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ORIGINAL PAPER

Beneficial Effects of Physical Activity and Crocin Against Adolescent Stress Induced Anxiety or Depressive-Like Symptoms and Dendritic Morphology Remodeling in Prefrontal Cortex in Adult Male Rats

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Abstract

Increasing evidence suggests that exposure to chronic stress during adolescent period may lead to behavioral and neuronal morphology deficits in adulthood. This study examined whether crocin, the main active saffron constituent, and voluntary exercise, alone or combined, could prevent the detrimental influences of chronic restraint stress during adolescent (postnatal days, PND, 30–40) on behavioral and morphological deficits in adult (PND60) male rats. Results showed that plasma corticosterone levels increased at PND40, but not PND60 in stressed rats. Moreover, stressed rats demonstrated enhanced anxiety levels and depression like behaviors in adulthood. These behavioral abnormalities were accompanied by a decline in apical dendritic length in both infralimbic and prelimbic regions and dendritic branches in infralimbic region of the prefrontal cortex. Treatment with crocin, exposure to wheel running activity, and the combined interventions alleviated both behavioral and morphological deficits induced by adolescent stress. Moreover, these treatments exerted positive neuronal morphological effects in the prefrontal cortex in non-stressed animals. Our findings provide important evidences that exercise as a non-pharmacological intervention and crocin treatment during pre-pubertal period can protect against adolescent stress induced behavioral and morphological abnormalities in adulthood.

Keywords Adolescent stress · Crocin · Running wheel exercise · Anxiety · Depression · Dendritic remodeling · Prefrontal cortex

Introduction

During the pre-pubertal and adolescence periods, the limbic system structures such as amygdala and hippocampus, and prefrontal cortex undergo functional and structural changes [1, 2]. These structures play an important role in stress reactivity because they contain high densities of corticosteroid receptors, which detect glucocorticoids and regulate the hypothalamic–pituitary–adrenal (HPA) axis [3]. The adolescent stress model is presented as a useful model to study in rodents different aspects of stress-related disorders such as anxiety and depression and neural mechanisms of vulnerability and resilience to stress [4, 5]. In this model, a variety of stressors such as restraint, foot-shock, forced swim and elevated platform exposure are administered to animals in a variable way between postnatal days (PND) 21 and 40 (adolescent period). Rats exposed to adolescent stress have a delayed rise of glucocorticoid levels and prolonged glucocorticoid release in response to several types of stressors as compared with adult rats [6], owing to incomplete maturation of negative-feedback systems [7, 8].

Several lines of studies suggest that adolescent stress leads to architectural changes in the rat prefrontal cortex (PFC). Chronic stress induced neural reorganiztion suh as dendritic branch points and length loss in the PFC and

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executive dysfunction in rodents [9, 10]. In contrast, dendrites in the amygdala expanded in response to chronic stress exposure [11, 12]. Adolescent stress induced pyramidal neuron atrophy in the prelimbic region of the PFC in both male and female rats, leading to emotional deficits [13].

There is an increasing demand for prevention and treatment of long lasting effects of adolescent stress on brain functions and development. Current research is focused on findings new substances with few or no side effects than the synthetic drugs. One such substance is crocin, the main active substitute of saffron, which has anti-anxiety [14], and anti-depressant effects in rodents including mice and rats [15, 16]. Saffron extract and crocin also can ameliorate scopolamine or ethanol-induced impairments of learning and memory [17], and prevent the impairment of learning and memory as well as the oxidative stress damage to the hippocampus induced by chronic stress in rats [18]. A recent human study has demonstrated that crocin tablets given with selective serotonin reuptake inhibitors (SSRI) lead to a greater improvement in symptoms of depression and anxiety when compared to SSRIs alone in patients with major depressive disorder [19]. These findings demonstrate the beneficial effects of crocin against stress and affective disorders. Thus, it would be interest to investigate therapeutic effects of crocin against stress induced deficits in adolescent stressed rats.

Physical exercise is another alternative non-pharmacological intervention for alleviating the detrimental adolescent stress on brain health with no apparent side-effects. Human studies suggest that exercise could have benefits for overall health and cognitive functions [20, 21]. Exercise is currently advocated as a behavioural intervention to improve neurological disorders in several neurodegenerative diseases [22, 23]. Human and animal studies have shown that exercise improves learning and memory, and reduces the risk of neurodegenerative diseases [24]. The positive effects of exercise on cognitive activity might be mediated through increased neurogenesis, neuronal remodeling, synaptic plasticity and release of neurotrophic growth factors including BDNF in brain structures such as the hippocampus [25–30]. Exercise also changes the morphology of neurons. For example, it has been observed that exercise increases density of dendritic branch points in the dentate gyrus, CA1 pyramidal neurons, and entorhinal cortical layer III pyramidal neurons [31]. Exercise partially reverses neonatal alcohol-induced defects in spine density and dendritic complexity of the PFC cortex in male adolescent rats [32]. Additionally, exercise has anti-stress effects. For example, voluntary wheel running and certain pharmacological treatments, especially fluoxetine (a serotonin reuptake inhibitor) and reboxetine (a norepinephrine reuptake inhibitor), significantly alleviate anxiety- and depression-like behaviors in stressed male rats [33], and voluntary exercise could reduce anxiety and depression-like behaviors in rodents experienced uncontrollable foot shocks [34] and repeated social stress [35].

The present study aimed to examine the effects of physical activity as non-pharmacological intervention and pharmacological intervention with crocin, and particularly the combined intervention for a possible synergistic effect on adolescent stress induced behavioral and neuronal morphology abnormalities. Thus, in the first part of the present study, we studied the effect of adolescent chronic stress on anxiety and depression-like behaviors and corticosterone levels as well as neuronal morphological changes in the PFC in adulthood. In the second part, we examined whether the exercise regimen, crocin treatment and the combined intervention during pre-pubertal period would ameliorate adolescent stress induced behavioral and morphological neuronal deficits in adulthood.

Materials and Methods

Animals

Thirty-day-old male Wistar rats (80 ± 12 g) obtained from the breeding colony of the Semnan University of Medical Sciences (SUMS), Semnan, Iran. Animals were housed (4–5 rats per cage) in cages ($50 \times 26 \times 25$ cm) on a 12-h light/dark cycle at 22–24 °C, with food and water ad libitum. All experiments were executed in accordance with the Guide for Care and Use of Laboratory Animals (National Institute of Health Publication No. 80-23, revised 1996) and were approved by SUMS (IR.SEMUMS.REC.1394.211).

Stress Induction

Starting on PND30, rats were exposed to a 2-h restraint stress between 10:00 a.m. and 12:00 a.m. for 10 days [11, 36]. During the restraint, rats were placed into plastic restraint containers for 120 min without access to either food or water. The size of the container was adjusted to the growth of the rats.

Drug

Crocin was provided from Sigma-Aldrich and it was dissolved in a physiological saline and injected intraperitoneally (IP) in a volume of 2 ml/kg. Crocin was injected at doses of 25 and 50 mg/kg for 15 days in treatment groups. These doses were chosen based on previous studies [18, 37].

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

(diameter = 35 cm, width = 9.5 cm). Animal movements were automatically recorded by an electronic system that linked to running wheel (model 2021.PN; Tajhiz Gostar Company, Tehran, Iran). The running wheels were removed from the cages after the 15 days period. Animals were single-housed while exposed to the running wheel. In order to control the potential confounding effects of single housing stress during 15 days wheel running on subsequent animal behaviors, the corresponding sedentary rats were confined to similar cages with no access to a wheel.

Anxiety Test

Elevated plus maze (EPM) was used to measure anxiety levels in rats. The apparatus was made of two open arms (50 cm × 10 cm) and two enclosed arms (50 cm × 10 cm, surrounded by 40-cm high wooden walls) raised 50 cm above the floor. All arms were joined in the central neutral area (10 × 10 cm) of the maze so that rats could freely pass from one arm to another. The open arms were bordered by a 0.5-cm high Plexiglas edge to avoid falls. Animal behaviors were recorded by an overhead camera connected to a PC running video tracking software. Rats were moderately located in the neutral area facing one of the open arms and given 5 min to discover the maze. The duration and number of entries into open and enclosed arms were recorded. A total of four paws inside of an arm were used as criteria for entrance. Anxiogenic influences selectively decrease open arm entries (OAE) and/or open arms time (OAT) and, in contrast, anxiolytic influences selectively increase the OAE and/or OAT [38, 39]. The number of total arm entries was used as a measure of spontaneous locomotor activity.

Force Swimming Test (FST)

In this test a cylindrical swimming tank made of glass (45 cm high and 25 cm diameter) was filled with 25 °C tap water up to 35 cm where rats could not touch the bottom of the cylindrical swimming tank. The test involved 2 days. On the first day, rats were placed in water for a 15-min. Then, they were removed from water, dried and cleaned with a towel and returned to their cages. In the second day, rats were retested for 5 min under the same condition. Swimming behavior (active movement of the forepaws with goal-directed horizontal actions, such as crossing between quadrants of the cylinder and turning), climbing (upward goal-directed movements of the forepaws along the side of the cylindrical container), and duration of immobility behavior (floating in water with only movement necessary to keep the head above water) were recorded. Reduction in swimming duration and climbing duration and increase in immobility duration represent depression-like behaviors in rats [33].

Corticosterone Measurements

For corticosterone levels measurement, three blood samples were obtained from tail of conscious rats at the PND30 (before stress application), PND40 (after the conclusion of stress), and PND60 (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 a.m. and 1 p.m. 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.

Morphological Data Analysis

After completion of behavioral tests, all groups of rats were killed under deep anesthesia. Brains were removed and processed using Golgi stain. The brain was immersed in the Golgi-Cox solution (five volume parts of 5% potassium dichromate solution, five volume parts of 5% mercuric chloride solution, four volume parts of 5% potassium chromate solution, ten volume parts of H_2O) in the dark for 14 days. Brains then were dehydrated through a graded series of ethanol at about 24-h intervals (50% ethanol, 1 h; 70% ethanol, 1 h; 95% ethanol, 1 h and 100% ethanol, 1 h), and cleared in xylene. Samples then were placed inside molten paraffin (50 °C) to penetrate into the tissue, and coronal sections were cut in the thickness of 150 µm on a sliding microtome [40, 41]. Pyramidal neurons of the PFC were selected on the basis of the following criteria: (1) the cell type must be identical; (2) neurons must be dark and consistently sliver impregnated throughout the extent of all dendrites; (3) dendrites must be untruncated; and (4) stained neurons must be relatively free from the neighboring impregnated neurons. Pyramidal neurons were defined by the presence of a basilar dendritic tree, a distinct, single apical dendrite, and dendritic branch points [42, 43].

Since chronic juvenile stress retracts apical dendritic length or branch points, and basal dendritic length or branch points remain intact [13], the present study analyzed only morphology of apical dendrites of pyramidal neurons in both IL and PL regions of the PFC. Analysis of apical dendritic length and branch points of pyramidal neurons was performed using an image J (1.48 version) software. For each animal, an average apical dendritic length and branch points of 6–8 selected pyramidal neurons was calculated. From each experimental group, five animals randomly were selected for morphology analysis.

Statistical Analysis

Behavioral, biochemical and anatomical data are expressed as the mean ± standard error of the mean (S.E.M.). Data from Experiment 1 analyzed with two-way ANOVA with repeated measurement on days. Data from Experiment 2 were analyzed with two way ANOVA as stress (two levels: control or stress) and treatments (three levels: crocin, voluntary exercise, and crocin + voluntary exercise) as between subjects variables. SPSS software (version 24) was used for data analysis. Statistical differences were considered significant when $p < 0.05$.

Experiments

Experiment 1: Effect of Adolescent Chronic Stress on Plasma Corticosterone Levels

This experiment investigated the effect of 10 days chronic stress during adolescent on corticosterone levels. Rats were divided into two saline + no-stress (SAL-NS) and saline + stress (SAL-S) groups (n = 6 in each group). Corticosterone levels were measured on before stress in PND30 and on the last day of restraint stress in PND40 and again in adulthood (PND60) as methods described the above.

Experiment 2: Effect of Adolescent Chronic Stress on Emotional Behaviors and Neuronal Morphology of the PFC: Beneficial Effects of Crocin and Voluntary Exercise

The aim of this experiment was to examine the effects of adolescent chronic stress on anxiety or depressive-like symptoms, and neuronal morphology of the PFC, and therapeutic effects of crocin, voluntary exercise, and the combined treatments against behavioral and morphological consequences of chronic stress. Animals were randomly divided into 12 experimental groups as follows (n = 7–10 in each group): saline + no-stress (SAL-NS); exercise + no-stress (EXC-NS), crocin (25 mg/kg) + no-stress (C25- NS); crocin (50 mg/kg) + no-stress (C50-NS); exercise + crocin (25 mg/kg) + no-stress (EXC/C25-NS); exercise + crocin (50 mg/kg) + no-stress (EXC/C50-NS); saline + stress (SAL-S), exercise + stress (EXC-S); crocin (25 mg/kg) + stress (C25-S); crocin (50 mg/kg) + stress (C50-S); exercise + crocin (25 mg/kg) + stress (EXC/C25-S) and exercise + crocin (50 mg/kg) + stress (EXC/C50-S). The first six groups received systemic administrations of physiological saline, crocin or exposed to running wheel for 15 days in PND41-55. The last six groups were stressed in Plexiglass tubes 2 h/day for 10 days in PND30-40 and then received systemic injections of saline, crocin and or exposed to running wheel in PND41-55. On days 60–62, all rats were subjected to behavioral tests. After the end of behavioral testing, animals were decapitated, and their brains removed and dendritic morphology (dendritic length and number of branch points) of pyramidal neurons in the PFC was examined. The adrenal glands also were removed and weighed (Fig. 1).

Results

Corticosterone Levels

Figure 2 shows the plasma corticosterone levels in two experimental groups. A two-way repeated measures ANOVA indicated significant effects of stress ($F_{1,10} = 4.77$, $p = 0.035$), and day ($F_{2,20} = 42.48$, $p = 0.0001$) and significant interaction between both factors ($F_{2,20} = 8.051$, $p = 0.003$) significant effect statistical analysis indicated significant differences among days ($F_{2,30} = 42.48$, $p = 0001$). Post-hoc comparison indicated that restraint stress significantly ($p < 0.0001$) increased the plasma corticosterone concentrations in the SAL-S group than the SAL-NS group in PND40.

Anxiety Profile

A two-way ANOVA on the percent of open arm time (Fig. 3a) demonstrated no significant main effects of stress ($F_{1,100} = 1.195$, $p = 0.277$), significant main effects of treatment ($F_{5,100} = 3.886$, $p = 0.003$) and an interaction between stress and treatment ($F_{5,100} = 3.065$, $p = 0.013$).

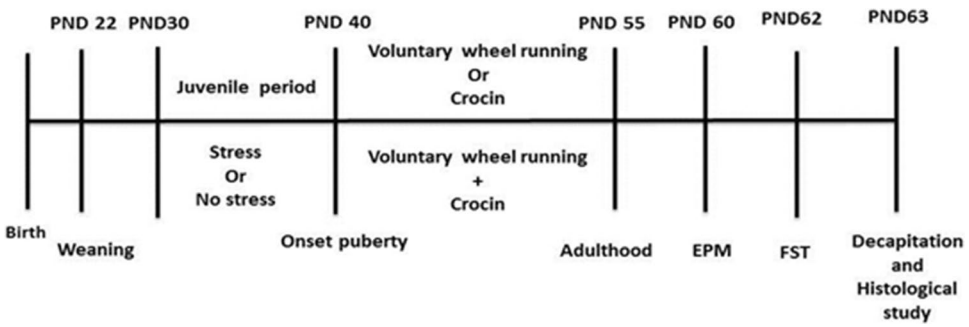

Fig. 1 Timeline of experiment 2 (see "Materials and Methods" section for more detail)

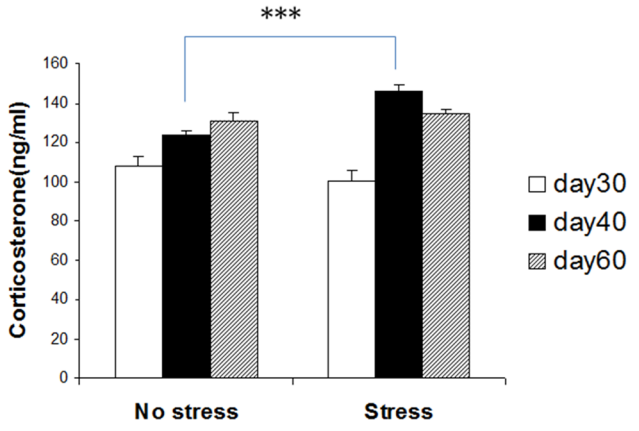

Fig. 2 Total serum corticosterone (ng/ml) levels in male rats exposed to 10 days chronic restraint stress during adolescence period. Corticosterone was measured in PND 30 (baseline), PND40 (at the end of stress period), and PND60 (adulthood). Corticosterone level in the stressed group was significantly higher than the control group in PND30 ($p < 0.001$), but not PND60. ***$p < 0.0001$ than the No-Stress group. *PND* postnatal day

The percent of open arm time was significantly decreased in the SAL-S group than the SAL-NS group ($p = 0.024$). The percent of open arm time significantly increased in the C25-S ($p = 0.032$), C50-S ($p = 0.001$) and EXC/C25-S ($p = 0.01$) groups than the SAL-S group. Data of open arm entry in the EPM are illustrated in Fig. 3b. A two-way ANOVA showed no significant main effects of stress ($F_{1,100} = 1.347$, $p = 0.249$), significant main effects of treatment ($F_{5,100} = 4.992$, $p = 0.0001$) and an interaction between stress and treatment ($F_{5,100} = 4.322$, $p = 0.001$). Moreover, open arm entry significantly decreased in the SAL-S group than the SAL-NS group ($p = 0.001$). Open arm entry significantly increased in the C25-S ($p = 0.0001$), C50-S ($p = 0.0001$), EXC/C25-S ($p = 0.046$), EXC/C50-S ($p = 0.005$) groups than the SAL-S group.

Analysis of total arm entry data (not shown) showed no significant main effects of stress ($F_{1,100} = 2.386$, $p = 0.126$), but significant main effects of treatment ($F_{5,100} = 3.283$, $p = 0.009$) and no significant interaction between stress and treatment ($F_{5,100} = 1.86$, $p = 0.109$). Only, the difference between the EXC-NS and C50-NS group was significant ($p = 0.013$).

Depression-Like Behaviour

A two-way ANOVA on immobility time (Fig. 4a) showed significant main effects of stress ($F_{1,100} = 34.68$, $p = 0.0001$) and treatment ($F_{5,100} = 14.52$, $p = 0.0001$), but no interaction between stress and treatment ($F_{5,100} = 1.87$, $p = 0.105$). The immobility time of the SAL-S group was significantly higher than that of the SAL-NS group ($p = 0.001$). As

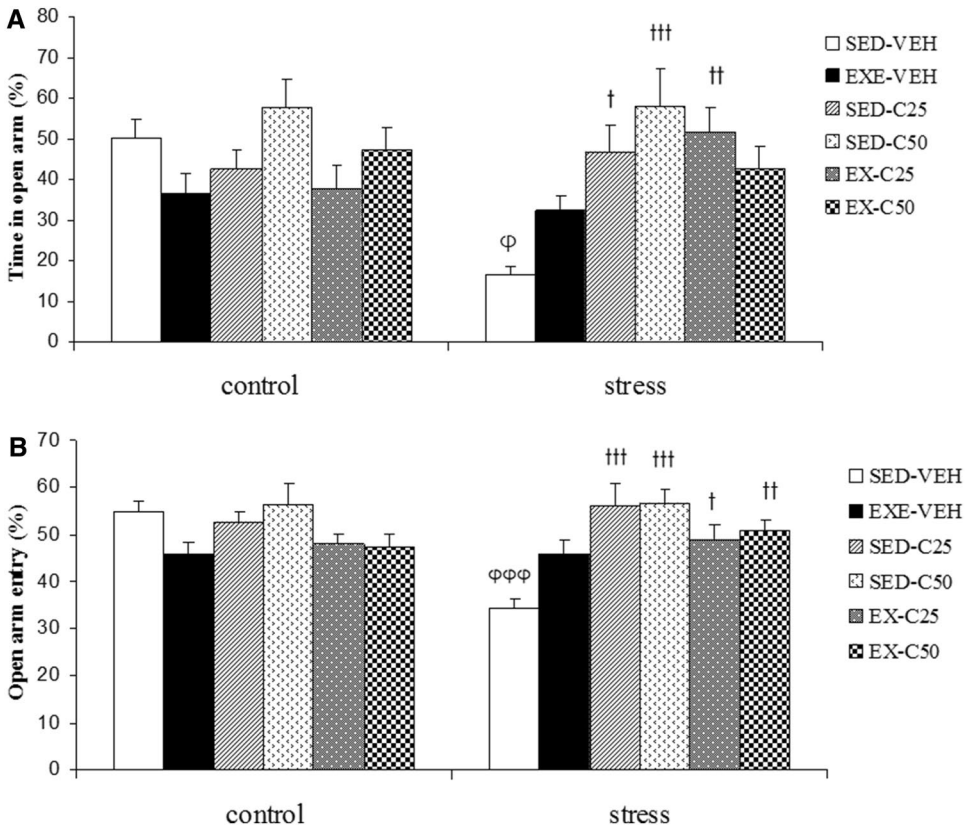

Fig. 3 Effects of voluntary exercise, crocin and the combined treatment on anxiety-like behaviors induced by adolescent chronic restraint stress. **a** Time spent in open arm, and **b** open arm entry. Adolescent chronic stress enhanced anxiety as the stressed rats spent showed less activity (time spent and number of entries) in the open arms than control group. Crocin or the combined treatment inhibited the stress induced anxiety-like behaviors. $^{\Phi}p < 0.05$, $^{\varphi\varphi\varphi}p < 0.001$ than the control group; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ than the stressed SED-VEH group

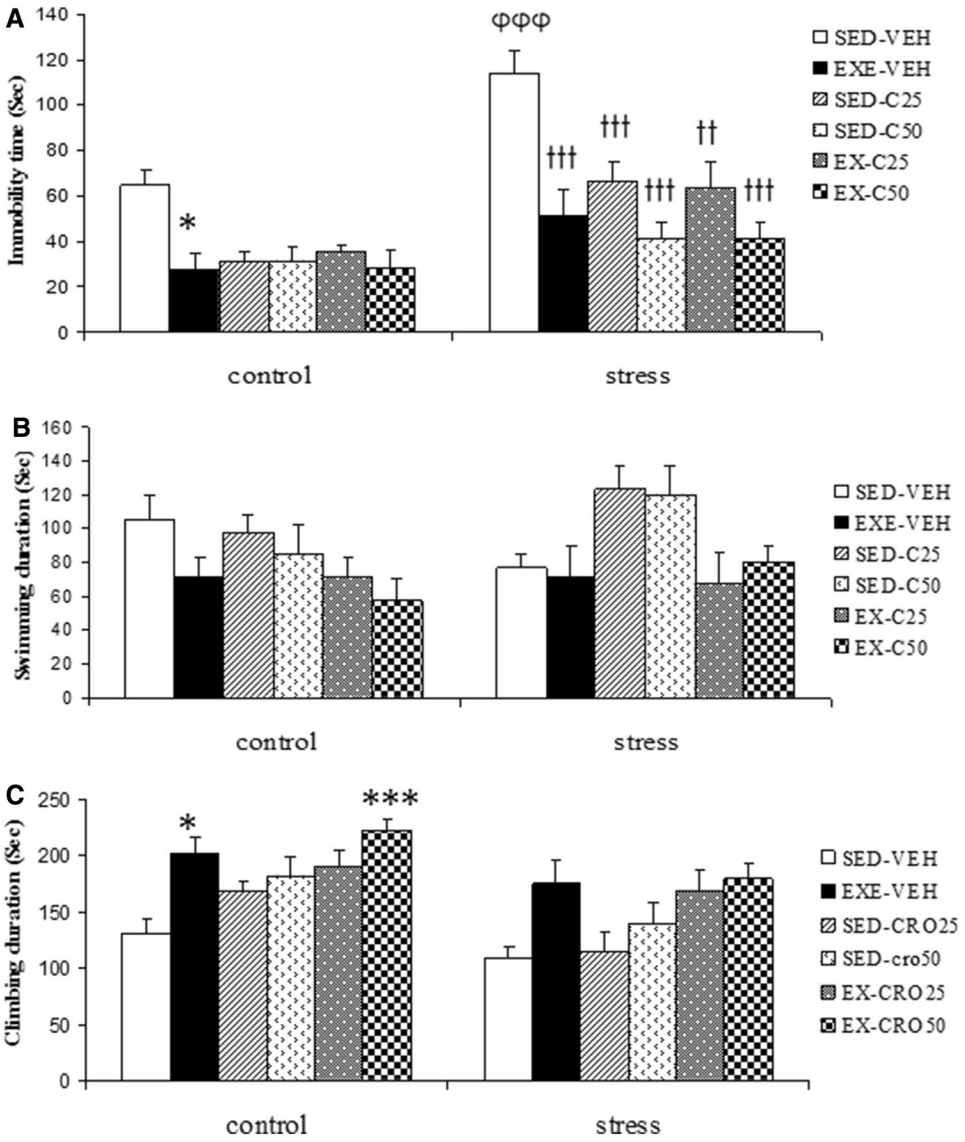

Fig. 4 Effects of voluntary exercise, crocin and the combined treatment on depression-like behaviors induced by adolescent chronic restraint stress. **a** Immobility time (s), **b** swimming duration, and **c** climbing duration in force swimming test. Adolescent chronic stress enhanced depression-like behaviors as the stressed rats showed increased immobility time than control group. Voluntary exercise, crocin and the combined treatment prevented inhibited the stress response. $^{\varphi\varphi}p<0.01$ than the control SED-VEH group. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ than the stressed SED-VEH group

shown in Fig. 4a, the duration of immobility in the EXC-S ($p=0.0001$), C25-S ($p=0.001$), C50-S ($p=0.0001$), EXC/C25-S ($p=0.002$) and EXC/C50-S ($p=0.0001$) groups was significantly lower than that of the SAL-S group. Also, the difference between the SAL-NS and EXC-NS ($p=0.027$) was significant.

Analysis of swimming duration (Fig. 4b) showed no significant main effects of stress ($F_{1,100}=1.234$, $p=0.269$), and significant main effects of treatment ($F_{5,100}=3.46$, $p=0.006$) and no significant interaction between stress and treatment ($F_{5,100}=1.638$, $p=0.157$).

Analysis of climbing duration (Fig. 4c) showed significant main effects of stress ($F_{1,100}=16.86$, $p=0.001$), significant main effects of treatment ($F_{5,100}=8.87$, $p=0.0001$) and no significant interaction between stress and treatment ($F_{5,100}=0.502$, $p=0.774$). The difference between the SAL-S and the EXC/C50-S groups was significant ($p=0.038$). Also, the climbing time of EXC/NS ($p=0.029$) or EXC/C50-NS ($p=0.001$) was significantly longer than that of the SAL-NS group.

Dendritic Morphology in the PFC

Dendritic Morphology in the IL Region of the PFC

Data from dendritic length and branch points of pyramidal neurons in the IL region of the PFC are shown in Fig. 5. Analysis of the dendritic length data (Fig. 5a) showed significant main effects of stress ($F_{1,60}=19.1$, $p=0.0001$) and treatment ($F_{5,60}=87.7$, $p=0.0001$) and a significant interaction between stress and treatment ($F_{5,60}=18.3$, $p=0.0001$). The dendritic length in the SAL-S group was significantly shorter than SAL-NS group ($p=0.0001$). Also, the dendritic length in all stressed groups treated with crocin or exercise

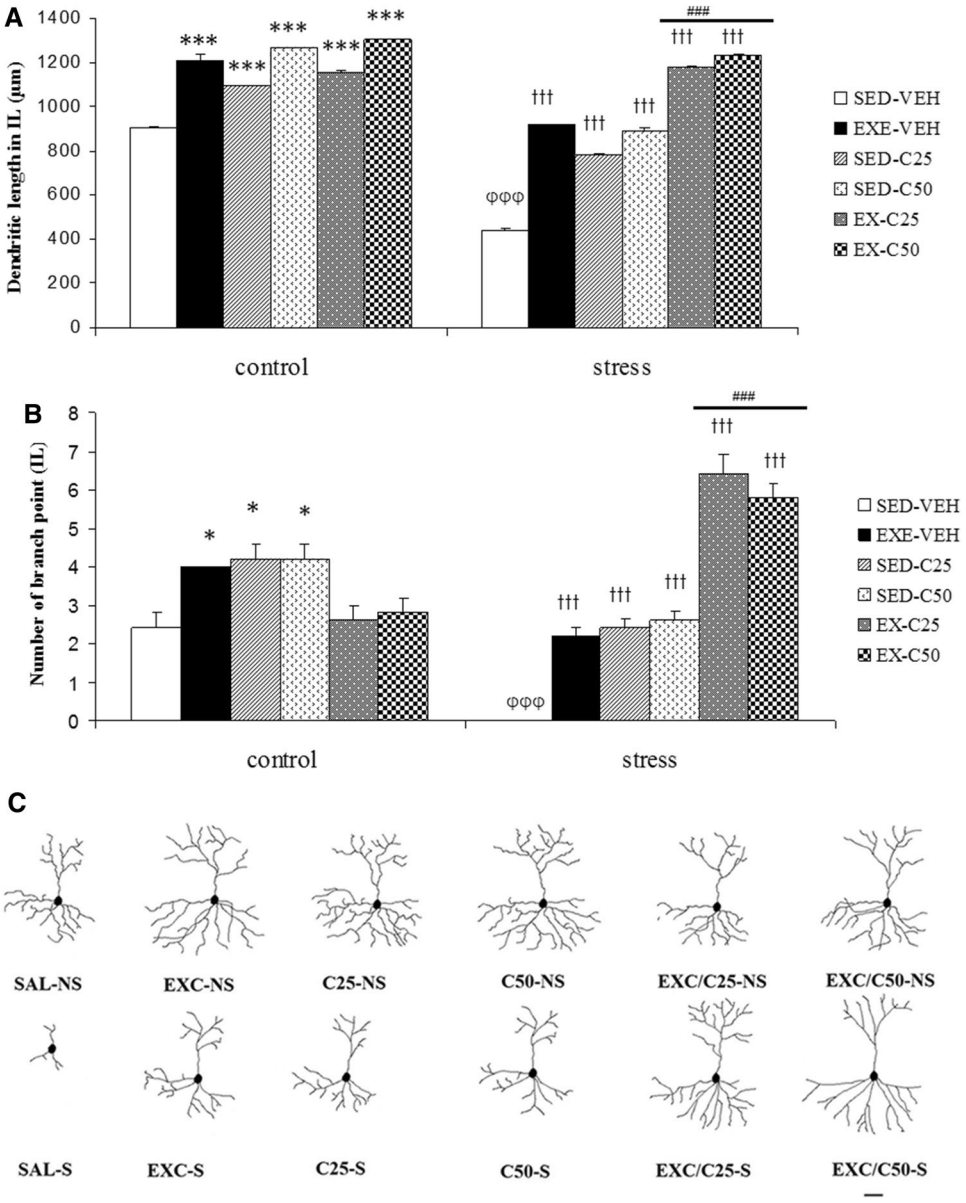

Fig. 5 Effects of voluntary exercise, crocin and the combined treatment on neuronal remodeling in the IL region of the prefrontal cortex induced by adolescent chronic restraint stress. **a** dendritic length, and **b** dendritic branch points. Adolescent chronic stress reduced dendritic length branch points. All treatments were able to reverse the effects of stress. $^{\varphi}p<0.05$, $^{\varphi\varphi}p<0.01$, $^{\varphi\varphi\varphi}p<0.001$ than the control SED-VEH group; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ than the stressed SED-VEH group.*$p<0.05$, **$p<0. 01$, ***$p<0.001$ than the control SED-VEH group and $^{\#\#\#}p<0.001$ than EXE-VEH, SED-C25, and SED-C50 groups. **c** Computer-assisted reconstructions of Golgi impregnated pyradmial neurons from the IL region of the prefrontal cortex in unstressed (above) and stressed (below) groups exposed to crocin, exercise and the combined treatments. These neurons were selected because they are representative of dendritic lengths near their respective group means. Scale bar: 50 µm

was longer than the SAL-S group (all, $p<0.01$). The EXC/C25-S, and EXC/C50-S groups had significantly higher dendritic length than the EXC-S, C25-S and C50-S groups (all, $p<0.001$), showing a potentiating effect between exercise and crocin on dendritic length. The differences between the SAL-NS and all other sham-treated groups were significant (all, $p<0.001$).

ANOVA on dendritic branch points (Fig. 5b) showed no significant main effects of stress ($F_{1,60} = 0.492$, $p=0.486$), but significant main effects of treatment ($F_{5,60} = 25.477$, $p=0.0001$) and a significant interaction between stress and treatment ($F_{5,60} = 35.2$, $p=0.0001$). Dendritic branch points of the IL neurons in the SAL-S group were significantly lower than that in the SAL-NS group ($p=0.0001$). Also, dendritic branch points in the stress groups treated with crocin stress ($F_{1,60} =$ or exercise were higher than the SAL-S group (all, $p<0.001$). The EXC/C25-S, and EXC/C50-S groups had significantly higher dendritic branch points than the EXC-S, C25-S and C50-S groups (all, $p<0.001$).The differences between the SAL-NS group with the C25-NS ($p=0.05$), and C50-NS ($p=0.015$) groups were significant.

Dendritic Morphology in the PL Region of the PFC

Analysis of the PL (Fig. 6a) data showed significant main effects of stress ($F_{1,60} = 63.1$, $p=0.0001$), and treatment ($F_{5,60} = 35.13$, $p=0.0001$) and a significant interaction between stress and treatment ($F_{5,60} = 98.6$, $p=0.0001$). The dendritic length of the PL neurons in the SAL-S group was significantly shorter than the SAL-NS group ($p=0.0001$).

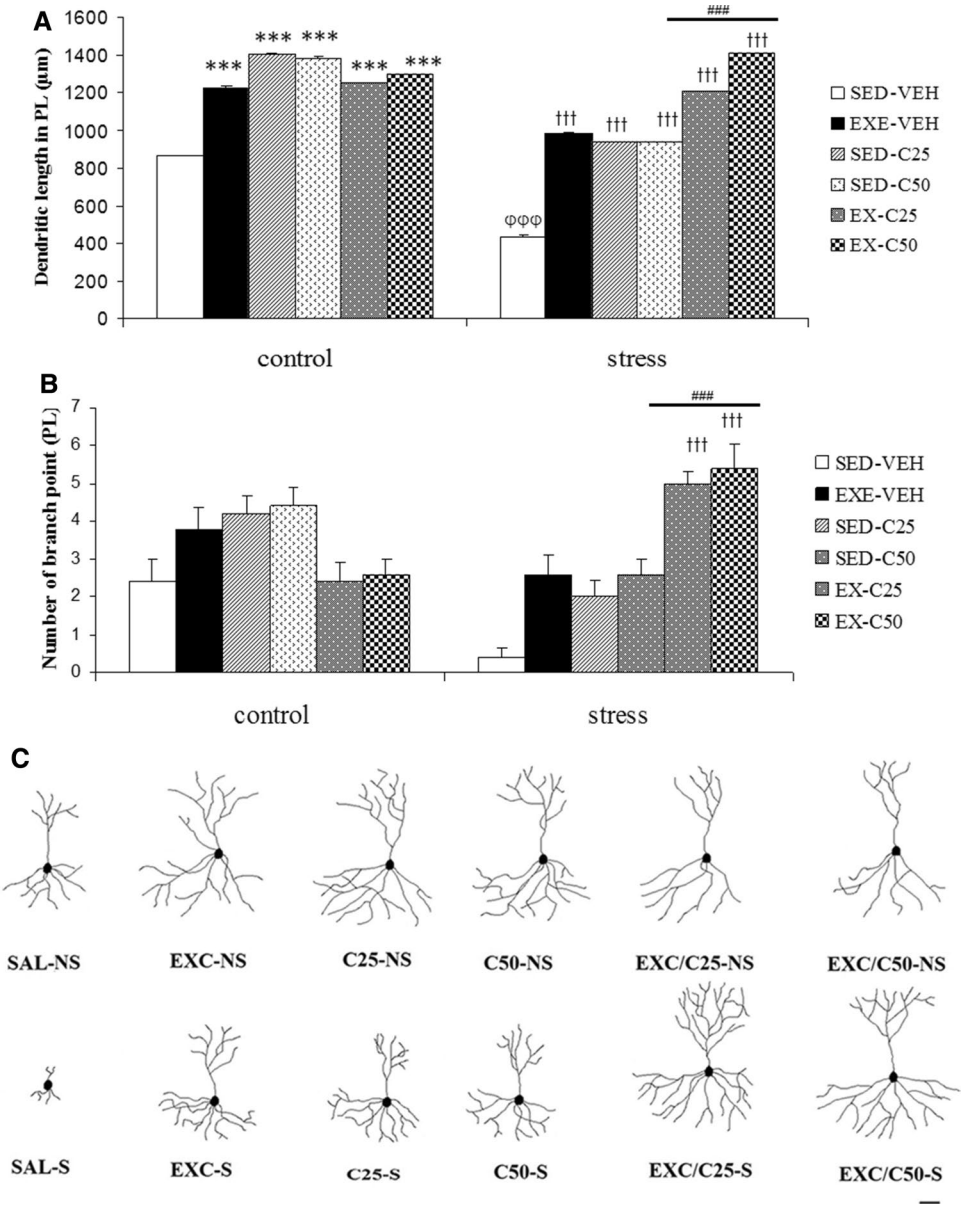

Fig. 6 Effects of voluntary exercise, crocin and the combined treatment onneuronal remodeling in the PL region of the prefrontal cortex induced by adolescent chronic restraint stress. **a** dendritic length, and **b** dendritic branch points in the PL. Adolescent chronic stress reduced dendritic length. All treatments were able to reverse the effects of stress. $^{\varphi}p<0.05$, $^{\varphi\varphi}p<0.01$, $^{\varphi\varphi\varphi}p<0.001$ than the control SED-VEH group; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ than the stressed SED-VEH group.*$p<0.05$, **$p<0. 01$,***$p<0.001$ than the sham SED-VEH group, and $^{\#\#\#}p<0.001$ than EXE-VEH, SED-C25, and SED-C50 groups. **c** Computer-assisted reconstructions of Golgi impregnated pyramidal neurons from the PL region of the prefrontal cortex in unstressed (above) and stressed (below) groups exposed to crocin, exercise and the combined treatments.These neurons were selected because they are representative of dendritic lengths near their respective group means. Scale bar: 50 µm

The dendritic length in the PL neurons in the stress groups treated with crocin or exercise was longer than the SAL-S group (all, $p<0.01$). Moreover, the EXC/C25-S, and EXC/C50-S groups showed significantly longer dendritic length than the EXC-S, C25-S and C50-S groups (all, $p<0.001$). The differences between the SAL-NS group and all other sham-treated groups were significant (all, $p<0.01$).

Analysis of dendritic branch points of the PL neurons (Fig. 6b) showed no significant main effects of stress ($F_{1,60} = 1.133.758$, $p=0.292$), a significant effect of treatment ($F_{5,60} = 7.074$, $p=0.0001$) and a significant interaction between stress and treatment ($F_{5,60} = 11.572$, $p=0.0001$). No significant difference was found between the SAL-S and SAL-NS groups. Dendritic branch points in the EXC/C25-S, and EXC/C50-S groups were significantly higher than the SAL-S group (both, $P=0.0001$). Moreover, the EXC/C25-S, and EXC/C50-S groups showed higher dendritic branch points than the EXC-S, C25 - S and C50-S groups (all, $p<0.001$).

These findings together indicate that adolescent stress caused a decline in dendritic length, and branch points in the PFC, and crocin treatment, exercise and the combined treatment alleviated adolescent stress-induced behavioral and structural abnormalities.

Adrenal Weight

A two-way ANOVA on the mean adrenal gland weight (mg/100 g body weight) (Fig. 7) demonstrated no significant main effects of stress ($F_{1,100} = 2.197$, $p=0.145$),

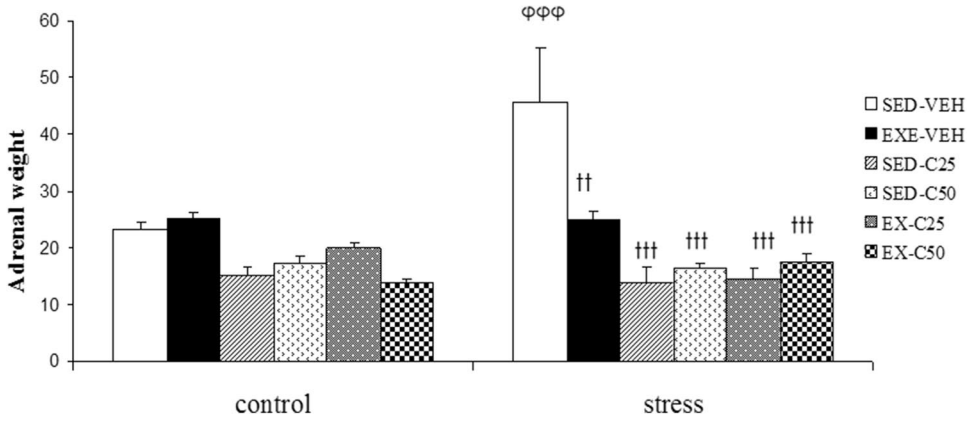

Fig. 7 Effects of voluntary exercise, crocin and the combined treatment onincreased adrenal size (mg/100 g body weight) induced by adolescent chronic restraint stress. Adolescent stress significantly increased the adrenal weight. All treatments were able to recover this response. $^{\varphi\varphi\varphi}p < 0.001$ than the control SED-VEH group; $^{\dagger\dagger\dagger}p < 0.001$ than the stressed SED-VEH group

significant main effects of treatments ($F_{5,100} = 10.234$, $p = 0.0001$) and significant interaction between stress and treatments ($F_{5,100} = 4.31$, $p = 0.002$). Moreover, adrenal gland weight significantly increased in the SAL-S group than the SAL-NS group ($p = 0.001$). The percent of adrenal weight significantly decreased in the EXC-S ($p = 0.005$), C25-S ($p = 0.0001$) and CO50-S ($p = 0.0001$), EXC/C25-S ($p = 0.001$) and EXC/C50-S ($p = 0.001$) groups than the SAL-S group.

Discussion

Our study showed rats exposed to chronic stress during adolescence exhibited anxiety and depression like behaviors, enhanced the corticosterone levels and adrenal weight in adulthood. These changes were accompanied by a significant decrease in the dendritic length in both IL and PL and dendritic branch points in the IL regions of the PFC in adulthood. Crocin treatment, exposure to wheel running and the combined crocin and exercise alleviated adolescent stress-induced behavioral and structural abnormalities.

Adolescent Stress Enhances Corticosterone Levels

Evidences evidence that immobile stress in adolescent period can increase the corticosterone level in rats [44, 45]. The present study showed that chronic stress can significantly elevate the baseline corticosterone levels in adolescent (the end of stress in PND40) (Fig. 2). However, this enhanced response was not found in adulthood (PND60), confirming the findings of other studies showing that exposure to chronic restraint stress during adolescence did not influence baseline corticosterone levels in adult male rats [45]. On the other hands, animals exposed to forced swim, elevated platform and restraint stress between PND27 and 29 displayed increased basal corticosterone levels as adults [46].

Adolescent Stress Induces Anxiety or Depression Like Symptoms in Adulthood: Beneficial Influences of Crocin and Exercise

The present study indicated adolescent stressed rats exhibited anxiogenic and depression like behaviors in adulthood as assessed by the EPM (Fig. 3) and FST (Fig. 4), respectively. The stressed rats exhibited significantly less time in open arms and fewer open arms entries than the control group. However, there was no significant difference in number of the total arm entries between the two groups, suggesting that increased anxiogenic-like behaviors in the stressed rats were not due to hypo-activity or motor impairment. The stressed group also showed increased immobility time in the FST than the non-stress group in adulthood. These findings are consistent with other studies showing that stress can induce the anxiety and depression like behavior in different lifespan [8]. Recent studies have demonstrated that the experience of variable stress in the juvenile period increased anxiety and depression like behaviors, and up-regulated hippocampal MR mRNA expression, and remodeled cortico-limbic architecture, and altered HPA axis activity and L1-CAM expression in adulthood [13, 33, 47–49]. It is reported that an increase in anxiety like-behavior in the restraint stressed rodents was correlated with the elevated plasma levels of adrenocorticotropic hormone and corticosterone [50]. Moreover, prolonged and repetitive exposure to glucocorticoids during juvenile development produced neurotoxic effects on several brain regions related to anxiety and depression, such as hippocampus, amygdala, and PFC [8], which would help explain the depressive- and anxiety-like behavior observed later in life.

The current work demonstrated that treatment with crocin and the combined crocin and exercise led to anti-anxiety and antidepressant effects as demonstrated by increased open arm time and entry in the EPM (Fig. 3) and reduced the immobility time in the FST (Fig. 4). Exercise alone resulted in antidepressant-like behavior as shown by reduced the immobility time in the FST. The observed anti-anxiety and

depressant effects of crocin are supported by the recent studies. For example, a recent study has shown that that subacute administration of crocin in different doses decreased the immobility time of rats in the FST, and increased the protein levels of cAMP response element binding (CREB), brain derived neurotrophic protein (BDNF), and vascular endothelial growth factor (VEGF) in the hippocampus [37]. VEGF plays an important role in hippocampal neurogenesis [51] and response to stress [52], as well as neuroprotective effects [53]. The neuropeptide VEGF is down-regulated by animal models of depression such as FST and infusion of VGF in the brain results in antidepressant-like behavior in the FST, suggesting a role for VEGF in depression [54]. Also, the effectiveness of BDNF as anti-depressant agent in the FST model of depression has demonstrated. In fact, a single bilateral injection of BDNF into the dentate gyrus of hippocampus produced an antidepressant effect in both the learned helplessness and FST tasks that was comparable in magnitude with repeated systemic administration of a chemical antidepressant [55]. Moreover, both exercise and antidepressants up-regulated the genes of BDNF and VEGF, whose transcriptions also are dependent on cAMP response element binding protein (CREB) [54]. As mentioned above, both systemic crocin and exercise increase BDNF and VEGF in the hippocampus in rats. Thus, it seems likely these neurotrophic factors mediate the observed beneficial effects of crocin and voluntary exercise against adolescent stress induced anxiety or depressive-like symptoms. Further work is needed to examine this assumption.

Changes in HPA axis activity is another important mechanism that may mediate the anxiolytic or antidepressant-like effects of crocin, exercise and the combined treatment in stressed rats. Previous studies have shown that chronic stress or corticosterone administration of adolescent rats increased anxiety in the EPM and decreased immobility in the FST [56]. The high similarity between the effects of chronic treatment with corticosterone and chronic stress on anxiety- and depression-like behaviors highlight the role of glucocorticoids in the effects of stressors [56]. We recently reported crocin can prevent spatial learning and memory deficits as well as oxidative stress damage to the hippocampus, and reduce serum corticosterone levels in rats exposed to chronic restraint stress [18]. It was reported that both saffron and crocin treatment reduced the lipid oxidation in the hippocampus of stressed rats [57], and crocin reduced the neuron number loss in the hippocampus of diabetic rats [58]. A recent study also has shown that systemic injection of crocin was able to reverse the impairing effects of chronic stress on spatial memory and to lower corticosterone levels in the hippocampus and frontal cortex in rats [59]. Additionally, the present study showed that treatment with crocin, exposure to wheel running activity, and the combined interventions completely suppressed chronic stress induced adrenal hypertrophy (a typical stress response in living organisms) (Fig. 7), indicating anti-stress effects of these interventions. Despite that chronic exercise increases CORT levels in a similar manner to chronic stress, it is proposed that elevated CORT acts to elevate dopamine in the mPFC under chronic exercise, but not chronic stress, and the mPFC dopaminergic system may, at least in part, exert various beneficial effects of exercise on cognition, mood, and the brain [60]. These findings together suggest that the interaction with the HPA axis is might be an important mechanism that mediates the protective effects of crocin or exercise against the harmful effects of chronic stress on brain functions and structures.

Adolescent Stress Induces Abnormal Neuronal Remodeling in the PFC in Adulthood: Beneficial Influences of Crocin and Exercise

Chronic adolescent stress not only changes behaviors but also induces neuronal remodeling such as retraction of neuritis. For example, it has been reported that adolescent chronic stress induced changes in HPA reactivity, depressive-like behavior, and remodeling of pyramidal neurons in the hippocampus, PFC and amygdala in both males and females rats [13]. The present work showed that adolescent stress caused a significant decrease in the dendritic length and in the PFC sub-regions and dendritic branch points in the IL area in adulthood (Figs. 5, 6). Several previous studies indicated that chronic stress in adulthood induced dendritic retraction and a reduction in spine number in the pyramidal neurons in layers II/III of the PFC [61, 62]. Moreover, pyramidal neurons in layer III of the PL and IL regions of the PFC in male rats were affected by chronic stress [63]. Also, apical dendritic branches were reduced in stressed male rats [64], which is accompanied by spine loss [65]. A recent study indicated that chronic adolescent stress can induce pyramidal cell atrophy in the pre-limbic regions of the PFC in both male and female rats [13]. It seems that glucocorticoids plays an important role in the mediating the effects of chronic stress on the mPFC remodeling. The mPFC contains a high density of adrenal steroid receptors [66], which become activated during stress and mediate the stress response [67]. Glucocorticoids also directly stimulate release of excitatory amino acids which, in turn, are mediators of the stress-induced remodeling in the PFC as NMDA receptor blockade alters stress-induced dendritic remodeling in medial prefrontal cortex [68]. Moreover, glucocorticoids regulate release of endocannabinoids which also mediate stress induced structural remodeling in the PFC [69].

The mPFC plays a central role in the regulation of emotional behaviors as the reduced activity of the mPFC results in depression- and anxiety-like behaviors in mice [70]. The present study showed that adolescent chronic stress led to dendritic retractions and loss in the mPFC;

these changes appear to be the morphological correlate of stress-induced impairment of PFC-dependent behavior(s). The present study showed that crocin, wheel running and the combined treatment alleviated adolescent stress induced behavioral deficits and structural abnormalities in the PFC. Also, a synergistic effect was found between crocin and exercise on dendritic length and branching (Figs. 5, 6). Moreover, these treatments had positive effects on the PFC morphology in non-stressed rats.

Previous studies have shown the positive effects of voluntary exercise during adolescence on brain structure and functions. For example, voluntary exercise for 13 days in adolescence (PND30–42) produced increases in both cell proliferation and survival within the DG in male rats [71]. Moreover, voluntary exercise for 4 weeks during adolescence (starting PND31) increased recognition memory performance in the novel object in male rats [72]. The mechanism underlying the effects of exercise on brain function and structure during adolescence period is not well defined. However, changes in the activity of the endocannabinoids system and HPA axis, and increases in BDNF levels are some possible mechanisms that may mediate the effects of exercise during adolescence period [73].

In conclusion, our study is the first to demonstrate the beneficial effects of crocin, exercise and the combined crocin and exercise against behavioral deficits and abnormal morphological remodeling in the PFC induced by adolescent stress in adulthood. Moreover, these treatments exerted positive morphological effects on this area, which is involved in emotion and cognition, in non-stressed rats. Adolescence may represent a critical period for which chronic stress can induce long-lasting behavioral and morphological modifications extending in adulthood. Thus, the appropriate interventions such as exercise and healthy diet (saffron) during this critical time may ameliorate stress-induced deficits in brain function and structure and even improve brain performance in healthy (non-stressed) subjects.

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Author Contributions MG-S and ARP designed the overall study and wrote the paper. MG-S, SN, BY, AAV conducted the research, collected data and carried out the lab work. MG-S and ARP carried out the statistical analysis and mostly drafted the manuscript. ARP coordinated and supervised the study. All authors approved the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors report no biomedical financial interests or potential conflicts of interest regarding this work.

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