



Evodiamine Reduces Caffeine-Induced Sleep Disturbances and Excitation in Mice

Yong-Hyun Ko[†], Kyu-Yeon Shim[†], Seok-Yong Lee and Choon-Gon Jang*

Department of Pharmacology, School of Pharmacy, Sungkyunkwan University, Suwon 16419, Republic of Korea

Abstract

Worldwide, caffeine is among the most commonly used stimulatory substances. Unfortunately, significant caffeine consumption is associated with several adverse effects, ranging from sleep disturbances (including insomnia) to cardiovascular problems. This study investigates whether treatment with the *Evodia rutaecarpa* aqueous extract (ERAE) from berries and its major molecular component, evodiamine, can reduce the adverse caffeine-induced sleep-related and excitation effects. We combined measurements from the pentobarbital-induced sleep test, the open field test, and the locomotor activity test in mice that had been dosed with caffeine. We found that ERAE and evodiamine administration reduced the degree of caffeine-induced sleep disruption during the sleep test. Additionally, we found that evodiamine significantly inhibits caffeine-induced excitation during the open field test, as well as decreasing hyperlocomotion in the locomotor activity test. Additional *in vitro* experiments showed that caffeine administration decreased the expression of γ -aminobutyric acid (GABA)_A receptor subunits in the mouse hypothalamus. However, evodiamine treatment significantly reversed this expression reduction. Taken together, our results demonstrate that ERAE and its major compound, evodiamine, provide an excellent candidate for the treatment or prevention of caffeine-induced sleep disturbances and excitatory states, and that the mechanism of these beneficial effects acts, at least in part, through the GABA_A-ergic system.

Key Words: Evodiamine, *Evodia rutaecarpa*, Caffeine, γ -aminobutyric acid receptor, Sleep

INTRODUCTION

Caffeine, a constituent of coffee and many other common beverages, is one of the most widely consumed psychoactive substances around the world and has mild reinforcing activities (Fredholm *et al.*, 1999; Jain *et al.*, 2005). Unfortunately, caffeine also induces several adverse behavioral effects, including convulsive activity, the promotion of wakefulness, and locomotor stimulation even at relatively low doses (Garrett and Holtzman, 1994). Additionally, the acute or chronic intake of caffeine in order to improve daily performance is commonly associated with sleep disturbances and insomnia, as well as other side effects including cardiovascular problems (Mabunga *et al.*, 2015). In particular, previous studies have shown that caffeine exerts an allosteric effect on the γ -aminobutyric acid (GABA)_A receptors and that it may reduce GABA neurotransmitter levels in the central nervous system (CNS) (Kardos and Blandl, 1994; Fredholm *et al.*, 1999). Finally, it has been well established that caffeine induces excitation in the brain by antagonizing the adrenergic receptors (Dunwiddie

and Masino, 2001).

The GABA pathway is the main inhibitory neurotransmitter pathway, and as such it plays an essential role regulating sleep in the CNS (Kwon *et al.*, 2017). Specifically, pharmacological evidence has made it clear that GABA_A receptors contribute to sleep regulation through their activity in the hypothalamus (Nitz and Siegel, 1996). Several recent studies that investigated potential treatment options for insomnia and other sleep problems found that several positive results were achieved by modulating the GABA_A-ergic systems (Turek and Losse-Olson, 1986). Several known sedative-hypnotics for sleep disruption have been found to act on GABA_A receptors (Mohler *et al.*, 1995). GABA_A receptors have several subunits, including the α (1-6), β (1-4), γ (1-3), δ (1), ε (1) subunits (Seifi *et al.*, 2014). The common pentameric GABA_A receptor is composed of two α subunits, two β subunits, and either a γ or δ subunit (Hammer *et al.*, 2015). Each GABA_A receptor subunit plays a distinct role depending on the brain region, with the hypothalamus exhibiting its own pattern of functionality (Wafford and Ebert, 2006).

Open Access <https://doi.org/10.4062/biomolther.2017.146>

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received Jul 17, 2017 Revised Sep 11, 2017 Accepted Sep 14, 2017

Published Online Jan 9, 2018

*Corresponding Author

E-mail: jang@skku.edu

Tel: +82-31-290-7780, Fax: +82-31-292-8800

[†]The first two authors contributed equally to this work.

Evodia rutaecarpa is a berry-producing plant that has been used in traditional medicine to treat inflammatory diseases throughout Korea, China, and Japan. Previous studies have shown that *E. rutaecarpa* and its major constituent, evodiamine, hold several pharmacological properties that have anti-inflammatory and anti-allergic effects (Shin *et al.*, 2007; Zhao *et al.*, 2014). Additionally, it has been found that evodiamine extracted from *E. rutaecarpa* berries enhances learning and memory in transgenic mouse models of Alzheimer's disease (Yuan *et al.*, 2011). Evodiamine also has anti-depressant effects on a chronic unpredictable mild stress (CUMS) rat model (Jiang *et al.*, 2015). Despite these documented benefits, the effects of *Evodia rutaecarpa* aqueous extract (ERAE) and its major compound, evodiamine, on caffeine-induced behaviors have yet to be fully investigated.

To address this shortcoming, this study seeks to investigate the potential beneficial effect of treatment with ERAE and its major compound, evodiamine, on reducing negative caffeine-induced behaviors such as sleep disruption and locomotor activity in mice. We hypothesized that ERAE and evodiamine could provide beneficial effects in reversing the effects of caffeine on sleep onset, sleep duration, and locomotor stimulation. We also assessed the effect of evodiamine on the GABA_A-ergic systems in the mouse hypothalamus by performing Western blot analysis.

MATERIALS AND METHODS

Preparation of *E. rutaecarpa* aqueous extract (ERAE)

Evodia rutaecarpa dried berries were purchased from Kyung-Dong Oriental medicine market (Seoul, Korea), were originally collected in China in September 2015, and were confirmed by professor Ki-Hyun Kim (School of Pharmacy, Sungkyunkwan University, Suwon, Korea). Berries were cut into small pieces and extracted three times in hot water using reflux extraction equipment (70–80°C) with a cooling system (40°C) for 1 h, after which point the aqueous extract was filtered (Whatman No. 2, USA). Supernatants were concentrated under reduced pressure with a vacuum rotary evaporator (EYELA, N-1000, Japan) followed by lyophilization. From 193 g of dried fruit of *E. rutaecarpa*, 31 g of dried extract was obtained (16.06% yield) and stored at -20°C until use.

Chemicals and reagents

Evodiamine, caffeine, anti- β -actin antibody, dimethyl sulfoxide (DMSO), and Tween 80 were purchased from Sigma Chemicals (St. Louis, MO, USA). Rabbit anti-GABA_A receptor subunit α 4, anti-GABA_A receptor subunit β 1, anti-GABA_A receptor subunit γ 3, and anti-glutamic acid decarboxylase (GAD_{65/67}) antibodies were purchased from Abcam Company (Cambridge, MA, USA). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc (West Grove, PA, USA). All other chemicals were of analytical grade.

Animals

Male CD-1 mice (aged 4 weeks, 20–23 g) were purchased from Koatech Co., Ltd (Pyongtaek, Korea). Mice were housed 12 per cage (26×42×18 cm), allowed access to water and food *ad libitum* and maintained at constant temperature (23 ± 1°C) and humidity (55 ± 5%) under a 12-h light/dark cycle (lights on 07:00 to 19:00 h). All experiments were in accor-

dance with the NIH Guide for the Care and Use of Laboratory Animals and with the approval of Sungkyunkwan University's Institutional Animal Care and Use Committee (SKKUIA-CUC-2016-03-0002).

Pentobarbital-induced sleep test

The pentobarbital-induced sleep test was performed according to the method described by Hong *et al.* (2016) with some modifications, and will be briefly outlined here. Mice were treated with the ERAE (50, 100, and 200 mg/kg) and its major components, evodiamine (5, 10, and 20 mg/kg) one hour before each test. Sleep disturbance was induced by the administration of caffeine (50 mg/kg) (Cho *et al.*, 2014) 30 minutes before administering sodium pentobarbital (40 mg/kg), which marks the start of the sleep test. Mice treated with pentobarbital were placed in individual cages and we measured the time to sleep onset and the length of sleep duration. Sleep was identified as occurring in mice that remained immobile for at least 3 min. The time to sleep onset was defined as being the time elapsed after pentobarbital administration until the first positive instance of sleep. Sleep duration was defined as the difference between time of loss and recovery of the righting reflex. Animals that failed to fall asleep within 15 min after pentobarbital treatment were excluded from this study.

Open field test

We measured locomotor activity using the open field test. Briefly, the open field consisted of an opaque plastic box (30×30×30 cm) divided into 16 (4×4) identical sectors (7.5×7.5 cm), which were subdivided into peripheral and central sectors. The central sector comprised the 4 central squares (2×2) and the peripheral sector comprised the remaining squares (Li *et al.*, 2008; Sakata *et al.*, 2010). Mice were treated with evodiamine (10 and 20 mg/kg) one hour before each test. Mice were then administered caffeine (10 mg/kg) (Kayir and Uzbay, 2004; Zhang *et al.*, 2011) 30 min prior to the test, after which point they were placed into the center of the open field and allowed to explore for 5 min under dim light. After this initial period, we used a video tracking system (NeuroVision, Pusan, Korea) to record the total distance traveled over 60 min, which we used as an indicator of locomotor activity. The time spent within, as well as the number of entries into, the central sector were used as an anxiolytic indicator (Prut and Belzung, 2003). The open field arena was thoroughly cleaned with 70% ethanol between each test.

Western blot analysis

Western blot analyses were performed using previously published methods (Hong *et al.*, 2016). Briefly, we isolated hypothalamus tissue from mouse brains by promptly excising and homogenizing using a rotary homogenizer with 200 μ L of ice-cold lysis T-per tissue protein extraction buffer (Thermo Scientific, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, GmbH, Mannheim, Germany). All samples were incubated on ice for 30 min. After centrifugation at 10,000×g for 15 min, we separated and stored the supernatant at -70°C. Protein concentration was measured using a protein assay kit (Thermo Scientific). Each homogenate (15 μ g of protein) was subjected to 8–12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corporation, Pen-

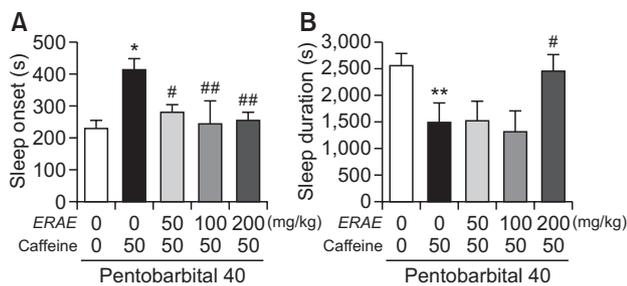


Fig. 1. Effects of *Evodia rutaecarpa* aqueous extract (ERAE) on caffeine-induced sleep disruptions as measured during the pentobarbital-induced sleep test. Mice were treated with ERAE (50, 100, and 200 mg/kg, p.o.) or vehicle solution 60 min before the tests (A, B). 30 min before the test, mice were treated with caffeine (50 mg/kg, i.p.). At the start of the test each mouse was injected with pentobarbital (40 mg/kg, i.p.). Data are presented as the mean \pm SEM (n=12). * p <0.001 and ** p <0.05 compared to the vehicle-control group. # p <0.05 and ### p <0.01 compared to the caffeine-only group.

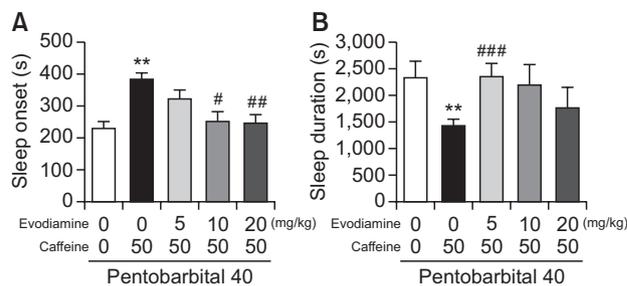


Fig. 2. Evodiamine's effects on caffeine-induced sleep disruptions as quantified through the pentobarbital-induced sleep test. Mice were treated with evodiamine (5, 10, and 20 mg/kg, p.o.) or vehicle solution 60 min prior to the test (A, B). Thirty minutes before the test, each mouse was treated with caffeine (50 mg/kg, i.p.). The test began with the injection of pentobarbital (40 mg/kg, i.p.). Data are presented as the mean \pm SEM (n=12). * p <0.001 and ** p <0.05 compared to the vehicle-control group. # p <0.01, ## p <0.001 and ### p <0.05 compared to the caffeine-only group.

sacola, FL, USA) dissolved in transfer buffer (25 mM Tri-HCl buffer (pH 7.4) containing 192 mM glycine and 20% v/v methanol), and blocked using a solution (5% non-fat milk in 0.5 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. Each membrane was incubated in the relevant primary antibodies overnight at 4°C; anti-GABA_A receptor subunit α 4 (1:1000), anti-GABA_A receptor subunit β 1 (1:1000), anti-GABA_A receptor subunit γ 3 (1:1000), and anti-GAD_{65/67} (1:1000). Membranes were then washed 5 times with Tris-buffered saline containing 0.1% Tween 20 (TBST). Each blot was incubated in horseradish-peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature. Blots were washed again with TBST to determine band density and enhanced chemiluminescence (ECL) was quantified by immersing the probed membrane in a 1:1 mixture of ECL reagents A and B (Animal Genetics Inc., Suwon, Korea) for 5 min. Finally, membranes were exposed to photographic film for a few minutes. Protein bands were quantified using densitometric analysis with ImageJ software obtained from the NIH (Bethesda, MD, USA).

Statistical analyses

All data are presented as the mean \pm standard error of mean (SEM) and analyzed using Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Results from the pentobarbital-induced sleep test, locomotor activity, open field test, and Western blot were compared using one-way analysis of variance (ANOVA) followed by Newman-Keuls test. Comparisons were determined to be statistically significant at a threshold of p <0.05.

RESULTS

ERAE improves sleep onset and sleep duration in caffeine-induced sleep disturbance mouse model

We used the pentobarbital-induced sleep test to assess whether ERAE reduced the degree of sleep disturbances caused by caffeine intake (50 mg/kg). We found that sleep onset time was significantly longer in the caffeine-treated group compared to the vehicle control (Fig. 1A, $F_{(4, 29)}=4.984$,

p <0.001). Additionally, caffeine administration significantly decreased sleep duration compared to the vehicle control (Fig. 1B, $F_{(4, 30)}=3.435$, p <0.05). Importantly, the caffeine-induced increases in sleep disruptions were significantly reversed by pre-treatment with ERAE (50, 100, and 200 mg/kg) (p <0.05, p <0.01 and p <0.01, respectively). Our results demonstrate that caffeine intake increases sleep disruption in mice, and that pre-administration of ERAE reverses this effect.

Evodiamine advances sleep onset and increases sleep duration in a caffeine-induced sleep disturbance mouse model

In order to assess the effects of evodiamine treatment on caffeine-induced sleep disruption, we again relied upon the pentobarbital-induced sleep test. Caffeine-treated mice exhibited significantly increased time to sleep onset compared to the vehicle control (Fig. 2A, $F_{(4, 46)}=6.373$, p <0.001). Moreover, caffeine-treated mice showed significantly lower sleep duration compared to the vehicle control (Fig. 2B, $F_{(4, 45)}=1.746$, p <0.05). Mice that were pre-treated with evodiamine (5, 10, and 20 mg/kg), however, significantly recovered from the caffeine-induced increases in sleep disruption, restoring normal sleep behaviors compared to the caffeine-treated group (p <0.05, p <0.01, and p <0.001, respectively).

Evodiamine effect on the open field test for caffeine-treated mice

We used the open field test to determine the effects of evodiamine on caffeine-induced excitation in mice. We found that caffeine (10 mg/kg)-treated mice exhibited increased activity (in terms of center-count occupancy time) (Fig. 3A, 3B, $F_{(3, 28)}=3.035$ and $F_{(3, 26)}=2.467$, p <0.01 and p <0.05, respectively). Treatment with evodiamine (20 mg/kg), however, significantly reversed this behavioral effect on excitation as compared to the caffeine-treated group (p <0.05, respectively).

Effect of evodiamine on hyperlocomotion in caffeine-treated mice

We next evaluated the effect that evodiamine treatment had on caffeine-induced locomotor activity in mice. We assessed locomotor activity by measuring the total distance travelled in a locomotor chamber over a 60-min period. We found that

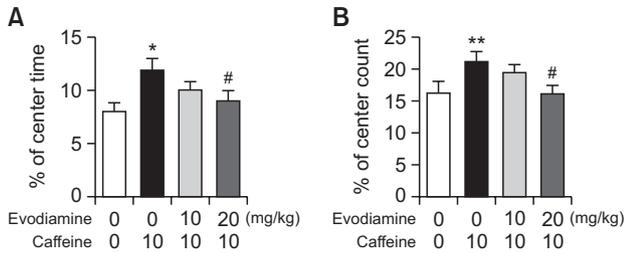


Fig. 3. Evodiamine's effect on caffeine-induced excitation as quantified using the open field test. Mice were treated with evodiamine (10 and 20 mg/kg, p.o.) or vehicle 60 min before each test (A, B). Thirty minutes prior to each test, each mouse was treated with caffeine (10 mg/kg, i.p.). Excitation was determined by the amount of time each mouse occupied the central quadrants. Data are presented as the mean \pm SEM (n=12). * p <0.01 and ** p <0.05 compared to the vehicle-control group. # p <0.05 compared to the caffeine-only group.

caffeine administration (10 mg/kg) significantly increased locomotor activity (Fig. 4, $F_{(3,27)}=15.97$, p <0.001) to a level we defined as hyperlocomotion. We found that prior injection with evodiamine (20 mg/kg) inhibited caffeine-induced hyperlocomotion (p <0.05).

Evodiamine restores normal GABA_A receptor expression in the hypothalamus of caffeine-induced mice

Having shown that evodiamine reverses caffeine-induced increases in sleep disruption, we confirmed caffeine's effect on expression of several GABA_A receptor subunits (α 4, β 1, and γ 3) using Western blot analysis. We found that treatment with caffeine leads to lower expression of GABA_A receptor subunit α 4 (Fig. 5A, $F_{(3,20)}=16.25$, p <0.001), β 1 (Fig. 5B, $F_{(3,20)}=7.713$, p <0.05), and γ 3 (Fig. 5C, $F_{(3,20)}=10.64$, p <0.001) in the hypothalamus compared to the vehicle control. Evodiamine treatment (10 and 20 mg/kg) in caffeine-treated mice significantly reversed caffeine's effect on GABA_A receptor subunit α 4 and β 1 expression (though not γ 3 expression) compared to the caffeine-only group (p <0.001, respectively).

DISCUSSION

Several pharmacological benefits have previously been reported for *E. rutaecarpa* and its major compound evodiamine in both *in vitro* and *in vivo* models. There have yet to be, however, any detailed studies evaluating the effects of *E. rutaecarpa* and evodiamine on caffeine-induced sleep and locomotor behavior. This study seeks to address this shortcoming by investigating the potential benefits of ERAE- and evodiamine-treatment in terms of caffeine-induced effects such as sleep disruption and hyperlocomotion in mice. In the course of this work, we also confirmed that evodiamine reverses the caffeine-induced reduction in GABA_A receptor subunit (α 4, β 1, and γ 3) expression in the hypothalamus of a mouse model.

The basis of our investigation into ERAE and evodiamine's effect on caffeine-induced sleep disruption (including sleep onset delay and decreased sleep duration) were measurements from the pentobarbital-induced sleep test. Sleep onset time and sleep duration quantified from the pentobarbital-induced sleep test are known to provide a useful measure of sleep behavior and of the GABAergic systems (Liao et

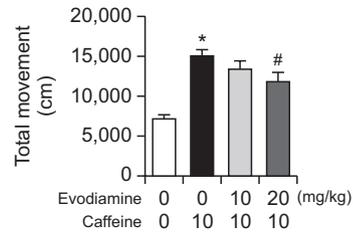


Fig. 4. Evodiamine's effect on caffeine-induced hyperlocomotion. Mice were treated with evodiamine (10 and 20 mg/kg, p.o.) or vehicle 60 min before each test. Thirty minutes prior to each test, each mouse was treated with caffeine (10 mg/kg, i.p.). Locomotor activity was defined as the total distance traveled over 60 min. Data are expressed as the mean \pm SEM (n=12). * p <0.001 compared with the vehicle-control group. # p <0.05 compared with the caffeine-only group.

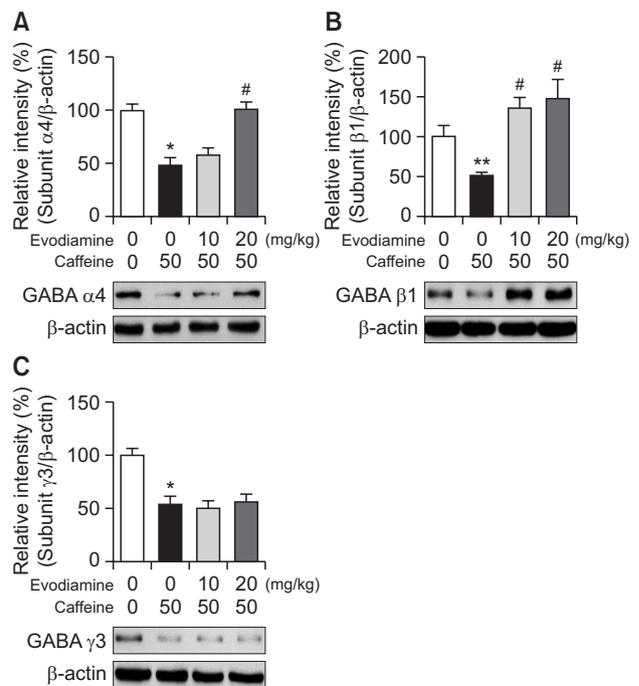


Fig. 5. Evodiamine's effects on the expression of GABA_A receptor subunits α 4 (A), β 1 (B), and γ 3 (C) in the hypothalamus. Mice were decapitated 60 min after the pentobarbital-induced sleep test and the hypothalamus was dissected for Western blot analysis. Data are presented as the mean \pm SEM (n=3). * p <0.001 and ** p <0.05 compared to the vehicle-control group. # p <0.001 compared to the caffeine-only group.

al., 1998). Pentobarbital enhances the effects of GABA and induces sleep in a predictable fashion (Ticku and Maksay, 1983). Sedative-hypnotics or anti-anxiety agents have also been shown to increase pentobarbital-induced sleep duration (Ma et al., 2008). Treatment with caffeine, however, suppresses the expression of GABAergic systems and results in sleep disturbances (Mukhopadhyay and Poddar, 2000; Li et al., 2004; Mabunga et al., 2015). In this study, we demonstrate that administration of caffeine (50 mg/kg) causes significant sleep disruptions during the pentobarbital-induced sleep test. Pre-treatment with ERAE (50, 100, and 200 mg/kg) and evo-

diamine (5, 10, and 20 mg/kg), however, significantly reduced the sleep disrupting effects of caffeine administration in mice. These results indicate that ERAE and its major component, evodiamine, significantly ameliorate the effects of caffeine administration on sleep disruption. The GABAergic systems may be involved as a possible mechanism of action for ERAE and evodiamine. A dose-dependent effect on sleep onset time was not observed, and only 200 mg/kg ERAE demonstrated a significant effect on sleep duration. This indicates that ERAE affects sleep onset and sleep duration differently, possibly due to the different mechanisms involved in sleep onset and sleep duration. Sleep onset is more likely to be affected by the opening of the GABA channel, and sleep duration is affected by the elongation of the channel opening. Another factor is that the extract of *Evodia rutaecarpa* berries is composed of numerous compounds, not a single compound. As seen in Fig. 2, evodiamine had a large effect on sleep duration at a dose of 5 mg/kg. However, this activity was slightly reduced in the 10 mg/kg and 20 mg/kg treatment groups. This could be because the elongation of the channel opening may be reduced at doses of 10 or more mg/kg. In addition, high doses of evodiamine may affect other receptors in a non-specific manner.

To further elucidate evodiamine's effects on caffeine-induced excitation, we also performed the open field test and assessed locomotor activity in our mouse model. The time in the central sector of the open field is an indicator of anxiety (Lalonde and Strazielle, 2010), and locomotor activity (defined as the total distance traveled over 60 min) provides a surrogate measure of motor function and degree of excitation (Misra *et al.*, 1986; Job, 2016). Previous studies have shown that caffeine is one of the most frequently consumed behaviorally active drugs, and is correlated with decreased anxiety-related behaviors (Solinas *et al.*, 2002; Sweeney *et al.*, 2016). For example, prior work has shown that low doses of caffeine intake induce anxiolytic and stimulatory behavior, including hyperlocomotion (Sudakov *et al.*, 2015; Hughes and Hancock, 2017). It has been hypothesized that caffeine enhances motor activity, at least in part, by blocking adenosine receptors (Powell and Holtzman, 1998). Adenosine interacts with dopamine receptors at both pre- and post-synaptic regions in order to inhibit dopaminergic activity (Prediger *et al.*, 2005). Moreover, the GABAergic systems play a direct role regulating locomotor activity via the dopaminergic and adenosinergic interaction (Mukhopadhyay and Poddar, 1995). Our results are consistent with previous reports (Kayir and Uzbay, 2004; Zhang *et al.*, 2011; Sweeney *et al.*, 2016; see Fig. 2, Fig.1, and Fig. 7, respectively) and indicate that caffeine (10 mg/kg) administration reduces anxiety-like behavior in mice. Caffeine-administered mice showed significantly increased central occupancy in the open field test, indicating greater excitation. Additionally, caffeine administration significantly increased total locomotion compared to the control group. These caffeine-induced effects on the open field and locomotor excitatory and stimulatory activity were significantly reduced after evodiamine treatment (20 mg/kg).

Lastly, we sought to characterize the likely molecular mechanism of caffeine-induced sleep disturbance. We measured the expression of GABA_A receptor subunits in the hypothalamus of untreated, caffeine-treated, and evodiamine-/caffeine-treated mice. It has been suggested that the activation of GABA_A-ergic systems could provide a valuable mechanism for preventing and treating sleep disruption (Kwon *et al.*, 2017).

Previous work has shown that general sedative effects are mainly mediated by the GABA_A-ergic systems (Chagraoui *et al.*, 2016). Additionally, the hypothalamus has been particularly associated with the regulation of sleep or wakefulness by acting through the GABA_A receptor (Zecharia *et al.*, 2009; Yanovsky *et al.*, 2012). Previous work has shown that drugs or other active compounds can enhance sleep duration by increasing some GABA_A receptor subunit expression, such as α (3-5), β (1-2), and γ 3 in rodents (Yanovsky *et al.*, 2012; Shah *et al.*, 2014; Kwon *et al.*, 2017). It is also known, however, that the GABA_A receptor (or GABA/benzodiazepine receptor) site interactions that have been associated with sedative-hypnotic activity can also be reversed by caffeine treatment (Roca *et al.*, 1988; Kardos and Blandl, 1994). Our results indicate that caffeine treatment (50 mg/kg) significantly inhibits the expression of GABA_A receptor subtypes, including α 4, β 1, and γ 3, in the mouse hypothalamus. Additionally, we show that evodiamine treatment (10 and 20 mg/kg) significantly recovers the expression of subtype α 4 and β 1, though it failed to recover the expression of subtype γ 3 and glutamic acid decarboxylase (GAD_{65/67}) (data not shown). Together, these findings demonstrate that evodiamine likely acts to reverse caffeine-induced behaviors by acting, at least in part, through the GABA_A-ergic systems in the hypothalamus of our mouse model. To the best of our knowledge, this is the first study that has demonstrated that ERAE and evodiamine improve caffeine-induced sleep disturbances and excitation states in mice. It is clear, however, that further molecular characterization extending from this study is required in order to clarify evodiamine's precise activity on sleep and excitation behavior after caffeine consumption.

In conclusion, our results have demonstrated that ERAE and its major compound, evodiamine, exert an ameliorating effect on caffeine-induced sleep and excitation behaviors, and that it may have important therapeutic benefits in counteracting the sleep disturbances and over-excitation induced by caffeine. Additionally, evodiamine's mechanism of action on caffeine-induced sleep disruption is likely, at least in part, due to its activation of the activation of GABA_A-ergic systems in the hypothalamus. We suggest that our findings indicate that ERAE and evodiamine could act as a potential therapeutic agent to treat insomnia or sleep problems that have been connected to caffeine intake.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ACKNOWLEDGMENTS

This research was supported by grants (NRF-2012R1A5A2A 28671860 and NRF-2016R1D1A1A09919739) from the Basic Science Research Program through the National Research Foundation (NRF). It was also supported by a grant (HI12C0035) from the Korean Health Technology R&D project, Ministry of Health & Welfare, Korea.

REFERENCES

- Cho, S., Yang, H., Yoon, M., Kim, J., Kim, J. and Kim, S. B. (2014) Arousal inhibitory effect of phlorotannins on caffeine in pentobarbital-induced mice. *Fish Aquat. Sci.* **17**, 13-18.
- Dunwiddie, T. V. and Masino, S. A. (2001) The role and regulation of adenosine in the central nervous system. *Annu. Rev. Neurosci.* **24**, 31-55.
- Fredholm, B. B., Bättig, K., Holmén, J., Nehlig, A. and Zvartau, E. E. (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* **51**, 83-133.
- Garrett, B. E. and Holtzman, S. G. (1994) D₁ and D₂ dopamine receptor antagonists block caffeine-induced stimulation of locomotor activity in rats. *Pharmacol. Biochem. Behav.* **47**, 89-94.
- Chagraoui, A., Skiba, M., Thuillez, C. and Thibaut, F. (2016) To what extent is it possible to dissociate the anxiolytic and sedative/hypnotic properties of GABA_A receptors modulators? *Prog. Neuropsychopharmacol. Biol. Psychiatry* **71**, 189-202.
- Hammer, H., Bader, B. M., Ehnert, C., Bundgaard, C., Bunch, L., Hoestgaard-Jensen, K., Schroeder, O. H., Bastlund, J. F., Gramowski-Voß, A. and Jensen, A. A. (2015) A multifaceted GABA_A receptor modulator: functional properties and mechanism of action of the sedative-hypnotic and recreational drug methaqualone (quaalude). *Mol. Pharmacol.* **88**, 401-420.
- Hong, S. I., Kwon, S. H., Hwang, J. Y., Ma, S. H., Seo, J. Y., Ko, Y. H., Kim, H. C., Lee, S. Y. and Jang, C. G. (2016) Quinpirole increases melatonin-augmented pentobarbital sleep via cortical ERK, p38 MAPK, and PKC in mice. *Biomol. Ther. (Seoul)* **24**, 115-122.
- Hughes, R. N. and Hancock, N. J. (2017) Effects of acute caffeine on anxiety-related behavior in rats chronically exposed to the drug, with some evidence of possible withdrawal-reversal. *Behav. Brain Res.* **321**, 87-98.
- Jain, N. S., Hirani, K. and Chopde, C. T. (2005) Reversal of caffeine-induced anxiety by neurosteroid 3- α -hydroxy-5- α -pregnane-20-one in rats. *Neuropharmacology* **48**, 627-638.
- Jiang, M. L., Zhang, Z. X., Li, Y. Z., Wang, X. H., Yan, W. and Gong, G. Q. (2015) Antidepressant-like effect of evodiamine on chronic unpredictable mild stress rats. *Neurosci. Lett.* **588**, 154-158.
- Job, M. O. (2016) Injection of cocaine-amphetamine regulated transcript (CART) peptide into the nucleus accumbens does not inhibit caffeine-induced locomotor activity: implications for CART peptide mechanism. *Pharmacol. Biochem. Behav.* **148**, 8-14.
- Kardos, J. and Blandl, T. (1994) Inhibition of a gamma aminobutyric acid A receptor by caffeine. *Neuroreport* **5**, 1249-1252.
- Kayir, H. and Uzbay, I. T. (2004) Evidence for the role of nitric oxide in caffeine-induced locomotor activity in mice. *Psychopharmacology (Berl.)* **172**, 11-15.
- Kwon, Y. O., Hong, J. T. and Oh, K. W. (2017) Rosmarinic acid potentiates pentobarbital-induced sleep behaviors and non-rapid eye movement (NREM) sleep through the activation of GABA_A-ergic systems. *Biomol. Ther. (Seoul)* **25**, 105-111.
- Lalonde, R. and Strazielle, C. (2010) Relations between open-field, elevated plus-maze, and emergence tests in C57BL/6J and BALB/c mice injected with GABA- and 5HT-anxiolytic agents. *Fundam. Clin. Pharmacol.* **24**, 365-376.
- Liao, J. F., Huang, S. Y., Jan, Y. M., Yu, L. L. and Chen, C. F. (1998) Central inhibitory effects of water extract of *Acori graminei* rhizoma in mice. *J. Ethnopharmacol.* **61**, 185-193.
- Li, S., An, J., Sun, C. K. and Li, Z. W. (2004) Inhibitory effect of caffeine on GABA-activated current in acutely isolated rat dorsal root ganglion neurons. *Sheng Li Xue Bao* **56**, 384-388.
- Li, S., Wang, C., Wang, W., Dong, H., Hou, P. and Tang, Y. (2008) Chronic mild stress impairs cognition in mice: from brain homeostasis to behavior. *Life Sci.* **82**, 934-942.
- Mabungu, D. F., Gonzales, E. L., Kim, H. J. and Choung, S. Y. (2015) Treatment of GABA from fermented rice germ ameliorates caffeine-induced sleep disturbance in mice. *Biomol. Ther. (Seoul)* **23**, 268-274.
- Ma, Y., Ma, H., Jo, Y. J., Kim, D. S., Woo, S. S., Li, R., Hong, J. T., Moon, D. C., Oh, K. W. and Eun, J. S. (2008) Honokiol potentiates pentobarbital-induced sleeping behaviors through GABA_A receptor Cl⁻ channel activation. *Biomol. Ther. (Seoul)* **16**, 328-335.
- Misra, A. L., Vadlamani, N. L. and Pontani, R. B. (1986) Effect of caffeine on cocaine locomotor stimulant activity in rats. *Pharmacol. Biochem. Behav.* **24**, 761-764.
- Mohler, H., Benke, D., Benson, J., Lüscher, B. and Fritschy, J. M. (1995) GABA_A-receptor subtypes *in vivo*: cellular localization, pharmacology and regulation. *Adv. Biochem. Psychopharmacol.* **48**, 41-56.
- Mukhopadhyay, S. and Poddar, M. K. (1995) Caffeine-induced locomotor activity: possible involvement of GABAergic-dopaminergic-adenosinergic interaction. *Neurochem. Res.* **20**, 39-44.
- Mukhopadhyay, S. and Poddar, M. K. (2000) Long-term caffeine inhibits Ehrlich ascites carcinoma cell-induced induction of central GABAergic activity. *Neurochem. Res.* **25**, 1457-1463.
- Nitz, D. and Siegel, J. M. (1996) GABA release in posterior hypothalamus across sleep-wake cycle. *Am. J. Physiol.* **271**, R1707-R1712.
- Powell, K. R. and Holtzman S. G. (1998) Lack of NMDA receptor involvement in caffeine-induced locomotor stimulation and tolerance in rats. *Pharmacol. Biochem. Behav.* **59**, 433-438.
- Prediger, R. D., Da Cunha, C. and Takahashi, R. N. (2005) Antagonistic interaction between adenosine A2A and dopamine D2 receptors modulates the social recognition memory in reserpine-treated rats. *Behav. Pharmacol.* **16**, 209-218.
- Prut, L. and Belzung, C. (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: A review. *Eur. J. Pharmacol.* **463**, 3-33.
- Roca, D. J., Schiller, G. D. and Farb, D. H. (1988) Chronic caffeine or theophylline exposure reduces γ -aminobutyric acid/benzodiazepine receptor site interactions. *Mol. Pharmacol.* **33**, 481-485.
- Sakata, K., Jin, L. and Jha, S. (2010) Lack of promoter IV-driven BDNF transcription results in depression-like behavior. *Genes Brain Behav.* **9**, 712-721.
- Seiff, M., Brown, J. F., Mills, J., Bhandari, P., Belelli, D., Lambert, J. J., Rudolph, U. and Swinny, J. D. (2014) Molecular and functional diversity of GABA-A receptors in the enteric nervous system of the mouse colon. *J. Neurosci.* **34**, 10361-10378.
- Shah, V. K., Choi, J. J., Han, J. Y., Lee, M. K., Hong, J. T. and Oh, K. W. (2014) Pachymic acid enhances pentobarbital-induced sleeping behaviors via GABA_A-ergic systems in mice. *Biomol. Ther. (Seoul)* **22**, 314-320.
- Shin, Y. W., Bae, E. A., Cai, X. F., Lee, J. J. and Kim, D. H. (2007) *In vitro* and *in vivo* antiallergic effect of the fructus of *Evodia rutaecarpa* and its constituents. *Biol. Pharm. Bull.* **30**, 197-199.
- Solinas, M., Ferré, S., You, Z. B., Karcz-Kubicha, M., Popoli, P. and Goldberg, S. R. (2002) Caffeine induces dopamine and glutamate release in the shell of the nucleus accumbens. *J. Neurosci.* **22**, 6321-6324.
- Sudakov, S. K., Nazarova, G. A., Alekseeva, E. V. and Kolpakov, A. A. (2015) Peripheral administration of a μ -opioid receptor agonist DAMGO suppresses the anxiolytic and stimulatory effects of caffeine. *Bull. Exp. Biol. Med.* **158**, 295-297.
- Sweeney, P., Levack, R., Watters, J., Xu, Z. and Yang, Y. (2016) Caffeine increases food intake while reducing anxiety-related behaviors. *Appetite* **101**, 171-177.
- Ticku, M. K. and Maksay, G. (1983) Convulsant/depressant site of action at the allosteric benzodiazepine-GABA receptor-ionophore complex. *Life Sci.* **33**, 2363-2375.
- Turek, F. W. and Losse-Olson, S. (1986) A benzodiazepine used in the treatment of insomnia phase-shifts the mammalian circadian clock. *Nature* **321**, 167-168.
- Wafford, K. A. and Ebert, B. (2006) Gaboxadol—a new awakening in sleep. *Curr. Opin. Pharmacol.* **6**, 30-36.
- Yanovsky, Y., Schubring, S., Fleischer, W., Gisselmann, G., Zhu, X. R., Lübbert, H., Hatt, H., Rudolph, U., Haas, H. L. and Sergeeva, O. A. (2012) GABA_A receptors involved in sleep and anaesthesia: β 1- versus β 3-containing assemblies. *Pflugers Arch.* **463**, 187-199.
- Yuan, S. M., Gao, K., Wang, D. M., Quan, X. Z., Liu, J. N., Ma, C. M., Qin, C. and Zhang, L. F. (2011) Evodiamine improves cognitive abilities in SAMP8 and APP(swe)/PS1(Δ E9) transgenic mouse models of Alzheimer's disease. *Acta Pharmacol. Sin.* **32**, 295-302.
- Zecharia, A. Y., Nelson, L. E., Gent, T. C., Schumacher, M., Jurd, R., Rudolph, U., Brickley, S. G., Maze, M. and Franks, N. P. (2009) The involvement of hypothalamic sleep pathways in general anesthesia: testing the hypothesis using the GABA_A receptor β ₃N265M knock-in mouse. *J. Neurosci.* **29**, 2177-2187.

Zhang, Q., Yu, Y. P., Ye, Y. L., Zhang, J. T., Zhang, W. P. and Wei, E. Q. (2011) Spatiotemporal properties of locomotor activity after administration of central nervous stimulants and sedatives in mice. *Pharmacol. Biochem. Behav.* **97**, 577-585.

Zhao, T., Zhang, X., Zhao, Y., Zhang, L., Bai, X., Zhang, J., Zhao,

X., Chen, L., Wang, L. and Cui, L. (2014) Pretreatment by evodi-amine is neuroprotective in cerebral ischemia: up-regulated pAkt, pGSK3 β , down-regulated NF- κ B expression, and ameliorated BBB permeability. *Neurochem. Res.* **39**, 1612-1620.