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Combined Administration of Cannabidiol and L-Theanine Improves Sleep-Related Outcomes in Caffeine-Induced Sleep Disturbances Mouse Model

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Abstract

Sleep disruption is associated with increased risks of metabolic, cardiovascular, and neuropsychiatric disorders. Cannabidiol (CBD) and L-theanine have attracted attention as potential sleep-supporting agents due to their neuroactive properties. This study investigated the individual and combined effects of CBD and L-theanine in a caffeine-induced sleep disturbance mouse model using behavioral assessments and neurochemical analyses. Mice were orally administered CBD and L-theanine at ratios of 1:1, 2:1, or 1:2 prior to pentobarbital-induced sleep testing. Co-administration significantly reduced sleep onset latency and increase total sleep duration compared with single treatments under sleep-disturbed conditions. Combination index analysis indicated a synergistic interaction at lower doses, whereas such interaction was not observed at higher doses under normal conditions. To further characterize associated neurochemical changes, serum serotonin and melatonin levels, as well as cortical gamma-aminobutyric acid (GABA), were measured using enzyme-linked immunosorbent assay. CBD increased serotonin levels in a dose-dependent manner, while L-theanine elevated serotonin at lower doses. Combination treatment was associated with increased serotonin and melatonin levels which rose by more than 40% relative to the caffeine-treated group. Cortical GABA levels were also elevated following combination treatment. Collectively, these findings indicate that co-administration of CBD and L-theanine improves sleep-related outcomes and is associated with neurochemical changes, although the precise mechanisms remain to be determined. This study suggests the potential of CBD and L-theanine co-administration as an alternative approach for managing stimulant-induced sleep disturbances.

Key Words: Cannabidiol, L-theanine, Sleep disturbance, Caffeine-induced model, Neurochemical changes, Synergistic interaction

INTRODUCTION

Sleep deprivation has been a topic of interest since the early 1900s. Today, sleep deprivation is a global public health issue caused by lifestyle, technology, and work demands. Sleep deprivation is primarily connected to multiple health conditions, especially cognitive decline (Killgore, 2010; Ren *et al.*, 2025), mood disorders (Rumble *et al.*, 2015), and metabolic dysfunction (Duan *et al.*, 2023). Additionally, previous reports suggest it is associated with health risks, including cardiovascular, respiratory, neurological, immunological, and endocrine-related conditions (Briançon-Marjollet *et al.*, 2015; Pan *et al.*, 2023; Rault *et al.*, 2020). Sleep deprivation can occur due to stress, a shift in the work environment, or a change in food and drinking habits. These factors contribute to the physiological alteration of melatonin secretion. Melatonin is a hormone produced to regulate the circadian rhythm (sleep–wake cycle). If its production is reduced, the body does not receive a strong sleep signal, which makes it harder to fall asleep or to have meaningful sleep rest. Chronic disruption in melatonin levels contributes to insomnia and circadian rhythm disorders (Ackermann *et al.*, 2013). Generally, sleep regula-

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tion involves complex homeostatic processes governed by neurotransmitters and hormones. Specifically, adenosine contributes to sleep promotion by dampening neuronal excitability via binding to A1 and A2a, which increases sleep pressure and cognitive fatigue. Also, chronic sleep deprivation desensitizes the pathways associated with adenosine and impairs sleep recovery, leading to mood and attention deficits (Huang *et al.*, 2024; Lazarus *et al.*, 2019). Hypnosis has been used to manage sleep disorders, where Benzodiazepines, such as diazepam or chlorodiazepoxide, are commonly used as hypnotic treatments for sleep deprivation and insomnia (Edinoff *et al.*, 2021). However, its efficacy is still debated. Interestingly, the development of Benzodiazepine receptor agonists (BZRAs) has led to improved sedation and anxiolytic effects. Specifically, BZRAs enhance the effect of the neurotransmitter GABA at the GABA-A receptor, which is selectively expressed in multiple regions of the brain. Thus, they are considered molecular targets for most hypnotic treatments approved against insomnia (de Mendonça *et al.*, 2023; Riemann and Perlis, 2009). However, reports indicate that BZRAs cause dependence, tolerance, and cognitive side effects. Furthermore, serotonin, a precursor of melatonin, is linked to mood regulation and prevents depressive symptoms. The upregulation of serotonin synthesis leads to increased melatonin production via enzymatic conversion by serotonin N-acetyltransferase and hydroxyindole-O-methyltransferase. This leads to a synergistic effect of serotonin and melatonin in improving mood regulation and the mitigation of depressive symptoms (Ferracioli-Oda *et al.*, 2013; Reiter *et al.*, 2014). In addition, serotonin contributes to improved pain tolerance (Jouvet, 1999).

Recently, CBD, a non-psychoactive compound from *Cannabis sativa*, garnered scientific attention for its potential role in sleep regulation (D'Angelo and Steardo, 2024). Thus, several preclinical investigations conducted over the past decade have demonstrated beneficial effects on sleep (Karimi-Haghighi and Haghparast, 2018; Murillo-Rodríguez *et al.*, 2011). Also, CBD is known to regulate euphoria, anxiety, inflammation, and stress, thereby improving sleep quality (Azarfarin *et al.*, 2025; Buchanan-Peart *et al.*, 2020; Jantsch *et al.*, 2025; Liu *et al.*, 2024; Singh *et al.*, 2025; Skelley *et al.*, 2020). Recent reports suggest that CBD could alleviate sleep deprivation by modulating neural and circadian pathways (Lafaye *et al.*, 2019; Miranda *et al.*, 2024). CBD is known to interact with the endocannabinoid system by modulating G-protein-coupled cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2), facilitating the maintenance of sleep–wake balance (Ralevic, 2003). Besides, CBD triggers adenosine signaling, which alters serotonin receptors that help in reducing anxiety and improving sleep quality (Kesner and Lovinger, 2020). Apart from CBD, several other compounds modulate neurotransmitter systems that ameliorate sleep deprivation (Murillo-Rodriguez *et al.*, 2017; Zhu *et al.*, 2024). L-theanine is one of the known compounds that has been widely studied for its role in modulating the glutamatergic system, a critical pathway in sleep regulation (Dasdelen *et al.*, 2022; Kim *et al.*, 2019a). It acts as a glutamate receptor antagonist and reduces excitatory neurotransmission, which regulates psychological stress (Kakuda, 2011; Kim *et al.*, 2009). Additionally, it improves sleep quality primarily through the GABAergic system. Moreover, L-theanine enhances brain levels of serotonin, dopamine, and GABA, thereby promoting relaxation, stabilizing mood, and reducing stress (Zhang *et al.*, 2021). These positive effects support improving sleep quality and recovery from sleep deprivation. Together, CBD and L-theanine offer a promising avenue for therapeutic interventions aimed at improving sleep quality and addressing insomnia.

Collectively, treatments for sleep disorders include pharmacological, behavioral, and emerging natural therapies. Conventionally, benzodiazepines and BZRAs induce sleep but carry a risk of dependence, tolerance, and cognitive side effects. On the other hand, melatonin supplements are known to control circadian rhythm-related insomnia, but are limited by side effects like mood changes, irritability, or hormonal effects. However, technological advancements offer some of the frameworks that help to explore drug–target–disease interactions. This study explores the potential effects of CBD and L-theanine against sleep disorders using sleep-deprived mice, focusing on phenotypic outcomes rather than direct mechanistic validation.

MATERIALS AND METHODS

Chemicals

CBD was obtained from Averix Bio, LLC (Wilson, NC, USA) through a domestic supplier in the Republic of Korea, and L-theanine was obtained from MedChemExpress (MCE) (New Jersey, NY, USA). Diazepam was purchased from Samjin Pharmaceutical Co., Ltd. (Seoul, Korea), and pentobarbital was obtained from Hanlim Pharm CO., Ltd. (Seoul, Korea).

Animal care and experimental design

Seven-week-old ICR mice weighing 28-32 g were purchased from Samtako (Osan, Korea). Mice were housed and maintained at the animal facility of Jeonbuk Medical School under typical living conditions (22°C ± 2°C; 45% relative humidity), which included a 12 h light-dark cycle with sufficient food and water. All the animal tests were performed in accordance with regulations set by the Institutional Animal Care and Use Committee at Jeonbuk National University Hospital (JBUH-IACUC-2022-8, JBUH-IACUC-2023-26, JBUH-IACUC-2025-38). The reporting in this manuscript follows the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Kilkenny *et al.*, 2010). Animals were acclimatized for 3 days, then randomly divided into 10 groups, consisting of 8-12 mice each. The final number of mice in the caffeine-treated group was seven, as one mouse failed to fall asleep and was therefore excluded from analysis (Supplementary Fig. 2). For the pharmacological investigations, L-theanine and diazepam (positive control) were suspended in saline, while CBD was suspended in a solution containing 5% EtOH, 5% Tween 20, and 90% DDW. In this study, the experimental groups are as follows: Control (0.9% saline); CBD (CBD 5 or 10 mg/kg) L-theanine (L-theanine 5 or 10 mg/kg); Combination treatment of CBD and L-theanine 1:1 ratio (CBD 10 and L-theanine 10 mg/kg); CBD and L-theanine 2:1 ratio (CBD 10 and L-theanine 5 mg/kg); CBD and L-theanine 1:2 ratios (CBD 5 and L-theanine 10 mg/kg); diazepam-positive control (2 mg/kg). Besides, the sleep disorder model was designed to determine the synergistic effect of CBD and L-theanine on caffeine-induced sleep disturbance relative to single-agent treatments. Here, mice were treated with 80 mg/kg of caffeine via intraperitoneal injection (i.p.), except for the vehicle group. In this model, the experimental groups are as follows: control

(0.9% saline); Caffeine control (80 mg/kg); Caffeine and CBD (CBD 2.5 or 5 mg/kg following caffeine 80 mg/kg); Caffeine and L-theanine (L-theanine 2.5 or 5 mg/kg following caffeine 80 mg/kg); Caffeine and combination treatment of CBD and L-theanine 1:1 ratio (CBD 2.5 and L-theanine 2.5, CBD 5 and L-theanine 5 mg/kg); CBD and L-theanine 2:1 ratio (CBD 5 and L-theanine 2.5 mg/kg); CBD and L-theanine 1:2 ratio (CBD 2.5 and L-theanine 5 mg/kg); Diazepam positive control (2 mg/kg). All the control/test solutions were administered via oral gavage. Following a 30-min reaction period, animal behavior was analyzed using the Open Field Test (OFT) and Rotarod test. In the second experiment, animals received a solution under the same conditions as in the first experiment, following pentobarbital administration via intraperitoneal injection (i.p.). For euthanasia, mice were administered intraperitoneal (i.p.) with ketamine (Yuhan, Seoul, Korea) at a dose of 50 mg/kg and xylazine (Hongik Medicare, Seoul, Korea) at a dose of 10 mg/kg based on their body weight for anesthesia. Whole blood samples from mice were obtained before animals were sacrificed. For analysis, whole blood was collected, and serum was separated and stored at –80°C until use. All the experimental designs of investigations are shown in Fig. 1A-1B.

Pentobarbital-induced sleep test (Sleep induction)

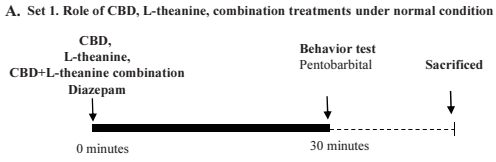
The sleep evaluation method was based on the prolongation of pentobarbital-induced sleeping time. In this test, CBD, L-theanine, their combinations at varying concentrations, and diazepam (2 mg/kg) were administered via oral gavage (p.o.). On the other hand, a 42 mg/kg solution of sodium pentobarbital in saline was administered intraperitoneally 30 min post-treatment with the test compounds to induce sleep. Mice were considered asleep when they remained immobile and lost the righting reflex upon being placed on their backs. Sleep latency and duration were assessed based on the loss and recovery of the righting reflex. Sleep latency was defined as the time interval between pentobarbital injection and the onset of sleep, indicated by the loss of the right reflex. Sleep duration was measured as the time from sleep onset to the recovery of the right reflex, with a maximum observation period of 2 h.

Animal behaviour tests

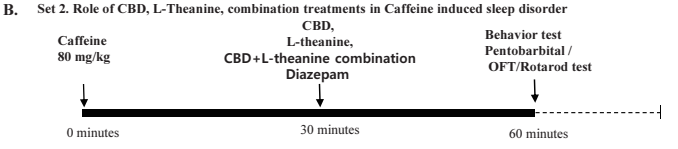
Open field test (OFT): General locomotor activity was examined using the OFT as reported earlier (Monarca *et al.*, 2025). Prior to the examination, mice underwent a familiarization period within the designated habituation area consisting of two consecutive days of unrestricted locomotion sessions, each lasting 12 min. Following this acclimation phase, the mice were administered either control or test compounds to evaluate their effects. For evaluation, the mouse was placed in the center of the apparatus, which comprised a square area surrounded by black acrylic walls (41×41×40 cm). The movements of the mice were tracked and recorded over 12 min with the help of a camera. All distances travelled by mice are represented in meters. The obtained data were analysed with EthoVision XT video tracking software (RRID: SCR_000441, Noldus, Netherlands). This application facilitated the analysis of the total distance covered and the time duration spent in the central or peripheral zones.

Rotarod test

To evaluate the motor coordination of the mice, the rotarod test was performed as described in previous studies (Shiotsuki *et al.*, 2010). Initially, mice were trained for 3 days, with a session of 10 min to make the animal familiar with the apparatus. On test day, mice were placed on the resting drum (3 cm diameter) of a rotarod apparatus (Ugo Basile, Varese, Italy) for at least 1 min. Then, the speed of the rotarod was accelerated to 36 revolutions per minute (rpm). The mice were subjected to three trials with 10 min intervals between trials. The retention time of the rod in each trial was recorded. The latency to



Group name	Oral administration	Pentobarbital-induced sleep or OFT/ Rotarod test
Control	Vehicle	Pentobarbital (42 mg/kg) (i.p.) OFT for 12 minutes Rotarod for 10 minutes
CBD	CBD only	Pentobarbital (42 mg/kg) (i.p.) OFT for 12 minutes Rotarod for 10 minutes
L-theanine	L-theanine only	Pentobarbital (42 mg/kg) (i.p.) OFT for 12 minutes Rotarod for 10 minutes
Caffeine + Combination treatments	CBD+ L-thenine 1:1 (CBD 10: L-theanine 10) 2:1 (CBD 5:L-theanine 2.5) 1:2 (CBD 2.5:L-theanine 5)	Pentobarbital (42 mg/kg) (i.p.) OFT for 12 minutes Rotarod for 10 minutes



Group name	I.P injection	Oral administration	Pentobarbital-induced sleep or OFT/ Rotarod test
Control	0.9 % saline	5 % EtOH + 5 % Tween 20 + DDW	Pentobarbital (42 mg/kg) (i.p.) OFT for 12 minutes Rotarod for 10 minutes
Caffeine control	Caffeine	5 % EtOH + 5 % Tween 20 + DDW	Pentobarbital (42 mg/kg) (i.p.) OFT for 12 minutes Rotarod for 10 minutes
Caffeine + CBD	Caffeine	CBD (2.5, 5 mg/kg)	Pentobarbital (42 mg/kg) (i.p.) OFT for 12 minutes Rotarod for 10 minutes
Caffeine + L- theanine	Caffeine	L- theanine (2.5, 5 mg/kg)	Pentobarbital (42 mg/kg) (i.p.) OFT for 12 minutes Rotarod for 10 minutes
Caffeine + Combination treatments	Caffeine	CBD+ L-thenine (mg/kg) 1:1 (CBD 2.5:L-theanine 2.5 CBD 5, L-theanine 5) 2:1 (CBD 5:L-theanine 2.5) 1:2 (CBD 2.5:L-theanine 5)	Pentobarbital (42 mg/kg) (i.p.) OFT for 12 minutes Rotarod for 10 minutes

Fig. 1. Schematic representation of the experimental designs for assessing sleep-related behavior in mice treated with CBD, L-theanine, or their combination. (A) Experimental schedule for evaluating the role of CBD, L-theanine, and their combination under normal conditions. Behavioral analyses, including the open field test (OFT) and rotarod test, were performed in pentobarbital-induced mice to evaluate sleep-related effects. (B) Experimental schedule for assessing the influence of CBD, L-theanine, and their combination in a caffeine (80 mg/kg, i.p.)-induced sleep disorder model. Behavioral analyses on pentobarbital-induced mice were performed to evaluate sleep quality.

first fall and falling frequencies were recorded.

Melatonin Quantification

Plasma melatonin levels were assessed at two different zeitgeber times (ZT) to account for circadian variations in endogenous secretion. Blood samples were first collected at 6:00 PM, after the pentobarbital sleep test. To assess melatonin secretion at the circadian peak, an additional set of blood samples was collected at ZT18, corresponding to the onset and nocturnal peak of endogenous melatonin release. All animals were maintained under a controlled 12:12 h light-dark cycle (light on at ZT0; light off at ZT12). During blood withdrawal at ZT18, animals were handled under dim red light to prevent light-induced suppression of melatonin secretion. Blood samples were collected by cardiac puncture under anaesthesia, immediately centrifuged at 3000×g for 10 min at 4°C, and the plasma was stored at –80°C until analysis. Melatonin levels were quantified using a commercially available ELISA kit (#MBS263465, MyBioSource, San Diego, CA, USA) following the manufacturer's instructions. Briefly, the samples were prepared by diluting the serum with the sample diluent buffer. Standard solutions and diluted samples were added to the pre-coated 96-well ELISA plate, followed by the addition of biotinylated detection antibody. The plate was incubated at 37°C for 60 min, washed three times with wash buffer, and incubated with enzyme conjugate at 37°C for 30 min. After an additional washing step, the color reagent was added and incubated in the dark for 30 min at 37°C. The reaction was stopped using the provided color reagent C, and absorbance was measured at 450 nm using a FlexStation 3 Multi-mode Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA). The melatonin concentration in the samples was calculated based on the standard curve generated using the provided melatonin standards. All samples and standards were measured in duplicate. The results were expressed as pg/mL of melatonin.

Serotonin Quantification

Serotonin levels were quantified using a commercially available ELISA kit (#MBS1601042, MyBioSource, San Diego, CA, USA) following the manufacturer's instructions. Briefly, the supernatant from serum samples was collected and added with 10 μL of anti-ST antibody and 50 μL of HRP-conjugated streptavidin and incubated at 37°C for 60 min. Following incubation, the reaction mixture was washed and incubated in the dark at 37°C for 15 min. Later, the reaction was stopped using a stop solution, and absorbance was measured at 450 nm within 10 min. Serotonin levels were quantified using a standard curve generated from serotonin standards, and results are expressed as pg/mL of serotonin.

Combination Index (CI) calculation

The combined effects of CBD and L-theanine on sleep duration were evaluated using a Combination Index (CI) approach conceptually based on the Chou-Talaly equation additivity principle (Chou, 2006). Sleep duration obtained from single-compound treatment (CBC or L-theanine) and combination treatments at predefined doses were used for a single-dose-based, exploratory assessment of interaction, rather than for classical media-effect analysis using full-dose-response curves. CI values were calculated using the following formula:

$$\mathbf{CI} = \frac{\boldsymbol{D_1}}{\boldsymbol{D_{x1}}} + \frac{\boldsymbol{D_2}}{\boldsymbol{D_{x2}}}$$

Where D_1 and D_2 represent the dose of CBD and L-theanine used in combination, and D_{x1} and D_{x2} represent the doses of each compound alone producing comparable effects under the same experimental conditions. Since full dose-response curves were not generated, D_{x1} and D_{x2} were approximated based on observed effects at the tested doses. CI values were interpreted descriptively (CI<1: synergistic tendency; CI: 1 additive effect, and CI>1: antagonistic tendency).

EEG recording

EEG and EMG were recorded according to previously described methods (Kim *et al.*, 2019b). Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and a headmount (Pinnacle Technology, Inc., Lawrence, KS, USA) equipped with EEG and EMG electrodes was surgically implanted for polysomnographic recordings. The anterior portion of the headmount was positioned 3 mm anterior to the bregma. Subsequently, four screws were inserted into the skull for EEG recording, and two wire electrodes were placed into the nuchal muscle for EMG recording. After a one-week postoperative recovery period, EEG and EMG recordings were conducted in a custom-designed cage that allowed free movement. One day prior to treatment, mice were acclimatized to the EEG apparatus for 4 h without recording. On the experimental day, mice were connected to the recording system during the light phase (09:50-14:00), allowed a 10-min habituation period, and recordings were then obtained for 4 h (Pinnacle Technology, Inc., Lawrence, KS, USA). Mice were initially treated with saline or caffeine, followed 30 min later by administration of CBD, L-theanine, a combination of CBD and L-theanine, or vehicle. Mean absolute power (μV2) was calculated for alpha (8.4-4 Hz) and beta (13-30 Hz) frequency bands.

Statistical analysis

GraphPad Prism 10.6.1 (GraphPad Software Inc., San Diego, CA, USA) was used for all the statistical analyses. One-way analysis of variance (ANOVA), followed by Tukey's or Dunnett's post hoc test was performed for multiple comparisons. *P*-values of <0.05, <0.01, and <0.001 were considered statistically significant. All results are presented as mean ± SD unless otherwise stated; EEG data are presented as mean ± SEM.

RESULTS

Co-administration of CBD and L-theanine additively increased the duration of pentobarbital-induced sleep

The effects of CBD, L-theanine, and their combination were evaluated by measuring sleep latency, sleep onset time, and total sleep duration. In the pentobarbital-induced sleep test, administration of CBD or L-theanine at various concentrations significantly increased total sleep duration and decreased sleep latency compared to the vehicle group (Supplementary Fig. 1). L-theanine at doses of 10, 20, and 40 mg/kg showed comparable effects, reducing latency and increasing total sleep time (Supplementary Fig. 1A). Similarly, CBD treatment at doses of 5, 10, 20, and 40 mg/kg significantly reduced sleep latency and prolonged sleep duration (Supplementary Fig.

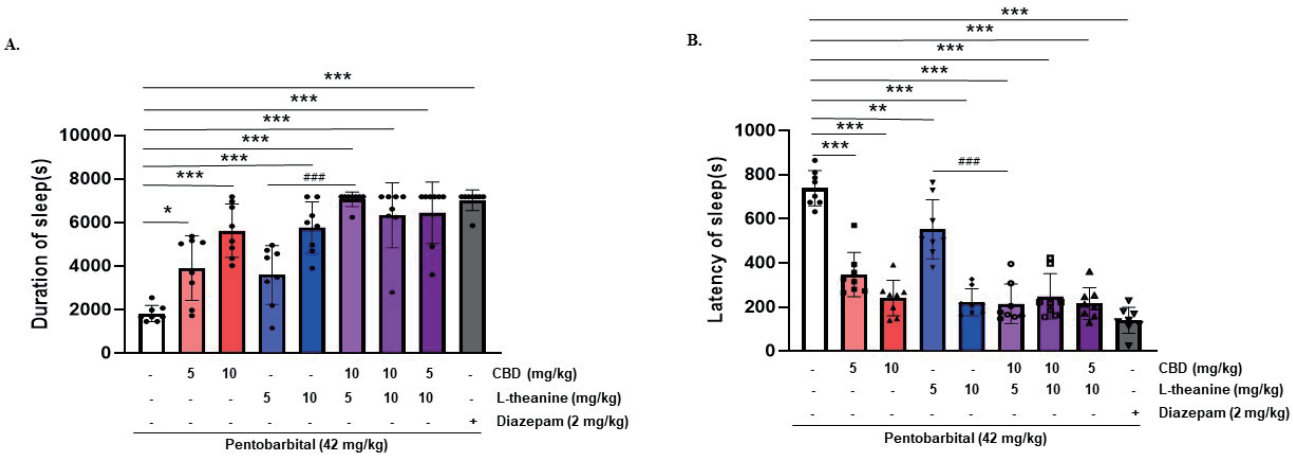


Fig. 2. Effects of CBD, L-theanine, and their combination on sleep duration and sleep latency in a pentobarbital-induced sleep under normal conditions. Mice were pretreated orally (p.o.) with CBD (5, 10 mg/kg), L-theanine (5, 10 mg/kg), or a combination of CBD and L-theanine in ratios of 1:1, 1:2, and 2:1. Thirty minutes later, all mice received an intraperitoneal injection of pentobarbital (42 mg/kg). Diazepam (2 mg/kg, p.o.) was used as a positive control. Sleep duration (A) and sleep latency (B) were measured (n=8 per group). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons. Statistical significance is indicated as follows: *p<0.05, ***p<0.001 vs. control; $^{\#\#\#\#}p$<0.001 vs. single treatment with L-theanine.

1B). To evaluate the combined effects of CBD and L-theanine, mice received one of the following treatments: vehicle (control), CBD alone (5, 10 mg/kg), L-theanine alone (5, 10 mg/kg), or CBD+L-theanine at 1:1 (10 mg/kg each), 2:1 (CBD 10 mg/kg: L-theanine 5 mg/kg) or 1:2 (CBD 5 mg/kg: L-theanine 10 mg/kg) ratios. Both individual treatments at doses ≥5 mg/kg and all combination treatments significantly improved sleep latency and total sleep duration (Fig. 2A-2B). However, sleep latency or duration was better in combined doses, especially with CBD (10 mg/kg) and L-theanine (5 mg/kg) (Fig. 2A-2B). Among the combination groups, the 2:1 ratio (CBD 10 mg/kg+L-theanine 5 mg/kg) demonstrated the most pronounced enhancement in sleep parameters. The positive control, diazepam (2 mg/kg), also significantly increased pentobarbital-induced sleep compared to the control group. Compared to single treatments, the 2:1 combination extended sleep duration more effectively, although it did not reduce sleep latency. These results suggest that the combined administration of CBD and L-theanine, particularly at the 2:1 ratio, has complementary and additive effects on sleep promotion in the pentobarbital-induced sleep model.

Ratio-dependent interactions between CBD and L-theanine on sleep in a caffeine-induced sleep disturbance model

To establish a mouse model of sleep disturbance, caffeine was administered intraperitoneally at concentrations of 40 and 80 mg/kg one hour prior to a pentobarbital-induced sleep test. Caffeine at 40 and 80 mg/kg significantly increased sleep latency and reduced total sleep duration compared to the vehicle group, confirming the successful induction of a sleep-disturbed state (Supplementary Fig. 2). Based on these findings, 80 mg/kg caffeine was selected as the optimal dose for subsequent experiments to evaluate the effects of CBD and L-theanine on sleep. Following intraperitoneal administration of caffeine (80 mg/kg, i.p.) and a 30-min reaction period, CBD and L-theanine were administered orally, either as monotherapies (2.5 or 5 mg/kg) or in combination at various ratios. To test the combined effect, the following ratios (CBD: L-theanine) were tested: 1:1 (2.5 mg/kg: 2.5 mg/kg and 5 mg/kg: 5 mg/kg), 2:1 (5 mg/kg: 2.5 mg/kg), and 1:2 (2.5 mg/kg: 5 mg/kg). Thirty minutes after oral administration, sleep latency and total sleep duration were measured using the pentobarbital-induced sleep. Among the combination groups, the 2:1 ratio of CBD to L-theanine significantly reduced sleep latency and increased total sleep duration in the caffeine-induced sleep disturbance model (Fig. 3), indicating improved sleep quality. Under normal conditions (Fig. 2), the 2:1 ratio of CBD to L-theanine was also the most effective combination; however, in caffeine-induced sleep disturbance models, even low-dose combinations produced greater improvements in sleep. Compared with single treatments with either CBD or L-theanine, combination treatments enhanced sleep duration in a concentration ratio-dependent manner. Notably, the combination of CBD 5 mg/kg with L-theanine 2.5 mg/kg at a 2:1 ratio exhibited a synergistic interaction as indicated by a CI value of 0.74 (Fig. 3A, Table 1). In contrast, other tested combinations showed additive or antagonistic interactions. Collectively, these findings suggest that the combined administration of CBD and L-theanine modulates caffein-induced sleep disturbance in a ratio-dependent manner, with combination treatments generally producing greater improvements than single treatments, and a synergistic interaction observed under specific dosing conditions. Consistent with its well-established arousal-promoting effects, caffeine administration increased EEG power in higher-frequency bands associated with cortical activation. In contrast, co-administration of CBD and L-theanine reduced caffeine-induced EEG alteration, as evidenced by significant reductions in alpha band (8.4-14 Hz) absolute power and a concomitant downward trend in beta-band (13-30 Hz) power (Supplementary Fig. 3).

Behavioral effects of CBD and L-theanine combinations in the caffeine-induced sleep disturbance model

To evaluate the behavior and motor effects of CBD and L-theanine under caffeine-induced sleep disturbance, mice were

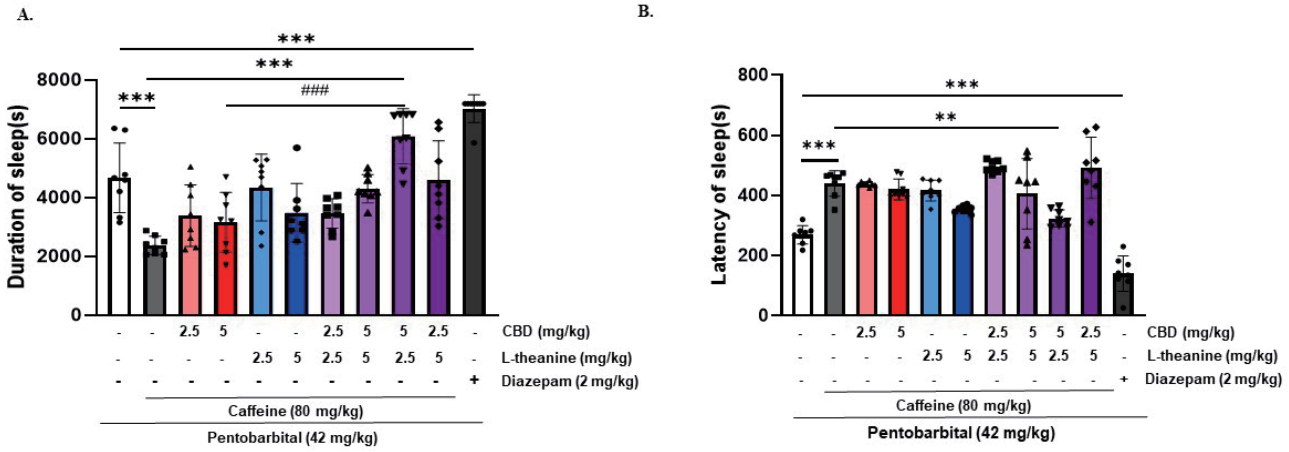


Fig. 3. Effects of CBD, L-theanine, and their combination on caffeine-induced sleep disruption in the pentobarbital-induced sleep model in mice. Caffeine (80 mg/kg, i.p.) was administered 1 h before the pentobarbital sleep test to induce a sleep-disturbed state. After 30 min of caffeine treatments, mice received orally (p.o.) CBD, L-theanine, either individually (2.5 or 5 mg/kg) or in combination as different ratios (1:1, 1:2, or 2:1). Thirty minutes after pretreatment, all mice received an intraperitoneal injection of pentobarbital (42 mg/kg). Sleep duration (A) and sleep latency (B) were measured (n=8 per group). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons. Statistical significance is indicated as follows: $^{**}p<0.01$, $^{***}p<0.001$, vs. caffeine treatments. $^{###}p<0.001$ vs. single treatment with CBD.

Table 1. Chou-Talalay combination index (CI) analysis of co-treatment with cannabidiol (CBD) and L-theanine on duration of sleep in a caffeine-induced sleep disruption model

Group name	Combination index (CI)	Chou–Talalay method
CBD 5 mg/kg+L-theanine 2.5 mg/kg	0.74**	Synergistic (CI<1)
CBD 2.5 mg/kg+L-theanine 5 mg/kg	0.95*	Additive (CI=1)
CBD 2.5 mg/kg+L-theanine 2.5 mg/kg	2.74	Antagonistic
CBD 5 mg/kg+L-theanine 5 mg/kg	0.98*	Additive (CI=1)

CI values were calculated using a single-dose–based, exploratory approximation of the Chou–Talalay additivity principle, as described in the Methods and Supplementary Methods. Sleep duration data from single-compound and combination-treated groups were normalized to fractional effects (Fa) to account for caffeine-induced sleep suppression. CI values are presented as descriptive indicators of interaction, Here, CI<1 indicates a synergistic tendency, CI=1 indicates an additive interaction, and CI>1 indicates an antagonistic tendency.

intraperitoneal injected with caffeine (80 mg/kg), followed by oral administration of CBD and L-theanine either individually or in combination at various ratios: 1:1 (2.5 mg/kg and 2.5 mg/kg) or (5 mg/kg and 5 mg/kg), 2:1 (5 mg/kg and 2.5 mg/kg), and 1:2 (2.5 mg/kg and 5 mg/kg). After 30 min, behavioral performance was assessed using the OFT and Rotarod test. In the OFT test, caffeine treatment alone failed to increase locomotor activity as anticipated. However, diazepam (positive control) significantly suppressed overall locomotor activity due to its sedative effects. In contrast, combination treatment with CBD and L-theanine, especially at the 1:1 (5 mg/kg), 2:1 and 1:2 ratios, resulted in a mild but significant reduction in total travel distance compared to caffeine-treated mice, indicating attenuation of caffeine-induced arousal without inducing excessive sedation (Fig. 4A, Supplementary Fig. 4A). Similarly, in the Rotarod test, caffeine alone did not significantly affect motor coordination compared to the negative control group. However, combination treatment with CBD and L-theanine at all tested ratios (1:1, 2:1, and 1:2) significantly decreased the latency to fall, indicating a reduction in motor endurance (Fig. 4B, Supplementary Fig. 4B). Notably, this reduction was less severe than that observed in the diazepam-treated group, which exhibited marked impairment in motor performance due to its muscle relaxant properties. Importantly, administration of CBD or L-theanine alone did not significantly affect either locomotor activity or motor coordination. The effects were evident only in the combination groups, indicating a synergistic role of CBD and L-theanine in modulating behavioral arousal. Caffeine administration did not induce significant hyperactivity in the OFT under our experimental conditions. Nevertheless, treatment with CBD and L-theanine produced a modest reduction in locomotor activity and motor endurance, without causing the profound motor impairments typically observed with conventional sedatives such as diazepam.

Synergistic effects of CBD and L-theanine on sleep-related neurochemical changes associated with serotonin and melatonin

To investigate the neurochemical changes associated with the sleep-promoting effects of CBD and L-theanine, serum serotonin, melatonin, and cortical GABA levels were examined using ELISA. Caffeine administration significantly reduced blood serotonin and melatonin levels, as well as cortical GABA levels, compared to the control group. CBD treatment alone increased blood serotonin levels in a dose-dependent manner, whereas L-theanine increased serotonin levels at a lower concentration (2.5 mg/kg). Among the combination treatments, co-administration of CBD and L-theanine at a 1:1

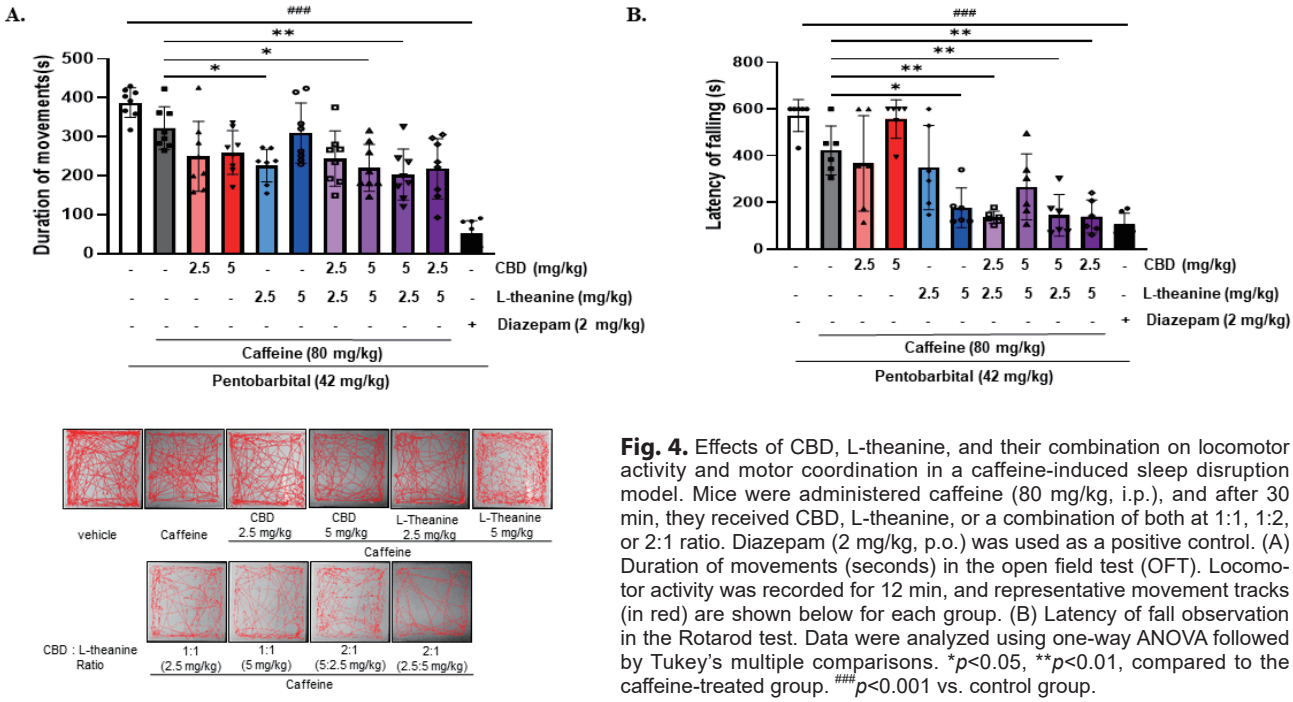


Fig. 4. Effects of CBD, L-theanine, and their combination on locomotor activity and motor coordination in a caffeine-induced sleep disruption model. Mice were administered caffeine (80 mg/kg, i.p.), and after 30 min, they received CBD, L-theanine, or a combination of both at 1:1, 1:2, or 2:1 ratio. Diazepam (2 mg/kg, p.o.) was used as a positive control. (A) Duration of movements (seconds) in the open field test (OFT). Locomotor activity was recorded for 12 min, and representative movement tracks (in red) are shown below for each group. (B) Latency of fall observation in the Rotarod test. Data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons. *p<0.05, **p<0.01, compared to the caffeine-treated group. $^{###}p$<0.001 vs. control group.

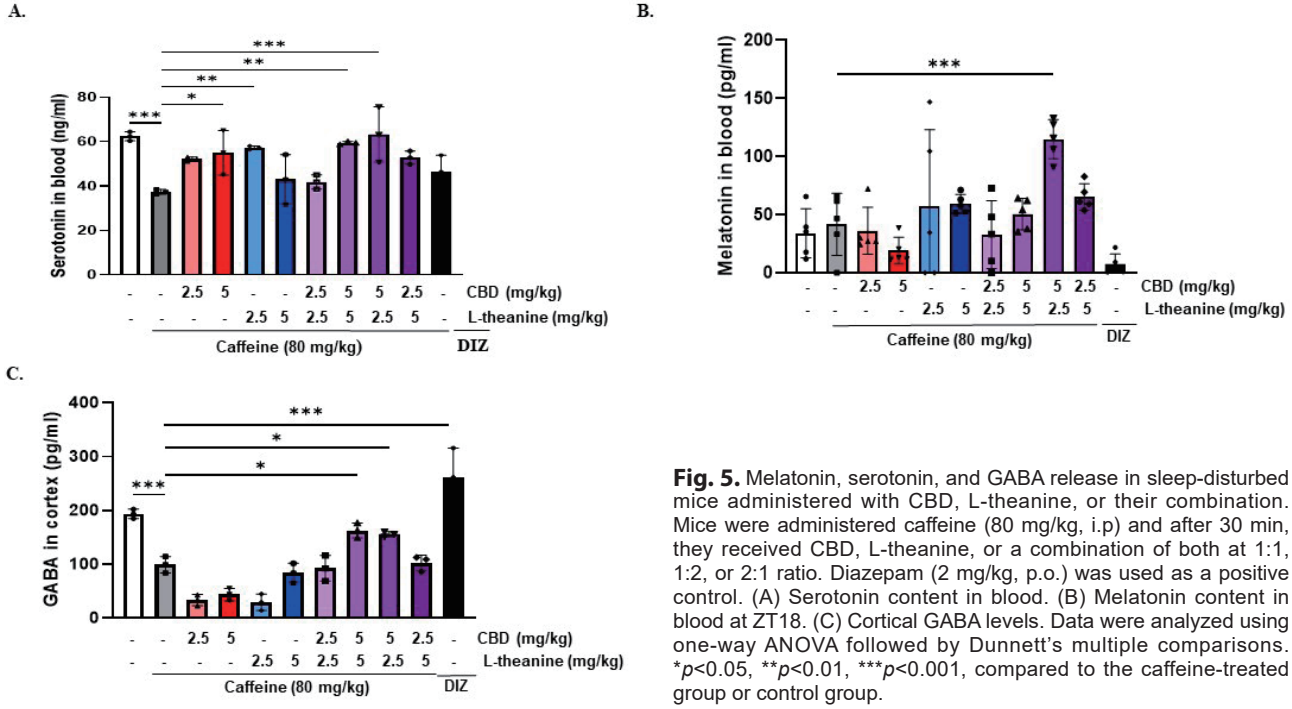


Fig. 5. Melatonin, serotonin, and GABA release in sleep-disturbed mice administered with CBD, L-theanine, or their combination. Mice were administered caffeine (80 mg/kg, i.p) and after 30 min, they received CBD, L-theanine, or a combination of both at 1:1, 1:2, or 2:1 ratio. Diazepam (2 mg/kg, p.o.) was used as a positive control. (A) Serotonin content in blood. (B) Melatonin content in blood at ZT18. (C) Cortical GABA levels. Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, compared to the caffeine-treated group or control group.

ratio (5 mg/kg each) and a 2:1 ratio (CBD 5 mg/kg, L-theanine 2.5 mg/kg) significantly elevated serotonin levels compared to the caffeine-only group (Fig. 5A). To assess treatment-related changes under conditions of physiologically elevated melatonin secretion, plasma melatonin levels were primarily measured at ZT18, corresponding to peak phase of the endogenous melatonin secretion. Individual administration of CBD or L-theanine did not restore melatonin levels suppressed by caffeine. In contrast, co-administration at ratios of 1:2 (CBD 2.5 mg/kg, L-theanine 5 mg/kg) and 2:1 (CBD 5 mg/kg, L-theanine 2.5 mg/kg) significantly elevated melatonin concentrations. Notably, at the 2:1 ratio, melatonin secretion was increased by more than 40% relative to the caffeine-treated group, approaching levels observed in the negative control

(Fig. 5B). The combination treatment resulted in the greatest increase in circulating melatonin levels compared with both the control and single treatment groups. Plasma melatonin levels measured at ZT12 showed a similar trend (Supplementary Fig. 5A). In the cortex, GABA levels were significantly increased in mice treated with CBD and L-theanine combinations, particularly at the 1:1 (CBD 5 mg/kg, L-theanine 5 mg/kg) and 2:1 (CBD 5 mg/kg, L-theanine 2.5 mg/kg) ratios (Fig. 5C). In blood, GABA levels were significantly increased following single treatment with either CBD or L-theanine, whereas no significant changes were observed following combination treatment (Supplementary Fig. 5B). Taken together, these findings indicate that while CBD and L-theanine individually modulate distinct neurochemical profiles, their combination is associated with enhanced peripheral serotonin, melatonin, and cortical GABA levels, which may be related to the observed improvements in sleep parameters in caffeine-induced sleep disturbance model.

DISCUSSION

Sleep deprivation impairs cognitive performance, immune function, and mental well-being, underscoring the need for effective, safer sleep-promoting interventions. Although pharmacological hypnotics are widely prescribed, their clinical utility is limited by risks of tolerance, dependence, and cognitive or motor impairment. Therefore, there is a growing interest in alternative strategies that improve sleep by modulating arousal rather than forcibly inducing deep sleep. In this context, the present study investigated the effects of CBD and L-theanine, alone and in combination, using a mouse model of caffeine-induced sleep disturbance, with a primary focus on phenotypic outcomes rather than direct mechanistic validation.

Caffeine, one of the most commonly consumed psychoactive substances is widely used to enhance alertness and concentration. However, caffeine intake, particularly within several hours before sleep, is strongly associated with delayed sleep onset, reduced total sleep time, increased nighttime awakenings, and impaired next-day functioning (Gardiner *et al.*, 2023). Consistent with previous reports, administration of caffeine at 80 mg/kg significantly increased sleep latency and reduced sleep duration in the pentobarbital-induced sleep test without inducing overt abnormal behaviors. Importantly, although caffeine did not produce excessive hyperlocomotion, it significantly increased alpha-band EEG power and showed an upward trend in beta-band EEG power, reflecting enhanced cortical arousal and vigilance at the neurophysiological levels. These findings support the validity of the caffeine-treated condition as an arousal-based model of sleep disturbance. Although caffeine has been reported to influence anxiety-related behaviors, the present study was not designed to assess affective or emotional states directly. Accordingly, the observed EEG changes are interpreted as markers of cortical arousal relevant to sleep disruption rather than anxiety-related behaviors per se.

In this sleep-deprived mouse model, both CBD and L-theanine individually improved sleep parameters at relatively high doses (>10 mg/kg). Notably, co-administration of low-dose CBD (5 mg/kg) and L-theanine (2.5 mg/kg) at a 2:1 ratio effectively restored sleep onset and duration impaired by caffeine (Fig. 3). Combination index analysis yielded a CI value of 0.74, suggesting a synergistic interaction at this specific dose ratio. Importantly, the low-dose combination produced effects comparable to or greater than those observed with higher-dose single treatments, supporting the potential advantage of combination therapy. Such an approach may allow efficacy to be maintained while minimizing the risk of dose-related effects reported with higher doses of CBD or L-theanine administered individually (Borzelleca *et al.*, 2006; Huestis *et al.*, 2019; Machado Bergamaschi *et al.*, 2011; Türközü and Şanlier, 2017). EEG spectral analysis provided important insight into the neurophysiological basis of these behavioral effects. Co-administration of CBD and L-theanine significantly attenuated caffeine-induced increases in alpha-band power, while beta-band activity showed a decreasing trend without reaching statistical significance. (Supplementary Fig. 3). Because alpha and beta activity are closely associated with cortical arousal and alertness, the findings indicate a reduction in hypearousal rather than direct enhancement of deep sleep. Consistently, the combination treatment did not robustly increase delta power, a hallmark of deep NREM sleep, indicating that the observed sleep-facilitating effects are more likely associated with the suppression of arousal-related cortical activity, rather than direct induction of slow-wave sleep. Additional studies examining sleep architecture and sleep quality will be necessary to further elucidate the underlying mechanisms.

The ratio-dependent effects observed in this study may reflect differences in the pharmacokinetic and pharmacodynamic profiles of the two compounds. L-theanine is rapidly absorbed and has a relatively short half-life, allowing for fast but transient modulation of arousal-related pathways (Sanchez de Medina *et al.*, 2023; Yamaura *et al.*, 2024), whereas CBD undergoes slower hepatic metabolism and exhibits prolonged systemic exposure (Mazur *et al.*, 2009). Although the precise mechanisms remain unclear, these differences may contribute to the observed ratio-dependent interactions. The antagonistic interaction observed at a low-dose 1:1 ratio (CI>2) suggests that, under certain dosing condition, the combined effect may be less effective than expected from additivity. At low concentrations, the individual effects of each compound may be insufficient to elicit a substantial sleep-promoting response, and their combination may therefore fail to produce additive or synergistic benefits. Alternatively, partial overlap in the modulation of arousal-related pathways at subthreshold doses may constrain positive interactions. Together, these observations highlight the importance of doses-ratio optimization in achieving favorable outcomes with combination therapy. This observation is particularly relevant in the context of caffeine-induced arousal. Caffeine enhances alertness by antagonizing adenosine receptors and increasing dopaminergic signaling (Hardy *et al.*, 1988). L-theanine alone has minimal sleep-promoting effects, especially in the presence of caffeine, and its efficacy has been shown to depend on interactions with other tea components (Jang *et al.*, 2012; Kim *et al.*, 2025). CBD, while generally associated with anxiolytic and sedative properties, can also modulate dopaminergic signaling (Renard *et al.*, 2017; Zuardi *et al.*, 2017). Thus, an equal ratio of CBD and L-theanine may be suboptimal for counteracting caffeine-induced arousal, whereas a higher proportion of CBD appears to provide more consistent sleep related benefits.

Neurochemical analyses further supported the behavioral and EEG findings. Co-administration of CBD and L-theanine significantly increased melatonin, serotonin, and cortical

GABA levels compared with caffeine-treated mice, with effects exceeding those observed under single-compound administration. Notably, cortical, but not plasma-GABA levels were elevated, suggesting region-specific modulation of central GABAergic activity. Previous studies have demonstrated that GABA plays a critical role in sleep initiation and maintenance, particularly within cortical and hypothalamic circuits (Brown *et al.*, 2012; Saper *et al.*, 2005). Given the established role of GABA in sleep initiation and maintenance, these findings may be relevant to the observed sleep-related outcomes.

Melatonin secretion, a key regulator of sleep onset and circadian rhythms, was significantly enhanced by the combination treatment when measured at ZT18, a physiologically relevant time point corresponding to the peak of endogenous melatonin release (Gandhi *et al.*, 2015; Nakahara *et al.*, 2003). While this increase may be related to enhanced serotonin availability, a precursor of melatonin, or modulation of GABAergic signaling pathways, previous studies have suggested that melatonin synthesis can be influenced by serotonergic input and GABAergic regulation of pineal circadian pathways (Hardeland, 2008; Klein, 2007; Liu and Reppert, 2000). However, the present study did not directly assess pineal function, receptor-levels interaction, or circadian regulatory mechanisms. Therefore, the involvement of melatonin signaling should be interpreted cautiously as a plausible, but not directly demonstrated, mechanism.

In addition, behavioral assessments indicated that the combination treatment produced mild reductions in locomotor activity and motor coordination. In the OFT, locomotor activity was modestly reduced compared with caffeine-treated mice, while the magnitude of this effect was substantially smaller than that observed with diazepam. Similarly, rotarod performance showed a mild but detectable decrease following combination treatment. Although these effects were less pronounced than those induced by diazepam, suggesting a relatively favorable motor safety profile, although further evaluation is warranted.

Several limitations of the present study should be acknowledged. Detailed analyses of sleep architecture, including NREM and REM bout structure, were not performed, and molecular analyses were limited to set of neurochemical markers. Furthermore, direct assessment of melatonin signaling pathways was not conducted. Thus, future studies incorporating comprehensive sleep staging and mechanistic investigations are necessary to further elucidate the underlying processes.

In summary, this study demonstrates that a low-dose combination of CBD and L-theanine produces a synergistic interaction in improving sleep-related outcomes in a caffeine-induced sleep disturbance model. These effects are associated with attenuation of cortical hyperarousal and changes in serotonergic, GABAergic, and melatonin-related neurochemical profiles. Notably, the combination achieved efficacy comparable to or greater than that of higher-dose single treatments, while maintaining relatively mild motor and behavioral effects. While the precise mechanisms remain to be determined, the findings suggest that CBD and L-theanine may differentially influence components of the sleep regulatory system. Collectively, these results support the potential of CBD and L-theanine co-administration as an alternative strategy for managing stimulant-induced sleep disturbances and warrant further mechanistic and translational investigation.

CONFLICT OF INTEREST

The authors declared that they have no competing interests.

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AUTHOR CONTRIBUTIONS

Conception and design: JS. Kim and HJ. Chae; Methodology development: JS. Kim and JH. Cheong; Data acquisition: JS. Kim, YJ. Yu, HK. Kim, A.U, MH. B, and Nicole B.C; Data analysis and interpretation: JS. Kim and HJ Kim. Cheong; Manuscript writing: JS. Kim, HJ Kim and HJ Chae; Administrative, technical, or material support: JS. Kim, HJ. Shim, JH. Cheong, and HJ Chae; Study supervision: JH. Cheong and HJ. Chae. All the authors read and approve the final manuscript.

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