Article

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Spinosin Ameliorates PCPA-Induced Insomnia and Anxiety-Like Behaviors via a TrkB-Dependent Restoration of Hypothalamic GABAergic Signaling

Hong Yu[1,†], Ye Wang[1,†], Hang Sun[1], Xiaoyan Ma[1], Lili Huang[1,*]

[1]School of Pharmacy, Heilongjiang University of Chinese Medicine, 150040 Harbin, Heilongjiang, China
*Correspondence: huanglili@hljucm.edu.cn (Lili Huang)
[†]These authors contributed equally.
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Background: Insomnia commonly presents alongside hyperarousal and anxiety and is often accompanied by a fundamental imbalance between inhibitory γ-aminobutyric acid (GABA) and excitatory glutamate (Glu) tissue levels. Current therapeutics often lack efficacy in addressing the neuroplastic deficits associated with chronic sleep disturbances. Spinosin is a C-glycoside flavonoid derived from Ziziphi Spinosae Semen with reported sedative activity, yet whether it improves insomnia-associated anxiety and which neurotrophic pathways are involved remain unclear. This study aimed to evaluate spinosin's effects on sleep indices and anxiety-like behaviors and tested whether activation of the Brain-derived neurotrophic factor (BDNF)-Tropomyosin receptor kinase B (TrkB)-cAMP response element-binding protein (CREB) axis contributes to recovery of hypothalamic GABAergic function.

Methods: Insomnia-like phenotypes were established in male C57BL/6J mice using *p*-chlorophenylalanine (PCPA), after which spinosin was administered orally (10–40 mg/kg) once daily for 10 days. Sleep behavior was assessed with a pentobarbital challenge, and anxiety-related behaviors were evaluated via the open field, light-dark box, elevated plus maze, and novelty-suppressed feeding tests. Neurotransmitter levels, protein expression, and gene transcription in the hypothalamus were analyzed. To probe causality, we used corticosterone-injured HT22 cells and pharmacologically blocked TrkB with ANA-12 *in vitro*.

Results: Across the tested doses, spinosin reduced sleep latency, prolonged sleep duration, and mitigated anxiety-like behaviors in PCPA-treated mice without impairing motor coordination. Mechanistically, spinosin restored the hypothalamic Glu/GABA balance by upregulating glutamate decarboxylase 67 (GAD67) and GABA_A receptor expression. These effects were accompanied by the reactivation of the hypothalamic BDNF-TrkB-CREB signaling pathway. Importantly, ANA-12 largely eliminated these spinosin-mediated changes *in vitro*, supporting a TrkB-dependent component.

Conclusion: Spinosin effectively ameliorates insomnia and comorbid anxiety by re-establishing hypothalamic excitatory and inhibitory neurotransmitter levels. This therapeutic action is fundamentally driven by the activation of BDNF-TrkB signaling, which subsequently reinforces GABAergic signaling. Together, the findings suggest that spinosin may act as a multi-target candidate for insomnia accompanied by hyperarousal.

Keywords: spinosin; insomnia; anxiety; GABA; BDNF-TrkB signaling; hypothalamus

Introduction

Insomnia is widespread, with epidemiological studies commonly reporting prevalence in the approximately 10–30% range worldwide [1,2]. This condition is rarely isolated, frequently accompanied by hyperarousal states and psychiatric comorbidities, particularly anxiety and depression, creating a bidirectional cycle of sleep deprivation and emotional dysregulation [3,4]. Current pharmacological interventions predominantly rely on benzodiazepines and non-benzodiazepine receptor agonists (Z-drugs), which act as positive allosteric modulators at the type-A receptor for γ-aminobutyric acid (GABA_A) [5]. While efficacious for acute management, these agents are often associated with adverse effects, including tolerance, rebound insomnia, cognitive impairment, and alteration of sleep parameters [6,7]. Consequently, there is a growing imperative to identify novel therapeutic agents from natural products that possess favorable safety profiles and multi-target mechanisms capable of addressing both sleep disturbances and associated hyperarousal.

The neurobiological underpinnings of sleep-wake regulation involve a complex interplay between sleep-promoting and arousal-promoting systems, primarily located in the hypothalamus [8]. Sleep–wake regulation is organized by anatomically and functionally distinct hypothalamic subnuclei, including sleep-promoting GABAergic neurons in the ventrolateral preoptic area and arousal-

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promoting populations in the lateral hypothalamus (e.g., orexin neurons). In this study, hypothalamic tissue was analyzed at the regional level to assess whether spinosin is associated with restoration of inhibitory-excitatory balance and Brain-derived neurotrophic factor (BDNF)-Tropomyosin receptor kinase B (TrkB) signaling in hypothalamic homogenates. However, subnucleus-specific mechanisms require further investigation. A core pathophysiological feature of insomnia involves a shift in the excitatory–inhibitory (E/I) equilibrium, wherein GABAergic tone is functionally insufficient to counterbalance glutamatergic drive [9]. The *p*-chlorophenylalanine (PCPA) model, which depletes serotonin (5-HT) and subsequently induces a state of insomnia and anxiety-like hyperarousal, effectively mimics this neurochemical imbalance and has been widely utilized to evaluate potential sedative-hypnotic candidates [10,11]. Restoring the hypothalamic neurotransmitter homeostasis, particularly through enhancement of GABAergic signaling, remains a primary therapeutic strategy.

Emerging evidence suggests that neurotransmitter regulation is intricately linked to neurotrophic signaling. Among such mediators, brain-derived neurotrophic factor (BDNF) and its cognate receptor tropomyosin receptor kinase B (TrkB) serve dual roles: sustaining synaptic plasticity while also calibrating sleep–wake homeostasis and affective tone [12,13]. Notably, circulating BDNF titers are consistently lower in individuals suffering from persistent insomnia [14]. At the mechanistic level, BDNF/TrkB activity supports maturation of inhibitory circuits and can promote expression of GAD67, a key enzyme involved in GABA synthesis [15]. Subsequent engagement of the transcription factor cAMP response element-binding protein (CREB) consolidates this neurotrophic and synapse-stabilizing cascade [16]. Thus, pharmacological agents that activate the BDNF-TrkB-CREB axis may offer a fundamental restoration of GABAergic function, distinct from the transient receptor modulation provided by conventional hypnotics.

Spinosin, a major C-glycoside flavonoid isolated from Ziziphi Spinosae Semen (Suan Zao Ren), has demonstrated significant sedative and anxiolytic properties in preclinical models [17,18]. Pharmacokinetic studies have shown that spinosin is detectable in brain tissue, including hypothalamic regions [19]. While prior research has attributed its effects to the modulation of serotonin (5-HT_1A) receptors or GABA_A receptors [18,20], it remains unclear whether spinosin can ameliorate PCPA-induced insomnia and anxiety-like behaviors through the modulation of neuroplasticity signals. Specifically, the causal involvement of the BDNF-TrkB pathway in spinosin-mediated GABAergic restoration has not been definitively established using specific pharmacological inhibitors.

We investigated the therapeutic effects of spinosin on sleep parameters and anxiety-like behaviors in a PCPA-induced insomnia mouse model in this study. We hypothesized that spinosin ameliorates insomnia-like phenotypes by restoring hypothalamic GABAergic signaling by activating the BDNF-TrkB pathway. To test this, we combined behavioral assessments, biochemical analyses, and an *in vitro* blockade experiment using the selective TrkB antagonist ANA-12 [21]. Our findings provide novel insights into the neuromolecular mechanisms of spinosin, positioning it as a promising candidate for treating insomnia and comorbid hyperarousal states.

Materials and Methods

Animals

We purchased male C57BL/6J mice (aged 8–10 weeks old, body weight 22–25 g) from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were group-housed under standard environmental conditions (temperature: 22 ± 2 °C; humidity: 50–60%) with a reversed 12:12-h light-dark cycle (illumination onset: 07:00), and had unrestricted access to standard chow and tap water except where noted. A minimum acclimatization period of seven days preceded all experimental procedures.

Experimental protocols received ethical approval from the Institutional Animal Care Committee at Heilongjiang University of Chinese Medicine (Approval No. 2024121322) and were conducted in compliance with the guidelines of the National Institutes of Health for the humane treatment of laboratory animals.

Drugs and Treatment

To induce insomnia-like phenotypes, *p*-chlorophenylalanine (PCPA; C6506, Sigma-Aldrich, St. Louis, MO, USA) was prepared in sterile saline and administered intraperitoneally at 300 mg/kg once daily for three consecutive days. Controls received matched saline volumes.

Spinosin (purity ≥99%; A0585, CHENGDU MUST BIO-TECHNOLOGY Co., Ltd., Chengdu, China) was suspended in carboxymethylcellulose sodium (CMC-Na; 0.5%; 30036365, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and administered by oral gavage at doses of 10, 20, and 40 mg/kg, with Zolpidem (10 mg/kg, p.o.; Z103, Sigma-Aldrich, St. Louis, MO, USA) as a reference compound [22]. All treatments were delivered at 0.01 mL/g body weight. Pharmacological intervention commenced 24 h following the final PCPA administration and continued once daily for ten successive days. To minimize circadian variation as a confounding variable, all doses were administered within a fixed two-hour window (09:00–11:00). All mice received equal volumes of vehicle (0.5% CMC-Na).

Dose selection was informed by published evidence that spinosin (10–40 mg/kg) prolongs pentobarbital-evoked sleep and augments non-rapid eye movement (NREM) duration in rodents while sparing motor function [17,18]. Accordingly, three escalating dose levels (10, 20, and 40

mg/kg) were adopted to capture the full dose–response relationship of spinosin against PCPA-evoked insomnia-like behavior and to delineate the underlying neurobiological substrates.

Experimental Design

Mice were randomly assigned to the following groups (n = 8 per group): Control, PCPA model, PCPA + zolpidem, and PCPA + Spinosin (low, medium, and high doses). Behavioral tests were conducted after completion of drug treatment in a fixed order from low-stress to high-stress paradigms. Investigators were blinded to group allocation during behavioral testing and data analysis.

All behavioral tests were performed in the full cohort (six groups, n = 8/group). For molecular and biochemical assays, a pre-specified subset of four groups (Control, PCPA, zolpidem, and medium-dose spinosin) was analyzed to reduce experimental costs while prioritizing mechanistic validation.

Behavioral Tests

All behavioral assays were performed after the drug regimen had concluded. Behavioral tests were conducted in a fixed sequence from least to most stressful to minimize carryover effects, and every session fell within the 09:00–15:00 window in a dedicated, low-noise environment. Prior to each assay, animals were given $\geq$30 min of room acclimatization. Apparatus surfaces were decontaminated with 75% ethanol between subjects to eliminate residual olfactory information. The operator was blinded to group identity throughout data acquisition and scoring.

The behavioral test sequence was as follows: rotarod test, open field test, light–dark box test, elevated plus maze, and novelty-suppressed feeding test. The pentobarbital-induced sleep test was conducted 48 h after the novelty-suppressed feeding test to avoid interference from acute stress.

Rotarod Test: To assess motor coordination and postural balance, an accelerating rotarod paradigm (Model XR-6C, Shanghai XinRuan Information Technology Co., Ltd., Shanghai, China) was employed. Animals received one familiarization session 24 h before the formal test. During the trial, rod velocity linearly increased from 4 to 40 rpm over a 300-s ramp, and the duration each mouse maintained its position (fall latency) was recorded. Three consecutive attempts separated by $\geq$15-min rest intervals were averaged per animal.

Open Field Test (OFT): Voluntary exploration and anxiety-related indices were recorded in a square open-field enclosure (50 $\times$ 50 cm, 35-cm opaque sidewalls). Ambient illumination was maintained at 30–50 lux (target $\approx$ 40 lux at the floor surface), verified before each session with a calibrated photometer (Model TES-1330A, TES Electrical Electronic Corp., Taipei, Taiwan, China) to guarantee uniform spatial lighting. Individual mice were introduced to the center and permitted to explore undisturbed for 5 min. An automated video-tracking platform (SuperMaze v2.0, Shanghai XinRuan Information Technology Co., Ltd., Shanghai, China) captured total path length and dwell time within the designated central zone.

Light–Dark Box Test (LDB): A bipartite shuttle box (44 $\times$ 21 $\times$ 21 cm) was partitioned into a brightly lit zone (~400 lux; occupying roughly two-thirds of the total floor) and a dim zone (~4 lux, red illumination; one-third of the floor). Each animal was positioned in the lit zone at trial onset and given 5 min of unobstructed locomotion. Two outcome measures were collected: cumulative time in the illuminated sector and frequency of inter-compartment crossings.

Elevated Plus Maze (EPM): The plus-maze comprised two walled (closed) arms and two unwalled (open) arms (each 30 $\times$ 5 cm) radiating from a shared central platform, positioned 50 cm above the floor; enclosed arms featured 20-cm opaque barriers. Subjects were released onto the central junction with their head oriented toward an open arm and allowed 5 min of free exploration. Open-arm entry counts and cumulative open-arm occupancy time served as anxiety-related endpoints.

Novelty-Suppressed Feeding Test (NSF): Stress-evoked hyponeophagia was evaluated with a novelty-suppressed feeding paradigm. Following a 24-h food restriction (water ad libitum), each mouse was introduced to a corner of the previously described open-field enclosure (50 $\times$ 50 cm) in whose geometric center a single food pellet had been placed. Feeding onset latency was timed up to a 600-s ceiling. Animals were promptly returned to their home cages upon trial completion, and a 30-min home-cage chow intake measurement was performed to rule out appetite-related confounds.

Pentobarbital-Induced Sleep Test: Sleep sensitivity was detected 48 h after the NSF session. Sodium pentobarbital (40 mg/kg, 0.01 mL/g; P3761, Sigma-Aldrich, Shanghai, China) was prepared in physiological saline and injected intraperitoneally; each mouse was then transferred to an individual clean cage. Onset of sleep was defined as an uninterrupted loss of the righting reflex (LORR) persisting $\geq$60 s, with latency calculated from injection time to the first qualifying LORR event. Sleep duration was defined as the interval from sustained LORR to recovery, operationalized as the animal successfully righting itself three consecutive times within 60 s of being placed in the supine position.

Measurement of Home-Cage Intake

Immediately after the novelty-suppressed feeding test, mice were returned to their home cages and provided with pre-weighed food pellets. Home-cage food intake was measured over a 30-min period by weighing the remaining food and calculated as the difference between the initial and final food weights. This measurement was used to exclude potential alterations in appetite or metabolic status.

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Tissue Collection

At 24 h post-behavioral testing, animals received a terminal dose of sodium pentobarbital (50 mg/kg, i.p.; P3761, Sigma-Aldrich, Shanghai, China) and were euthanized by cervical dislocation. Brains were promptly excised, and the hypothalamus was micro-dissected over ice with reference to a standardized murine brain atlas. Harvested specimens were snap-frozen in liquid nitrogen and maintained at –80 °C pending subsequent analysis. Hypothalamic samples were dissected according to a standard atlas and analyzed as whole hypothalamic homogenates; subnuclei were not isolated separately.

Determination of Neurotransmitter Levels in the Hypothalamus

Hypothalamic GABA (E-BC-K852-M, Elabscience, Wuhan, China) and glutamate (Glu) (ab83389, Abcam, Cambridge, UK) concentrations were quantified using commercial kits following the manufacturer's protocols. Tissue was homogenized in chilled phosphate-buffered saline, centrifuged (12,000 ×g, 10 min, 4 °C), and the clarified supernatant was reserved. For the GABA assay, phenol and sodium hypochlorite reacted with GABA to generate a blue-green chromophore, and absorbance was measured at 640 nm. For the Glu assay, glutamate was specifically recognized by the enzyme mix to produce proportional color development, and absorbance was measured at 450 nm. Both readings were acquired on a microplate spectrophotometer (Model Multiskan FC, Thermo Fisher Scientific, Vantaa, Finland), and analyte levels were interpolated from the respective calibration curves. Final values were corrected for total protein content (BCA method) and reported as nmol/mg protein.

Evaluation of Macroscopic Glutamate and GABA Pools

To provide a preliminary biochemical estimation of the neurochemical environment in the hypothalamus, the macroscopic pool of excitatory versus inhibitory neurotransmitters was evaluated. The relative Glutamate/GABA ratio was calculated by dividing the absolute glutamate concentration (nmol/mg protein) by the corresponding GABA concentration (nmol/mg protein) obtained from ELISA assays for each whole-hypothalamus homogenate. It should be noted that this calculation reflects the total tissue neurotransmitter pool rather than dynamic synaptic release.

Western Blotting

Hypothalamic specimens were lysed in radioimmunoprecipitation assay (RIPA) buffer (P0013B, Beyotime Biotechnology, Shanghai, China) supplemented with a protease/phosphatase inhibitor cocktail (P1045, Beyotime Biotechnology, Shanghai, China), and protein yield was quantified by bicinchoninic acid (BCA) colorimetry (P0012, Beyotime Biotechnology, Shanghai, China). Equalized protein loads were resolved on SDS–polyacrylamide gels (P0015; Beyotime, Shanghai, China) and electro-transferred onto polyvinylidene difluoride membranes (IPVH00010, Millipore, Burlington, MA, USA). Primary antibodies (1:1000 dilution) against GAD67 (ET1703-71; HuaBio, Hangzhou, China), GABA_A receptor subunit (α1) (12410-1-AP; Proteintech, Wuhan, China), BDNF (28205-1-AP; Proteintech, Wuhan, China), TrkB (80878-6-RR; Proteintech, Wuhan, China), phosphorylated TrkB (p-TrkB; ET1610-35; HuaBio, Hangzhou, China), CREB (ET1601-15; HuaBio, Hangzhou, China), phosphorylated CREB (p-CREB; 28792-1-AP, Proteintech, Wuhan, China), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 60004-1-Ig; Proteintech, Wuhan, China) were used.

Membranes were subsequently probed with horseradish peroxidase (HRP)-labeled secondary antibodies (1:2000; ZB-2305, ZB-2301; Zhongshan Golden Bridge, Beijing, China). Immunoreactive signals were detected via enhanced chemiluminescence (P0018S, Beyotime Biotechnology, Shanghai, China), and band densities were quantified in ImageJ (v1.53, National Institutes of Health, Bethesda, MD, USA); phosphoprotein signals were expressed as ratios to the cognate total-protein band.

Quantitative Real-Time PCR (qPCR)

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (DP424; Tiangen, Beijing, China). RNA was reverse-transcribed into cDNA using a reverse transcription kit (KR116; Tiangen, Beijing, China). Quantitative PCR was performed using SYBR Green Master Mix (FP205, Tiangen, Beijing, China) on a real-time PCR system (Model CFX96 Touch, Bio-Rad Laboratories, Hercules, CA, USA). The relative expression levels of Glutamate decarboxylase 1 (*Gad1*, encoding GAD67), Gamma-aminobutyric acid type A receptor subunit alpha1 (*Gabra1*, encoding GABA_A receptor α1 subunit), Bdnf, and neurotrophic tyrosine receptor kinase 2 (Ntrk2, encoding TrkB) were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH. Table 1 presents the sequences of primers.

Cell Culture and Drug Treatment

Murine hippocampal HT22 neurons (Cell Bank, Chinese Academy of Sciences, Shanghai, China) were propagated in Dulbecco's Modified Eagle Medium (DMEM; C11995500BT, Gibco, Thermo Fisher Scientific, Grand Island, NY, USA) contained 10% heat-inactivated fetal bovine serum (10099141, Gibco, Thermo Fisher Scientific, Grand Island, NY, USA) and antibiotics (penicillin 100 U/mL; streptomycin 100 µg/mL; C0222, Beyotime Biotechnology, Shanghai, China) at 37 °C under a 5% CO_2 humidified environment. Cell identity was verified by Short Tandem Repeat (STR) genotyping, and routine mycoplasma screening returned negative results. To estab-

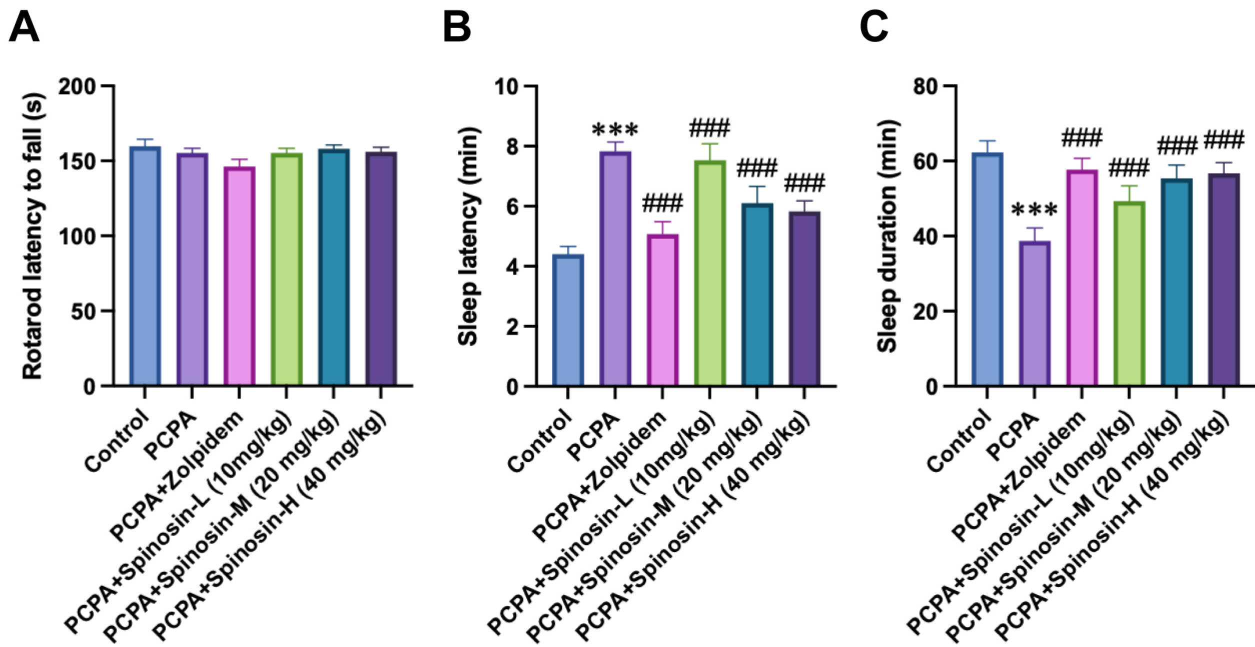

Fig. 1. Spinosin attenuated *p*-Chlorophenylalanine (PCPA)-induced insomnia-like behaviors without affecting motor coordination. (A) Effects of spinosin on motor coordination assessed by the rotarod test. (B,C) Effects of spinosin on pentobarbital-induced sleep parameters, including sleep latency (B) and sleep duration (C). N = 8. ***$p < 0.001$ vs. Control group; ###$p < 0.001$ vs. PCPA group.

Table 1. The primer sequences used for quantitative real-time PCR.

Primers	Sequences (5′-3′)
mus-*Gad1*-F	TACTCCTGTGACAGAGCCGA
mus-*Gad1*-R	CGTAGCAACCAACACTCCCT
mus-*Gabra1*-F	AAGTGAGACTTCGGACCACG
mus-*Gabra1*-R	CTACAACCACTGAACGGGCT
mus-*Bdnf*-F	GCCATCCACACGTGACAAAA
mus-*Bdnf*-R	ACCCAGTATACCAACCCGGA
mus-*Ntrk2*-F	TTCTGGAGTTTCTGCCCCTG
mus-*Ntrk2*-R	GGTGGATCTCCCTGGACTCT
mus-*Gapdh*-F	TGTCTCCTGCGACTTCAACA
mus-*Gapdh*-R	GGTGGTCCAGGGTTTCTTACT

Gad1 (encoding GAD67), Glutamate decarboxylase 1; *Gabra1* (encoding GABA_A receptor α1 subunit), Gamma-aminobutyric acid type A receptor subunit alpha1; *Bdnf*, Brain-derived neurotrophic factor; *Ntrk2* (encoding TrkB), neurotrophic tyrosine receptor kinase 2; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase.

lish an *in vitro* injury model, cells were exposed to corticosterone (150 µM; HY-B1618, MedChemExpress, Monmouth Junction, NJ, USA) for 24 h [23]. Spinosin was added to the culture medium at a concentration of 10 µM for 24 h, either alone or in combination with the TrkB inhibitor ANA-12 (10 µM; HY-12497, MedChemExpress, Monmouth Junction, NJ, USA) [21], which was applied 30 min prior to spinosin treatment.

Cell Viability Assay

Viability was quantified with the Cell Counting Kit-8 reagent (C0038, Beyotime, Shanghai, China). Following treatment, 10 µL of CCK-8 working solution was dispensed into each 96-well culture well and incubated for 2 h (37 °C). Optical density at 450 nm was recorded, and survival rates were calculated relative to untreated controls.

Western Blot Analysis of Cultured Cells

Cell lysates were prepared in inhibitor-supplemented RIPA buffer and processed by immunoblotting exactly as for the tissue specimens. Band intensities were normalized to GAPDH and presented as fold-changes versus the untreated control.

Statistical Analysis

Results are reported as mean ± SEM (standard error of the mean). Statistical analyses were performed using GraphPad Prism (v9.5.1, GraphPad Software, San Diego, CA, USA). Prior to analysis, we utilized the Shapiro-Wilk test to assess normality and the Brown-Forsythe test to evaluate homogeneity of variances. After careful verification of our dataset, all the data presented in the figures met the assumptions of normal distribution and homoscedasticity. Group differences were assessed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A *p*-value < 0.05 was considered statistically significant.

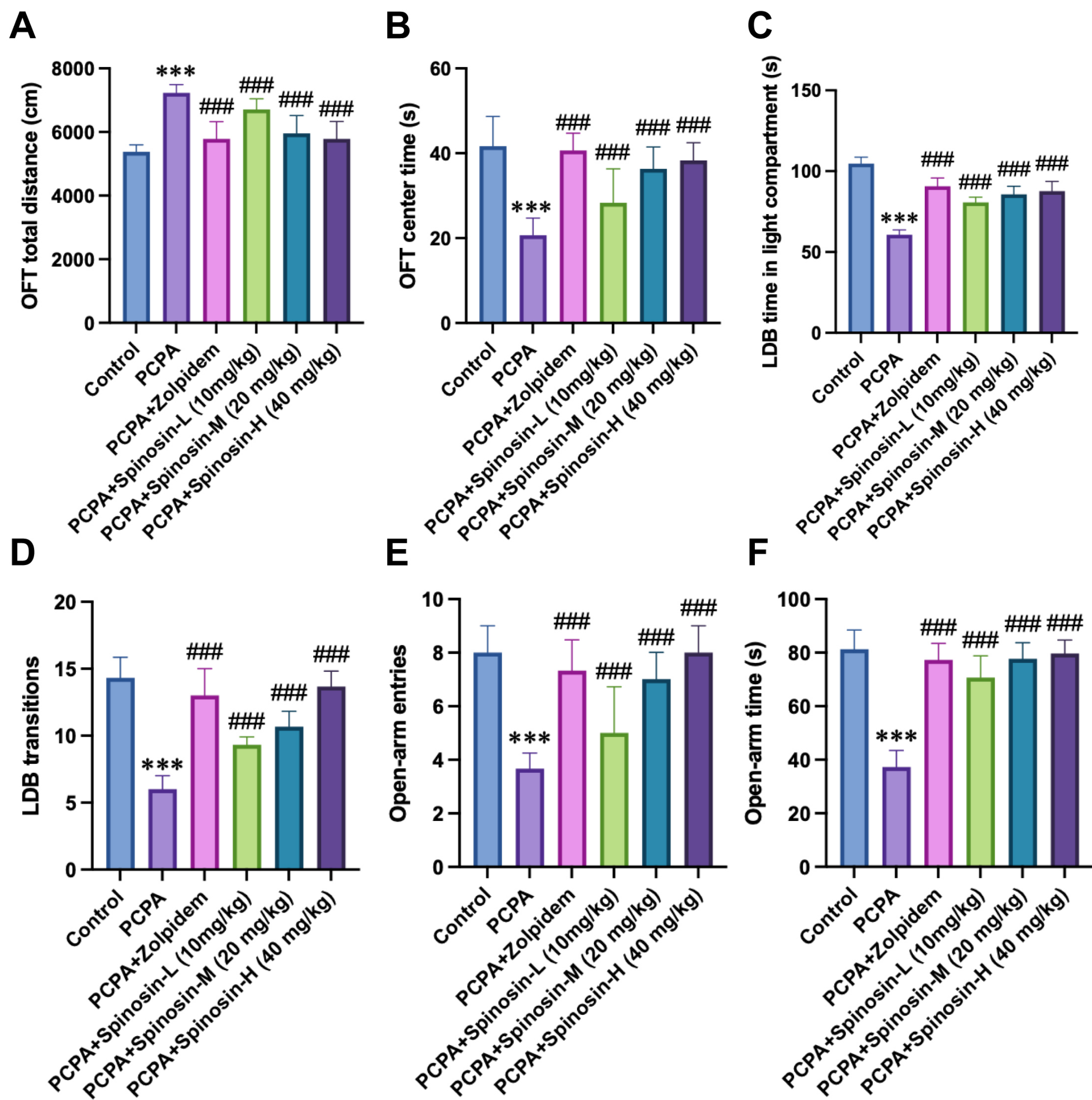

Fig. 2. Spinosin reduced hyperarousal- and anxiety-like behaviors in PCPA-treated mice. (A) Total distance traveled in the open field test performed under dim light conditions (30–50 lux). (B) Time spent in the central area of the open field. (C) Time spent in the light compartment in the light–dark box test (light compartment: ~400 lux; dark compartment: ~4 lux). (D) Number of transitions between light and dark compartments. (E) Number of open-arm entries in the elevated plus maze. (F) Time spent in the open arms of the elevated plus maze. N = 8. ***$p < 0.001$ vs. Control group; ###$p < 0.001$ vs. PCPA group.

Results

Spinosin Attenuated PCPA-Induced Insomnia-Like Behaviors Without Affecting Motor Coordination

To exclude the possibility that the behavioral effects of spinosin were secondary to motor impairment or non-specific sedation, motor coordination was evaluated using the rotarod test. As shown in Fig. 1A, no significant difference was observed in the latency to fall among the Control, PCPA, zolpidem, or spinosin-treated groups, indicating that neither PCPA nor spinosin affected motor performance.

Pentobarbital-induced sleep was further assessed to evaluate sleep sensitivity. As shown in Fig. 1B,C, PCPA-treated mice exhibited a significantly prolonged sleep latency and a shortened sleep duration compared with the Control group ($p < 0.001$). Treatment with zolpidem markedly reduced sleep latency and prolonged sleep duration ($p < 0.001$). Notably, spinosin administration dose-dependently shortened sleep latency and increased sleep duration in PCPA-treated mice ($p < 0.001$), with the medium and high doses producing effects comparable to those of zolpidem.

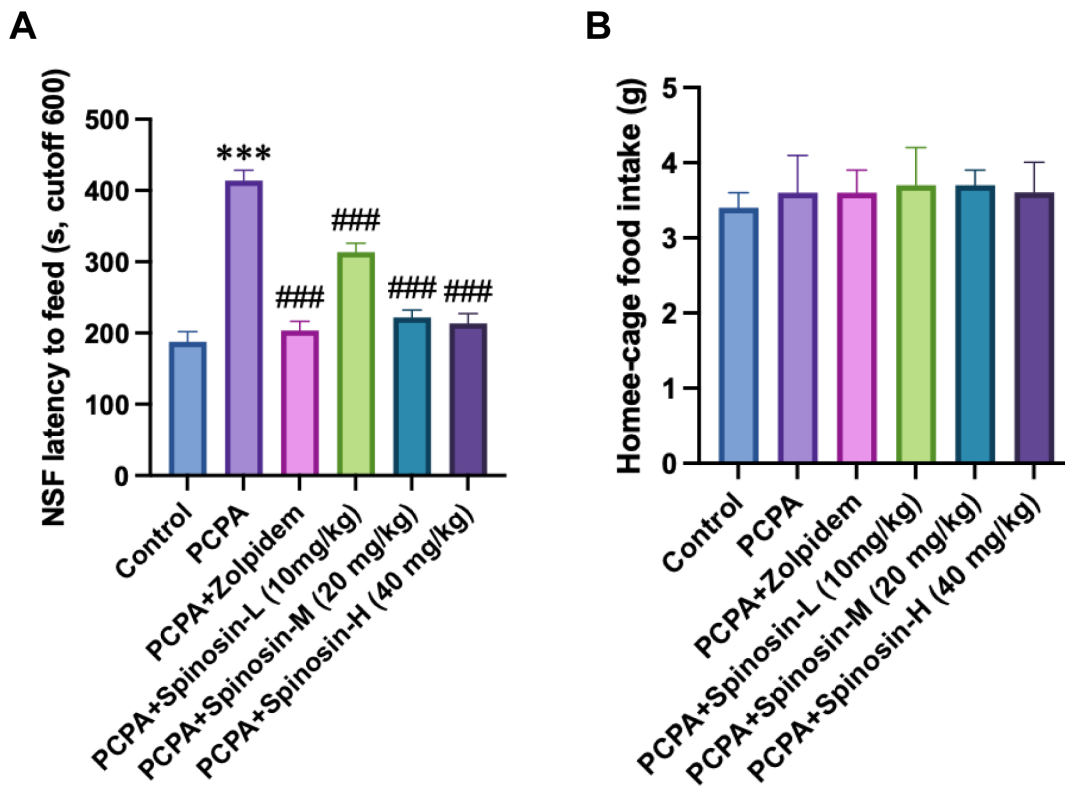

Fig. 3. Spinosin alleviated stress-induced hyponeophagia in the novelty-suppressed feeding test. (A) Latency to feed in a novel environment with a cutoff time of 600 s. (B) Home-cage food consumption measured immediately after the test. N = 8. ***$p < 0.001$ vs. Control group; ###$p < 0.001$ vs. PCPA group.

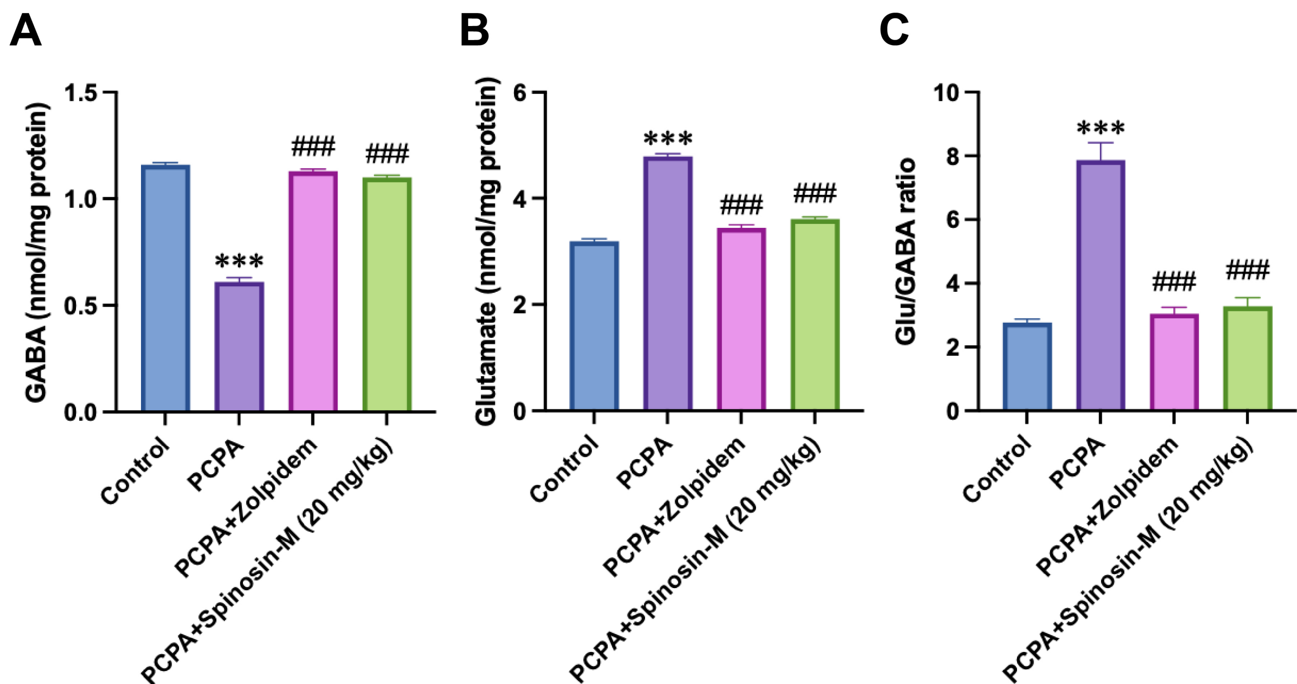

Fig. 4. Spinosin modulated the macroscopic pool of excitatory and inhibitory neurotransmitters in the hypothalamus of PCPA-treated mice. (A) γ-Aminobutyric acid (GABA) levels in the hypothalamus. (B) Glutamate (Glu) levels in the hypothalamus. (C) Relative ratio of total glutamate to GABA content in the hypothalamus. N = 8. ***$p < 0.001$ vs. Control group; ###$p < 0.001$ vs. PCPA group.

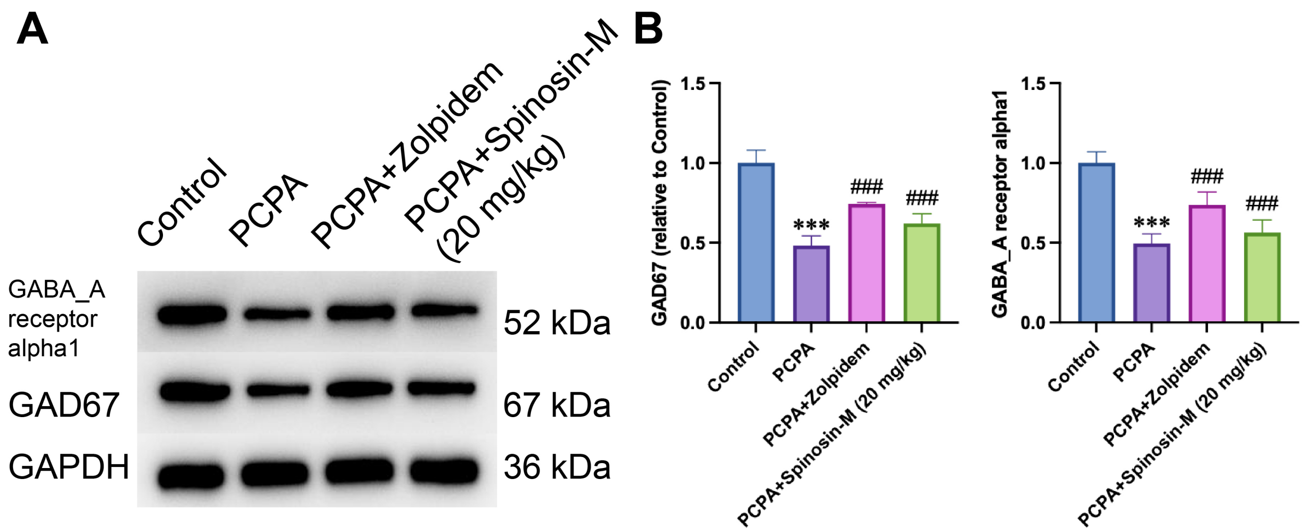

Fig. 5. Spinosin enhanced hypothalamic GABAergic signaling by upregulating GAD67 and GABA_A receptor expression. (A) Representative Western blot images of GABA_A receptor subunit (α1) and glutamate decarboxylase 67 (GAD67) in the hypothalamus. (B) The quantitative analysis of protein expression. N = 8. ***$p < 0.001$ vs. Control group; ###$p < 0.001$ vs. PCPA group.

Spinosin Reduced Hyperarousal- and Anxiety-Like Behaviors in PCPA-Treated Mice

To assess hyperarousal and anxiety-like behaviors, mice were subjected to the open field test, light-dark box test, and elevated plus maze. In the open field test (Fig. 2A), PCPA-treated mice displayed a significant increase in total distance traveled compared with Control mice ($p < 0.001$), suggesting a hyperarousal-like state. This increase was attenuated by zolpidem and spinosin treatment ($p < 0.001$), particularly at the medium and high doses. Analysis of anxiety-related behavior revealed that PCPA-treated mice spent significantly less time in the central area of the open field (Fig. 2B; $p < 0.001$). Spinosin treatment significantly increased center time compared with the PCPA group ($p < 0.001$), indicating an anxiolytic-like effect.

Consistently, in the light-dark box test, PCPA-treated mice spent less time in the light compartment and exhibited fewer transitions between compartments (Fig. 2C,D). These changes were significantly reversed by spinosin administration ($p < 0.001$). In the elevated plus maze, PCPA-treated mice showed a marked reduction in both the number of open-arm entries and the time spent in the open arms (Fig. 2E,F; $p < 0.001$), whereas spinosin treatment significantly restored these parameters toward control levels.

Spinosin Alleviated Stress-Induced Hyponeophagia in the Novelty-Suppressed Feeding Test

The novelty-suppressed feeding test was performed to further evaluate stress-related behavioral alterations. As shown in Fig. 3A, PCPA-treated mice exhibited a significantly prolonged latency to feed in a novel environment compared with Control mice ($p < 0.001$), indicating enhanced stress-induced hyponeophagia. Treatment with zolpidem or spinosin significantly reduced the latency to feed ($p < 0.001$), with medium and high doses of spinosin producing robust effects. Importantly, no significant difference was observed in home-cage food consumption among groups (Fig. 3B), suggesting that the changes in feeding latency were not attributable to alterations in appetite or metabolic status.

Spinosin Modulated the Macroscopic Pool of Excitatory and Inhibitory Neurotransmitters in the Hypothalamus

Given the critical role of hypothalamic neurotransmission in sleep-wake regulation, levels of GABA and Glu were quantified in the hypothalamus. As shown in Fig. 4A, PCPA treatment significantly reduced hypothalamic GABA levels compared with the Control group, whereas spinosin administration markedly increased GABA levels ($p < 0.001$).

Conversely, PCPA-treated mice exhibited significantly elevated glutamate levels (Fig. 4B), which were reduced by spinosin treatment ($p < 0.001$). Consequently, the Glu/GABA ratio was markedly increased in the PCPA group and significantly normalized following spinosin administration (Fig. 4C), indicating a modulation of the relative excitatory and inhibitory neurotransmitter pools at the whole-tissue level.

Spinosin Enhanced Hypothalamic GABAergic Signaling by Upregulating GAD67 and GABA_A Receptor Expression

To further investigate the effects of spinosin on GABAergic signaling, the expression of key GABA-related proteins in the hypothalamus was examined. Representative immunoblots are shown in Fig. 5. Quantitative analysis revealed that PCPA treatment significantly reduced the

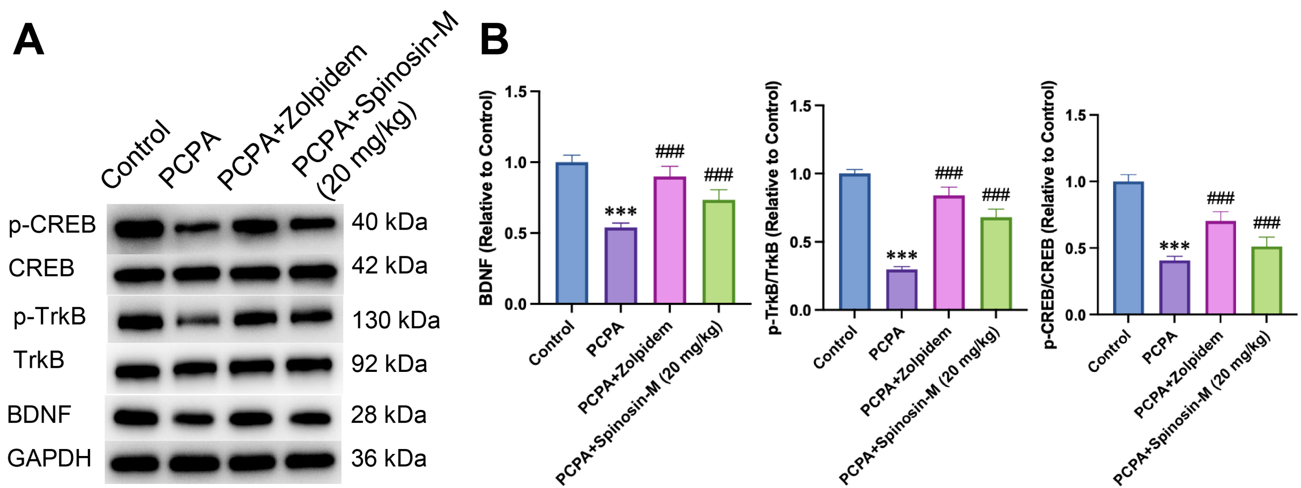

Fig. 6. Spinosin activated the Brain-derived neurotrophic factor (BDNF)-Tropomyosin receptor kinase B (TrkB)-cAMP response element-binding protein (CREB) signaling pathway in the hypothalamus. (A) Representative Western blot images of brain-derived neurotrophic factor (BDNF), phosphorylated TrkB (p-TrkB), total TrkB, phosphorylated CREB (p-CREB), and total CREB. (B) Protein expression of BDNF, ratio of p-TrkB/TrkB and p-CREB/CREB. N = 8. ***$p < 0.001$ vs. Control group; ###$p < 0.001$ vs. PCPA group.

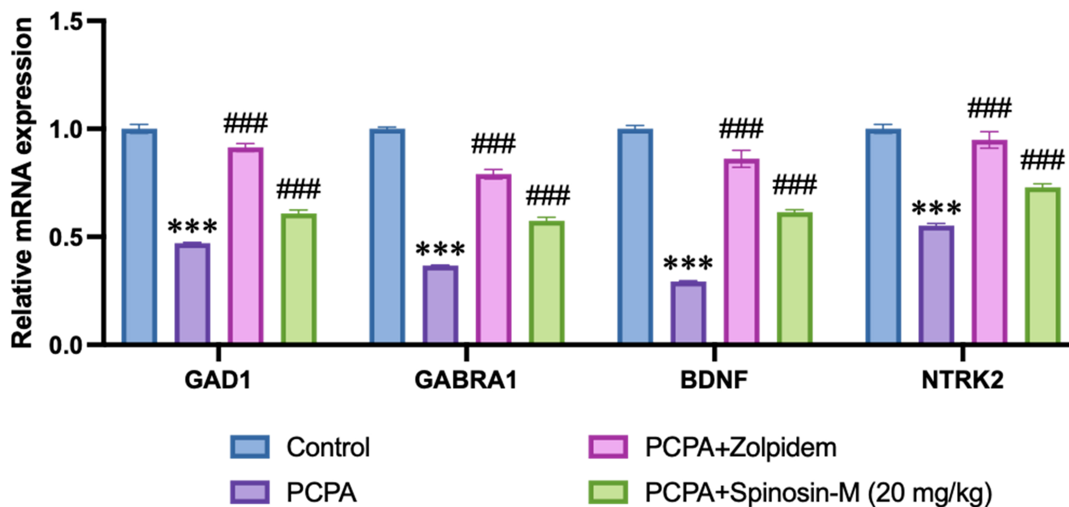

Fig. 7. Spinosin regulated GABAergic- and neurotrophic-related gene expression in the hypothalamus. Relative mRNA expression levels of Glutamate decarboxylase 1 (*Gad1*, encoding GAD67), Gamma-aminobutyric acid type A receptor subunit alpha1 (*Gabra1*, encoding GABA_A receptor α1 subunit), *Bdnf*, and neurotrophic tyrosine receptor kinase 2 (*Ntrk2*, encoding TrkB) were determined by quantitative real-time PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). N = 8. ***$p < 0.001$ vs. Control group; ###$p < 0.001$ vs. PCPA group.

protein levels of GAD67 and the GABA_A receptor subunit compared with the Control group ($p < 0.001$). Spinosin significantly increased the expression of both GAD67 and the GABA_A receptor subunit in PCPA-treated mice, suggesting an enhancement of GABA synthesis and receptor availability ($p < 0.001$).

Spinosin Activated the BDNF-TrkB-CREB Signaling Pathway in the Hypothalamus

Given the involvement of neurotrophic signaling in sleep regulation and emotional behaviors, the BDNF-TrkB-CREB pathway was examined. As shown in Fig. 6, PCPA-treated mice exhibited a significant reduction in hypothalamic BDNF protein expression compared with Control mice ($p < 0.001$). Furthermore, the ratios of phosphorylated TrkB to total TrkB and phosphorylated CREB to total CREB were significantly decreased following PCPA treatment (Fig. 6; $p < 0.001$). Spinosin administration significantly restored BDNF expression and enhanced TrkB and CREB phosphorylation ($p < 0.001$), indicating activation of the BDNF-TrkB-CREB signaling pathway in the hypothalamus.

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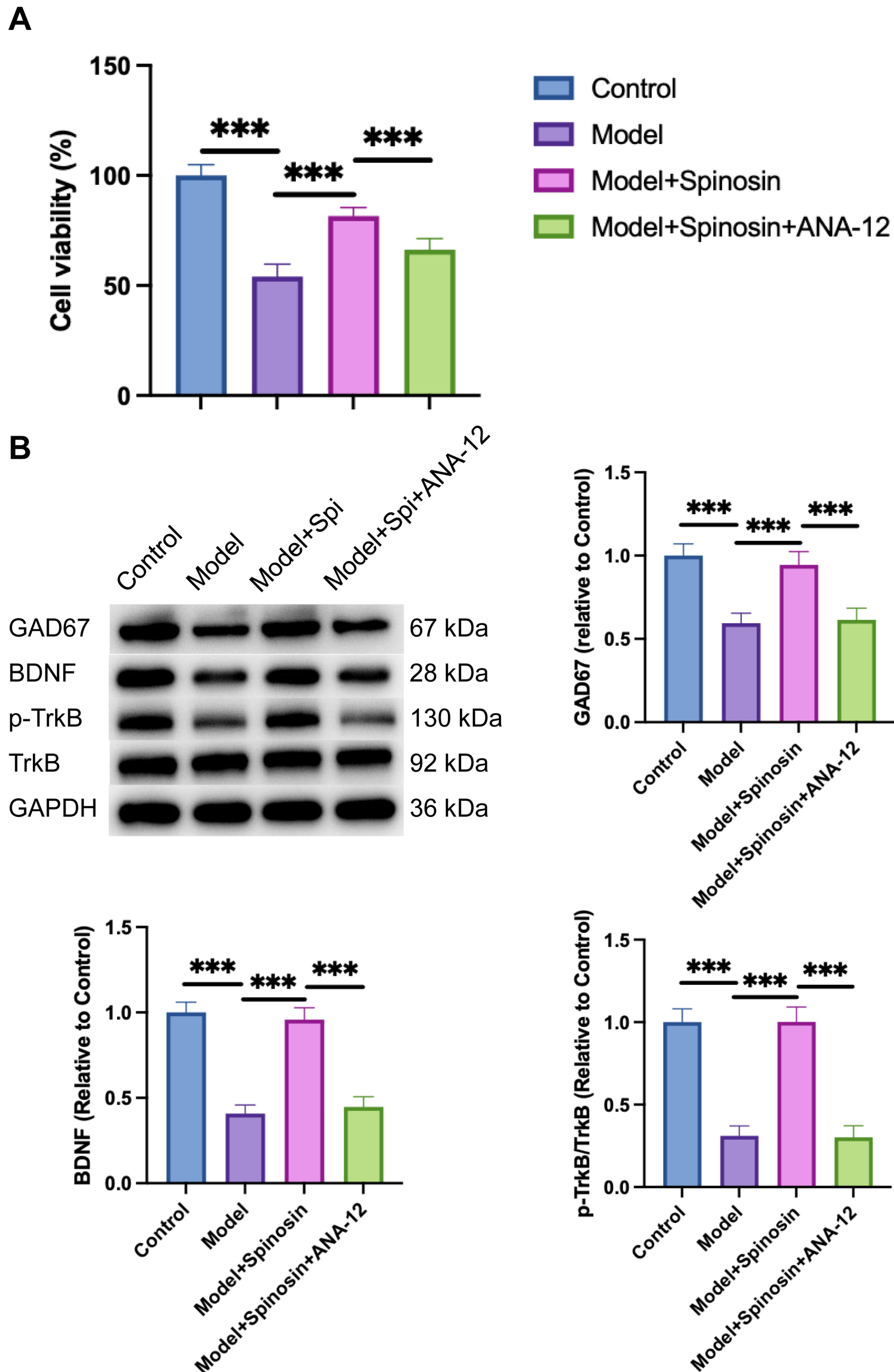

Fig. 8. Blocking TrkB signaling attenuated the neuroprotective effects of spinosin *in vitro*. (A) Cell viability assessed by CCK-8 assay following corticosterone exposure with or without spinosin and TrkB inhibitor treatment. (B) Representative Western blot images of GAD67, BDNF, p-TrkB, and TrkB or expression and quantitative analysis of protein expression levels. N = 8. ***$p < 0.001$ vs. Control group.

Spinosin Regulated the Expression of GABAergic- and Neurotrophic-Related Genes in the Hypothalamus

To determine whether spinosin also affected gene expression, mRNA levels of GABAergic- and neurotrophic-related genes were analyzed by quantitative real-time PCR. As shown in Fig. 7, PCPA treatment significantly downregulated the mRNA expression of GAD1, GABRA1, BDNF, and NTRK2 (TrkB) in the hypothalamus ($p < 0.001$). Spinosin treatment significantly reversed these transcriptional changes ($p < 0.001$), further supporting its regulatory effects on GABAergic neurotransmission and BDNF-TrkB signaling at the transcriptional level.

Blocking TrkB Signaling Attenuated the Neuroprotective Effects of Spinosin in Corticosterone-Injured HT22 Cells

To further explore the causal involvement of TrkB signaling in the neuroprotective effects of spinosin, an *in vitro* model utilizing HT22 mouse hippocampal neuronal cells was employed. While our *in vivo* biochemical analyses primarily focused on the hypothalamus, HT22 cells were selected as the *in vitro* tool because the hippocampus is highly vulnerable to stress-related hyperarousal, and this cell line provides a robust, mouse-derived CNS model for evaluating corticosterone-induced injury and BDNF-TrkB signaling. As shown in Fig. 8A, exposure to corticosterone (to simulate a stress-induced hyperarousal state) significantly reduced the viability of HT22 cells, whereas spinosin treatment markedly increased cell survival ($p < 0.001$). Importantly, co-treatment with the specific TrkB inhibitor significantly attenuated the protective effects of spinosin on cell viability (Fig. 8A; $p < 0.001$). Consistently, the spinosin-induced upregulation of BDNF, p-TrkB or GAD67 expression in these hippocampal neurons was partially reversed by TrkB inhibition (Fig. 8B; $p < 0.001$), directly indicating that TrkB signaling is functionally required for the neuroprotective actions of spinosin.

Discussion

The present study provides the first evidence that spinosin, a flavonoid derived from Ziziphi Spinosae Semen, ameliorates PCPA-induced insomnia and comorbid anxiety-like behaviors via restoration of hypothalamic GABAergic signaling mediated by the BDNF-TrkB pathway. We demonstrated that spinosin not only corrected the sleep-wake architecture and hyperarousal state but also reestablished the excitatory and inhibitory neurotransmitter homeostasis in the hypothalamus. Crucially, using the specific TrkB inhibitor ANA-12, we confirmed that the neuroprotective and GABA-modulating effects of spinosin are functionally dependent on TrkB receptor activation, offering a novel pharmacological perspective on its therapeutic potential.

The PCPA model is widely recognized for mimicking the etiology of clinical insomnia, where serotonin depletion leads to a state of persistent hyperarousal rather than simple sleep loss [24]. Our behavioral data showed that PCPA administration resulted in fragmented sleep, reduced exploration in open environments, and prolonged latency in the novelty-suppressed feeding test, reflecting a phenotype of "anxious insomnia" [25]. While traditional hypnotics such as zolpidem promote sleep by positively modulating GABA_A receptors at the benzodiazepine site, chronic insomnia is increasingly conceptualized as a stress-related condition associated with allostatic overload and impaired neuroplasticity, which may not be fully normalized by symptomatic hypnotic treatment alone [26]. Spinosin exhibited a dual therapeutic profile: it acted as a sedative by shortening sleep latency and an anxiolytic by increasing open-arm entries in the EPM. This broadly consistent efficacy suggests that spinosin operates through a mechanism distinct from simple sedation, potentially targeting the upstream regulation of emotional and arousal networks [27].

At the neurochemical level, the transition from wakefulness to sleep requires the dominance of GABAergic inhibition over glutamatergic excitation within the hypothalamus, particularly in the ventrolateral preoptic nucleus (VLPO) [28,29]. In the present study, PCPA-treated mice displayed an impaired neurochemical environment characterized by glutamate excitotoxicity and deficits in GABAergic signaling. Spinosin treatment effectively reversed this imbalance, decreasing the Glu/GABA ratio. This restoration was underpinned by the upregulation of GAD67 (the rate-limiting enzyme for GABA synthesis) and the GABA_A receptor alpha1 subunit. Given that reduced GAD67 expression is a well-documented feature of chronic stress and sleep deprivation models [30], the ability of spinosin to restore GAD67 levels suggests that it promotes the resynthesis of GABA, thereby re-establishing the "inhibitory brake" required for sleep maintenance.

A pivotal finding of this study is the identification of the BDNF-TrkB pathway as the upstream driver of these GABAergic improvements. Sleep homeostasis is intricately linked to neurotrophic support; sleep deprivation has been shown to rapidly decrease BDNF levels in the hypothalamus and hippocampus [31,32]. Consistent with this, we observed suppressed BDNF expression and reduced phosphorylation of TrkB and CREB in PCPA-treated mice. Spinosin administration reactivated this signaling axis. The phosphorylation of CREB is of particular importance, as p-CREB acts as a transcriptional enhancer for both *Bdnf* and *Gad1* (the gene encoding GAD67) [33]. This data implies that spinosin may initiate a positive feedback loop: activating CREB to transcribe BDNF, which subsequently binds to TrkB to maintain neuronal survival and synaptic function.

The most significant innovation of our work lies in the causal verification of this pathway. Previous studies on spinosin largely focused on receptor binding affinities or

observational changes in protein levels [27]. By employing the TrkB antagonist ANA-12 in our *in vitro* corticosterone-injury model, we demonstrated that blocking TrkB signaling abrogated the protective effects of spinosin on cell viability and prevented the recovery of GAD67 expression. This aligns with neurodevelopmental evidence that BDNF-TrkB signaling promotes the maturation of GABAergic interneurons and supports the stability of inhibitory synaptic architecture [34,35]. Consequently, we propose that spinosin does not merely act as a GABA_A receptor agonist, but rather as a neurotrophic modulator that reinforces the structural and functional integrity of the GABAergic system through a TrkB-dependent mechanism.

Notwithstanding the strengths of the current work, certain methodological boundaries should be acknowledged. First, we analyzed the whole hypothalamus; however, sleep regulation is mediated by specific hypothalamic subnuclei, including the sleep-promoting ventrolateral preoptic area (VLPO) and the lateral hypothalamus (LHA), which contains key wake–sleep modulatory populations such as orexin neurons [36]. Future studies utilizing immunofluorescence or stereotactic microinjection could precisely localize the action of spinosin. Second, while we confirmed the involvement of TrkB, the direct molecular target of spinosin upstream of BDNF remains to be elucidated. It is possible that spinosin engages GPCR pathways (e.g., 5-HT_1A signaling), which can, in principle, transactivate TrkB; additionally, adenosine receptor signaling shows strong functional coupling with TrkB in plasticity-related contexts [37–39]. The systemic administration of spinosin implies that its effects may also involve peripheral metabolic modulation, which was not assessed here. Because the hypothalamus contains both sleep-promoting and wake-promoting nuclei, regional homogenates may mask subnucleus-specific changes. Third, while sleep disruption is profoundly associated with memory impairment, the current study primarily focused on the hyperarousal and anxiety-like phenotypes of insomnia. Although we did not include specific behavioral paradigms for memory assessment (such as the Morris water maze), our *in vitro* data demonstrated that spinosin exerts robust neuroprotective effects on HT22 hippocampal neurons against stress-induced injury. Given the central role of the hippocampus in memory consolidation and existing literature reporting the memory-ameliorating properties of spinosin [20], future studies incorporating specific cognitive behavioral tests and hippocampal molecular analyses are warranted to comprehensively evaluate the efficacy of spinosin on the "insomnia-cognitive decline" axis. Thus, while our results indicate hypothalamic-region alterations in GABAergic markers and BDNF-TrkB-CREB signaling, future studies using subnucleus-resolved approaches will be needed to determine whether the primary locus involves VLPO, lateral hypothalamus, or their interactions within the sleep-wake switch circuitry. While our biochemical data indicate a normalization of total GABA and glutamate levels, a major limitation of the present study is the reliance on whole-tissue homogenates via ELISA. This macroscopic evaluation does not differentiate between synaptic, metabolic, and glial neurotransmitter pools and therefore cannot directly represent the functional synaptic excitatory/inhibitory (E/I) balance. The exact functional state of GABAergic transmission requires validation through electrophysiological whole-cell recordings, such as measuring miniature inhibitory and excitatory postsynaptic currents (mIPSCs and mEPSCs). Future investigations employing patch-clamp techniques within specific hypothalamic subnuclei (e.g., the ventrolateral preoptic nucleus) are imperative to conclusively demonstrate the real-time synaptic efficacy mediated by spinosin.

Conclusion

Our study demonstrates that spinosin alleviates insomnia and anxiety-like behaviors in a serotonin-depleted mouse model. The therapeutic efficacy of spinosin is attributed to the rectification of hypothalamic neurotransmitter dysregulation, driven by the activation of the BDNF-TrkB-CREB signaling cascade. By establishing the TrkB-dependency of spinosin-mediated GABAergic restoration, these findings provide a robust scientific basis for the clinical application of spinosin as a multi-target agent for the management of complex insomnia disorders.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

HY, YW and LLH designed the research study. XYM and HS performed the research. HY and YW analyzed the data. LLH drafted the article. All authors contributed to important editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures were approved by the Animal Ethics Committee of Heilongjiang University of Chinese Medicine (Approval No. 2024121322) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines 2.0.

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Conflict of Interest

The authors declare no conflict of interest.

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