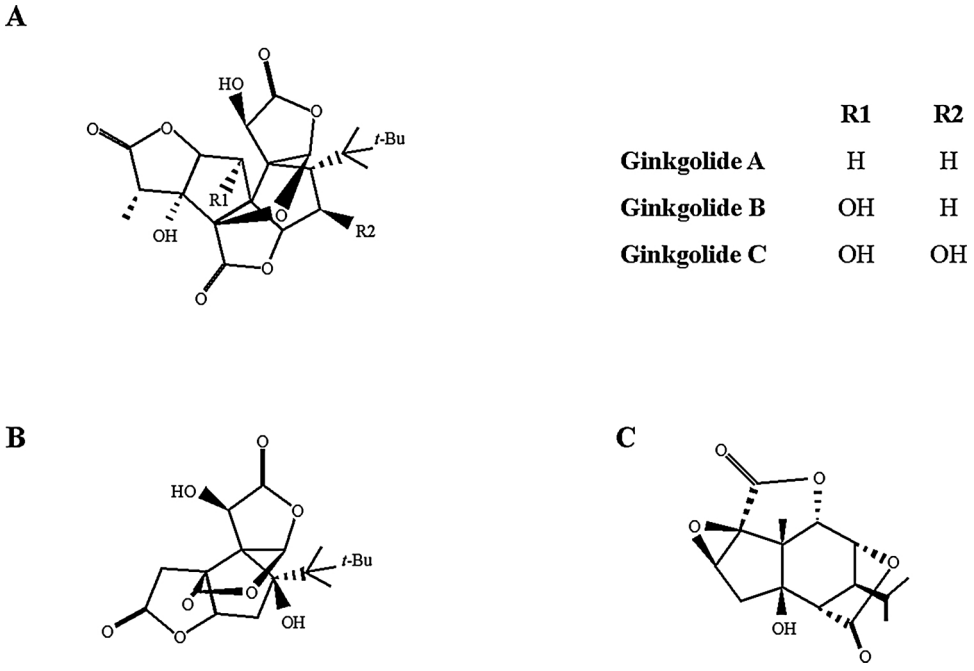
https://doi.org/10.1016/j.neulet.2020.134832
Received 10 December 2019; Received in revised form 6 February 2020; Accepted 7 February 2020
Available online 09 February 2020
0304-3940/



	R1	R2
Ginkgolide A	H	H
Ginkgolide B	OH	H
Ginkgolide C	OH	OH

Fig. 1. The structures of ginkgolide A, B, and C (**A**), bilobalide (**B**), and picrotoxinin (**C**). Ginkgolides and bilobalide, which are classified as terpene lactones, possess three lactones and a *tert*-butyl group. Furthermore, the structures of ginkgolides and picrotoxinin are similar in that they both form an equal-sized cave structure and the positions of lactone groups.

ginkgolides (A, B, and C) and bilobalide are shown in Fig. 1. The ginkgolides exert neuroprotective effects on neuronal cell damage-induced ischemic stroke, as well as inflammatory and neurodegenerative diseases, such as dementia and the Alzheimer's disease [10,15]. In particular, ginkgolide B is the most potent selective and competitive platelet-activating factor receptor antagonist, possessing antioxidant and anti-inflammatory properties [10,16].

It has been revealed that ginkgolides (A, B, and C) and bilobalide demonstrate antagonistic action at the gamma-aminobutyric acid type A ($GABA_A$) and glycine receptors in the CNS [17–20]. However, few studies have investigated the association between the *Ginkgo biloba* extract and sleep-wake function [21,22]. Although it was shown that the *Ginkgo biloba* extract counteracts sleep induced by anesthetics, there were no systematic sleep measures performed in these studies. In the current study, we evaluated the effects of ginkgolides (A, B, and C) and bilobalide on the vigilance state, core body temperature and locomotor activity using sleep electroencephalography (EEG), electromyography (EMG), and a telemetry implant in healthy C57BL/6 mice.

2. Materials and methods

2.1. Materials

Ginkgolides A, B, and C were purchased from Sigma–Aldrich, (St. Louis, MO, USA), while bilobalide was purchased from Selleckchem (Houston, TX, USA). Ginkgolide A and C, and bilobalide were stored at −20 °C. Ginkgolide B was stored at 2 °C – 8 °C.

2.2. Animals

C57BL/6 male mice, aged 15 weeks (Jackson laboratory, Bar Harbor, ME, USA), were maintained on a 12-h light/dark cycle with *ad libitum* access to food and water at an ambient temperature of 23 °C ± 1 °C. Each mouse was individually housed in a recording cage (22 cm × 16 cm × 12 cm). This study was approved by and performed according to the Stanford University Administrative Panel on Laboratory Animal Care Guidelines (APLAC-21646).

2.3. Surgical procedures for telemetry implant and headstage

A telemetry implanting device (G2 E-Mitter; Mini Mitter OR, Oakmont, PA, USA) was intraperitoneally implanted under 3 % isoflurane anesthesia to evaluate the core body temperature and locomotor activity. The mice were surgically prepared for EEG and EMG recordings with a headstage attached to the cable recorder. Two of the four electrodes, which consisted of stainless-steel screws, were implanted into the skull 1.5 mm lateral and 1.5 mm anterior to the bregma (over the motor cortex). The other two electrodes were implanted 3 mm lateral and 1 mm anterior to the lambda (over the visual cortex) for the EEG. Two Teflon-coated stainless-steel wires were inserted into the neck extensor muscles on both sides for the EMG. The six electrodes were attached to one 2 × 3 pin header that was secured to the skull with dental acrylic. Postoperatively, the mice subcutaneously received an analgesic (carprofen, 3 mg/kg) and antibiotic (enrofloxacin, 25 mg/kg), and were allowed to recover for 2 weeks prior to the experiments [23].

2.4. Procedure of data collection

After 2 weeks of postoperative recovery period, the mice were individually moved into experimental cages specifically modified to include plastic micro-isolation cages fitted with a low-torque slip-ring commutator (Biella Engineering, Irvine, CA, USA). Each cage was placed in the custom-designed recording chamber with individual ventilated compartments. The following day, the headstages of the mice were connected to the EEG/EMG recording cables, which consisted of the slip ring commutator through a 15–20 cm of lightweight six-strand shielded signal cable (NMUF6/30-4046SJ; Cooner Wire, Chatsworth, CA, USA). The commutator output was amplified. The mice had *ad libitum* access to food and water in the experimental cages. The room temperature was maintained at 23 °C ± 1 °C. A 12-h light/dark cycle was implemented throughout the experiment. After 1 week of habituation to the experimental conditions, consecutive EEG/EMG, core body temperature and locomotor activity recordings were performed.

The EEG/EMG signals were amplified using a Grass Instrument model 12 (West Warwick, RI, USA), and digitally filtered (30 Hz Low Pass Filter for EEG; 10–100 Hz Band Pass Filter for EMG). Subsequently, the EEG signals were captured at 256 Hz using a data acquisition

software (Vital Recorder; Kissei Comtec Co. Ltd., Matsumoto, Japan). EEG signals collected with ipsilateral bipolar EEG electrodes placed over motor and visual cortices, together with the bipolar EMG signals, were recorded for sleep scoring [24].

2.5. Intraperitoneal injection of ginkgolides and bilobalide

Eight mice were included in each of the four groups of ginkgolide A, B, and C or bilobalide. Ginkgolide A, B, or C or bilobalide in two doses (i.e., 0.5 mg/kg and 5.0 mg/kg) and vehicle were intraperitoneally injected at *Zeitgeber* Time (ZT) 2 on different days, with an interval of at least 48 h.

2.6. Sleep recording

We visually confirmed the sleep/wake stages on the basis of EEG and EMG signals in 10-s epochs, according to our standard criteria using the Sleepsign software (Kissei Comtec Co. Ltd.). We required the 50 % or more of a specific state in each epoch to score the epoch. Firstly, wakefulness was characterized by desynchronized, low-amplitude, and mixed-frequency (> 4 Hz) EEG with high EMG activity, which appears as a rhythmic theta/alpha (7–9 Hz) wave. Secondly, non-rapid eye movement (NREM) sleep was characterized by synchronized, high-amplitude, and low-frequency (0.25–4 Hz) EEG with low EMG activity. Thirdly, similar to wakefulness, rapid eye movement (REM) sleep was characterized by desynchronized, low-amplitude, and mixed-frequency EEG, whereas the EMG activity during REM sleep was low compared with that observed during NREM sleep. During REM sleep, some muscle fasciculation may be observed in the EMG trace. Typically, rhythmic theta/alpha (7–9 Hz) waves with reduced EMG activity are dominant [23]. Changes in the sleep state were considered when at least one 10-s epoch was scored as appearing a different sleep stage, and the duration of the state episode was determined as the duration of a continuous single state episode. We also analyzed the EEG power density of wake, REM sleep, and NREM sleep using the Sleepsign software after injection of vehicle, ginkgolides, and bilobalides. All sleep scoring was performed by a single observer (H.M.) who was blinded to animal information.

2.7. Sleep and wake data analyses

The rate (%) of each vigilance state (i.e., wake, REM sleep, and NREM sleep) for 6 h (ZT2–8, light period) after the injection of ginkgolides or bilobalide and vehicle was assessed and plotted with a 0.5-h interval. Moreover, the cumulative amount (min) for 6 h after injection was also assessed and plotted with a 0.5-h interval.

2.8. Core body temperature and locomotor data analyses

The plastic cages were individually placed on the telemetry receiver (Series 4000, Mini Mitter) that transmitted the signals for core body temperature and locomotor activity every 1 min. Subsequently, the data were acquired using the Vital View software (Mini Mitter, OR). The 0.5 -hly mean value of core body temperature (°C) after the injection of ginkgolides or bilobalide and vehicle was firstly calculated. Then, the difference of temperature between before (baseline) and after the injection was assessed and plotted with a 0.5-h interval for 6 h (ZT2–8, light period) to adjust the variability of baseline. Furthermore, the area under the curve was also assessed in each compound group for 6 h after injection. The mean value of locomotor activity (counts/h) for 6 h after the injection was assessed and plotted with a 1-h interval. The cumulative amount (counts/h) for 6 h after injection was also assessed and plotted with a 1-h interval.

2.9. Statistical analysis

All data were expressed as the mean ± standard error of the mean. All statistical analyses were performed using the SPSS version 22 software (IBM Corp., Armonk, NY, USA). Changes in the total amounts of sleep/wake, core body temperature, and locomotor activity were analyzed through repeated one-way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons. The time course changes in the amounts of sleep/wake, core body temperature, and locomotor activity as well as EEG power density distributions were analyzed through repeated two-way ANOVA (compound group [vehicle, 0.5 mg/kg, and 5 mg/kg], time, and compound × time). The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Sleep/wake amounts and time courses

Initially, we investigated the effects of ginkgolides (A, B, or C) or bilobalide on wake, REM sleep, and NREM sleep for 6 h (ZT2–8, light period) after intraperitoneal injection of 0.5 mg/kg and 5.0 mg/kg of each compound (Fig. 2). Although there were no significant differences in the amounts of sleep/wake in the ginkgolide A and C, and bilobalide groups (all $P > 0.05$, Fig. 2A, C, and D, respectively), we found dose-dependent significant differences in the total amounts of wake and NREM sleep for 6 h among the vehicle and ginkgolide B group through repeated one-way ANOVA [$F(2, 14) = 9.72$, $P = 0.002$ and $F(2, 14) = 4.93$, $P = 0.024$, respectively. (Fig. 2B)]. A *post hoc* analysis showed that the amount of wake in the 5 mg/kg of ginkgolide B group was significantly increased (14.9 %) compared with that in the vehicle group ($P = 0.005$, Fig. 2B). In addition, the amount of NREM sleep in the 5 mg/kg of ginkgolide B group was significantly decreased compared with that observed in the vehicle group ($P = 0.022$, Fig. 2B).

We also showed the time course and the cumulative amounts of wake, REM sleep, and NREM sleep up to 6 h after injection in the ginkgolide B group (Figs. 3A and B, respectively). A high dose of ginkgolide B enhanced wakefulness and reduced REM and NREM sleep at several time points during the time course; however, the differences were not statistically significant (all $P > 0.05$; Fig. 3A). By performing cumulative amount analyses, we found significant differences in the amounts of wake and NREM sleep using repeated two-way ANOVA [compound × time; $F(22, 154) = 2.84$, $P < 0.001$ and $F(22, 154) = 2.23$, $P = 0.002$, respectively; Fig. 3B]. Ginkgolide B did not change the EEG power densities of wake, REM sleep, and NREM sleep compared with those at the vehicle session (all $P > 0.05$; Fig. 3C).

3.2. Core body temperature and locomotor activity

We subsequently investigated the effects on core body temperature and locomotor activity for 6 h after the injection of ginkgolides (A, B, or C) or bilobalide (Figs. 4 and 5, respectively). We revealed the time course differences in core body temperature from pre-injection (baseline) up to 6 h after the injection of vehicle and 0.5 mg/kg and 5 mg/kg of ginkgolides or bilobalide (Fig. 4A). There were no statistically significant effects on core body temperature in the ginkgolides and bilobalide groups (all $P > 0.05$; Fig. 4B). Next, we showed the time course of locomotor activity up to 6 h after the injection of vehicle and 0.5 mg/kg and 5 mg/kg of ginkgolides or bilobalide (Fig. 5A). Although there were no significant differences in the cumulative amounts of locomotor activity for 6 h after the injection in the ginkgolide A, B, and C groups (all $P > 0.05$; Fig. 5B and C), we found a significant difference in the bilobalide group through repeated two-way ANOVA [compound × time; $F(12, 84) = 1.89$, $P = 0.047$; Fig. 5B]. We found a significant, short-lasting reduction in the cumulative amount of locomotor activity by bilobalide using repeated one-way ANOVA [$F(2, 14) = 10.7$, $P = 0.002$]. A *post hoc* analysis showed that locomotor

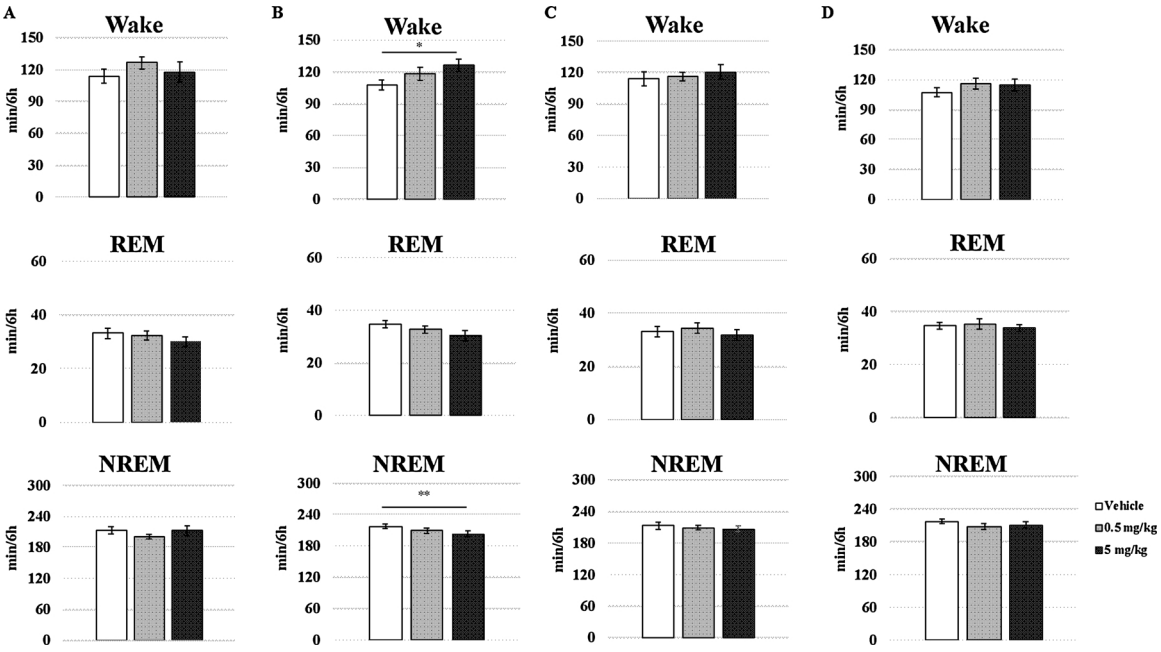


Fig. 2. Total amount (min) of each of the three sleep stages composed of wake, REM sleep, and NREM sleep for 6 h (ZT2–8, light period) after intraperitoneal injection of vehicle and 0.5 mg/kg and 5.0 mg/kg of ginkgolide A (**A**), ginkgolide B (**B**), ginkgolide C (**C**), or bilobalide (**D**). Error bars denote standard error of the mean. REM, rapid eye movement; NREM, non-rapid eye movement; ZT, *Zeitgeber* Time. * $P < 0.01$, ** $P < 0.05$.

activities were significantly decreased by injection of 5 mg/kg bilobalide compared with those recorded after injection of vehicle and 0.5 mg/kg bilobalide ($P = 0.003$ and $P = 0.006$, respectively; Fig. 5B).

4. Discussion

We evaluated the effects of ginkgolides (A, B, and C) and bilobalide on the sleep, core body temperature, and spontaneous locomotor activity in C57BL/6 mice. We found that ginkgolide B increased the amount of wake and decreases that of NREM sleep during the first 6 h after intraperitoneal injection in a dose-dependent manner. In contrast, ginkgolides A and C and bilobalide did not induce significant differences in the amounts of sleep. Interestingly, the wake-promoting effect of ginkgolide B was not associated with increases in core body

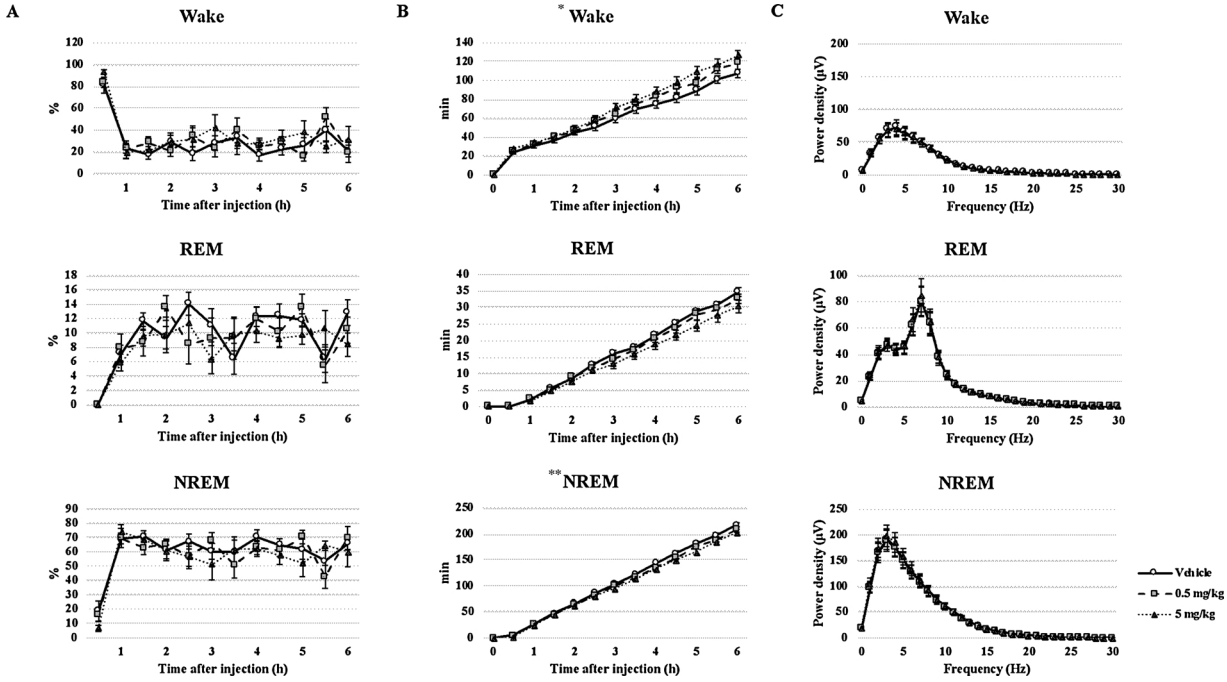


Fig. 3. (**A**) The rate (%) of wake, REM sleep, and NREM sleep for 6 h (ZT2–8, light period) after intraperitoneal injection of vehicle and 0.5 mg/kg and 5.0 mg/kg of ginkgolide B plotted with a 0.5-h interval. (**B**) The cumulative amount (min) of wake, REM sleep, and NREM sleep for 6 h (ZT2–8, light period) after intraperitoneal injection of vehicle and 0.5 mg/kg and 5.0 mg/kg of ginkgolide B plotted with a 0.5-h interval. (**C**) The EEG power density (μV) of wake, REM sleep, and NREM sleep for 6 h (ZT2–8, light period) after intraperitoneal injection of vehicle and 0.5 mg/kg and 5.0 mg/kg of ginkgolide B. Error bars denote standard error of the mean. REM, rapid eye movement; NREM, non-rapid eye movement; ZT, *Zeitgeber* Time. * $P < 0.001$, ** $P < 0.01$.

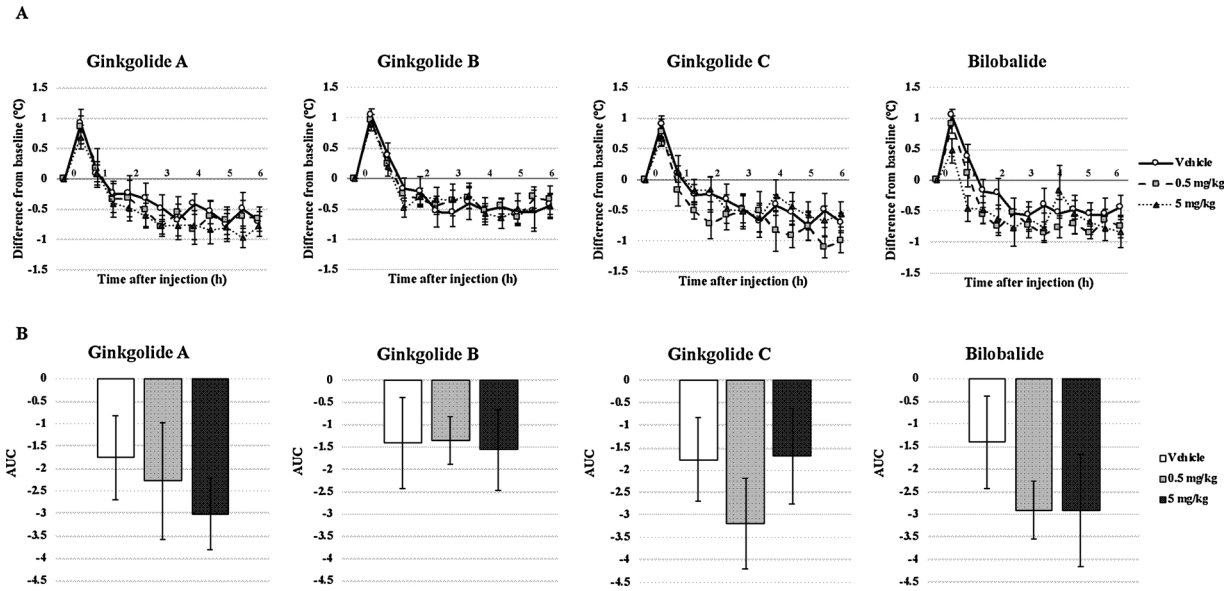


Fig. 4. (**A**) The difference of core body temperature (°C) between before (baseline) and after intraperitoneal injection of vehicle and 0.5 mg/kg and 5.0 mg/kg of ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide, respectively, for 6 h (ZT2–8, light period) plotted with a 0.5-h interval. (**B**) The AUC of core body temperature for vehicle and 0.5 mg/kg and 5.0 mg/kg of ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide, respectively, for 6 h (ZT2–8, light period). Error bars denote standard error of the mean. REM, rapid eye movement; NREM, non-rapid eye movement; ZT, *Zeitgeber* Time; AUC, area under the curve. All $P > 0.05$.

temperature and locomotor activity. Injection of ginkgolide B did not change the EEG power density distributions in any sleep/wake stages.

Two previous studies have reported that ginkgolides and bilobalide shortened the duration of sleep in anesthetized mice [21,22]. The intraperitoneal injection of 1 mg/kg of ginkgolide B reduced the barbital-induced sleep duration in mice compared with vehicle. The second study also demonstrated that 1, 2, and 5 mg/kg of ginkgolide B prolonged the barbital-induced sleep latency compared with vehicle. These findings suggested that ginkgolide B may counteract the $GABA_A$-mediated anesthetic actions of barbiturates [21]. In the current study, through systematic sleep recordings, we demonstrated that ginkgolide B promotes wakefulness in physiological sleep/wake cycles.

Ginkgolides and bilobalide have been shown to negatively modulate the function of GABA and glycine [17,20,25,26]. Thus, the wake-promoting effects of ginkgolide B may be mediated by counteracting with these inhibitory neurotransmitters in the CNS. It is well demonstrated that GABA and glycine are involved in sleep-inducing mechanisms in health and diseases. Moreover, $GABA_A$-enhancing compounds and

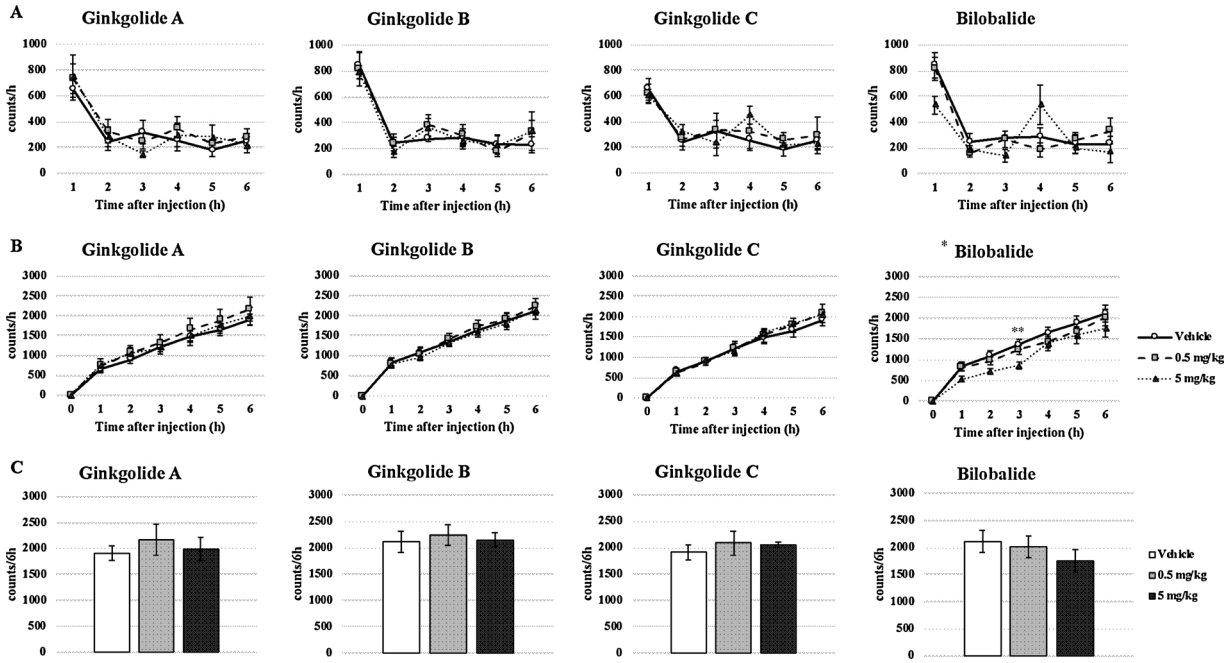


Fig. 5. (**A**) Each locomotor amount (counts) for 6 h (ZT2–8, light period) after intraperitoneal injection of vehicle and 0.5 mg/kg and 5.0 mg/kg of ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide, respectively, plotted with a 1-h interval. (**B**) The cumulative locomotor amount (counts) for 6 h (ZT2–8, light period) after intraperitoneal injection of vehicle and 0.5 mg/kg and 5.0 mg/kg of ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide, respectively, plotted with a 1-h interval. (**C**) Total locomotor amount (counts) for 6 h (ZT2–8, light period) after intraperitoneal injection of vehicle and 0.5 mg/kg and 5.0 mg/kg of ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide, respectively. Error bars denote standard error of the mean. ZT, *Zeitgeber* Time. * $P < 0.05$, ** $P < 0.01$.

glycine have been used as hypnotics and sleep aids [27,28]. In particular, we recently demonstrated that the administration of glycine alleviates acute insomnia in a rat model [28]. Furthermore, Rye et al. recently showed that $GABA_A$-mediated mechanisms may also be involved in the pathophysiology of some forms of hypersomnia (i.e., idiopathic hypersomnia) in humans [29]. Therefore, it is of interest to evaluate the wake-promoting effects of ginkgolide B in humans and identify compounds that alleviate sleepiness in various situations. This is particularly important, considering that the use of ginkgolide B in the clinical setting has not been associated with the occurrence of serious adverse effects [30].

Although the exact modes of action of ginkgolide B are not elucidated, direct interactions of $GABA_A$ and glycine receptors may be involved. $GABA_A$ and glycine receptors are classified as members of the ligand-gated ion channel superfamily. The $GABA_A$ receptor, which is a pentameric transmembrane receptor composed of five combined subunits (e.g., $\alpha1\beta2\gamma2$) arranged around a central pore, is the molecular target for several drugs (e.g., barbiturates, benzodiazepines, and neurosteroids), ethanol, and certain anticonvulsants [31]. These drugs elicit their potency through interaction with the $GABA_A$ receptor. The glycine receptor is also a pentameric transmembrane receptor composed of 48 kDa α and 58 kDa β subunits [32,33]. Some compounds, such as picrotoxin (the equimolar mixture of picrotoxinin and picrotin), are noncompetitive antagonists for $GABA_A$ and glycine receptors [34,35]. Picrotoxin inhibits the chloride ion flow at the site within the ion channel of the $GABA_A$ receptor, rather than at the GABA recognition site. Consequently, picrotoxin elicits antagonistic action against GABA-enhanced drugs, such as barbiturates and benzodiazepines [35]. Similarly, picrotoxin also acts as an antagonist for the glycine receptor by binding to the site within the ion channel pore [34,36].

The antagonistic effects of ginkgolide B on both $GABA_A$ and glycine receptors may be equivalent to those of picrotoxinin, owing to several structural similarities between ginkgolide B and picrotoxinin [17,25,26]. Ginkgolide B and bilobalide act at the overlapped binding site of picrotoxinin at the $GABA_A$ receptor [20,37]. However, ginkgolide B and bilobalide contain a hydroxyl group close to the lipophilic *tert*-butyl group and the hydroxy lactone group but not close to picrotoxinin. These groups may be associated with the absence of convulsant action of ginkgolide B and bilobalide as opposed to picrotoxinin (Fig. 1) [20,26]. Previous studies also showed differences in pharmacological effects. Unlike picrotoxinin, ginkgolide B potently inhibits the glycine receptor with a β subunit [19,32]. Collectively, this evidence demonstrates that, if the increased amount of wake induced by ginkgolide B is associated with negative modulation at the $GABA_A$ and glycine receptors, the functional mechanisms involved are not the same as those of picrotoxinin.

5. Conclusion

We showed that ginkgolide B dose-dependently increased the amount of wake and decreased that of NREM sleep in physiological sleep/wake cycles in mice. These effects may be achieved through the negative modulation of the activity of GABA and glycine at the $GABA_A$ and glycine receptors, respectively.

It has been demonstrated that GABA and glycine are involved in sleep-inducing mechanisms in healthy and disease statuses. Therefore, it is of interest to evaluate the wake-promoting effects of ginkgolide B in humans and identify compounds that alleviate sleepiness in natural and pathological situations.

CRediT authorship contribution statement

Shohei Nishimon: Validation, Formal analysis, Data curation, Writing - original draft. **Mai Yamaguchi:** Conceptualization, Methodology, Investigation. **Hisae Muraki:** Formal analysis. **Noriaki Sakai:** Conceptualization, Methodology, Validation, Investigation. **Seiji Nishino:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

All authors declare no conflicts of interest.

Acknowledgment

We acknowledge Dr. Atsufumi Manabe (Showa University School of Dentistry) for the continuous supports to the SCN lab. We also thank Drs. Toshikazu Shiba and Yumi Kawazoe (RegeneTiss, Inc.) for useful discussions.

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