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Journal of Natural & Ayurvedic Medicine

Effects of *Withania somnifera* on Cholinergic Signaling in the Cerebral Cortex and Memory Function in the Aging Rat Brain

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Research Article

Volume 2 Issue 1

Received Date: December 16, 2017

Published Date: January 09, 2018

Abstract

Objectives: *Withania somnifera* Dunal (WS, ashwagandha) has been traditionally used as an adaptogen in Ayurveda. Administered over a long-term, it has demonstrated its potential as a remedy against age- and stress-related cognitive dysfunction.

Aims of the Study: In this long-term study, the effects of WS alone and WS combined with whole food diet and voluntary wheel-running on age-related alterations in acetylcholine esterase (AchE) levels and in the density of the cholinergic muscarinic receptor subtype 1(M_1GPCR), combined with the downstream transcription factor phospho-CREB (P-CREB) were evaluated. Additionally, index of memory function of the animals (measured by Novel object recognition test, NOR) and spatial learning ability (Barnes Maze) was correlated to the aforementioned molecular outcomes.

Methods: WS root powder was tested in the live rats by oral administration of 100mg/kg for 19 months, followed by novel object recognition (NOR) and Barnes Maze tests at 20 months of age. AchE activity in frontal cortex was measured via colorimetric assay; M_1-GPCR and P-CREB/total CREB density via Western blot (WB).

Results: WS herbal supplement alone or in combination with additional lifelong lifestyle interventions did not lead to an increase in the levels of muscarinic acetylcholine receptor in the frontal cortex of the aged rats, did not affect AchE activity significantly, nor did it alter expression of P-CREB in the frontal cortex of the aged rat. However, aerobic exercise and healthy diet treatments led to significant decrease in AchE activity combined with an increase in P-CREB and M_1GPCR immuno reactivity. In addition, via NOR Discrimination Index (DI), WS-treated rats have the best object memory, have above average hippocampus-dependent spatial memory and frontal-cortex mediated learning flexibility; combined intervention did not improve object discrimination while it preserved comparable spatial memory. Additionally, exercised rats showed adequate mental flexibility in special tasks, but their object memory was below average. Linear regression analysis revealed a significant correlation between M_1 receptor density and memory index; between M_1 receptor density and AchE activity in WS and Combo-treated rats, but failed to reach significance for inverse correlation with latency to complete Barnes Maze spatial navigation task.

Conclusions: Elevated cortical density of M_1-GPCR, and P-CREB in rats exercising over their life span, supports a protective or enhancing role of aerobic exercise against age-related decline and validates its use as a potential therapeutic intervention to protect neuronal health throughout animal lifespan. The mechanism of muscarinic receptor promotion may involve AchE. Improved object memory, hippocampus- and frontal cortex dependent spatial learning helps to compel evidence of existing physiological targets for WS's biologically active compounds. However, the mechanism of WS physiologic action remains elusive. The effect of combined lifestyle interventions on the cholinergic function are less definitive and require further analysis.

Keywords: Adaptogen; Ayurveda; Aged; Learning; Memory; Acetylcholine Esterase; CREB; Exercise

Abbreviations: AchE: Acetylcholine Esterase; Combo: Combination Treatment; DI: Discrimination Index; GPCR: G-Protein Coupled Receptor; P-CREB: Phospho-Cyclic Adenosine Monophosphate Response Element Binding; M_1: Muscarinic Type I Receptor; NOR: Novel Object Recognition; rv: Reversal; t: Trial; WFD: Whole Food Diet; WS: *Withania somnifera*

Introduction

Withania somnifera Dunal (WS) has been widely used in Ayurvedic medicine, for its functioning as an adaptogenand a vitalizer because of it's very little known toxicity and apparent ability to protect multiple body systems against mental, physical and environmental stressors while preserving physiological homeostasis [1-6].

WS, especially its root, possesses anxiolytic and anti-depressant properties comparable to those of benzodiazepine and imipramine respectively [7,8]. Its root extract has demonstrated restoration of neuronal function in the basal ganglia in animal models of Huntington's disease, counteracted induced retrograde amnesia and significantly alleviated anxiety in human studies of pre-mature aging [9-11]. However, the molecular events of action for these properties are not fully understood, due in part, to lack of long-term studies.

There is evidence that WS inhibits acetylcholine esterase (AchE), while enhancing GPCR M_1muscarinic receptor binding in the basal forebrain area and pre-frontal cortex [12,13]. *In vitro* and *in vivo*, AchE inhibition by various fractions (specifically aqueous extract) and crude extracts of WS has been measured in the cortical and basal areas of rat brains, and supplementation by WS notably reduced levels of hypobaric hypoxia induced AchE levels [12-14]. The cholinergic signaling system within the cerebral cortex plays an important role in cognition, memory formation and retention and successful learning of new information [15-18]. Thus, acetylcholine is markedly reduced in patients diagnosed with Alzheimer's type dementia and that cognition is also impaired in normal individuals by choline antagonists [19,20]. Because blocking muscarinic receptors interferes with memory and cognitive acuity in humans and experimental animals, enabling muscarinic receptors and enhancing cholinergic neurotransmission might do just the opposite to memory loss and adult-onset cognitive disorders – specifically, treat them and reverse them [21,22]. Therefore, the observed role of WS in capacitation of cerebral cholinergic signaling fits well with our hypothesis in preventing and alleviating memory impairment and cognitive dysfunction [13].

The essential role of CREB in formation of long-term memory and the induction of CREB-dependent transcription of proteins for long-term potentiation and synaptic plasticity has been fully established [23-30]. Following administration of inhibitors and modulators targeting this transcription factor, rapidly decaying memories were observed [31,32]. Furthermore, impairments in initial CREB phosphorylation paralleled by forgetfulness patterns have been found associated with the aging process and behavioral deficiencies [27,33,34]. Stress and depression (often precipitating factors for premature brain aging), synaptic degradation and neuronal atrophy, are accompanied by low or negative cortical expression of P-CREB compared to the subjects successfully treated for depression pharmaceutically or via lifestyle interventions such as voluntary and involuntary physical exercise [35,36]. Lifestyle interventions, such as physical activity and a whole food balanced diet, enhance neurocognitive function and improve the lifespan in older adults [37-42]. Older rodents that utilized voluntary wheel running confirmed

the positive effects of exercise on behavioral performance on learning and memory tasks [40,43,44]. Following physical exercise, animals demonstrated faster acquisition and greater retention of the platform location on the Morris Water Maze, had more newborn neurons, and more intense neuronal proliferation in the hippocampal region of medial temporal lobe [44-47]. Physical exercise also provides benefits for the neural activity and induces long-term potentiation [48].

Because physiological homeostasis depends on the metabolism of ingested foods, Ayurveda advocates the consumption of fresh, natural and minimally processed food [49,50]. Thus, it can be concluded that balanced nutritional intake can definitely have a direct and a sustained effect on neuronal health. Hence, our goal was to evaluate the synergistic effects of the whole food diet, regular voluntary aerobic exercise and long-term herbal supplementation on the levels of muscarinic cholinergic receptors, P-CREB and AchE activity in the frontal cortex of rat brain.

The long-term (the longest conducted to date) nature of the current study makes it novel in the investigation of an herbal treatment to offset the neurodegeneration associated with normal aging. Because it has been established that the rat brain undergoes significant age-associated decline in expression of the proteins that determine cognitive integrity, the goal of this study was to test the possibility that WS plays a role in preserving memory and cognition in aged rats [51].

It is hypothesized that long-term treatment with WS alone and a combined application of WS, whole food diet and voluntary exercise can preserve cholinergic function through inhibition of the esterase, while upregulating the muscarinic GPCR through increased CREB phosphorylation in aged rats.

Materials and Methods

Plant Material

Withania somnifera Dunal is a perennial shrub, Nightshade (Solanaceae) family, distributed in India and Middle East (plants.usda.gov; itisreport.gov, taxonomic serial No: 505824); also known as ashwagandha in Sanskrit. The whole desiccated root powder was grown and harvested in India; supplied by Banyan Botanicals located in Albuquerque, NM, USA. Batch: 417063.

WS from Banyan Botanicals is certified by New Mexico's Department of Agriculture, with quality control testing for organoleptic properties and presence of contaminants. Quality control data is the following: Test Method Specification Result TPC USP (2021; 2022) o10,000,000 CFU/g 520,000 CFU/g Total coliforms AOAC 991.14o10,000 CFU/g o100,000 CFU/g o100,000 CFU/g 2000 CFU/g Arsenic (As) ICP-MSr0.01 mg/d 0.118 ppm Cadmium (Cd) ICP-MSr0.006 mg/d 0.020 ppm Lead (Pb) ICP-MSr0.02 mg/d 0.185 ppm Mercury (Hg) ICPMSr0.02 mg/d 0.004 ppm Organoleptic QC-010 See Organoleptic Spec. Complies Identity FTIR Banyan Method [52].

Antibodies

The rabbit polyclonal anti-bodies to $mGPCR_1$were purchased from Abcam (Cambridge, MA, USA), anti-phospho-CREB, anti-CREB and anti-tubulin was purchased from EMD Millipore (Temecula, CA, USA). Secondary antibodies, rabbit anti-IgG and anti-mouse IgG, were purchased from GE Healthcare UK Limited (Little Chalfont Buckinghamshire).

Experimental Animals

Male albino Sprague-Dawley rats were purchased at 5 weeks old from Charles River Laboratories, (Wilmington, MA, USA). All rats were kept at 12/12 reverse light cycle in temperature and humidity – controlled environment, single housed, with water and food ad libitum. Rats were housed individually in polycarbonate cages (l = 31 cm, w = 16 cm, ht = 13 cm) and were taken out of the cage for weight measurements and handling once per week. Controls (n=8; two did not survive) did not receive any treatment and were fed standard lab chow (Mouse Diet 9F, 5020) supplied by Lab Diet (St. Louis, MO, USA). WS group (n=9; one did not survive), received WS root powder orally (100 mg/kg), mixed in 2 g of an organic apple sauce as a vehicle throughout their life span daily [53]. The whole-food diet (WFD) group was fed a specialized rat chow "Fiesta Max" (Kaytee, Inc.), which contained many whole (unprocessed) foods, such as nuts, seeds, dried fruits and dried vegetables. "Fiesta Max" was introduced gradually into the rats' regimen one week after their arrival into the vivarium. The above regimen was supplemented with 2.5 g of fresh fruit and one vegetable per day, 5 times per week. The exercise group (n=10) had free access to running wheels in their cages. Finally, the combination group (WS + voluntary running exercise + whole food diet; n=10) received all three interventions in the manner described above. Throughout the 19-month daily dose of WS, no significant side-effects or adverse drug reactions (ADR) were observed, except weight gain and some porphyria around the eyes and nose, both of which may be because of aging and/or the

stress associated with living their entire lives in a little cage (unpublished observations).

Behavioral tests (see following) were conducted at 19 months of age, at the end of which, rats were anaesthetized using isofluorane and then immediately sacrificed by decapitation. Brains were excised and initially preserved at -70°C. All rats were handled in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and CSULA's Institutional Animal Care and Use Committee Protocol 13-2. All federal regulations such as the Public Health Service Policy, and the Good Laboratory Practice Act, Animal Welfare Act were followed.

Behavioral Tests

Novel Object Recognition Test – working memory

Novel object recognition test (NOR) has been designed as a measure of assessing working memory function and has been well validated using animal models [54,55]. Rat's behaviors were evaluated using the following measures: 1. Exploration time for each object (seconds); 2. Discrimination index (DI), which is the difference between time spent exploring novel and familiar objects during test phase divided by the total time spent on both objects [54]. The index value can vary from-1 (indication of preference for familiar object) to +1 (preference for novel object), with 0 indicating null preference [56,57]. A higher DI indicates better memory retention. Discrimination Index is a more sensitive measure, making it possible to adjust for differences in the rat's exploratory activity (total time spent on both objects) relative to the absolute time devoted to each object [54].

Barnes Maze Test - Spatial Memory: Barnes' dry-land Maze was developed by Carol Barnes in 1979 to assess spatial memory and learning ability in rats [58]. This test relies on rodent's natural tendency to escape bright light and exposed space, retreating into a dark, secluded space. No additional external stimulus is employed. Procedural protocol, adapted from Sunyer, et al. consisted of three phases: habituation (1 day), acquisition (7 to 8 days) and reversal (3 days) [58]. Briefly, habituation phase lasts a day and is used to introduce the rat to the table, the escape hole, for maximum of 3 minutes. During acquisition phase (8 days; 3 trials/day x 3 min/trial) animal is trained to use spatial memory to locate the target hole. During reversal phase (reversal day 1, 2 and 3; 3 trials/day x 3 min/trial), escape box location is moved 90˚ relative to the original position and extra-maze cues are included to guide the rat. Thus, spatial memory (short term and long term reference memory) and non-spatial cues are used by the rat for escape strategy. Primary latency to reach the escape hole, and the errors made (head pokes into the wrong hole) were measured for each trial via Noldus Etho Vision XT (Sacramento, CA) video tracking system. Results are reported as a mean day latency (sec/day) and the standard error of the mean (SEM), where a mean signifies the average of three trials/rat during that day.

Molecular Tests

Acetylcholine Esterase measurement: For detection and quantification of AchE in the frontal cortex, Ellman's spectrophotometric method was adopted [59]. Enzyme activity in samples was determined using a commercial colorimetric assay kit (Biovision, San Jose, CA, USA) that quantifies AchE activity in nmol/min/ml of wet sample. These values were subsequently normalized to total protein concentration in the samples previously obtained by Bradford total protein assay [60,61]. Final enzyme activity is expressed in nmol/mg.

Western blot – M_1GPCR, P-CREB: Thirty micrograms of rat frontal cortical tissue was applied in triplicate to 10% SDS-PAGE gels, followed by electro transfer of proteins to nitrocellulose membranes. Specific procedures and antibody dilutions were performed according to the manufacturer's instructions. Nitrocellulose membranes were then incubated with Western blotting enhanced chemiluminescence (ECL) detection reagents (Amersham, Pharmacia-Biotech, Piscataway, NJ, USA), followed by opposition to Hyperfilm (Amersham, Piscataway, NJ, USA). To control for inadvertent differences in protein loading and variability in transfer efficiency, all blots first probed with anti-M_1GPCR or anti-P-CREB were stripped and re-probed with anti-α-tubulin (1:10,000 dilution) or anti-CREB, respectively, in accordance with the manufacturer's instructions, followed by re-probing with anti-mouse IgG (1:1,000 dilution) again followed by ECL. Western blotting assays were performed at least twice.

Lightly exposed bands on Western blot films were quantified using MCID image processing system (St. Catherine's, Ontario, Canada) software, which measured optical density. All gray values were within the range determined by a standard curve. Optical densities of M_1Ach GPCR bands were divided by those of alpha-tubulin, whereas optical densities of P-CREB were divided by those of CREB.

Statistical Analysis

Two-way analysis of variance on SPSS 22.2 and GraphPad Prism 7.02 statistical software were used to analyze latency in Barnes Maze, time of novel object exploration while one-way ANOVA was used to analyze DI in NOR to assess the treatment effect on the cognition in the aged animals. One-way ANOVA followed by LSD test was performed to measure the effects of the treatments on AchE and the expressed amounts of M_1GPCR and P-CREB. Significance levels were set at $p < 0.05$. Values were expressed as a mean ± SEM.

Results

Novel Object Recognition Test–Short-Term Memory

Two-way analysis of variance (ANOVA, treatment x object), followed by least significant differences (LSD) multiple-comparisons test, revealed statistically significant effects of both treatment and object on the exploration time (Figure 1A). Significantly higher preferences for the novel object over the familiar one was demonstrated by WS, combo- and vehicle-treated animals. The results of a one-way ANOVA analysis followed by LSD multiple comparisons test revealed significant effect of the treatment on DI, a more sensitive measure normalizing for the total exploration time (Figure 1B). WS-treated rats showed significantly higher discrimination of the novelty than control ($p = 0.05$), running, combo- and WFD-treated groups, while the running group scored significantly lower than all groups.

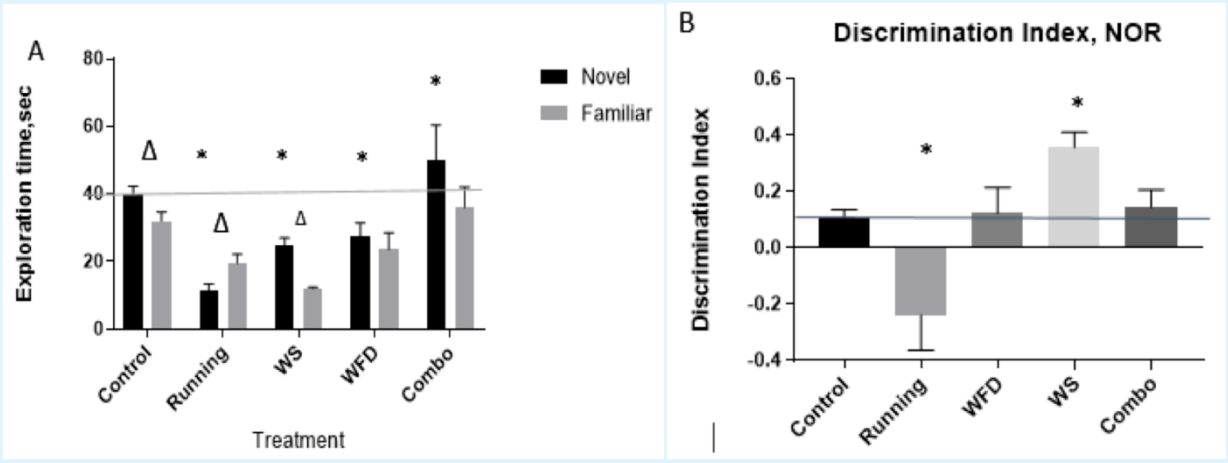

Figure 1: Novel object recognition test results. For both panels: Δ marks significant difference ($p \leq 0.05$) in exploration time between novel/familiar objects; * marks significant difference ($p \leq 0.05$) from controls in novel object exploration time/DI. Panel (A): Novel and familiar objects relative exploration times for each group. Results are reported in seconds as mean ± SEM, time spent exploring the novel object: controls, 39 ± 2 sec; running 11.4± 2.0 sec; WS, 26 ± 2 sec; WFD, 27.6±3.8 sec; combo, 51 ± 5.4 sec. The combo-treatment group explored novel object significantly longer than controls did ($p= 0.007$), whereas WS- ($p=0.001$), running- ($p = 0.036$) and WFD- ($p = 0.000$) treated groups showed significantly lower novel object exploration time than controls did. There is significant effect of both treatment ($F(4, 84) = 12$, $p < 0.0001$) and object ($F(1, 84) = 4.25$, $p = 0.038$) on exploration time. Significantly higher preferences for the novel object over the familiar one was demonstrated by three groups: controls ($p = 0.0263$), combo ($p = 0.015$) and WS ($p = 0.0001$); higher preference for familiar was demonstrated by the running group ($p = 0.003$). However, total mean exploration time of both objects by control (71 ± 4 sec) and by combo-treatment animals (87 ± 11 sec) was significantly higher ($p < 0.0001$) than that of WS- (38 ± 3 sec), running (30.9±2.4 sec), and WFD- (51±14 sec) treated rats. Panel (B): Discrimination Index (DI). One-way ANOVA followed by LSD multiple comparisons test revealed significant main effect of treatment ($F(4,42) = 6.68$, $p = 0.000$) on DI. WS-treated rats showed significantly higher discrimination of the novelty than did controls, running and WFD groups ($p = 0.05$), while the running group scored significantly lower than all other groups (difference from controls, $p = 0.007$). DI for controls (0.107 ± 0.03), running (-0.24±0.12), WFD (0.12 ±0.09), WS (0.358 ± 0.05), combo (0.146 ± 0.05).

Barnes Maze Test-Spatial Memory

Two-way ANOVA, repeated measures (treatment x trial day) in aged rats revealed statistically significant effect of treatment ($F(4, 462) = 65.3$, $p=0.000$) and trial day ($F(10, 462) = 15.24$, $p=0.000$)on the latency to locate the escape hole during acquisition (t)and reversal (rv)phases. Acquisition: two-way ANOVA revealed significant treatment effects ($F(4, 322) = 7.35$, $p<0.0001$) and trial effects($F(7, 322) = 17.61$, $p<0.0001$). Reversal: two-way ANOVA revealed significant treatment effects ($F(4, 136) = 11.8$, $p<0.0001$) and trial effects($F(2, 92) = 0.7406$, $p=0.048$). Rats' exploratory behavior throughout the duration of the experiment was significantly affected by the interaction between treatment x trial in the linear regression model ($F(40,517)=11.62$, $p <0.0001$).A significant effect of treatment and trial during both phases indicates that learning occurred for the experimental groups at different rates as well as that performance was affected by the reversal of the escape location. Moreover, each of the experimental group's behavior depended simultaneously on the trial day and the treatment they received; hence, the heterogeneity in latency among all groups. Significant distribution in latency, effectively the treatment effect, was observed on trial 3, where the combo-and WFD-treated groups found the escape hole faster than did the rest of the groups, whereas the running group took the longest time. That is, the running group maintained latency to locate the goal at a higher level until the end of the acquisition phase. WS-treated rats showed shorter latency than vehicle treated rats (controls) during the initial four trials of the acquisition, with fluctuating levels thereafter. The declining trend for the combo-treatment rats' latency to target continued into the 4th day where it significantly outperformed the other four groups. However, on day 5 of acquisition, combo-treated rats' latency exceeded that of the WFD-treated group and reached that of controls. On day 6, WFD-fed animals showed the shortest latency among all the groups on that day. During the final four trials (t5 - t8), combo-treated rats repeated the trend of the initial four trials decreasing the latency levels and ending well below that of WS- and running-treated groups; running animals showed the highest latency throughout the acquisition phase. Controls, WFD- and combo-treated groups performed very similarly on day 8. However, the treatment effect ($F (4, 42) = 40.25$, $p=0.0001$) on final trial 8 led to two distinct patterns: WS took as long as the running animals to locate the escape hole while WFD-treated and controls reduced their latency to levels comparable to that of the combo-treated group on t 8. Interestingly, the WS -treated group was significantly slower than controls or combo -treated group in finding the escape hole on the final day (t8). However, on the reversal day 1 (when hole location was moved 90° relative to the original), WS-treated group located the escape hole significantly faster than the combo-treated and control groups, and maintained this speed throughout the reversal phase. The similarity in performance shared between WS-treated and running groups was evident on t8 and rv1.The time running group took on rv1 was similar to the time WS-treated animals took; however running rats did not maintain the high speed, but rather, increased the latency. On the other hand, the combo-treated rats took significantly longer to find the escape hole on reversal day 1 than they did on the number of the acquisition days, as well as than both running and WS-treated groups took on rv1; combo-treated rats were also able to learn the new location at a rate comparable to that during acquisition, and significantly outperformed their controls, WFD- and running counterparts, but not WS-treated group. WFD-treated group performed behind controls on rv1 (highest time to target) and remained behind combo-group (but ahead of controls) until the end of the reversal phase. Effect of treatment on rv1: $F (4, 42) = 9.021$, $p < 0.0001$. Rv2 treatment effect: $F (4, 42) = 15.62$, $p<0.0001$.WFD, WS and combo are significantly different from controls ($F (3, 33) = 10.96$, $p<0.0001$). Rv3: effect of treatment: $F (4, 42) = 47.42$, $p<0.0001$. WS- and combo-treated are significantly lower than controls, $p =0.006$. Notably, the WS-, WFD- and combo-treated groups, but not controls and running, required only four trials of acquisition phase, rather than eight, to reach their respective lowest latency times.

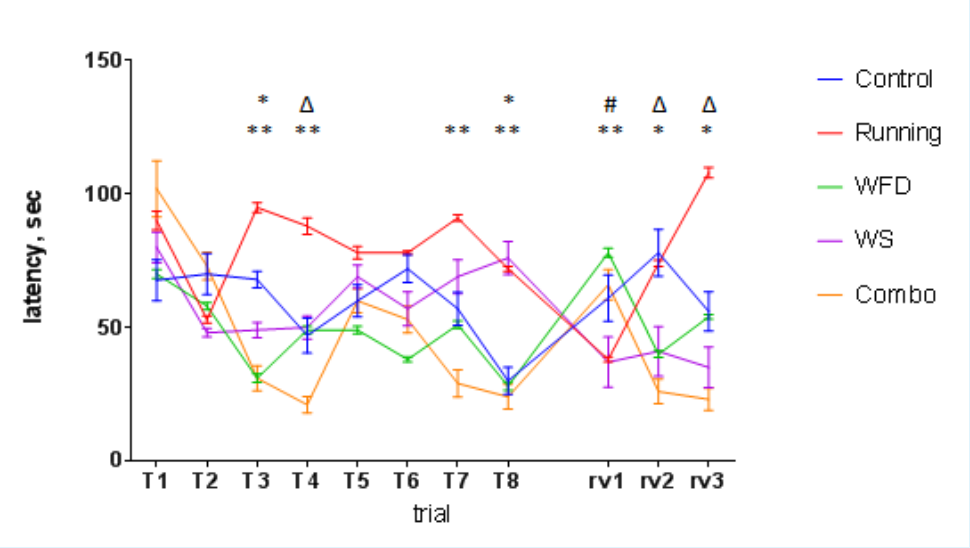

Figure 2: Barnes Maze learning curve for acquisition and reversal phases. A two-way ANOVA repeated measures revealed significant treatment effect on acquisition ($p < 0.001$) and reversal phases ($p < 0.001$). *, WS-treated group is different from controls; Δ, combo-treated group is different from control; **, WS-treated group is different from combo; #, difference from previous trials. Significant latencies distributions appear on the 3rdtraining trial: combination (31±4 sec) and WFD (31±1.2 sec) rats take significantly less time to find target than controls ($p < 0.001$,68± 3 sec) or WS ($p = 0.003$, 48±2.8 sec), WS shows lower latency than control ($p = 0.002$), running groups shows the highest latency 95 ±2 sec. 4th trial: combo-treated group is faster than controls and WS-treated ($p = 0.001$, 21 ±3 sec); running group is the slowest (88±3 sec). 7thtrial: combo treatment shows the smallest latency of 29±5 sec, the running group – the longest 91±1 sec. 8thtrial (last): WS/running are slower than control, combo and WFD-treated ($p < 0.000$). WS-treated performed significantly better on rv1 (rv2, rv3) than T8 ($p = 0.003$; 37 ± 9sec; 78 ± 8; 56 ±7sec), significantly better ($p = 0.0001$) than combo (66 ±6 sec), WFD (78±1.6 sec), control (61±8 sec). Running animals performed very similar to WS-treated on rv1, latency 38±1 sec. combo performed worse on rv1 than T8 ($p < 0.000$; 66 ±6 sec). End of reversal phase: combo and WS are faster than controls ($p = 0.002$ and 0.038, respectively), than running group ($p = 0.000$). Mean latencies, acquisition phase: T3: combo, 31± 4 sec; WS, 48± 2.8 sec; control, 68± 3 sec; WFD, 31±1.2 sec; running, 95± 2 sec. T4: combo, 21 ± 3 sec; WS, 47± 4 sec; control, 50±6.6 sec; WFD, 49± 1.45 sec; running, 88±3 sec. T8: combo, 24±4 sec; WS, 76± 6 sec; control, 30±5 sec; WFD, 28± 1.6 sec; running, 72±1 sec. Rv1: combo, 66 ± 6 sec; WS, 37 ± 9 sec; control, 61±8 sec; WFD, 78± 1.6 sec; running, 38±1sec.Rv2: control, 78 ± 8 sec; combo, 26 ± 4 sec; WS, 41 ± 5 sec; WFD, 40± 1.3 sec; running, 74±1sec. Rv3: combo, 23 ± 4 sec; WS, 35 ± 7.5 sec; control, 56 ±7 sec; WFD, 54± 1.0 sec; running,108±2sec.

Acetylcholine Esterase Measurement

There were significant effects of treatment on the levels of AchE activity within the frontal cortex of the experimental groups ($F(4,39) = 7.329$, $p<0.0001$). Animals with access to running wheels expressed the least amount of active AchE in the frontal cortex, being significantly less active than in controls($p < 0.0001$), WFD ($p< 0.044$) and WS ($p <0.0001$) groups (Figure 3). Rats fed whole food diet only during their life time at an advanced age showed enzyme activity higher than running animals ($p = 0.044$) yet significantly below that of WS-treated animals ($p =0.009$). AchE activity within the frontal cortices of WS-treated animals, on the other hand, did not statistically exceed that of controls; however WS-treated rats AchE activity is markedly higher than in running ($p < 0.0001$), WFD- ($p = 0.009$) and combo-treated ($p = 0.02$) groups. Lifetime treatment with combo effectively decreased active AchE, as compared to that in the WS-treated group possibly due to inhibiting effects of running and/or whole food diet. The major finding of this assay is that herbal treatment has no clear inhibitory and/or stimulatory effect on AchE activity in frontal cortex of aged rats. Additionally, the ability of running exercise to decrease enzyme activity in the frontal cortex independently and in combination is quite potent.

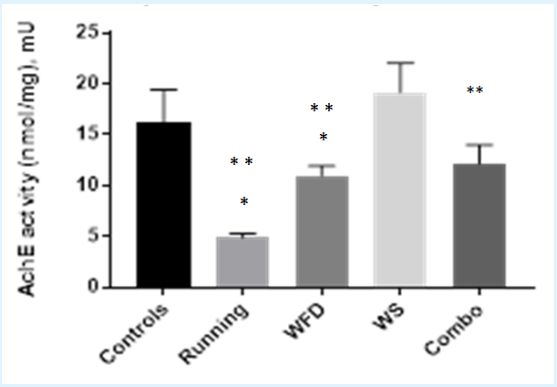

Figure 3: Acetylcholinesterase activity analysis. Statistically significant effect of treatment on enzyme activity within frontal cortices of aged rats was detected by one-way ANOVA and post-hoc ($F(4,39) = 7.329$, $p < 0.0001$). * marks significant difference from controls; **, from WS treatment. mean ± SEM in mU: controls, 16 ± 3 (n = 8); running, 4.915 ± 0.359 (n=10); WFD, 10.978 ± 0.9522 (n=8); WS, 19 ± 2.8 (n = 9); combo, 12.155 ± 1.83629 (n =9). Running exercise markedly decreased AchE activity from baseline control level ($p < 0.0001$). Combo ($p = 0.02$), running ($p < 0.0001$) and WFD ($p = 0.009$) levels of AchE were significantly lower than that of WS-treated group.

Western Blot – M_1GPCR, P-CREB

Expression of the protein was assessed by means of Western blot (Figure 4). One-way ANOVA with main effects of treatment on protein expression levels revealed that treatment had no statistically significant overall effect on M_1Ach GPCR immunoreactivity ($F(4,39) = 1.383$, $p = 0.257$) (Figure 4A).However, only running rats expressed significantly more receptor than the controls did ($p = 0.046$). WFD-, WS- and combo-treated (which includes running) groups did not have a significantly enhancing effect on the density of acetylcholine M_1Ach GPCR, which is believed to be one of the cognitive function mediators.

As it was anticipated, there were significant differences in levels of P-CREB in frontal cortex of treatment groups ($F(4,36) = 9.217$, $p < 0.000$).Running rats activated significantly more CREB than did each of the other four groups; in addition, the WFD-treated group exhibited significantly for P-CREB than did controls.

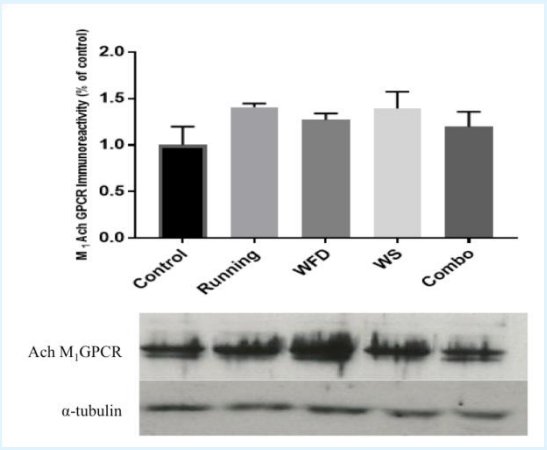

Figure 4A

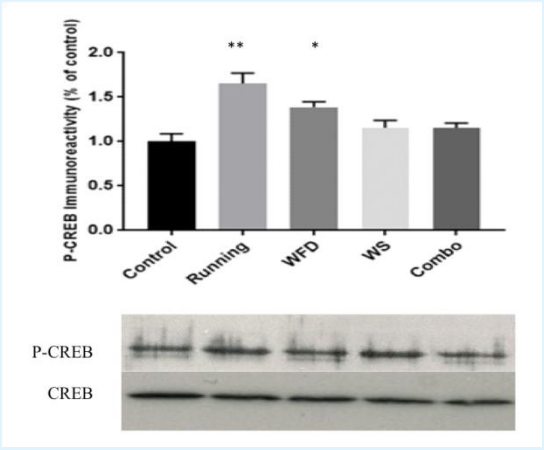

Figure 4B

Figure 4: Panel (A): Western blot: acetylcholine muscarinic GPCR receptor (type 1) in rat frontal cortex: There are significant treatment effects on the expression of M_1GPCR across groups($p = 0.001$). Post-hoc LSD specified higher receptor concentrations in the frontal cortices of running rats (n = 10) relative to (*) vehicle (n = 8) $p = 0.046$. Panel (B): Western blot: Phospho-CREB expression in rat frontal cortex, where P-CREB levels in the running group (** marks $p < 0.000$) and WFD-treated group (* marks $p = 0.003$) are significantly higher than in controls. The running group also activates significantly more CREB than do the WFD- ($p = 0.032$),WS- ($p < 0.000$) and combo- ($p < 0.000$) treated groups (not marked on the graph). Optical density values were normalized along a standard curve of gray values to correct for differences in film appearance.

Behavioral and Molecular Results are differentially Correlated

Correlation between DI in the NOR and latency to find the target hole in the Barnes Maze on the trial 4 indicated significant inverse correlation for all five treatment groups combined($p = 0.0008$, 2-tailed,$R^2 = 0.2241$, Figure 5, A1). Similar inverse correlation exists between DI (NOR) and latency to find target hole on Barnes Maze on reversal day 3 (final evaluation, Figure 2) ($p = 0.0003$, 2-tailed, $R^2=0.2597$, Figure 5, A2). Analogous analysis was performed to assess the correlation of DI vs P-CREB and M_1GPCR expression in rats' frontal cortices. Levels of M_1 ACh receptors were found to be positively related to the DI in NOR ($p = 0.0136$, 2-tailed, $R^2= 0.1365$, Figure 5, A3) and levels of P-CREB are correlated ($p = 0.0497$, 2-tailed, $R^2= 0.09515$, Figure 5, A4). Latency to find target on acquisition day 4 is positively related to P-CREB expression ($p = 0.0029$, 2-tailed, $R^2= 0.2051$, Figure 5, B1). On the other hand, latency to find target on reversal day 1 is negatively related to P-CREB expression in rats' frontal cortices ($p = 0.0304$, 2-tailed, $R^2= 0.1067$, Figure 5, B2). AchE activity in the frontal cortex is positively correlated to MıGPCR density ($p=0.03$, $R^2=0.2$; graph not shown) and to DI of NOR ($p=0.003$, 2-tailed, $R^2= 0.200$).

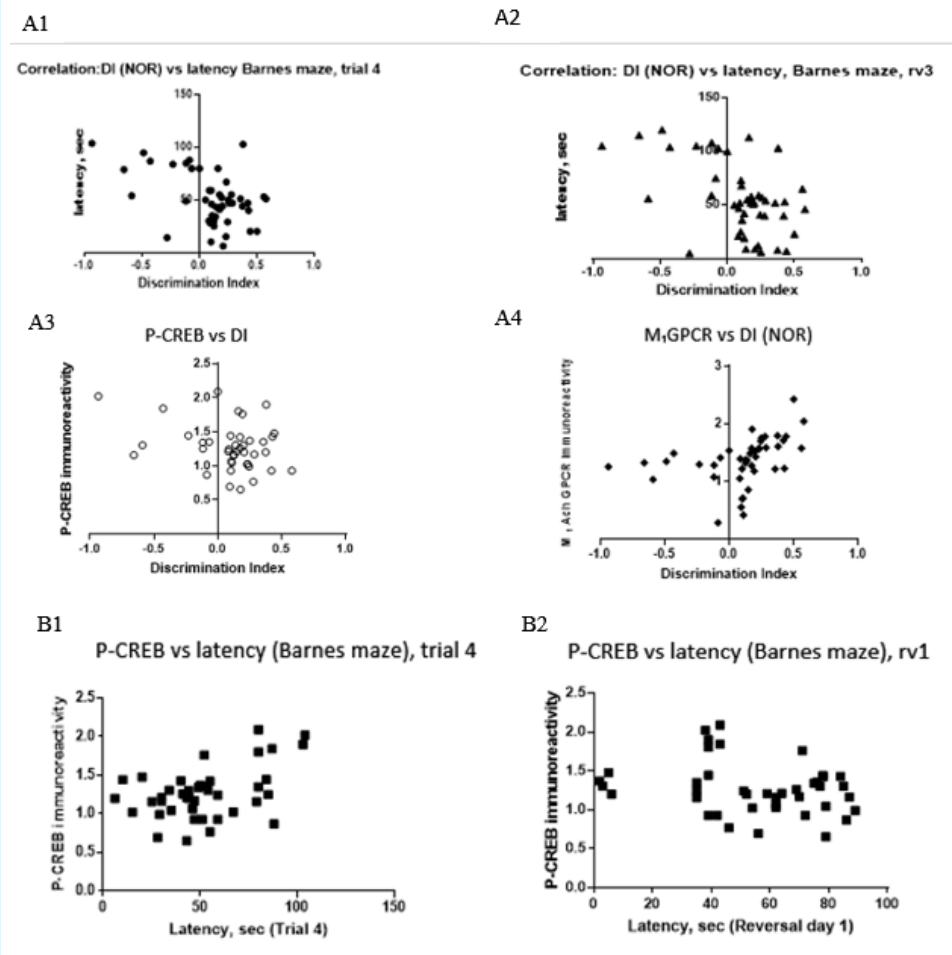

Figure 5: Panel (A1): Correlation between latency on trial 4 in the Barnes Maze and DI in NOR. A2: Correlation between latency on reversal day 3 (Barnes Maze) and DI (NOR). A3: levels of expressed P-CREB in frontal cortex vs DI (NOR). A4: levels of expressed M_1 GPCR vs DI(NOR). Linear model Regression analysis revealed (A1) significant inverse correlation $p=0.0008$ and (A2) $p =0.0003$; (A3) levels of P-CREB are negatively correlated, $p = 0.0497$ (2-tailed) (A4) significant positive correlation, $p = 0.0136$ (2-tailed). Panel (B1): Correlation between frontal cortex P-CREB expression and latency to find target (Barnes Maze, acquisition, trial 4), significant positive correlation, $p = 0.0029$ (2-tailed). B2: levels of P-CREB expression in frontal cortex are negatively related to latency to locate the escape hole (Barnes Maze, reversal day 1), $p = 0.0304$ (2-tailed). Linear model regression analysis at 95% confidence interval was used on all of the tests.

Discussion

Animals underwent two distinct behavioral tests designed to assess different cognitive functional levels: long-term working memory (NOR) on one hand, and spatial memory, spatial navigation ability, and mental flexibility (Barnes Maze) on the other. According to the results of NOR, long-term administration of WS and access to running wheels as separate interventions led to a significantly shorter total exploration time of both, familiar and novel objects. Rats that received combined treatment showed the longest total exploratory time. Total exploratory activity of the animal is a valuable index of cognitive function, because it reflects the long-term effects of treatments on the cognitive motivational processes and anxiety levels. However, it falls short, as it does not provide enough information to estimate recognition memory. Because the main focus of this test is the recognition (remembering) of the object, which is demonstrated by discriminatory behavior, DI was more critical in assessing the animals' memory of the object. Rats receiving WS herbal treatment showed better object recognition, as measured by DI, compared to that of control-, combination-, whole food diet-treated, and running groups. Inversely, life-long purely voluntary aerobic exercise led to the lowest among the experimental groups (below zero) DI, which is the sign of poorly functioning declarative memory. Given the similarity in the length of total exploratory time, the difference between WS (0.35) and running (-0.24) groups' DI is striking, as it serves to demonstrate the importance of DI in distinguishing simple motivation to explore the environment from mere recognition of the object; it also aids in depicting the independent nature of these two processes.

Furthermore, significant improvement in the ability of animals receiving combined treatment to find the target over the four initial consecutive days on Barnes Maze is indicative of a functioning spatial memory and learning rate. At the same time, the ability of running and WS-treated rats, unlike control and WFD, to successfully perform on the reversal phase (at a rate faster than acquisition days, e.g. on the first day) is suggestive of the employment of an effective egocentric (internal self-movement cues such as path integration) and allosteric (external using distal cues) spatial search strategy acquired by the animals during the training phase [62].

The observation that WS demonstrated the ability to locate the escape chamber on the reversal day 1 significantly faster than on the last day of training, and maintained this speed throughout the reversal phase, tentatively supports the fact that learning for WS rats occurred before the official end of the acquisition phase (T8), and certain spatial navigation strategies were readily in use. Additionally, ability to incorporate new external cues into the spatial leaning on the reversal day 1 are suggestive of the mental flexibility beyond simply good memory. At the same time, significant, but short-lived, improvement in latency to locate the escape chamber by running group on the first day of reversal, as compared to any day of the acquisition phase, may indicate the anxiogenic nature of the target reversal, more so than the task of running on the platform surface for seven consecutive days with target location being constant. This rather successful performance by the running group on the reversal day 1 superimposed on to the continuous highest latency levels throughout the acquisition phase lead us to believe that besides the spatial learning hippocampal- dependent component, there is also the possibility of being faced with a combination of hippocampal-independent abilities and motivations [63]. Additional analysis of the learning curves suggests that combo-treated animals immediately attained significantly shorter latencies (than all groups with exception of WFD on t5, t6) during the training phase; however, they performed far from perfectly on reversal day 1. On the other hand, during training, WS rats showed less fluctuating learning curve. Moreover, WS animals showed more superior navigation skill (shorter latency and a stable curve) during reversal phase. Striking differences in regards to finding the target hole emerge for groups on day 1 of reversal phase. Ability to master the navigation during the training phase, to remember the specific location of the invisible target is controlled by the hippocampal and entorhinal regions of the brain; while mental flexibility required for successful adaptation to the reversal conditions is the domain of pre-frontal cortex [36,64-69]. This data, therefore, may be useful in pinpointing the target brain areas of herbal supplement alone and in combination with exercise and a healthy unprocessed diet. Moreover, performance on Barnes Maze may also is influenced by a number of noncognitive factors such as anxiety, thigmotaxis, immobility, or exploratory activity [70-72].

WS treatment did not significantly increase the concentration of M_1GPCR, nor did it significantly affect AchE activity in the frontal cortex of the aged rats (notice the upward trend that fails to reach significance), nor alter expression of P-CREB in this brain region. Hence, the mechanism of action by which WS administered alone improves declarative and special memory function

remains unclear. The observed positive correlation between M1ACh receptor concentrations and AchE activity in the frontal cortices of animals leads us to believe that the levels of these two parameters may be somehow correlated via the compensatory mechanism: levels of AchE rise adapting to up-regulation of muscarinic receptor to avoid overstimulation [23]. Unlike severely demented, pathology-free aged animals do not display cholinergic deficit: upregulation of acetylcholine receptors is a simple consequence of the elevated acetylcholine concentration, which requires higher AchE activity [17,74-76]. These apparently conflicting results may be explained by supposition that AchE changes its activity with respect to the levels of acetylcholine, which, in turn, is tightly regulated by the necessity to simultaneously perform cognitive, immune and physical activities [76]. While inhibition of AchE is used to rescue the neuronal function in Alzheimer's Disease-affected basal forebrain, non-pathological brains do not necessarily require inhibition of AchE to cognitively function; conversely, AchE may rise in response to elevated release of acetylcholine [19,75-79]. Thus, our hypothesis about the inverse correlation between memory and AchE activity may be untenable.

Our initial hypothesis relied on the potency of WS to enhance/mimic the effects of cholinergic binding without affecting the levels of acetylcholine itself. Contrary to expectations, these results did not help us introduce cholinergic receptors into the breadth of the possible physiological targets for WS's biologically active compounds and mechanism of action remains unclear. Nevertheless, improved memory in animals receiving WS over their life span, is suggestive of a protective role of WS against CNS age-related decline. Our supposition is based on a positive correlation between the amounts of M_1 subtype of muscarinic receptor and long-term memory function because Hu, et al. had previously reported that in normally aged rats that were treated with an extract of sarpogenin from the Chinese medicinal plant *Rhizomaanemarrhenae*, the memory impairment closely resembled cholinergic system damage [80].

In the current study, the increased P-CREB levels within the frontal cortex in response to long-term exercise stands out in stark contrast to shorter term hippocampal *in vivo* and *in vitro* studies using brain slices to evaluate long-term potentiation and tissue culture [23,81].

The results of our study clearly support a role of voluntary aerobic exercise in activating one important element of the cholinergic signaling pathway – the M1GPCR. Importantly, the memory-enhancing role of herbal supplement, both alone and in combination with exercise, has been supported on the behavioral levels by two tests designed to evaluate spatial memory and long-term memory. No synergistic effects, however, were among WS, physical exercise and a healthy diet on the molecular measures; as a result, this part of hypothesis was refuted.

Limitations of the Study

The current study suffers from some limitations: (1) because WS was administered in organic apple sauce as vehicle, an additional control group receiving only the organic apple sauce should have been included. This might have shed some additional light on the mode of action if the vehicle itself is at least partially responsible for the observed effects, inasmuch as it might have several chemicals, such as polyphenols, that could have interfered with the compounds in WS itself. (2) In light of the putative benefits that exercise has on cognition and memory, unlimited access to running wheel might have masked or even limited the beneficial effects of WS. It might have been, therefore, more prudent to limit their exercise activity. (3) Although we used frontal cortices in which to evaluate AchE, M1GPCR and P-CREB, it might have been more appropriate to use some of the forebrain areas known to be involved in aging and neurodegenerative diseases to study cholinergic signaling.(4) Arguably not necessarily a limitation, although the current study shows that WS root powder is beneficial in recognition memory, it is still not known which component(s) of this highly heterogeneous mixture is responsible. A recent study reported increased neuronal survival signaling in the presence of withanolide A, a putative active ingredient of WS [82]. Thus, possible interaction of two or more active ingredients may undermine accurate determination of the duration or half-life of any one chemical within the complex mixture of WS.

Conclusions

While it is evident from our long-term study that WS may help to prevent age-associated behavioral deficits, clearly, more studies are needed to reach a firm conclusion regarding its complex physiological mechanism of action. Additional support for the hypothesis that cholinergic signaling pathway is involved in the observed behavioral effects would come from utilization of specific inhibitors administered during behavioral test in addition to the current treatments:

AchE inhibitor physostigmine and muscarinic acetylcholine receptor antagonist scopolamine; both administered by injection [83]. This might allow us to evaluate the degree of acetylcholine involvement and gain insight into how WS works both alone and in combination with wholefood diet and voluntary exercise. This long-term study yielded some valuable information in regards to the role of WS and aerobic exercise in improving memory function and spatial learning capability in aged rodents. It provides a solid foundation for further investigation into the implications that WS might have for the neuroprotection of the aging organism.

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