Ashwagandha and its active ingredient, withanolide A, increase phosphorylation of TrkB in cultured hippocampal neurons

ABSTRACT

Aims: To primary rat embryonic hippocampal neurons in culture, ashwagandha or one of its active ingredients, withanolide A were added in the presence or absence of nutrient supplementation and then assayed for activity of the BDNF receptor, TrkB.

Study design: Primary hippocampal neurons were cultured and grown in nutrient-rich or nutrient-poor medium. Ashwagandha or withanolide A were then be added to both types of media with or without an inhibitor ofTrkB or either the PI-3K or MAPK pathway.

Place and Duration of Study: Department of Biological Sciences, California State University, Los Angeles, CA, USA, between July 2021 and August 2022.

Methodology: Rat embryos were removed by cesarean section from mother rats at 18 days' gestation and the hippocampi of the former dissected, plated into culture dishes, and treated with the appropriate drug(s) (see Study Design above). After 4 days, neurons were harvested for Western blotting. Optical density of Western blot bands were quantified and statistically analyzed in a 2-way ANOVA, using a level of statistical significance at $P < .05$.

Results: Under normal conditions (with N2 supplement), ashwagandha, but not withanolide A, increased phospho-TrkBimmunoreactivity when compared to the effects of vehicle (controls, $F_{(11, 24)} = 22.48$, $P < .001$), although withanolide A did not quite reach statistical significance ($P = .069$) when compared to that of the controlled condition. Likewise, under nutrient-deprived conditions, both ashwagandha and withanolide A also increased phosphorylation of TrkB when compared to that of vehicle-nutrient-deprived conditions ($P < .0001$). The same results were obtained in the presence of inhibitors of TrkB itself and the PI-3K (ashwagandha, $P < .001$; withanolide A, $P < .001$) and MAPK (ashwagandha, $P = .027$; withanolide A, $P = .045$) pathways.

Conclusion: Ashwagandha or withanolide AactivatesTrkB, in nutrient-deprived hippocampal neurons, underscoring its role in neuronal survival signaling.

Keywords: Ashwagandha, depression, stress, hippocampal neurons, TrkB, BDNF, PI-3K, MAPK

1. INTRODUCTION

By now, it is well-known that in the central nervous system (CNS), the hippocampus is one of structures that is most affected by the ravages of

mood disorders [1,2,3] although this effect is not universal [4], depending on the type of mood disorder [1]. Over the past several decades, a wealth of evidence has emerged, showing that such mood disorders may be ameliorated by physical exercise, which is a putative releaser of circulating neurotransmitters [5,6,7,8]. Indeed, their extended residence time in central synapses and the release of these neurotransmitters into the systemic circulation is the basis of antidepressant medications [9] and the antidepressant effects of physical exercise [10,11,12]. The binding of these neurotransmitters to their respective receptors has been shown to activate pro-survival neuronal signaling of brain-derived neurotrophic factor (BDNF) [3, 13,14], Akt [15,16,17], MAPK [13,18,19,20], and CREB [13,20,21], which are decreased in depression [20] and which are part and parcel of neurite outgrowth [8,22] and neuronal survival [22].

Several years ago, our laboratory provided evidence that in tissue culture, embryonic hippocampal neurons can be stressed by depriving them of certain nutrients [23,24]. These nutrients comprise a mixture called N2, which consists of insulin, progesterone, putrescine, selenium dioxide and human transferrin. After being allowed to acclimate for several hours following culturing, neurons were deprived of N2. It was shown that the addition of various compounds known to increase cell survival, such as norepinephrine [13,23], serotonin [24], or antidepressants [24] could be neuroprotective by increasing BDNF expression and activating the Akt/PI-3K and MAPK cascades and subsequent phosphorylation of CREB [16], and increased dendritic lengths and neuronal survival [2,22,25]. Thus, we proposed that this model is analogous to the dendritic pruning and neuronal death that hippocampal neurons undergo in the wake of mood disorders [2].

Ashwaghanda (Withaniasomnifera, WS, Indian ginsing) has been shown to improve cognition and mood in depressed people by decreasing cortisol levels [26,27]. Because there is much evidence that WS has ameliorative properties in both peripheral [28] and central [29,30] disorders (also see [22] and references cited therein), we evaluated its cell survival-promoting effects in our tissue culture model of nutrient deprivation stress.

More recently, our laboratory reported that, in hippocampal neurons in cuture, BDNF and Akt, MAPK, and CREB phosphorylation, as well as neuronal survival, are decreased in response to nutrient-deprived conditions, while WS and one of its most potent active ingredients, withanolide A (WA), was able to increase the immunoreactivity or phosphorylation of the afore-mentioned variables under both nutrient-supplemented and –deprived condtions relative to those of controls [22].

The current study is an addendum to Hwang et al. [22], where herein, TrkB phosphorylation was evaluated in response to nutrient-supplemented or

–deprived, and WS- or WA-supplemented conditions under PI-3K/Akt or MAPK cascade blockade in embryonic hippocampal neurons. Others have noted that, not surprisingly, that expressison of BDNF and its receptor covary [3,31] in terms of activation [32]. Specifically, we hypothesize that TrkB phosphorylation will be decreased under nutrient-deprived conditions, as well as under conditions of TrkB, PI-3K and MAPK inhibition, but will be ameliorated in the presence of WS or WA.

2. MATERIALS AND METHODS

2.1. Plant material used

Withaniasomnifera (L.)Dunal is a member of the family Solanaceae, which is listed in www.theplantlist.org and the World Checklist of Selected Plant Families (WCSP). For this study, we used an ashwagandha tincture in methanol.

2.2. HPLC analysis of Withanolide A and Ashwagandha

Analysis of WS and WA were conducted using HPLC and were taken from the same stock, stored at -20°C, as performed previously [22].

2.3. Animal Care and Handling

All rat 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. All experiments were approved by the California State University Institutional Review Board and the Animal Care Committee.

2.4. Antibodies, chemicals and assays

N2 was purchased from Invitrogen (Grand Island, NY, USA), is composed of insulin, progesterone, putrescine, selenium dioxide and human transferrin and dissolved in minimal essential medium (MEM) with Earle's salts at a final culture concentration of 1% as previously described [24]. Anti-phospho-TrkB and anti-TrkB were both purchased from Cell Signaling Technologies (Danvers, MA, USA). Anti-mouse IgG and anti-rabbit IgG were

purchased from Amersham Pharmacia-Biotech (Piscataway, NJ, USA). WA was purchased from Sigma (St. Louis, MO, USA); K252a was purchased from Calbiochem (LaJolla, CA, USA); LY294002 and PD98059 were purchased from Promega (Madison, WI, USA). Finally, WS was purchased from Sprouts grocery store (Whittier, CA, USA). 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 40 µg/ml [22] and has been stored at -20°C.

2.5. 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 [24]. Briefly, pregnant female rats (Sprague-Dawley, 2 months of age, 250 g, Charles River, Wilmington, MA, USA) at 18 days gestation were sacrificed with an overdose of isofluorane, followed by rapid decapitation and the embryos removed via cesarean section. Both hippocampi were dissected from each embryo into Ca-Mg-free DMEM and then rinsed twice into 15-ml conical tubes to which 0.125% trypsin was added for 5-10 min in a 37°C water bath with gentle inversion every 2-3 minutes. The reaction was then quenched in 10% fetal bovine serum (FBS)/DMEM in two volumes to stop the reaction. Hippocampi were then centrifuged at 200 g for 5 min and the resultant pellet resuspended in 2-5 ml DMEM. The hippocampi were then triturated with glass Pasteur pipettes to mechanically dissociate the neurons from each other and connective tissue. They were then filtered through a 40-µm cell strainer (Falcon) into new conical tubes and then resuspended up to 5 ml DMEM. Cells were then counted in a hemacytometer to quantify and then plated at a density of 50,000 cells/cm2 in a total volume of 1 ml.

2.6. Procedures

Three days following plating, in half of 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 by K252a (200 nM, [13], LY294002 (20 µM, [22]) or PD98059 (10 µM, [22]) and/or WS (40 µg/ml, [22]) or WA (20 µM, [33]). Twenty-four hr later, cells were lysed and harvested for subsequent SDS-PAGE and Western blotting (see below).

2.6.1. Harvesting Cultured E18 Hippocampal Neurons

Cultured hippocampal neurons were harvested exactly as described previously [24]. Briefly, the media was aspirated off and the cells scraped with a cell scraper, each well reconstituted in a total volume of 0.5 ml hot cell lysis buffer (10 mMtris-base, pH 7.4, 1% SDS). Each sample was then boiled for 5 min, triturated through a 26-G needle to sheer genomic DNA, centrifuged and the volume measured. An equal volume of gel loading buffer was then added to each sample [34]. Samples were then stored at -20°C till electrophoresis and Western blotting.

2.6.2. SDS-PAGE/Western Blotting

Electrophoreses and subsequent Western/immunoblotting were performed exactly as described previously [24]. Briefly, after protein concentrations were determined using the method of Lowry [35], 10% polyacrylamide gels were cast and then 30 µg protein of each sample was loaded therein. Following electrophoresis, proteins were electrotransferred at 80 V to nitrocellulose for 2 hr at room temperature. Blots were then probed with anti-phospho-TrkB according to manufacturer's specifications (Cell Signaling Technologies). Blots were then exposed to enhanced chemiluminescence (ECL, Amersham-Pharacia-Biotech, Piscataway, NJ, USA). Blots were then stripped (in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mMtris-HCl, pH 6.7, 55°C, 10 min, while agitating) and then re-probed with anti-TrkB according to manufacturer's specifications (Cell Signaling Technologies) and again exposed to ECL.

2.6.3. Statistical Analysis

Quantification of immunoreactive bands was performed using ImageJ (Rasband, W., NIH, USA), to quantify lightly exposed Western blotting bands within the linear range of a standard curve. The optical density of each phospho-TrkB band was divided by that of its respective loading control, TrkB, to yield the corrected band intensity. Each sample had been loaded onto the gel in triplicate and each Western blotting experiment for each sample performed 2-3 times.

For the Western blotting experiments, statistical analyses entailed a two-way analysis of variance (ANOVA) to examine the main effects nutrient supplement (N2) (enriched vs. deprived media) x compound (WS, WA,K252a, LY292002, and/or PD98059) on phospho-TrkB). Results were

considered statistically significant at $P<.05$. 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.

HPLC analysis showed that WS and WA were both of high purity, showing sharp and consistent peaks at 227 nm as shown previously [22].

3.2 Both Ashwagandha and WithanolideA Increase TrkB Phosphorylation

Overall, there are statistically significant main effects for whether nutrient (N2) was present ($F(1, 24) = 158.64$, $P< .001$) and the compound(s) used ($F(5, 24) = 2.82$, $P = .039$), as well as a statistically significant interaction between nutrient and compound ($F(5, 24) = 14.91$, $P< .001$). Under normal conditions (with N2 supplement), WS, but not WA increased phospho-TrkBimmunoreactivity when compared to the effects of vehicle (Veh+, Fig. 1, $F(11, 24) = 22.48$, $P< .001$), with WA not quite reaching statistical significance ($P = .069$) when compared to that of the Veh+ condition. Likewise, under deprived conditions (N2-), both Ws and WA also increased phosphorylation of TrkB when compared to that of vehicle (Veh-, Fig. 1, $P< .0001$). Moreover, true to its function as a phospho-Trk inhibitor, K252a suppressed phosphorylation of TrkB compared to those of vehicle under both nutrient-supplemented ($P< .001$) and deprived ($P = .027$) conditions (Fig. 1). When combined with either WS or WA, K252a significantly inhibited the phosphorylation of TrkB, compared to that of WS ($P< .001$) or WA ($P< .001$) alone, respectively, under both nutrient-supplemented ($P = .006$) and deprived ($P =.047$) conditions.

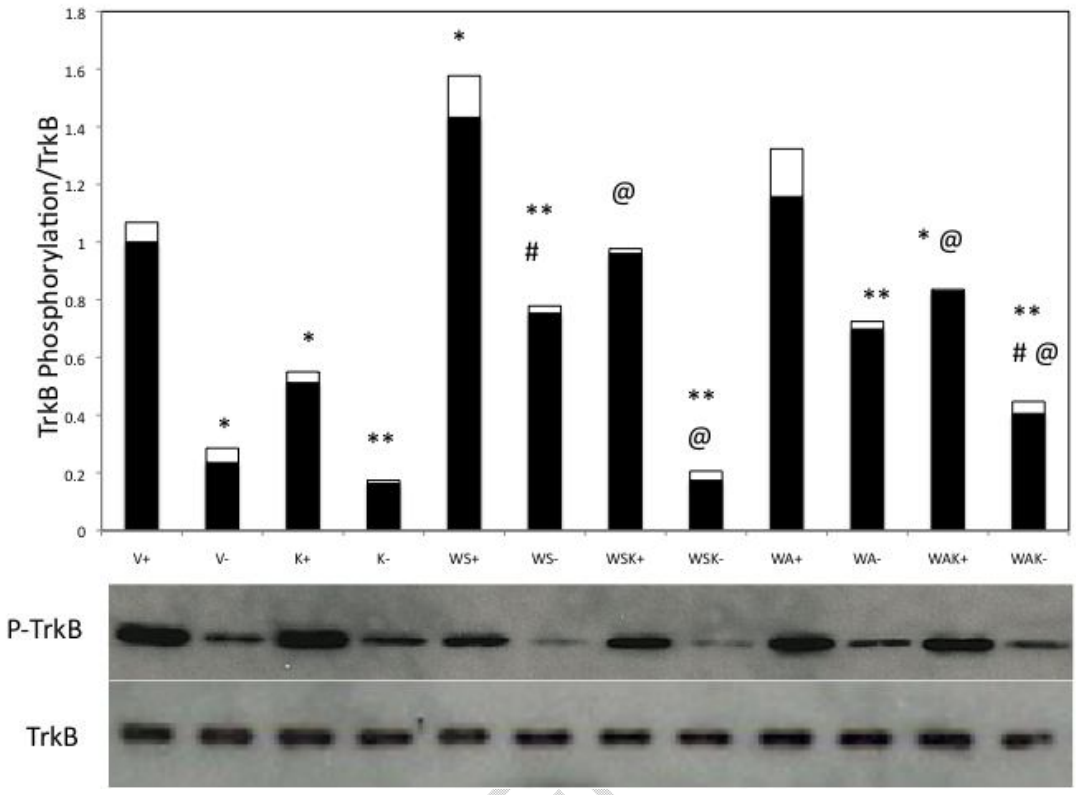

Figure 1. * Significantly different from V+; # Significantly different from V-; ** Significantly different from corresponding (+) condition; @ Significantly different from corresponding condition without inhibitor. All at *P*< .05.

3.3 Both Ashwagandha and WithanolideA Increase TrkB Phosphorylation Through the MAPK Pathway

Overall, there are statistically significant main effects for whether nutrient (N2) was present (F(1, 24) = 189.04, *P*< .001) and the compound(s) used (F(5, 24) = 6.35, *P*< .001), as well as a statistically significant interaction between nutrient x compound (F(5, 24) = 13.25, *P*< .001). As in Figure 1, under either nutrient-supplemented or –deprived conditions, WS, but not WA, increased phospho-TrkBimmunoreactivity when compared to the effects of vehicle (Veh+, Fig. 2, F(11, 24) = 26.09, *P*< .001), with WA not quite reaching statistical significance (*P* = .061) when compared to that of the Veh+ condition. Likewise, under deprived conditions (N2-), both WS and WA also increased phosphorylation of TrkB when compared to that of vehicle (Veh-, Fig. 2, *P*< .0001). Under normal N2-suppplemented conditions, PD98059 resulted in statistically similar levels of TrkB activation, whereas in N2- conditions, there was a huge significant decrease, compared to that of

N2+ (P < .001). When combined with either WS or WA under N2+ conditions, PD98059 failed to inhibit TrkB phosphorylation, bringing its levels comparable to those of Veh+, but only slightly decreased activation of the receptor in N2-deprived conditions, although these decreases were still statistically lower than their respective N2+ counterparts (WSPD+, P = .027 and WAPD+, P = .045) (Fig. 2).

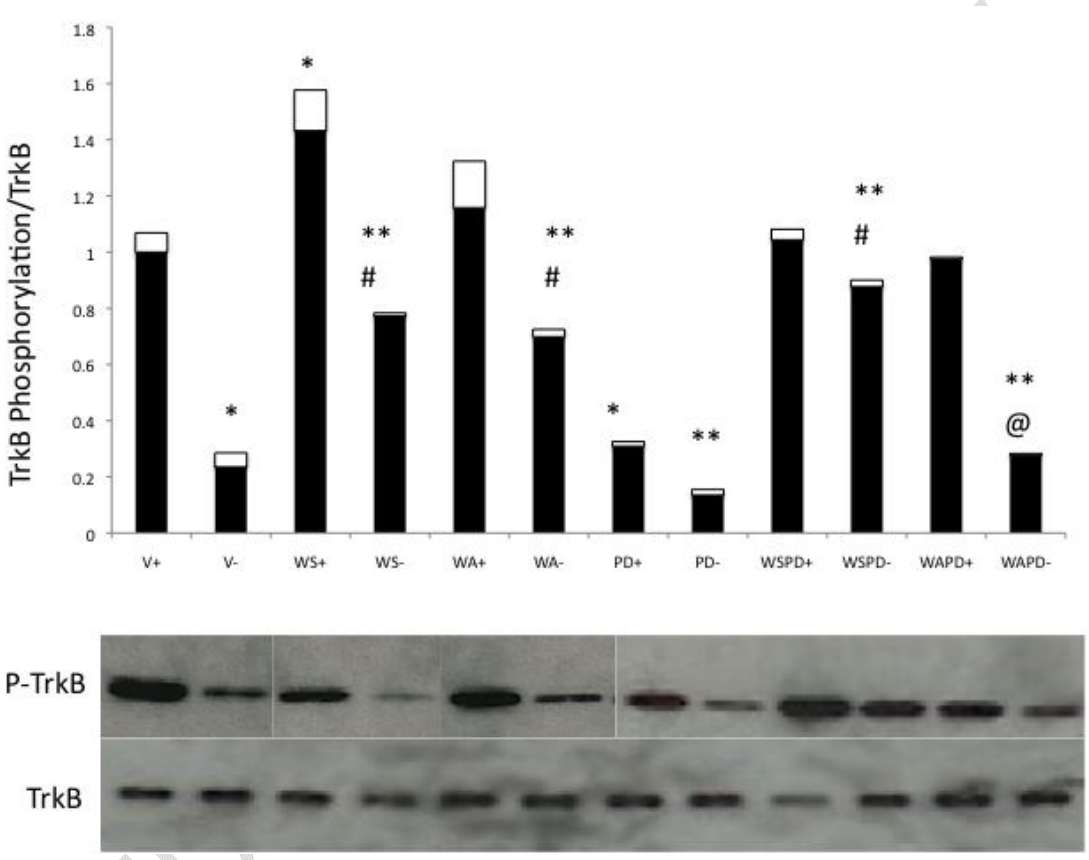

Figure 2. * Significantly different from V+; # Significantly different from V-; ** Significantly different from corresponding (+) condition. @ Significantly different from corresponding condition without inhibitor. All at P< 0.05.

3.4 Both Ashwagandha and WithanolideA Increase TrkB Phosphorylation Through the PI-3K/Akt Pathway

Overall, there are statistically significant main effects for whether nutrient (N2) was present ($F(1, 24) = 155.98$, $P < .001$) and the compound(s) used ($F(5, 24) = 8.64$, $P < .001$), as well as a statistically significant interaction between nutrient and compound ($F(5, 24) = 22.37$, $P < .001$) As in Figure 1, under either nutrient-supplemented or –deprived conditions, WS, but not WA, increased phospho-TrkBimmunoreactivity when compared to the effects of vehicle (Veh+, Fig. 3, $F(11, 24) = 28.28$, $P < .001$), with WA not quite reaching statistical significance ($P = .063$) when compared to that of the Veh+ condition. Likewise, under deprived conditions (N2-), both compounds also increased phosphorylation of TrkB when compared to that of vehicle (Veh-, Fig. 3, $P < .0001$), but again, WA did not reach statistical significance when compared to that of the Veh+ conditions. Being a potent inhibitor of the Akt pathway, LY294002 significantly inhibited phosphorylation of TrkB, compared to Veh+ conditions ($P < .001$), but under nutrient-deprived conditions, LY294002 did not lower the levels of TrkB activation, compared to that of Veh- conditions ($P = .078$). Even when combined with LY294002 under N2+ conditions, WS and WA raised TrkB phosphorylation to levels comparable to those of Veh+ and significantly higher than that of LY294002 alone ($P < .001$ for WS; $P < .001$ for WA). Under N2- conditions, even when combined with LY294002, WS, but not WA, slightly, but significantly ($P = .037$) raised TrkB activity, relative to that of LY294002-alone conditions (Fig. 3).

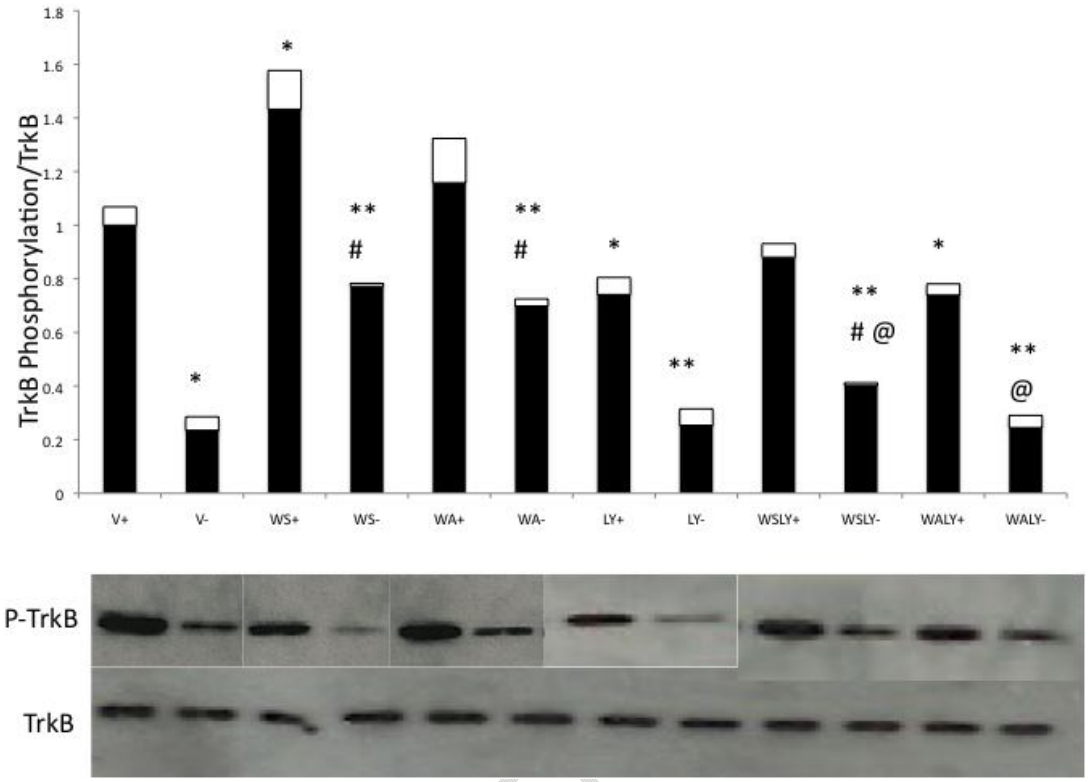

Figure 3. * Significantly different from V+; # Significantly different from V-; ** Significantly different from corresponding (+) condition. @ Significantly different from corresponding condition without inhibitor. All at $P < 0.05$.

3.5 Both Ashwagandha and WithanolideA Increase TrkBPhosphorylation - A Comparison Among the three Inhibitors

Recall from the Results that the *P* level is higher for the main effects of compound (*P* = .039). In the presence of N2, WS is able to increase the phosphorylation of TrkB in the presence of PD98059 more than in the presence of either K252a or LY294002; without N2, Ws is able to increase the phosphorylation of TrkB in the presence of PD90059 more than in the presence of either K252a or LY294002 (*P* = .035, Fig. 4). In the presence of N2, WA is able to increase the phosphorylation of TrkB in the presence of PD98059 more than in the presence of either K252a or LY294002; without N2, WA is able to increase the phosphorylation of TrkB in the presence of K252a more than in the presence of either PD98059 or LY294002 (*P* = .029, Fig. 4).

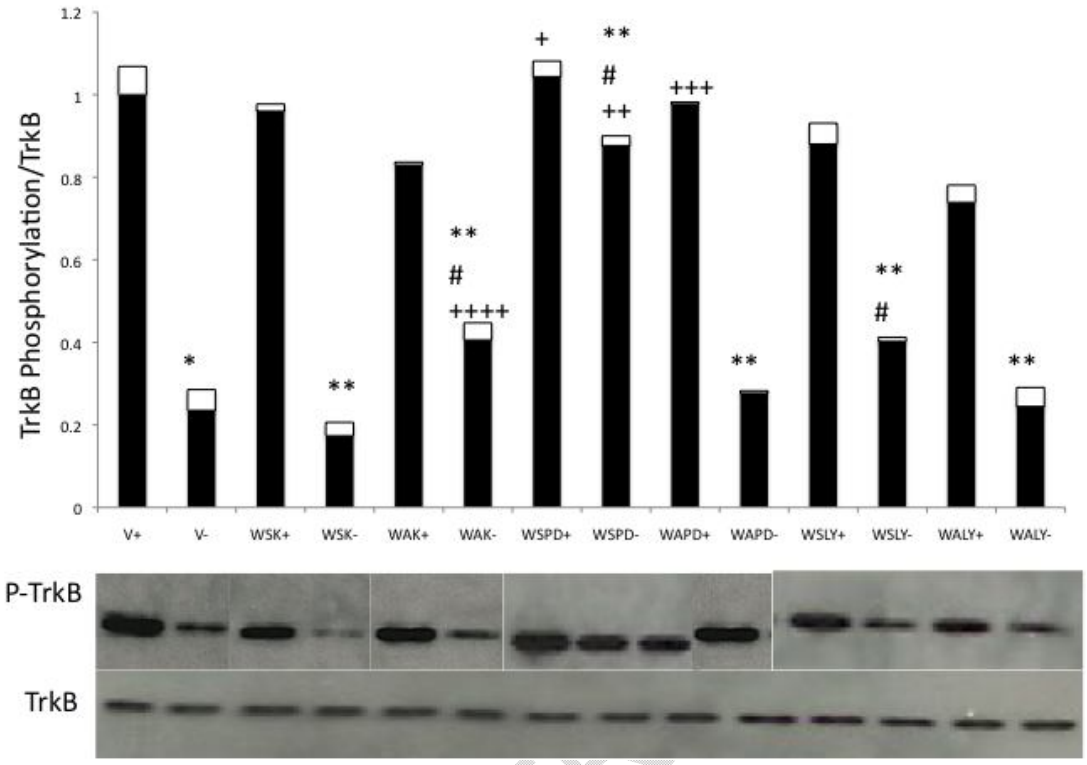

Figure 4. * Significantly different from V+; # Significantly different from V-; ** Significantly different from corresponding (+) condition. +, Significantly greater than WSK+ or WSLY+; ++, Significantly greater than WSK- or WSLY-; +++, Significantly greater than WAK+ or WALY+; ++++, Significantly greater than WAPD- or WALY-. All at $p < 0.05$.

4. DISCUSSION

Consistent with earlier studies showing that WS increased BDNF levels [22,36], the current study found that both WS and WA increase the levels of its activated receptor as well both with and without nutrient supplementation. Reliably, inhibition of TrkB was exacerbated with its inhibitor, K252a, under nutrient-deprived conditions. Likewise, the same pattern of inhibition was statistically significant when WS or WA were combined with either PD98059 or LY294002.

Thus, activation of the PI-3K/Akt cascade is bourne out by our observations that when WS or WA were combined with LY294002, TrkB phosphorylation was increased to levels comparable to those of controls, but not quite as high as that of WS or WA alone (Fig. 1). Even in the presence of the two suppressing conditions of PD90959 and N2 deprivation, both WS or

WA managed to increase the levels of activated receptor to slightly lower than that of controls (vehicle +N2), but certainly much higher than that of controls, WS, or WA without N2 (Fig. 2). On the other hand, when the two inhibiting conditions of LY294002 and N2 deprivation were present, both WS and WA increased the levels of activated receptor to lower than that of controls (vehicle +N2), but higher than that of controls and WS without N2 (Fig. 3). WA, however, did not increase the doubly inhibiting conditions of –N2 and LY294002 (Fig. 3). When the combination treatments of Figs. 1, 2, and 3 were re-configured and re-analyzed to statistically compare the triple treatments with each other (WS or WA plus inhibitor plus +/- N2), WS increased TrkB phosphorylation in the presence of PD98059 more than in the presence of the other two, indicating that the MAPK pathway plays a smaller role in maintenance of cell survival (Fig. 4); but in the absence of N2, this effect was exacerbated, indicating that the MAPK pathway plays an even smaller role in cell survival. That is, WS is able to more easily overcome the inhibitory effects of PD98059 and promote cell survival to a greater extent (Fig. 4). Conversely, inhibition of the receptor or of the PI-3K pathway was able to prevail over the pro-survival effects of WS (Fig. 4). In the presence of N2, WA increased TrkB phosphorylation in the presence of PD98059 more than in the presence of either of the other two, indicating that the MAPK pathway plays a smaller role in maintenance of cell survival (Fig. 4); but in the absence of N2, WA is able to more easily overcome the inhibitory effects of K252a and promote cell survival to a greater extent than with either PD98059 or LY294002 (Fig. 4), indicating that it is perhaps the de novo synthesis of BDNF that has a more pronounced effect on cell survival. Recall that although the MAPK and Akt/PI-3K cascades are activated by TrkB phosphorylation, the respective inhibitors used in this study underscore their influence on CREB phosphorylation and BDNF synthesis [13,22]. Thus, with less BDNF synthesized, there is less binding to TrkB and therefore, less phosphorylation. The greater influence of the Akt/PI-3K cascade on TrkB phosphorylation is consistent with earlier findings that both WS and WA increased the phosphorylation of Akt, but not of MAPK, under either nutrient-supplemented or nutrient-deprived conditions [22].

Our results show that (1) all three inhibitors (one TrkB inhibitor and two cell survival signaling pathway inhibitors) are able to suppress TrkB phosphorylation and that this suppression is exacerbated by nutrient deprivation conditions. (2) Our tissue culture model is again validated as a viable screening procedure to evaluate how much WS or WA can maintain TrkBphosporylation even in light of nutrient deprivation and/or TrkB or survival pathway inhibition. And (3) our prior HPLC analyses that indicated that WA was present in the WS tincture [22], supporting out observations that both the WS tincture and WA exerted the same effects on intracellular signaling. In our previous study [22], we found the same results with regard to BDNF, Akt and CREB activation, in response to WS and WA, not to

withaferin A, which is the reason for not including the latter in the current study.

Although the current study was conducted in tissue culture in rat embryonic hippocampal neurons, the results reported herein nevertheless provide a plausible molecular mechanism underlying recent clinical findings that WS improves psychological and cognitive effects in depressed patients [26,38] possibly through the hypothalamus-adrenal-pituitary axis [29]. Moreover, Manchanda and Kaur [39] have reported that hippocampal BDNF mRNA and phospho-Akt was increased in rats fed WS leaf extracts, relative to those fed a low-fat diet, albeit not statistically so. Hippocampal TrkB mRNA, however, was significantly higher in such rats compared to their low-fat diet counterparts [39]; this does not necessarily mean that pro-survival pathways prevail in rats fed WS leaf extracts, as (1) transcription levels are not always correlated with translation levels [40,41] and (2) TrkB expression is not activation, as the receptor must be phosphorylated to be activated [42,43].

In light of the previous [22] and the current findings, the next obvious question to ask is how does WS (and WA) promote neuronal survival. Although our data show that BDNF, phospho-TrkB and phospho-Akt/PI-3K pathway are up-regulated, what does WS do to initiate these pathways? We had shown earlier, for example, that application of norepinephrine to hippocampal neurons resulted in increased BDNF expression and increased activation of the phospho-Akt/PI-3K and MAPK pathways and CREB phosphorylation [13]. Norepinephrine binds to its G-protein-coupled receptor, following the protein kinase A pathway [44], leading to the phosphorylation of CREB, which takes part in the transcription of BDNF and its binding to TrkB, which is then autophosphorylated, and finally, activating the pro-survival Akt-PI-3K and MAPK cascades [45]. But, what about WS (and WA)? What do they bind to promote cell survival through these same cascades? It is possible that they, such as WA, promote cell survival [46,47] not via a receptor-mediated pathway, but through other means, such as anti-oxidation [48,49].

It is well known that antidepressants or anti-anxiety medications bind to noradrenergic, serotonergic or GABAergic receptors, acting like agonists at these receptors or reuptake inhibitors (see [13], and references cited therein; [9,50]). Alternatively, Casarotto et al. [51] recently provided evidence that antidepressants bind directly to TrkB, thereby initiating a conformational change that facilitates BDNF binding and subsequent cell survival signaling, perhaps through plastic changes [52]. Using both tissue culture incubation with antidepressants, such as fluoxetine, and molecular modeling calculations and simulations, Casarotto et al. [51] provided compelling evidence that the BDNF receptor itself could be a binding site for

antidepressants. It is possible, therefore, that TrkB could also offer an amenable binding site for one or more of the active ingredients of WS; WA perhaps could promote neurite outgrowth [53] via direct interaction with TrkB. Future studies must be undertaken to determine if this is feasible.

Figure Captions:

Figure 1.TrkB inhibitor, K252a, significantly decreased phosphorylation of TrkB under both N2+ and N2- conditions, when compared to their respective vehicle controls. Conversely, WS, but not WA, significantly increased phosphorylation of TrkB, compared to that of Veh+ controls. Without nutrient supplementation, WS significantly increased phosphoryation above that of Veh control conditions. And when WS and K252a were combined, phosphorylation of TrkB was significantly higher than that of K252 alone in the nutrient-supplemented condition, but not in the deprived condition. Finally, under N2 conditions, when the inhibitor was combined with WA, TrkB was significantly higher than that of K252a alone and significantly lower than that of WA alone. And under N2- conditions, the combination was also significantly higher than that of K252a alone and significantly lower than that of WA alone. TrkB phosphorylation levels as indicated are the optical density of the respective bands were divided by those of TrkB. Values are means +/- SEM. Abbreviations: WSK, WS-plus-K252a; +, in the presence of N2; -, in the absence of N2.

Figure 2. The MAPK inhibitor, PD98059, significantly decreased phosphorylation of TrkB under both N2+ and N2- conditions, when compared to their respective vehicle controls. Conversely, WS, but not WA, significantly increased phosphorylation of TrkB, compared to that of Veh+ controls. Without nutrient supplementation, WS significantly increased phosphoryation above that of Veh (N2-) control conditions. And when WS and PD98059 were combined, phosphorylation of TrkB was significantly higher than that of PD98059 alone in both the N2+ and N2- conditions. Finally, when PD98059 was combined with WA, TrkB was significantly higher than that of PD98059 alone in both the N2+ and N2- conditions. TrkB phosphorylation levels as indicated are the optical density of the respective bands were divided by those of TrkB. Values are means +/- SEM. Abbreviations: WSPD, WS-plus-PD98059; +, in the presence of N2; -, in the absence of N2.

Figure 3. The PI-3K inhibitor, LY294002, significantly decreased phosphorylation of TrkB under N2+, but not under N2- conditions, when

compared to their respective vehicle controls. Conversely, WS, but not WA, significantly increased phosphorylation of TrkB, compared to that of Veh+ controls. Without nutrient supplementation, WS significantly increased phosphoryation of TrkB above that of Veh (N2-) control conditions. And when WS and LY294002 were combined, phosphorylation of TrkB was significantly higher than that of LY294002 alone in both the N2+ and N2- conditions. Finally, under N2+ conditions, when the inhibitor was combined with WA, TrkB activation was significantly higher than that of LY294002 alone, but not in the N2- conditions. TrkB phosphorylation levels as indicated are the optical density of the respective bands were divided by those of TrkB. Values are means +/- SEM. Abbreviations: WSLY, WS-plus-LY294002; +, in the presence of N2; -, in the absence of N2.

Figure 4.Statistical re-analysis of each of the three inhibitors when combined with WS or WA with or without N2. In the presence or absence of N2 supplementation, WS increased TrkB phosphorylation was still higher in the presence of PD98059, compared to that in the presence of K252a or LY294002. In the presence, but not in the absence of N2 supplementation, WA increased TrkB phosphorylation was still higher in the presence of PD98059, compared to that in the presence of K252a or LY294002; without N2, TrkB phosphorylation is higher than with the two cascade inhibitors. TrkB phosphorylation levels as indicated are the optical density of the respective bands were divided by those of TrkB. Values are means +/- SEM. Abbreviations: WSK, WS-plus-K252a; WSPD, WS-plus-PD98059; WSLY, WS-plus-LY294002; +, in the presence of N2; -, in the absence of N2.

5. CONCLUSION

In the current study, we have extended previous findings that ashwagandha and one of its active ingredients, withanolide A, indeed activates the BDNF receptor, TrkB, in nutrient-deprived hippocampal neurons, underscoring its role in neuronal survival signaling.

CONSENT

Not applicable.

ETHICAL APPROVAL

All rat handling were conducted in strict accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (1996).All experiments were approved by the California State University Institutional Review Board and the Animal Care Committee.
All authors hereby declare that "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.

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DEFINITIONS, ACRONYMS, ABBREVIATIONS

BDNF, brain-derived neurotrophic factor; P-CREB, phospho-cyclic adenosine monophosphate response element binding; DMEM, Dubecco's Modified Eagles's Medium; HPLC, high-performance liquid chromatography; LY, LY294002; MAPK, mitogen-activated protein kinase; NE, norepinephrine; PD, PD98059; PI-3K, phosphatidylinositol-3'-kinase; TrkB, tyrosine receptor kinase B; veh, vehicle; WS, Withaniasomnifera; WA, withanolide A