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Beneficial effects of *Spirulina platensis*, voluntary exercise and environmental enrichment against adolescent stress induced deficits in cognitive functions, hippocampal BDNF and morphological remolding in adult female rats

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ABSTRACT

Chronic exposure to stress during adolescent period has been demonstrated to impair cognitive functions and the dendritic morphology of pyramidal neurons in the rat hippocampal CA3 area. The present study investigated the combined protective effects of *Spirulina platensis* (SP), a supplement made from blue-green algae with neuroprotective properties, voluntary exercise (EX) and environmental enrichment (EE) against cognitive deficits, alternations in hippocampal BDNF levels, and abnormal neuronal remodeling in adult female rats (PND 60) induced by exposure to chronic restraint stress during adolescent period (PND 30–40). Rats were exposed to restraint stress (2 h/day for 10 days, PND 30–40). Then, the animals were subjected to treatment with SP (200 mg/kg/day), EX, EE and the combined treatments (SP + EX, and SP + EE) between PND 41 and 55 of age. Following the interventions, spatial learning and memory, passive avoidance performance, hippocampal dendritic morphology and BDNF levels were assessed. Results showed that plasma corticosterone levels increased at PND 40 and remained elevated at PND 55 and 70 in the stressed rats. Stressed rats showed deficits in spatial learning and memory and passive avoidance performance, decreased BDNF levels in the hippocampus, and reduced apical dendritic length and branch points of the CA3 pyramidal neurons. These deficits were alleviated by the SP, EX and EE, and the combined treatments, which accompanied with a decline in serum corticosterone in stressed animals. Some treatments even enhanced cognitive functions, and BDNF levels and neuroanatomical remodeling in the hippocampus of non-stressed animals. Our findings provide important evidences that physical activity, exposure to EE, and the SP treatment during adolescent period can protect against adolescent stress induced behavioral, biochemical and neuroanatomical impairments in adulthood.

1. Introduction

Under normal conditions, stress is the response of an organism to a threat, to maintain body homeostasis (Radley et al., 2005). However, upon the exposure to stress for a long period, the central nervous system can be cumulatively damaged (De Kloet et al., 2005). Activation of the hypothalamus-pituitary-adrenal (HPA) axis during stressful situations leads to release of glucocorticoids (GCs), which involve in regulating various physiological functions (Akirav and Maroun, 2013) by modulating physiological and behavioral processes (Joëls et al., 2006). Chronic stress has been shown to emerge detrimental effects on learning and memory (Yuen et al., 2012). Changes in spatial learning as a result of morphological changes in the various hippocampal regions are linked to the prolonged period of stress (Conrad, 2006; Tata and Anderson, 2010). Behaviorally, both human and animal studies have found the impairment of various hippocampal-dependent memory tasks during exposure to stress (Kim et al., 2006).

Morphology of cortico-limbic structures including the hippocampus,

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amygdala, and prefrontal cortex seems to be especially sensitive to the profound effects of stress (Cook and Wellman, 2004; Vyas et al., 2002). Chronic stress produces alterations such as the retraction of the apical dendrites in the CA3 region of the hippocampus (Conrad et al., 2007). It also induces the modification in hippocampal dendritic length and branching points (Donohue et al., 2006; McLaughlin et al., 2005). These modifications correspond to the deficits expected from impaired hippocampal function (Starkman et al., 2003). Further, repeated stress in rats has been shown to lead to reduced cell proliferation and neuron number in the dentate gyrus along with reduced its volume (Pham et al., 2003).

Exposure to stress during adolescent phase has many effects on brain development, and these effects may include learning and memory deficits in adulthood (Brown et al., 2013; Brydges et al., 2014b; Dallé and Mabandla, 2018). There is evidence suggesting that early exposure to stressors leads to long-term changes in the BDNF pathway (Jawahar et al., 2015; Murgatroyd et al., 2015). BDNF is the most abundant neurotrophin in the brain that is involved in neural development, neuronal plasticity and neuroprotection and it has a critical role in dendritic remodeling in both hippocampus and BLA (Barha et al., 2011; McEwen et al., 2016). Previous studies have shown that stress through reducing the expression of some genes such as BDNF gene causes a series of physiological and pathophysiological changes, which can provide the basis for the development of degenerative brain diseases (Vollmayr et al., 2001).

Chronic stress exposure is closely related to the development of psychiatric disorders that occur more frequently in woman than men (Bangasser and Valentino, 2014). Animal studies demonstrate a similar female sensitivity to stress. For example, it has been shown that female rats exposed to chronic adolescent stress showed decreased sucrose consumption, hyperactivity in the elevated plus maze, decreased activity in the forced swim test, and a blunted corticosterone response to an acute forced swim stress than controls during both adolescence and adulthood. Male littermates exposed to chronic adolescent stress did not manifest significant changes in behavior as adolescents or adults (Bourke and Neigh, 2011). Pre-pubertal stress has been shown to impaired contextual fear responses in males and enhanced performance in spatial navigation in females in adulthood, showing sex-specific effects of pre-pubertal stress on hippocampal-dependent behaviors (Brydges et al., 2014b). It is now well accepted that females typically have higher baseline and stress hormone responses (Kajantie and Phillips, 2006; Rhodes and Rubin, 1999; Wang et al., 2007).

There is a growing body of information about the positive effects of exercise on cognitive functions in humans and experimental animals (Ghodrati-Jaldbakhan et al., 2017; Kramer et al., 2006). Exercise regimens as a non-pharmacological treatment of stress have been used recently to improve neurological deficits (Kramer et al., 2006; Penedo and Dahn, 2005) levels of BDNF (Vaynman et al., 2004), neurogenesis (Van Praag et al., 2005), synaptic plasticity (Patten et al., 2013) and also learning and memory (Niehues da Cruz et al., 2012; Vaynman et al., 2004). Recently, studies have focused on the enhancing effects of exercise on cognition, specifically the combination of exercise and dietary management as an effective strategy to treatment of cognitive disorders (Gomez-Pinilla and Hillman, 2013).

Besides exercise, environmental enrichment (EE) has been demonstrated to improve the detrimental effects of prolonged stress on hippocampal structure (Will et al., 1977; Winocur, 1998). EE can improve chronic stress-induced spatial memory deficits when started during adulthood (Wright and Conrad, 2008). EE has also been shown to exert profound effects on brain function in anatomical and molecular levels (Baroncelli et al., 2010) and leads to a significant change in the expression of the genes involved in neuronal structure, synaptic transmission and plasticity (Rampon et al., 2000). Several studies have also shown that EE enhances hippocampal neurogenesis and long-term potentiation (LTP), as the cellular basis for memory formation (Ahmadalipour et al., 2018; van Praag et al., 2000).

Importantly, the effects of exposure to EE as a non-invasive therapy in producing robust changes in neuronal morphology and behavior are well documented (McCreary et al., 2016). For example, stress causes dendritic shrinkage and loss of branch points in the hippocampus (McEwen et al., 2016) or modification of dendritic spine number and shape (Diamond et al., 2006; McLaughlin et al., 2005), and these consequences were rescued by EE. Beneficial effects of EE are powerful to restore dendritic and synaptic morphology (Peng et al., 2011), and lead to the larger cell proliferation and neuronal density (van Praag et al., 2000).

Current research is focused towards the discovery of new substances with neuroprotective properties with little or no side effects. *Spirulina*, a supplement made from blue-green algae, is well known to contain various antioxidants, especially phycocyanin and basic necessary nutrients has been used as a food source (Capelli and Cysewski, 2010). Some studies suggest that *Spirulina* has the protective effects in the treatment of neurodegenerative disorders (e.g., Parkinson, and Alzheimer diseases (Chattopadhyaya et al., 2015; Koppula et al., 2012; Pabon et al., 2012)). *Spirulina* exerts a significant protective effect on hippocampus neural progenitor cells against lipopolysaccharide induced acute systemic inflammatory (Bachstetter et al., 2010). *Spirulina* also reduces oxidative stress in the hippocampus and protects against damaging neurobehavioral effects of systemic kainic acid (Pérez-Juárez et al., 2016). In recent years, *Spirulina* has gained more attention from medical scientists for the treatment of a wide spectrum of diseases. Considering that *Spirulina* presents neuroprotective properties (Ozdemir et al., 2004; Romay et al., 2003), it would be an interest to study its effects against chronic stress induced behavioral and biochemical abnormalities.

The aims of the present study were to investigate the effects of *Spirulina platensis* (SP), EE, and EX and their combined interventions on behavioral, biochemical and morphological alternations induced by adolescent stress in adult female rats. Findings of the present study are important to open new therapeutic ways against the harmful effect of stress on brain functions and morphology.

2. Materials and methods

2.1. Animals

Wistar female pup rats (30 days old, 60–70 g) obtained from the breeding colony of the Semnan University of Medical Sciences (SUMS), Semnan, Iran. Animals were maintained at 12-h light/dark cycle and in a controlled room temperature (22 ± 2 °C) and, housed 5 to 6 per cage given free access to food and water ad libitum. All experiments were conducted between 10:00 and 12:00 h. The Animal Ethics Committee of Semnan University of Medical Sciences (SUMS) (IR.SEMUMS.REC.1394.208) approved all experiments. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

SP (Setendorf Company, Jask, Bandar Abbas, Iran) was dissolved in distilled water shortly before use and administrated at a dose of 200 mg/kg per day by oral gavage for 15 days. The control rats received distilled water alone and solutions were refreshed daily. This dose of SP was chosen based on dose-response effects of SP on inhibitory avoidance memory in the present study.

2.3. Stress induction and corticosterone measurement

Starting on PND 30, rats were exposed to a 2-h restraint stress between 10:00 AM and 12:00 AM for 10 days (PND 30–40) (Nibuya et al., 1999; Vyas et al., 2002). During the restraint, each rat pup was restrained in a clear polyethylene cylinder. To achieve complete

immobilization, the cylinder size was adjusted according to the size of the pup rats. A hole in the front of the cylinder was set to allow the animals to breathe freely.

For corticosterone levels measurement, three blood samples were obtained from tail of conscious rats at the PND 40 (after the termination of stress), PND 55, and in PND 70 (adulthood). Blood was collected in tubes and centrifuged (3000 ×*g*, 20 min) and the plasma was stored at −70 °C until used for the corticosterone assay. All samples were collected between 10 AM and 1 PM. Serum corticosterone levels were measured using the commercially available ELISA kit (E20160505043, Hangzhou Eastbiopharm Co. Ltd., Zhejiang Province, China) following the manufacturer's instructions. The sensitivity of the assay was 2.51 ng/ml.

2.4. Voluntary wheel running exercise

Each of the exercising rats was given all day/night access to a cage that was equipped with a running wheel. An electronic system that linked to the running wheel (model 2021.PN) automatically recorded animal movements (Tajhiz Gostar Company, Tehran, Iran). The running wheels were removed from the cages after the 15 days period (PND 41–55). The corresponding sedentary rats were confined to similar cages with no access to a wheel.

2.5. Environmental enrichment

Rats were randomly assigned to the Plexiglas cages or EE (100 cm × 100 cm × 50 cm) (10 animals per cage) equipped with the running wheel, a raised platform and enriched with a complex of plastic tunnels, steel chains, plastic balls and toys in different size, which were changed every 5–6 days. Changing the location of objects produces novelty and excites the rats exploring sense. EE housing began after exposure to chronic stress before adulthood from PND 41–55.

2.6. Inhibitory avoidance training

For evaluation of associative memory in rats, inhibitory avoidance training was conducted using a shuttle box (two equally sized light and dark compartments, which was separated with a guillotine door between them) (Technique Azma Company, Tabriz, Iran). The floor of the apparatus was embedded with parallel stainless steel bars (3 mm in diameter), which were separated by a distance of 1.2 cm, and connected to an electric shock generator. At first, all experimental animals became habituated to the equipment without shock delivery. For habituation, animals were placed in the light compartment, facing away from the moving door, and 5 s later, the guillotine door was raised. Upon entering the rat into the dark chamber, the door was closed and then animal was returned into the home cage. Thirty minutes later, the habituation trial was repeated. The acquisition trial was performed followed by the same interval, and animals received a 0.5 mA constant shock for 3 s immediately after entering to the dark compartment. After 10 s rat was returned to home cage. The acquisition trial was finished if rat remained in the light compartment for 120 consecutive seconds. Two days later, the rat was placed in the light chamber and the guillotine door opened 5 s later and the latency of entering the dark compartment (step-through latency, STL) and the time spent there during 10 min were recorded. 600 s was considered as the cutoff point to entering into the dark chamber and was recorded if occurred.

2.7. Morris water-maze

Water maze (WM) apparatus comprises a black circular pool (120 cm in diameter and 60 cm high), was used to testing of spatial learning and memory (Manufacturing of Technique Azma Company, Tabriz, Iran). The pool is filled with water to a depth of 25 cm at a temperature of 22 °C. A Plexiglas platform (9 × 9 × 9 cm) was placed in the center of one of the four cardinal points of the compass (N, E, S, and W) approximately 1 cm below the water surface. Visual cues comprise several wall posters installed in the room. The whole experiment carried out in three phases: (A) Habituation: 24 h before training, rats were allowed to swim in the pool without the escape platform for 5 min. (B) Training: In this phase the position of the platform was fixed throughout the experiments. Animals randomly were placed in the pool from one of the four quadrants to swim until it climbed onto the escape platform and then stay on the platform for 10 s. If rats did not spontaneously find the platform during the 60 s, they were gently placed on it at the end of the trial. The variables such as escape latency to find the platform, the total distance, swimming velocity and the percentage of time the animal passes through each quarter of the pool was measured. Training procedure for each rat was repeated for 4 sessions (days) and each session occurred over the 4 trials in a day. After the 4th trial in each session, the animal was towel-wiped and returned to the home cage under a heat lamp. (C) Retrieval test: The day after the last training session, a probe test was performed to assessment the spatial memory. In this test the platform was removed, and each rat was released into water for 60 s. The swimming velocity, relative time spent in the target quadrant and opposite quadrants and the latency to reach the platform location for first time were measured (Maei et al., 2009).

2.8. BDNF measurements

The rats were decapitated, and the right hippocampus was dissected and was then immediately frozen at −70 °C until used for preparation of homogenates with a homogenizer (Polytron PT 2100, KINEMATICA AG, Switzerland). The tissues were homogenate in cold lysis buffer and the supernatants, which were obtained after centrifugation at 12,000*g* for 20 min at −4 °C. The BDNF protein levels were assessed using Rat BDNF ELISA kits (Hangzhou Eastbiopharm Co., LTP) according to the manufacturer's recommendations. The sensitivity of the assay was 0.01 ng/ml. The level of total protein in supernatants was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.9. Golgi histology and dendritic analysis

At the end of behavioral tests, all groups of rats were sacrificed under deep anesthesia with isoflurane decapitated, and then unperfused brains were rapidly removed. The block containing the dorsal hippocampus was dissected and processed for rapid Golgi staining methods according to procedures described previously (Vyas et al., 2002). Previous studies have shown that dendrites of the CA3 pyramidal neurons are the most sensitive to chronic stress than the dendrites of the CA1 or dentate gyrus neurons (Magarin and McEwen, 1995; Watanabe et al., 1992); thus, the present study examined apical dendritic length and branch points of pyramidal neurons in the CA3. The dorsal hippocampus (Bregma −2.6 to −4) was cut into 120-μm-thick sections in the horizontal plane using a sledge microtome. Sections were collected serially, dehydrated in absolute alcohol, cleared in xylene and coverslipped. Slides were coded prior to the quantitative analysis, and the code was broken only after the analysis was completed. Neurons were selected based on the following criteria: (1) cell was located in the CA3 region of the hippocampus; (2) cell was relatively isolated from the surrounding neurons; (3) cell body and dendrites were fully impregnated and untruncated. The dendrites of pyramidal neurons were defined by the presence of two or more basilar dendritic arbors, a distinct, single apical dendrite extending from the apex of the soma towards the pial surface of the cortex, and dendritic branch points (Cook and Wellman, 2004; Wellman, 2001). In six sections evenly spaced through the entire dorsal hippocampus, all pyramidal neurons meeting these criteria were identified. Each selected neuron was traced at 400× magnification using a light microscope with a camera lucida

drawing tube attachment.

Analysis of the apical dendritic length and branch points of pyramidal neurons in the CA3 area was performed using an image J (1.48 version) software. For each animal, an average apical dendritic length and branch points within a 120-μm thick section of each dendritic tree of 6–8 randomly selected pyramidal neurons was calculated. From each experimental group, five animals randomly were selected for morphology analysis.

2.10. Experiments

2.10.1. Experiment 1: effect of adolescent chronic stress on plasma corticosterone levels

This experiment was designed to determine the effect of 10 days restraint stress during adolescent phase on corticosterone levels. Rats were divided into two control (no-stress), and restraint stress groups ($n = 10$ in each group). Corticosterone levels were measured on after the termination of stress in PND 40 and on the last day of interventions in PND 55 and again in adulthood (PND 70) as methods described the above.

2.10.2. Experiment 2: effect of different doses of SP on passive avoidance performance

This experiment was designed to determine the dose-response effects of SP on passive avoidance performance, and to find the most effective dose of SP for the next experiment. Rat pups (PND 41) were divided into control and treatment groups. The treatment groups received doses of 50, 100, 200 and 400 mg/kg SP for 15 days (PND 41–55) by gavage tube. The control group received the same volume of saline. Then, passive avoidance training and testing was examined in adulthood (PND 60) according to the procedures described the above.

2.10.3. Experiment 3: effect of adolescent chronic stress on cognitive functions, brain BDNF and neuronal morphology of the hippocampus: beneficial effects of SP, EX, EE and the combined treatment

The aim of this experiment was to examine the effects of adolescent chronic stress on spatial learning and memory, emotional memory, and neuronal morphology and BDNF levels in the hippocampus and therapeutic effects of SP, EX, EE, and the combined treatments against chronic stress. Rats randomly were distributed into 12 following groups (10 animals in each group). Control group: rats received only oral physiological saline using gavage tube; EX group: rats exposed to the running wheel for 15 days; SP group: rats received 200 mg/kg SP per day for 15 days using gavage tube, EE group: rats exposed to the enriched environment; SP + EX group: rats exposed to the running wheel and received SP; SP + EE group: rats exposed to the enriched environment for 15 days and received SP; Stress group: rats exposed to 15 days restraint stress; Stress + SP group: rats exposed to stress and then received SP; Stress + EX group: rats received stress and then exposed to the running wheel; Stress + EE group: rats received stress and then exposed to EE; Stress + SP + EX group: rats received stress and then exposed to the running wheel and SP; and Stress + SP + EE group: rats received stress and then exposed to EE and SP. On PND 55, a blood sample was taken from the tail of the half animals of each group to measure corticosterone levels. From PND 60, all groups were subjected to passive avoidance and spatial tests. After behavioral testing, animals were decapitated, and their brains removed, and morphology of the CA3 pyramidal neurons (dendritic length and the number of branch points) was examined (Fig. 1).

2.11. Statistical analysis

Results are expressed as mean ± SEM. Data from Experiment 1 were analyzed by two-way repeated measures ANOVA. Data from Experiment 2 were analyzed using one-way ANOVA. Data of passive avoidance, spatial memory, BDNF levels and dendritic length and branch points form Experiment 3 were analyzed with two-way ANOVA, and data of spatial learning were analyzed with a three-way ANOVA with repeated measurement on training days. In all cases, Tukey post hoc test was used for multiple comparisons. The accepted level of significance for all tests was $P < 0.05$. Effect sizes were determined by eta squared for ANOVA results and Cohen's d analysis was used to evaluate the effect sizes between all pair-wise comparisons, which is the difference between means divided by standard deviation.

3. Results

3.1. Experiment 1

Two-way ANOVA on corticosterone levels (Fig. 2) across three time points (PND 40, 55 and 70) revealed significant effects of stress ($F_{1,18} = 145.51$, $P < 0.001$, $\eta^2 = 0.55$), of days ($F_{2,36} = 3.645$, $P < 0.05$, $\eta^2 = 0.16$) and a significant interaction between two factors ($F_{2,36} = 3.824$, $P < 0.05$, $\eta^2 = 0.17$). Paired comparisons showed that corticosterone levels in the restraint group was significantly higher than the control group in PND 40 ($P < 0.001$, d = 2.01), PND 55 ($P < 0.001$, d = 2.89) and PND 70 ($P < 0.01$, d = 2.29).

3.2. Experiment 2: dose-response effects of the SP treatment on passive avoidance performance

Fig. 3 shows the dose-response effects of SP on passive avoidance performance. One way ANOVA showed a significant difference among the groups ($F_{4,45} = 8.965$, $P < 0.001$, $\eta^2 = 0.44$). Between-group comparisons showed the SP at doses of 200 mg/kg ($P < 001$, d = 6.40), and 400 mg/kg ($P < 0.01$, d = 5.80) significantly improved memory retention than the control group. The difference of 50 mg/kg SP with doses of 200 mg/kg ($P < 0.01$, d = 5.99) and 400 mg/kg ($P < 0.01$, d = 5.39), and of the dose of 100 mg/kg SP with 200 mg/kg SP ($P < 0.05$, d = 4.24) was significant. Thus, the dose of 200 mg/kg SP was chosen for the next experiment.

3.3. Experiment 3

3.3.1. Passive avoidance testing

The results of the STL data collected during the retention test are shown in Fig. 4. An ANOVA revealed a main significant effect of stress ($F_{1,108} = 8.624$, $P < 0.01$, $\eta^2 = 0.22$), of treatment ($F_{5,108} = 48.889$, $P < 0.001$, $\eta^2 = 0.64$), and an interaction between the treatment and group ($F_{1,108} = 10.928$, $P < 0.01$, $\eta^2 = 0.52$). The post hoc analysis revealed that the STL of the stress group was significantly lower than the control group ($P < 0.01$, d = 1.83), showing that stress leads to passive avoidance memory impairment, whereas treatments with SP ($P < 0.001$, d = 5.79), EX ($P < 0.001$, d = 4.57), and EE ($P < 0.001$, d = 4.31) individually or the combined SP + EX ($P < 0.001$, d = 7.45) and SP + EE ($P < 0.001$, d = 9.61) significantly increased the STL in the stress group. The STL of the EE ($P < 0.001$, d = 9.72), SP ($P < 0.001$, d = 8.76), SP + EX ($P < 0.001$, d = 5.32) and SP + EE ($P < 0.001$, d = 6.25) groups was significantly higher than the control group, suggesting that SP, housing in EE or the combined treatment improved passive avoidance memory.

3.3.2. Spatial learning and memory

Escape latency data of the experimental groups during the 4 days of training in the water maze are illustrated in Fig. 5. All groups learned to locate the platform during the 4 successive days of training as indicated by decreasing escape latencies over the progression of training. Mixed repeated measure ANOVA on latency to find the platform showed a significant effect of day ($F_{3,324} = 115.383$, $P < 0.001$, $\eta^2 = 0.65$), a significant effect of stress ($F_{1,108} = 29.683$, $P < 0.001$, $\eta^2 = 0.56$), a significant effect of treatment ($F_{5,108} = 30.144$, $P < 0.001$, $\eta^2 = 0.30$). The interactions between stress and treatment ($F_{5,108} = 41.01$,

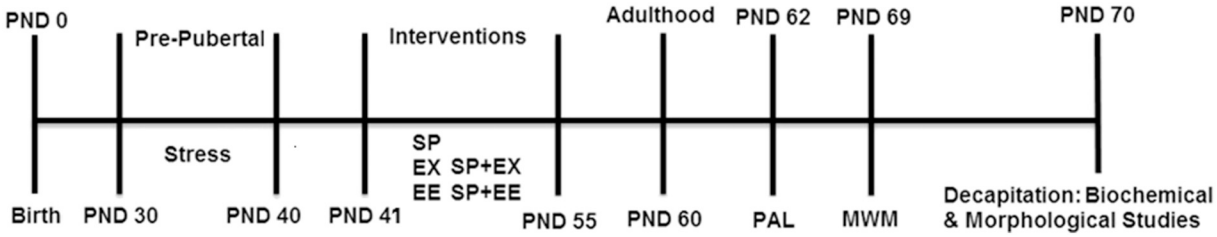

Fig. 1. Timeline of Experiment 3. Rats were exposed to restraint stress (2 h/day for 10 days, PND 30–40). Then, the animals were subjected to different treatments between PND 41 and 55 of age. Following the interventions, passive avoidance performance, spatial learning and memory in the water maze and hippocampal dendritic morphology and BDNF levels were examined. PND: postnatal day; SP: *Spirulina platensis*; EX: voluntary exercise; EE: enriched environment; PAL: passive avoidance learning; MWM: Morris water maze.

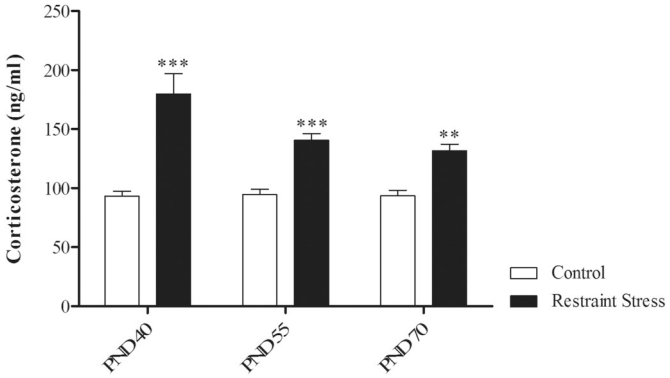

Fig. 2. Total serum corticosterone (ng/ml) levels in female rats exposed to 10 days chronic restraint stress. Corticosterone was measured at the PND 40 (after the termination of stress), PND 55 and in PND 70 (adulthood). Corticosterone level in the stressed group was significantly higher than the control group in PND 40, PND 55 and PND 70. ***$P < 0.001$; **$P < 0.01$ significant different from the control group.

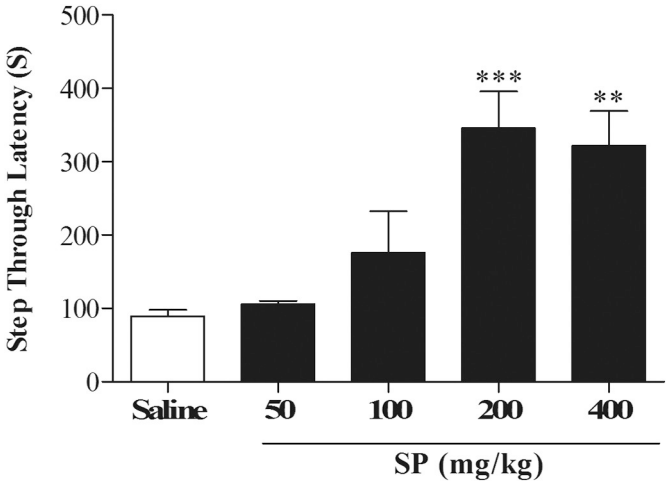

Fig. 3. Effects of different doses of *Spirulina platensis* on passive avoidance performance in female rat pups (PND 40). ***$P < 0.001$; **$P < 0.01$ significant different from the control group.

$P < 0.001$, $\eta^2 = 0.41$), day and stress ($F_{3,324} = 2.76$, $P < 0.045$, $\eta^2 = 0.01$), day and stress and treatment ($F_{15,324} = 2.355$, $P < 0.01$, $\eta^2 = 0.036$) and day and treatment ($F_{15,324} = 1.73$, $P < 0.05$, $\eta^2 = 0.016$) were all significant. Between-group comparisons indicated that escape latency of the stress group was significantly higher than the control group on the last day (day 4) of training ($P < 0.01$, d = 1.13). In all training days, the escape latencies of the SP ($P < 0.001$, d = 4.97), EX ($P < 0.001$, d = 5.13), EE ($P < 0.001$, d = 6.03), and the combined SP + EX ($P < 0.001$, d = 5.99) and SP + EE ($P < 0.001$, d = 6.12) groups were significantly shorter than the stress group, indicating that stressed rats demonstrated slowed down learning rate than the stressed rats receiving the treatments (SP, EX, EE and the combined). Additionally, the escape latencies of the EE, and SP + EE groups in day 1 ($P < 0.01$, d = 4.18 and $P < 0.01$, d = 3.52 respectively), and day 2 ($P < 0.01$, d = 5.08 and $P < 0.01$, d = 6.28 respectively), SP + EE group in day 3 ($P < 0.01$, d = 3.79), and EE group in day 4 ($P < 0.05$, d = 2.54) were significantly shorter than the control group, indicating that these treatments (EE exposure and the combined SP + EE) accelerated spatial learning in the control (non-stressed) rats.

The data for the time spent in the target area are illustrated in Fig. 6A. An ANOVA on the time spent in the target area showed a significant effect of stress ($F_{1,108} = 3.967$, $P < 0.05$, $\eta^2 = 0.19$), a significant effect of treatment ($F_{5,108} = 9.358$, $P < 0.001$, $\eta^2 = 0.35$) and an interaction between both factors ($F_{6,108} = 2.430$, $P < 0.05$, $\eta^2 = 0.27$). The post hoc analysis indicated that the time spent in the target area of the stress group was significantly lower than that of the control group ($P < 0.01$, d = 5.17). The time in the target zone of the stress groups treated with the EX ($P < 0.001$, d = 6.54), SP ($P < 0.001$, d = 6.96), EE ($P < 0.001$, d = 7.11), SP + EX ($P < 0.001$, d = 7.14) and SP + EE ($P < 0.001$, d = 8.36) was significantly lower than the stress group.

The data for the platform location latency are shown in Fig. 6B. An ANOVA on the platform location latency showed a significant effect of stress ($F_{1,107} = 22.16$, $P < 0.001$, $\eta^2 = 0.61$), a significant effect of treatment ($F_{5,107} = 15.199$, $P < 0.001$, $\eta^2 = 0.31$) and a significant interaction between both factors ($F_{5,107} = 2.972$, $P < 0.05$, $\eta^2 = 0.15$). Post hoc comparisons showed that the platform location latency of the stress group was significantly higher than that of the control group ($P < 0.001$, d = 6.40). The platform location latencies of the stress groups treated with the EE ($P < 0.001$, d = 10.38), EX ($P < 0.001$, d = 9.06) and SP ($P < 0.001$, d = 7.83) treated groups were significantly lower than the stress group.

3.3.3. Corticosterone levels after the interventions

Serum corticosterone levels (ng/ml) in stressed or non-stressed rats subjected to SP, EX, EE and combined treatment on PND 55 are shown in Fig. 7. A two-way ANOVA on corticosterone levels revealed a significant effect of stress ($F_{1,48} = 12.541$, $P < 0.01$, $\eta^2 = 0.41$), a treatment effect ($F_{5,48} = 7.847$, $P < 0.001$, $\eta^2 = 0.37$) and no significant interaction between group and treatment ($F_{5,48} = 1.249$, $P = 0.301$, $\eta^2 = 0.058$). The post hoc analysis showed that corticosterone levels in the stress group was significantly higher than the control group ($P < 0.01$, d = 5.21). Corticosterone levels in the stress group treated with the SP ($P < 0.01$, d = 4.42), EX ($P < 0.01$, d = 5.76), EE ($P < 0.01$, d = 6.38), and the combined EX + SP ($P < 0.01$, d = 6.59), and EE + SP ($P < 0.01$, d = 7.19) was significantly lower than the stress group.

3.3.4. Hippocampal BDNF

An ANOVA on hippocampal BDNF levels (Fig. 8) showed a main

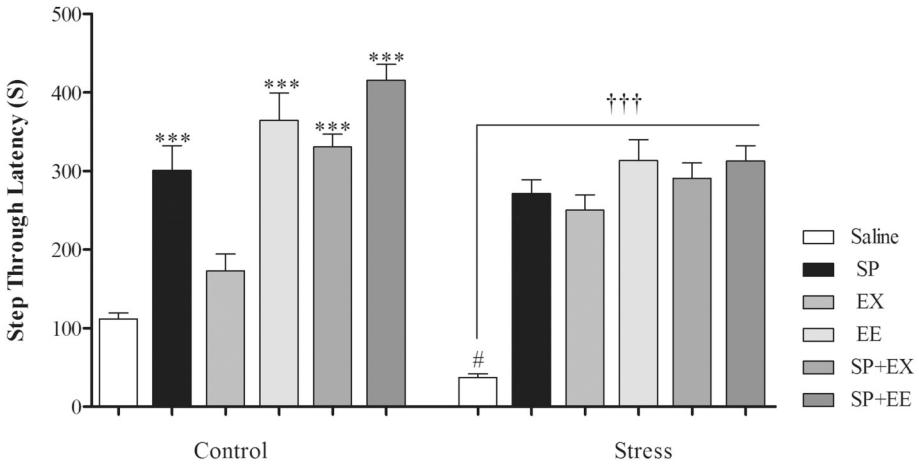

Fig. 4. Inhibitory avoidance performance in stressed or non-stressed rats subjected to SP, EX, EE, and the combined treatment. Data are expressed as mean ± SEM of step-through latencies during a 10-min retention test. ***$P < 0.001$ compared to control in non-stressed groups, $^{\#}P < 0.05$ compared to control, $^{\dagger}P < 0.001$ between group comparison in stressed rats. SP: *Spirulina platensis*; EX: voluntary exercise; EE: enriched environment.

effect of stress ($F_{1,48} = 77.810$, $P < 0.001$, $\eta^2 = 0.43$), a treatment effect ($F_{5,48} = 9.663$, $P < 0.001$, $\eta^2 = 0.26$), and a significant interaction between both factors ($F_{5,48} = 4.061$, $P < 0.05$, $\eta^2 = 0.12$). Between-group comparisons indicated that BDNF levels in the stress group was significantly lower than the control group ($P < 0.001$, d = 7.72). BDNF levels in the stress group treated with the combined SP + EE ($P < 0.001$, d = 8.41) and SP + EX ($P < 0.05$, d = 4.36) was significantly higher than the stress group, indicating that these combined treatment increased BDNF in the hippocampus of the stress group.

3.3.5. Morphology of hippocampus CA3

An ANOVA on the dendritic length of CA3 neurons (Fig. 9A) revealed a significant effect of stress ($F_{1,48} = 47.782$, $P < 0.001$, $\eta^2 = 0.44$), a treatment effect ($F_{5,48} = 49.719$, $P < 0.001$, $\eta^2 = 0.77$) and an interaction between group and treatment ($F_{5,48} = 13.457$, $P < 0.001$, $\eta^2 = 0.21$). The post hoc analysis showed that dendritic length of CA3 neurons was significantly reduced in the stress group than the control group ($P < 0.001$, d = 26.11). Dendritic length of CA3 neurons in the SP ($P < 0.001$, d = 41.27), EX ($P < 0.001$, d = 28.49), EE ($P < 0.001$, d = 40.86), and the combined EX + SP ($P < 0.001$, d = 90.52), and EE + SP ($P < 0.001$, d = 86.54) groups was significantly higher than the stress group. The SP treatment ($P < 0.001$, d = 97.4), EX ($P < 0.001$, d = 51.67), EE ($P < 0.001$, d = 46.31) and the combined SP + EX ($P < 0.001$, d = 83.01), and SP + EE ($P < 0.001$, d = 74.40) significantly increased dendritic length of the CA3 neurons in the control group. In the stress groups, dendritic length of the CA3 neurons in the combined (EX + SP and EE + SP) groups was significantly longer than the SP ($P < 0.001$, d = 86.20 and $P < 0.001$, d = 102.3, respectively), EX ($P < 0.001$, d = 104.1 and $P < 0.001$, d = 106.4, respectively) and EE ($P < 0.001$, d = 118.6 and $P < 0.001$, d = 87.83, respectively).

An ANOVA on the dendritic branch points of CA3 neurons (Fig. 9B) revealed a significant effect of stress ($F_{1,48} = 10.933$, $P < 0.001$, $\eta^2 = 0.21$), a significant effect of treatment ($F_{5,48} = 15.790$, $P < 0.001$, $\eta^2 = 0.32$) and an interaction between group and treatment ($F_{5,48} = 22.\ 465$, $P < 0.001$, $\eta^2 = 0.46$). The post hoc analysis showed that apical dendritic branch points of CA3 neurons was significantly reduced in the stress group than the control group ($P < 0.01$, d = 5.25). Dendritic branch points of CA3 neurons in the SP ($P < 0.001$, d = 7.17), EX ($P < 0.01$, d = 5.73), EE ($P < 0.05$, d = 5.25), and the combined SP + EX ($P < 0.001$, d = 8.45) and SP + EE ($P < 0.001$, d = 9.33) groups was significantly higher than the stress group. In stress groups, dendritic branch points in combined (EX + SP and EE + SP) groups was significantly higher than the SP ($P < 0.001$, d = 7.64 and $P < 0.001$, d = 5.37, respectively), EX ($P < 0.001$, d = 9.72 and $P < 0.001$, d = 8.04, respectively) and EE ($P < 0.001$, d = 9.56 and $P < 0.001$, d = 6.13, respectively). Representative camera lucida drawings of Golgi-impregnated CA3 pyramidal neurons from control and stress groups are depicted in Fig. 9C.

4. Discussion

The present study investigated the combination effects of *Spirulina platensis*, voluntary exercise and environmental enrichment on cognitive-behavioral deficits, hippocampal BDNF and morphological changes induced by exposure to adolescent stress in adult female rats. Findings showed that adolescent stress impaired passive avoidance performance, spatial learning and memory and induced dendrite retraction of the

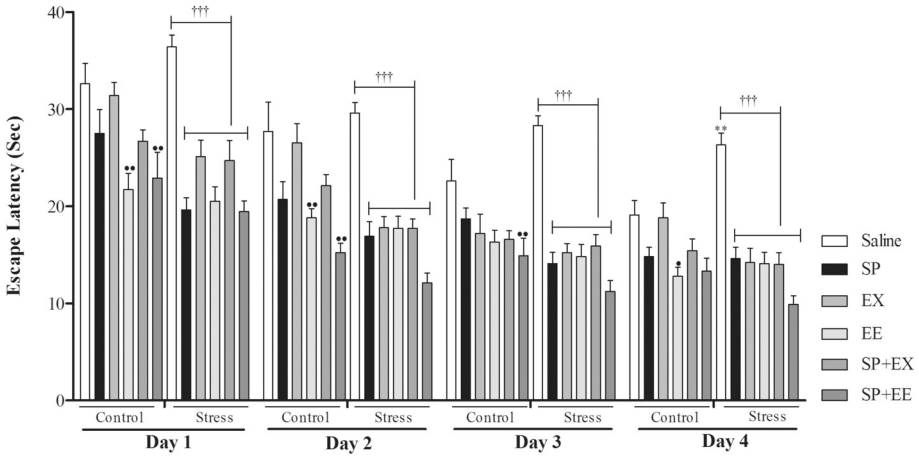

Fig. 5. Spatial learning performance in stressed or non-stressed rats subjected to SP, EX, EE and combined treatment. Chronic stress impaired spatial learning as the stressed animals had longer escape latencies than their non-stressed groups in the last training day. Data are expressed as the mean ± S.E.M. $^{\dagger\dagger\dagger}P < 0.001$ than the stress group. $^{¨}P < 0.01$, $^{\cdot}P < 0.05$ than the control group on the same day.**$P < 0.01$ than the control group on the same day. SP: *Spirulina platensis*; EX: voluntary exercise; EE: enriched environment.

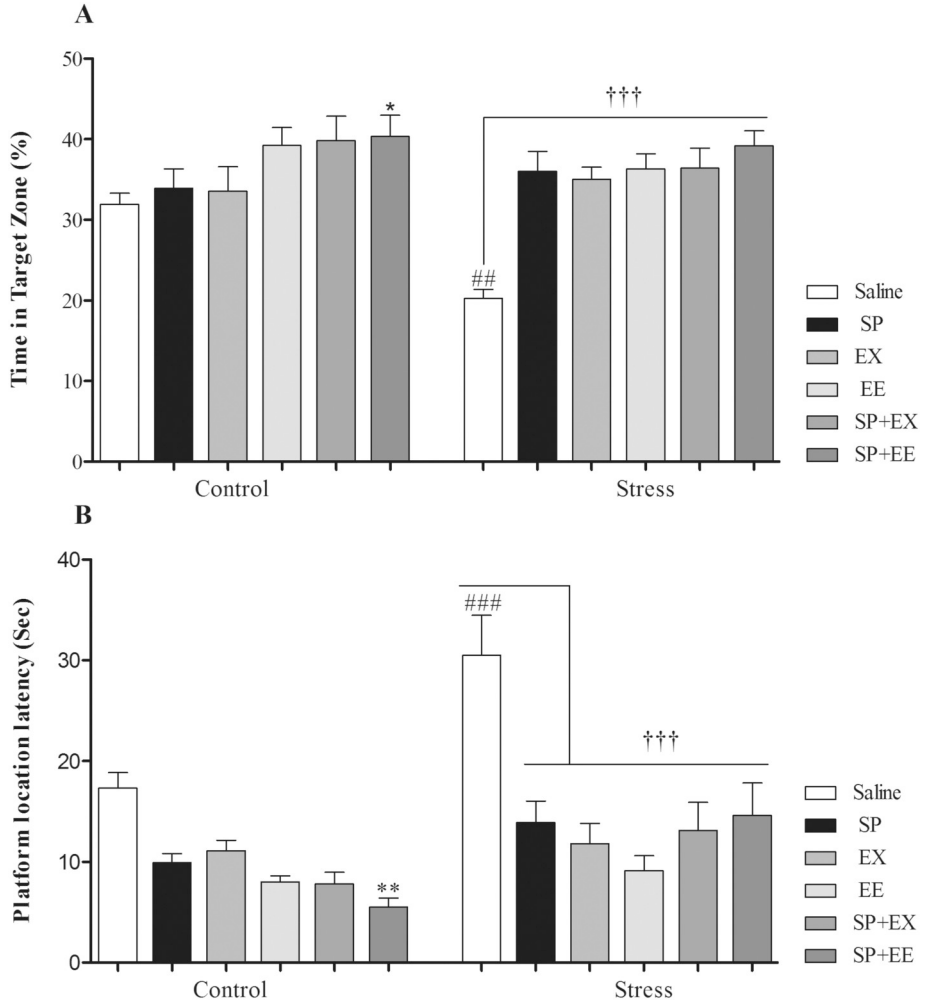

Fig. 6. Spatial memory performance in stressed or non-stressed rats subjected to SP, EX, EE and combined treatment. Chronic stress impaired memory retention as the stressed animals had reduced time spent in the target zone (A) and longer platform location latencies (B) during probe test. Data are expressed as the mean ± S.E.M. SP: *Spirulina platensis*; EX: voluntary exercise; EE: enriched environment. In A: $^{\#\#}P < 0.01$ than the corresponding control group; $^{\dagger}P < 0.001$ than the stressed group. $^{*}P < 0.05$ than the control group. In B: $^{\#\#\#}P < 0.001$ than the corresponding control group; $^{\dagger}P < 0.001$ than the stressed group. $^{**}P < 0.01$ than the control group.

CA3 subregion of the hippocampus in adulthood. In addition, adolescent stress significantly decreased the BDNF levels in the hippocampus. Treatment with the *Spirulina platensis*, voluntary exercise and environmental enrichment and the combined treatment could prevent these harmful effects of chronic stress.

4.1. Exposure to adolescent stress enhances corticosterone levels in adult female rats

Our findings showed that adolescent restraint stress increased serum corticosterone levels, which remained at a high level even in adulthood.

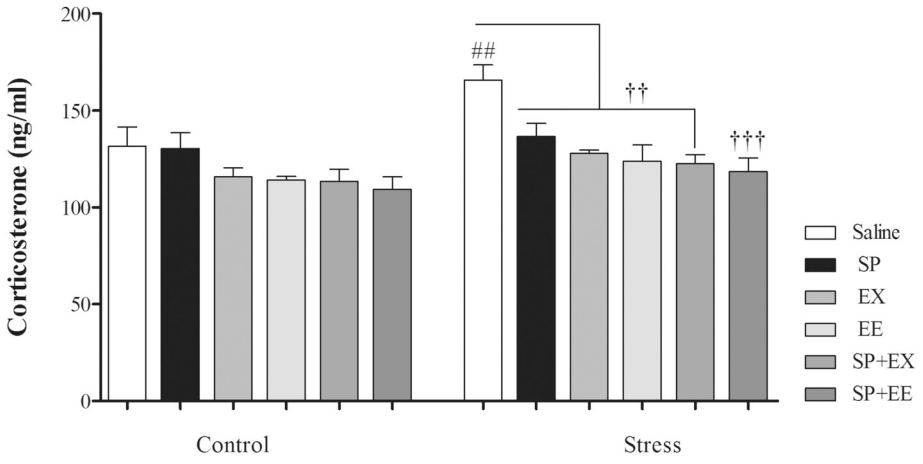

Fig. 7. Serum corticosterone levels (ng/ml) in control and stressed rats subjected to SP, EX, EE and combined treatment. Corticosterone was measured at the PND 55 (after the interventions). Corticosterone level in the stressed group was significantly higher than the corresponding control group in PND 55. Data are expressed as the mean ± S.E.M. $^{\#\#}P < 0.01$ than the corresponding control group; $^{\dagger\dagger\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.01$ than the stress group.

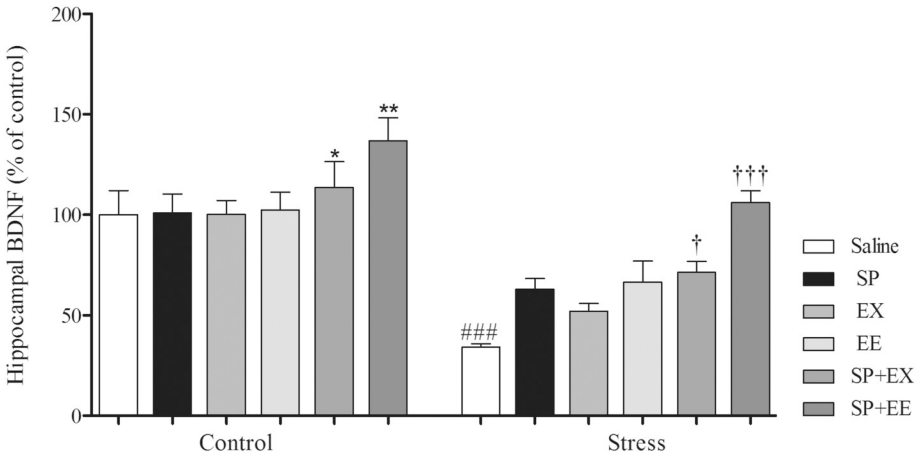

Fig. 8. Hippocampal BDNF levels in control and stressed rats subjected to SP, EX, EE and combined treatment. Chronic stress reduced hippocampal BDNF, and the combined treatment of SP + EE and SP + EX restored this deficit. Data are expressed as the mean ± S.E.M. SP: *Spirulina platensis*; EX: voluntary exercise; EE: enriched environment. $^{\#\#}P < 0.01$ than the corresponding control group; $^{\dagger}P < 0.001$; $P < 0.01$ than the stressed group.

This shows that exposure to stressors during juvenility increased HPA axis basal activity as showed by increased resting corticosterone levels on PND 55 and 70 (15 and 30 days after the termination of stress, respectively). This finding is in agreement with reported elevated levels of resting corticosterone two months following exposure to adolescent stress (Grigoryan et al., 2015; Ilin and Richter-Levin, 2009). Adolescent stress also showed increased levels of corticosterone when challenged in either juvenility or adulthood (Jacobson-Pick and Richter-Levin, 2010). These findings together show that adolescent stress alters HPA axis activity in basal and challenged conditions in juvenility or adulthood. Elevated corticosterone levels may encompass the impact of adolescent stress on the brain, behavior, and cognition in juvenility or adulthood (Lupien et al., 2009; McEwen, 2008).

Although there is a wealth of information on the effects of prenatal

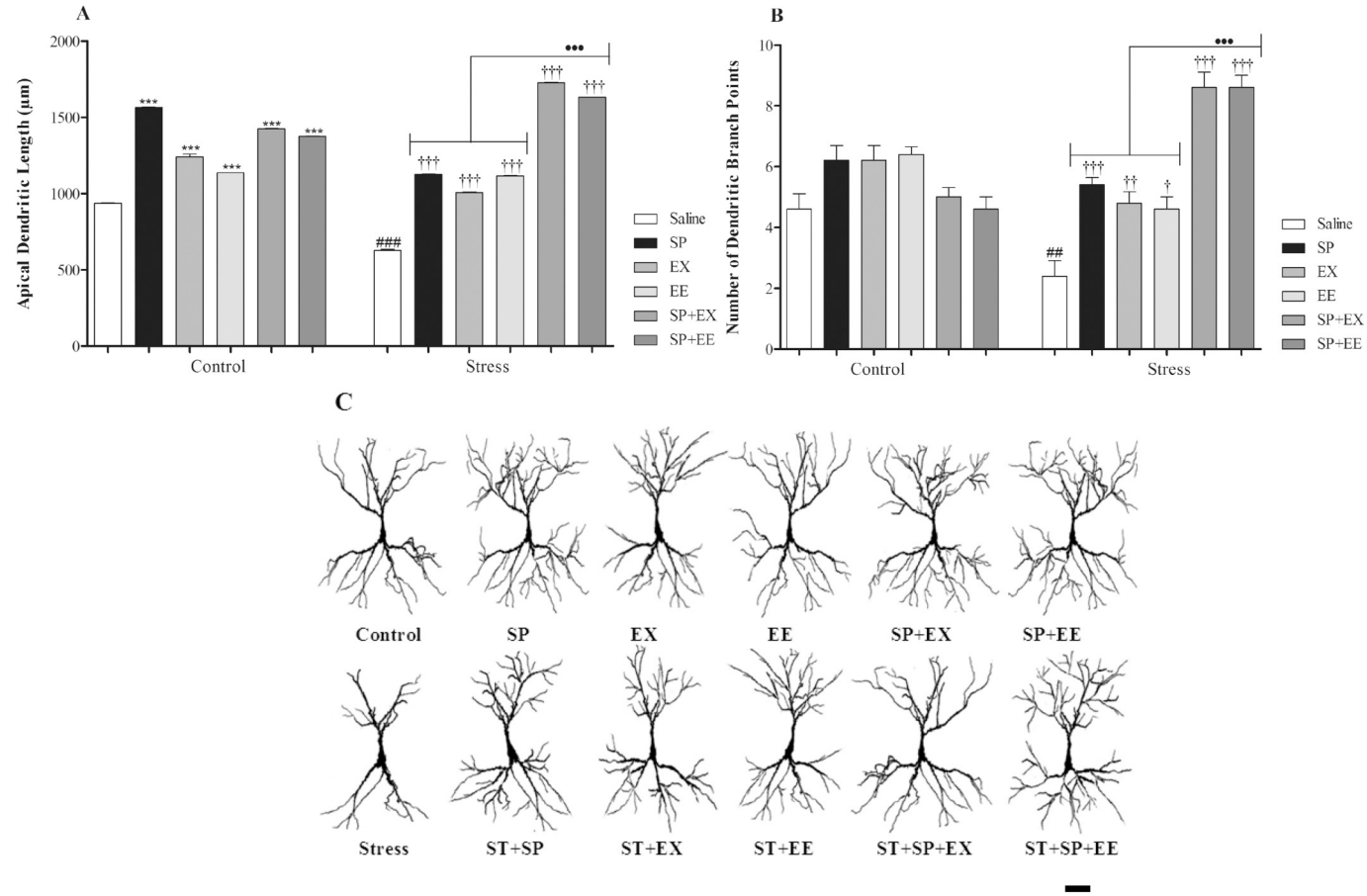

Fig. 9. Apical dendritic remodeling of the CA3 region of the hippocampus in stressed or non-stressed rats subjected to SP, EX, EE and combined treatment. $^{\#\#\#}P < 0.001$ than the corresponding control group; $^{\dagger\dagger\dagger}P < 0.001$ than the stressed group; $^{\dddot{}}P < 0.01$ than the SP, EE and EX gropus, and $^{***}P < 0.001$ than the control group. In B: $^{\#\#}P < 0.01$ than the control group; $^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ than the stress group, and $^{\dddot{}}P < 0.01$ than the SP, EE and EX groups. C: Computer-assisted reconstructions of Golgi impregnated pyramidal neurons from the CA3 region of the dorsal hippocampus in unstressed (above) and stressed (below) groups exposed to SP, EX, EE and combined treatment. These neurons were selected because they are representative of dendritic lengths near their respective group means. Scale bar: 50 µm.

SP: *Spirulina platensis*; EX: voluntary exercise; EE: enriched environment.

stress on adulthood, comparatively little is known about the effects of adolescent (adolescent or childhood) stress. Adolescent period is a time of considerable development at the level of behavior, cognition and the brain (Brydges, 2016; Brydges et al., 2012; Brydges et al., 2014a; Lupien et al., 2009). A large body of research has revealed females are susceptible to stress effects (Brydges et al., 2014a; Sotiropoulos et al., 2015) and increased risk of depressive disorders in females is well documented (Lupien et al., 2009). It is now well accepted that females typically have higher baseline and stress hormone responses (Kajantie and Phillips, 2006; Rhodes and Rubin, 1999; Wang et al., 2007).

4.2. *Spirulina platensis* enhances passive avoidance performance in a dose-dependent manner

Data from Experiment 2 showed that treatment with *Spirulina platensis* improved passive avoidance performance in a dose-dependent manner in intact rats, which is a novel finding. *Spirulina platensis* is a blue-green algae that grows in both salty and fresh water and it is high in proteins and is also a rich source of essential fatty acids, vitamins and minerals. Although *Spirulina platensis* has many beneficial effects on health, but its effect on learning and memory has not yet been examined. As far as we are aware, this is the first study to focus on the dose-response effects of *Spirulina platensis* on memory retention. We found the dose-response effects of *Spirulina platensis* on memory retention and with the maximum response in a dose of 200 mg/kg. Although the mechanisms whereby *Spirulina platensis* enhances passive avoidance performance are not known, it could have the memory-enhancing effects through several mechanisms, one of which involves providing critical amino acids for protein synthesis in neural cells of brain structures involved in memory formation. In addition, *Spirulina platensis* has neuroprotective effects through reducing oxidative stress and antioxidant properties (Capelli and Cysewski, 2010).

4.3. Adolescent stress impairs passive avoidance performance and spatial learning and memory and induces dendritic retraction in hippocampal CA3 subregion: beneficial effects of Spirulina platensis, voluntary exercise and environmental enrichment

We have found that the chronic stress during the adolescent phase impairs passive avoidance performance and spatial learning and memory in adulthood. In passive avoidance test, which measures contextual and emotional memory, stressed rats showed impairment on a 48-h memory retention test. In the water maze, stressed rats able to learn the platform location similar to control rats in the first three days, but in the last day of training a significant difference was found between the stressed and non-stressed rats, suggesting a slower learning rate in stressed rats. In probe test, stressed rats showed memory impairment as evidenced by increased platform location latency and less time in the target zone than non-stressed rats. Our findings are in agreement with other studies showing impairment of learning and memory in juvenile and adult rats exposed to chronic stress (Isgor et al., 2004; Moreira et al., 2016; Radecki et al., 2005; Tzanoulinou et al., 2014).

We found that treatment with *Spirulina platensis*, voluntary exercise and environmental enrichment, and the combined treatments could recover both emotional memory, and spatial learning and memory in stressed rats. No synergic or addictive effects were found between treatments. All treatments alone recovered stress-induced learning and memory deficits. In the control rats (non-stressed), all treatments except exercise enhanced passive avoidance performance and the environmental enrichment alone and in the combination with the *Spirulina platensis* improved spatial learning and memory in non-stressed animals, showing the beneficial effects of these interventions on learning and memory performance in physiological conditions.

To date, there is a little information about the effects of *Spirulina platensis* on learning and memory. Many studies have shown that *Spirulina platensis* displays a wide range of effects such as neuroprotective, hepatoprotective, anti-inflammatory and anti-oxidative properties (Ozdemir et al., 2004; Romay et al., 2003). Because of potent neuroprotective properties of *Spirulina platensis*, the findings of this study suggest that the application of *Spirulina platensis* is useful for improvement of memory deficits induced by stress. The protective effects of the enriched environment and physical activity against the cognitive impairment induced by adolescent stress are in line of earlier reports indicating that early exposure of animals to environmental enrichment or physical activity can change neural structure and function leading to enduring improvements in learning and memory and improve memory deficits (Belz et al., 2003; Chapillon et al., 1999; Fox et al., 2006; Gobbo and O'Mara, 2005; Hamm et al., 1996; Larsen et al., 2010; Pacteau et al., 1989; Pena et al., 2009; Rhodes and Rubin, 1999; Schmidt and Duman, 2010; Simpson and Kelly, 2011; Wainwright et al., 1993; Will et al., 1977; Wolfer et al., 2004). Enriched environment and physical may negate the deficits produced by a chronic stress on hippocampal-dependent spatial learning and memory tasks by altering many properties of the hippocampus (Conrad et al., 2007). Some studies have shown that enriched environment and exercise increased levels of BDNF, dendritic branching, and neurogenesis and improved cellular plasticity (Gobbo and O'Mara, 2004; Kuma et al., 2004; Mello et al., 2009; Molteni et al., 2002; Nithianantharajah and Hannan, 2006; Radahmadi et al., 2016).

There is increasing evidence to suggest that chronic stress alters hippocampal structure via changing the hippocampal morphology in male and female rats, including modification of dendrites in the CA3 region (Kleen et al., 2006) and remodeling of dendritic spine shape and number (Diamond et al., 2006; Eiland et al., 2012; McLaughlin et al., 2005; McLaughlin et al., 2010; Vyas et al., 2002). These findings are consistent with our morphologic results from the CA3 area, suggesting that chronic restraint stress decreased the length and branch points of apical dendrites. These harmful effects of chronic stress might be mediated via a decline in BDNF production. Several lines of studies showed that synaptic plasticity depend on BDNF, and the reduced level of BDNF following chronic stress is accompanied by the impairment of hippocampal function and volume (Chao et al., 2006; Kuipers and Bramham, 2006). In addition, several other mediators including corticosterone, serotonin and glutamate may contribute to remodeling of dendrites in this brain region (McEwen, 2016).

The present study showed that *Spirulina platensis*, enriched environment, voluntary exercise, and combined treatment alleviated adolescent stress induced dendritic retraction of the CA3 pyramidal neurons. An interesting novel finding was the existence of a positive synergistic effect between *Spirulina platensis* and enriched environment or exercise on dendritic length and branching in stressed rats. These treatments had positive effects on morphology of the CA3 pyramidal neurons in non-stressed rats, showing their beneficial effects on neuronal morphological remodeling in physiological conditions. It is well established that adult hippocampal neurogenesis enhanced through exposure to an enriched environment equipped with a running wheel (van Praag et al., 2000). As expected, findings obtained from our enriched environment and physical activity paradigms are consistent with a recent study showing that running is a critical neurogenic stimulus when introduced with an enriched environment (Kobilo et al., 2011). Additional evidence for a role of protective effects of the enriched environment comes from studies which refers to opposite effect of the enriched environment on morphological measures such as dendritic spine density, neuronal morphology, and hippocampal neurogenesis (van Praag et al., 2000). Exposure to the enriched environment during the adolescent period has been reported to completely reverse the effects of maternal separation on both HPA and behavioral responses to stress (Francis et al., 2002). In addition, some studies have observed that exercise can reverse the negative effects of stress on hippocampal cell proliferation (Kannangara et al., 2009; Nakajima et al., 2010), even in aged animals (Kannangara et al., 2011). A positive effect of exercise

on chronic stress consequences is associated with the extensive literature showing potent properties of exercise in neurogenesis (Van Praag et al., 2005), morphological and functional changes, and cognitive improvement (Cotman and Berchtold, 2002; Kondo and Shimada, 2015). Consistent with these findings, we have showed that physical activity or exposure to the enriched environment especially when combined with the *Spirulina platensis* improved dendritic morphology in the CA3 region. The positive effects of these combined treatments against adolescent stress induced deficits might be mediated through increasing in BDNF levels (see the below). The mechanisms underlying the protective effects of *Spirulina platensis* against morphological remodeling induced by exposure to adolescent stress in female rats are unknown. As aforementioned, *Spirulina platensis* has potent neuroprotective and antioxidant properties (Ozdemir et al., 2004; Romay et al., 2003), which may mediate the beneficial effects of against adolescent stress induced deficits. In the *Experiment 1* the elevation of corticosterone level was observed even in PND 50–70, suggesting the continuous activation of HPA axis. Our data showed that corticosterone levels in stressed animals were decreased after all interventions, suggesting that the protective effects of treatments against adolescent stress might be mediated by calming down the HPA axis, or by the other downstream mechanisms, such as the enhancement of the neuronal circuit surveillance. Further studies are required to test these assumptions.

4.4. Adolescent stress decreases BDNF levels in hippocampus

We have shown that exposure to chronic stress during the adolescent phase reduced BDNF levels in the hippocampus as measured in the adult period. Although *Spirulina platensis*, environmental enrichment and exercise alone did not increase significantly hippocampal BDNF levels in the control rats and stressed rats, the combined treatment of *Spirulina platensis* with either environmental enrichment or physical exercise significantly increased the BDNF levels in both groups, suggesting a synergistic effect between treatments. Previous studies have shown that exercise or environmental enrichment enhance hippocampal BDNF (McCreary et al., 2016; Vaynman et al., 2004) and adolescent male and female rats (Ahmadalipour et al., 2015; Uysal et al., 2015). The present study did not show the effect of single treatments of exercise or environmental enrichment on hippocampal BDNF in the control and stressed groups. Two possibilities may explain these observed results. One probability is that number of animals per group ($n = 5$) is not enough to detect statistically significant differences between groups. Another possibility is the time point of BDNF measurement. We measured BDNF levels at the time point of two weeks after the end of the interventions. At this time, BDNF protein may return to basal levels or reduce to an undetectable level. Further studies are needed to test these assumptions.

Previous studies have shown that BDNF mediates the beneficial effects of exercise on brain functions, particularly learning and memory (Vaynman et al., 2004), neurogenesis (Van Praag et al., 2005), and synaptic plasticity (Patten et al., 2013). BDNF also play an important role in mediating the beneficial effects of environmental enrichment on brain functions (Chourbaji et al., 2012). Voluntary exercise induces a selective increase in BDNF in the apical dendrites of the CA3 neurons, promoting an increase in dendritic length and spine density of the CA3 pyramidal neurons (Baj et al., 2012). Thus, the increased BDNF levels in the hippocampus may mediate the enhancing effects of the combination of *Spirulina platensis* and voluntary exercise or environmental enrichment on dendritic remodeling of the CA3 neurons in the control and stressed rats found in the present study.

One limitation of the present study was single housing of the animals during exposure to voluntary exercise, which can be stressful for rodents, especially during the adolescent period. However, to reduce the potential confounding effects of a single housing stress during physical activity 15 days on the results of experiments, the corresponding sedentary rats were confined to similar cages with no access to a wheel.

In summary, the present study shows the beneficial effects of *Spirulina platensis*, environmental enrichment, voluntary exercise, and their combined therapy against cognitive deficits, the reduction in BDNF levels, and dendritic retraction of the CA3 region induced by exposure to adolescent stress in adult female rats. No enhancement in learning and memory was found in response to the combination of Spirulina with exercise or environmental enrichment compared to each of Spirulina, exercise and environmental enrichment alone. However, there were synergistic effects of the combined treatments (*Spirulina platensis* with environmental enrichment or exercise) on hippocampal BDNF levels and dendritic morphology. Findings of the present study could have important implications for the development of novel strategies for the treatment of adolescent stress-related disorders.

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Authors' contributions

N.M-K. and A.R.P. designed the overall study and wrote the paper. N.M-K. conducted the research, collected data and carried out the lab work. N.M-K. and A.R.P. carried out the statistical analysis and mostly drafted the manuscript. A.R.P. coordinated and supervised the study. All authors approved the manuscript.

Conflict of interest

We attest that we have herein disclosed any and all financial or other relationships that could be construed as a conflict of interest and that all sources of financial support for this study have been disclosed.

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