**Regular Article**

*Nelumbo nucifera* Seed Extract Promotes Sleep in *Drosophila melanogaster*

Kyungae Jo, a Hyeon-Son Choi, b SangDuck Jeon, c Chang-Won Ahn, c and Hyung Joo Suh*a,a

a Department of Public Health Science, Korea University; Seoul 02841, Republic of Korea; b Department of Food Science and Technology, Seoul Women’s University; Seoul 01797, Republic of Korea; and c Research and Development Center, Nong Shim Co., Ltd.; Seoul 07057, Republic of Korea.

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The sleep-promoting effects of the water extract of *Nelumbo nucifera* seeds (NNE) were investigated in an invertebrate model. The effects of NNE on the subjective nighttime activity, sleep episodes, and sleep time were determined using *Drosophila melanogaster* and locomotor activity monitoring systems in basal and caffeine-induced arousal conditions. The movements of fruit flies were analyzed using the Noldus EthoVision-XT system, and the levels of neuromodulators were analyzed using HPLC. Expression of neuromodulator receptors was analyzed using real-time PCR. NNE was shown to contain neurotransmission-related components; γ-aminobutyric acid (GABA) (2.33±0.22 mg/g), tryptophan (2.00±0.06 mg/g), quinidine (0.55±0.33 mg/g), and neferine (0.16±0.01 mg/g). The total activity of flies during nighttime was decreased by 52% with 1.0% NNE treatment. In the individual and collective conditions, the subjective nighttime activities (45/38%) and sleep bouts (20/14%) of flies was significantly decreased with NNE treatment, while total sleep times (10/27%) were significantly increased. This sleep-promoting effect is more pronounced in caffeine-treated conditions; the nighttime activity of flies was reduced by 53%, but total sleep time was increased by 60%. Our video-tracking analysis showed a significant decrease of the moving distance and velocity of flies by NNE. This NNE-mediated sleep-promoting effect was associated with up-regulation of GABA_A/GABA_B and serotonin receptors. The NNE-mediated increase of GABA content was identified in flies. These results demonstrate that NNE effectively promotes sleep in flies by regulating the GABAergic-serotonergic neuromodulators, and could be an alternative agent for sleep promotion.

**Key words** *Nelumbo nucifera*; insomnia; sleep; caffeine; γ-aminobutyric acid; *Drosophila melanogaster*

*Nelumbo nucifera* Gaertn. is known as the Indian Lotus and belongs to the family Nymphaeaceae or Nelumbonaceae. N. nucifera is a large aquatic herb widely cultivated and used as food or medicine in eastern Asia. The herb is known as an ornamental plant due to its beautiful flowers and delicate fragrance. All parts of this plant are used in different ways (e.g., seeds in food or medicine, leaves in food or plate [thali], rhizomes in food, thalamus in fruit, stalks in pickles, and petals in natural pigments. In particular, the *N. nucifera* seed, after peeled, can be eaten fresh or roasted. The powdered seed can be mixed with honey to improve its taste and is used in bread preparation. It has been reported to have therapeutic potentials and further approved for use as both food and medicine in China. *N. nucifera* has been used as a folk medicine to treat many illnesses, including insomnia, nervous disease, restlessness, tissue inflammation, cardiovascular disease, skin disease, and cancer. In particular, *N. nucifera* has been suggested to have sedative-hypnotic and anxiolytic properties. In addition, the seed of *N. nucifera* has been reported to exert pharmacological effects including anti-inflammatory, hypoglycemic, anti-parkinsonian, and hepatoprotective activity. Several studies have shown that the embryo of the *N. nucifera* seed has an improving effect on nervous-related disorders. However, systematic studies of *N. nucifera* seed on sleep and its behavior in fruit flies has not been reported yet.

Sleep is defined as a natural state of body, which is characterized by lowered sensory functions and reduced responses to surroundings, at least superficially. However, it is not an unconscious passive state, but an active state that is maintained via interactions of the neuron network in the brain. Sleep is vital to maintaining fundamental functions of the body in animals, including humans, who spend one-third of their lives sleeping. In childhood, proper sleep is very important to maintain normal growth, development, mental health, and immune function. Sleep also actively affects the physiological function, recovery of mind and body fatigue, and improvement of memory and cognitive ability via complicated and systematic signaling transduction. However, the lack of sleep causes various mental and physical problems. Insufficient sleep can result in daytime behavioral/functional difficulties, including tiredness, memory failure, and lethargy. The long-term lack of sleep increases the potential of various serious diseases. In particular, insomnia, a sleep disorder, is known to be associated with heart problems, diabetes, cardiovascular disease, headaches, and depression. In recent years, the population with sleep disorder or insomnia is increasing continually. Approximately, 30% of the general population has been known to have a sleep disorder. In the United States, 50–70 million adults are known to be struggling with sleep disorders. In China, 37.75% of people aged 60 years and older have insomnia. Generally, the treatment of insomnia has involved the use of pharmacological agents or medicines such as benzodiazepine type medications, barbiturates, and anti-psychotics. Benzodiazepine hypnotic agents are known to affect insomnia, by targeting one of the γ-aminobutyric acid (GABA)_A receptors. However, the prolonged use of these medications causes many side effects including oversedation, hypnolnesia, depression, and drug dependence. Therefore, a natural source-based solution with reduced side effects is needed to study, develop, and use. Several stud-
ies showed the positive effect of natural products on the sleep promotion and sedation; valerian (*Valeriana officinalis*), hops (*Humulus lupulus*), and German chamomile (*Matricaria recutita*) are well-known for their sedative and sleep-promoting effects. In addition, *N. nucifera* seeds have been found to have an effect on the central nervous system in the vertebrate model. However, no study has studied the association between the sleep promotion effect of *N. nucifera* seeds with changes in the sleep behavior and neuromodulators.

The fruit fly *Drosophila melanogaster* has been one of the invertebrate model systems for biological, physiological, and neurological research. Circadian rhythms in *Drosophila* are similar to that of humans. Flies and human share the basic neuronal transmission systems and mechanisms, which show complex behavioral aspects. The sleep parameters of *Drosophila*, such as total sleep time and number of sleep bouts, were easily analyzed based on both the large amount and the intensity of locomotor activity.

This study was designed to investigate the effect of *N. nucifera* seeds on sleep and its behavior using various analyzing systems in normal and caffeine-mediated sleep disturbance conditions. The sleep promotion effects of *N. nucifera* seeds are described with an analysis of the neuromodulators and their receptors, suggesting the potential of *N. nucifera* as a natural sleep-promoting agent.

### MATERIALS AND METHODS

#### Preparation of Freeze-Dried Samples

Fifty grams of dry *N. nucifera* seeds (Korea oriental medicine industry association, Seoul, South of Korea) were suspended in distilled water (500 mL) and boiled at 60°C for 2 h. This process was repeated for 1 h with fresh distilled water, and the combined aqueous extracts were then filtered, concentrated by vacuum rotary evaporator (R-100, BUCHI Labortechnik AG, Flawil, Switzerland), freeze-dried, and stored at 4°C.

#### Analysis of GABA, Tryptophan, Quinidine, and Neferine

GABA, tryptophan, quinidine, and neferine from *N. nucifera* seed extract (NNE) were analyzed by HPLC. GABA was detected by using the Waters AccQ-Tag column (3.9 × 150 mm) and fluorescence (250 nm of excitation and 395 nm of emission). The detection mobile phase A, B, and C consisted of the Water AccQ-Tag Eluent A (acetate-phosphate buffer), acetonitrile, and Milli-Q Water, respectively.

1-Tryptophan was measured using the Phenomenex LUNA C18 column (4.6 × 150 mm). The detection of 1-trypophan was achieved by native fluorescence with excitation at 220 nm and emission at 320 nm. Mobile phase A and B consisted of 0.05% aqueous trifluoroacetic acid (TFA)–methanol (97.5:2.5) and 0.05% aqueous TFA–methanol (40:60), respectively. The flow rate was 1.0 mL/min. The gradient of mobile phase solvents was as follows: 100% A for 1.0 min, 50% A and 50% B for 16 min, and 100% A for 16–20 min.

The HPLC analysis of quinidine and neferine was conducted on the Phenomenex LUNA C18 column (4.0 × 250 mm) with a mobile phase (pH 9.2) as methanol–0.2 M potassium dihydrogen phosphate (KH₂PO₄)–0.2 M NaOH–trimethylamine (71:17:12:0.002). The flow rate was 0.8 mL/min. The samples were examined at a wavelength of 282 nm.

#### Fly Stocks

Wild-type *D. melanogaster* Canton-S strain flies were purchased from the blooming Drosophila Stock Center (Indiana University, Bloomington, IN, U.S.A.). The flies feed on a medium containing sucrose, cornmeal, dried yeast, agar, propionic acid, and *p*-hydroxybenzoic acid methyl ester solution. The flies were maintained at 25°C and 60% relative humidity with a 12 h:12 h light:dark cycle. The *N. nucifera* seed samples were added to the standard medium with the indicated doses. Each 2–5-d-old male fly was anesthetized using CO₂ and collected for proper analysis.

#### Determination of Sleep Behavior

NNE powder was mixed in a sucrose-agar media containing 5% sucrose and 2% agar for the locomotor activity determination. NNE (0.25, 0.5, 1.0%) and alprazolam (0.01%) were used as the sample and positive control, respectively. In the *Drosophila* activity monitoring system (DAM; TriKinetics, Waltham, MA, U.S.A.), flies were kept and analyzed in individual glass tubes to examine the behavior patterns of individual flies. The collective activity of the flies was examined by the locomotor activity monitoring system (LAM, TriKinetics) to analyze the locomotor activity with the social behavior of the flies. All the experiments were performed in triplicate (DAM: 10 flies per group, LAM: 30 flies per group). Flies were acclimated for 3 d in the tubes, and the activities were then recorded every 1 min for 5–8 d under darkness at room temperature. Caffeine (0.1%) was used as a stimulant for the wakeful state. GABA, benzodiazepine (BDZ) receptor antagonist, 0.01% flumazenil (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.) was used to inhibit GABAergic action. Data were produced using the DAM management software (TriKinetics). The number of interruptions was recorded using an infrared detector at each time interval, in which the data were visualized using the Actogram J software. The sleep analyses in tested flies were performed during the time of darkness. The sleep parameters (e.g., total activity, duration of sleep and sleep bout) were analyzed using the R statistical software.

The open field test was executed using the video-tracking system with some modifications of a previous study. Caffeine-treated or normal flies (10 flies per group) were used in the presence or absence of NNE and allowed to adapt to the chamber for 1 min, after which the activity of flies was video recorded for 5 min at 1 h before subjective nighttime. The experimental chamber was a circular arena (8 mm in diameter and 0.1 mm in height) with a white background. The mobility of the animals was analyzed using the Noldus EthoVision-XT system (Noldus Information Technology, the Netherlands).

#### Determination of Neuromodulators from Fruit Flies

GABA, 5-hydroxytryptophan (5-HTP), and serotonin contents from the fruit flies were analyzed using HPLC-fluorescence detection system, which consisted of the Phenomenex LUNA C18 column (4.6 × 150 mm), a column heater (40°C), and a fluorescence detector. The experiments were performed in triplicate in which 50 flies per group were used. After starvation for 2 h, the flies were administered the NNE samples for 3 h and collected. The heads of the flies were homogenized in 150 mL 0.1 M trichloroacetic acid (TCA) solution, which contained 100 mM sodium acetate, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM isoproterenol (as an internal standard), and 10.5% methanol (pH 3.8). Samples were centrifuged at 12000×*g* for 10 min, and the supernatant was collected and filtered through a 0.45 μm polyvinylidene fluoride (PVDF) syringe filter. Each of the neuromodulators (*i.e.*, GABA, 5-HTP, and serotonin), which were detected in the HPLC system, was
mRNA Expression of Neurotransmitter Receptors

NNE powder (0.5, 1.0%) was mixed in sucrose-agar media and treated with flies for 14 d. The extraction of total RNA from the heads of the flies (17–20-d old) was performed using TRIzol® reagent (Invitrogen, CA, U.S.A.). Extracted RNA samples were controlled in quality (A260/A280 >1.8) and treated with RQI ribonuclease (RNase)-free deoxyribonuclease (DNase) I (Promega, WI, U.S.A.) to remove DNA contaminants. Reverse transcription was performed using 1 µg of total RNA, SuperScript® III Reverse Transcriptase (Invitrogen), nucleotides, and oligo d(T). The targeted cDNAs were amplified using the Power TaqMan PCR Master Mix kit (Applied Biosystems, CA, U.S.A.). The cycle conditions for real-time PCR were as follows: 95°C for 10 min (1 cycle) and 95°C for 15 s /60°C for 1 min (50 cycles). The quantitative analyses of genes were performed using the StepOne plus Software V 2.0 (Applied Biosystems, Foster City, CA, U.S.A.), and the relative comparisons of genes were achieved using the $2^{-\Delta\Delta C_T}$ method with the normalization to a validated control gene, RpL32 (NM_001144655.3). The primer information of the tested neurotransmitter receptor genes for quantitative (q) RT-PCR was as follows: GABA A-R Rdl (NM_001274688.1), GABA B-R1 (NM_001259104.1), GABA B-R2 (NM_079714.2), and 5-hydroxytryptamine 1A (5-HT1A) (NM_166322.2).

Statistical Analyses

All data were statistically analyzed using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago, IL, U.S.A.). Differences among the groups were determined using the one-way ANOVA and Tukey’s multiple tests. Data are represented as the mean ± the standard error of the mean (S.E.M.). The Student’s t-tests were also used to analyze differences.

RESULTS

Contents of GABA, Tryptophan, Quinidine, and Nefer-
The contents of GABA, tryptophan, and neferine, which are recognized to be sedative substances, from NNE were analyzed using an HPLC system, as shown in Fig. 1. Each compound was detected using a different analytical condition in the columns, composition of mobile phases, and detection ranges, producing different chromatogram profiles of each compound (Fig. 1). Table 1 shows the contents of GABA, tryptophan, quinidine, and neferine, which are found in NNE. GABA and tryptophan of NNE were 2.33 and 2.00 mg/g of the extract, respectively; the contents of quinidine and neferine of NNE were 0.55 and 0.16 mg/g of the extract, respectively. These results showed that NNE might positively contribute to the promotion of sleep.

Effects of NNE on Locomotor Activity

Actograms show that NNE effectively inhibited the locomotor activity of fruit flies at two time zones (daytime and nighttime) (Fig. 2). The inhibitory effect was more significant for 0.5 and 1.0% NNE than for 0.25% NNE. Administration of 1.0% NNE decreased locomotor activities by 33 and 52%, respectively, during the subjective nighttime and daytime (p<0.001) (Fig. 2). This result showed a similar effect to (BDZ, 0.01%), a positive control, which showed a significant inhibition of locomotion activity with a single dose. Figure 3 shows the effects of NNE on the subjective nighttime activity, sleep episodes, and nighttime sleep duration in DAM and LAM systems. In these systems, the subjective nighttime activities and sleep bouts of flies showed a significant decrease with NNE treatment, while total sleep durations were significantly increased. Nighttime activities of the 1% NNE-treated group were reduced by 45 and 38%, respectively, in both individual and collective conditions (Figs. 3A, D), and sleep bouts were also decreased by 20% and 14% (Figs. 3B, E), respectively; whereas sleep times increased by 10 and 27% (Figs. 3C, F), respectively. In addition, the administration of BDZ induced similar effects in the 1.0% NNE-treated group (p<0.05) in the subjective nighttime activity, sleep episodes, and subjective nighttime sleep duration. This result showed that NNE effectively contributed to the sleep promotion of fruit flies by inhibiting locomotion activity and enhancing sleep time in both individual and collective systems.

Effects of NNE on Locomotion Activity in a Caffeine-Induced Awake Model

To examine the effect of NNE on locomotor activity in the insomnia model, caffeine (0.1%) was mixed with the sucrose-agar media. In the DAM system, the

| Component | GABA (mg/g of extract) | L-Tryptophan (mg/g of extract) | Quinidine (mg/g of extract) | Neferine (mg/g of extract) |
|-----------|------------------------|-------------------------------|-----------------------------|---------------------------|
| **Content** | 2.33±0.02              | 2.00±0.06                     | 0.55±0.03                   | 0.16±0.01                 |
caffeine-induced group caused a significant increase in the subjective nighttime activity (30%) \((p<0.05)\) and number of sleep episodes (32%) \((p<0.01)\) and reduced the total subjective nighttime sleep (7%) \((p<0.05)\). The 0.01% BDZ with 0.1% caffeine-treated group showed a significant decrease in this caffeine-induced increase in the subjective nighttime activity \((p<0.05)\) and number of sleep episodes \((p<0.01)\) and but showed an increase in the caffeine-induced decrease of the total nighttime sleep duration \((p<0.05)\). In addition, all the concentrations of NNE in 0.1% caffeine-treated conditions demonstrated a significant suppression on the caffeine-induced changes in sleep behaviors of fruit flies (Figs. 4A–C). A high-dose of NNE (1%) showed a similar effect as BDZ. The nighttime activity and sleep bouts of 1% NNE were decreased by 53 and 62%, respectively, compared with the only caffeine-treated group, and the total nighttime sleep duration was increased by around 7%. In the LAM system, the caffeine-treated group also showed a statistically significant increase in subjective nighttime activity and number of sleep episodes, and decreased total night time sleep \((p<0.05)\). As in
the DAM system, the administration of 1.0% NNE significantly decreased the subjective nighttime activity \((p<0.01)\) and number of sleep episodes \((p<0.05)\), together with a significant increase of total nighttime sleep, when compared to caffeine treatment only \((p<0.01)\). One percent NNE treatment showed the similar effects to BDZ regarding the nighttime activity, sleep bouts, and nighttime sleep duration. In particular, 1% NNE treatment led to a 53%-increase in the total nighttime sleep duration compared with the caffeine-only-treated group. This result showed that NNE has a sleep-promoting effect even in the caffeine-induced sleep disturbance condition.

**Effects of NNE on Movement of Fruit Flies**

The movement of flies treated with NNE was analyzed using the Noldus EthoVision-XT system in the normal and caffeine-associated conditions (Fig. 5). We determined moving distance, velocity, activity (moving center point), and inactivity (unmoving center point) by establishing a threshold for spatial migration. In this experiment, caffeine increased the distance and velocity of the flies by approximately two-fold, compared with the normal condition \((p<0.05)\), together with a significant increase of total nighttime sleep, when compared to caffeine treatment only \((p<0.01)\). One percent NNE treatment showed the similar effects to BDZ regarding the nighttime activity, sleep bouts, and nighttime sleep duration. In particular, 1% NNE treatment led to a 53%-increase in the total nighttime sleep duration compared with the caffeine-only-treated group. This result showed that NNE has a sleep-promoting effect even in the caffeine-induced sleep disturbance condition.

**Effect of NNE on Neurotransmitter Receptors mRNA Expression**

To determine how NNE led to sleep promotion in flies, major neurotransmitter receptors were examined at the mRNA level. Rdl, a GABA\(_A\) receptor, was significantly increased with NNE treatment in a dose-dependent manner (Fig. 6A). The high dose (1%) of NNE showed a two-fold increase in the Rdl mRNA expression compared with the control \((p<0.01)\). R1 and R2, which are GABA\(_B\) receptors, and 5-HT1A, which is a serotonin receptor, were also significantly increased in the 1% NNE treatment group (Figs. 6B–D). The 5-HT1A mRNA level was increased by approximately 50% in the 1% NNE treatment group (Fig. 6D). This result showed that NNE induced sleep promotion in flies by up-regulating the GABAergic and serotonergergic receptors.

**GABA Level in the Brain of Fruit Flies Administered with NNE**

GABA neuromodulator level was increased with NNE exposure in flies compared with control (Fig. 7). Flies exposed to NNE for 1h showed more than a six-fold increase in change of the GABA level \((2.2\,\mu g/mg\,protein)\) compared with the normal control (Fig. 7). This change of GABA level was maintained until 3h after exposure, but its enhanced change almost disappeared after 6h. However, 5-HTP and serotonin levels were barely detected, and the difference of

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**Fig. 5. Effects of NNE on the Movement of Fruit Flies**

This movement test was executed on a white background for 5min in 1h before subjective nighttime. The (A) moving distance (cm), (B) velocity (cm/s), (C) the center point for movement (cm/s), (D) the center point for immobility (cm/s) of experimental fruit flies were analyzed using the Noldus EthoVision-XT system in the control group (sucrose-agar media group), 0.1% caffeine group, and NNE-treated groups \((0.25, 0.5, 1.0\%)\) with the 0.1% caffeine. Values are presented as the mean movements±S.E.M. Different letters indicate significant differences \((p<0.05)\) between groups.
their levels in the NNE treatment and normal control groups was not significant (data not shown). This result indicated that the GABA level increase in the flies originated from the NNE, and NNE-derived GABA induces the mRNA expression of the GABA receptors.

Effects of Flumazenil on NNE-Mediated Sleep Behavior

Fig. 6. Effects of NNE on Rdl, GABA\textsubscript{A}-R1, GABA\textsubscript{B}-R2, and 5-HT1A mRNA Expression in Fruit Flies

Fly heads were collected after 12h:12h light:dark cycles for 2 weeks. Total RNA was extracted according to the method, and each mRNAs were analyzed using the qPCR system. Values are the mean±S.E.M. from 150 fruit flies per group. The symbol indicates statistically significant (*p<0.05, **p<0.01, and ***p<0.001) differences from the control group. NNE, Nelumbo nucifera seed extract; Rdl, GABA\textsubscript{A} receptor; GABA\textsubscript{B}-R1, GABA\textsubscript{B} receptor 1; GABA\textsubscript{B}-R2, GABA\textsubscript{B} receptor 2; 5-HT1A, 5-hydroxytryptamine 1A.

in Fruit Flies

GABA\textsubscript{A}-BDZ receptor antagonist flumazenil (0.01%) was shown to inhibit the effect of NNE on the subjective nighttime activity, sleep episodes, and nighttime sleep duration in DAM system (Fig. 8). Flumazenil-treated group did not change the behavior of flies when compared to the control group. In the 1% NNE-treated group, subjective nighttime activities (45%) ($p<0.01$) and sleep bouts (20%) ($p<0.05$) of flies were significantly decreased, but total sleep duration (10%) ($p<0.05$) was significantly increased. When flumazenil (0.01%) and NNE (1%) were treated together, NNE-mediated nighttime activities and sleep bouts were significantly increased (36%, 19%, respectively) ($p<0.05$) and sleep time was significantly reduced (6%) ($p<0.05$). In particular, sleep bouts and sleep time were reversed to normal level by flumazenil. This result showed that the effect of NNE on sleep behavior is blocked by flumazenil, suggesting that the sleep-promoting effect of NNE is due to GABAergic action.

DISCUSSION

N. nucifera (lotus) is a perennial aquatic medicinal plant that has been widely used for centuries in oriental medicine. Almost all parts of the lotus, including flowers, leaves, leaf stalks, seeds, and rhizomes, are utilized as both food and herbal medicine. In this study, we demonstrated that NNE effectively improved the sleep behaviors including sleep durations in fruit flies. The effect of 1% NNE on the sleep behaviors was almost similar to that of benzodiazepine, a sleep-inducing drug (Figs. 3, 4). In particular, a favorable effect of NNE on sleep behaviors was more obvious in caffeine-treated
condition than in the normal state (Figs. 4, 5).

Caffeine is one of the most widely used psychoactive compounds to promote alertness.\(^{26}\) It is also a main component in coffee, which is one of the most popular beverages over the world.\(^{27}\) The increase of coffee consumption is associated with the increase of sleep disorders.\(^{28}\) Our data showed that NNE reversed the caffeine-mediated reduction of sleep (Figs. 4, 5), indicating that NNE has a potential to offset the caffeine-induced adverse effect on the sleep.

The previous study showed that \(N.\ nu cifera\) seeds were involved in the increase of sleep time.\(^{3}1)\) Sugimoto et al. showed that neferine, a compound derived from the \(N.\ nu cifera\) seed, increased the sleep time in the rat model. Therefore, neferine could be recognized as one of the active compounds responsible for the sleep-promoting effect in the \(N.\ nu cifera\) seed based on the previous data.\(^{3}1)\) The current study also showed that neferine was contained in NNE via HPLC analysis (Table 1). Furthermore, our data showed that \(N.\ nu cifera\) seeds also contain the other sleep-related compounds, including GABA, tryptophan, and quinidine. Among them, GABA is one of the best-known sleep-promoting agents. GABA, the ligand for the GABA receptors, plays a suppressive role in the neuronal excitability of the central nervous system.\(^{29}\) It is generally contained in natural plants including \(Schisandra chinensis\) and as part of food in our regular diet such as in tea and rice.\(^{3}0,31)\)

The GABA content was 2.33 mg/g distilled water (DW) in our extract sample (Table 1). In comparison to the other edible plants, its level was higher than that observed in \(Angelica gigas, Eleutherococcus senticosus,\) and \(Thuja orientalis,\) but lower than that in \(Schisandra chinensis\) and \(Polygonum multiflorum.\)\(^{3}2)\) In addition, a trial to enhance the contents of GABA has been executed.\(^{3}2)\) However, this exogenous GABA administration has been thought not to directly bind to the GABA receptors in the brain because it cannot pass the blood–brain barrier.\(^{3}3,34)\) Nevertheless, studies have reported that the GABA administration increases the GABAergic system in the body and improves the sleep and its behaviors.\(^{3}5,36)\) Furthermore, many studies have reported the permeability of GABA on the blood–brain barrier, even if it is a small amount.\(^{37–39}\)

Therefore, dietary GABA is thought to be able to play a role in the GABAergic system in the interior or periphery of the brain, and NNE-derived GABA is expected to directly/indirectly contribute to the sleep promotion effect of NNE. This is supported by the results of the Fig. 7 showing that GABA content of fly brain increased with the NNE treatment. Actually, GABA has been known to be associated with the synthesis of melatonin, a sleep-inducing hormone. It promotes the catabolism to synthesize the \(N\)-acetylserylserotonin, a precursor of melatonin, from serotonin.\(^{40}\) Tryptophan is also a candidate molecule to affect NNE-derived sleep promotion because it is a precursor of the serotonin and melatonin.\(^{41}\) Several studies showed that tryptophan administration is helpful to improve sleep behaviors.\(^{41,42}\) Quinidine (or quinine) has been known to be a chemical to increase sleep time, in particular, it was shown to increase the duration of action of pentobarbital by inhibition of microsomal enzyme.\(^{33}\) These chemicals derived from NNE are expected to influence the effect of NNE-derived sleep promotion. In addition, \(N.\ nu cifera\) is a natural product, containing complex mixtures of chemicals, and it can contain other chemicals to affect sleep physiology. Thus, these chemicals could contribute to the NNE-mediated sleep promotion autonomously or in company with GABA and tryptophan. As natural plant-derived chemicals, flavonoids have been described to be linked to GABA receptors.\(^{29}\) The \(N.\ nu cifera\) seed has been known to contain various flavonoids including rutin, luteolin, and catechin.\(^{3,44}\) In particular, rutin has been observed as a depressant of the central nervous system, which increased the sleep time and decreased the locomotive activity in mice.\(^{45}\) A recent study showed that luteolin mediates the anti-depressant effect of \(Cirsium japonicum.\)\(^{46}\) Besides, many studies showed the sedative effects of flavonoids via a modulation of the GABA receptors in mice.\(^{46–49}\)

Therefore, these flavonoids in NNE could, at least in part, contribute to the NNE-derived sleep promotion effect, and further studies would be extensively performed on NNE-derived flavonoids in the next study.

Many studies have reported on natural substances to improve sleep behavior.\(^{50,51}\) Ecklonia cava Kjellman extracts induced sleep via a positive allosteric modulation of the GABA\(_A\)-BDZ receptor.\(^{50}\) In addition, passionflower (\(Passiflora incarnata\)) herbal tea has exhibited an enhanced sleep quality in human.\(^{51}\) Most of the natural compounds have been observed to modulate GABA\(_A\) receptor to exhibit sleep promotion effects.\(^{48,50,52}\) However, our data showed that NNE increased expressions of the GABA receptors as well as the 5-HT1A receptors (Fig. 6). In the GABA receptors, there are two classes, GABA\(_A\) and GABA\(_B\). The GABA\(_A\) receptor, a fast ionotropic receptor, is involved in the action of GABA for sleep-promoting effect and activation has been shown to decrease sleep latency for sleep promotion, whereas the GABA\(_B\) receptor, a metabotropic receptor, was not involved in the sleep latency, but shown to be associated with the sleep maintenance of deep sleep.\(^{53}\) Our data showed NNE upregulated both of GABA\(_A\) and GABA\(_B\) receptors (Fig. 6), indicating that...
NNE-mediated sleep promotion could include fast and long-lasting effects on sleep promotion. Furthermore, NNE also increased the expression of another neurotransmitter receptor, 5-HT1A receptor (Fig. 6D). 5-HT1A receptor has a serotonin as a ligand, which is a precursor of tryptophan, an amino acid. This increase of 5-HT1A mRNA expression is thought to be contributed to tryptophan in NNE. As seen in the current data, NNE led to the upregulation of multiple neurotransmitter receptors (Fig. 6). These data indicated that the NNE-derived sleep promotion effect could be the result of the actions of multiple principles (or chemicals) via multiple mechanisms. Our data showed that GABA content in the brain was increased with the NNE treatment (Fig. 7). This data correlated with the NNE-mediated up-regulation of the mRNA expression of the GABA receptors (Fig. 6). Since the direct action of NNE-derived GABA cannot be ruled out, GABA in the fruit fly brain is considered to originate from NNE-derived GABA. We also tried to analyze the serotonin and 5-HTP levels in the fly brain. However, these chemicals were barely detectable in our analytical systems, and the differences in the levels among the groups were not significant (data not shown). Serotonin and 5-HTP might be quickly metabolized to the other metabolites at a negligible level. Based on our data, the NNE-derived sleep promoting effect is shown to mainly be involved in the GABAergic action via an interaction of GABA and GABA receptors. This is supported by the fact that flumazenil, the GABA\textsubscript{A}-BDZ receptor antagonist, inhibit GABAergic action of NNE on sleep behavior (Fig. 8). However, serotonin and 5-HTP neurotransmitters would have to be further examined to approach the detailed mechanism and active principle of NNE for sleep promotion in the future.

In conclusion, the current study showed the sleep-promoting effect of NNE in 

Drosophila melanogaster. NNE effectively increased the sleep duration but decreased the locomotor activity in the DAM and LAM systems. In particular, the NNE-derived effect was obvious in the caffeine-associated arousal condition. This NNE-mediated sleep-promoting effect was linked to the modulation of the neurotransmitter receptors, including the GABA receptors. NNE-mediated increase of GABA in the fly brain and the inhibition of flumazenil on GABA-mediated sleep behaviors indicates that dietary NNE promotes sleep through the activation of the GABAergic system.

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