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RESEARCH

Withania somnifera Regulates Mitochondrial Biogenesis and Energetics in Rat Cortical Neurons: Role of BDNF and SIRT1

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Abstract

Withania somnifera, a psychoactive plant with putative neuroprotective actions, is used in Indian traditional medicine for the treatment of neuropsychiatric and neurodegenerative disorders. However, the key mechanisms underlying the pleiotropic actions of *Withania somnifera* on the nervous system remain poorly understood. Given converging evidence suggests a critical role for mitochondrial dysfunction in the pathophysiology of neuropsychiatric and neurodegenerative diseases, we hypothesized that *Withania somnifera* may exert pleiotropic effects via targeting mitochondria. Treatment with *Withania somnifera* root extract (RE) or the withanolide-withanoside rich fraction (WLS) enhanced cellular ATP levels in rat cortical neurons *in vitro* and in the neocortex *in vivo*. *In vivo* respirometry performed on mitochondria isolated from the neocortex following RE or WLS treatment revealed increased mitochondrial respiration and OxPhos efficiency. Furthermore, WLS treatment evoked increases in mitochondrial mass, and RE and WLS treatments enhanced expression of brain derived neurotrophic factor (BDNF) and Sirtuin 1 (SIRT1), both *in vitro* and *in vivo*. Pharmacological inhibitor studies support an important role for BDNF and SIRT1 in the mitochondrial effects of *Withania somnifera*. Experiments with distinct phytochemical components of WLS identified withanolide A and withanoside IV as key constituents that enhance mitochondrial biogenesis and neuroenergetics. The neuroprotective actions of WLS, withanolide A and withanoside IV against corticosterone-induced neuronal cell death *in vitro*, required signaling via BDNF and SIRT1. Collectively, these results indicate that *Withania somnifera* root extract and specific phytochemical constituents robustly influence mitochondria in cortical neurons, contributing to stress adaptation and neuroprotection via BDNF and SIRT1 signaling.

Keywords Ashwagandha · Mitochondria · Neuroprotection · Withanolide A · Withanoside IV · Neocortex

Introduction

Ancient cultures have traditionally used selected plants for medicinal, nutritive, religious and ceremonial practices [1]. Inspired by their prevalent usage in socio-cultural and healing contexts, plants and their natural products were studied pharmacologically to gain insights into the physiological basis for their mechanism of action and also as a valuable starting source for potential drug leads [2–8]. Many modern drugs derive their sap from the knowledge domain of plant-derived natural products [6, 9] with famous examples including the anti-inflammatory, acetylsalicylic acid (aspirin), derived from salicin extracted from the willow tree (*Salix alba L.*) bark, the cardiotonic glycoside, digitoxin derived from foxglove (*Digitalis purpurea L*) and the anti-malarial quinine, which is obtained from the bark of *Cinchona succirubra* [10]. In the context of plant and fungi-derived natural products with psychoactive effects, there has been a recent resurgence of interest in their therapeutic utility to treat neuropsychiatric and neurodegenerative disorders [11–13]. The modern medicine mindset of a clear preference for single molecule

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therapeutics, has been widened to consider the benefits of multi-targeted therapy [14]. This opens the possibility to consider the approach of 'reverse pharmacology' which capitalizes on transdisciplinary insights based on the empirical heritage of knowledge from Ayurveda and other traditional systems of medicine to identify potential phytoactives for further study in vitro and in vivo as a path to accelerate drug development [6].

Withania somnifera (Ashwagandha, Indian ginseng) a psychoactive plant indigenous to the Indian subcontinent, has been used for centuries in Ayurveda as a 'rasayana' or adaptogen, that boosts energy, stress resilience and well-being, and is a putative neuroprotective agent [15, 16]. Ethnopharmacological evidence indicates wide usage as a nootropic to enhance memory function, as an anxiolytic and antidepressant agent, in the treatment of sleep disorders, and with empirical evidence of beneficial effects in the amelioration of neurodegenerative disorders [17–20]. Despite the evidence of these pleiotropic therapeutic actions of *Withania somnifera* in neuropsychiatric and neurodegenerative disorders, the underlying cellular mechanisms and the identification of the key beneficial bioactives that mediate these putative neuroprotective effects of *Withania somnifera* remain unclear. Given the well-characterized, key role of mitochondrial dysfunction in the pathogenesis of neurodegenerative conditions [21–23], as well as an emerging body of evidence linking mitochondrial dysfunction to neuropsychiatric conditions [24–27], and the regulation of mitochondria by polyphenols in the context of stress resilience and aging [28–30], we hypothesized that the pleiotropic therapeutic effects of *Withania somnifera* may involve the targeting of neuronal energetics and mitochondria.

Here we have used in vitro and in vivo approaches, to evaluate the influence of *Withania somnifera* root extract (RE) and its phytochemical constituents on mitochondrial biogenesis and energetics in the neocortex. We have also assessed the effects of *Withania somnifera* RE and its phytochemical constituents on the expression of regulators of mitochondrial biogenesis and function, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC- 1α), NAD^+-dependent deacetylase sirtuin1 (SIRT1), nuclear respiratory factor 1 (NRF1) and transcription factor A, mitochondrial (TFAM) [31–35]. Furthermore, we examined the contributions of the trophic factor brain derived neurotrophic factor (BDNF) and SIRT1, in mediating the mitochondrial and neuroprotective effects of *Withania somnifera*. Our findings identify a hitherto unknown role for the *Withania somnifera* RE and its phytoactive chemical constituents as regulators of mitochondrial biogenesis and energetics in cortical neurons, and suggest that these mitochondrial effects may contribute to neuroprotection against corticosterone-induced stress via BDNF and SIRT1 signaling.

Materials and Methods

Animals

Sprague–Dawley rats bred in the Tata Institute of Fundamental Research (TIFR) animal facility were group housed and maintained on a 12 h light–dark cycle with ad libitum access to food and water. *In vitro* experiments, utilized timed pregnant Sprague–Dawley dams to generate rat cortical cultures from E18.5 embryos. Male Sprague–Dawley rats (3 months) were used for all *in vivo* experiments. All animal experiments were carried out in accordance with the Committee for the Control and Supervision of Experiments on Animals (CCSEA), Government of India, and approved by the TIFR Institutional Animal Ethics committee (56/GO/ReBi/S/1999/CCSEA).

Primary Cortical Culture

Primary cortical neuron cultures were derived from E18.5 rat embryos as described previously [36]. Briefly, Sprague–Dawley dams were euthanized with CO_2, embryos collected and cortices dissected in ice cold minimum essential medium. The cortices were dissociated in 0.05% trypsin/EDTA for 10 min, triturated to obtain a single cell suspension and plated in neurobasal medium supplemented with 2% B27 and 0.5 mM L-glutamine, at a density of 1 million cells per 9.6 cm^2, in dishes coated with poly-D-lysine (0.1 mg/ml, Sigma-Aldrich, USA). Neuron cultures were maintained at 37 °C, 5% CO_2 and 95% humidity with a half-medium change every alternate day. Cortical neurons were allowed to adhere, differentiate and arborize *in vitro* prior to treatment on day *in vitro* (DIV) 10 or DIV 13 as indicated. All cell culture reagents were purchased from Thermo Fisher Scientific, (USA).

Drug Treatment Paradigms

Rat cortical neurons were treated with *Withania somnifera* root extract (RE) (10, 25 and 50 µg/ml) or withanolide—withanoside rich fraction (WLS) (1, 2.5 and 5 µg/ml) for 72 h for dose response studies assessing mitochondrial DNA levels and cellular ATP levels. In experiments evaluating the constituents of the WLS extract, on assessing mitochondrial DNA levels and cellular ATP levels, cortical neurons were treated with withanolide A (WLA, 1 µM), withanoside IV (WSIV, 1 µM), withanolide B (WLB, 1 µM), withanoside V (WSV, 1 µM), 12-deoxy-withastramonolide (DWS, 1 µM) and withaferin A (WA, 1 µM) for 72 h. Cortical cultures were treated with varying doses of RE (10 and 50 µg/ml) or WLS (1 and 5 µg/ml), for durations of 4 h, 6 h, 8 h or 72 h

as indicated, to assess the impact of differential durations of treatment on gene expression. Cortical cultures were treated with RE (10 and 50 µg/ml), WLS (1 and 5 µg/ml), WLA (1 µM) or WSIV (1 µM) for 4 h to determine effects on mature BDNF (mBDNF) and SIRT1 expression and mBDNF release. In experiments with TrkB receptor antagonists or the SIRT1 inhibitor, rat cortical neurons were treated with RE (50 µg/ml), WLS (5 µg/ml), WLA (1 µM) or WSIV (1 µM) for 4 or 72 h in the presence or absence of the TrkB receptor antagonist, ANA- 12 (1 µM), the TrkB receptor antagonist, cyclotraxin B (1 µM), or the SIRT1 inhibitor EX- 527 (10 µM). Rat cortical neurons were treated with BDNF (50 ng/ml) for 72 h to assess the influence of BDNF on mitochondrial DNA content, cellular ATP levels and gene expression.

For experiments evaluating the influence of WLS, WLA and WSIV on cell viability in response to corticosterone (Cort)-induced stress, rat cortical neuron cultures received chronic Cort treatment (1 µM, 12 days) which overlapped for the last 72 h with WLS (5 µg/ml), WLA (1 µM) or WSIV (1 µM) administration. To evaluate the contribution of BDNF and SIRT1 to the effects of WLS, WLA and WSIV on viability in neuron cultures challenged with chronic Cort treatment, the TrkB receptor antagonist, ANA- 12 (1 µM), the TrkB receptor antagonist, cyclotraxin B (1 µM), or the SIRT1 inhibitor EX- 527 (10 µM) were used.

The RE and WLS extracts, WLA, WSIV, WLB, WSV, DWS and WA, TrkB receptor antagonists ANA- 12, cyclotraxin B, SIRT1 catalytic activity inhibitor EX- 527 and Cort were prepared in DMSO. Controls (Ctl) involved treatment of cultures with vehicle 0.1% DMSO unless specified. BDNF was prepared in water and corresponding control (Ctl) cultures were treated with water.

For *in vivo* experiments, Sprague–Dawley rats (3 months), received vehicle (water) or RE (250 mg/kg) or WLS (250 mg/kg) administered in drinking water for a duration of 15 days. Vehicle, RE and WLS containing drinking water were replaced every alternate day and measured to determine water consumption. Animals were sacrificed on the last day of treatment and the neocortex was dissected for further experiments.

WLA, WSIV, WLB, WSV, DWS, WA, EX- 527 and Cort were purchased from Sigma-Aldrich (USA) and BDNF, ANA- 12 and cyclotraxin B were purchased from Tocris Bioscience (United Kingdom). The RE and WLS extracts, were prepared at Pharmanza Herbal Pvt. Ltd. Briefly, the roots of the *Withania somnifera* plant used in this study were procured from Madhya Pradesh, India and voucher specimens were deposited and authenticated at the Botanical survey of India, Jodhpur. The dried roots were pulverized and extracted twice with hydro-alcohol 40:60 by ultrahigh-performance liquid chromatography as described previously [37]. After filtration, the extract was concentrated under reduced pressure and spray dried to get the powdered *Withania somnifera* root extract (RE). RE was further purified by multiple extraction steps with alcohol to obtain the withanolide—withanoside rich fraction (WLS). The composition of the RE and WLS extract are provided in Supplementary Table 1.

Quantitative Real Time Polymerase Chain Reaction

RNA was extracted from cells or tissue using Tri Reagent (Sigma-Aldrich, USA), or using the commercially available RNeasy Mini kit (Qiagen) according to the manufacturer's protocols. Briefly, 50 ng of RNA per sample was reverse transcribed to complementary DNA (cDNA) using random hexamers and the Superscript IV reverse transcription kit (Invitrogen, USA). This was followed by amplification of cDNA using gene specific primers and the KAPA SYBR® FAST Universal 2X qPCR Master Mix (Kapa Biosystems), by quantitative real time PCR in a Light Cycler 96 (Roche Applied Science, Switzerland) real time PCR system. The expression level of each gene was normalized to the endogenous 18S ribosomal RNA per sample, and the relative fold change between control and treated samples was determined by the ΔΔCt method, as described previously [36]. Data are represented as fold change ± SEM as compared to the control. Primer details are provided in Supplementary Table 2.

Mitochondrial DNA Levels

Total DNA was extracted from cells or tissue using the commercially available Pure link genomic DNA extraction kit (Invitrogen, USA) or the All Prep DNA/RNA Mini kit (Qiagen, Germany). To evaluate mitochondrial DNA (mtDNA) levels in control versus treated samples, levels of cytochrome B—a mitochondrial genome encoded gene were normalized to levels of a nuclear encoded gene cytochrome C by quantitative real time PCR. Relative mitochondrial DNA content between control and treated groups was computed by the ΔΔCt method as described previously [36]. Primer details are provided in Supplementary Table 2.

Western Blot Analysis

Control and treated cells or tissues were lysed in ice cold Radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris–Cl (pH 8.0), 1 mM EDTA, 1% NP- 40, 0.1% sodium deoxycholate, 0.1% SDS, 0.5 mM EGTA, 140 mM NaCl), with protease and phosphatase inhibitors added immediately before use (Roche Applied Science). Sample lysates were centrifuged at 13,000 rpm and protein content of the supernatant was determined using the QuantiPro BCA (Bicinchoninic Acid) assay kit (Sigma-Aldrich). Samples were resolved by sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE), followed by transfer of proteins by western blotting to polyvinylidene fluoride (PVDF, Merck Millipore, MA, USA) membranes. Membranes were blocked in 5% milk or 5% BSA (bovine serum albumin) in Tris Buffered Saline-Tween (0.1%) and probed with primary antibodies in 2.5% BSA overnight at 4 °C. Primary antibodies included rabbit anti-VDAC (1:1000, Abcam), mouse anti-ATP5A (1:1000, Abcam), rabbit anti-β actin (1:5000, Abclonal), rabbit anti-BDNF (1:1000, Abcam), rabbit anti-phospho-TrkA (Tyr490)/TrkB (Tyr516) (1:500, Cell Signaling Technology), rabbit anti-TrkB (1:1000, Millipore), rabbit anti-phospho Akt (Thr308) (1:1000, Cell Signaling Technology), rabbit anti-Akt (1:1000, Cell Signaling Technology), rabbit anti-SIRT1 (1:1000, Millipore).

Blots were washed in Tris Buffered Saline-Tween (0.1%) and incubated with goat anti-rabbit IgG peroxidase labelled or goat anti-mouse IgG peroxidase labelled (1:5000, Abclonal) secondary antibodies for 1 h at room temperature. The blots were washed and the chemiluminescent signal was detected using a kit (Thermo Fisher Scientific), and bands were captured on the Amersham ImageQuant 800 imager. The relative density of bands was quantitated using ImageJ software (NIH, USA) and normalized to the loading control actin.

Cellular ATP

Cells or tissue were lysed in boiling water and centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was collected and cellular ATP levels were determined using the ATP bioluminescent assay kit (Sigma-Aldrich), by mixing the luciferin substrate and luciferase enzyme mix with equal volumes of sample supernatant in a 96 well plate. The light emitted was measured using a luminometer (Berthold Technologies, Germany) and is proportional to the ATP consumed in the reaction. ATP levels were normalized to the protein content of each sample, estimated using the BCA protein assay kit (Sigma-Aldrich). Data are represented as fold change ± SEM as compared to the control.

Isolation of Cortical Mitochondria and Respiration Analysis

Mitochondria were isolated from the cortex of vehicle, RE and WLS treated Sprague Dawley rats by differential centrifugation, as described previously [36]. Cortex tissue was minced using six strokes in a dounce homogenizer (Sigma Aldrich) in 1 ml of ice-cold mitochondrial isolation buffer (MSHE + BSA, containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA, and 0.5% (w/v) fatty acid-free BSA, pH 7.2). The lysate was centrifuged at 800 g for 10 min at 4 °C, and the supernatant was centrifuged at 8,000 g for 10 min (4 °C). The pellet was recovered and suspended in 500 µl of ice-cold MSHE + BSA. This centrifugation step was repeated twice to wash the pellet. The mitochondrial pellet was suspended in 500 µl of ice-cold mitochondrial assay buffer (MAS + BSA, containing 220 mM mannitol, 70 mM sucrose, 10 mM KH_2PO4, 5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA and 0.5% (W/V) fatty acid-free BSA, pH 7.2). The protein content of each sample was estimated using the BCA assay, and 10 µg of mitochondrial preparations were used for respiration analysis on the Seahorse XFe24 analyzer. 10 µg of mitochondria were plated per well in a 50 µl volume of MAS + BSA buffer containing substrates, pyruvate (10 mM) and malate (2 mM). The plate was centrifuged at 2000 g for 20 min at $4^{O}C$, substrate containing MAS + BSA (450 µl) was slowly added to each well and the plate was loaded into the Seahorse XFe24 analyzer.

Oxygen Consumption rate (OCR) (pmol min^{-1}) was measured initially under limiting endogenous ADP and succinate concentrations and is a measure of basal State 2 respiration, primarily Complex-I dependent. This was followed by an injection of 2 µM rotenone + 10 mM Succinate + 4 mM ADP, with the post injection OCR reading providing a measure of Complex- II dependent state 3 respiration. Oligomycin (2 µM) was injected next which induced state 4 respiration, followed by an injection of the mitochondrial uncoupler FCCP (8 µM) which induced maximal respiration and antimycin A (8 µM) injection which resulted in non-mitochondrial respiration. State 2 (Complex-I/-II dependent) and state 3 (Complex-II dependent) OCR were depicted graphically as fold change of control. The oligomycin sensitive reduction in OCR was calculated as the ATP production rate represented as fold change of control.

Statistical Analysis

Data were subjected to statistical analysis using GraphPad Prism 10 software (GraphPad Software Inc, USA). To determine significance in experiments with two groups, Student's unpaired *t*-test was performed and for multiple group comparisons, one-way, two-way or three-way ANOVA analysis was performed followed by Tukey *post-hoc* group comparisons. Statistical significance was determined at $p < 0.05$.

Results

We hypothesized that *Withania somnifera* may influence mitochondrial biogenesis and energetics, thus contributing to the effects of *Withania somnifera* on stress adaptation and neuronal survival. To test this hypothesis, we have capitalized on studies using both *in vitro* cortical neuronal cultures, as well as *in vivo* approaches with adult Sprague–Dawley rats. We directly assessed the influence of *Withania somnifera* root extract (RE) and the withanolide—withanoside rich

fraction (WLS) on mitochondrial biogenesis and energetics in cortical neurons, and also evaluated the contribution of specific neuronal signaling pathways to the neuroprotective effects of *Withania somnifera* in response to corticosterone-induced stress.

Withania somnifera RE and WLS Fraction Regulate Mitochondrial DNA Content and ATP Levels in Cortical Neurons

We examined the influence of RE and WLS on mitochondrial mass in cortical neurons *in vitro* by assessing alterations in mitochondrial DNA (mtDNA) content. We treated cortical neurons with increasing doses of RE and WLS (Fig. 1A), and observed a significant dose-dependent increase in mtDNA content with the enriched WLS extract, while no effects were noted with RE (Fig. 1B) (one-way ANOVA: $F_{(8, 27)} = 14.17$, $p < 0.0001$). We next determined if RE and WLS alter cellular ATP levels in cortical neurons, and observed a significant dose-dependent increase in ATP levels following treatment with both RE and WLS (Fig. 1C) (one-way ANOVA: $F_{(8, 27)} = 64.13$, $p < 0.0001$). These results indicate an increase in mitochondrial mass with the enriched WLS extract, and an increase in cellular ATP production with RE and WLS.

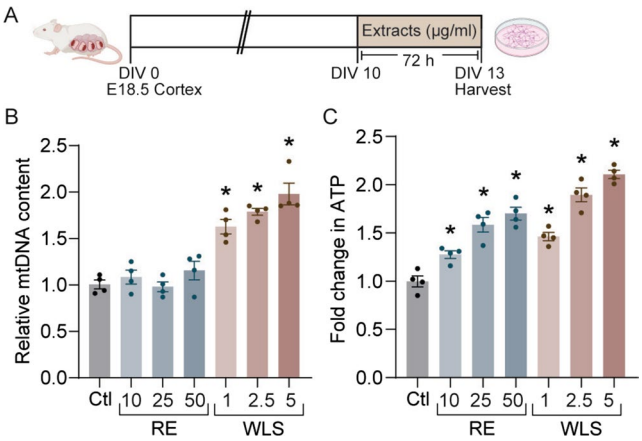


Fig. 1 *Withania somnifera* RE and WLS fraction regulate mitochondrial DNA content and ATP levels in cortical neurons. (**A**) Shown is a schematic depicting the treatment paradigm with increasing doses of *Withania somnifera* root extract (RE) (10, 25, 50 µg/ml) and withanolide—withanoside rich fraction (WLS) (1, 2.5, 5 µg/ml), of rat cortical neuron cultures, commencing day *in vitro* (DIV) 10 for a duration of 72 h. (**B**) Graph represents qPCR analysis for mtDNA content from control, RE and WLS treated cortical neurons. Data are represented as relative mtDNA content ±SEM. ($n = 4$ per treatment group/N = 2, $^*p < 0.05$ as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test). (**C**) Graph represents cellular ATP levels from control, RE and WLS treated cortical neurons. Data are represented as fold change of control ±SEM. ($n = 4$ per treatment group/N = 2, $^*p < 0.05$ as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test)

Withania somnifera RE and WLS fraction regulate mitochondrial DNA content, ATP levels, mitochondrial marker protein expression and mitochondrial oxygen consumption rate in the rat neocortex

Given we noted robust effects on mtDNA and ATP production in rat cortical neurons *in vitro*, we evaluated the influence of RE and WLS administered in drinking water on mitochondria *in vivo* in the neocortex of Sprague Dawley rats (Fig. 2A). We observed a significant increase in mtDNA levels (Fig. 2B) (one-way ANOVA: $F_{(2, 21)} = 7.74$, $p = 0.003$), in the neocortex of WLS, but not RE, treated rats, in agreement with our in vitro data. Cellular ATP levels were also significantly upregulated in the neocortex (Fig. 2C) (one-way ANOVA: $F_{(2, 21)} = 11.94$, $p = 0.0003$) of RE and WLS treated rats. We observed a significant increase in the cortical expression of the mitochondrial outer membrane marker protein, VDAC, in WLS, but not RE, treated rats, (Fig. 2D, E) (one-way ANOVA: $F_{(2, 11)} = 9.86$, $p = 0.004$). The cortical expression of the mitochondrial protein, ATP5A, a catalytic subunit of the mitochondrial ATP synthase complex, was significantly enhanced in RE and WLS treated rats (Fig. 2F, G) (one-way ANOVA: $F_{(2, 9)} = 32.99$, $p < 0.0001$). We next addressed if RE and WLS altered oxidative phosphorylation and electron transport chain (ETC) efficiency using Seahorse analysis in mitochondria isolated from the neocortex. Oxygen consumption rate (OCR) measurements (Fig. 2H) on isolated mitochondria from RE and WLS treated rats, demonstrated increased state 2 respiration (complex I and II dependent, basal respiration) (Fig. 2I) (one-way ANOVA: $F_{(2, 10)} = 5.60$, $p = 0.023$), state 3 respiration (complex II dependent, ADP-coupled respiration) (Fig. 2J) (one-way ANOVA: $F_{(2, 10)} = 41.83$, $p < 0.0001$) and ATP production rate (Fig. 2K) (one-way ANOVA: $F_{(2, 10)} = 6.64$, $p = 0.015$). Given that OCR is normalized to equal amounts of mitochondria loaded per well, our results of enhanced state 2 and state 3 respiration suggest increased ETC efficiency following RE and WLS treatment. Taken together, our *in vitro* and *in vivo* data indicate an increase in mitochondrial mass with the enriched WLS extract, and enhanced mitochondrial energetics and oxidative phosphorylation efficiency with the RE and WLS extract.

Withania somnifera RE and WLS Fraction Alter the Expression of Regulators of Mitochondrial Biogenesis and Function in Cortical Neurons

Mitochondrial biogenesis and oxidative phosphorylation require the coordinated expression of nuclear and mitochondrial encoded genes, orchestrated by regulators such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC- 1α), NAD^+-dependent deacetylase sirtuin1

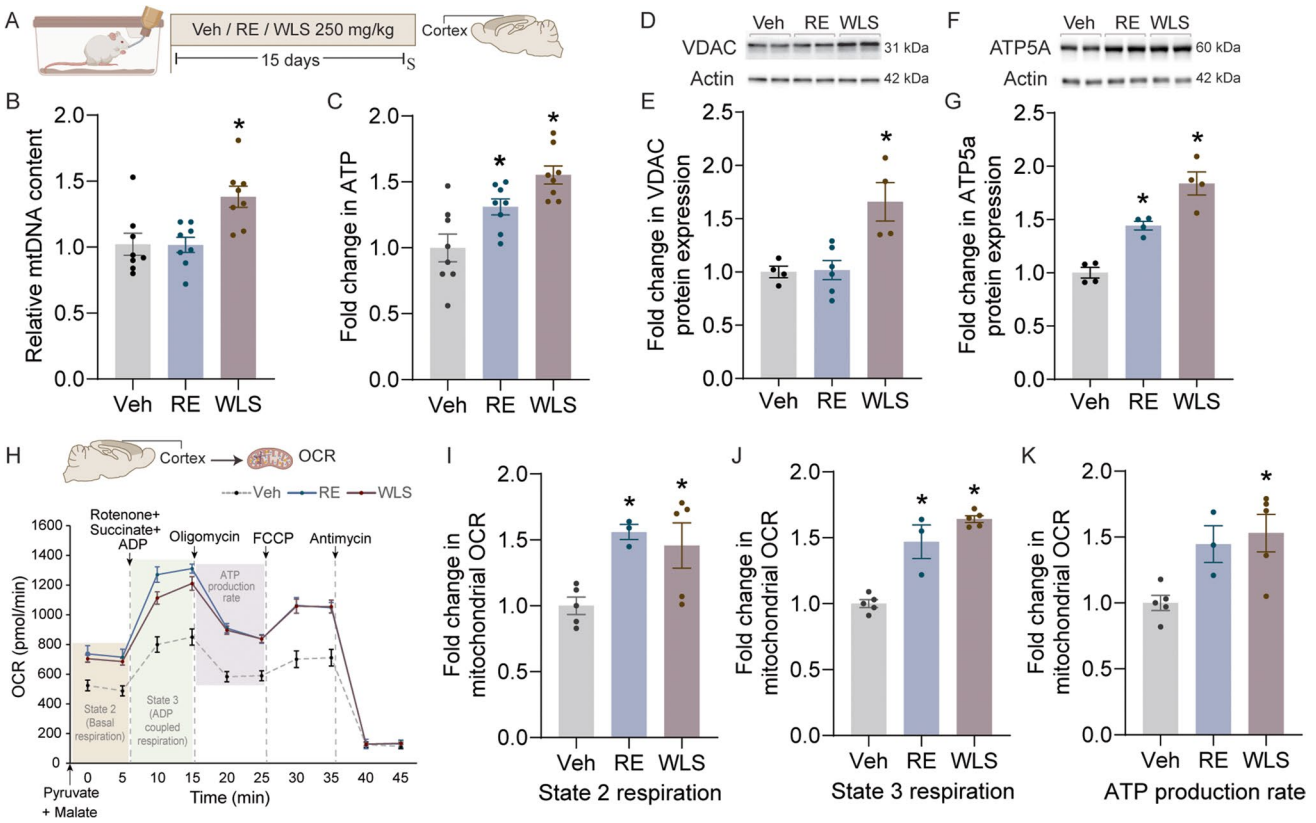


Fig. 2 Withania somnifera RE and WLS fraction regulate mitochondrial DNA content, ATP levels, mitochondrial marker protein expression and mitochondrial oxygen consumption rate in the rat neocortex. (**A**) Shown is a schematic depicting the treatment paradigm with vehicle (Veh), *Withania somnifera* root extract (RE) (250 mg/kg), and withanolide—withanoside rich fraction (WLS) (250 mg/kg), administered in drinking water to adult Sprague–Dawley rats for a duration of 15 days and sacrificed (S) on the last day of treatment. (**B** and **C**) Graphs represent mtDNA content (**B**) and cellular ATP levels (**C**) in cortices derived from vehicle, RE and WLS treated rats. Data are represented as fold change of vehicle ± SEM. (n = 8 animals per treatment group, $*p < 0.05$ as compared to vehicle, one-way ANOVA, Tukey's *post-hoc* test). (**D** and **E**) Shown is a representative immunoblot for VDAC protein levels with actin as the loading control (**D**) and the bar graph depicts quantitative densitometric analysis of VDAC protein levels normalized to actin (**E**) in cortices derived from vehicle, RE and WLS treated rats. Data are expressed as fold change of vehicle ± SEM. (n = 4 animals per treatment group/N = 1, $*p < 0.05$ as compared to vehicle, one-way ANOVA, Tukey's *post-hoc* test). (F and G) Shown is a representative immunoblot for ATP5A protein levels with actin as the loading control (F) and the bar graph depicts quantitative densitometric analysis of ATP5A protein levels normalized to actin (**G**) in cortices derived from vehicle, RE and WLS treated rats. Data are expressed as fold change of vehicle ± SEM. (n = 4–6 animals per treatment group/N = 1, $*p < 0.05$ as compared to vehicle, one-way ANOVA, Tukey's *post-hoc* test). (**H**) Shown is a trace for oxygen consumption rate (OCR) measurements from isolated mitochondria (10 µg) derived from the cortices of vehicle, RE and WLS treated rats, using the Seahorse XFe24 analyzer. OCR measurements include state 2 basal respiration, followed by injection of 2 µM rotenone + 10 mM Succinate + 4 mM ADP to measure state 3, ADP-coupled respiration, with successive injections of oligomycin (2 µM) to induce state 4 respiration, FCCP (8 µM) to induce maximal respiration and antimycin A (8 µM) to induce non-mitochondrial respiration. (**I**—**K**) Graphs represent quantitation of state 2 (via complex- 1/II) respiration (**I**) state 3 (complex II dependent) respiration (**J**) and ATP production rate (K), on isolated mitochondria (10 µg) derived from the cortices of vehicle, RE and WLS treated rats, measured on the Seahorse XFe24 analyzer. Data are expressed as fold change of vehicle ± SEM. (n = 3–5 animals per treatment group/N = 1, $*p < 0.05$ as compared to vehicle, one-way ANOVA, Tukey's *post-hoc* test)

(SIRT1), nuclear respiratory factor 1 (NRF1) and transcription factor A, mitochondrial (TFAM) [31–33, 35, 38]. PGC-1α is a transcriptional coactivator of NRF1, that regulates transcription of OxPhos and mitochondrial transcription genes, including TFAM that mediates mtDNA replication [31–35, 38]. SIRT1 deacetylates and activates PGC- 1α, and promotes the increased expression of PGC- 1α, and several mitochondrial and OxPhos associated genes [31–33, 38–42]. These regulators respond to external cues and integrate these with physiological status to modulate mitochondrial biogenesis and energetics [31, 33]. We observed a dose-dependent and significant increase in *Sirt1* (one-way ANOVA: $F_{(4, 15)} = 24.47$, $p < 0.0001$), *Ppargc1a* (one-way ANOVA: $F_{(4, 15)} = 36.06$, $p < 0.0001$), *Tfam* (one-way ANOVA: $F_{(4, 15)} = 21.01$, $p < 0.0001$), and *Nrf1* (one-way ANOVA: $F_{(4, 15)} = 33.82$, $p < 0.0001$), mRNA expression following treatment of cortical neurons with RE and WLS as early as 4 h (Fig. 3A,B). We next examined the influence of RE and WLS on the

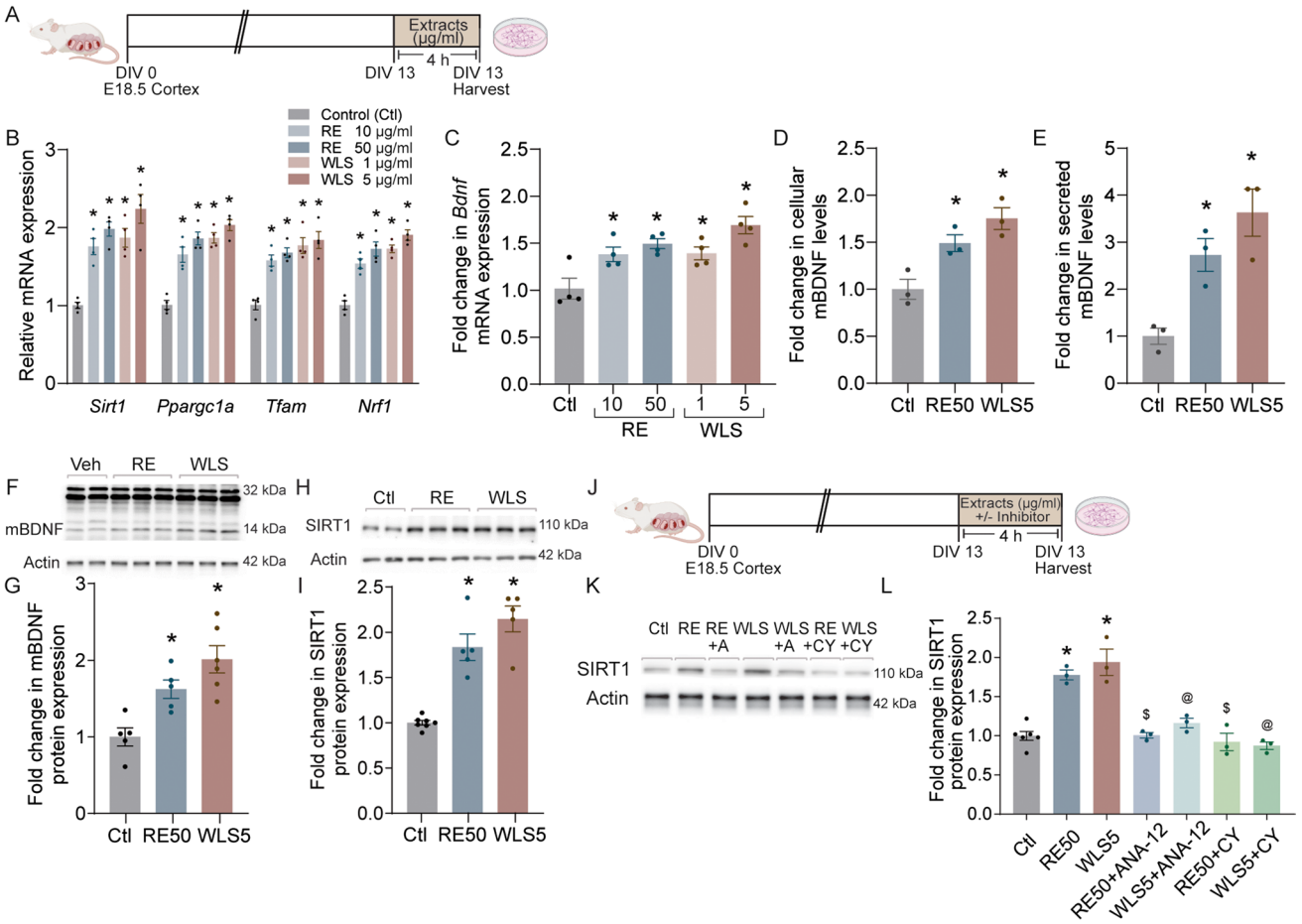


Fig. 3 Withania somnifera RE and WLS fraction enhance expression of regulators of mitochondrial biogenesis and function in cortical neurons. (**A**) Shown is a schematic depicting the treatment paradigm with *Withania somnifera* root extract (RE) (10, 50 µg/ml) and withanolide—withanoside rich fraction (WLS) (1, 5 µg/ml), of rat cortical neuron cultures for a duration of 4 h on DIV 13. (**B** and **C**) Graphs depict mRNA expression levels of regulators of mitochondrial biogenesis and function, *Sirt1*, *Ppargc1a*, *Tfam* and *Nrf1* (**B**) and the trophic factor *Bdnf* (**C**) in control, RE (10, 50 µg/ml) and WLS (1, 5 µg/ml) treated cortical neurons. Data are represented as fold change of control ± SEM. ($n = 4$ per treatment group/N = 2, $^{*}p < 0.05$ as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test). (**D** and **E**) Graphs depict quantitative enzyme-linked immunosorbent assay (ELISA) analysis for mature BDNF (mBDNF) protein expression levels in cortical neuron culture lysates (**D**) and mBDNF release in culture medium (**E**) of control, RE (50 µg/ml) and WLS (5 µg/ml) treated cortical neurons. Data are expressed as fold change of control ± SEM. ($n = 3$ per treatment group/N = 2, $^{*}p < 0.05$ as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test). (**F** and **G**) Shown is a representative immunoblot for mature BDNF (mBDNF) protein levels with actin as the loading control (**F**) and the bar graph depicts quantitative densitometric analysis of mBDNF protein levels normalized to actin (**G**) in control, RE (50 µg/ml) and WLS (5 µg/ml) treated cortical neurons. Data are expressed as fold change of control ± SEM. ($n = 5$–6 per treatment group/N = 2, $^{*}p < 0.05$ as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test). (**H** and **I**) Shown is a representative immunoblot for SIRT1 protein levels with actin as the loading control (H) and the bar graph depicts quantitative densitometric analysis of SIRT1 protein levels normalized to actin (**I**) in control, RE (50 µg/ml) and WLS (5 µg/ml) treated cortical neurons. Data are expressed as fold change of control ± SEM. ($n = 5$–7 per treatment group/N = 2, $^{*}p < 0.05$ as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test). (**J**) Shown is a schematic depicting the treatment paradigm with RE (50 µg/ml) or WLS (5 µg/ml), in the presence or absence of the TrkB receptor antagonist, ANA- 12 (1 µM), the TrkB receptor antagonist, cyclotraxin B (1 µM), or the SIRT1 inhibitor EX- 527 (10 µM) of rat cortical neuron cultures for a duration of 4 h on DIV 13. (**K** and **L**) Shown is a representative immunoblot for SIRT1 protein levels with actin as the loading control (**K**) and the bar graph depicts quantitative densitometric analysis of SIRT1 protein levels normalized to actin (**L**) in cortical neurons treated with RE or WLS in the presence or absence of the TrkB receptor antagonists, ANA- 12, cyclotraxin B or the SIRT1 inhibitor EX- 527. Data are expressed as fold change of control ± SEM. ($n = 3$–6 per treatment group/N = 2, $^{*}p < 0.05$ as compared to control (Ctl), $^{\$}p < 0.05$ as compared to RE treated group, $^{@}p < 0.05$ as compared to WLS treated group, one-way ANOVA, Tukey's *post-hoc* test)

expression of BDNF, a neurotrophic factor that promotes neuronal survival, as well as structural and functional plasticity [43–46]. Prior reports also indicate a role for BDNF in enhancing neuronal mitochondrial biogenesis and energetics [46–51] and our results further support this observation, wherein treatment of cortical neurons with BDNF for

72 h (Fig. S1A) enhanced mtDNA content (Fig. S1B) ($p = 0.0005$), cellular ATP levels (Fig. S1C) ($p < 0.0001$) and upregulated *Ppargc1a* (Fig. S1D) ($p = 0.0007$), and *Sirt1* (Fig. S1E) ($p = 0.0008$) expression. We addressed the influence of RE and WLS treatment on BDNF expression in cortical neurons and observed a dose-dependent upregulation of *Bdnf* mRNA (Fig. 3B) (one-way ANOVA: $F_{(4, 15)} = 9.51$, $p = 0.0005$) at 4 h.

We then examined the influence of longer duration treatment with RE and WLS (6 and 8 h) on the transcriptional regulation of *Bdnf*, *Sirt1*, *Ppargc1a*, *Tfam* and *Nrf1*. We noted enhanced *Bdnf* (one-way ANOVA: $F_{(4, 15)} = 12.75$, $p = 0.0001$), *Sirt1* (one-way ANOVA: $F_{(4, 15)} = 13.59$, $p < 0.0001$), *Ppargc1a* (one-way ANOVA: $F_{(4, 15)} = 11.24$, $p = 0.0002$), *Tfam* (one-way ANOVA: $F_{(4, 15)} = 7.28$, $p = 0.0018$), and *Nrf1* (one-way ANOVA: $F_{(4, 15)} = 26.32$, $p < 0.0001$) expression at 6 h with RE and WLS, which was not observed in the 8 h treatment group (Fig. S2 A,B). Furthermore, an increase in *Bdnf* (one-way ANOVA: $F_{(4, 15)} = 20.40$, $p < 0.0001$), *Sirt1* (one-way ANOVA: $F_{(4, 15)} = 14.30$, $p < 0.0001$), *Tfam* (one-way ANOVA: $F_{(4, 15)} = 7.08$, $p = 0.0021$), *Nrf1* (one-way ANOVA: $F_{(4, 15)} = 17.52$, $p < 0.0001$) but not *Ppargc1a* mRNA levels was also noted when cortical neurons were treated with RE and WLS for 72 h, suggestive of a later wave of transcriptional regulation on sustained exposure (Fig. S2 C,D).

We next performed quantitative enzyme-linked immunosorbent assay (ELISA) analysis to detect mature BDNF (mBDNF) levels, and demonstrated a significant ~ 1.4 fold increase in mBDNF levels in RE-treated cortical neurons, and ~ 1.7 fold increase in mBDNF levels in WLS-treated cortical neurons (Fig. 3D) (one-way ANOVA: $F_{(2, 6)} = 13.30$ $p = 0.006$). On measuring mBDNF levels in the medium of RE- and WLS-treated cortical neurons, we observed an ~ 2.7 and ~ 3.6 fold increase in mBDNF released into the culture medium respectively (Fig. 3E) (one-way ANOVA: $F_{(2, 6)} = 13.28$, $p = 0.006$). We next quantitated the influence of RE and WLS treatment on mBDNF and SIRT1 protein levels by western blotting, and observed a significant increase in mBDNF (Fig. 3F, G) (one-way ANOVA: $F_{(2, 13)} = 12.01$, $p = 0.001$) and SIRT1 (Fig. 3H, I) (one-way ANOVA: $F_{(2, 14)} = 34.55$, $p < 0.0001$) levels.

We then sought to investigate whether the RE- and WLS-evoked increase in SIRT1 levels, involves a role for BDNF signaling using pharmacological antagonists (ANA- 12 and cyclotraxin B) of tyrosine receptor kinase B (TrkB), the BDNF receptor (Fig. 3J). The RE- and WLS-mediated increase in SIRT1 expression in cortical neurons was abolished in the presence of ANA- 12 and cyclotraxin B (Fig. 3K, L) (one-way ANOVA: $F_{(6, 17)} = 24.45$, $p < 0.0001$). Tukey's multiple comparisons test indicated that the RE-evoked upregulation in SIRT1 levels ($p < 0.0001$) was abrogated in the presence of ANA- 12 ($p = 0.0002$) and cyclotraxin B ($p < 0.0001$), and the WLS evoked upregulation in SIRT1 levels ($p < 0.0001$) was abrogated in the presence of ANA-12 ($p = 0.0002$) and cyclotraxin B ($p < 0.0001$), indicating BDNF signaling contributes to the RE- and WLS-mediated increase in SIRT1 levels.

Withania somnifera RE and WLS Fraction Enhance Expression of Regulators of Mitochondrial Biogenesis and Function in the Rat Neocortex

We investigated the influence of RE and WLS on expression of regulators of mitochondrial biogenesis and function *in vivo,* in the neocortex of Sprague–Dawley rats (Fig. 4A). We observed a significant upregulation in *Sirt1* (one-way ANOVA: $F_{(2, 21)} = 21.88$, $p < 0.0001$), *Ppargc1a* (one-way ANOVA: $F_{(2, 21)} = 20.77$, $p < 0.0001$), *Tfam* (one-way ANOVA: $F_{(2, 21)} = 88.85$, $p < 0.0001$), and *Nrf1* (one-way ANOVA: $F_{(2, 21)} = 114.7$, $p < 0.0001$) mRNA expression (Fig. 4A,B) in the neocortex of RE- and WLS-treated rats. Further, we observed a robust increase in *Bdnf* (Fig. 4C) (one-way ANOVA: $F_{(2, 21)} = 10.56$, $p = 0.0007$) transcript levels in the neocortex of RE- and WLS-treated rats. We then evaluated mBDNF protein levels by western blotting analysis, and noted a significant increase (Fig. 4D, E) (one-way ANOVA: $F_{(2, 8)} = 11.56$, $p = 0.004$) in the neocortex of RE- and WLS-treated rats. We assessed the impact of RE and WLS treatments *in vivo* on downstream signaling cascades recruited by BDNF, and noted an increase in pTrkB/TrkB levels (Fig. 4F – H) (one-way ANOVA: $F_{(2, 11)} = 43.50$, $p < 0.0001$), as well as pAkt/Akt levels (Fig. 4F, G, I) (one-way ANOVA: $F_{(2, 11)} = 10.62$, $p = 0.003$) in the neocortex. Tukey's multiple comparisons test reveal a significant increase in pTrkB/TrkB levels: RE ($p < 0.0001$), WLS ($p < 0.0001$) and a significant increase in pAkt/Akt levels: WLS ($p = 0.002$). We also examined the influence of RE and WLS treatment *in vivo* on SIRT1 protein levels, and noted a significant increase in SIRT1 expression in the neocortex of RE- and WLS-treated rats using western blotting analysis (Fig. 4J, K) (one-way ANOVA: $F_{(2, 9)} = 7.34$, $p = 0.013$). Our *in vivo* studies are in agreement with our *in vitro* observations that RE and WLS treatments enhance expression of several regulators of mitochondrial biogenesis and function and also enhance BDNF levels and TrkB-Akt signaling in the neocortex.

BDNF and SIRT1 Contribute to the Effects of *Withania somnifera* RE and WLS Fraction on Mitochondria

We investigated the contribution of BDNF to the mitochondrial effects of RE and WLS. To address this, cortical neuron cultures were treated with RE or WLS, in the presence of TrkB receptor antagonists, ANA- 12 or cyclotraxin B

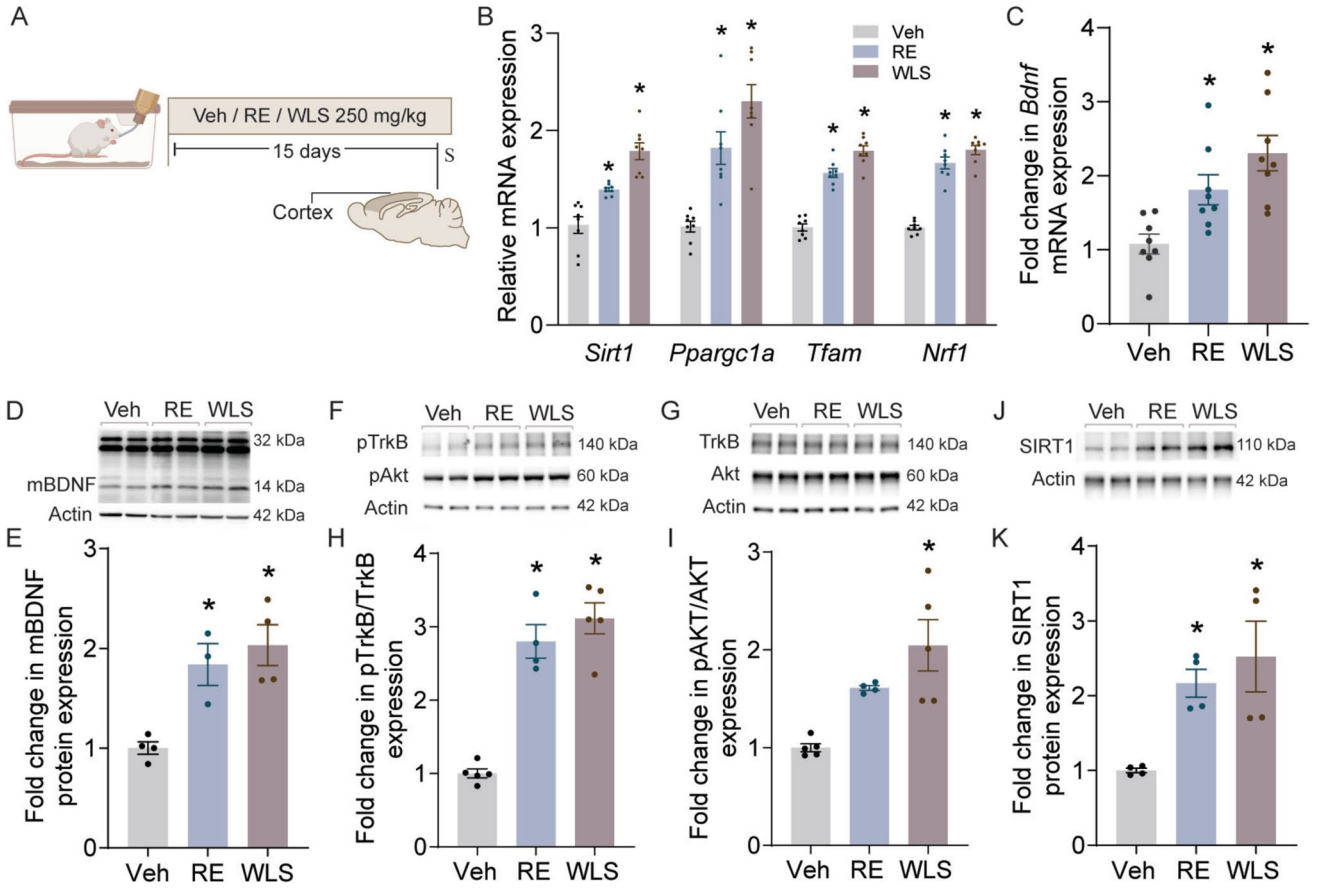


Fig. 4 Withania somnifera RE and WLS fraction enhance expression of regulators of mitochondrial biogenesis and function in the rat neocortex. (**A**) Shown is a schematic depicting the treatment paradigm with vehicle (Veh), *Withania somnifera* root extract (RE) (250 mg/kg), and withanolide—withanoside rich fraction (WLS) (250 mg/kg), administered in drinking water to adult Sprague–Dawley rats for a duration of 15 days and sacrificed (S) on the last day of treatment. (**B** and **C**). Graphs depict mRNA expression levels of regulators of mitochondrial biogenesis and function *Sirt1*, *Ppargc1a*, *Tfam* and *Nrf1* (**B**) and the trophic factor *Bdnf* (**C**) in the neocortex of vehicle, RE and WLS treated rats. Data are represented as fold change of vehicle ± SEM. ($n = 8$ animals per treatment group/N = 1, $^*p < 0.05$ as compared to vehicle, one-way ANOVA, Tukey's *post-hoc* test). (**D** and **E**) Shown is a representative immunoblot for mature BDNF (mBDNF) protein levels with actin as the loading control (**D**) and the bar graph depicts quantitative densitometric analysis of mBDNF protein levels normalized to actin (**E**) in cortices derived from vehicle, RE and WLS treated rats. Data are expressed as fold change of vehicle ± SEM. ($n = 3$–4 animals per treatment group/N = 1, $^*p < 0.05$ as compared to vehicle, one-way ANOVA, Tukey's *post-hoc* test). (**F** and **G**) Shown is a representative immunoblot of pTrkB (**F**) and TrkB (**G**) and pAkt (**F**) and Akt (**G**) protein levels with actin as the loading control (**F** and **G**) from cortices derived from vehicle, RE and WLS treated rats. (**H** and **I**) Quantitative densitometric analysis of pTrkB/TrkB (H), and pAkt/Akt (I) protein levels with ratios normalized to actin as loading controls from cortices derived from vehicle, RE and WLS treated rats. Data are expressed as fold change of vehicle ± SEM. ($n = 4$–5 animals per treatment group/N = 1, $^*p < 0.05$ as compared to vehicle, one-way ANOVA, Tukey's *post-hoc* test). (**J** and **K**) Shown is a representative immunoblot for SIRT1 protein levels with actin as the loading control (J) and the bar graph depicts quantitative densitometric analysis of SIRT1 protein levels normalized to actin (K) in cortices derived from vehicle, RE and WLS treated rats. Data are expressed as fold change of vehicle ± SEM. (n = 4 animals per treatment group/N = 1, $^*p < 0.05$ as compared to vehicle, one-way ANOVA, Tukey's *post-hoc* test)

(Fig. 5A). We noted a significant two-way ANOVA interaction of WLS x ANA- 12 ($F_{(1, 12)} = 10.19$, $p = 0.007$) and WLS x cyclotraxin B ($F_{(1, 12)} = 15.64$, $p = 0.002$) for mtDNA content in cortical neurons (Fig. 5B). *Post-hoc* Tukey comparison analysis revealed that the WLS-mediated increase in mtDNA was abrogated on treatment with ANA- 12 and cyclotraxin B. The mtDNA content differed significantly between the WLS treated neurons and WLS + ANA- 12 treated neurons ($p = 0.001$) and between the WLS treated neurons and WLS + cyclotraxin B treated neurons ($p < 0.0001$) (Fig. 5B). We noted significant two-way ANOVA interactions of RE x ANA- 12 ($F_{(1, 12)} = 11.69$, $p = 0.005$), and RE x cyclotraxin B ($F_{(1, 12)} = 14.86$, $p = 0.002$), for ATP levels in cortical neurons (Fig. 5C). Further, we also noted significant two-way ANOVA interactions of WLS x ANA-12 ($F_{(1, 12)} = 56.15$, $p < 0.0001$) and WLS x cyclotraxin B ($F_{(1, 12)} = 63.02$, $p < 0.0001$), for ATP levels in cortical neurons (Fig. 5C). *Post-hoc* Tukey comparison analysis revealed

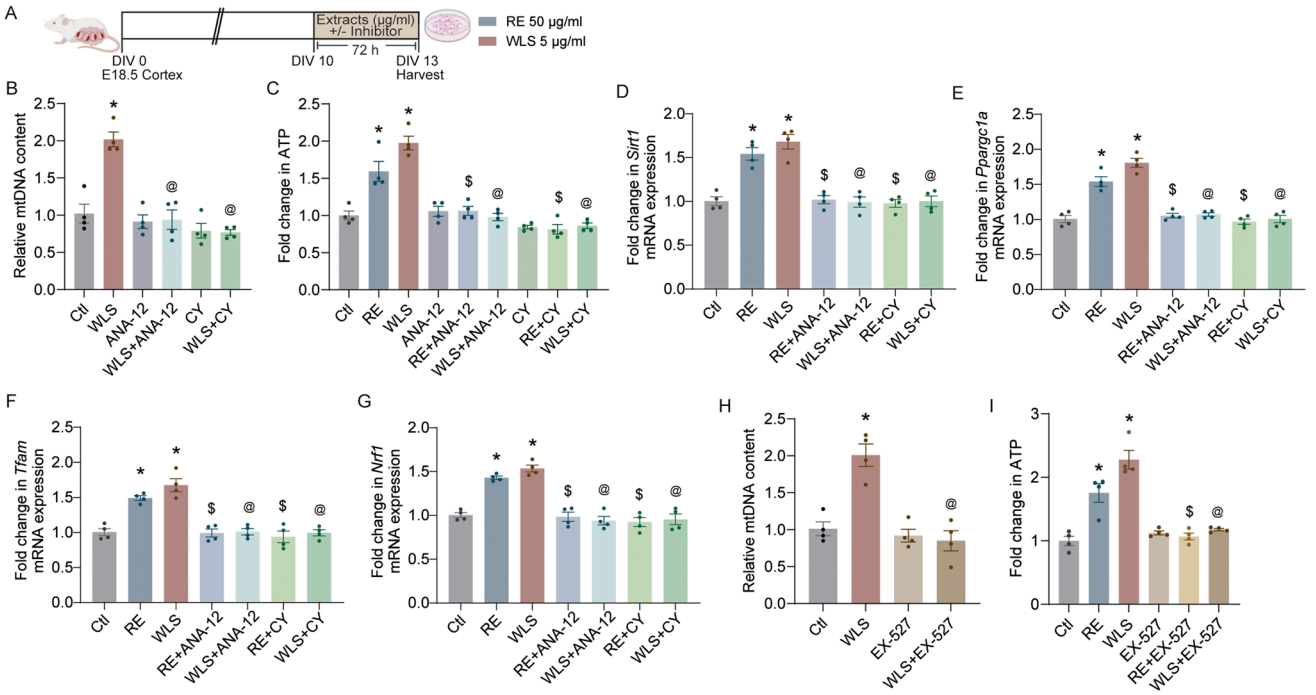


Fig. 5 BDNF and SIRT1 contribute to the effects of Withania somnifera RE and WLS fraction on mitochondria. (**A**) Shown is a schematic depicting the treatment paradigm with *Withania somnifera* root extract (RE) (50 µg/ml) or withanolide—withanoside rich fraction (WLS) (5 µg/ml), in the presence or absence of the TrkB receptor antagonist, ANA- 12 (1 µM), the TrkB receptor antagonist, cyclotraxin B (1 µM), or the SIRT1 inhibitor EX- 527 (10 µM) of rat cortical neuron cultures commencing DIV 10 for a duration of 72 h. (**B**) Graph represents qPCR analysis for mtDNA content from cortical neurons treated with WLS in the presence or absence of the TrkB receptor antagonists, ANA- 12 or cyclotraxin B. Data are represented as relative mtDNA content ± SEM. (n = 4 per treatment group/N = 2, $^{*}p < 0.05$ as compared to control (Ctl), $^{@}p < 0.05$ as compared to WLS treated group, two-way ANOVA, Tukey's *post-hoc* test). (**C**) Graph represents cellular ATP levels from cortical neurons treated with RE or WLS in the presence or absence of the TrkB receptor antagonists, ANA- 12 or cyclotraxin B. Data expressed as fold change of control ± SEM. (n = 4 per treatment group/N = 2, $^{*}p < 0.05$ as compared to control (Ctl), $^{\$}p < 0.05$ as compared to RE treated group, $^{@}p < 0.05$ as compared to WLS treated group, two-way ANOVA, Tukey's *post-hoc* test). (**D**-**G**) Graphs represent mRNA expression levels of regulators of mitochondrial biogenesis and function *Sirt1* (**D**), *Ppargc1a* (**E**), *Tfam* (**F**) and *Nrf1* (**G**) from cortical neurons treated with RE or WLS in the presence or absence of the TrkB receptor antagonists, ANA- 12 or cyclotraxin B. Data are expressed as fold change of control ± SEM. (n = 4 per treatment group/N = 2, $^{*}p < 0.05$ as compared to control (Ctl), $^{\$}p < 0.05$ as compared to RE treated group, $^{@}p < 0.05$ as compared to WLS treated group, one-way ANOVA, Tukey's *post-hoc* test). (H) Graph represents qPCR analysis for mtDNA levels from cortical neurons treated with WLS in the presence or absence of the SIRT1 inhibitor EX- 527. Data are represented as relative mtDNA content ± SEM. (n = 4 per treatment group/N = 2, $^{*}p < 0.05$ as compared to control (Ctl), $^{@}p < 0.05$ as compared to WLS treated group, two-way ANOVA, Tukey's *post-hoc* test). (**I**) Graph represents cellular ATP levels from cortical neurons treated with RE or WLS in the presence or absence of the SIRT1 inhibitor EX- 527. Data are expressed as fold change of control ± SEM. (n = 4 per treatment group/N = 2, $^{*}p < 0.05$ as compared to control (Ctl), $^{\$}p < 0.05$ as compared to RE treated group, $^{@}p < 0.05$ as compared to WLS treated group, two-way ANOVA, Tukey's *post-hoc* test)

that treatment with ANA- 12 or cyclotraxin B abrogated the RE and WLS- mediated increase in ATP levels. The ATP levels differed significantly between the RE treated neurons and RE + ANA- 12 treated neurons ($p = 0.004$) and between the RE treated neurons and RE + cyclotraxin B treated neurons ($p < 0.0001$). We also noted that the ATP levels differed significantly between the WLS-treated neurons and WLS + ANA- 12 treated neurons ($p < 0.0001$) and between the WLS-treated neurons and WLS + cyclotraxin B treated neurons ($p < 0.0001$) (Fig. 5C).

We observed a significant increase in the expression of *Sirt1* (Fig. 5D), *Ppargc1a* (Fig. 5E), *Tfam* (Fig. 5F) and *Nrf1* (Fig. 5G), transcripts following RE and WLS treatments, which was prevented in the presence of ANA- 12 or cyclotraxin B (Fig. 5D- 5G) [one-way ANOVA: *Sirt1*: ($F_{(6, 21)} = 20.37$, $p < 0.0001$); *Ppargc1a*: ($F_{(6, 21)} = 43.59$, $p < 0.0001$); *Tfam*: ($F_{(6, 21)} = 17.87$, $p < 0.0001$), *Nrf1*: ($F_{(6, 21)} = 21.99$, $p < 0.0001$]. *Post-hoc* Tukey comparison analysis revealed that transcript levels differed significantly between the RE treated neurons and RE + ANA- 12 treated neurons (*Sirt1:* $p = 0.0002$; *Ppargc1a:* $p < 0.0001$; *Tfam:* $p = 0.0005$; *Nrf1:* $p = 0.0002$) and between the RE treated neurons and RE + cyclotraxin B treated neurons (*Sirt1:* $p <$ 0.0001; *Ppargc1a:* $p < 0.0001$; *Tfam:* $p < 0.0001$; *Nrf1:* $p <$

0.0001) (Fig. 5D- 5G). We also noted that transcript levels differed significantly between the WLS-treated neurons and WLS + ANA- 12 treated neurons (*Sirt1:* $p < 0.0001$; *Ppargc1a:* $p < 0.0001$; *Tfam:* $p < 0.0001$; *Nrf1:* $p < 0.0001$) and between the WLS-treated neurons and WLS + cyclotraxin B treated neurons (*Sirt1:* $p < 0.0001$; *Ppargc1a:* $p < 0.0001$; *Tfam:* $p < 0.0001$; *Nrf1:* $p < 0.0001$) (Fig. 5D- 5G). These observations indicate that BDNF via TrkB receptor signaling contributes to the RE- and WLS-evoked effects on mtDNA levels and ATP production, as well as the RE- and WLS-evoked regulation of *Sirt1*, *Ppargc1a*, *Tfam* and *Nrf1* mRNA expression in cortical neurons.

We next evaluated the contribution of SIRT1 to the mitochondrial effects of RE and WLS by simultaneously delivering the SIRT1 catalytic activity inhibitor, EX- 527, to RE and WLS-treated cortical neurons. We noted significant two-way ANOVA interactions of WLS x EX- 527 ($F_{(1, 12)} = 11.62$, $p = 0.005$) for mtDNA content in cortical neurons (Fig. 5H). *Post-hoc* Tukey comparison analysis revealed that treatment with EX- 527 abrogated the WLS-mediated increase in mtDNA and the mtDNA content differed significantly between the WLS-treated neurons and WLS + EX- 527 treated neurons ($p = 0.0008$) (Fig. 5H). We noted significant two-way ANOVA interactions of RE x EX- 527 ($F_{(1, 12)} = 21.73$, $p = 0.0005$) and WLS x EX-527 ($F_{(1, 12)} = 53.13$, $p < 0.0001$) for ATP levels in cortical neurons (Fig. 5I). Post hoc Tukey comparison analysis revealed that treatment with EX- 527 abrogated the RE and WLS- mediated increase in ATP levels. The ATP levels differed significantly between the RE treated neurons and RE + EX- 527 treated neurons ($p = 0.0006$) and between the WLS treated neurons and WLS + EX- 527 treated neurons ($p < 0.0001$) (Fig. 5I). Our results indicate that SIRT1 signaling contributes to the RE- and WLS-evoked effects on mitochondrial DNA content and ATP production *in vitro*.

Mitochondrial Effects of WLS Fraction are Mediated via the Phytoactive Constituents Withanolide A and Withanoside IV

We next sought to determine the phytoactive constituents of the WLS fraction that mediate the effects on mitochondrial biogenesis and function in cortical neurons. We treated cortical neuron cultures with specific WLS constituents: withanolide A (WLA), withanoside IV (WSIV), withanolide B (WLB), withanoside V (WSV), 12-deoxy-withastramonolide (DWS) and withaferin A (WA) and assessed their influence on mtDNA content and cellular ATP levels (Fig. 6A, B). We observed that treatment with WLA and WSIV enhanced mtDNA content (Fig. 6C) (one-way ANOVA: $F_{(7, 24)} = 26.97$, $p < 0.0001$) and ATP levels (Fig. 6D) (one-way ANOVA: $F_{(7, 24)} = 15.12$, $p < 0.0001$) in cortical neurons, comparable to the scale of effect noted with the WLS extract. *In vitro* treatment with WLB, WSV, DWS and WA did not appear to influence mtDNA content and ATP levels in cortical neurons (Fig. 6C, D). We then examined the influence of treatment with WLA and WSIV on BDNF and SIRT1 in cortical neurons (Fig. 6E). Quantitative ELISA analysis for mBDNF levels, revealed an increase in both cellular mBDNF levels (Fig. 6F) (one-way ANOVA: $F_{(3, 8)} = 23.92$, $p = 0.0002$) and mBDNF levels detected in the culture medium (Fig. 6G) (one-way ANOVA: $F_{(3, 8)} = 8.164$, $p = 0.008$), of cortical neuron cultures treated with WLA and WSIV. Elevated levels of the SIRT1 protein were observed following treatment of cortical neurons with WLA and WSIV (Fig. 6H, I) (one-way ANOVA: $F_{(2, 8)} = 15.33$, $p = 0.002$). Taken together, these results demonstrate that WLA and WSIV are the phytoactive constituents of WLS, that enhance BDNF and SIRT1 levels and mediate the mitochondrial effects of WLS on mtDNA content and ATP production.

WLS Fraction, Withanolide A and Withanoside IV Exert Neuroprotective Effects Against Corticosterone-Induced Stress via BDNF and SIRT1

We next sought to examine the potential neuroprotective effects of the WLS fraction, withanolide A (WLA) and withanoside IV (WSIV) in buffering corticosterone-mediated stress in cortical neurons *in vitro*. Rat cortical neurons were exposed to high concentrations of corticosterone (Cort) (1 μM), within a physiological range for twelve days, with an overlap in treatment with WLS, WLA or WSIV during the last 72 h to assess for potential neuroprotective effects (Fig. 7A). We noted significant two-way ANOVA interactions of: (1) WLS x Cort ($F_{(1, 12)} = 30.95$, $p = 0.0001$); (2) WLA x Cort ($F_{(1, 12)} = 23.22$, $p = 0.0004$) and (3) WSIV x Cort ($F_{(1, 12)} = 23.76$, $p = 0.0004$) for cell viability in cortical neurons (Fig. 7B). *Post-hoc* Tukey comparison analysis revealed that treatment with WLS, WLA and WSIV abrogated the Cort-mediated reduction in cell viability. Cell viability as assessed by the MTT test differed significantly between the (1) Cort treated neurons and WLS + Cort treated neurons ($p = 0.0002$); (2) Cort treated neurons and WLA + Cort treated neurons ($p = 0.0002$) and (3) Cort treated neurons and WSIV + Cort treated neurons ($p = 0.0014$) (Fig. 7B). These results indicate that WLS, WLA and WSIV mediate neuroprotective effects and enhance cell survival in response to chronic Cort-induced stress.

Given our results of a role for BDNF and SIRT1 in mediating the mitochondrial effects of WLS, we tested the contribution of BDNF and SIRT1 to the neuroprotective effects of WLS, WLA and WSIV, using the TrkB receptor antagonists, ANA- 12 and cyclotraxin B, as well as the SIRT1 inhibitor EX- 527. Cell viability was assessed in cortical neuron cultures challenged with or without chronic Cort treatment for twelve days and co-treated with WLS, WLA or WSIV

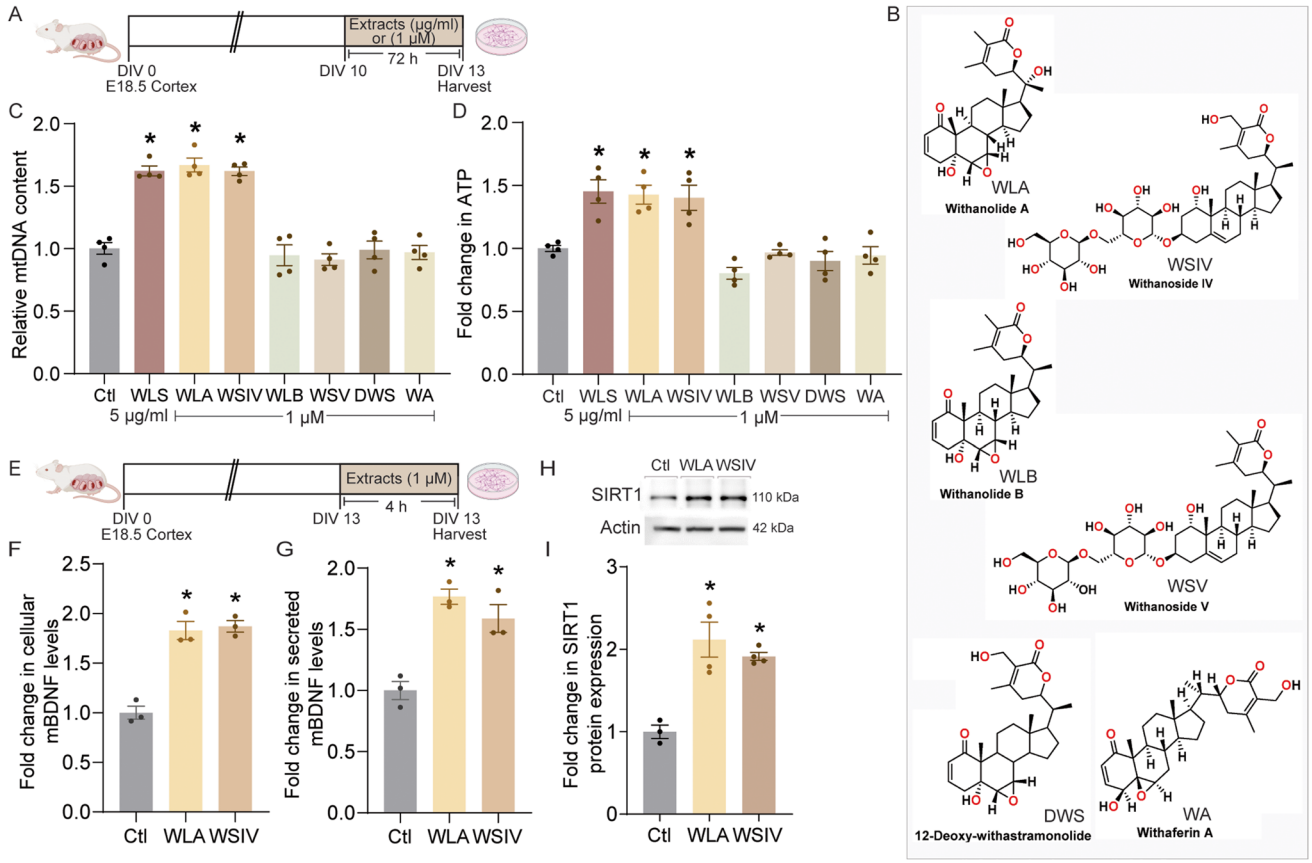


Fig. 6 Mitochondrial effects of the WLS fraction are mediated via the phytoactive constituents withanolide A and withanoside IV. (**A**) Shown is a schematic depicting the treatment paradigm with withanolide—withanoside rich fraction (WLS) (5 µg/ml), withanolide A (WLA, 1 µM), Withanoside IV (WSIV, 1 µM), withanolide B (WLB, 1 µM), withanoside V (WSV, 1 µM), 12-deoxy-withastramonolide (DWS, 1 µM) and withaferin A (WA, 1 µM) of rat cortical neuron cultures commencing DIV 10 for a duration of 72 h. (**B**) The panel depicts the chemical structures of WLS constituents: WLA, WSIV, WLB, WSV, DWS and WA. (**C**) Graph represents qPCR analysis for mtDNA content from cortical neurons treated with WLS, WLA, WSIV, WLB, WSV, DWS and WA. Data are represented as relative mtDNA content ± SEM. ($n = 4$ per treatment group/N = 2, $^*p < 0.05$ as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test). (**D**) Graph represents cellular ATP levels from cortical neurons treated with WLS, WLA, WSIV, WLB, WSV, DWS and WA. Data are expressed as fold change of control ± SEM. ($n = 4$ per treatment group/N = 2, $^*p < 0.05$ as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test). (**E**) Shown is a schematic depicting the treatment paradigm with withanolide A (WLA, 1 µM) or Withanoside IV (WSIV, 1 µM) of rat cortical neuron cultures for a duration of 4 h on DIV 13. (**F** and **G**) Graph depicts quantitative enzyme-linked immunosorbent assay (ELISA) analysis for mature BDNF (mBDNF) protein expression levels in cortical neuron culture lysates (**F**) and mBDNF release in culture medium (**G**) of control, WLA and WSIV treated cortical neurons. Data are expressed as fold change of control ± SEM. ($n = 3$ per treatment group/N = 2, $^*p < 0.05$ as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test). (**H** and **I**) Shown is a representative immunoblot for SIRT1 protein levels with actin as the loading control (**H**) and the bar graph depicts quantitative densitometric analysis of SIRT1 protein levels normalized to actin (**I**) in control, WLA and WSIV treated cortical neurons. Data are expressed as fold change of control ± SEM. ($n = 3$–4 per treatment group/N = 2, $^*p < 0.05$ as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test)

in the presence or absence of the TrkB receptor antagonist, ANA- 12, cyclotraxin B or the SIRT1 inhibitor EX- 527 for the final 72 h of treatment (Fig. 7C).

We noted significant three-way ANOVA interactions of WLS x Cort x ANA- 12 ($F_{(1, 24)} = 12.13$, $p = 0.001$) (Fig. 7D), WLS x Cort x cyclotraxin B ($F_{(1, 24)} = 8.52$, $p = 0.007$) (Fig. 7E) and WLS x Cort x EX- 527 ($F_{(1, 24)} = 11.92$, $p = 0.002$) (Fig. 7F) for cell survival of cortical neurons. *Post-hoc* Tukey comparison analysis indicated that treatment with ANA- 12, cyclotraxin B or EX- 527 prevented the WLS-mediated increase in cell viability against chronic Cort-induced cell death. *Post-hoc* Tukey group comparisons revealed that the cell survival differed significantly between (1) the WLS + Cort + ANA-12 treated neurons and WLS + Cort treated neurons ($p < 0.0001$) (Fig. 7D), (2) the WLS + Cort + cyclotraxin B treated neurons and WLS + Cort treated neurons ($p = 0.0001$) (Fig. 7E) and the (3) WLS + Cort + EX- 527 treated neurons and WLS + Cort treated neurons ($p < 0.0001$) (Fig. 7F).

We noted significant three-way ANOVA interactions of WLA x Cort x ANA- 12 ($F_{(1, 24)} = 4.42$, $p = 0.046$) (Fig. 7G), WLA x Cort x cyclotraxin B ($F_{(1, 24)} = 6.97$, $p = 0.014$) (Fig. 7H) and WLA x Cort x EX- 527 ($F_{(1, 24)} = 7.15$, $p = 0.013$) (Fig. 7I) for cell survival of cortical neurons. Post hoc Tukey comparison analysis indicated that treatment with ANA- 12, cyclotraxin B or EX- 527 prevented the WLA mediated increase in cell viability against chronic Cort-induced cell death. *Post-hoc* Tukey group comparisons revealed that the cell survival differed significantly between (1) the WLA + Cort + ANA- 12 treated neurons and WLA + corticosterone treated neurons ($p = 0.0007$) (Fig. 7G), (2) the WLA + Cort + cyclotraxin B treated neurons and WLA + Cort treated neurons ($p = 0.0006$) (Fig. 7H) and the (3) WLA + Cort + EX- 527 treated neurons and WLA + Cort treated neurons ($p < 0.0001$) (Fig. 7I).

We noted significant three-way ANOVA interactions of WSIV x Cort x ANA- 12 ($F_{(1, 24)} = 13.33$, $p = 0.001$) (Fig. 7J), WSIV x Cort x cyclotraxin B ($F_{(1, 24)} = 4.30$, $p < 0.05$) (Fig. 7K) and WSIV x Cort x EX- 527 ($F_{(1, 24)} = 9.36$, $p = 0.005$) (Fig. 7L) for cell survival of cortical neurons. *Post-hoc* Tukey comparison analysis indicated that treatment with ANA- 12, cyclotraxin B or EX- 527 prevented the WSIV mediated increase in cell viability against chronic Cort-induced cell death. *Post-hoc* Tukey group comparisons revealed that the cell survival differed significantly between (1) the WSIV + Cort + ANA- 12 treated neurons and WSIV + Cort treated neurons ($p < 0.0001$) (Fig. 7J), (2) the WSIV + Cort + cyclotraxin B treated neurons and WSIV + Cort treated neurons ($p = 0.0005$) (Fig. 7K) and the (3) WSIV + Cort + EX- 527 treated neurons and WSIV + Cort treated neurons ($p = 0.0002$) (Fig. 7L).

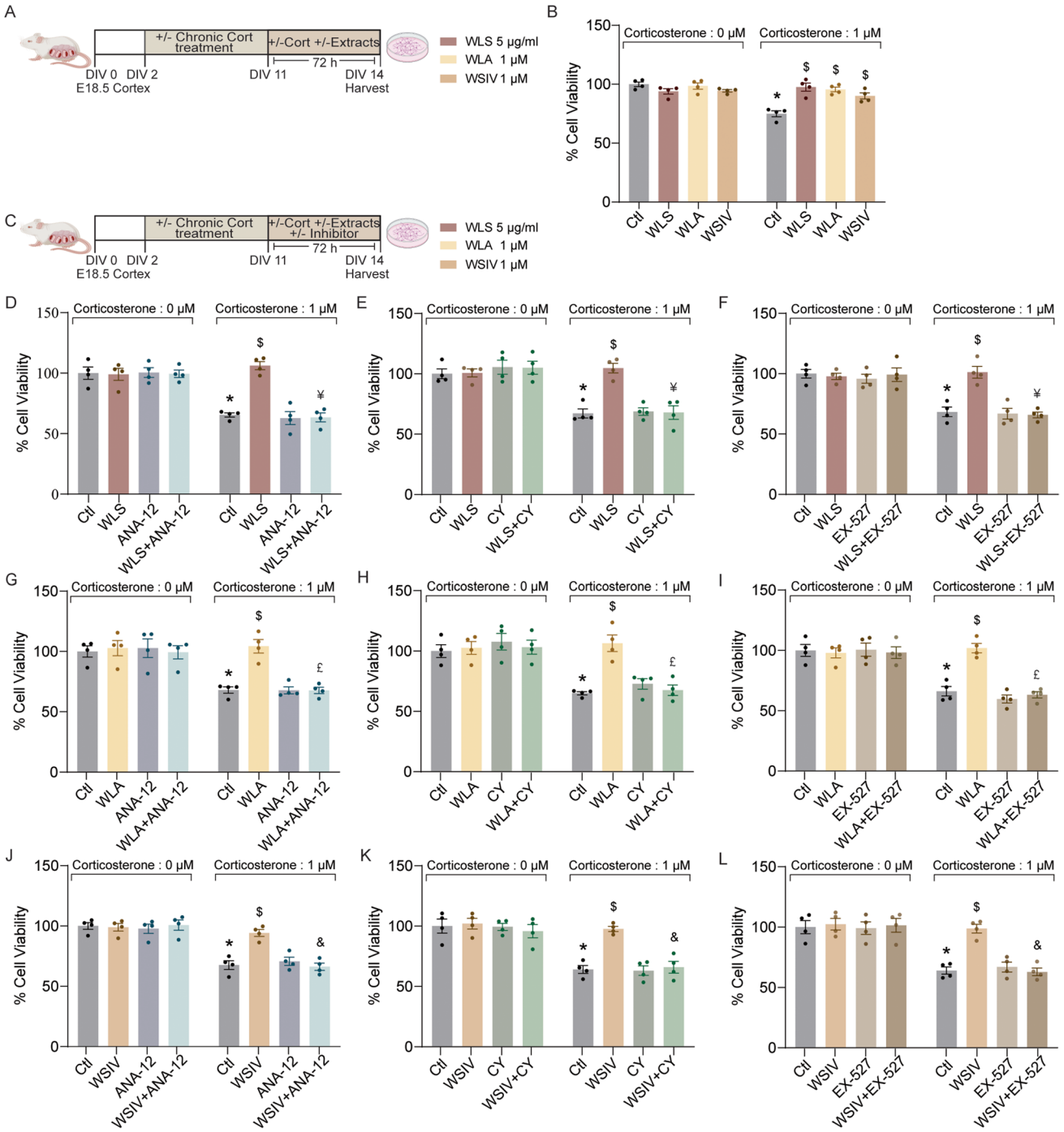
Collectively, these pharmacological inhibitor studies implicate BDNF and SIRT1 in contributing to the robust neuroprotective effects of WLS and its constituents WLA and WSIV, in the context of chronic Cort-induced stress *in vitro*.

Discussion

Here, we show that *Withania somnifera* RE and the withanolide – withanoside (WLS) rich fraction, enhanced mitochondrial biogenesis and energetics in rat cortical neurons *in vitro,* and the neocortex *in vivo,* with significant increases in mitochondrial respiration and OxPhos efficiency. RE and WLS drive a transcriptional program enhancing the expression of several regulators of mitochondrial biogenesis and function, namely *Sirt1*, *Ppargc1a*, *Tfam* and *Nrf1*. Furthermore, RE and WLS increase BDNF expression and release from cortical neurons. We find that the RE and WLS-evoked transcriptional program is dependent on enhanced BDNF-TrkB signaling. Furthermore, the mitochondrial effects of RE and WLS are completely abolished by both TrkB receptor antagonists and a catalytic inhibitor of SIRT1 in cortical neurons, demonstrating that BDNF and SIRT1 signaling are key to the mitochondrial effects of RE and WLS. A systematic evaluation of distinct WLS phytoactives identified withanolide A (WLA) and withanoside IV (WSIV) as the important active constituents of the WLS fraction that mediate mitochondrial effects in cortical neurons, and revealed a key role for BDNF-SIRT1 in the neuroprotective actions of these phytoactives (Fig. 8). Ethnopharmacological evidence indicates that *Withania somnifera* exerts pleiotropic neuroprotective effects against diverse neurotoxic insults [52–56], enhances cognition and mood, and is used to treat neuropsychiatric and neurodegenerative disorders in traditional Indian medicine [15, 16, 18, 57–60]. In this regard, the findings of our study provide novel evidence that mitochondrial biogenesis and neuroenergetics are a major mechanistic target for *Withania somnifera,* thus targeting a central pathway that could serve to endow neurons with enhanced capacity to buffer cellular stressors and respond effectively to heightened energetic demands.

While previous studies have not systematically assessed the impact of *Withania somnifera* on mitochondrial biogenesis and function in neurons, a few reports suggest enhanced ATP levels, following treatment in Parkinsonian and Huntington's disease rodent models [61, 62]. In this regard, our observations reveal striking effects on mitochondrial respiration, with enhanced mitochondrial basal (state 2) and ADP-coupled (state 3) respiration, as well as ATP production rate in the neocortex of RE and WLS-treated rats indicative of enhanced OxPhos efficiency, which could in turn help to buffer heightened energy demands. While our *in vitro* studies suggest that the mitochondrial effects of RE and WLS may arise due to direct actions on cortical neurons, in the absence of studies with direct local delivery of RE/WLS into the neocortex *in vivo,* it is currently not possible to rule out a contribution of non-direct effects via potential ascending peripheral pathways.

Both *in vitro* and *in vivo* experiments revealed that RE and WLS evoke a transcriptional program with enhanced cortical expression of several genes associated with regulation of mitochondrial biogenesis and function, including *Ppargc1a*, *Sirt1*, *Tfam* and *Nrf1* [31–33]. SIRT1 deacetylates and activates the master regulator of mitochondrial biogenesis PGC- 1α, driving the expression of several OxPhos associated genes [31–33, 39]. SIRT1 plays a critical role in RE and WLS-evoked mitochondrial effects, which are totally abrogated by EX- 527, a catalytic inhibitor of SIRT1. We find that BDNF signaling appears to lie upstream of the recruitment of the SIRT1- PGC- 1α axis, with both RE and WLS enhancing expression and release of BDNF, and driving TrkB-Akt signaling in cortical neurons. Pharmacological TrkB antagonists prevented the influence of RE and



WLS on the transcriptional program (*Ppargc1a*, *Sirt1*, *Tfam* and *Nrf1),* enhanced SIRT1 protein levels, and the increase in mitochondrial biogenesis and function. The evidence that both BDNF and SIRT1 are major targets for *Withania somnifera* in the neocortex, and contribute to the RE and WLS mediated regulation of mitochondrial biogenesis and function, sets up the possibility that these hub proteins may serve as key components that contribute to the pleiotropic actions of *Withania somnifera* that extend beyond an impact on neuroenergetics and survival, and hold implications for well-documented effects of *Withania somnifera* on neuronal arborization, neuroplasticity, mood and aging [16, 20, 57, 59, 63–65].

BDNF besides its well documented role in promoting neuronal survival and plasticity [43–46], has also been implicated in regulating neuronal mitochondrial biogenesis and energetics [46–51] and influencing mitochondrial docking at synapses [66]. Our results corroborate the mitochondrial

◂**Fig. 7** WLS fraction, withanolide A and withanoside IV exert neuroprotective effects against corticosterone-induced stress via BDNF and SIRT1**.** (A) Shown is a schematic depicting the treatment paradigm of rat cortical neuron cultures with or without chronic corticosterone (cort) (1 μM) treatment for 12 days commencing from DIV 2, with cotreatment with withanolide—withanoside rich fraction (WLS) (5 μg/ml), withanolide A (WLA) (1 μM) or withanoside IV (WSIV) (1 μM) for the last 72 h. (B) The bar graph depicts cell viability assessed by the MTT assay in cortical neurons challenged with or without chronic corticosterone, with cotreatment with WLS, WLA or WSIV for the last 72 h. Data are expressed as percent of untreated control (Ctl) cell viability ± SEM. (n = 4 per treatment group/N = 2, $*p < 0.05$ as compared to untreated control Ctl, $^{\$}p < 0.05$ as compared to 1 μM corticosterone Ctl treated group, two-way ANOVA, Tukey's *post-hoc* test). (C) Shown is a schematic depicting the treatment paradigm of rat cortical neuron cultures with or without chronic corticosterone (cort) (1 μM) treatment for 12 days commencing from DIV 2, with cotreatment with withanolide—withanoside rich fraction (WLS) (5 μg/ml), withanolide A (WLA) (1 μM) or withanoside IV (WSIV) (1 μM) in the presence or absence of the TrkB receptor antagonist, ANA- 12 (1 μM), the TrkB receptor antagonist, cyclotraxin B (1 μM), or the SIRT1 inhibitor EX- 527 (10 μM) for the last 72 h. (D—F) The bar graph depicts cell viability assessed by the MTT assay in cortical neurons challenged with or without chronic corticosterone, with cotreatment with WLS in the presence or absence of the TrkB receptor antagonist, ANA- 12 (D), the TrkB receptor antagonist, cyclotraxin B (E), or the SIRT1 inhibitor EX- 527 (F) for the last 72 h. Data are expressed as percent of untreated control (Ctl) cell viability ± SEM. ($n = 4$ per treatment group/N = 2, $*p < 0.05$ as compared to untreated control Ctl, $^{\$}p < 0.05$ as compared to 1 μM corticosterone Ctl treated group, $^{¥}p < 0.05$ as compared to WLS + 1 μM corticosterone treated group, three-way ANOVA, Tukey's *post-hoc* test). (G—I) The bar graph depicts cell viability assessed by the MTT assay in cortical neurons challenged with or without chronic corticosterone, with cotreatment with WLA, in the presence or absence of the TrkB receptor antagonist, ANA- 12 (G), the TrkB receptor antagonist, cyclotraxin B (H), or the SIRT1 inhibitor EX- 527 (I) for the last 72 h. Data are expressed as percent of untreated control (Ctl) cell viability ± SEM. ($n = 4$ per treatment group/N = 2, $*p < 0.05$ as compared to untreated control Ctl, $^{\$}p < 0.05$ as compared to 1 μM corticosterone Ctl treated group, $^{£}p < 0.05$ as compared to WLA + 1 μM corticosterone treated group, three-way ANOVA, Tukey's *post-hoc* test). (J—L) The bar graph depicts cell viability assessed by the MTT assay in cortical neurons challenged with or without chronic corticosterone, with cotreatment with WSIV, in the presence or absence of the TrkB receptor antagonist, ANA- 12 (J), the TrkB receptor antagonist, cyclotraxin B (K), or the SIRT1 inhibitor EX- 527 (L) for the last 72 h. Data are expressed as percent of untreated control (Ctl) cell viability ± SEM. ($n = 4$ per treatment group/N = 2, $*p < 0.05$ as compared to untreated control Ctl, $^{\$}p < 0.05$ as compared to 1 μM corticosterone Ctl treated group, $^{\&}p < 0.05$ as compared to WSIV + 1 μM corticosterone treated group, three-way ANOVA, Tukey's *post-hoc* test)

effects of BDNF on cortical neurons and reveal robust effects on biogenesis, ATP levels and gene expression of *Ppargc1a* and *Sirt1*. Interestingly BDNF is known to be targeted by polyphenols [30] and diverse plant-derived phytoactives [67]. Prior studies indicate that *Withania somnifera* leaf extract enhances BDNF expression in rodent models of amnesia and metabolic stress [68, 69]. Further, in silico studies [70] suggest that major constituents of *Withania somnifera* including WLA, could directly target TrkB and PI3 K/Akt signaling. At present the underlying mechanisms via which RE and WLS enhance BDNF expression, secretion and signaling are unclear. One possibility is that *Withania somnifera* phytoactives may influence neuronal activity [71] thus driving activity-dependent regulation of BDNF expression and release. Alternatively, BDNF expression could be influenced via the activation of intracellular signaling pathways [72] that target transcription factors such as CREB [73], or nuclear factor kappa-B [74]. Our findings demonstrate a central role for BDNF in contributing to the effects of RE and WLS on mitochondrial biogenesis and energetics. This raises the intriguing possibility that through the recruitment of a trophic factor signaling cascade, *Withania somnifera* may exert diverse downstream effects, such as targeting neuronal bioenergetics, structural and functional plasticity, and evoking neuroprotective actions.

Amongst the most commonly described uses of *Withania somnifera* in traditional medicine is as a stress adaptogen [19]. Stress-evoked allostatic load is posited to involve a compromise of neuronal mitochondrial function [25, 26], suggesting the intriguing possibility that through the targeting of these mitochondrial pathways, *Withania somnifera* may ameliorate specific stress-related consequences. We find that the phytoactives of *Withania somnifera* root extract—WLS, WLA and WSIV protect against a chronic corticosterone induced decline in neuronal survival in both a BDNF and SIRT1 dependent manner. Chronic corticosterone treatment of neurons results in impaired mitochondrial function and enhanced cell death [30, 75]. The results of the present study indicate that phytoactives from *Withania somnifera* (WLS, WLA and WSIV), via the recruitment of BDNF and SIRT1 as vital intermediaries, endow neurons with enhanced survival when challenged with chronic corticosterone. This motivates future studies to address underlying mechanisms through which *Withania somnifera* phytoactives promote the ability of cortical neurons to effectively buffer cellular stress, via the hub proteins BDNF and SIRT1, and a modulation of mitochondria and neuronal energetics. Furthermore, our work motivates future studies *in vivo* to ascertain the neuroprotective and behavioral impact of the targeting of mitochondrial biogenesis and function by *Withania somnifera*.

In conclusion, a substantial body of literature, including preclinical and clinical studies, as well as ethnopharmacological evidence of long-term indigenous usage, suggest potential therapeutic applications for *Withania somnifera* as an anxiolytic and antidepressant [19], as a neuroprotective agent against diverse stressors [76–78], in mild traumatic brain injury [74], in models of excitotoxicity [73, 79] as well as in Alzheimer's, Parkinson's and Huntington's disease [15, 17, 54]. Given the breadth of neuropathological conditions for which *Withania somnifera* is widely used, points to the possible targeting of a common central core mechanism at the cellular level that could contribute to the pleiotropic

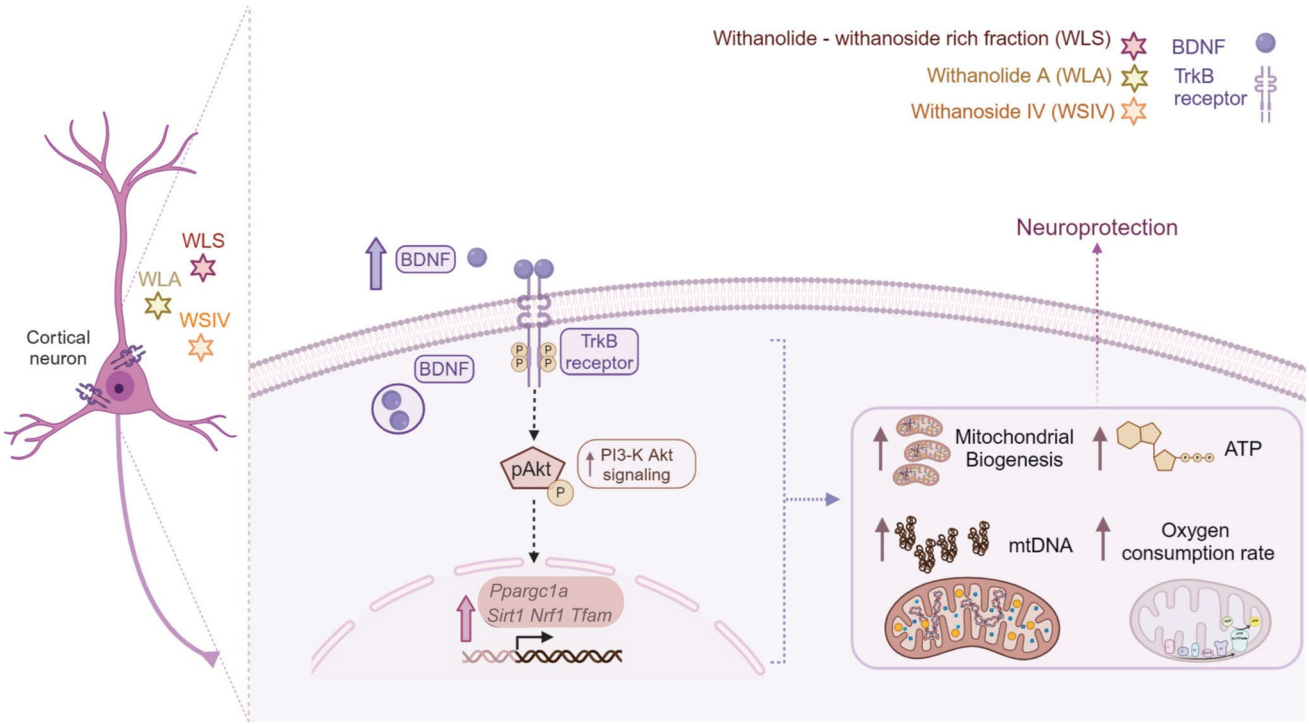


Fig. 8 Schematic depicting the putative mechanism for the WLS fraction, withanolide A and withanoside IV effects on mitochondria. Shown is a schematic illustrating a putative mechanism for the effects of the withanolide—withanoside rich fraction (WLS) and its phytoactives withanolide A (WLA) and withanoside IV (WSIV), in mediating effects on mitochondria in cortical neurons. WLS, WLA and WSIV evoke an increase in mature BDNF (mBDNF) expression in cortical neurons and enhanced release of mBDNF. This results in mBDNF evoked activation and phosphorylation of the TrkB receptor and downstream phosphorylation of protein kinase B (Akt). This activation of the TrkB-Akt signaling pathway, probably via a multiple step process, results in enhanced expression of several regulators of mitochondrial biogenesis, *Ppargc1a*, *Sirt1*, *Nrf1*, *Tfam.* We hypothesize that RE and WLS via BDNF signaling drive a transcriptional program that serves to enhance mitochondrial biogenesis, reflected through increased markers of mitochondrial proteins and mtDNA expression, and heightened mitochondrial energetics including increased OxPhos efficiency and ATP production. Our results indicate that BDNF and SIRT1 are critical to these effects on mitochondrial biogenesis and energetics. WLS, WLA and WSIV exhibit increased cell survival against corticosterone-induced stress evoked cell death in cortical neurons, an effect that requires BDNF and SIRT1. This increase in mitochondrial biogenesis and function may contribute to the effects of WLS, WLA and WSIV in mediating neuroprotection and stress adaptation against corticosterone-induced stress

therapeutic use. In this regard, our findings that *Withania somnifera* phytoactives enhance mitochondrial biogenesis and neuroenergetics within cortical circuits raises the intriguing possibility that the targeting of neuroenergetics may be such a central mechanism that could contribute to the reported pleiotropic effects in diverse pathophysiological contexts, spanning from enhancing stress adaptation to neurodegenerative disorders.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12035-025-04920-7.

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Author contributions SEF, LH, ABV and VAV designed research; SEF, KK and UG performed research; LH synthesized and contributed plant extracts; UK contributed analytic tools and input; SEF, ABV and VAV analysed data; SEF, ABV and VAV wrote the paper; all authors read and approved the final manuscript; VAV supervised the project.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests KK, UG, UK, ABV and VAV have no relevant financial or non-financial interests to disclose. SEF serves as a consultant to Beckley Psytech and has no other competing interests to declare relevant to the content of this article. LH synthesized and provided the extracts of Withania somnifera used in this study at Pharmanza Herbal Pvt. Ltd. Kasturba Health Society-MRC receives grant support from Pharmanza Herbal Pvt. Ltd.

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Supplementary Information

Withania somnifera regulates mitochondrial biogenesis and energetics in rat cortical neurons via BDNF and SIRT1

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Supplementary Figures

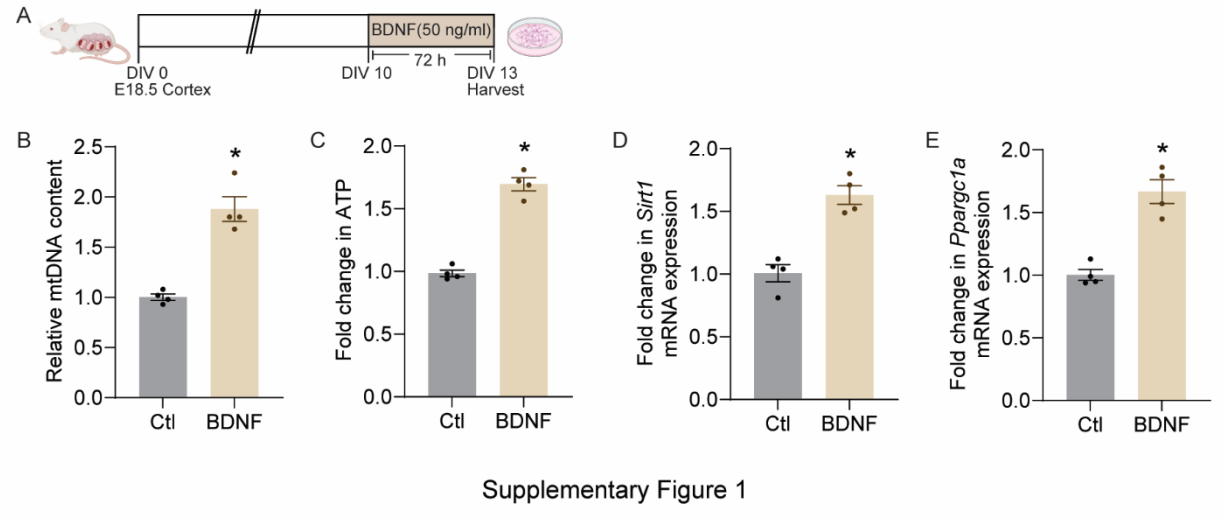

Figure S1: Supplementary Figure 1 *Brain derived neurotrophic factor (BDNF) regulates mitochondrial DNA content, ATP levels and expression of regulators of mitochondrial biogenesis and function in cortical neurons*

(A) Shown is a schematic depicting the treatment paradigm with Brain Derived Neurotrophic Factor (BDNF) (50 ng/ml) of rat cortical neuron cultures, commencing day *in vitro* (DIV) 10 for a duration of 72 h. (B) Graph represents qPCR analysis for mtDNA content from control and BDNF treated cortical neurons. Data are represented as relative mtDNA content ± SEM. (n = 4 per treatment group/N = 2, **p* < 0.05 as compared to control (Ctl), unpaired Student's *t*-test). (C) Graph represents cellular ATP levels from control and BDNF treated cortical neurons. Data are represented as fold change of control ± SEM. (n = 4 per treatment group/N = 2, **p* < 0.05 as compared to control (Ctl), unpaired Student's *t*-test). (D and E) Graphs depict mRNA expression levels of regulators of mitochondrial biogenesis and function *Sirt1*(D) and *Ppargc1a* (E) in control and BDNF treated cortical neurons. Data are represented as fold change of control ± SEM. (n = 4 per treatment group/N = 2, **p* < 0.05 as compared to control (Ctl), unpaired Student's *t*-test).

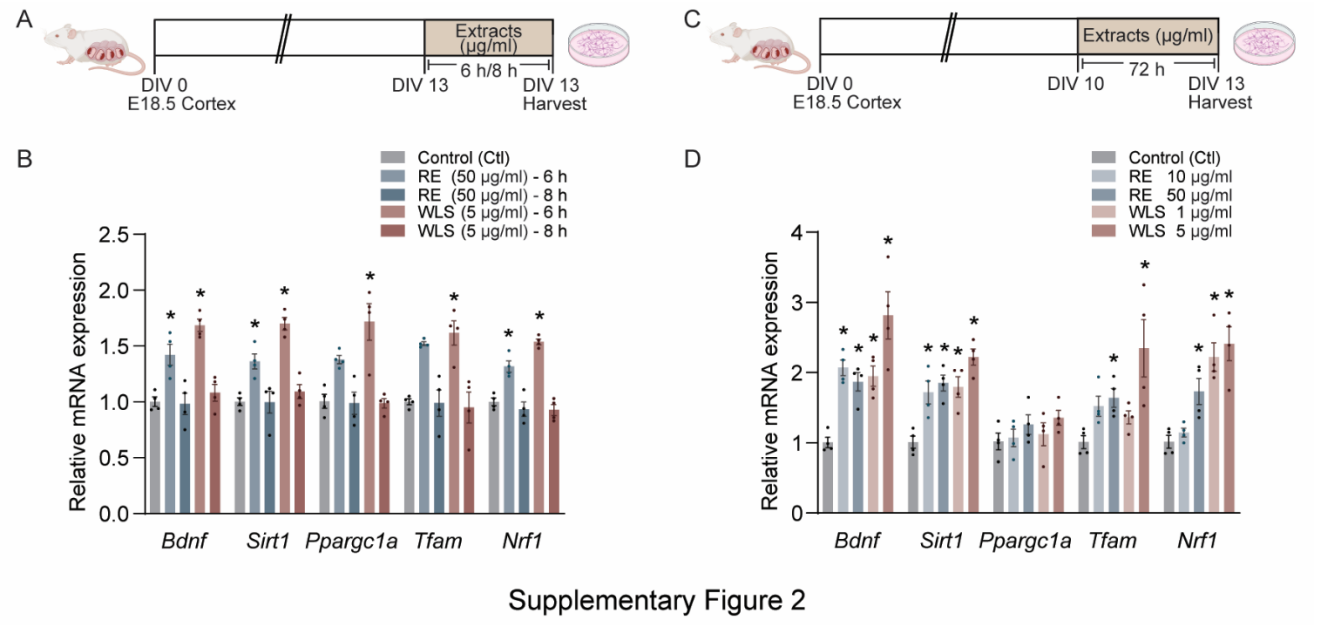

Figure S2: Supplementary Figure 2 *Withania somnifera RE and WLS fraction regulate expression of regulators of mitochondrial biogenesis and function in cortical neurons*

(A) Shown is a schematic depicting the treatment paradigm with *Withania somnifera* root extract (RE) (50 µg/ml) and withanolide - withanoside rich fraction (WLS) (5 µg/ml), of rat cortical neuron cultures for 6 h and 8 h and lysed synchronously at DIV 13. (B) Quantitative qPCR analysis of *Bdnf, Sirt1, Ppargc1a, Tfam* and *Nrf1* expression levels in control, RE and WLS treated cortical neurons at 6 h and 8 h. Data are represented as fold change of control ± SEM. (n = 4 per treatment group/N = 2, **p* < 0.05 as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test). (C) Shown is a schematic depicting the treatment paradigm with increasing doses of *Withania somnifera* root extract (RE) (10, 50 µg/ml) and withanolide - withanoside rich fraction (WLS) (1, 5 µg/ml), of rat cortical neuron cultures, commencing day *in vitro* (DIV) 10 for a duration of 72 h. (D) Quantitative qPCR analysis of *Bdnf, Sirt1, Ppargc1a, Tfam* and *Nrf1* expression levels in control, RE and WLS treated cortical neurons at 72 h. Data are represented as fold change of control ± SEM. (n = 4 per treatment group/N = 2, **p* < 0.05 as compared to control (Ctl), one-way ANOVA, Tukey's *post-hoc* test).

Supplementary Table 1: Extracts, Pure Compounds and Drugs

Extracts, Pure Compounds, Drugs			Dose	Source
1.	*Withania somnifera* hydroalcoholic (40:60) root extract (RE)	1.Withanoside-IV = 0.59% 2.Withanoside-V = 0.50 % 3.Withaferin-A = 0.19 % 4.12-deoxy-withastramonolide = 0.39% 5.Withanolide- A = 0.14 % 6.Withanolide B = 0.070 % 7.Withanone = 0.0099 % Total withanolides withanosides = 1.8 %	10, 25, 50 μg/ml	Pharmanza Herbal
2.	Withanolide-Withanoside rich fraction (WLS)	1. Withanoside-IV = 2.84 % 2. Withanoside-V = 4.62 % 3. Withaferin-A = 4.69 % 4. 12-deoxy-withastramonolide = 2.43% 5. Withanolide- A = 0.52 % 6. Withanolide B = 0.44 % 7. Withanone = Not determined Total withanolides withanosides =15.5 %	1, 2.5, 5 μg/ml	Pharmanza Herbal
3.	Withanolide A (WLA)		1 μM	Sigma-Aldrich
4.	Withanoside IV (WSIV)		1 μM	Sigma-Aldrich
5.	Withanolide B (WLB)		1 μM	Sigma-Aldrich
6.	Withanoside V (WSV)		1 μM	Sigma-Aldrich
7.	12-Deoxy-withastramonolide (DWS)		1 μM	Sigma-Aldrich
8.	Withaferin A (WA)		1 μM	Sigma-Aldrich
9.	ANA-12 (A)	Tyrosine protein kinase B (TrkB) receptor non-competitive antagonist preventing activation by BDNF	1 μM	Tocris Bioscience
10.	Cyclotraxin B (CY)	Tyrosine protein kinase B (TrkB) receptor antagonist, allosterically alters TrkB receptor conformation and inhibits BDNF-induced TrkB receptor activity	1 μM	Tocris Bioscience
11.	EX-527	Selective Sirtuin 1 (Sirt1) inhibitor	10 μM	Sigma-Aldrich
12.	Corticosterone (Cort)		1 μM	Sigma-Aldrich
13.	Brain derived neurotrophic factor (BDNF)		50 ng/ml	Tocris Bioscience

Supplementary Table 2: List of Primer sequences

Primer sequences used for quantitative PCR analysis of rat cDNA			
Gene name	**Description**	**Primer**	**Sequence (5'-3')**
Bdnf	Brain derived neurotrophic factor	Forward	TCTTGCTGTGGTCTCTTTTTGG
		Reverse	CCACAGACATTTACTTACAGTTTCAATG
Sirt1	sirtuin 1	Forward	AGAACCACCAAAGCGGAAA
		Reverse	ACAGCAAGGCGAGCATAAA
Ppargc1a	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Forward	TGAACTACGGGATGGCAACT
		Reverse	GAAGAGCAAGAAGGCGACAC
Nrf1	nuclear respiratory factor 1	Forward	CACACACAGCATAGCCCATC
		Reverse	TTTGTTCCACCTCTCCATCA
Tfam	transcription factor A, mitochondrial	Forward	GCTAAACACCCAGATGCAAA
		Reverse	GCTTCCTTCTCTAAGCCCATC
Primer sequences used for relative mitochondrial DNA (mtDNA) analysis in rat			
Gene name	**Description**	**Primer**	**Sequence (5'-3')**
mt-Cytb	cytochrome b, mitochondrial	Forward	ACGCTCCATTCCCAACAAAC
		Reverse	GTTGGCCTCCGATTCATGTT
Cycs	cytochrome c, somatic	Forward	CTGGACCAAACCTCCATGGTC
		Reverse	ACCTGATAAGTCTGCATTGGTT

List of abbreviations

1. 12-deoxy-withastramonolide - DWS
2. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide - MTT
3. 6-Chloro-2,3,4,9-tetrahydro-1H-Carbazole-1-carboxamide - EX-527
4. Brain-derived neurotrophic factor – BDNF
5. Carbonylcyanide-p-trifluoromethoxyphenylhydrazone – FCCP
6. Corticosterone - Cort
7. Cyclotraxin B - CY
8. Dimethyl sulfoxide – DMSO
9. Mitochondrial DNA - mtDNA
10. Mitochondrial α-F1 subunit of ATP synthase - ATP5A
11. N-[2-[[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide - ANA-12 or A
12. Nuclear respiratory factor 1 – NRF1
13. Oxidative phosphorylation – OxPhos
14. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha – PGC-1α
15. Phosphatidylinositol 3-kinase – PI3K
16. Protein kinase B (PKB) – Akt
17. Sirtuin 1 – SIRT1
18. Transcription factor A, mitochondrial – TFAM
19. Tyrosine receptor kinase B - TrkB
20. Voltage-dependent anion channel - VDAC
21. Withaferin A - WA
22. *Withania somnifera* root extract – RE
23. Withanolide - withanoside rich fraction - WLS
24. Withanolide A - WLA
25. Withanolide B - WLB
26. Withanoside IV - WSIV
27. Withanoside V - WSV