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RESEARCH

Treadmill Exercise Improves Behavioral and Neurobiological Alterations in Restraint-Stressed Rats

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Abstract

Stress is a state that is known to impact an organism's physiological and psychological balance as well as the morphology and functionality of certain brain areas. In the present work, chronic restraint stress (CRS) model rats treated with treadmill exercise were used to examine anomalies associated to emotion and mood as well as molecular changes in the brain. Forty male Sprague-Dawley rats were divided into control, stress, exercise, and stress+exercise groups. CRS were exposed to stress group rats and exercise group underwent a chronic treadmill exercise. Depressive-like behavior was evaluated with the forced swim test (FST) and tail suspension test (TST). For assessing anxiety-like behavior, the light-dark test (LDT) and the open field test (OFT) were used. The Morris water maze test (MWMT) was used for testing memory and learning. Brain's monoamine level and the expression of genes related to stress were measured. It was discovered that CRS lengthens latency in the MWMT, increases immobility in the FST and TST, decreases time in the light compartment, and causes hypoactivity in the OFT. CRS reduced the dopamine levels in the nucleus accumbens(NAc). Brain-derived neurotrophic factor (BDNF), dopamine receptors, and serotonin receptor (HTR2A) gene expression in the prefrontal cortex, corpus striatum, and hypothalamus were decreased by CRS. Exercise on a treadmill leads to increase NAc's dopamine and noradrenaline levels and prevented behavioral alterations. Exercise increased the alterations of BDNF expressions in the brain in addition to improving behavior. As a result, CRS-induced behavioral impairments were effectively reversed by chronic treadmill exercise with molecular alterations in the brain.

Keywords Chronic restraint stress · Treadmill exercise · Anxiety · Depression · Memory · Monoamin

Introduction

Stress helps the body adapt situations that require survival and challenges to homeostasis (De Kloet et al. 2005; Jols and Baram, 2009). However, ongoing stress has a detrimental effect on mental and behavioral health (Lupien et al. 2009; McEwen 2012). According to the research by Sosa and Almeida (2012) and Godsil et al. (2013), chronic stress has been associated with behavioral alterations in rodents and humans, including depressive-like symptoms, anxiety, and memory and learning impairments. These alterations are frequently connected to aberrant gene expression, neuroendocrine dysfunction, and structural alterations in different parts of the brain. Although abnormal levels of monoamine neurotransmitters are known to affect how anxious and depressed symptoms manifest, the underlying neurobiological mechanisms are still not fully understood (Heninger et al. 1996; Akter et al. 2019; Wang et al. 2019).

Chronic restraint stress (CRS), an experimental model of continuous movement restraint, is used to create long-term psycho-emotional stress, which results in depressive and anxious-like behaviors, impairments in memory and learning, and neuronal damage (Sousa et al. 2018). On the other hand, exercise is being recommended as the greatest way to postpone the start of problems related to restriction stress.

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For instance, it is undeniable that creatures that are physically active are more stress-resistant. In fact, a growing body of evidence from studies on both humans and animals suggests that leading an active lifestyle helps with stress management and quick recovery from its negative effects (Greenwood and Fleshner 2008, 2011; Van Praag et al. 2014). It has also been shown that regular light to moderate exercise improves mental and cognitive health (Dishman et al. 2006; Van Praag et al. 2014). Exercise is said to relieve stress, but the precise reasons why are uncertain. This is partly because it changes the signaling of monoamines in the brain.

Long-term exercise alters the serotonin or 5-hydroxytryptamine, 5-HT, and dopamine (DA) systems, which may alter the activity of these neurotransmitters in stress-resistant regions, according to studies using rodent models (Foley and Fleshner 2008; Greenwood et al. 2012). A deeper comprehension of the mechanisms through which exercise fosters stress resistance may aid in the discovery of new targets for the treatment or prevention of stress-related disorders.

The precise neurobiological mechanisms underlying the anxiolytic and antidepressant properties of physical activity as well as its positive effects on cognitive functioning are still unknown, despite the identification of a variety of neuroadaptive changes brought on by exercise (Greenwood and Fleshner 2011). The discovery of these pathways might promote exercise as a treatment and preventative tool for depression, anxiety, and cognitive decline. It may also provide some insight into potential new approaches to treating mental diseases.

In this study, we put up the hypothesis that certain biochemical alterations in the rat model's brain may be responsible for the depression, anxiety, and learning deficiencies brought on by CRS. Uncertainty exists regarding the relationship between exercise's positive effects on CRS-induced behavioral changes and either the monoamine cycle in the brain, the expression of different neurotransmitter receptors, or both. Rats exposed to CRS were utilized in this work to assess behavioral responses, the function of exercise in these responses, and neuromodular effects on a variety of indicators.

Materıals and Methods

Animals

Forty male Sprague-Dawley rats, aged 3 months, were randomly assigned ($n = 10$) to the following groups at the Firat University Experimental Research Center: (1) control group (C), (2) stress group (S), (3) exercise group (E), (4) stress+exercise group (S+E). All of the rats were kept in controlled environments with free access to standard chow food and water (12 h of light each day between 07:00 and 19:00, temperatures between 22 and 25 °C, and humidity levels between 40 and 55% with continuous air circulation).

Induction of Chronic Restraint Stress (CRS)

As shown in Table 1, throughout the first, second, and third 15 days, the animals in the CRS group were restrained for 1, 2, and 3 h daily. Animals were restricted in a plexiglass tube with good ventilation but no access to food or water. The rat is held firmly in place and has its normal bodily mobility restricted by the size-appropriate restrainer tube without any harm being done to the animal. The restrainer tube was put in a different room (14:00–17:00 h) to keep upset animals separated from other animals throughout the stressful time. After experiencing restraint stress, the animals in the CRS group were returned to their cages at home for the remainder of the day.

Table 1 Experimental design.

Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Exercise speed (m/min)	15	15	15	15	15	-	-	20	20	20	20	20	-	-	20
Exercise duration (min)	15	15	20	20	20	-	-	20	20	25	25	25	-	-	30
Stress duration (h)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Days	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
Exercise speed (m/min)	20	20	20	20	**-**	20	20	25	**TST**	25	25	25	**LDT**	25	25
Exercise duration (min)	30	35	35	35	**-**	40	40	40	**TST**	40	40	40	**LDT**	45	45
Stress duration (h)	2	2	2	2	**-**	2	2	2	**TST**	2	2	2	**LDT**	2	2
Days	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**
Exercise speed (m/min)	25	**FST**	25	25	25	**OFT**	25	25	25	25	25	25	25	**MWMT**	-
Exercise duration (min)	45	**FST**	45	45	50	**OFT**	50	50	50	50	50	50	50	**MWMT**	-
Stress duration (h)	3	**FST**	3	3	3	**OFT**	3	3	3	3	3	3	3	**MWMT**	-

"-" not applicable, *FST* forced swimming test, *LDT* light/dark test, *MWM* Morris water maze test, *OFT* open field test, *TST* tail suspension test

Exercise Training on Treadmill

Animals were exposed to chronic treadmill exercise for 34 days until day 45, with periodic increases in speed and duration as shown in Table 1. Rats in the exercise groups were forced to run at a constant inclination of 0° throughout the experiment using a motorized treadmill (May TME 0805, St. Louis, MO, USA). The treadmill speed was gradually increased from 15 to 25 m/min 5 days a week, excluding the day of the behavior tests, and the duration of each running session was increased from 15 to 50 min. After each run, the treadmill was cleaned with a 70% ethanol solution. By contacting a metal grid, a tiny electric shock stimulation was given to the rats to encourage them to run. A mild electric shock stimulus (0.2–0.5 mA) was given by touching a metal grid to encourage the rats to run.

Behavioral Assessment

Open Field Test (OFT)

Rats' anxiety levels and overall locomotor activity are measured using the open field test (Prut and Belzung 2003). Animals were treated to OFT using an $80 \times 80 \times 40$ cm opaque white plexiglass apparatus on day 36. The apparatus had 9 and 16 equal squares at the center and the periphery, respectively. The animals were brought into the test room at least 30 min before the test. With a video camera put on top of the device, each rat was placed in the compartment's center and given 10 min to freely explore the space. During the 10 min of recording, a number of factors were examined, including grooming time, the number of rearings, the number of defecations, and the number of lines crossed (locomotor activity).

Light/Dark Test (LDT)

Rats' levels of anxiety were measured using the LDT, which has two compartments made of plexiglass. The walls of one compartment were black, while the walls of the other compartment were transparent and brightly lit from the top by a 60-W bulb. The compartments were connected by an opening located at the floor level in the center of the compartments. In the test, animals were placed in the light compartment with the direction of the opposite side of the dark compartment. For 5 min, the number of transitions between the two compartments and the amount of time spent in each compartment were captured on camera (Rock et al. 2017). Rats were thought to be less anxious the more time they spent in the light compartment and the more transitions they underwent.

Forced Swimming Test (FST)

The forced swimming test is a method for evaluating depressive-like behavior in rodent models. The test is composed of 2 sessions (Yankelevitch-Yahav et al. 2015). The first session's pre-test period lasts for 15 min. Animals were placed within a transparent plexiglass cylinder (height: 60 cm, diameter: 25 cm), which was covered with a black wooden device and contained a 40-cm water reservoir that was kept at a temperature of 23 to 25 °C. After the initial session, 24 h pass until the test stage, which lasts 5 min. Rats were placed in cylindrical containers for 5 min and subjected to a variety of movements, including swimming (active forepaw movement with goal-directed motion), immobility/floating (rats floating on the surface with only movement sufficient to keep the head above water), and climbing (upward goal-directed movements of the forepaws along the wall of the cylinder to escape from the tank). Times were captured on video.

Tail Suspension Test (TST)

On day 24, rats were given TST to assess any depressive-like behavior. In the experiment, a metal rod mounted 50 cm above the ground and spaced 1 cm from the tip of the tail was used to hold each animal separately upside down by the tail using a 20 cm long adhesive tape. Each rat was hung for 6 min, during which time its activity was observed. The duration of the animals' immobility was timed and utilized as a gauge of depressive-like behavior. The animals' immobility was seen as a symptom of behavioral despair (Can et al. 2012).

Morris Water Maze Test (MWMT): Spatial Learning and Spatial Memory Assessment

A hidden platform with a diameter of 10 cm was placed 2 cm below the surface of a black tank (height: 50 cm, diameter: 120 cm) that was filled with 40 cm of water at 24 ± 2 °C. The tank was divided into four equal-sized quadrants: East, West, North, and South. The platform was placed in the same spot on each test day. Between days 40 and 44 of the trials, animals were placed in each quadrant and given 60 s to find the platform as part of a 5-day reference memory test phase. If they did not find it, the animals were pulled out of the water and placed on the platform. The platform was hard to find, and it took a while. The time spent to find the platform was recorded to measure the spatial learning and memory performance, which were recorded for 5 days. Animals were put through a probe trial at the end of day 5 to assess the memory consolidation (Tuzcu and Baydas 2006). Basically, the platform was removed from the tank, animals were placed at the center of the tank, and behavior

was captured for 60 s. For the purpose of evaluating spatial learning and memory, the amount of time spent in the target quadrant was recorded.

RNA Isolation and Gene Expression Analyses by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

At the end of the study, all animals were administered intraperitoneally 10% ketamine HCl (Ketalar® flacon, Pfizer Inc., Istanbul, Turkey) and xylazine HCl (2.5 mg/kg-Rompun ®-IM) and then sacrificed (cervical dislocation). After that, their brains were quickly removed, and approximately 30 mg of brain tissue was homogenized in 1 mL Trizol (Invitrogen, Foster City, CA, USA) using an 18-gauge needle and incubated for 5 min at room temperature. Two hundred microliters of chloroform was added on top of the homogenate, vortexed for 30 s, and incubated at room temperature for an additional 2–3 min. The samples were then centrifuged at 10,000 × g for 20 min at 4 °C. The colorless aqueous phase was transferred to a fresh tube, and equal volume of isopropanol was added, vortexed, and incubated at room temperature for 10 min. The samples were centrifuged at 10,000 × g for 20 min at 4 °C and, supernatant was discarded. RNA pellet was washed with 1 mL 75% ethanol twice and centrifuged at 10,000 × g for 5 min at 4°C. The ethanol was completely removed, and RNA pellet was dissolved in 30 µl nuclease-free water and stored at –80 °C for further analyses. RNA was quantified using a BioSpec-nano (Shimadzu) spectrophotometer and reverse transcribed using Multiscribe™ Reverse Transcriptase (Invitrogen, Foster City, CA, USA) according to the manufacturer's instructions. The DRD1, DRD2, BDNF, HTR2A, and HTR2C gene expression levels were determined by using RT^2qPCR Primer Assay (330,001, Qiagen, Hilden, Germany) on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). GAPDH was used as the housekeeping transcript. Cycling parameters were comprised of the initial denaturation of 15 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C, amplification at 60–65 °C for 30 s, and elongation at 72 °C for 30 s. Relative gene expressions were determined by the $2^{-\Delta\Delta CT}$ method.

Nucleus Accumbens Isolation for Monoamin Analysis

The prefrontal cortex (PFC) region was isolated after the animals were anesthetized, and their brains were rapidly removed. The nucleus accumbens (NAC) region was carefully removed using the micropunching technique in accordance with the Paxinos and Watson atlas (Paxinos and Watson 2006). To eppendorf tubes containing NAC as internal standards, 50 µl of dihydroxybenzylamine hydrobromide (DHBA) and 100 µl of 0.1M hydrochloric acid (HCl) were added. After mixing for 5-6 min until homogenized by vortexing, the samples were placed in the centrifuge. The clear supernatant that was still in the upper half of the tubes after centrifugation for 10 min at +4 °C and 4000 rpm was collected with a micropipette and put in 0.5 ml Eppendorf tubes. These liquid fractions were then stored at −20 °C for analysis in a high-performance liquid chromatography with electrochemical detection (HPLC-ECD; Waters Corp, Milford, MA, USA) system.

Determination of Total Protein Levels

Precipitates that remained after centrifugation in tubes containing NAC samples were used to quantify protein quantities. Two hundred ten microliters of 0.1 M NaOH solution was added to the tubes, and they were subsequently incubated at +4 °C. The following day, each sample was combined in a vortex for 1 min before being placed into two spectrophotometer cuvettes. One hundred milliliters, 0.9 ml of distilled water, and 1 ml of Coomassie Blue (Pierce, Illinois, USA) were placed in the cuvettes. Readings at a wavelength of 595 nm in a spectrophotometer (Jasco V-530, Tokyo, Japan) were used to calculate absorbance values. For each sample, the average of the two outcomes was determined. The total protein concentrations were determined as µg by substituting "*y*" for the average absorbance in the formula that was generated using bovine serum albumin (Sigma Chemicals Co., St. Louis, USA).

High-Performance Liquid Chromatography (HPLC) Analysis

In order to investigate the precise neurochemical mechanisms underlying memory impairments, anxiety- and depression-like effects, and antidepressant and anxiolytic effects of treadmill exercise, the levels of central monoamine neurotransmitters, such as noradrenaline (NA), dopamine (DA), and its metabolite dihydroxyphenylacetic acid (DOPAC), in the NAC were detected using HPLC-ECD system after behavioral assessments. Because it was extremely stable and did not react with any of the biogenic amines in the samples, DHBA was employed in the investigation as an internal standard. Data were quantified (Sigma) using the Waters HPLC (Breze Waters) software tool and external standards. Following the analyses, the concentrations of monoamine and metabolites were calculated using the formula "pg amine/mg protein".

Statistical Analysis

The statistics package SPSS version 22 was used to conduct the statistical analysis. Data are expressed as mean ± standard deviation (SD). A one-way ANOVA was

used to compare the mean values of the four groups, and Tukey's multiple comparison test was used to find any differences. In all cases, a p value of 0.05 or lower was regarded as statistically significant.

Results

The Effect of Treadmill Exercise on the Depressive-Like Behavior of Rats with CRS

To evaluate depressive-like behaviors, we performed the FST and TST behavior tests. According to TST, exercise significantly decreased the immobility time. The S group's mean time of immobility was significantly greater than the C group's (179.9 ± 20.7, 131.2 ± 20.04, $p = 0.001$, respectively) as shown in Fig. 1. Exercised and exercise-trained stress rats (SE group) showed significantly less immobility time compared to the S group (99.1 ± 34.1, $p < 0.0001$, and 117.2 ± 27.1, $p < 0.0001$, respectively). The floating (immobility) time increased significantly in the S group (57.3 ± 6.03, $p < 0.0001$), while it decreased in the exercised rats (36.4 ± 6.05, $p < 0.0001$) and the S+E group (42 ± 8.08, $p < 0.0001$) (Fig. 2). This suggests that restraint stress exposure resulted in an effect on the FST that was similar to depression. As illustrated, there was no significant difference among the groups in the time spent climbing and swimming.

The Effect of Treadmill Exercise on the Anxiety-Like Behavior of Rats with CRS

The effect of treadmill exercise on locomotor activity in OFT is depicted in Fig. 3A. The S group exhibited fewer crossed lines than the exercise group ($p = 0.006$). Furthermore, it was discovered that the exercise group had significantly more rearings than the S group (29.5 ± 10.4, 18.5 ± 8.07, $p = 0.01$), but not in the stress+exercise group (22.5 ± 6.6). In Fig. 3C, there were no differences between the groups in terms of grooming time or number of defecations (Fig. 3B, D).

According to the LDT that was used to measure the animals' levels of anxiety, the animals in the stress group spent significantly more time in the dark compartment than the control group (235.2 ± 35.3, 109.4 ± 50.1, $p < 0.0001$) (Fig. 4). Running effectively reduced stress-induced anxiety in male rats, as evidenced by the fact that exercise considerably decreased the length of time male rats spent in the dark compartment (143.6 ± 61.5, $p < 0.0001$). The S+E group spent more time in the light compartment (156.4 ± 61.5, $p = 0.005$) as compared to the S group. There was no obvious difference between the groups in the number of transitions from a light to a dark compartment (data not shown).

Chronic Restraint Stress-Induced Cognitive Impairment in Adult Male Rats

Spatial learning and memory performance were evaluated during the 5 days of training period. For this, the time spent

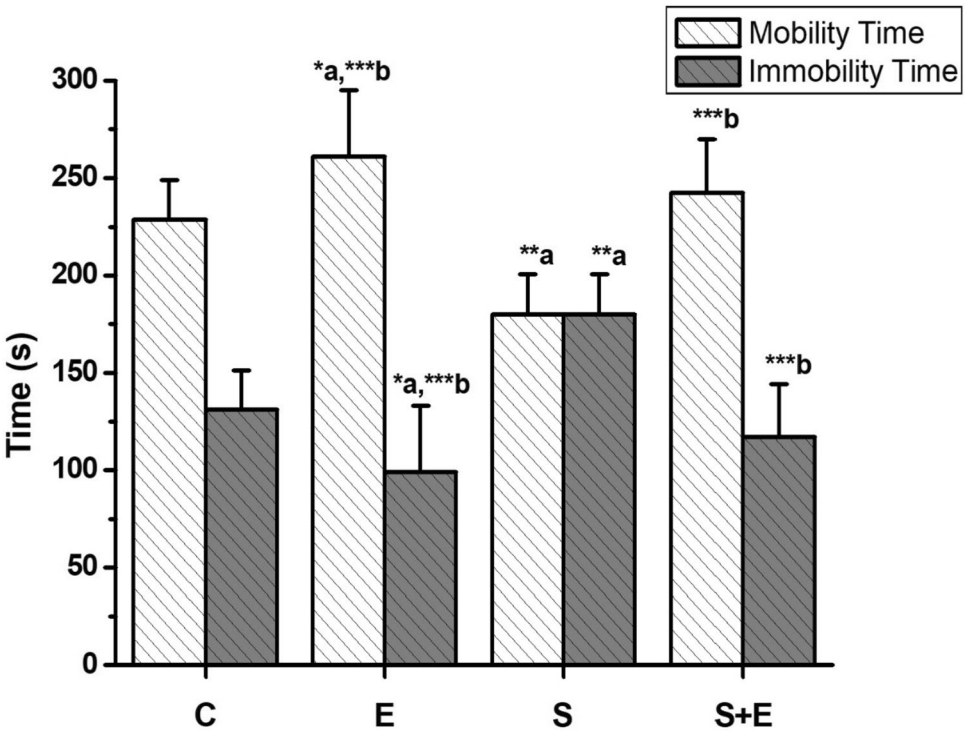

Fig. 1 Tail suspension test. Effect of exercise and stress on mobility and immobility time in tail suspension test ($n = 10$). *$p = 0.04$; **$p = 0.001$; ***$p < 0.0001$; a: vs. C; b: vs. S

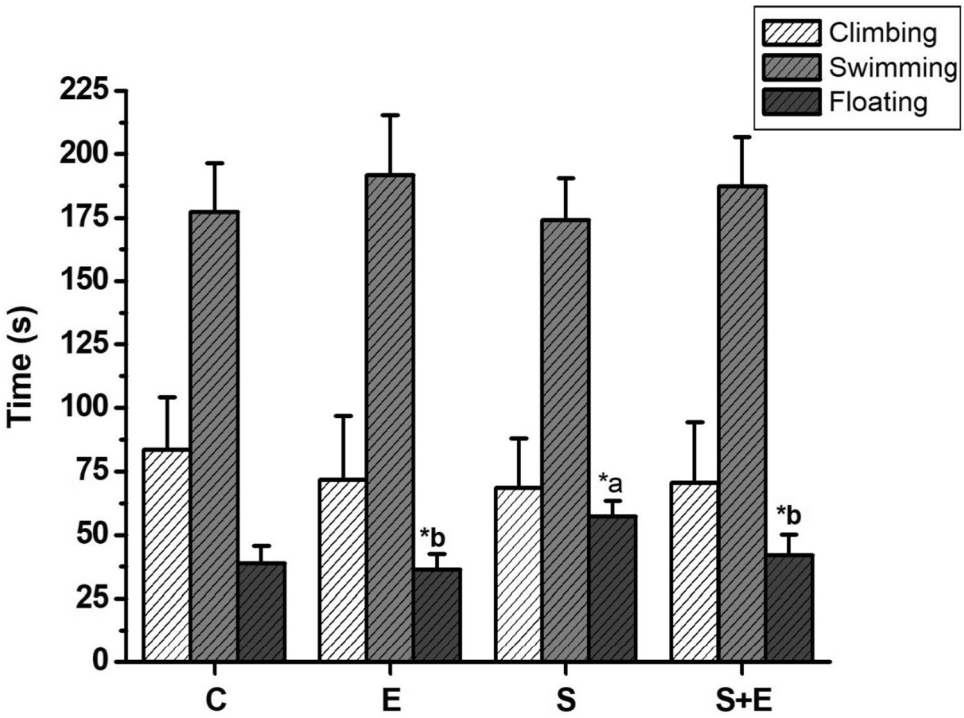

Fig. 2 Forced swimming test. Effect of exercise and stress on time spent climbing, swimming, and floating ($n=10$). *$p<0.0001$; a: vs. C; b: vs. S

finding the platform was recorded. A probe test was performed to evaluate the extent of memory consolidation. The time spent in the target quadrant was used to display the results of the MWM test, which measured the degree of memory consolidation that occurs after learning. Figure 5 demonstrates that the S group spent much less time than the C group in the target quadrant ($p<0.0001$), which suggests cognitive and memory impairment. Compared to the S group, rats in the E and S+E groups spent a lot more time in the target quadrant ($p<0.0001$). Between the C, E, and S+E groups, there were no differences.

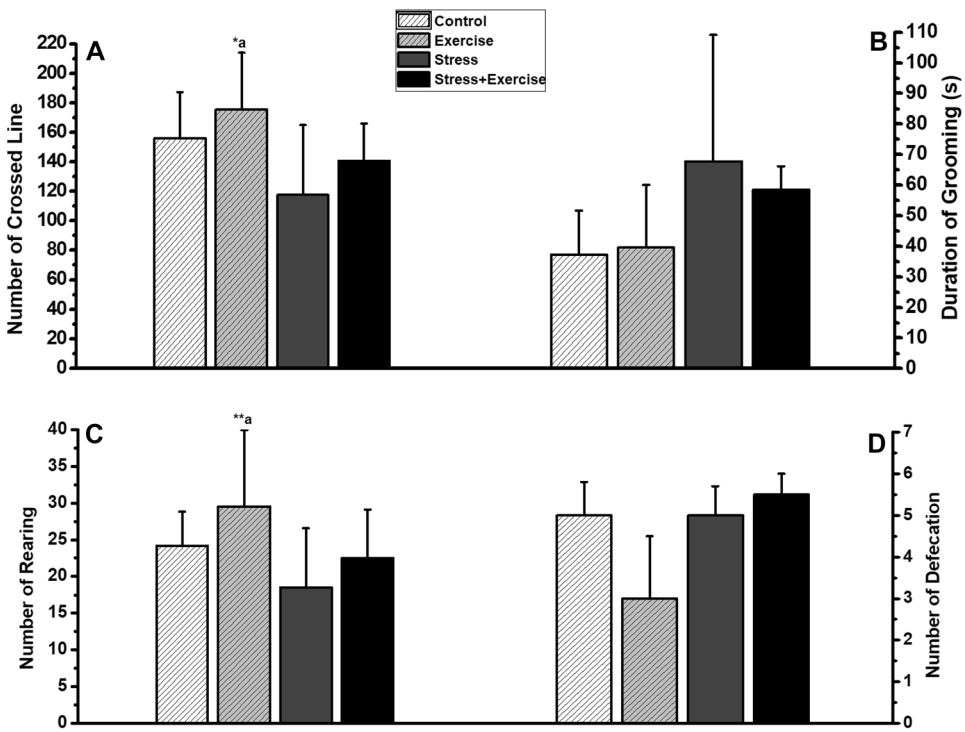

Fig. 3 Open field test. Effect of exercise and stress on number of lines crossed **A** duration of grooming time **B** number of rearings **C** and number of defecation **D** ($n=10$). *$p=0.006$ and **$p=0.01$; a: vs. S

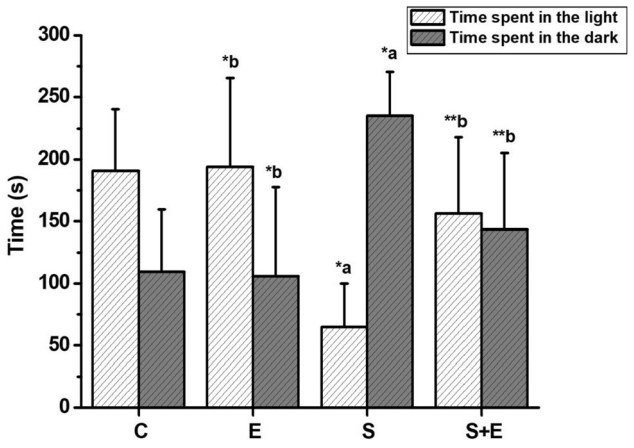

Fig. 4 Light-dark test. Effect of exercise and stress on the total time spent in the light and dark compartment ($n=10$). *$p<0.0001$ and **$p=0.005$; a: vs. C; b: vs. S

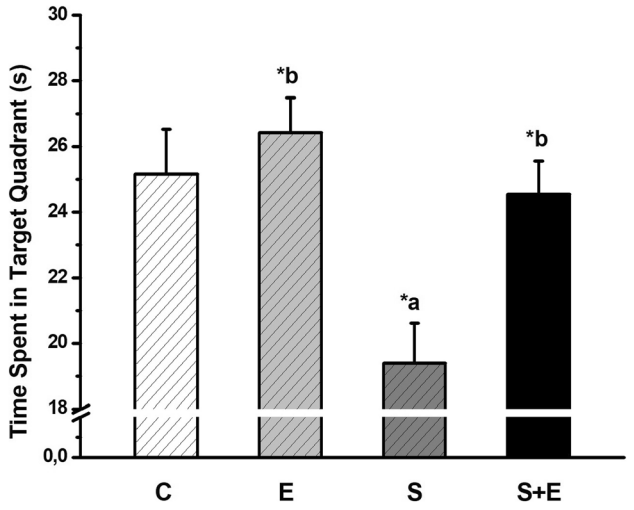

Fig. 5 Morris water maze test. Effect of exercise and stress on the time spent in the target quadrant. *$p<0.0001$; a: vs. C; b: vs. S

CRS Reduced Protein Expression in the Brain

Drd1, *Drd2*, *Bdnf*, *Htr2a*, and *Htr2c* gene expression levels were assessed by RT-PCR analysis in the hypothalamus, hippocampi, PFC, and corpus striatum areas. Tables 2 and 3 show the effects of CRS and chronic exercise on these gene expression levels. *Htr2a* expression was significantly downregulated in the hypothalamus and PFC of all groups compared to the C group, even though it did not differ significantly in the PFC of the S group animals. While *Htr2a* expression was significantly higher in all locations in the E group than in the S group, it was significantly lower in the animals in the S+E group (Tables 2 and 3). Drd1 expression, on the other hand, was shown to be significantly lower in the hypothalamus of the S+E group animals compared to the control and to be significantly higher in the hypothalamus, hippocampus, and PFC of the exercise group rats compared to other groups (Tables 2 and 3). *Drd1* expression increased in the E group by 0.9 fold in the hypothalamus, 5.2 fold in the hippocampus, and 2.8 fold in the PFC when compared to the comparable C groups. *Drd1* and *Drd2* expressions in the corpus striatum of the rats did not differ across groups, while *Drd2* levels in the E group's hypothalamus, hippocampus, and PFC were significantly greater. Following CRS exposure, the expression of BDNF in the hypothalamus was significantly downregulated when compared to C rats, but it was significantly up-regulated in the PFC and hypothalamus of exercise animals when compared to S and S+E group animals, as well as in the hypothalamus when compared to C and S group animals (Tables 2 and 3).

Effect of Stress and Exercise on Monoamine Neurotransmitters in NAC

Figure 6 depicts a representative chromatogram with NA, DOPAC, and DA retention times (min) and responses (mV). The effects of prolonged stress and exercise on the levels of

Table 2 Fold exchange expression of the genes in the hypothalamus and hippocampus

	Hypothalamus			Hippocampus		
Genes	**E**	**S**	**S+E**	**E**	**S**	**S+E**
Htr2a	0.4^{ac}	0.3^{a}	0.08^{ab}	0.3^{ac}	0.5	0.1^{ab}
Htr2c	1.2	0.9	0.6	0.5	0.8	0.7
Drd1	0.9^{c}	0.4	0.2^{a}	5.2^{abc}	1.6	1.1
Drd2	2.4^{abc}	0.6	0.3^{a}	3.5^{abc}	1.1	0.7
Bdnf	1.4^{bc}	0.3^{a}	0.4^{a}	1.5	1.1	1.4

[a]vs. C
[b]vs. S
[c]vs. S+E

Table 3 Fold exchange expression of the genes in the prefrontal cortex and corpus striatum

Genes	Prefrontal cortex			Corpus striatum		
	E	S	S+E	E	S	S+E
Htr2a	0.4[ac]	0.9	0.1[ab]	0.4[c]	0.5	0.1[b]
Htr2c	1.3	0.8	0.9	0.8	1	0.9
Drd1	2.8[abc]	1	0.9	0.9	0.8	0.8
Drd2	2.2[bc]	0.9	0.4[a]	0.8	0.8	0.6
Bdnf	2.4[ab]	0.8	1.1	1.2	0.8	0.9

[a]vs. C

[b]vs. S

[c]vs. S+E

DA, NA, and DOPAC in the nucleus accumbens as well as the DOPAC/DA ratio are shown in Table 4. Stress or exercise had no impact on the DOPAC and NA levels in the brain region under investigation. The NA level increased in the E and S+E groups, despite the fact that this was not statistically significant. The NAC's NA turnover was equivalent to the pertinent control levels. The level of DA was significantly ($p=0.02$) lower in the S group compared to the C and E groups. The DOPAC/DA ratio showed that the stress-induced group DA turnover was significantly higher than the E group's [$F(3.11)=3.765$, $p=0.04$].

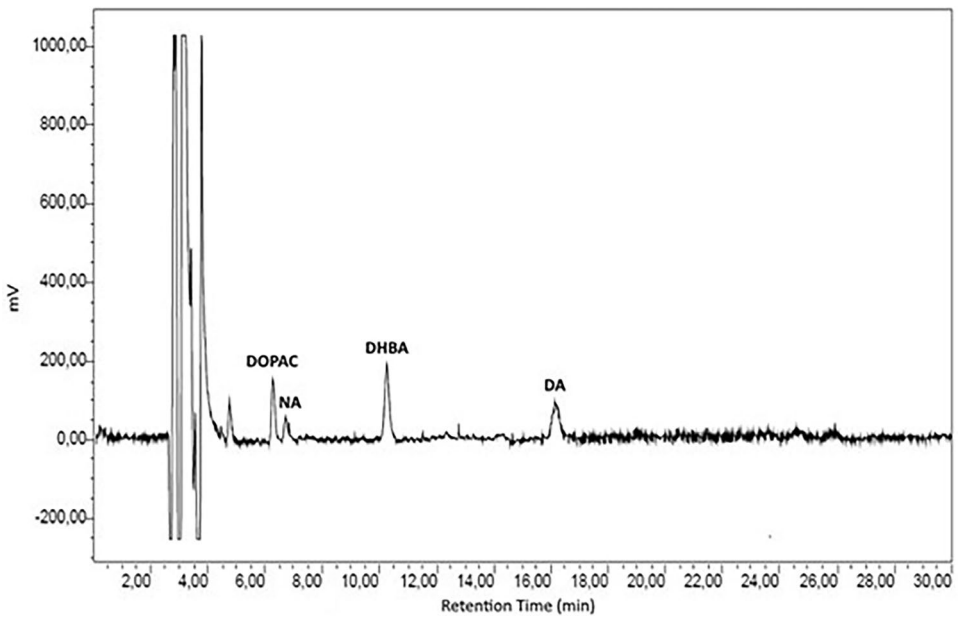

Fig. 6 Representative chromatogram showing retention time (min) and response (mV) of NA, DOPAC, and DA. DHBA, 3,4-dihydroxybenzylamine (internal standard); NA, noradrenaline; DOPAC: dopamine metabolite; DA, dopamine

Table 4 Effect of stress and exercise on monoamine neurotransmitters in nucleus accumbens (NAC)

	C	E	S	S+E
NA (pg/mg)	0.6 ± 0.38	1.1 ± 0.99	0.8 ± 0.31	1.4 ± 1.38
DOPAC (pg/mg)	1.2 ± 0.44	0.7 ± 0.09	1.5 ± 0.6	0.99 ± 0.5
DA (pg/mg)	22.3 ± 4.3	23.4 ± 3.9	**14.5 ± 1.6**[*b]	**12.9 ± 3.2**[*b,**a]
DOPAC/DA	0.05 ± 0.02	0.03 ± 0.009	**0.09 ± 0.03**[***b]	0.07 ± 0.04

NA noradrenaline, *DOPAC* dopamine metabolite, *DA* dopamine. Data are shown as mean ± SD

[*]$p=0.01$; [**]$p=0.02$; [***]$p=0.03$

[a]vs C

[b]vs E (one-way ANOVA)

Discussion

The development of anxiety- and depressive-like behaviors, as well as a deficiency in male rats' spatial learning and memory, demonstrates that restraint stress was a key component in the study's impacts on numerous elements of brain function. This study demonstrated how exercise alters behavior under continuing constraint stress and its underlying mechanisms. We were able to demonstrate the positive benefits of treadmill exercise on behavioral changes brought on by CRS as well as levels of monoamine and neurotransmitters in the brain. The chemical processes underlying these improvements in rat brain function were the focus of our study.

In this study, CRS significantly decreased spatial learning memory and elevated symptoms of anxiety- and depression-like behaviors. The TST revealed that the animals in the stress group were more immobile than the animals in the control group. Furthermore, compared to the animals in the stress+exercise group, which were more active during FST, the animals in the stress group floated for a longer period of time. According to LDT data, the stressed group spent much more time in the dark compartment, but exercise increased the amount of time spent in the light compartment without affecting the number of tunnels separating the two. The OFT inferred that exercise marginally, but not significantly, increased the locomotor activity of the stressed rats.

Studies have explored the physiological advantages of exercise, particularly its anxiolytic qualities (Kim and Han 2016). Additionally, a recent study (Lapmanee et al. 2017) found that exercising before CRS induction reduced anxiety and depression symptoms. It was demonstrated that treadmill exercise had an impact on CRS-induced rats that was comparable to an antidepressant.

In the MWM test, CRS rats showed a decrease in time spent in the target quadrant, a sign of spatial memory or the capacity to recall previously acquired information, but trained animals' spatial memory appeared to be reinforced. The MWM test results confirmed earlier research by demonstrating that physical exercise corrected losses in spatial learning and memory brought on by stress (Lapmanee et al. 2017). A deficit in spatial learning brought on by the CRS (Lapmanee et al. 2017) or a jumble of stressors (Zou et al. 2010; Lapmanee et al. 2017) has been found to be improved by exercise. The data above show that exercise reduces symptoms of anxiety and depression as well as stress-related deficits in spatial learning and memory, possibly via modulating the activity of the HPA axis (Pietrelli et al. 2018) or the serotonergic system (Shin et al. 2017).

The outcomes of behavioral experiments demonstrated a relationship between chronic stress and several monoaminergic neurotransmissions, including serotonin, dopamine, and norepinephrine. Chronic stress is known to alter central monoamine levels (Alghasham and Rasheed 2014; Akter et al. 2019). For instance, animals under chronic stress exhibit lower amounts of 5-HT in their PFC and hypothalamus (Oh et al. 2018). According to research (Torres et al. 2002; Mahar et al. 2014), stress alters the levels and metabolites of 5-HT and DA as well as the transmission of these chemicals in the PFC and hippocampus. Rats' hypothalamus, hippocampus, frontal cortex, and amygdala have been found to contain greater levels of the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) (Inoue et al. 1994). Serotonin and its metabolite 5-hydroxyindolacetic acid (5-HIAA) levels were also increased in the rat PFC, nucleus accumbens, hypothalamus, and amygdala. The effects of exercise, which are known to alter monoaminergic targets to guard against stress-induced anxiety, depression, and memory loss, are called into question by these findings. Exercise can reduce stress in part through changing the signaling of brain monoamines, while the precise mechanisms are unknown (Chaouloff 1989). Following acute exercise sessions, 5-HT and DA levels in several stress-sensitive rodent brain areas increased (Chaouloff 1989; Goekint et al. 2012). Furthermore, studies using rat models show that regular exercise promotes plasticity in the 5-HT and DA systems, which may change the level of activity of these neurotransmitters in parts of the brain that are resistant to stress (Greenwood et al. 2003, 2005; Foley and Fleshner 2008). Exercise is known to reduce the effects of stress on BDNF levels as well as the activity of the serotonergic and dopaminergic systems (Clark et al. 2015; Heijnen et al. 2015). The identification of novel targets for the treatment or prevention of illnesses linked to stress may result from a thorough understanding of the mechanisms that physical activity employs to guard against stress. According to Björklund and Dunnett (2007), dopamine plays a variety of functions in the central nervous system (CNS), including regulating locomotor activity, neuroendocrine secretion, motivation, emotional reactions, learning, and memory. Aerobic exercise has been shown in animal experiments to increase the levels of the neurotransmitter dopamine (DA) in the striatum, hypothalamus, midbrain, and brain stem, supporting the positive benefits of exercise on mood and memory (Foley and Fleshner 2008).

In the study, treadmill exercise successfully repaired the anxiety and depressive-like behaviors in the rats who had undergone CRS, as well as the deterioration of their spatial learning and memory. Aerobic exercise has been shown to enhance cognitive functions, specifically spatial memory, which is regulated by the hippocampus (Suwabe et al. 2018). Regarding the molecular mechanisms, it has been shown that BDNF is required for controlling exercise-induced brain plasticity, which enhances learning and memory (Voss et al. 2013). Lower BDNF levels are linked to increased susceptibility to stress and depression, according to studies (Zhang et al. 2014; Duman and Monteggia 2006). These studies also show that chronic stress and depressive-like symptoms are

correlated with a decrease in BDNF synthesis and activity in the hippocampus and frontal cortex.

In our study, the hypothalamus and PFC of the rats in the exercise group expressed BDNF at much higher levels. Although the corpus striatum and hippocampus showed an increase, this rise was not significant. Previous studies have demonstrated that stress greatly decreased the expression of BDNF, particularly in the hippocampus. Exercise-induced increases in BDNF have been linked to enhancements in learning and memory, better locomotion, and a decline in depressive and anxious-like behaviors. It is possible to argue, however, that exercise normalizes stress-induced alterations through a pathway unrelated to BDNF as well as through BDNF levels.

Two distressing emotional and behavioral effects, anxiety and panic, are highly correlated with the malfunctioning of the serotonergic system in the brain (Canteras and Graeff 2014). The major excitatory 5-HT receptors in the brain, known as 5-HT2A, play a crucial role in modulating the 5-HT response to stress (Carhart-Harris and Nutt 2017). The production and activation of cortical 5-HT2A receptors are increased by a variety of stresses (Murnane 2019). 5-HT-2C is one of the receptors that have a role in reactions to stress, anxiety, and fear (Martin et al. 2013; Marcinkiewcz et al. 2016). Studies on experimental animals demonstrate that activating these receptors contributes to the anxiogenic effects (Greenwood et al. 2012; Vicente and Zangrossi Jr 2012). It has been demonstrated that blocking the 5HT2C receptor has anxiolytic effects (Wood et al. 2001; Martin et al. 2002) and that anxiety is reduced in mice with a 5HT2C receptor deletion.

According to Jiang et al. (2009) and Martin et al. (2014), alterations in the activity of HTR2A and HTR2C serotonin receptors in response to stress have been connected to depressive-like behaviors. However, exercise was found to produce resistance to the anxiety- and depression-like behaviors brought on by HTR2C activation (Greenwood et al. 2012). Additionally, administering an HTR2A antagonist to the rat hippocampus area reduced the downregulation of BDNF mRNA brought on by immobilization stress (Vaidya et al. 1999). In our study, we found that the stress+exercise group animals had much lower levels of HTR2A expression than the stress group animals did in the hypothalamus, hippocampus, PFC, and corpus striatum, which suggests that 5-HT signaling has been downregulated. However, there were no discernible differences in HTR2C expression between the groups.

Chronic stress is known to alter the synaptic input and excitability of dopamine receptor D1 (DRD1) and dopamine receptor D2 (DRD2) cells (Anderson et al. 2019) as well as cause DRD1-expressing neurons to atrophy (Shinohara et al. 2018). Repeated stress paradigms impair working memory because DRD1-expressing pyramidal cells, especially those in the PFC, show lower activity (Arnsten 2015). In our study, we found that the exercise group rats had considerably higher levels of hippocampal DRD1 and DRD2 expression than the other groups. Animals in the exercise group showed a similar pattern in their hypothalamus, with the exception of DRD1 expression. Exercise did not significantly assist stressed animals, and the expression of DRD1 and DRD2 was downregulated in the hypothalamus of the stress+exercise group compared to the control group. Despite the current finding that exercise lowers stress-related behavioral abnormalities in rats, it can be rather surprising to observe that exercise negatively affects neurotransmitter levels in stress+exercise groups. Forced treadmill exercise could be the root of this issue. According to various research, forced exercise can cause psychopathological reactions like despair and anxiety in addition to the harmful physiological reactions linked to stress (Bakshi and Kalin 2000; Moraska et al. 2000). In one study, it was discovered that forcing rats to exercise raised their anxiety levels (Uysal et al. 2015). These indicators were lower in the stress+exercise group than in the stress group, which may be attributable to the co-development of the two parameters. In circumstances where chronic stress is first created and exercise is then started after the stress becomes chronic, this makes it possible to assess the consequences of chronic stress.

In addition to having preventive effects on memory dysfunctions through modulating neuroprotective activity through many monoamine and neurotransmitter pathways, our study has revealed that treadmill exercise has antidepressant and anxiolytic effects. Increased monoamine neurotransmitters may be a representation of the molecular and cellular processes underlying the antidepressant-like and anti-anxiolytic effects of exercise on the CRS-exposed rat.

In conclusion, exercise can counteract stress-induced anxiety and depressive-like behaviors as well as memory impairment by inducing long-term adaptations in various monoaminergic systems and expression of various proteins in the brain. Evidence suggests that in situations where future stress exposure is expected, voluntary moderate-intensity exercise may be helpful in postponing the onset of mood disorders and memory loss. For both preventing and treating emotional and cognitive issues in those who are under stress, exercise seems to be the ideal, affordable treatment.

Author Contribution Conception, design of research, acquisition, analysis or interpretation of data; and drafting of the work or revising it critically for important intellectual content: all authors. Supervision: H.K. All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval All experimental procedures were approved by the Local Ethics Committee for the Experimental Animal Research of Firat University (Approval no: 2014/34).

Conflict of Interest The authors declare no competing interests.

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