Maternal treadmill exercise during pregnancy decreases anxiety and increases prefrontal cortex VEGF and BDNF levels of rat pups in early and late periods of life

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ABSTRACT

In a previous study we demonstrated that, regular aerobic exercise during pregnancy decreased maternal deprivation induced anxiety. The purpose of this study is to investigate whether the positive effects of maternal exercise on the male and female offspring's early and late period of life. Half of the test subjects in each group were evaluated when they were 26 days old, and the other half were evaluated when they were 4 months old. The anxiety levels of maternally exercised groups were less than the control groups in both sexes and in both prepubertal and adult periods. The locomotor activity more increased in females. The prefrontal VEGF and BDNF levels were greater for both age groups and sexes in the maternally exercised group compared to control group. Moreover, there was a strong positive correlations between prefrontal cortex BDNF levels and results of open field tests; and VEGF levels and results of elevated plus maze tests. There was no difference in serum corticosterone levels between groups. These results indicate that maternal exercise during pregnancy may protect the pups from anxiety in early and late periods of life, and affects the prefrontal cortex positively.

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It is known that regular aerobic exercise improves certain brain functions by enhances cerebrovascular integrity and increases capillary growth and dendritic connections [9]. Aerobic exercise, in young and adult rats, increases the cognitive performance by improving the results of hippocampus related learning and memory tests [19,34,45].

Regular aerobic exercise during pregnancy improves the growth of the fetus and the placenta, and increases birth weight in humans [10]. In recent studies, it has been shown that exercise during pregnancy improved learning and memory of pups [18,19,23,30]. Regular aerobic exercise during pregnancy decreased maternal deprivation induced anxiety in our previous study [47]. However, it is not known whether the positive effects of maternal exercise has been continue to the adult period of pup's life.

Brain development in mammals starts in utero and continues until the end of the adolescent period [36]. Brain, throughout this development process, can easily be affected by internal and external factors (it has been observed that some events during this period affect the brain negatively, while others affect positively). For instance, stressful circumstances such as maternal deprivation in early developmental periods may cause various neuropsychiatric disorders which can affect the later periods of life, such as anxiety disorder, schizophrenia and depression [46]. Enriched environment and physical exercise, on the other hand, increase the number of brain cells and improve learning and memory [19,26,39].

Anxiety can be defined as an exaggerated emotional and dysfunctional state associated with hyper vigilance and increased behavioral responsively to fearful stimuli [38]. It has been shown that amygdala and the prefrontal cortex (PFC) have critical roles in emotional functions such as fear and anxiety. It is believed that the PFC regulates and controls the output of amygdale [33]. Therefore, the mutual relationship between amygdala and the PFC necessitates the examination of these brain areas separate from other brain regions. In recent studies, these two regions have been examined together and the structural and functional relation between them have been revealed [18].

Neurotrophic factors such as NGF, BDNF and VEGF are important agents in the growth, development and plasticity of the brain.

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It is known that BDNF and VEGF are associated with anxiety. BDNF deficiency causes aggressive behavior and anxiety disorders in animals and humans [8,14]. VEGF secretion was observed irregularities in anxious and depressed individuals [29]. It has been shown that exercise increased the neurotrophic factors such as BDNF and angiotrophic hormones such as VEGF in the hippocampus and the blood [37].

The aim of this study is to explore the effects of maternal aerobic exercise on the anxiety levels of the pups, and he prefrontal cortex BDNF and VEGF levels.

Fifty-six Wistar Albino rats were included in the study. The study group consisted of animals whose mothers performed regular aerobic exercise during pregnancy while the control group consisted of pups whose mothers were sedentary throughout the pregnancy. Each group was further divided into subgroups: male, female, prepubertal and adult, resulting in eight groups with 7 animals in each. The animals were maintained under standard colony conditions with a 12 h light/dark cycle (lights on 07:00 h), at constant room temperature ($22 \pm 1\,^{\circ}C$), and humidity (60%). Food and water were available ad lib. Experiments were carried out between 9.00 and 11.00 am in a sound-attenuated and air-regulated experimental room. All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylul University School of Medicine.

Mothers in the exercise group were familiarized to the treadmill, 1 week before the pregnancy, by 10 min/session a day at a speed of 5 m/min for 5 days. These mothers started exercising at 8 m/min for 30 min 5 days a week the day after mating. In the last week of pregnancy the speed was reduced to 6 m/min and the exercise was done for 30 min 5 days a week. This exercise type is regular mild treadmil exercise [45,47]. The anxiety levels of half of the group were evaluated when the pups were 26 days old (prepubertal group), and the other half when the pups were 4 months old (adult group), with open field arena and elevated plus maze apparatus. The recording and analysis of the anxiety test were done using the HVS video-tracking system.

The open-field consisted of an area of $1\,m \times 1\,m$ surrounded with a wall 50 cm in height, with a video camera installed 2.5 m above the apparatus. Each rat was placed in the center of the open-field and then locomotor activity (ambulation) was measured for 5 min in a sound-proof observation room illuminated with controlled light (100 lx).

Elevated plus maze apparatus consisted of a central platform ($5\,cm \times 5\,cm$) with two open arms (50 cm long, 10 cm wide and 0.5 cm high borders) and two closed arms (50 cm long, 10 cm wide with 40 cm high walls) that were elevated 50 cm above the ground. Rats were placed on the platform facing the open arm and were observed for 5 min. The total number of entries into the open and closed arms as well as the time spent in the open and closed arms were measured.

At the end of the behavior tests, after a light ether anesthesia, the blood of the subjects were drawn, and the PFC regions were separated after removing the brain tissue.

The serum corticosterone levels were measured with the radioimmunoassay method using a double antibody kit (ImmuChem, MP Biomedicals, Orangeburg, NY).

PFC tissue homogenate was analyzed by enzyme immunoassay for BDNF (Catalog Number EK0308, Boster Immunoleader, Wuhan, China) with assay sensitivity <2 pg/ml and range 31.2–2000 pg/ml and VEGF (Catalog Number EK0308, Boster Immunoleader, Wuhan, China) with assay sensitivity <1 pg/ml and range 15.6–1000 pg/ml.

PFC tissues were removed and fixed in 10% formalin in phosphate buffer for 24 h. The PFC was sectioned coronally into sequential 5 μm sections using a microtome. Each sample was subjected to the estimation of neuron density by taking three coronal sections through the prefrontal cortex that corresponded approximately to Plates 9, 11 in the rat atlas of Paxinos and Watson. All sections were stained by cresyl violet. The images were analyzed using a computer assisted image analyzer system consisting of a microscope (Olympus CX-41 Tokyo, Japan) equipped with a high-resolution video camera (Olympus DP71, Japan). The numbers of neurons in these regions were counted in $40\times$ mag. by a $16{,}800\,\mu m^2$ counting frame. The counting frame was placed randomly three times on the image analyzer system monitor and the numbers of neurons were counted and the average was taken. PFC neuron density was calculated.

Immunohistochemical staining was performed using the streptavidin/biotin method. The immunohistochemistry procedure for VEGF was performed. Tissue sections were incubated at $60\,^{\circ}C$ overnight then dewaxed in xylene for 30 min. After rehydrating through a decreasing series of alcohol, sections were washed in distilled water for 10 min. They were then treated with 10 mM citrate buffer (AP-9003-125, Labvision) at $95\,^{\circ}C$ for 5 min, to unmask antigens by heat treatment. Then slides washed in deionized water three times for 2 min. Sections were delineated using a Dako pen (Dako) and incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. They were then incubated with normal serum blocking solution for 30 min. Sections were incubated in a humid chamber for overnight at $+4\,^{\circ}C$ with antibodies to: VEGF (1/100 dilution: SC-7629, Santa-Cruz biotechnology). For negative controls, distilled water was used in place of the primary antibodies. They were washed three times for 5 min each with PBS, followed by incubation with biotinylated IgG and then with streptavidin–peroxidase conjugate (Zymed 85-9042). After washing three times for 5 min with PBS, sections were incubated with DAB substrate containing diaminobenzidine (Zymed 00-2020) for 5 min to detect immunoreactivity and then with Mayer's hematoxylin. Sections were covered with mounting medium. Three PFC sections were used for each sample in performing the immunohistochemical scoring. The qualitative intensity of staining for VEGF was assessed using a scale between 0 and +++. With 0 representing no detectable stain and +++ representing strongest stain [2].

Differences in the behavioral and biochemical parameters were performed using the three way ANOVA post hoc Sheefe comparisons. Correlation between open field and elevated plus maze test results and VEGF, BDNF ELISA results were calculated using Pearson correlation analysis. Results are presented as mean ± S.E.M. (significance level was set at $p \leq 0.05$).

The locomotor activities of rats whose mothers exercised during pregnancy increased in the open field test, compared to rats whose mothers were sedantary throughout pregnancy (increased locomotion; increased mobility time and walking path, both sexes and both ages, $p < 0.001$) (Fig. 1A and B). Moreover, both male and female pups in the study group spent more time on the open branches of elevated plus maze device, and their anxiety levels were lower, compared to control group (both age and both sex, $p < 0.001$) (Fig. 1C). The pups in the study group demonstrated significantly less aggressive behavior compared to pups in the control group. Finally, maternal exercise was associated with increased prefrontal BDNF and VEGF levels in both prepubertal and adult periods (BDNF; prepubertal males and females $p < 0.004$; adult males and females $p < 0.05$, VEGF; prepubertal females $p < 0.006$, adult females $p < 0.001$, prepubertal males $p < 0.001$, adult males $p < 0.008$) (Fig. 2A). VEGF immune staining and marking were increased in all maternal exercised groups (Fig. 2B and C). The cell numbers increased in prefrontal cortex of all maternal exercised groups (Fig. 2D). There was not any differences blood corticosterone levels between control and study groups.

There was a very strong positive correlations between prefrontal cortex BDNF levels and open field test result (the walking distance
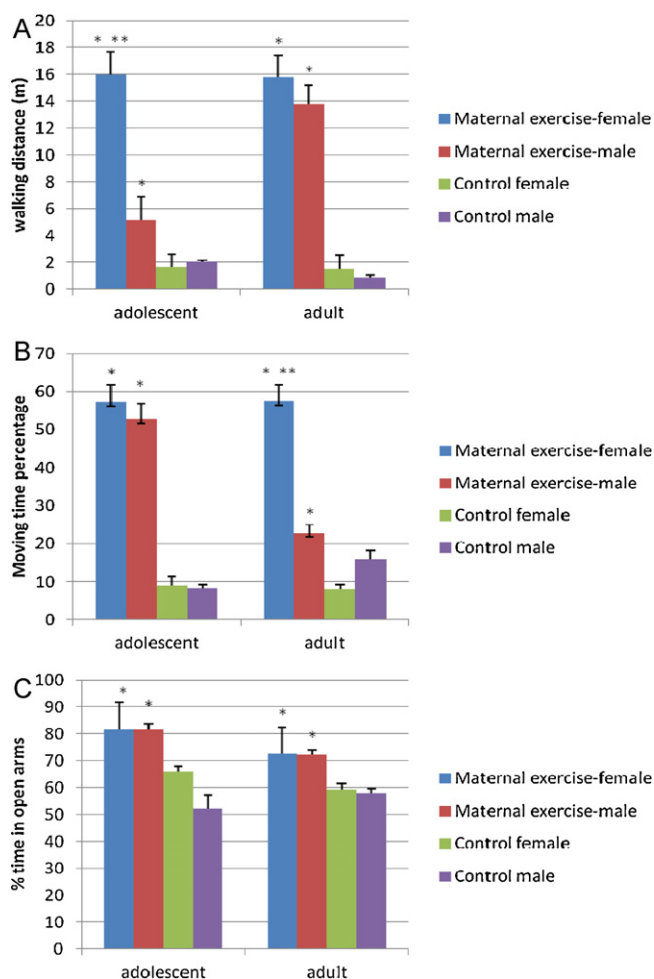
Fig. 1. Behavioral results of groups. (A) Walking distance in open field arena. (B) Percentage of time moving in open field test. (C) Elevated plus maze test results; *$p < 0.05$ compared to the same age and sex group, **$p < 0.05$ compared to the male pups of exercised mothers.

and BDNF; $r = 0.708$, $p = 0.000$, the moving time percentage and BDNF; $r = 0.718$, $p = 0.000$). Also, there was a strong positive correlation between prefrontal cortex VEGF levels and elevated plus maze test result (the time spent in the open arms and VEGF; $r = 0.606$, $p = 0.000$).

We have shown that pups whose mothers exercised during pregnancy took more risks in prepubertal and adult periods of life, they had more explorative behavior and lower levels of anxiety (they spent more time in open branches in elevated plus maze test, their locomotor activities were increased in the open field arena), and their prefrontal VEGF and BDNF levels were higher. To our knowledge, this is the first study about the effects of maternal exercise on prefrontal cortex of rats' prepubertal and adult periods of life. In the literature, most studies on the relation between exercise and anxiety investigated the effects of a single exercise period on the psychological state. These studies reported that exercise decreased anxiety in both normal and anxious individuals [5,25,40]. Regular swimming exercise decreased stress related anxiety, and regular treadmill exercise decreased stress induced anxiety, however they did not have any effect under normal conditions [5,40]. In children and adolescents, exercise decreased the anxiety score [22]. However, this decrease in the anxiety level is at its maximum 5–15 min after the end of the exercise period; the level returns to pre-exercise levels after 2–4 h [35]. On the other hand, the effects of long term exercise programs on anxiety are

not clear. We have demonstrated that regular maternal exercise decreased the anxiety levels of pups in both prepubertal and adult periods. BDNF, which has important role in neuronal survival, differentiation and synaptic plasticity, is an important molecule in mood disorder pathophysiology [6]. It has been previously shown that BDNF-deficient rats were more aggressive and had higher anxiety levels in open field and elevated-plus maze [8]. The humans who have less BDNF levels due to a variant gene have increased frequency of neuropsychiatric disorders such as depression, anxiety related dysfunctions and bipolar disorders [21]. It was also shown that angiotrophic factors (such as VEGF) have an important role, like neurotrophic factors, in the pathophysiology of psychiatric diseases. Dysregulation in VEGF signal was reported among individuals with anxious and depressive phenotype [14]. We found that the anxiety levels were low in groups with increased prefrontal cortex BDNF and VEGF levels. We also found a very strong positive correlation between the prefrontal cortex VEGF levels and open field test results, and prefrontal cortex BDNF levels and the elevated plus maze test results. It has been previously shown that maternal exercise increased the hippocampal BDNF levels [17,23,30]; however its effects on anxiety related prefrontal cortex BDNF and VEGF levels have not been reported.

It is known that PFC controls the emotional status and stress responses. Prefrontal cortex damage causes difficulty in emotional regulation [1]. Individuals with emotional regulation difficulties, also called the frontal disinhibition syndrome, had disproportionate emotional outburst due to provocation and took more risks [12]. PFC inhibited the autonomous and endocrine responses to stress [16]. The increase in PFC activity decreased anxiety [3].

Moreover, PFC regulates the dopamine release from the neurons in the mesocortical projection system. Dopamine is one of the significant neuromodulators of fear and anxiety [31]. The mesocortical dopaminerjic neurons originating from the midbrain ventral tegmental area and projecting to the prefrontal cortex have been implicated by many studies to be involved in the emotional responses to fear and anxiety. Cortical and limbic dopamine dysfunction causes emergence of anxiety [13]. Dopamine release from PFC was related to the regulation of anxiety and fear [41]. Increase of dopamine input in the nucleus accumbense from cortical structures and amygdala causes an exacerbation of symptoms of anxiety [7]. Exercise increases the dopamine synthesis in the brain by elevating the blood calcium level, thus increasing the brain calcium [42]. Therefore, increased dopamine levels may help in the treatment of diseases such as anxiety disorders and depression, which are associated with lower dopamine levels. In addition, dopamine is playing a major role in the control of locomotor activity. Stimulation of dopaminergic neurons causes an increase in locomotor activity [11]. In this study, the locomotor activity increased in maternally exercised rats.

Dopamine was induced by BDNF [43]. Anxiolytic effect of BDNF has trophic and modulatory effects on the growth and plasticity of dopaminergic neurons [6]. Stimulation of dopamine causes to increase in BDNF [20]. BDNF infusion to substantia nigra increases the dopamine turnover in striatum [27]; however, loss of BDNF expression causes downregulation in dopaminergic phenotype and dopaminergic neuronal death [32]. Also VEGF, which has decreased levels in anxiety, has a protective effect on dopaminergic neurons.

Anxiety disorders are more prevalent in girls than in boys, both in preadolescent period and adults [28]. The epidemiologic studies show that anxiety disorder is more prevalent in females than in males in adulthood [4]. However, the frequency of social anxiety disorder is the same for males and females [15]. In our study, we did not find any difference between the anxiety levels of females and males in prepubertal and adult periods in the elevated plus maze test. We also observed that the maternal
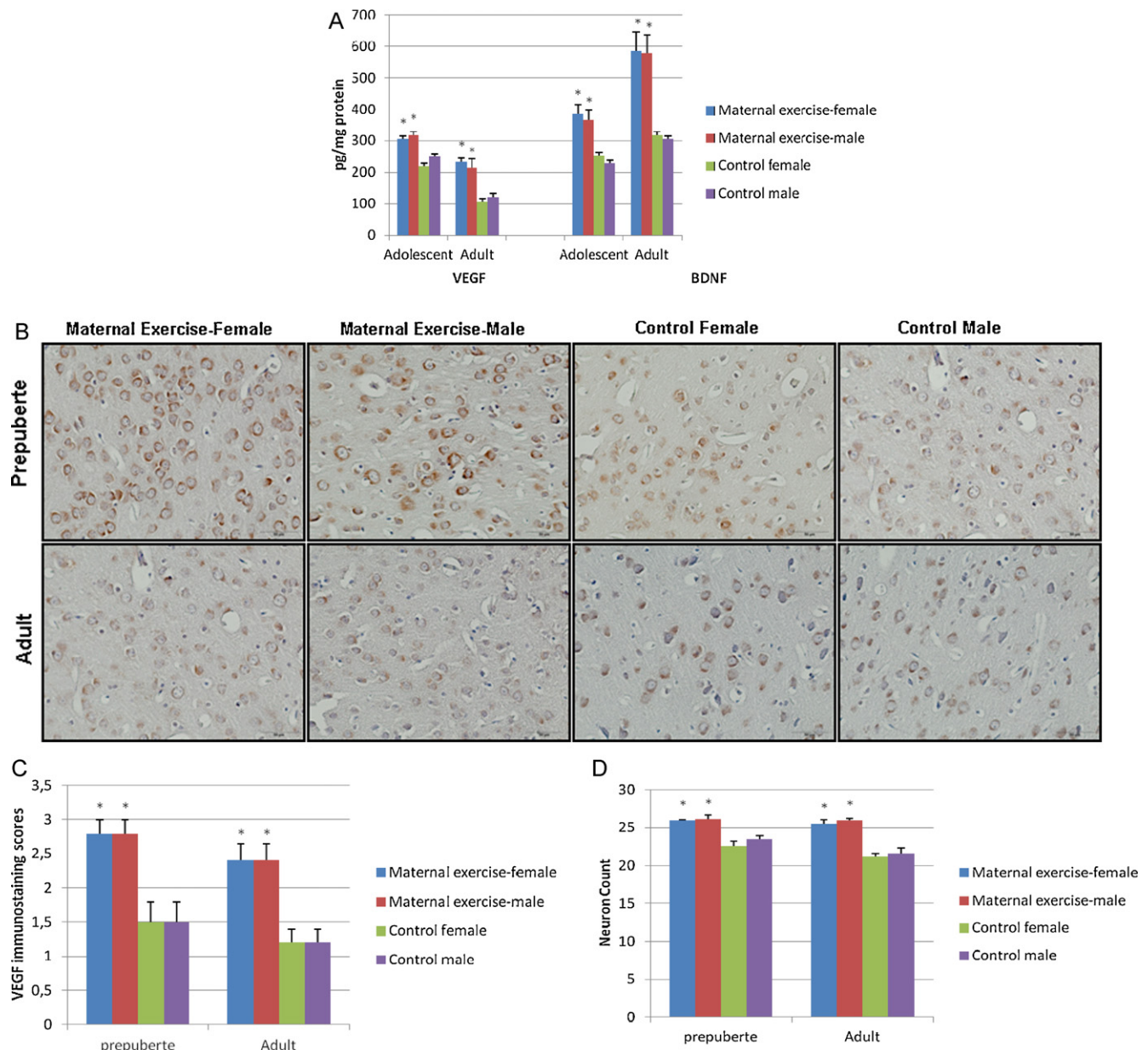
Fig. 2. The prefrontal VEGF and BDNF results of the groups. (A) The prefrontal VEGF and BDNF ELISA results of the groups. (B) VEGF+ immune staining cells. (C) Quantification in VEGF immunostaining scores. (D) Quantitative evaluation the effect of maternal exercise on the neuron numbers in prefrontal cortex. *$p < 0.05$ compared to the same age and sex group.

exercise increased the locomotor activity of females more than males, both in prepubertal and adult period in the open field test. It is known that, exercise increased monoamine levels such as tryptophan hydroxylase that is the precursor for serotonin synthesis [24]. Serotonin is well known that increase excitability of pre-motor and motor neurons. Stimulation of the motor cortex increases voluntary activation which can also be used to request voluntary activation [44]. In this study, increased locomotor activity in maternal exercised females may be resulted from increased serotonin levels.

In summary, maternal regular mild exercise during pregnancy increases the offsprings' prefrontal BDNF and VEGF levels in both prepubertal and adult periods in both sexes and thus decreases the anxiety levels. Further studies are needed to elucidate the role of BDNF and VEGF on the dopamine and serotonin metabolism that is neuromodulators of fear and anxiety in maternally exercised male and females.

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