Resveratrol attenuates chronic unpredictable mild stress-induced alterations in the SIRT1/PGC1α/SIRT3 pathway and associated mitochondrial dysfunction in mice

Sidra Tabassum
South China Normal University-Panyu Central Hospital Joint Laboratory of Translational Medical Research
Afzal Misrani
South China Normal University-Panyu Central Hospital Joint Laboratory of Translational Medical Research
Hui-xian Huang
South China Normal University-Panyu Central Hospital Joint Laboratory of Translational Medicine Research
Zai-yong Zhang
Guangzhou Panyu Central Hospital
Qiao-wei Li
Guangzhou Panyu Central Hospital
Cheng Long (longcheng@m.scnu.edu.cn)
South China Normal University College of Life Sciences https://orcid.org/0000-0002-0904-796X

Research Article

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Abstract

Environmental challenges, specifically chronic stress, have long been associated with neuropsychiatric disorders, including anxiety and depression. Sirtuin-1 (SIRT1) is a NAD^+-dependent deacetylase that is widely distributed in the cortex and is involved in stress responses and neuropsychiatric disorders. Nevertheless, how chronic stress modulates the SIRT1 pathway and associated signaling remains unclear. In this study, we first explored the impact of chronic unpredictable mild stress (CUMS) on the SIRT1/PGC1α/SIRT3 pathway, on GABAergic mechanisms, and on mitophagy, autophagy and apoptosis in mice. We also asked whether activation of SIRT1 by resveratrol (RSV) can attenuate CUMS-induced molecular and behavioral alterations. Two-month-old C57/BL6J mice were subjected to three weeks of CUMS and one week of RSV treatment (30 mg/kg; i.p.) during the third week of CUMS. CUMS caused downregulation of the SIRT1/PGC1α/SIRT3 pathway leading to impaired mitochondrial morphology and function. CUMS also resulted in a reduction in numbers of parvalbumin-positive interneurons and increased oxidative stress leading to reduced expression of autophagy- and mitophagy-related proteins. Strikingly, activation of SIRT1 by RSV ameliorated expression of SIRT1/PGC1α/SIRT3, and also improved mitochondrial function, GABAergic mechanisms, mitophagy, autophagy and apoptosis. RSV also rescued CUMS-induced anxiety-like and depressive-like behavior in mice. Our results raise the compelling possibility that RSV treatment might be a viable therapeutic method of blocking stress-induced behavioral alterations.

Introduction

Stress is an everyday experience in daily life that can emotionally impact affected individuals. Stressful, unpredictable life events have been implicated in numerous psychiatric conditions, including anxiety and depression [1, 2]. Chronic unpredictable mild stress (CUMS) is widely used in animals to model mood disorders that imitate the environmental stressors people may experience daily [3]. According to numerous studies, chronic stress has detrimental effects on several brain areas, including the medial prefrontal cortex (mPFC) [4–6]. Stress can negatively impact emotional regulation [7] and play a crucial role in GABAergic and mitochondrial dysfunction [8, 9]. However, the exact molecular mechanisms underlying stress-induced anxiety and depression are largely unknown.

Sirtuin-1 (SIRT1) is an NAD^+-dependent class III protein deacetylase that deacetylates histones and epigenetically affects gene expression [10]. It is broadly distributed in the brain with high levels in the cortex [11, 12]. SIRT1 modulates the function of specific transcription factors and other proteins involved in gene expression by regulating their acetylation [13, 14]. Since it can deacetylate a wide range of substrates, SIRT1 is associated with multiple cellular processes, including energy metabolism, mitochondrial biogenesis and stress responses [14, 15]. A genome-wide study identified a locus at or near the *SIRT1* gene as a significant contributor to the risk of depression [16]. Clinical studies also noted a positive correlation of *SIRT1* downregulation with depression in patients [17, 18]. Furthermore, preclinical studies suggest the involvement of SIRT1 in anxiety-like and depressive-like behavior [19, 20]. SIRT1 deacetylates downstream peroxisome proliferator-activated receptor gamma coactivator 1-alpha

(PGC1α), thus modulating its functions [21]. Together SIRT1/PGC1α are reported to be involved in the regulation of oxidative stress and mitochondrial dysfunction [22]. SIRT1 and SIRT3 regulate mitochondrial activity and maintain energy homeostasis to coordinate cellular energy storage. SIRT3 translocates to the mitochondria in response to stress (e.g., DNA damage), where mitochondrial matrix processing peptidase cleaves it at its active site [23]. It has been reported that SIRT1/PGC1α/SIRT3 signaling plays a critical role in the regulation of mitochondrial biogenesis [24]. However, the influence of CUMS on this pathway and associated signaling remains unclear.

Gamma-aminobutyric acid (GABA) signals are critical for the regulation and orchestration of glutamatergic pyramidal neuronal assemblies and their function in the PFC [25]. Accumulating evidence indicates that loss of GABAergic transmission imbalances excitatory and inhibitory (E/I) neurotransmission, leading to anxiety and depression [26, 27]. Preclinical studies using chronic stress paradigms demonstrate a dysregulation of GABA receptors in certain mood disorders [28, 29]. GABAergic interneurons (INs) are also crucial for normal brain functioning since they have the ability to modulate the activity of principal neurons [30], and their dysfunction can impair higher brain functions [31]. GABAergic INs account for up to 25% of total neurons in the PFC [32], and include fast-spiking parvalbumin (PV)-INs, which are easy to characterize and have therefore proved popular as a research model for quantitative and comprehensive studies. These INs are critical for generating network oscillations and for feedback and feedforward inhibition [33]. Despite the fact that stress can cause GABAergic dysfunction, the impact of CUMS on PV-INs has not been studied.

Resveratrol (RSV) (3,4',5-trihydroxystilbene; $C_{14}H_{12}O_3$) is a natural polyphenolic phytoalexin, enriched in red grapes, wines, peanuts and berries, which exhibits pleiotropic effects in preclinical and clinical settings [34]. Extensive evidence has shown the protective effects of RSV primarily through the regulation of SIRT1 activity in pathological models [35, 36]. RSV has satisfactory bioavailability and robust blood-brain barrier permeability; consequently, the notion of RSV-based therapy has generated significant interest [34]. A recent report demonstrates that RSV alleviates sevoflurane-induced neuroinflammation in a SIRT1-dependent manner and ameliorates cognitive impairments in the developing brain [37]. This raises the possibility that RSV might also counteract other stress-induced molecular and behavioral alterations. Therefore, we first sought to examine the impact of CUMS on SIRT1/PGC1α/SIRT3 signaling and GABAergic mechanisms, then explored the influence of altered SIRT1 signaling and GABAergic mechanisms on mitochondrial morphology, mitophagy, autophagy and apoptosis in mice. Importantly, we also asked whether activation of SIRT1 by RSV can attenuate CUMS-induced molecular and behavioral alterations.

Material And Methods

Animal and study groups

C57BL/6J mice (two-month-old) were bought from the animal care facility of Guangdong Medical Laboratory and housed at the animal housing facility of the School of Life Sciences at South China

Normal University (SCNU). Mice were kept in a climate-controlled room (25°C) where they were allowed free access to rodent chow and fresh water, with a 12-h light-dark cycle. The experimental procedures were approved by the Animal Care and Use Committee of SCNU and followed the animal care guidelines established by the National Institutes of Health.

Chronic Mild Unpredictable Stress Procedure And Resveratrol Treatment

All study animals were randomly allocated to one of four groups: Control (Ctrl), CUMS, resveratrol (RSV), and CUMS plus resveratrol (CUMS + RSV). Animals in the CUMS and CUMS + RSV groups were exposed to a randomized series of well-validated and common stressors (two stressors/day) for 21 consecutive days, as described previously [38]. The given stressors were randomly selected from the following list: cage rotation (1 h); cage tilting at a 30° angle (overnight); cage shaking (3 h); cold (1 h at 4^{o}C); lights off during light phase (3 h); lights on during dark phase; restraint (1 h); forced swimming (10 min at 18^{o}C); radio noise (6 h); food deprivation (FD; overnight); water deprivation (overnight); wet bedding (overnight); soiled bedding (overnight); no bedding (overnight); crowding (overnight). Animals were kept under standard housing conditions with access to food and water, *ad libitum*.

RSV and CUMS + RSV group mice were given RSV (30 mg/Kg, CAS# 501-36-0, Crovell Biotech, China) intraperitoneally (i.p.). In contrast, Ctrl mice were given physiological saline (Sigma Aldrich) during the third week of the CUMS procedure.

Western Blotting

Western blotting
Brains were quickly dissected, and the mPFC tissue was removed on ice. Tissues were then homogenized in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% SDS, and protease inhibitor) using micro tissue grinders (Kimble) for 1 min. The supernatant was collected following centrifugation at 4℃ (12,000 rpm for 15 min) and then incubated at 75℃ for 20 min for protein denaturation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins, which were then electrotransferred to nitrocellulose membranes. After transfer, membranes were blocked with nonfat milk (5%) in TBST for 2 h and incubated with specific primary antibodies for 24 h at 4℃: SIRT1 (Abcam, ab110304; 1:3000), PGC1α (Abcam, ab54481; 1:2000), SIRT3 (Abcam, ab189860; 1:2000), ATG5 (Abcam, ab108327; 1:3000), Beclin1 (Abcam, ab207612; 1:1000), Pink1 (Santa Cruz, sc-517353; 1:1000), NLRP3 (Abcam, ab263899; 1:2000), PDHA1 (Abcam, ab168379; 1:3000), Drp1 (Abcam, ab184247; 1:1000), Mfn1 (Abcam, ab57602; 1:3000), Mfn2 (Abcam, ab56889; 1:3000), caspase-3 (Abcam, ab13847; 1:1000) or GAPDH (AF0006, Beyotime, Jiangsu, China). Blots were washed three times with TBST, and a horseradish peroxidase (HRP)-labeled secondary antibody (CWS, Taizhou, China) was added to 5% defatted milk in TBST for 2 h at room temperature (RT), followed by another five washes (5 min each) in TBST. Protein bands were revealed using the immobilon ECL Western System (Millipore,

Burlington, USA), then quantified and analyzed using GelPro analysis software (Media Cybernetics, MD, USA).

Immunofluorescent Staining

Mice were anesthetized by 20% urethane i.p. and perfused transcardially with saline (0.9%) and paraformaldehyde (PFA; 4%) in phosphate-buffered saline (PBS) (0.01 M, pH 7.4). The brains were quickly removed for fixation in PFA for 12 h, dehydrated in a 15% sucrose solution for overnight, and immersed in 30% sucrose for 48–72 h until the brain no longer floated. A freezing microtome (Leica CM30505, Germany) was used to cut serial coronal/sagittal brain sections of the mPFC (30 μm thickness). Sections were then preserved with 0.5% Triton-X 100 for membrane permeability before being blocked for 1.5 h at RT with 5% bovine serum albumin (BSA) in 0.01 M PBS, followed by incubation with primary antibody overnight for PV (Abcam, ab11427; 1:1000) and inducible nitric oxide (iNOS) (Abcam, ab178945; 1:1000) at 4°C. The following day, slices were washed with PBS, incubated with Alexa Fluor 488 donkey anti-rabbit (1:2000; Invitrogen) and Alexa Fluor 594 goat anti-mouse (1:2000; Invitrogen) for 60 min at room temperature, and then washed three times (10 min each time) in PBS. Following the washing steps, slices were then incubated in DAPI solution (1:5 for 30 min, Beyotime, C1005) for nuclear labeling and sequentially coverslipped with an anti-fade mounting medium. Each brain slice was imaged using a confocal microscope (LSM 900, Zeiss, Germany) under a 40x objective. Image J software (National Institutes of Health, Bethesda, MD, USA) was employed for analysis.

Whole-cell Patch-clamp Recordings

Whole-cell recordings were made from pyramidal neurons in the PFC, as described previously [39]. PFC slices were quickly cut using a vibratome (VT 1000S, Leica, Germany) in a chilled oxygenated cutting solution containing (in mM): 2.5 KCl, 10 $MgSO_4$, 93 HCl, 20 HEPES, 1.2 NaH_2PO_4, 2 thiourea, 30 $NaHCO_3$, 25 glucose, 93 N-methyl-D-glucamine, 5 (+)-sodium L-ascorbate, 0.5 $CaCl_2$, 3 sodium pyruvate, (pH 7.4, 300 mOsm). Slices were incubated in cutting solution for 10 min at 32 ± 0.5°C, then transferred to incubating solution for 1 h at RT. The incubating solution comprised (in mM): 92 NaCl, 2 MgSO4, 30 $NaHCO_3$, 2.5 KCl, 25 glucose, 20 HEPES, 5 (+)-sodium L-ascorbate, 2 thiourea, 2 $CaCl_2$, 3 sodium pyruvate, 1.2 NaH_2PO_4. For whole-cell recordings, artificial cerebral spinal fluid (aCSF) was used, which contains (in mM): 2.5 KCl, 124 NaCl, 2 $MgSO_4$, 2 $CaCl_2$, 1.2 NaH_2PO_4, 5 HEPES, 12.5 D-glucose, 24 $NaHCO_3$, (pH 7.3, 300 mOsm). Throughout the experiment, bubbles of 95% O_2 / 5% CO_2 were maintained in the incubating and recording solutions. An internal solution was used to fill pipettes (3–5 MΩ resistance) that comprised 10 mM tetraethylammonium chloride (TEA-Cl), 100 mM cesium methane sulfonate, 4 mM QX-314, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.3 mM NaGTP, 2 mM Mg-ATP (pH 7.3, 290 mOsm). To record miniature inhibitory postsynaptic currents (mIPSCs), 1 μM tetrodotoxin (TTX) was added to the aCSF. Data were amplified with an amplifier (Axopatch 700B; Molecular Devices, USA), low-pass filtered

at 2 kHz, and then digitized with pClamp10 (Molecular Devices, USA). Offline data analysis was done using Clampfit10 software (Molecular Devices, USA) or Mini Analysis (Synaptosft, USA).

Transmission Electron Microscopy (Tem)

For mitochondrial morphology analysis, PFC tissues were post-fixed for 12 h in 2.5% glutaraldehyde (v/v) and 1% OsO_4 (w/v) for 1 h. Sections were dehydrated using a series of ascending ethanol concentrations, then denatured in absolute alcohol (C_2H_5OH) and embedded in EPON resin. Fine hippocampal tissue sections (70 nm thickness) were cut from tissue slices using an ultramicrotome (UC7/FC7, Leica, Germany) and stained with uranyl acetate and lead citrate. Tissues were then imaged using a transmission electron microscope (JEM 1400 plus, JEOL, Japan) as described previously [40]. The mitochondrial ultrastructure of hippocampal pyramidal cells was studied at magnifications of 18,500x and 49,000x. The number of mitochondria and the ratio of mitochondrial length to width were calculated in ImageJ software from TEM-imaged microphotographs.

Behavioral Tests

Open field test

The open field test (OFT) assesses locomotor activity and anxiety-like behavior [44]. The OFT paradigm is a 60 cm × 60 cm open space enclosed by Plexiglas chambers with walls 50 cm high. Under standard room lighting conditions, each mouse was placed in the central zone of the apparatus and given 10 min to move freely. After each session, the floor was wiped with 75% alcohol. Mouse activity was recorded using an automated video tracking system (Zhenhua, Anhui province, China) and analyzed offline. The total distance traveled, time spent in the central area, and time spent immobile were calculated.

Elevated Plus Maze Test

Another behavioral test used to evaluate the effects of numerous anxiolytic or anxiogenic drugs is the elevated plus maze (EPM) test [45]. In this test, mice are exposed to height and open areas, and the natural tendency of animals to avoid dangerous situations is recorded. The EPM is a plus-shaped paradigm that is elevated one meter above the ground and consists of two opposing open arms (50 cm × 910 cm), two opposing closed arms (50 cm × 910 cm × 940 cm), and a central open region. Mice were placed individually in the central open space facing the open arms at the start of the experiment. Each mouse was permitted to explore the EPM for 5 min freely while the behavior of each mouse was captured on the video camera. Before putting the next animal in the maze, the apparatus was cleaned with 75% ethanol to ensure no olfactory cues remained from the previous animal. The number of times each mouse entered the open arms, how long they stayed in the open arms and how much time they spent immobile were all later examined in the video recordings of each mouse.

Forced Swim Test

The forced swim test (FST) is an extensively used technique for assessing depression-like behavior [41]. Briefly, mice were forced to swim in a cylinder (20 cm in diameter, 40 cm in height) filled with water (temperature 24–25°C) for 6 min. The immobility time and mouse activity were video recorded and analyzed afterwards. The first two minutes of recording were considered a preliminary test, while the remaining four minutes were analyzed. Immediately after the swimming session, the mice were taken out from the cylinder, dried, and kept warm under a lamp in their home cages. The mouse was considered immobile if it floated passively in the water and only made the movements needed to keep its nose or head above the water.

Tail Suspension Test

The tail suspension test (TST) is another paradigm that evaluates depression-like behavior [42]. Concisely, mice were suspended by their tails with adhesive tape (positioned about 1 cm from the tail tip), while their heads were 60 cm above the ground. This test was performed for six minutes, during which the immobility time (determined by the absence of movement) was recorded. The first two minutes of the recording were considered a preliminary test, while the remaining four minutes were analyzed.

Sucrose Preference Test

The sucrose preference test (SPT) is a reward-based test used to predict depressive-like behavior [43]. Mice were first acclimated to a 1% sucrose solution before the 72 h of the actual test. Each cage contained two bottles of a 1% sucrose solution. After 24 h, one bottle of 1% sucrose solution was exchanged with tap water. The SPT was then carried out. During the test session, mice remained in individual cages with free access to the two bottles containing 100 mL 1% sucrose solution and 100 mL tap water. The preference for sucrose was determined using the formula:

$$SPT = \frac{sucroseconsumed\,(g)}{sucroseconsumed\,(g) + waterconsumed\,(g)} * 100$$

Results

Resveratrol prevents CUMS-induced alterations in the SIRT1/PGC1α/SIRT3 pathway

To investigate the role of the SIRT1/PGC1α/SIRT3 signaling pathway in the pathophysiology of chronic stress-induced behavioral alterations, we first measured the levels of SIRT1, PGC1α and SIRT3 by western blotting. Our results showed reduced levels of SIRT1 protein in CUMS mice ($p = 0.020$; Ctrl vs CUMS; Fig. 1A- B); however, RSV alleviated this reduction ($p = 0.045$; CUMS vs CUMS-RSV). Since SIRT1 is

responsible for deacetylating and activating PGC1α, a principal regulator of mitochondrial function and oxidative stress [46], we hypothesized that the CUMS-induced reduction in SIRT1 levels might also affect PGC1α. Strikingly, we found reduced levels of PGC1α in CUMS mice ($p = 0.007$; Ctrl vs CUMS; Fig. 1A, C); again, this was prevented by RSV treatment ($p = 0.048$; CUMS vs CUMS-RSV). SIRT3 is another member of the sirtuin family involved in the regulation of mitochondrial function and plays a critical role in stress regulation; however, in our study, we found reduced expression of SIRT3 protein in response to stress ($p = 0.004$; Ctrl vs CUMS; Fig. 1A, D), whereas RSV treatment prevented this alteration ($p = 0.047$; CUMS vs CUMS-RSV). Pyruvate dehydrogenase A1 (PDHA1) is a mitochondrial protein that promotes the transformation of pyruvate to acetyl-CoA and CO_2 and is responsible for the ultimate link between glycolysis and the tricarboxylic acid (TCA) cycle. Thus, PDHA1 is important for energy metabolism. We found decreased expression of PDHA1 in CUMS mice ($p = 0.006$; Ctrl vs CUMS; Fig. 1A, E), which reflects impaired mitochondrial function in CUMS mice; RSV treatment prevented this reduction ($p = 0.030$; CUMS vs CUMS-RSV). These results suggest that CUMS causes a reduction in levels of SIRT1/PGC1α/SIRT3 pathway components, leading to a reducion in the expression of mitochondrial functional protein PDHA1, thus causing mitochondrial disruption. In contrast, RSV prevents all these effects by increasing the expression of the SIRT1 protein.

Resveratrol Ameliorates Cums-induced Alterations In Mitochondrial Morphology

Mitochondria undergo frequent changes in morphology, size, quantity, and location upon stress. We next asked whether altered SIRT3 and mitochondrial protein expression could also result in altered mitochondrial morphology. To answer this question, we analyzed TEM images of mitochondria. Our results revealed an increased number of mitochondria in CUMS mice ($p < 0.001$ Ctrl vs CUMS; Fig. 2A-B), whereas RSV treatment restored organelle numbers to control levels ($p = 0.008$; CUMS vs CUMS-RSV). Furthermore, the mitochondrial aspect ratio decreased in CUMS mice ($p = 0.036$; Ctrl vs CUMS; Fig. 2A-C), which was prevented by RSV treatment ($p = 0.046$; CUMS vs CUMS-RSV). Since mitochondrial morphology is regulated by mitochondrial dynamics [47], we next evaluated the proteins involved in mitochondrial fission (Drp1) and fusion (Mfn1 and Mfn2). Our results revealed increased levels of Drp1 in CUMS mice ($p = 0.001$; Ctrl vs CUMS; Fig. 2D-E), indicating increased mitochondrial fission in CUMS mice, whereas RSV treatment normalized this effect ($p = 0.009$; CUMS vs CUMS-RSV). However, Mfn1 and Mfn2 were unchanged in all groups (Fig. 2F-G), suggesting no direct effect of CUMS on mitochondrial fusion. These results reveal that CUMS altered mitochondrial dynamics protein levels, resulting in altered mitochondrial morphology, which could be prevented by RSV treatment.

Resveratrol Ameliorates Cums-induced Alterations In Pv-ins

PV-INs play a crucial role in stress-induced PFC dysfunction [31] and are regulated by PGC1α [48]. Since we found reduced expression of PGC1α in CUMS mice, we sought to investigate whether stress also

affects PV-INs. Our analysis revealed a significant reduction in PV^+ cell number in CUMS mice ($p < 0.001$, Ctrl vs CUMS; Fig. 3A-B), while RSV treatment prevented this CUMS-induced reduction ($p = 0.024$, CUMS vs CUMS + RSV). iNOS is an isoform of nitric oxide that is only expressed when the cell is induced or stimulated, typically by pro-inflammatory cytokines [49]. CUMS mice showed significantly increased relative iNOS intensity compared to Ctrls ($p < 0.001$, Ctrl vs CUMS; Fig. 3A, C), whereas RSV treatment mitigated this outcome ($p = 0.005$, CUMS vs CUMS + RSV). However, co-localization analysis showed that the number of $iNOS^+$ cells as a proportion of PV^+ cells increased significantly in CUMS mice ($p < 0.001$, Ctrl vs CUMS). This indicates an increase in oxidative stress in PV-INs, which is alleviated by RSV treatment ($p = 0.002$, CUMS vs CUMS + RSV; Fig. 3A, D). These results suggest that CUMS reduces the number of PV-INs due to increased oxidative stress in these cells, but that RSV treatment can prevent this effect.

Resveratrol Ameliorates Cums-induced Reduction In Mipsc Frequency In The Medial Prefrontal Cortex

Mitochondria play a vital role in presynaptic and postsynaptic neurotransmission via calcium buffering and other metabolic functions [50]. In neurons, mitochondrial fission regulates the movement of mitochondria within the axons and dendrites; however, alterations in the mitochondrial dynamics (fission/fusion) can cause disruptions in this mitochondrial transport, consequently leading to synaptic abnormalities and neuronal death [50, 51]. Therefore, we determined whether mitochondrial dysfunction, as revealed by abnormal levels of mitochondrial fission/fusion proteins and morphology, might impair synaptic transmission in the mPFC. The mIPSC amplitude correlates with the density and conductance of postsynaptic $GABA_A$ receptors [52]. In contrast, mIPSC frequency denotes the number of functional GABA synapses and/or the presynaptic probability of release at individual synapses [53]. The results showed no significant differences in mIPSC amplitude among groups (Fig. 3E-F). In contrast, mIPSC frequency was significantly decreased in CUMS mice ($p < 0.011$, Ctrl vs CUMS; Fig. 3E, G); RSV prevented this outcome ($p = 0.045$, CUMS vs CUMS + RSV). These results implicate a neural protective role of RSV, as it prevents a CUMS-induced reduction in inhibition.

Resveratrol Ameliorates Cums-induced Alterations In Autophagy- And Mitophagy-related Protein Levels

Autophagy is a highly conserved homeostatic mechanism that plays an important role in cellular adaptation to stress by removing damaged proteins and maintaining mitochondrial integrity [54]. *ATG5* is one of the important autophagy genes that modulate the immune system, regulate mitochondrial quality control and undergo crosstalk with apoptosis [55], while Beclin1 is involved in the recruitment of membranes to form autophagosomes [56]. To determine whether the above-mentioned CUMS-induced molecular alterations also affect the autophagy pathway, we analyzed the protein expression of ATG5 and Beclin1 by western blotting. Our results showed decreased levels of ATG5 ($p = 0.007$; Ctrl vs CUMS;

Fig. 4A-B), which were normalized by RSV treatment ($p = 0.045$; CUMS vs CUMS-RSV). The levels of Beclin1 were also decreased in CUMS mice ($p = 0.010$; Ctrl vs CUMS; Fig. 4A, C), and this effect was prevented by RSV ($p = 0.043$; CUMS vs CUMS-RSV). Mitophagy is a specialized form of autophagy crucial for mitochondrial quality control since it removes damaged mitochondria by lysosomal degradation [57]. The Pink1-mediated mitophagy pathway exerts a protective effect under stress conditions [58]. We found decreased levels of Pink1 in CUMS mice ($p = 0.004$; Ctrl vs CUMS; Fig. 4A, D), reflecting the altered mitophagy in CUMS mice; again, this was prevented by RSV treatment ($p = 0.04$; CUMS vs CUMS-RSV). The nod-like receptor pyrin domain-containing 3 (NLRP3) inflammasome is stimulated upon microglial activation and subsequent pro-inflammatory signaling and has emerged as a key contributor to neuroinflammation [59]. Accumulating evidence indicates that autophagy might control inflammasome activation via several mechanisms, and that impaired mitophagy enhances NLRP3 activation [60]. Since we found alterations in autophagy and mitophagy, we evaluated the levels of NLRP3 in CUMS mice, with the results demonstrating increased protein expression ($p = 0.014$; Ctrl vs CUMS; Fig. 4A, E); this was alleviated by RSV treatment ($p = 0.009$; CUMS vs CUMS-RSV). The NLRP3 inflammasome modulates inflammation and cell death by processing and activating caspases, and it has been reported that during apoptosis, cleaved caspase-3 is responsible for most of the proteolysis; therefore, it is considered a reliable marker for programmed cell death [61]. Conversely, neuroinflammation and oxidative stress can activate caspase-3, leading to apoptosis [62]. We therefore tested levels of cleaved caspase-3 and found these to be increased in mPFC ($p = 0.013$; Ctrl vs CUMS; Fig. 4A, F), indicating increased apoptosis in CUMS mice; once more, this effect was prevented by RSV treatment ($p = 0.023$; CUMS vs CUMS-RSV).

Resveratrol Ameliorates Cums-induced Anxiety-like Behavior

The effects of CUMS and RSV treatment on mouse exploratory and anxiety-like behavior were also tested using the OFT and the EPM. In the OFT, CUMS mice showed an increased immobility time compared to Ctrls ($p = 0.011$; Ctrl vs CUMS; Fig. 6A-B), consistent with impaired exploratory behavior in stressed animals [63, 64]. However, RSV treatment of CUMS mice (CUMS + RSV) prevented this behavioral alteration ($p = 0.036$; CUMS vs CUMS + RSV). CUMS mice spent less time in the central area of the OFT apparatus than Ctrls ($p = 0.025$; Ctrl vs CUMS; Fig. 5A, C), but RSV treatment was able to prevent this effect ($p = 0.039$; CUMS vs CUMS-RSV). Further evaluation of the distance traveled showed that CUMS mice traveled less than Ctrls ($p = 0.008$; Ctrl vs CUMS; Fig. 5A, D); however, RSV treatment exerted a beneficial effect by preventing this behavioral impairment ($p = 0.04$; CUMS vs CUMS + RSV). In the EPM test, CUMS mice spent significantly less time in the open arms ($p = 0.024$; Ctrl vs CUMS; Fig. 5E-F), and RSV treatment prevented this ($p = 0.035$; CUMS vs CUMS-RSV). Furthermore, CUMS mice made significantly fewer entries into the open arms ($p = 0.021$; Ctrl vs CUMS; Fig. 5E, G), while RSV treatment normalized this behavior ($p = 0.018$; CUMS vs CUMS-RSV). Finally, immobility time in the EPM showed a significant increase in CUMS mice ($p = 0.013$; Ctrl vs CUMS; Fig. 5E, H), which was prevented by RSV ($p = 0.040$; CUMS vs CUMS-RSV).

Resveratrol Ameliorates Cums-induced Depressive-like Behavior

Next, we evaluated depression-like behavior using three widely used tests. In the FST, there was a significantly increased immobility time in CUMS mice ($p < 0.001$; Ctrl vs CUMS; Fig. 6A-B), which was ameliorated by RSV treatment ($p = 0.034$; CUMS vs CUMS-RSV). The TST also indicated a significantly increased immobility time in CUMS mice ($p = 0.004$; Ctrl vs CUMS Fig. 6C-D), which was reversed by RSV ($p = 0.044$; CUMS vs CUMS-RSV). Finally, the SPT results revealed a reduced sucrose preference in CUMS mice compared to Ctrls ($p = 0.003$; Ctrl vs CUMS; Fig. 6E-F), while RSV treatment ameliorated this alteration ($p = 0.039$; CUMS vs CUMS-RSV). These results indicate that CUMS increases depressive-like and anxiety-like behavior in mice, while treatment with RSV prevents these behavioral deficits.

Discussion

In the present study, we evaluated the effects of RSV on CUMS-induced alterations in the molecular pathway that leads to anxiety-like and depressive-like behavior. As expected, we found that CUMS causes downregulation of SIRT1/PGC1α/SIRT3 signaling components, leading to impaired mitochondrial morphology and function. CUMS also reduces PV-IN numbers and increases oxidative stress, leading to reduced autophagy- and mitophagy-related-protein expression. Strikingly, activation of SIRT1 by RSV treatment normalized expression of SIRT1/PGC1α/SIRT3 proteins, thus reversing CUMS-induced alterations in mitochondrial function, GABAergic mechanism, mitophagy, autophagy, apoptosis to pre-stress conditions, and consequently rescuing CUMS-induced anxiety-like and depressive-like behavior in mice (Fig. 7).

SIRT1 is considered a critical therapeutic target for depression [65] and has been identified as one of the two significant genome-wide loci contributing to depression [16]. Existing literature shows a positive association of SIRT1 with depression in patients [17, 18], along with animal studies suggesting reduced SIRT1 activity in a chronic stress model of depression [66, 67]. In line with these reports, we found a reduction in the levels of SIRT1 in CUMS mice. SIRT1 is responsible for deacetylating PGC1α and plays an essential protective role in stress regulation [68, 69]. Accordingly, our results showed downregulation of PGC1α in CUMS mice. It has been demonstrated that SIRT1 regulates mitochondrial function by deacetylation and consequent regulation of transcription factors binding the SIRT3 promoter [70]. Furthermore, PGC1α itself also controls SIRT3 gene expression [71]. Consistent with this notion, we also observed a reduction in SIRT3 expression, while activation of SIRT1 by RSV enhanced the expression of PGC1α and SIRT3, confirming that this signaling mechanism is a downstream effector of SIRT1.

Mitochondria are essential for normal neuronal function, and mitochondrial dysfunction is prevalent in major depressive disorder patients [72]. Numerous mitochondrial functions, such as mitochondrial dynamics and bioenergetics, are intensely stress-regulated [73]. Normal mitochondrial morphology depends on the coordination of fission and fusion of the mitochondria, and increased Drp1-induced mitochondrial fission consequently increases fragmentation and abnormal mitochondrial morphology [74]. As SIRT3 is a mitochondrial sirtuin, it is a transcriptional coactivator of many mitochondrial genes,

governing various mitochondrial functions [75]. In line with this, SIRT3-knockdown in H9c2 cells showed decreased mitochondrial function [76]. In our study, the reduction in SIRT3 in CUMS mice subsequently reduced the levels of PDHA1 and caused an imbalance in mitochondrial fission and fusion that further led to impaired mitochondrial morphology and function. The beneficial effects of RSV on mitochondrial health in our study are in agreement with previous reports [77]. Additionally, RSV modulates mitochondrial function and dynamics by diverse mechanisms [77, 78], thus cause cytoprotective effects.

PV-INs synapse on the cell bodies of pyramidal neurons in the PFC and strongly control mPFC output by sustaining an appropriate E/I balance and coordinating the oscillatory activity required for efficient signaling [79]. It has been reported previously that impaired PGC1α activity is associated with mitochondrial dysfunction and PV-IN pathology [80]. Furthermore, SIRT3 haploinsufficiency in mutant transgenic AD mice results in a significant loss of cerebral cortical INs [81]. In CUMS mice, we found reduced PV-IN numbers associated with increased oxidative stress. Importantly, we also report for the first time that RSV ameliorates this CUMS-induced oxidative stress and the reduction in PV-IN numbers, possibly through the SIRT1/PGC1a/ SIRT3 pathway. A growing body of evidence suggests that loss of intracortical GABAergic transmission and the resulting imbalance in E/I neurotransmission in the PFC contribute to the etiology of a variety of psychological illnesses, including anxiety [82]. We found a reduced mIPSC frequency in CUMS mice. mIPSC frequency is determined by presynaptic mechanisms, while mIPSC amplitude and levels of postsynaptic GABA receptors represent postsynaptic properties [53]. We previously described alterations in presynaptic and postsynaptic GABAergic receptors in CUMS mice [83], and the present study suggests that CUMS induces dysfunction in GABAergic mechanisms. Moreover, we uniquely report here that enhancing SIRT1 signaling by RSV treatment can ameliorate CUMS-induced GABAergic pathology and is therefore a potential therapeutic target for other stress-induced alterations.

Autophagy and mitophagy are defensive mechanisms that allow cells to survive under stress conditions [84, 85]. Activation of autophagy removes damaged or redundant organelles and proteins, thus preserving cell homeostasis [86]. Dysfunctional GABA metabolism influences autophagy pathways [87], and SIRT1 also regulates autophagy through the deacetylation of key autophagy-related molecules [35, 36]. Furthermore, SIRT3 enhances mitophagy and inhibits apoptosis, thus protecting neurons against excitotoxic and metabolic stress [88]. Together, these findings suggest that SIRT1/SIRT3 signaling and GABAergic mechanisms are important for the regulation of autophagy and mitophagy. Therefore, the reduction in autophagy/mitophagy-related protein levels in CUMS could result from the disruptions mentioned above. Additionally, we also found increased NLRP3 inflammasome and caspase-3 levels in CUMS mice. Caspase 3 is one of the executioner caspases that induce apoptosis. On the other hand, NLRP3 is an intracellular sensor that perceives a wide range of endogenous threats and environmental irritants, subsequently leading to NLRP3 inflammasome activation [89], which is an important event in the cell death process. Nevertheless, mitochondrial dysfunction is an additional upstream event associated with NLRP3 activation [90]. Previous findings suggest that PGC1α/SIRT3 regulates cell apoptosis [71]; this might be the reason that we also observed increased apoptosis when this pathway was compromised due to CUMS. However, it has been proposed that RSV might also have a role in activating

cell cycle arrest, inducing apoptosis and autophagy [91], but there is no direct evidence for this. Strikingly, activation of the SIRT1 pathway by RSV revokes CUMS-induced alterations in autophagy, mitophagy and apoptosis.

The CUMS protocol is a well-studied animal model of depression, with a similar effect to that of environmental stressors on humans, which results in reduced spontaneous activity and depression-like behavior in mice [92]. Our results consistently demonstrate reduced spontaneous activity and increased anxiety-like behavior in CUMS mice. Moreover, the increased immobility time in the TST and FST, as well as the decreased sucrose preference, in CUMS mice indicates depressive-like behavior. These results show the successful replication of a mouse model of depression based on the CUMS protocol. Importantly, we demonstrate that RSV treatment can alleviate anxiety-like and depressive-like symptoms in mice. Previously, a study revealed that RSV ameliorates cognitive impairments in the developing brain [37]. However, ours is the first study to show that RSV can potentially mitigate CUMS-induced anxiety-like and depressive-like behavior.

Conclusions

Together, our findings suggest that downregulation of the SIRT1/PGC1α/SIRT3 pathway in CUMS mice alters mitochondrial and GABAergic mechanisms resulting in reduced autophagy and mitophagy, and increased apoptosis. Strikingly, RSV treatment alleviates CUMS-induced anxiety-like and depressive-like behavior by enhancing the SIRT1/PGC1α/SIRT3 pathway and mitochondrial and GABAergic function. The current study raises the compelling possibility that RSV therapy might provide a viable approach to the treatment of CUMS-induced molecular and subsequent behavioral alterations.

Declarations

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Sidra Tabassum, project initiation, experimental design, immunostaining, behavior, statistical analysis, and manuscript writing; Afzal Misrani, western blotting, TEM analysis, and manuscript writing; Hui-xian Huang, patch clamp recording; Zai-yong Zhang, critical review of the manuscript; Qiao-wei Li, supervision, Cheng Long, funding acquisition and supervision. All authors read and approved the final manuscript.

Data availability

All data generated or analyzed in this study are available from the corresponding author on reasonable request.

Ethics statement

This study was approved by the South China Normal University Institutional Review Boards. The use of animals in experiments was approved by the Institutional Animal Care and Use Committee (IACUC) and followed National Institutes of Health (NIH) guidelines.

Consent to participate

Not applicable.

Consent for Publication

All authors have read the manuscript and approved the final version of the manuscript.

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Figures

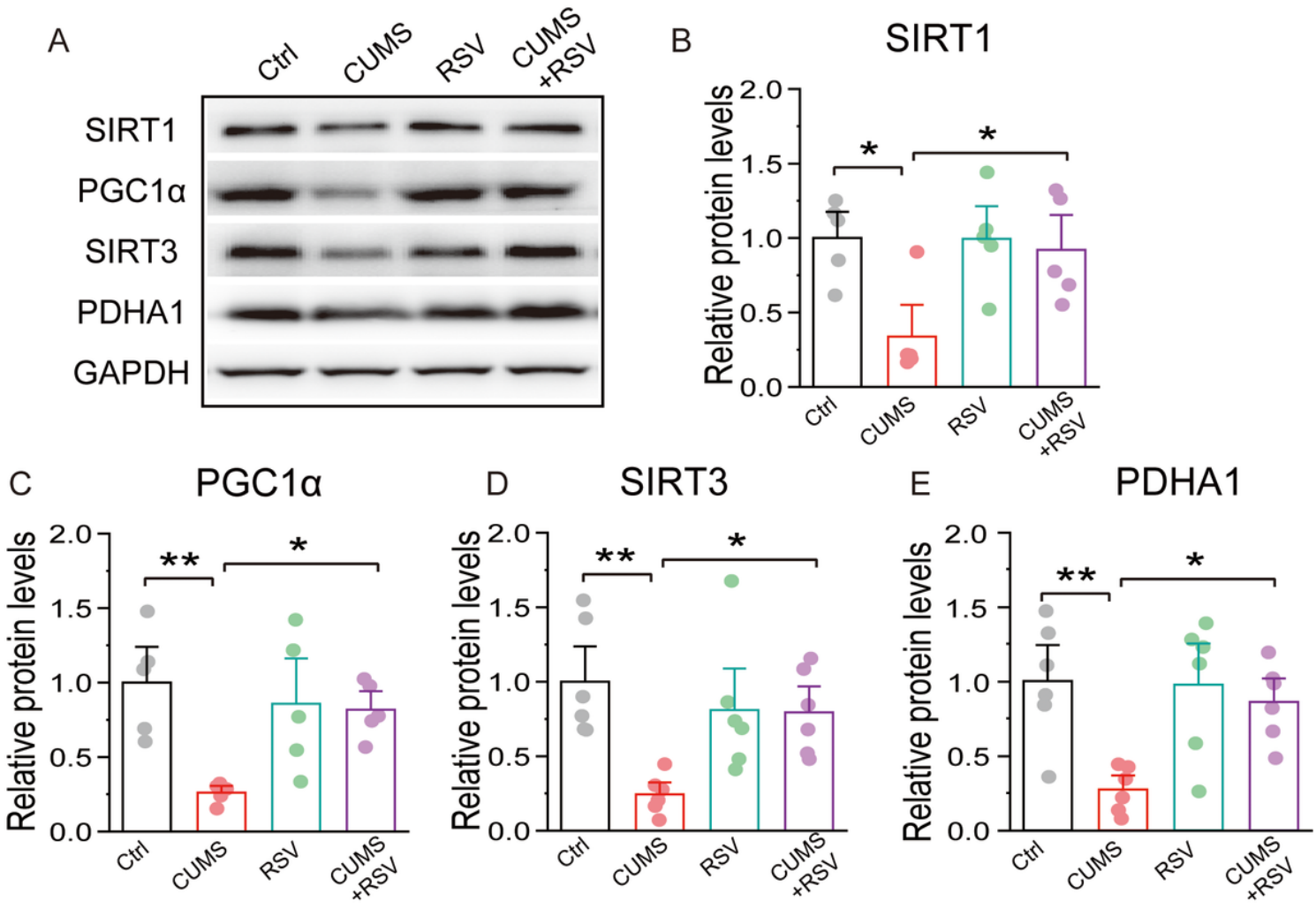

Figure 1

Resveratrol ameliorates CUMS-induced alterations in SIRT1/PGC1α/SIRT3 pathway components. (A) Representative immunoblotting images of mPFC extracts. **(B-E)** Quantification analysis of SIRT1, PGC1α, SIRT3 and PDHA1 (n = 5-6 mice per group), with two-way ANOVA followed by Tukey's post-hoc test. Each value represents the mean ± SEM; *p < 0.05, **p < 0.01.

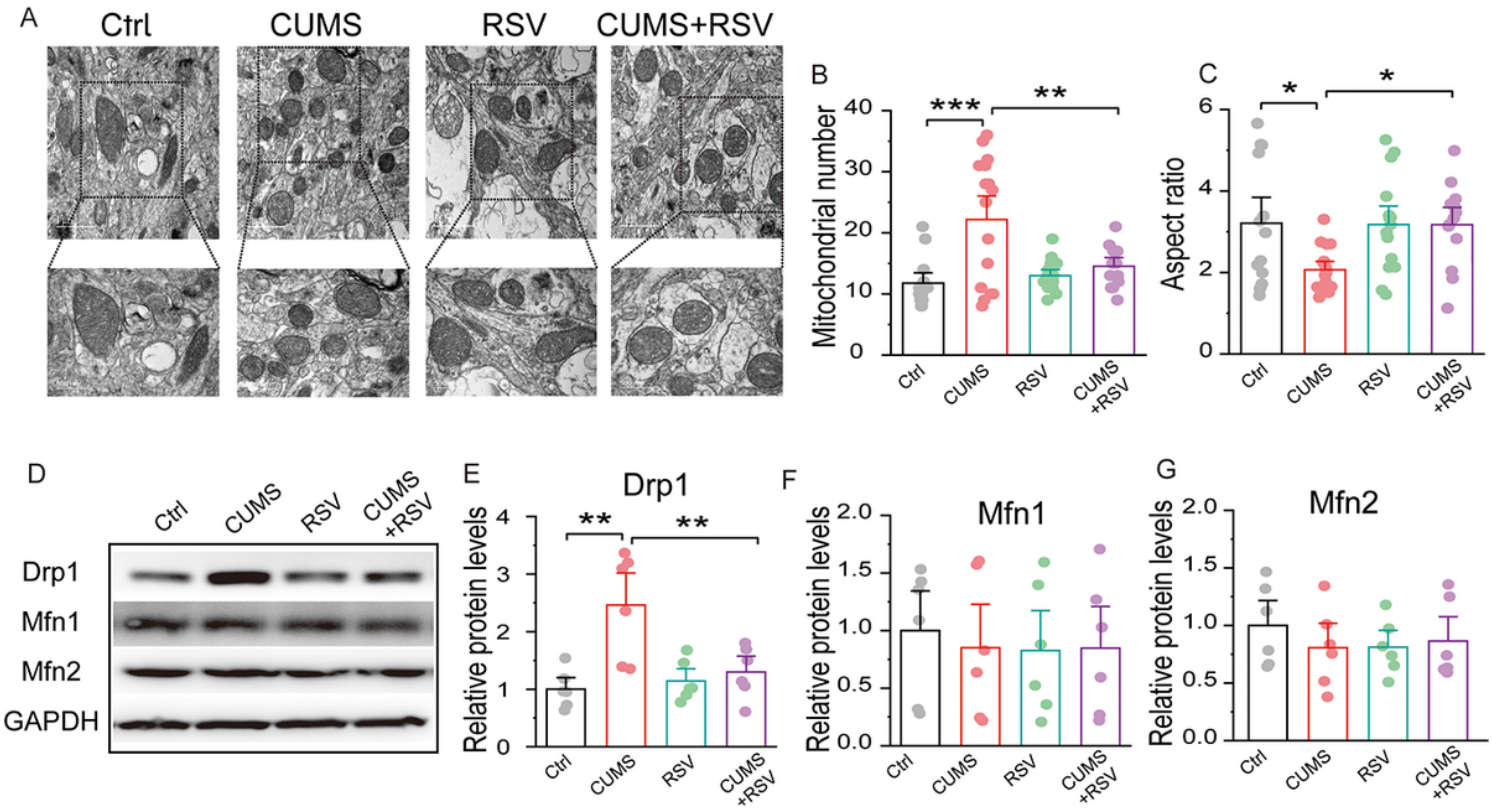

Figure 2

Resveratrol ameliorates CUMS-induced alterations in mitochondrial morphology and dynamics. (A) Representative TEM images of mitochondrial morphology in mPFC. **(B-C)**Analysis of mitochondrial number and aspect ratio (13-14 images /3 mice per group. **(D)** Representative immunoblotting images of mPFC extracts. **(E-G)** Quantification analysis of Drp1, Mfn1 and Mfn2 (n = 6 mice per group), with two-way ANOVA followed by Tukey's post-hoc test. Each value represents the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

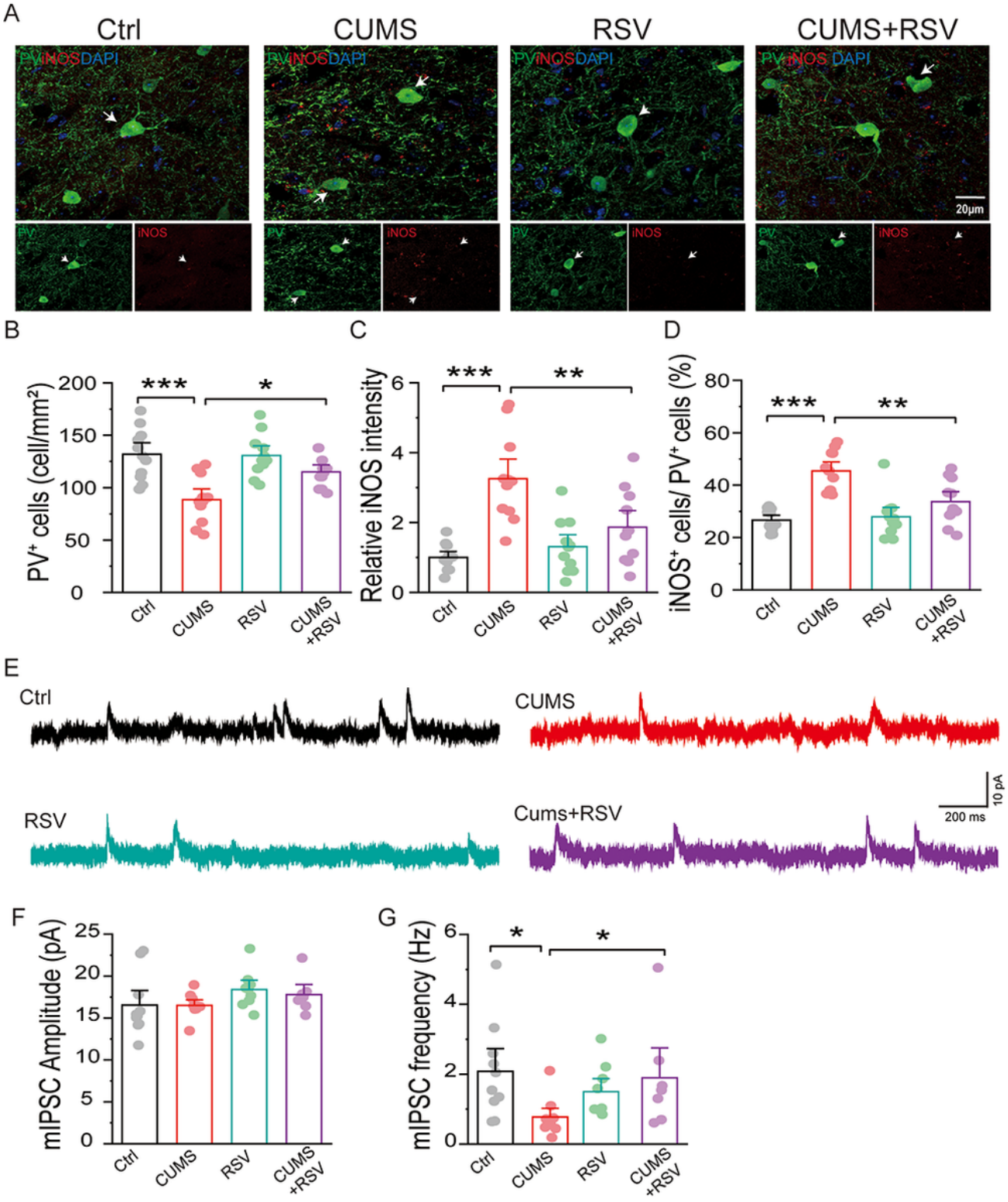

Figure 3

Resveratrol ameliorates CUMS-induced alterations in PV-IN numbers and mIPSC frequency. (A) Immunofluorescent staining of PV and iNOS in PFC brain sections; scale, 20 μm. **(B-D)** PV-IN density, iNOS intensity and co-expression of iNOS in PV-INs compared among groups (12 slices/ 4 mice in each group). **(E)**Representative mIPSC traces in pyramidal neurons. **(F)** Quantification of mIPSC amplitude and

(G) frequency (n = 8-10 neurons from 3 mice), with two-way ANOVA followed by Tukey's post-hoc test. Each value represents the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

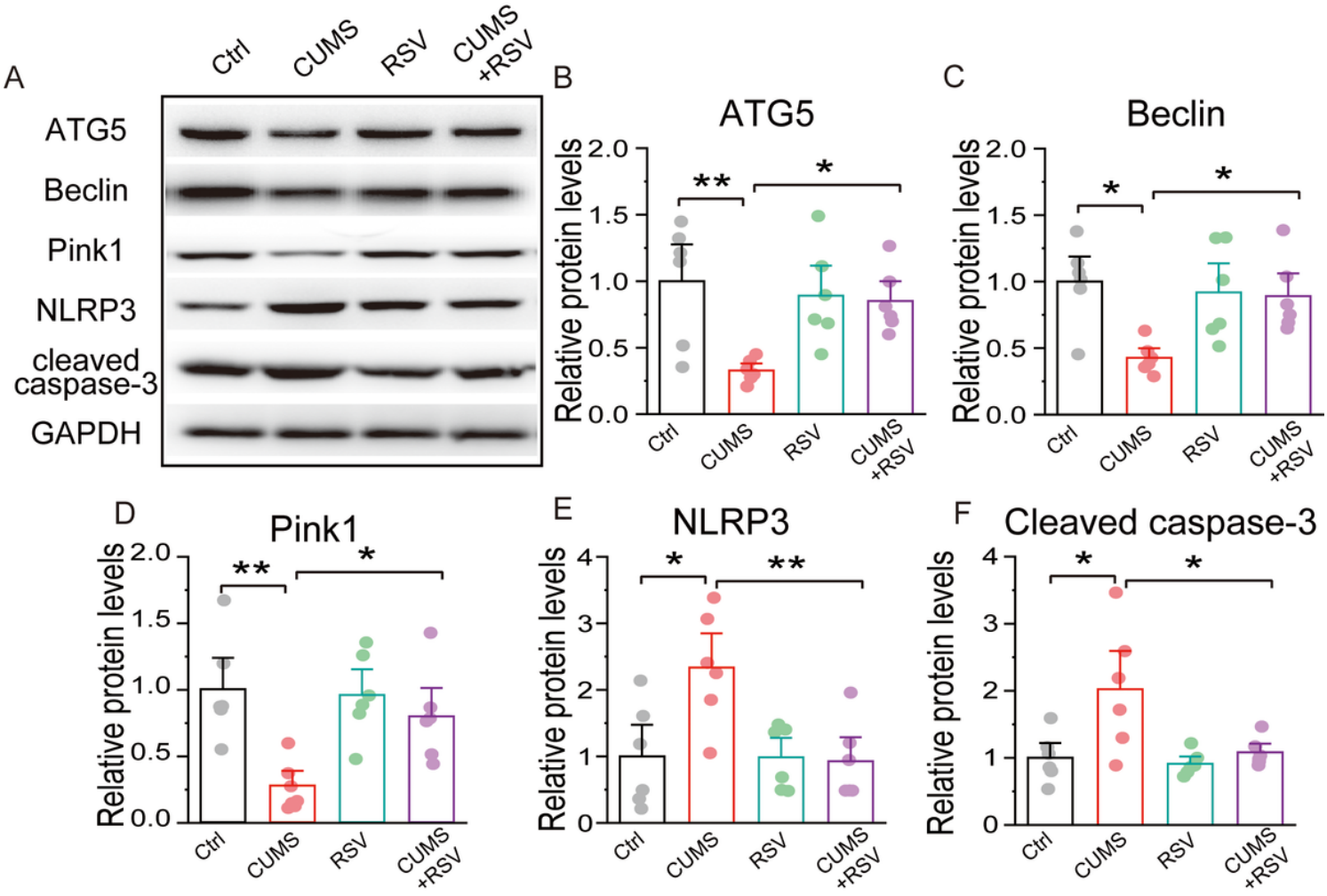

Figure 4

Resveratrol ameliorates CUMS-induced alterations in autophagy- and mitophagy-related protein levels. **(A)** Representative immunoblotting images of mPFC extracts. **(B-D)**Quantification analysis of ATG5, Beclin1, Pink1, NLRP3 and cleaved caspase-3 (n = 6 mice per group), with two-way ANOVA followed by Tukey's post-hoc test. Each value represents the mean ± SEM; *p < 0.05, **p < 0.01.

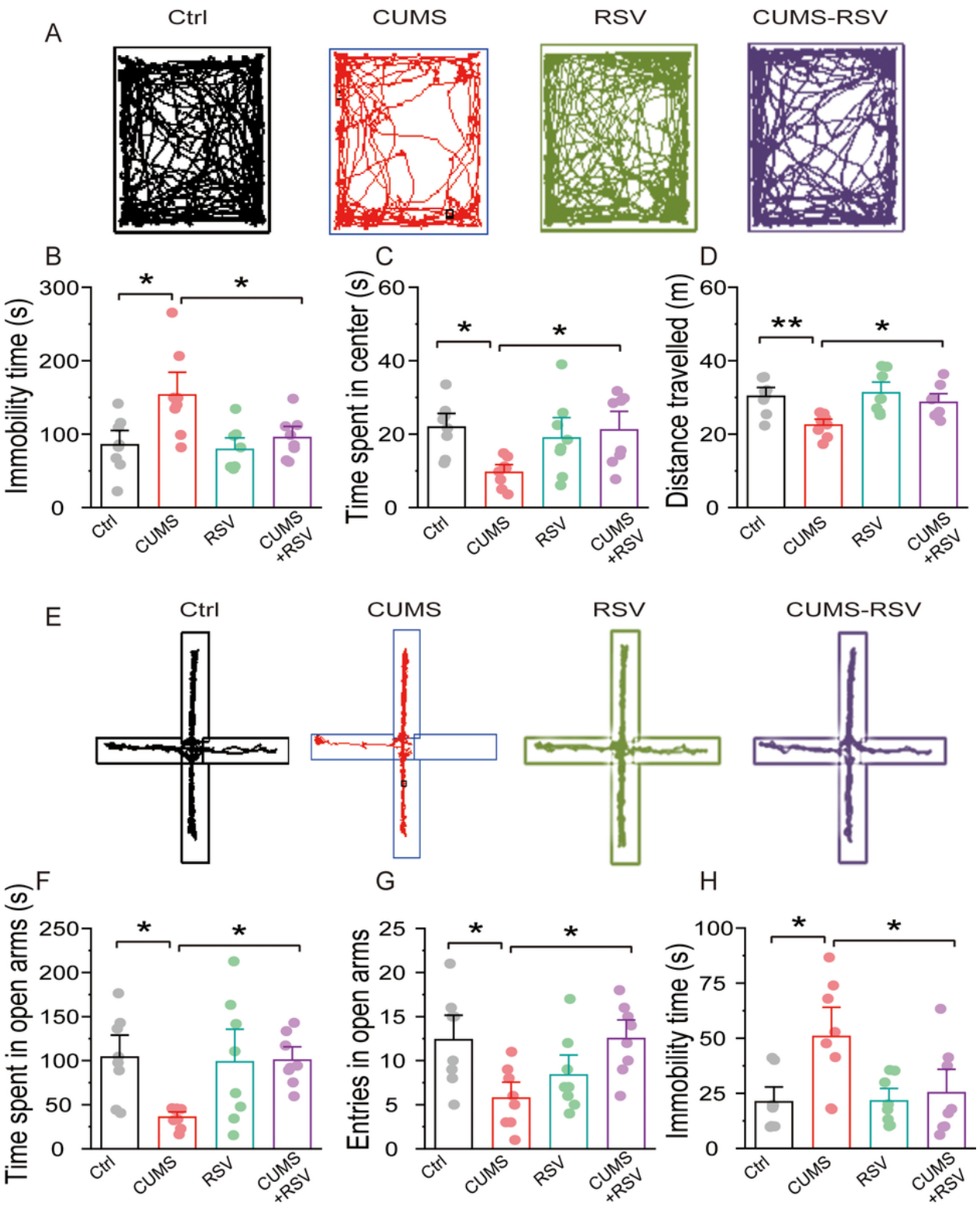

Figure 5

Resveratrol ameliorates CUMS-induced anxiety-like behavior. (A) Representative movement traces in the OFT.**(B-D)** Immobility time, time spent in the central area of the field, and total distance traveled in the open field are shown (n = 8 mice per group). **(E)** Representative movement traces in the EPM. **(F-H)** Time spent in and entries into the open arms, and immobility time were analyzed (n = 8 mice per group), with

two-way ANOVA followed by Tukey's post-hoc test.Each value represents the mean ± SEM; *p < 0.05, **p < 0.01.

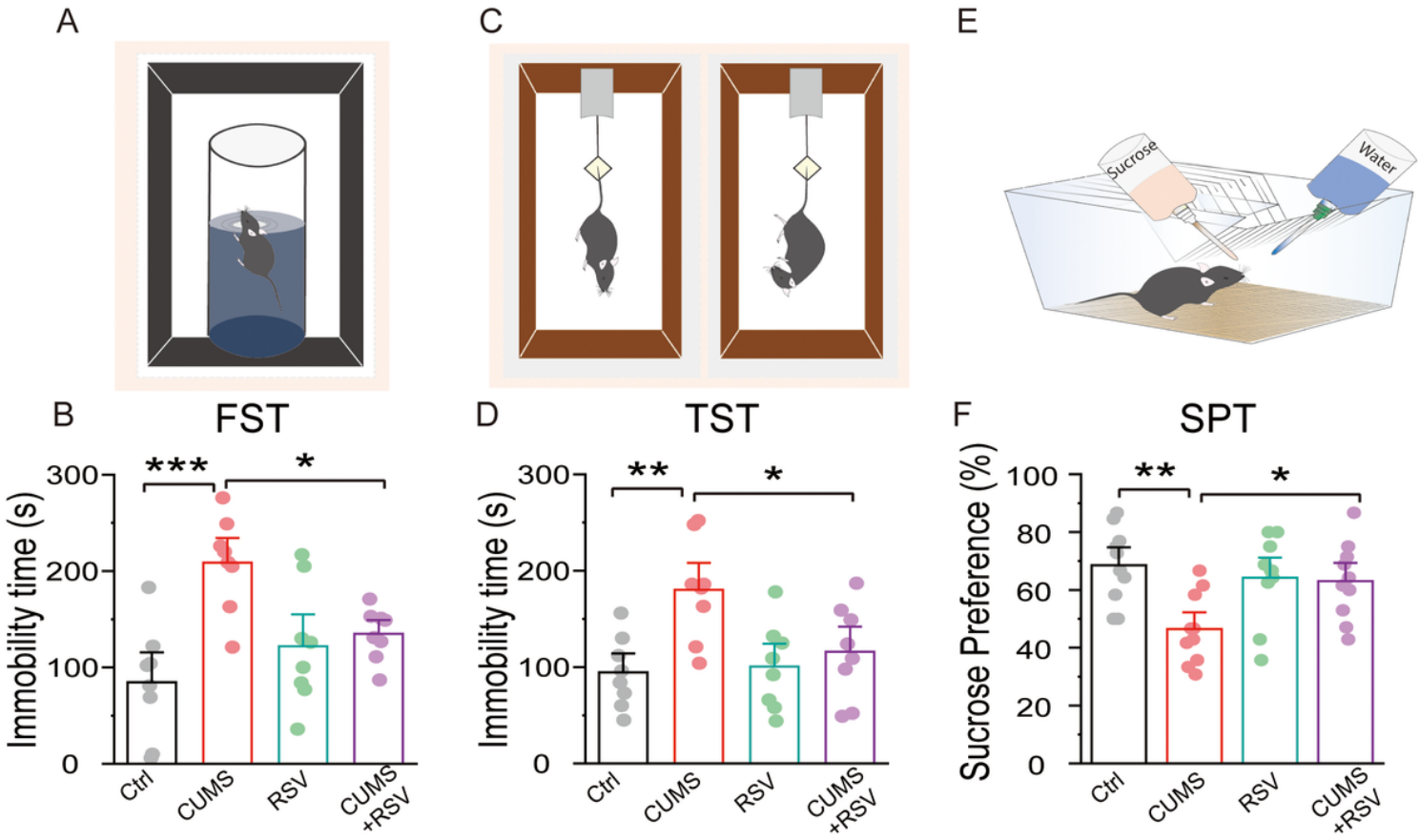

Figure 6

Resveratrol ameliorates CUMS-induced depressive-like behavior. (A, C, E) Schematic diagram of the experimental procedures. **(B, D, F)** Immobility time in the FST and TST and per cent sucrose consumption in the SPT are shown (n = 9-10 mice per group). Two-way ANOVA and a Tukey's post-hoc test were used for statistical analysis. Each value represents the mean ± SEM; *p < 0.05, **p < 0.01, ***p <0.001.

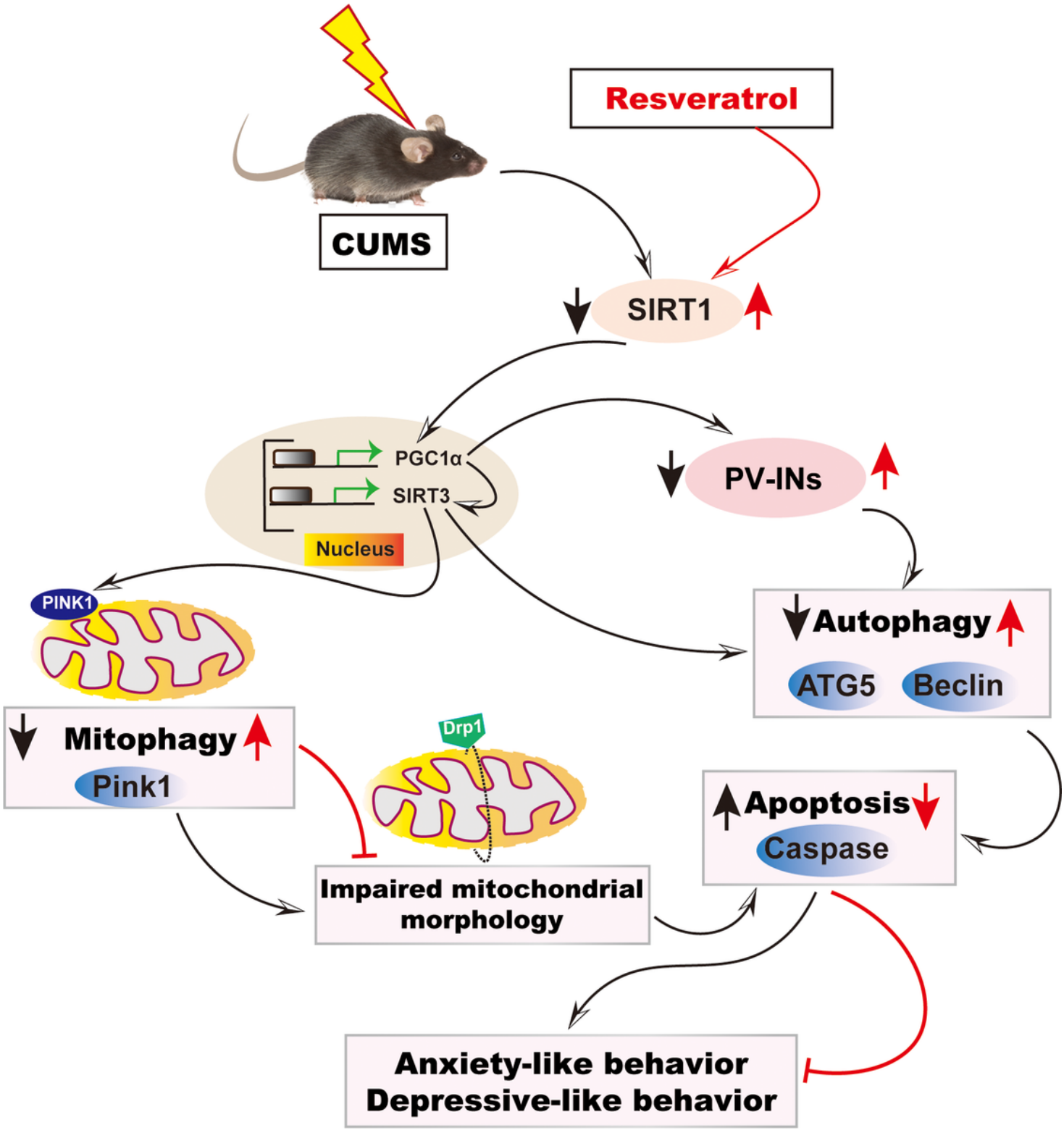

Figure 7

Schematic illustration depicting the effect of resveratrol on CUMS-induced alterations in the SIRT1/PGC1α/SIRT3 pathway and anxiety-like and depressive-like behavior. CUMS induces a reduction in SIRT1/PGC1α/SIRT3 pathway components and a decrease in the number of PV-INs; this impacts mitophagy and autophagy, thus impairing mitochondrial morphology. Collectively, these alterations increase apoptosis and lead to anxiety-like and depressive-like behavior. Conversely, RSV, by activating

the SIRT1 pathway, ameliorates all these CUMS-induced alterations. Black arrows indicate the effects of CUMS, while RSV effects are shown in red.