European Journal of Medicinal Plants

20(2): 1-19, 2017; Article no.EJMP.35355
ISSN: 2231-0894, NLM ID: 101583475

Ashwagandha and Its Active Ingredient, Withanolide A, Increase Activation of the Phosphatidylinositol 3' Kinase/Akt Cascade in Hippocampal Neurons

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Authors' contributions

This work was carried out in collaboration among all authors. Author DH performed half of the Western blotting experiments. Author MC dissected the embryonic hippocampal neurons, maintained their cultures and treated them with Ashwagandha and its compounds, performed the cell death assays, supervised the measurement of dendritic lengths, performed about half of the Western blottings and wrote the manuscript. Authors IV and LG measured the dendritic lengths of hippocampal neurons. Author HD determined the ideal conditions for the Western blotting experiments. Authors ALSA and SM performed the HPLC analyses of Ashwagandha, withanolide A and withaferin A. Author FZ supervised the HPLC analyses. Author ARN planned the overall study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2017/35355
Editor(s):
(1) Ghalem Bachir Raho, Biology department, Sidi Bel Abbes University, Algeria.
(2) Marcello Iriti, Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Rojita Mishra, Polosara Science College, India.
(2) M. Lukman, College of Pharmaceutical Sciences (STIFA) Nationality Makassar, Indonesia.
Complete Peer review History: http://www.sciencedomain.org/review-history/20658

Original Research Article

Received 9th July 2017
Accepted 22nd August 2017
Published 25th August 2017

ABSTRACT

Aims: To determine if whether, in a hippocampal neuron culture model subjected to nutrient deprivation stress (simulating degenerative disease state), Ashwagandha and/or two of its putative active ingredients, withanolide A or withaferin A, affect any of the following: neurite outgrowth, neuronal survival, activation of the pro-survival PI-3K/Akt and MAPK cascades, phosphorylation of CREB and expression of brain-derived neurotrophic factor (BDNF).

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Study Design: To primary rat embryonic hippocampal neurons in culture, half of which were subjected to nutrient deprivation stress, inhibitors of the PI-3K/Akt and MAPK cascade (LY294002 and PD98059, respectively) were applied, followed by Ashwagandha, withanolide A or withaferin A.
Methodology: Neuronal survival was determined by using fluorescently labeled markers for live *vs* dead cells and by lactate dehydrogenase assay. Average neurite length was measured under phase-contrast microscopy. And intracellular signal transduction activity was determined by Western blotting.
Results: Ashwagandha increased average neurite length. Ashwagandha, withanolide A and withaferin A all increased neuron survival in nutrient deprived conditions. Ashwagandha and withanolide A increased phosphorylation of Akt, but not MAPK, in both nutrient-adequate and nutrient-deprived conditions. Withaferin A increased BDNF expression under nutrient-deprived conditions, but decreased BDNF expression under adequate nutrient conditions; withaferin A still activated Akt under both types of nutrient conditions.
Conclusion: Using our model of nutrient deprivation stress, we showed that withaferin A helps cells adapt to stressful conditions, such as by increasing expression of BDNF, while withanolide A, continues to maintain cell survival and neural protection by increasing baseline levels of PI-3K/Akt. Our results are in agreement with extant literature on the effects of Ashwagandha or withaferin A on disease, such as cancer.

Keywords: Ashwagandha; ayurveda; depression; stress; signal transduction; hippocampal neurons.

1. INTRODUCTION

It is well known that neurons in the hippocampus are among the most easily influenced by genetic [1,2] and epigenetic [3,4] factors, which can have a profound impact on their survival. The hippocampus is one of the few places in the CNS capable of growing new neurons well into adulthood [5]. Such neurogenesis, confined to the subventricular and subgranular zones of the dentate gyrus (for review, [6]) must keep up with inevitable neuron death brought about by toxic insult [7-9], metabolism [10], disease [10-12] or simply aging [13]. Indeed, sheer numbers of survival of new neurons, as well as that of existing older ones, must outpace neuron death for overall brain health. However, inherent in this enhanced survival is the ability of neurons to also thrive, which critically depends on their ability to increase synaptic connections by promotion of neurite arborization [14]. To accomplish this, while sustaining neurogenesis, such neurons must receive adequate stimulation in the form of sympathetic nerve stimulation [15] through enhanced mental activation (learning) and/or physical activity [5,16-18], all of which, will then activate any number of a wide array of intracellular signaling cell survival cascades, such as anti-apoptotic Bcl2 [19,20], Akt/PI-3K [20] and MAPK [21,22]. These cascades then activate the transcription factor, cyclic AMP response element binding (CREB) protein [23], which, in turn, participates in the expression of neuronal and glial [24] brain-derived neurotrophic factor (BDNF) [25]. When BDNF binds its receptor, TrkB [26] the Akt/PI-3K and MAPK cell survival cascades and possibly others are activated in a positive-feedback type manner [27].

In clinical depression, it is well known that sub-optimal levels of circulating norepinephrine and serotonin are the basis behind the mechanism of many of the second- and third-generation antidepressant drugs [28]. Such drugs and/or physical exercise has been shown to enhance phospho-CREB [29], BDNF [30] and phospho-TrkB [30,31] levels through activation of the Akt/PI-3K survival cascade [32,33], thereby at least minimizing, if not completely reversing [34], hippocampal neuron atrophy or death [35].

To the extent that such neurite atrophy and withdrawal and neuron death are caused by chronic stress-induced depression [36,37], there is growing evidence that over-eating (nutrient overload), perhaps in response to such stress, is strongly linked to decline in cognitive functioning and lower BDNF levels [38-41]. This may help partially explain the co-morbidity of dementia, type II diabetes and cardiovascular disease [40].

Conversely, metabolic stress does not necessarily stem only from nutrient(s) excess, but also from its relative absence. We therefore developed an *in vitro* tissue culture model in which neurons are deprived of some critical nutrient in their growth media [42,43], thereby subjecting them to an alternate form of metabolic stress. We were able to show that various types of antidepressants protected hippocampal neurons from nutrient-deprivation stress and death [43].

Ashwagandha, derived from the plant, *Withania Somniferia* (WS), is a member of Indian Ayurvedic medicine and has been used for over 3,000 years [44] to treat a wide variety of medical problems [44-46], in both the periphery [47] and in the CNS [44,47-49], including Alzheimer's Disease [50,51], excitotoxicity [52,53], stress [37,49], mood disorders [44] and even aging [54].

To more clearly determine how WS may ameliorate the destructive effects of excitotoxicity, Bhatnager et al. [55] applied it to primary hippocampal neuron cultures treated with glutamate and found significant decreases in Ca^{2+} currents, cell death and reactive O_2 species, as well as significant elevations of both nerve growth factor and BDNF in response to WS; but the observed increases in BDNF occurred only when it was combined with the root extract of another plant, *Asparagus racemosus*.

Despite the foregoing, and because of its heterogeneous nature, how WS exerts its effects still unclear. Among its 40 or so active ingredients [56], only a few have received some attention, such as withanolide A, which has been shown to be a promotor of neurite extension [57-59], synaptogenesis [49,57] and memory improvement [58,60] and decreases β-amyloid production [61]. Another active compound, withaferin A, decreases inflammation associated with amyotrophic lateral sclerosis [62], but does not affect neurite outgrowth, at least in human neuroblastoma cell line [59]. It is not known, however, how these compounds, whether WS itself, or its individual components, affect neural plasticity.

In the current study, therefore, we hypothesize that application of WS or two of its active compounds (withanolide A and withaferin A) to our hippocampal cell culture model of nutrient deprivation stress would result in activation of the pro-survival Akt/PI-3K and MAPK cascades, CREB phosphorylation, BDNF production, and neuronal survival. This study is the first to evaluate the effects of Ashwagandha and two of its active ingredients on these two intracellular signal transduction cascades.

2. MATERIALS AND METHODS

2.1 Plant Material Used

Withania somnifera (L.) Dunal is a member of the family *Solanaceae*, and goes by several names, such as Winter Cherry (English), *Withania somnifera* (Latin) and Ashwagandha (Sanskrit). For this study, we used a tincture (purchased from Sprouts; see Section 2.1.1 below) in which Ashwagandha root had been ground to a fine powder and dissolved in 50% methanol/50% distilled water at a concentration of 500 mg/ml by the vendor/manufacturer. The plant root material itself was collected in March, 2011, in Pheonix, AZ by the vendor/manufacturer.

2.1.1 HPLC analysis of withanolide A in Ashwagandha

Analysis of Ashwagandha and withanolide A were conducted on the Shimadzu® LC-6AD Liquid Chromatograph and SPD-M20A Prominence Diode Array Detector (DAD) (Kyoto, Japan) with manual injection, using a Phenomenex Luna 5u C5 (150 x 4.60 mm 5 micron) column (Torrence, CA). Gradient elution consisting acetonitrile (Fisher Scientific® Optima®, Fair Lawn, NJ) and nanopure water was used with a flow rate of 1.0 ml/min. Mobile phase water was purified by Millipore Simplicity 185 water purification system (18.2 MΩ.cm). Prior to runs, the water was also vacuumed, removing any air bubbles. The gradient elution was achieved by 0 min – 15% acetonitrile, 5 min – 25% acetonitrile, 10 min – 45% acetonitrile, 12 min – 65% acetonitrile, 14 min – 70% acetonitrile, 17 min – 100% acetonitrile, 20 min – 100% acetonitrile, 21 min – 100% acetonitrile, 35 min – 25% acetonitrile, 40 min – 25% acetonitrile. Quality control data are the following: Measurements of Ashwagandha, a dried Ashwagandha root extract from Sprouts® (Pheonix, AZ) and withanolide A, from Sigma® (W2145-5MG, product of India) were detected with DAD at 254 nm. About 1 ml of Ashwagandha sample was filtered with BD 1-ml syringe and through Fisherbrand® 13-mm syringe filters. Five µl of filtered Ashwagandha sample was injected to obtain retention times. The filtered Ashwagandha was spiked with 40% withanolide A. Five µl of spiked Ashwagandha was injected; retention times were compared to those of Ashwagandha.

2.1.2 HPLC analysis of withaferin A in Ashwagandha

Wtihaferin A was tested under the same conditions as withanolide A.

2.2 Animal Care and Handling

All animal handling were conducted in strict accordance with the National Research Council's Guide for the Care and Use of Laboratory

Animals (1996). Maximal effort was placed on minimizing the suffering of any rats used in this study, as well as the number of rats used. All experiments were approved by the California State University Institutional Review Board and the Animal Care Committee (Protocol # 13-3).

2.3 Antibodies, Chemicals and Assays

N2 was purchased from Invitrogen (Grand Island, NY, USA) and is composed of insulin, progesterone, putrescine, selenium dioxide, human transferrin and minimal essential medium with Earle's salts as previously described [43]. Anti-BDNF was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA); anti-phospho-MAP kinase 1/2 (ERK 1/2), anti-MAPK 1/2, anti-α-tubulin, anti-phospho-Ser473-Akt, anti-Akt, and anti-phospho-Ser133-CREB, and anti-CREB were purchased from Millipore (Temecula, CA, USA). Anti-mouse IgG and anti-rabbit IgG were purchased from Amersham Pharmacia-Biotech (Piscataway, NJ, USA). Norepinephrine, withanolide A and withaferin A were purchased from Sigma (St. Louis, MO, USA, see Section 2.1.1) and LY294002 and PD98059 was purchased from Promega (Madison, WI, USA). Finally, Ashwagandha tincture (WS) was purchased from Sprouts grocery store (Whittier, CA, USA) (see Section 2.1.1). This tincture comes in 80 % methanol, which was evaporated off in a savant centrifuge till only a sticky brown/yellow pellet remained. This pellet was then reconstituted in DMEM to achieve the desired concentrations of 5, 10, 20, or 40 µg/ml.

2.4 Hippocampal Dissection at Embryonic Day 18 (E18) and Tissue Culture

Primary hippocampal neurons were obtained from rat embryos at 18 days in gestation exactly as described previously [43]. Briefly, female rats (Sprague-Dawley, 2 months of age, 250 g, Charles River, Wilmington, MA) were sacrificed by an overdose of isoflruorane and then decapitated on their 18th day of pregnancy. Their E18 embryos were removed by cesarean section and immediately placed on ice. Both hippocampi from each embryo were excised and cultured in strict accordance with the method of Banker and Cowan [63]. Neurons were dissected in Ca/Mg-free medium, to which, 0.125% trypsin was added and allowed to incubate for 10 min in a 37°C water bath. The reaction was then quenched in 10% fetal bovine serum/DMEM and then centrifuged for 5 min at 200 g. The supernatant was then carefully discarded, whereas the pellet was resuspended in 2 ml serum-free DMEM. Neurons were then triturated through a constricted glass pipette and then filtered through a 40-μm BD Falcon cell strainer (BD Biosciences Discovery Labware, San Jose, CA) into a fresh conical 50-ml tube. Ten microliters of trypan blue were then added to 10 μl of cells, which were then counted on a hemacytometer. Neurons were then plated in 12-well poly-/-lysine-coated plates at a density of 50,000 cells/cm^2 and 1 ml of serum-free Dulbecco's Modified Eagle's Medium (DMEM)/1% N2 supplement per well. Hippocampal neurons were treated in duplicate (see below).

2.5 Procedures

Three days following plating, in half the culture wells, the media was replaced with fresh DMEM/1% N2, while the other half of the culture wells received fresh DMEM only (-N2, nutrient deprivation). The cultures were allowed to adapt for six hr, followed LY294002 (20 µM) or PD98059 (10 µM) and/or WS, withanolide A or withaferin A and then lysed and harvested 24 hr later for subsequent SDS-PAGE and Western blotting. For neurons receiving norepinephrine, this compound was added two hr before harvesting [27]. To determine the optimal dose of WS to use for the cell signal transduction studies, a dose-response experiment was first performed for its effects of neuronal survival. WS concentrations used were 5, 10, 20 and 40 µg/ml. Withanolide A concentration was 20 µM [61]; and withaferin A concentration was 20 µM. Although Swarup et al. [62] used 1 µM withaferin A in microglial cultures, because glial outnumber neurons 10:1, 20 µM withaferin A was a comparable concentration.

2.5.1 Harvesting cultured E18 hippocampal neurons

Cultured hippocampal neurons were harvested exactly as described previously [43]. Briefly, neurons in each well, were scraped into microcentrifuge tubes containing 100 μl hot lysis buffer (10 mM Tris-base, 1% SDS, pH 7.4), boiled for 5 min, triturated through a 26-G syringe to sheer genomic DNA, and then an equal volume of Laemmli [64] gel-loading buffer added. Lysed neurons were then stored at -68°C till electrophoresis and Western blotting.

2.5.2 SDS-PAGE/western blotting

Electrophoreses and subsequent Western/immunoblotting were performed exactly as described previously [43]. Protein concentrations were determined using the method developed by Lowry [65]. Electrophoreses and subsequent Western/ immunoblotting were performed exactly as described previously [43]. Briefly, equal amounts of protein of each sample were applied to a single well of a 10% polyacrylamide gel and then electrophoresed. Proteins were then electro-transferred for two hr at 80 V, to nitrocellulose membranes (Amersham, Pharmacia-Biotech, Piscataway, NJ, USA) and Western blotting performed according to the instructions of the respective manufacturer of a particular phospho-antibody or anti-BDNF. Nitrocellulose blots were treated with the reagents for enhanced chemiluminescence (ECL, Amersham, Pharmacia-Biotech, Piscataway, NJ, USA) and apposed to hyperfilm (Amersham, Pharmacia-Biotech, Piscataway, NJ, USA). Each blot was then stripped in stripping buffer (100 mM 2-mercaptoenthanol, 2% SDS, 62.5 mM Tris-HCl, pH, 6.7, 55°C, 10 min, while agitating), and then re-probed with the total form of the respective antibody to control for inadvertent differences in protein loading.

2.5.3 Cell death assays

Cell death/cytotoxicity assay (Molecular Probes, Eugene, OR, USA) was performed exactly as described previously [43]. Briefly, three pre-determined fields in each tissue culture well were counted for the number of live and dead neurons determined by fluorescent fluorosceine isothocyanate and Texas red staining, respectively. Replicates comprised one-two repetitions. The lactate dehydrogenase (LDH) assay (Abcam, Cambridge, UK) was also used to evaluate cell viability.

2.5.4 Determining neuritic length

To functionally corroborate the HPLC results, (see HPLC analyses above), Ashwagandha's ability to elicit neurite growth, relative to that of vehicle-treated controls, was evaluated. The ability of Ashwagandha to grow neuritis has already been well documented by other investigators (see Introduction). Before harvesting neurons for Western blotting proteins, neurons treated with WS and vehicle were examined under phase contrast microscopy. Measurements of the lengths of neuritic processes were made by three counters blind to their treatment. In this way, the very same neurons used for determining neuritic length were also used for Western blotting evaluations.

2.6 Statistical Analysis

Quantification of immunoreactive bands was performed exactly as previously described [43]. Briefly, computer-assisted densitometry (MCID, Image processing system, St. Catherine's, Ontario, Canada) was used to quantify lightly exposed Western blotting bands within the linear range of a standard curve. The optical density of each band was divided by that of its respective loading control (total form for each phospho-form or anti-α-tubulin for BDNF) to yield the corrected band intensity. Each sample had been loaded onto the gel in triplicate and each Western blotting evaluation for each sample performed two-three times.

For the cell survival experiments, statistical analysis entailed a two-way ANOVA (drug x nutrient N2). For the neuritic length experiment, Student's t-test was used. For the Western blotting experiments, statistical analyses entailed a three-way analysis of variance (ANOVA) to examine the main effects nutrient supplement (N2) (enriched *vs.* deprived media) x WS compound (WS, withanolide A or withaferin A, from here on, referred to as "treatment") x inhibitor (LY292002 or PD98059) on cell survival, BDNF expression or cell survival signaling (phospho-forms). Results were considered statistically significant at $P < 0.05$. A significant F (ANOVA) was followed by a Fisher's post-hoc test of least significant differences (PLSD) to evaluate statistically significant differences among treatment groups.

3. RESULTS

3.1 Ashwagandha and withanolide A Co-elute

Quality control confirm the following: Retention time of filtered Ashwagandha was 15.74 minutes (Fig. 1A). The retention time of pure withanolide A was 15.86 minutes (Fig. 1B). The UV spectra of the peaks for both chromatograms were consistently similar, containing a detected peak at 227 nm. To finalize the confirmation, the filtered Ashwagandha was spiked with 40% withanolide A, eluding withanolide A at 16.00 minutes (Fig. 1C). The peak area of withanolide A in the spiked chromatogram increased and the UV spectra contained a peak at 226 nm. No

peaks of withaferin A were detected in the pure Ashwagandha tincture (Fig. 1D); in this case, Ashwagandha displayed a retention time of 15.86 minutes (Fig. 1A, 1B, 1C), whereas, withaferin A displayed a retention at 14.00 minutes.

3.2 Neuronal Survival and Process Length

WS promotes neuronal survival significantly above that of neurons treated with vehicle in the nutrient (N2+) supplemented condition by approximately 15-20 % (Fig. 2A). WS had the same effect in nutrient-deprived neurons (N2-), but increased neuronal survival by about 50 %. Norepinephrine, which was used only as a positive control, based on earlier studies in this tissue culture model [27], had no additional neuronal survival-promoting effect than WS alone in both the N2+ and N2- conditions (Fig. 2A). Therefore, norepinephrine as a positive control was not used any further in the Western blotting portions of this study.

Fig. 1A

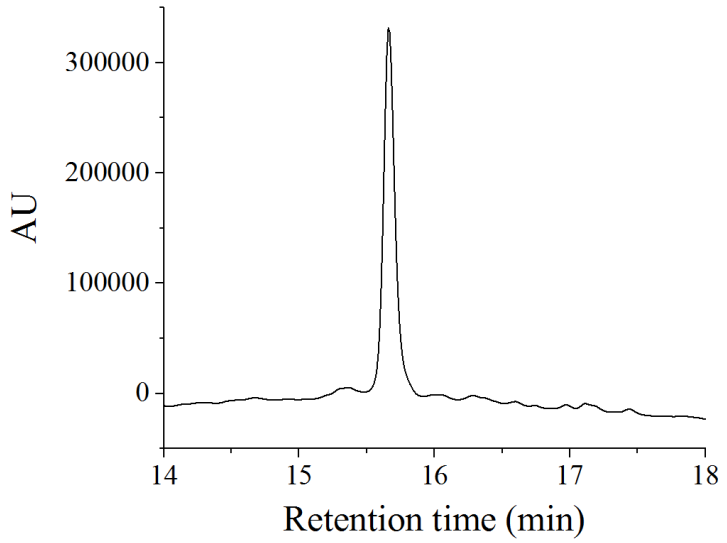

Fig. 1B

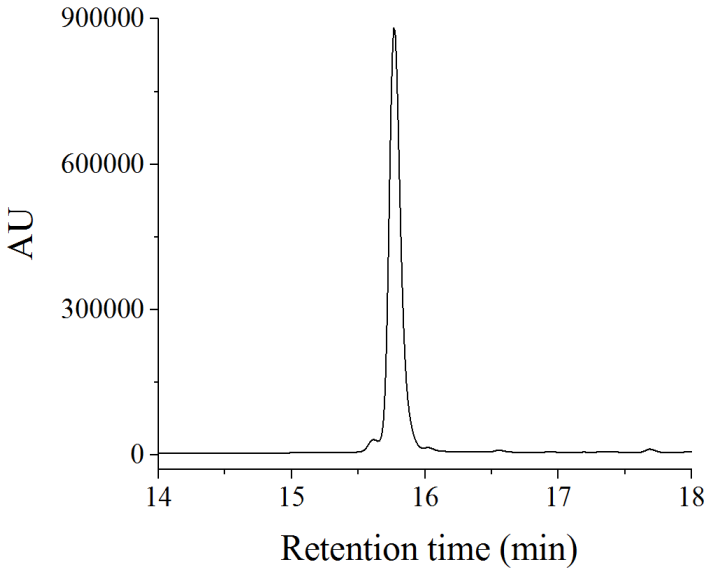

Fig. 1C

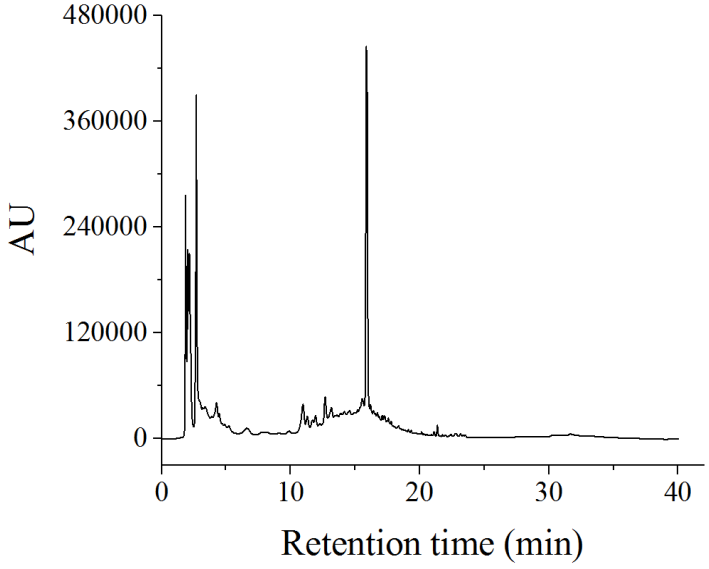

Fig. 1D

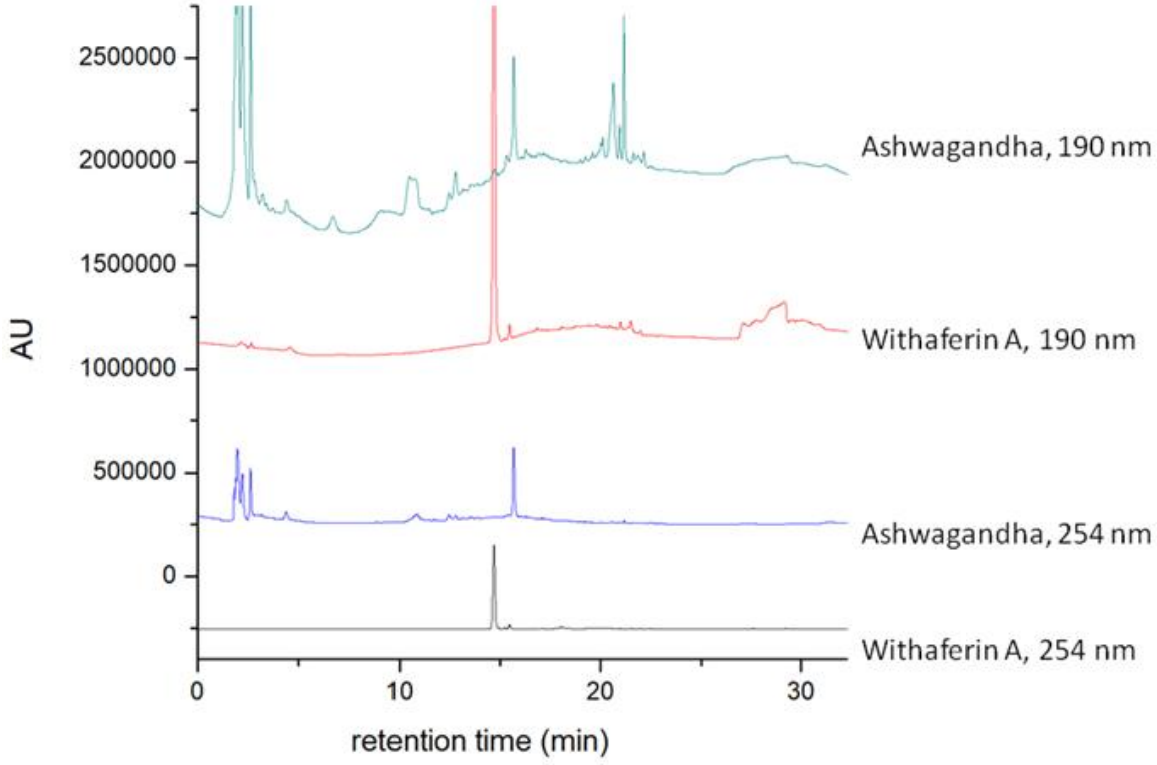

Fig. 1. HPLC chromatograms showing the purities of (A) Ashwagandha, (B) withanolide A, (C) confirmation that Ashwagandha co-elutes with withanolide A, and (D) withaferin A. To confirm that withaferin A was indeed not present in whole filtered Ashwagandha, detection was done at 190 nm and 254 nm

WS, withanolide A and withaferin A all promoted neuronal survival in nutrient-supplemented conditions (N2+), but in nutrient-deprived conditions (N2-), withanolde A and withaferin A,

increased neuronal survival (Fig. 2B). WS did not increase neuronal survival in nutrient-deprived conditions (N2-).

WS significantly increased dendritic length of hippocampal neurons above that of vehicle-treated neurons by approximately 75% (Fig. 2C).

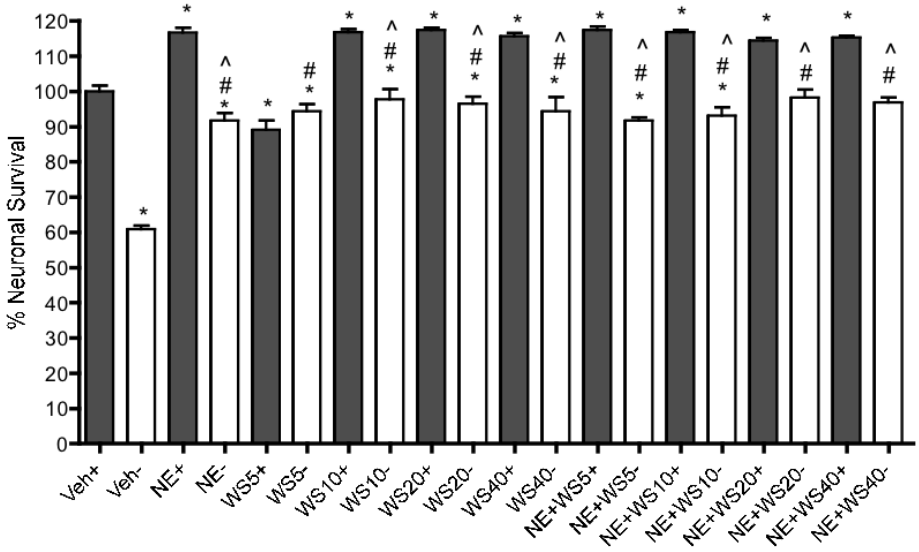

Fig. 2A. WS increases neuronal survival in a dose-dependent manner. (A) At 5 µg/ml, WS significantly decreased, while 10, 20 and 40 µg/ml WS increased, neuronal survival to comparable levels at approximately 15 % above that of Veh+-treated cells (*P* < .01). Likewise, under nutrient-deprived conditions (N2-), all four doses of WS significantly increased neuronal survival above that of vehicle-treated cells by approximately 30 %. Determined using the cell death/cytotoxicity assay

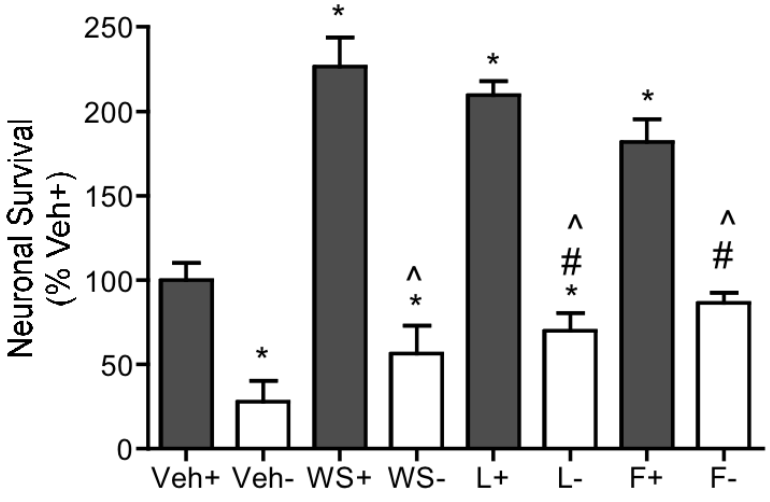

Fig. 2B. WS, withanolide A (L) and withaferin A (F) all significantly increased cell survival over that of vehicle controls (Veh+) under full nutrient conditions (*P* < .0001). However, for the nutrient-deprived conditions (N2-), withanolide A and withaferin A, but not WS (p = 0.065), significantly increased neuronal survival above that of Veh- (*P* = .05). Determined using the lactate dehydrogenase assay

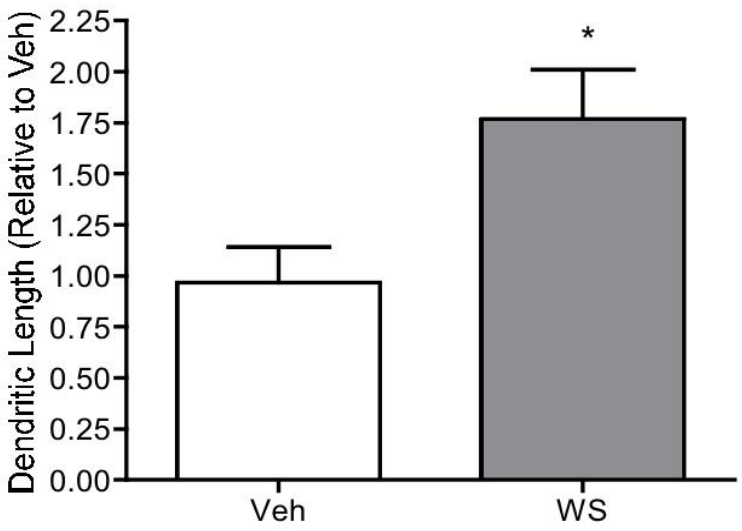

Fig. 2C. Neurons treated with WS (40 μg/ml) had on average 75% longer dendritic processes than those cells treated with only vehicle (*P* = .05). Original units were in microns. All treatments are expressed as a percentage relative to neurons treated with vehicle (DMEM) in the presence of N2 supplement (Veh+), which was numerically normalized to 100 for ease of comparison. Veh, vehicle; WS, *Withania somnifera* (Ashwagandha tincture, 5, 10, 20, 40 μg/ml); NE, norepinephrine (100 nM); *, significantly different from that of Veh+ at *P* < .001; #, significantly different from that of Veh- at *P* = .05; ^, significantly different from that of the corresponding N2+ supplement at *P* = .05

3.3 PI-3K/Akt Pathway

Under normal conditions (with N2 supplement), WS, withanolide A and withaferin A all increased phospho-Akt immunoreactivity when compared to the effects of vehicle (Veh+, Fig. 3A, $F_{(3, 28)}$ = 33.66, *P* < .0001). Likewise, under deprived conditions (N2-), all three compounds also increased phosphorylation of Akt when compared to that of vehicle (Veh-, Fig. 3A, $F_{(1, 28)}$ = 129.52, *P* < .0001). Moreover, all treatments, except for WS, resulted in higher P-Akt immunoreactivities in the N2+ than in the N2- conditions. Finally, the PI-3K inhibitor, LY294002, significantly decreased immuno-reactivity between identical treatments (*e.g.*, WS+ *vs*. WSLY+, Fig. 3A, $F_{(2, 28)}$ = 4.27, *P* = .024).

Under normal conditions (with N2 supplement), WS and withanolide A increased, but withaferin A decreased, BDNF levels when compared to the effects of vehicle (Veh+, Fig. 3B, $F_{(3, 28)}$ = 55.39, *P* < .0001). Likewise, under deprived conditions (N2-), WS, withanolide A and withaferin A all increased BDNF immunoreactivity when compared to that of vehicle (Veh-, Fig. 3B, $F_{(3, 28)}$ = 12.50, *P* < .0001). Moreover, each treatment, except for withaferin A, resulted in higher BDNF levels in the N2+ condition than in the corresponding N2- condition. Finally, LY294002 significantly decreased BDNF immunoreactivity between identical treatments (*e.g.*, WS+ *vs*. WS/LY+), but not when incubated with withaferin A in the presence of N2 (F+) or WS in its absence (WS-) (Fig. 3B, $F_{(2, 28)}$ = 11.023, *P* < .0001).

Under normal conditions (with N2), WS and withanolide A, but not withaferin A, significantly increased P-CREB immunorectivity above that of vehicle (Veh+, Fig. 3C, $F_{(3, 28)}$ = 10.18, *P* < .0001). Likewise, under deprived conditions (N2-), all three Ashwagandha compounds increased phosphorylation of CREB compared to that of vehicle (Veh-, Fig. 3C, $F_{(3, 28)}$ = 8.58, *P* < .0001). Moreover, each treatment resulted in significantly higher P-CREB levels in the N2+ condition than in the corresponding N2- conditions. Finally, LY294002 significantly decreased CREB phosphorylation in the WS-, L+ and L- conditions (Fig. 3C, $F_{(2, 28)}$ = 9.41, *P* = .001).

3.4 MAPK Pathway

Under full nutrient conditions, there were no significant increases in MAPK activation as a

result of any of our treatments. There was, however, a statistically significant decrease in P-MAPK immunorectivity levels following treatment with withaferin A (F+ *vs*. Veh+, Fig. 4A, $F_{(3, 28)} = 15.99$, $P < .0001$). However, under deprived conditions (N2-), only withaferin A resulted in significantly lower levels of MAPK phosphorylation, compared to that of vehicle (Veh-). Moreover, each treatment resulted in significantly higher P-MAPK levels in the N2+ condition than in the corresponding N2- condition. Finally, although the P-MAPK inhibitor, PD98059, significantly decreased P-MAPK levels, regardless of the presence of N2, the inhibitor significantly suppressed it activation under every similar treatment condition (*e.g.*, WS+ *vs*. WS/PD+, L+ *vs*. L/PD+, Fig. 3A, $F_{(2, 28)} = 8.91$, $P < .001$).

WS and withanolide A increased, while withaferin A decreased, BDNF immunoreactivity (compared to Veh+), but all three increased BDNF levels in the absence of N2 (compared to Veh-) (Fig. 4B, $F_{(3, 28)} = 4.2$, $P = .014$). Moreover, each treatment, except for withaferin A, resulted in higher BDNF levels in the N2+ condition than in the corresponding N2- condition. Finally, PD98059 significantly decreased BDNF immunoreactivity between identical treatments (*e.g.*, WS+ *vs*. WS/LY+), but not when incubated with withaferin A in the presence of N2 (F+ *vs*. F/PD+) (Fig. 4B).

Under normal N2-supplemented conditions, WS and withanolide A increased CREB phosphorylation (compared to that of Veh+, $F_{(3, 28)} = 8.47$, $P < .0001$). Likewise, under deprived conditions (N2-), all three Ashwagandha compounds barely increased phosphorylation of CREB compared to that of vehicle (Veh-, Fig. 4C, $F_{(3, 28)} = 2.87$, $P = .054$). Moreover, each treatment resulted in significantly higher P-CREB levels in the N2+ condition than in the corresponding N2- conditions. Finally, PD98059 significantly decreased CREB phosphorylation in the L- and F- conditions (Fig. 4C, $F_{(2, 28)} = 6.57$, $P = .005$).

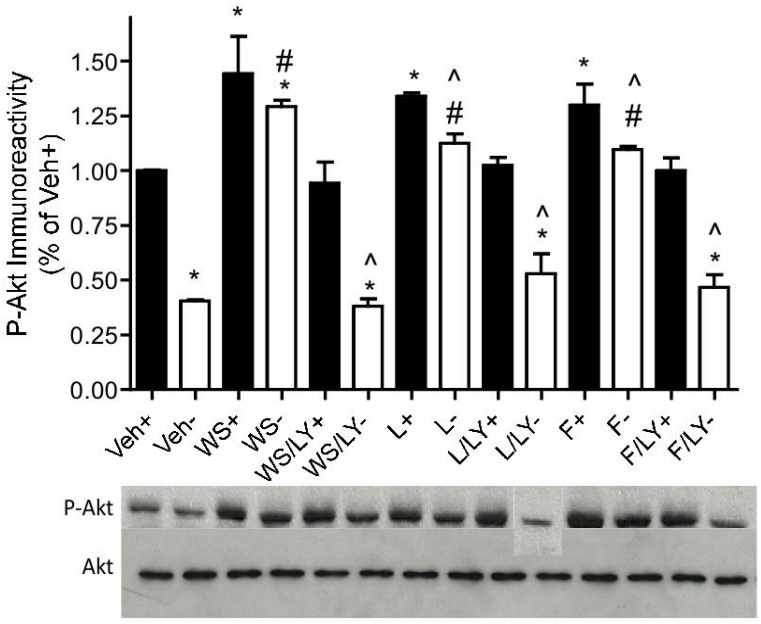

Fig. 3A. WS, withanolide A and withaferin A are PI-3K/Akt pathway-dependent. (A) WS, withanolide A and withaferin A increase phosphorylation of Akt in both nutrient-supplemented (compared to that of Veh+) and nutrient-deprived (compared to that of Veh-) conditions. P-Akt immunoreactivity was significantly lower in the presence of the PI-3K inhibitor, LY294002 (LY) for both the N2+ and N2- conditions; *i.e.*, WS+ > WS/LY+, WS- > WS/LY-, L+ > L/LY+, L- > L/LY-, F+ > F/LY+, F- > F/LY-, all at $P < .05$. P-Akt immunoreactivity (optical density of P-Akt/Akt) is evaluated following treatment of hippocampal neurons either in the presence (+) or absence (-) of N2 supplement

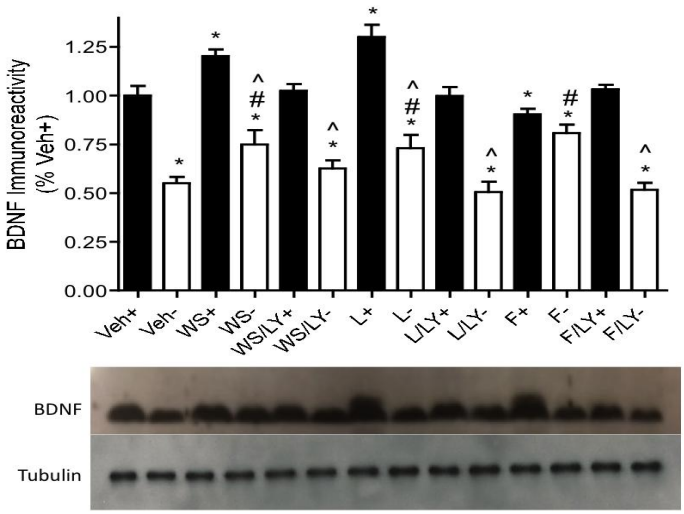

Fig. 3B. BDNF immunoreactivity was significantly increased as a result of WS or withanolide A (L), but decreased as a result of withaferin A (F), treatments (compared to Veh+), but all three increased BDNF levels in the absence of N2. BDNF immunoreactivity was significantly lower in the presence of LY294002 for both the N2+ and N2- conditions; *i.e.*, WS+ > WS/LY+, WS- > WS/LY-, L+ > L/LY+, L- > L/LY-, F- > F/LY-, all at *P* < .05, but not for the F+ *vs*. F/LY+ conditions. BDNF immunoreactivity (optical density of BDNF/tubulin) is evaluated following treatment of hippocampal neurons either in the presence (+) or absence (-) of N2 supplement

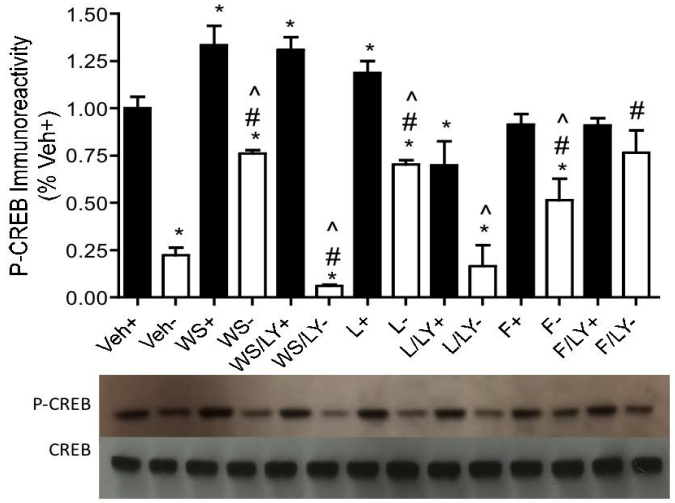

Fig. 3C. Phosphorylation of CREB was significantly increased as a result of WS or withanolide A (L), but not withaferin A (F) treatments (compared to that of Veh+), but all three increased P-CREB immunoreactivity without N2. P-CREB immunoreactivity was significantly lower in the presence of LY294002 for the WS-, L+ and L- conditions (*P* < .001). P-CREB immunoreactivity (optical density of P-CREB/CREB) is evaluated following treatment of hippocampal neurons either in the presence (+) or absence (-) of N2 supplement. All treatments are expressed as a percentage relative to neurons treated with vehicle (DMEM) in the presence of N2 supplement (Veh+), which was numerically normalized to 1.00. Veh, vehicle; WS, *Withania somnifera* (Ashwagandha tincture, 40 µg/ml, (Fig. 2A); LY, LY294002 (20 µM); L, withanolide A (20 µM, [61]); F, withaferin A (20 µM, [62]). *, significantly different from that of Veh+ at p < 0.05; #, significantly different from that of Veh- at *P* = .05; ^, significantly different from that of the corresponding N2+ supplement at *P* = .05

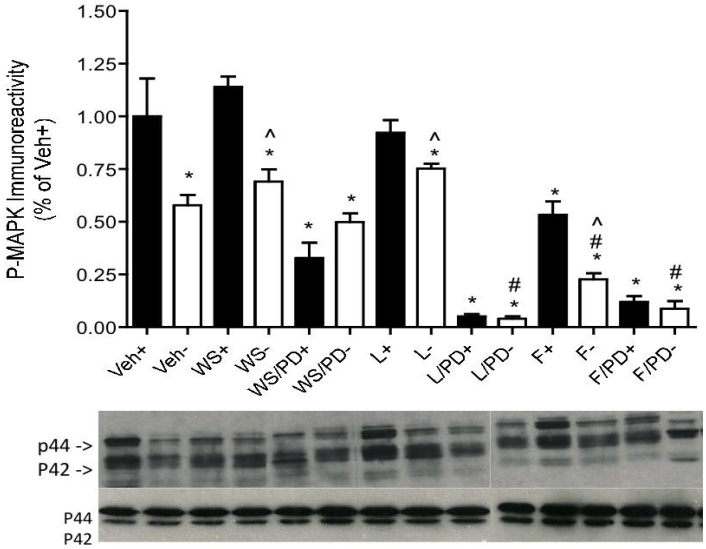

Fig. 4A. WS, withanolide A and withaferin A are not MAPK pathway-dependent. (A) Withaferin A decreased phosphorylation of MAPK in both nutrient-supplemented (compared to Veh+) and in nutrient-deprived (compared to Veh-) conditions. P-MAPK immunoreactivity was significantly lower in the presence of the P-MAPK inhibitor, PD98059 (PD) for both the N2+ and N2- conditions; *i.e.*, WS+ > WS/PD+, WS- > WS/PD-, L+ > L/PD+, L- > L/PD-, F+ > F/PD+, F- > F/PD-, all at *P* < .05. P-MAPK immunoreactivity (optical density of the average of p44 and p42 P-MAPK/average of p44 and p42 MAPK) is evaluated following treatment of hippocampal neurons either in the presence (+) or absence (-) of N2 supplement

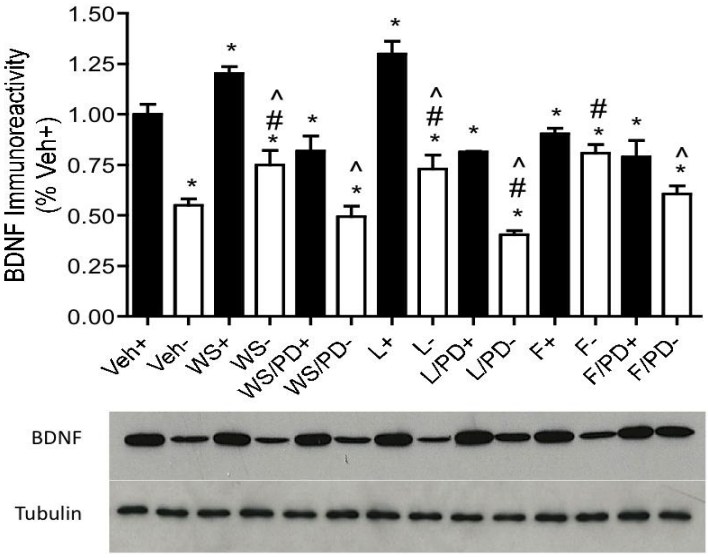

Fig. 4B. Withaferin A decreased, whereas WS and withanolide A increased, BDNF immunoreactivity (compared to Veh+), but all three increased BDNF levels in the absence of N2 (compared to Veh-). BDNF immunoreactivity was significantly lower in the presence of PD98059 for both the N2+ and N2- conditions; *i.e.*, WS+ > WS/PD+, WS- > WS/PD-, L+ > L/PD+, L- > L/PD-, F- > F/LD-, all at *P* < .05, but not for the F+ *vs*. F/PD+ conditions. BDNF immunoreactivity (optical density of BDNF/tubulin) is evaluated following treatment of hippocampal neurons either in the presence (+) or absence (-) of N2 supplement

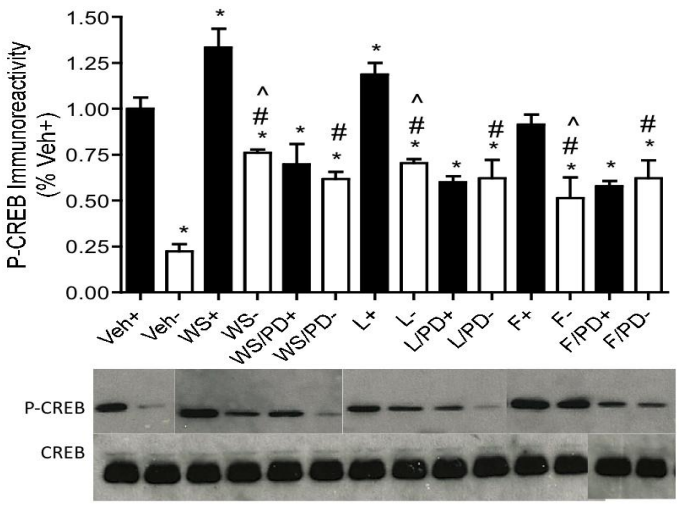

Fig. 4C. Phosphorylation of CREB was significantly increased as a result of WS or withanolide A (L), but not withaferin A (F) treatments (compared to that of Veh+), but all three increased P-CREB immunoreactivity without N2. P-CREB immunoreactivity was significantly lower in the presence of PD98059 for the L- (L- *vs*. L/PD-) and F- (F- *vs*. F/PD-) conditions (*P* < .05). P-CREB immunoreactivity (optical density of P-CREB/CREB) is evaluated following treatment of hippocampal neurons either in the presence (+) or absence (-) of N2 supplement. All treatments are expressed as a percentage relative to neurons treated with vehicle (DMEM) in the presence of N2 supplement (Veh+), which was numerically normalized to 1.00. Veh, vehicle; WS, *Withania somnifera* (Ashwagandha tincture, 40 µg/ml, Fig. 2A); PD, PD98059 (10 µM); L, withanolide A (20 µM, [61]); F, withaferin A (20 µM, [62]). *, significantly different from that of Veh+ at *P* < .05; #, significantly different from that of Veh- at *P* < .05; ^, significantly different from that of the corresponding N2+ supplement at *P* < .05

4. DISCUSSION

Although several other studies have investigated the molecular survival-promoting events underlying the chemical constituents of WS, such as increased anti-oxidant levels [66], decreased corticosterone levels and subsequent increased acetylcholine biosynthesis [67] and decreased NF-κB-mediated inflammation [68] and decreased cell death and increased BDNF [55], the current study presents evidence that Ashwagandha and two of its active ingredients mediate neuroprotection through the putative PI-3K/Akt cell survival-promoting cascade. Activation of these pathways may underlie how neuritigenesis (Fig. 2C, [57-59,61,70]) and neuronal survival (Fig. 2A, B; [46]) occur. In addition, withaferin A showed a particular selectivity for BDNF expression, which was increased under stressful conditions, but decreased under nutrient-adequate conditions; withaferin A inhibited the MAPK cascade under both conditions.

Activation of the PI-3K/Akt cascade is bourne out by our observations that all three Ashwagandha compounds stimulate Akt (Fig. 3A), whereas only withaferin A affects the MAPK cascade, resulting in a decrease in the phosphorylation of MAPK (Fig. 4A), regardless of the presence of the N2 supplement. In addition, both BDNF (Figs 3B, 4B) and P-CREB (Figs. 3C, 4C) levels were at least partially restored by all three Ashwagandha compounds in the nutrient-deprived conditions, thereby (1) further validating our tissue culture model of nutrient deprivation as something from which to be rescued [42,43], (2) corroborating our current findings that WS promotes neuronal survival (Fig. 2A, [49,50]) and neuritigenesis (Fig. 2C, [57,58,69]) and (3) corroborating our HPLC analyses that withanolide A was present in the Ashwagandha tincture, while withaferin A was not; that is, both the Ashwagandha tincture and withanolide A exerted the same effects on intracellular signaling. Taken together, our Western blotting results are summarized in Fig. 5, which show the effects of Ashwagandha and its two active ingredients on the PI-3K/Akt and MAPK cascades under both nutrient-supplemented (N2+) and -deprived (N2-) conditions. Note that the only difference between these two conditions is the effect of withaferin A

on BDNF: Stimulating its production in the N2- condition, but inhibiting its production in the N2+ condition (Figs. 3B, 4B). In addition, withaferin A nullifies the suppressing effects of both LY294002 and PD98059, specifically, the effects on BDNF (F+ *vs*. F/LY+ (Fig. 3B), F+ *vs*. F/PD+ (Fig. 4B)) and P-CREB (F+ *vs*. F/LY+ and F- *vs*. F/LY- (Fig. 3C), F- *vs*. F/PD- (Fig. 4C)).

Some insight into the apparently highly selective nature of withaferin A might be gleaned from recent evidence on its effects as an anti-cancer agent (see [71] for review). One of the key intracellular signaling pathways that are over-activated in neoplastic cells is the Ras oncogene MAPK/ERK cascade, which can be set into motion by the binding of BDNF to its TrkB receptor, although brain tumors can be activated by increased BDNF–induced activation of the PI-3K cascade as well [72]. Whether the context is cancer or stress/depression, our data indicating that under stressful conditions (N2-), withaferin A compensates, promoting cell survival and growth by increasing BDNF expression (Figs. 4B, 5) probably through Akt. Under adequate nutritional conditions (N2+), withaferin A-induced increases in BDNF may not be needed, again while the lactone is able to activate Akt (Figs. 3A, 5). And although CREB per sé is not activated by withaferin A (Figs. 3C, 5), its upstream activation of Akt would still lead to activation of CREB. It is possible that CREB activation is kept at a nominal baseline level under adequate conditions (N2+), but when conditions become stressful or inadequate (N2-), CREB activation is significantly increased (Figs. 3A, 3C, 4C). Note that the MAPK cascade is inhibited by withaferin A (Figs. 4A, 5), whether adequate nutrient is present or not, again indicating that neuroprotection is Akt- but not MAPK-dependent.

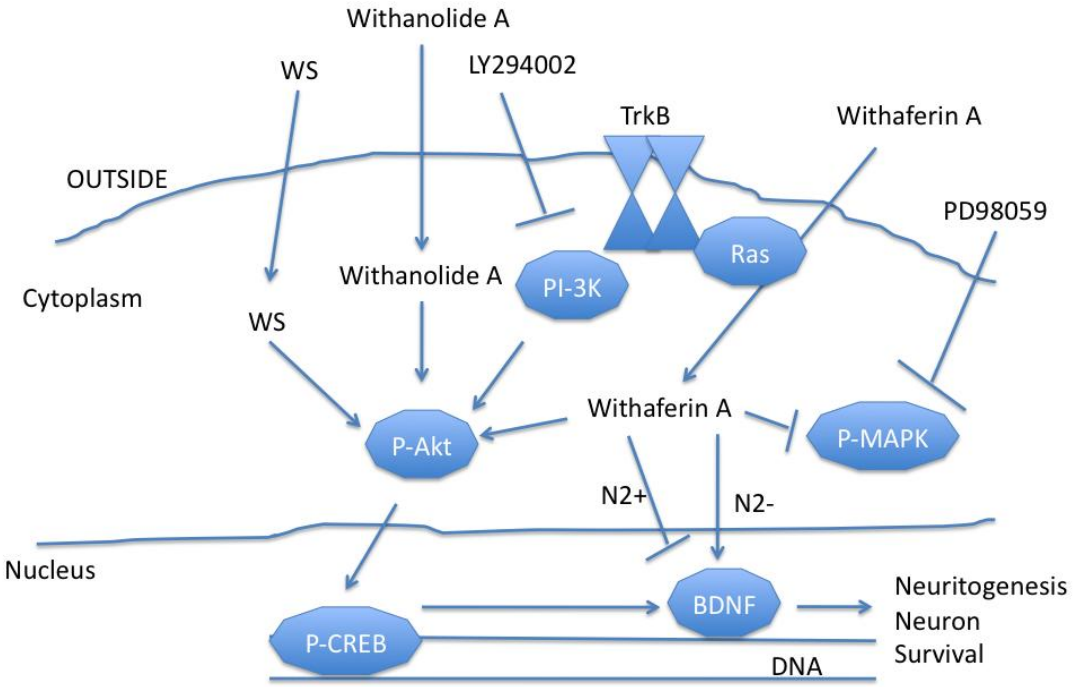

Fig. 5. Schematic representation of the intracellular signal transduction events explored in the current study. WS, withanolide A and withaferin A are all able to diffuse through the neuronal cell membrane into the cytoplasm wherein they exert their positive activating effects on the PI-3K/Akt pathway, thereby phosphorylating CREB, increasing expression of BDNF and ultimately, increasing elongation of neuronal processes and neuronal survival. Based on Western blotting results, the nutrient-supplemented (N2+) increased all of the foregoing to a greater extent than did the nutrient-deprived condition; in some cases, the latter decreased some of these measures. Note that the key difference between the two nutrient conditions is withaferin A, which inhibited BDNF expression under nutrient-supplemented conditions (N2+), but stimulated expression of the neurotrophin under deprived conditions (N2-). → indicates stimulatory effect; ⊥ indicates inhibitory effect

Both withanolide A and withaferin A are plant hormones, bearing at least some structural similarity to mammalian estrogens and androgens, which have long been known to be beneficial for CNS function [73,74]. Although withaferins have been shown to be anti-estrogens by down-regulating estrogen receptors as one of its anti-cancer actions [75], it is also possible that withanolides or withaferins themselves bind estrogen receptors, acting like an agonist and conferring at least some of the beneficial CNS effects reminiscent of estrogen (and testosterone) itself, which may ultimately culminate in changes in gene expression of critical proteins essential for neuroprotection, repair and survival [76,77]. In this analogous scenario, therefore, in stress or disease (*e.g.*, cancer), withaferin A may be beneficial, just as we demonstrated in the present study: Under nutrient deprived stress conditions, withaferin A up-regulated BDNF expression, which resulted perhaps from being an agonist to estrogen receptor beta [78]. This indirect up-regulation of the neurotrophin occurred through Akt, which was in addition to the separate action of withanolide A in activating Akt. In contrast, under normal or non-pathological or non-stressful conditions (N2+), withaferin A directly down-regulated BDNF expression, perhaps by acting as an estrogen receptor antagonist, or, at least, not as an agonist. Thus, both withanolide A and withaferin A led to up-regulation of BDNF, so as to maintain cell survival and promote neural protection. At least two of the individual ingredients of a greater whole in Ashwagandha, therefore, are differentially active, depending on the physiological state (nutrient conditions) and well-being of the cell.

5. CONCLUSIONS

Using our model of nutrient deprivation stress, we were able to show that Ashwagandha, withanolide A and withaferin A all increased neuron survival and average dendritic length. Consistently, Ashwagandha and withanolide A increased phosphorylation of Akt, but not MAPK, in both nutrient-adequate and nutrient-deprived conditions. Withaferin A increased BDNF expression under nutrient-deprived conditions, but decreased BDNF expression under adequate nutrient conditions; withaferin A still activated Akt under both types of nutrient conditions. Using our model of nutrient deprivation stress, therefore, we were able to show that withaferin A helps the cell adapt to stressful conditions, such as by increasing expression of BDNF, while the other active ingredient we analyzed, withanolide A, continues to maintain cell survival and neural protection by stimulating a baseline level of PI-3K/Akt activity. Our results show that two of the active ingredients of Ashwagandha differentially regulate intracellular signal transduction cascades, allowing the cell to adapt to physiological stressors.

Although withanolide A and withaferin A are isomers, their very different stereochemistries give rise to their rather different actions on intracellular cascades, as shown by the current results. Future studies should be aimed at systematically characterizing the various active ingredients of Ashwagandha in terms of their relative abundance (withanolide A is present in higher levels than is withaferin A [79], and therefore, relative potencies, method of extraction and purification [79] and characterization of their bond angles and degree of rotation, such as what Zhao et al. [59] did with several root-derived withanolides. And finally, molecular docking and simulation studies should be performed to detail the precise atomic alignments and geometric interactions that occur between, say, withaferin A and the estrogen receptor; and if there is indeed binding between them, between this complex and the estrogen response element.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

ACKNOWLEDGEMENTS

CSU-LSAMP is supported by the National Science Foundation under Grant # HRD-1302873 and the CSU Office of the Chancellor (for IV and LG), NIH-RISE Grant # GM61331 and CSULA Faculty-Student Achievement funds to ARN and by a scholarship from the Office of Graduate Studies to DH and also acknowledge National Science Foundation-Center for Research Excellence in Science and Technology (HRD-1547723) for FZ and A. Lopez.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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