

# Analysis of the effect of childhood stress on the expression of serotonin system genes in the hippocampus and cortex in adult male rats

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## Abstract

Reflects an interaction between genetic susceptibility and environmental factors such as stress are likely underlying neurobiology of major depression. Various factors have been proposed in anxiety behaviors caused by childhood stress, but the role of 5HT-3 and 5HT-2c receptors in its occurrence has been less investigated. Excessive expression of related genes in the brain is associated with the formation of anxiety behaviors (1-3). In this research, six pregnant rats were used. After giving birth, their babies experience separation stress from their mothers, called Maternal Neonatal Separation or childhood stress. This article aims to investigate the effect of childhood stress on 5HT-3 and 5HT-2c gene expression in the hippocampus and cortex and the possible effects of exercise on anxiety behaviors and 5HT-3 and 5HT-2c gene expression in the hippocampus and cortex in male rats. This article showed that voluntary exercise during adolescence can reduce the of these gene expression in the brain and has a protective role against stress.

**Keywords:** voluntary exercise, Wheel Running, Hippocampus, Nervous Central System, Stress, Serotonin system, Gene expression, hippocampus, cortex

## 1. Introduction

Early life experiences significantly influence children's neural, behavioral, and psychological development, with long-lasting effects across various domains. The Hippocampus and cortex are structured and sensitive to stress and are connected to anxiety (4-5). Evidence shows a disruption in the serotonin system in many mental illnesses such as depression, schizophrenia, Parkinson's, anxiety, epilepsy, etc. So far, many factors have been proposed to disrupt the serotonin system. Among serotonin receptors, 5HT-3 and 5HT-2c receptors are related to mental disorders, and their agonists and antagonists are used medically. Studies on animal models of depression, including MS, have shown that the gene

expression of these receptors has increased in the brain, especially in the Hippocampus and cortex. Hippocampus is a complex brain structure embedded deep into the temporal lobe (6). Sports activities and special aerobic exercises as influential non-drug factors in treating many diseases and helping reduce injuries have been studied by scientists for a long time (7). Experience shapes neural plasticity, and through this behavior and psychological processes throughout the lifespan (8), Recent research has determined that continuous exercise can change the metabolism and expression of serotonin system genes in people (9). On the other hand, it has been reported that childhood stress can change the function of the serotonin system in the brain, including the hippocampus. It is unclear whether continuous exercise during adolescence after childhood stress can affect the process of changes in the neurotransmitter serotonin and anxiety behaviors in adulthood by inhibiting the increase in the expression of the mentioned genes in the hippocampus and cortex. Recent studies have determined that the serotonin neurotransmitter system is decisive in the pathogenesis of anxiety behaviors. The serotonergic system in the central nervous system (CNS) plays a significant role in the brain's formation of appetite and sleep behavior (10-12). Environmental factors such as stress can disrupt the regulation and development of the serotonergic system. It has been proven disruption of this system is associated with a wide range of diseases, such as depression, memory and learning disorders, and anxiety. (13-15) The serotonergic system has many receptors that respond to serotonin or hydroxytryptamine. Among them, the 5HT-3 receptor is ionotropic, and the rest are membrane G-protein receptors. Among these receptors, 5HT-3 and 5HT-2c receptors play a role in anxiety behaviors (16,17). It has been found that gene overexpression of these receptors in some brain regions, such as the cortex and hippocampus, plays a role in anxiety behaviors (18). In addition, it has been reported that treatment with specific antagonists of these receptors can resolve anxiety behaviors (19,20). Using animal stress models has dramatically helped understand the effects of drugs and the mechanisms involved in stress-related diseases. One of the most reliable models of chronic stress is simulating childhood stress and trauma in the form of deprivation of parental attention. The animal model of separation from the mother (Maternal Separation or MS) is one of these models capable of disrupting the natural developmental process of the brain, especially during childhood, and leads to severe behavioral and cognitive disorders in adulthood (21-23).

Considering the importance of treating stress disorders such as anxiety, which improves the social and personal functioning of patients, and taking into account the side effects of drugs, researchers proposed sports activities as a non-pharmacological and effective occupational therapy method (24). In addition, Recent studies provide further evidence to support the notion that regular exercise reduces the risk of insulin resistance, metabolic syndrome, and type 2 diabetes. SI improves when individuals comply with exercise guidelines in a type of diabetes called type I, and insulin secretion stops. In this case, glucose remains high in the blood, so cells can't absorb it. Therefore, the cells are exposed to glucose deficiency. And the solution is to inject insulin into the bloodstream. Indeed, this injection should be completely controlled. Otherwise, too much insulin in the blood can cause death. (25,26) Among the sports exercises, the running wheel and treadmill are of particular importance because they are able to balance stress-related mechanisms such as the strong response of stress hormones (glucocorticoids) and neurotransmitter changes and balance blood sugar (27-29). But it is not clear whether the effects of exercise can be related to the regulation of the serotonergic system in the brain (especially anxiety behavior). Therefore, in this article, we investigate the effect of childhood stress on 5HT-3 and 5HT-2c gene expression in the hippocampus and cortex and the possible effects of exercise on anxiety behaviors and 5HT-3 and 5HT-2c gene expression in the hippocampus and cortex of rats.

## 1-1-Serotonin system and its relationship with anxiety

### Serotonin

Serotonin (serotonin) or hydroxy tryptamine is a type of biogenic monoamine acid neurotransmitter (30). According to biochemistry, it is a derivative of tryptophan. Serotonin has been found directly in the gastrointestinal tract, platelets, and central nervous system of animals and humans. This substance is popularly known as a "feel-good" agent. Almost 100% of the human serotonin body is in the enterochromaffin cells, which are dispersed in the membranes of the devices in the drugs, where they regulate bowel movements. A function less than serotonin is synthesized by the serotonergic nerve network of the central nervous system and has various parts. These functions include the regulation of mood, appetite, and sleep. Modulation of serotonin in synapses is the primary function of different classes of antidepressants. They make serotonin when released, which plays a role in homeostasis and blood coagulation by causing contraction. Serotonin has also been identified as one of the growth factors in specific cells for healing injuries. A type of tumor called carcinoid may be caused by an imbalance in the body's mass production, which is called carcinoid syndrome and causes complications such as inflammation, diarrhea, and heart problems. Due to the effect of serotonin on the heart's cardiac muscle, the patient may suffer damage from the suitable valve of the heart due to the heartbeat exceeding the limit of the cardiac muscle. Serotonin is a neurotransmitter that occurs in all organisms of the Bilateria branch, which functions as intestinal movements and the organism's perception of biological resources. Resources mean food availability in organisms with more superficial physical structures, such as invertebrates. In more advanced organisms such as arthropods and vertebrates, the definition of resources can include social preferences. In response to an organism's perception of comparison or abundance of resources, its growth, reproduction, or morale can be promoted or degraded. (31-33)

### 1-2-Serotonin receptors

Serotonin receptors, or 5HT, are serotonin receptors on the cell membrane of neurons and other cell types in animals. As an endogenous ligand-gated ion channel, they mediate the effects of serotonin and various drugs and hallucinogens. Except for the 5HT-3 receptor, the ligand gate of the ion channel, all 5HT receptors belong to the "heptahelical receptors" branch, which activates the intracellular secondary signaling system. Meanwhile, 5HT-1A plays a role in memory, learning, depression, and schizophrenia. 5HT-1B receptors play a role in vasoconstriction and aggressive behaviors. 5HT-2A and 5HT-2B receptors are involved in behavioral mechanisms and physiological regulation of brain processes, which include depression, anxiety, cardiac function, anxious behaviors, and the release of many neurotransmitters. The 5HT-4 receptor is involved in the food movement in the digestive system, memory and learning, and stimulation of intracellular processes dependent on adenylate cyclase. Other serotonin receptors, such as 5HT-5 and 5HT-7, play a role in cognitive function and mood. (34-36).

### 1-3-5HT-2c serotonin receptor

This serotonin on G proteins (GPCR) is responsible for transmitting excitatory neurotransmission. This protein is encoded by the 5HTR2C gene, which is located on the X chromosome. This receptor is scattered in the brain and body. Stimulation of these receptors reduces the function of dopamine and norepinephrine in the brain. These receptors are involved in anxiety, depression, appetite, and reproduction. This receptor controls dopamine release in the Striatum, Amygdala, Prefrontal cortex, and Nucleus Accumbens. Increased receptor expression is associated with depression and especially anxiety disorders, and most of the side effects of SSRI antidepressants are related to raising serotonin and ultimately stimulating this receptor.

On the other hand, the activity of these receptors is not dependent on the presence of serotonin, and they can function without their specific ligand. In this respect, they are considered constitutive receptors. Also, the increase in the expression of this gene is associated with the rise in the production of cytokines. On the other hand, serotonin plays a significant role in controlling the hypothalamus and pituitary hormones such as oxytocin, prolactin, and ACTH, and this control is done by 5HT-2A and 5HT-2c. Disruption of stress hormones and the central axis of hypothalamic-pituitary-adrenal stress (HPA axis) causes depression and anxiety. The response to stress and the release of stress hormones, such as CRH, are related to this receptor. Therefore, considering the role of this receptor in the occurrence of mental diseases, we investigated the effect of childhood stress on this receptor and the protective effects of exercise during adolescence on the anxiety behaviors of rats. We will discuss the consequences of childhood stress (37-39).

#### **1-4-5HT-3 receptor**

It is the only receptor of the serotonin system, an ion channel, and its function is specific to cations. It controls the brain's stimulation system and nerve cells' depolarization function. In response to serotonin, this receptor causes sodium, potassium, and calcium to enter the cells. This receptor is highly sensitive to sodium and potassium and is present in the interneurons of the cortex, hippocampus, and amygdala. This receptor is related to mood disorders and anxiety disorders, and antagonists of this receptor have anti-depressant and anti-anxiety effects(40,41) .

#### **1-5-Childhood stress and the serotonergic system**

Recent studies have shown that childhood stress and the serotonin system play a significant role in the formation of depression (20). Thus, childhood stress can change the biogenic synthesis of amines and serotonin in different parts of the brain. As a result, childhood stress reduces the expression of serotonin transporters in the brain and causes depression symptoms. In addition, this stress is capable of causing anxiety behaviors through disruption of the HPA axis. Also, studies have shown that childhood stress causes sensitivity to stress in adulthood, and these disorders are reduced by improving the environmental condition in childhood (Environmental Enrichment) (43,44).

Regarding anxiety disorders, the stress model of separation from the mother is an animal model proven to cause anxiety. Also, stress can change the function of the serotonergic system. As mentioned earlier, the serotonergic system plays a significant role in depression and anxiety disorders, but the role of 5HT-2C and 5HT-3 receptors is unknown.

#### **1-6-Early life stress models**

In mammals, including humans, monkeys, and rodents, maternal input has perhaps the most significant influence on the environment experienced during development (45-48). Thus, most animal models of early-life stress have manipulated maternal interaction, disrupting the quantity or quality of maternal care early in life [49-51]. For any model of early-life stress, detecting a behavioral outcome depends on several variables. The first set of variables pertains to the stress's timing, nature, and severity. Secondly, the age at which animals are tested, whether during adolescence, adulthood, or aging, can determine the outcome. Third, the type and difficulty of the test that is used to measure behavioral products are essential. For example, a rigorous test such as object location memory (OL) might uncover subtle deficits not apparent in a less challenging test, such as object recognition. (52) Deprivation of mother (lack): Separation from the mother is the most dangerous model for life stress. This model deals with the separation from the

mother in early life. In this model, in the first two weeks of life, the cubs are separated from the mother for 1 to 24 hours a day. Maternal separation increases the risk and occurrence of behavioral complications and HPA responses in adulthood (53,54)

### **1-7-The effects of exercise on the serotonergic system:**

According to the results of a new review, the beneficial effects of exercise can be related to its modulating effects on the serotonergic system. It has been found that physical activity can change the metabolism of serotonin, change the expression of serotonin receptors, and produce anti-anxiety and anti-depressant effects (55-58). The positive effects of exercise on the hippocampus, like neurogenesis, depend on serotonin's function (59). The role of 5HT-3 and 5HT-2c receptors in anxiety and mood disorders has been determined. (60,61) On the other hand, drugs related to these two receptors can produce anti-anxiety effects. (62,63) Considering the protective effect of exercise that is partially exerted through the serotonergic system, we consider that by using a valid animal model to investigate anxiety, we can detect changes in the gene expression of 5HT-3 and 5HT receptors. -2c in the hippocampus and cortex.

## **2. Materials and Methods**

Statistical population, research sample and sampling method in the present study, 10 Wistar female rats were purchased from the Pasteur Institute and placed separately in a cage (Animal Care Center, Sports Physiology Department, Tehran Branch, Islamic Azad University Center) after pregnancy, 60 male rats were selected as research subjects and separated from their mother for 180 minutes from the 2nd to the 14th day. Then, to determine the experimental and control groups, on the 21st day, these rats were randomly divided into 3 groups and were kept in cages in groups of 4 until the 28th day. The groups included the control, with the stress of separation from the mother and the Running wheel. During the research, the animals were kept in groups of 4 rats in polyethylene cages 15x15x30 made by Razi Rad Company, with a temperature of  $22 \pm 2^\circ\text{C}$  and humidity of  $50 \pm 5\%$  under light-controlled conditions (12 hours of darkness, 12 hours of light). were kept and had free access to water and standard food (10 grams per 100 grams of body weight) manufactured by Behpur company. The experimental protocol was based on guidelines for treatment and use of the laboratory. All the subjects were studied in the same environmental and time conditions. The measuring tools and instruments in this research were able to measure the studied variables.

### **2.1. Training Protocol**

Before implementing the training protocol, on the 21st day, the group got familiar with the running wheel for one week. Then, for 32 days until day 60, 2 rats were in each cage with two running wheels; each rat only had access to one of the running wheels (a Plexiglas glass sheet was placed between the two rats). Each was running well and was made of plexiglass (circumference = 105 cm, length = 10 cm, Navidan Teb company, Iran) and rotated quickly with a resistance of 50 g. Each wheel was connected to a magnetic switch that was connected to a counter located outside the animal's cage, and the number of rats had free access to the wheel of rodents 24 hours a day for 32 days until day 60, and the number of daily turns was kilometers. After being transferred laboratory environment and a week of familiarization new environment, the animals were randomly divided into three groups (each group including 12 rats, respecting the possible risk of the subjects in the research process), including control groups, with the stress of being separated from the mother and two wheels. The running wheel of rodents was used as a non-pharmacological treatment in the stress group.

### 2.1.1- Collection of hippocampus and cortex samples for gene expression analysis

On day 60, 4 rats from each group were selected to collect the hippocampus and cortex. The rats were killed after anesthesia, and the brain was separated on a cold surface. Hippocampus and cortex were isolated and frozen in liquid nitrogen. Then it is mixed with Trizol and homogenized in a homogenizer. After 5 minutes, 120% Alenda chloroform is added to the supernatant and vigorously shaken for 15 seconds. The product is centrifuged for 4.4min 15, 12000h, and the mineral and aqueous parts are separated; the part containing RNA is removed and mixed with isopropanol at a ratio of 1 to 5.0 and left for 10 minutes at room temperature and then at 4C. It was centrifuged at 12,000 g for 10 min. The pellet containing RNA was washed in ethanol and centrifuged again at 4C, 5 min, and 7500 g, and the supernatant solution was discarded, and then  $\mu$ 20L of Free-RNAs water was added to the plate, and it was placed in a water bath machine for 10 minutes at a temperature of 60 degrees. and finally, the extracted total RNA is kept at 70-70. RNA concentration is measured using a nanodrop device and the ratio of 260 to 280 between 8.1 and 2 is defined as optimal purity.

### 2.2.2-5HT-3 and 5HT-2c for cDNA synthesis

cDNA synthesis is done using 1hh of RNA and random hexamer primer, and Mmulv reverse transcriptase enzyme. The synthesis steps are as follows1  $\mu$ g RNA

1  $\mu$ l Random hexamer primer

10.5  $\mu$ l RNA- Free water

Placing at a temperature of 42 degrees for five minutes

Adding the following ingredients to the primary product

DNTP 2  $\mu$ l

Mmulv reverse transcriptase buffer 4  $\mu$ l

Ribolock Rnase inhibitor 0.5  $\mu$ l

Mulv reverse transcriptase 1  $\mu$ l

Place at 25 degrees for 10 minutes

Placing at a temperature of 42 degrees for 60 minutes

Placing at 72 degrees for 10 minutes

5HT-3 and 5HT-2c Real-time - PCR

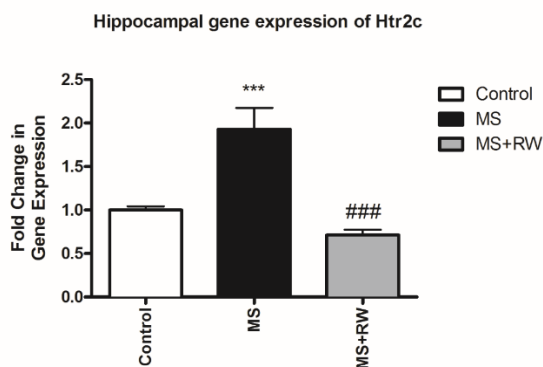
Gene	Sequence (5' → 3')	
	Forward	Reverse
HTR2c	TGGAGGTCTCCAGGTCAAATCT	TGTTTGCTGTGAGTCCCGAG
HTR3a	GCTGGTTGCAGAAAATGCCA	AGGAAGTACCTCTATGCAGGG
Hprt1	AGTTTGTTGTTGGATATGCCCTTG	TGTAGATTCAACTTGCCGCTGTC

At the beginning of the work, the optimal concentration of cDNA and primers related to each gene are determined using serial concentration tests for each of them separately so that the least amount of dimer is observed. Real-time PCR is performed using II Taq Ex Premix (2X) and a concentration of 100 ng :cDNA. The components of PCR-time Mix Real are as follows

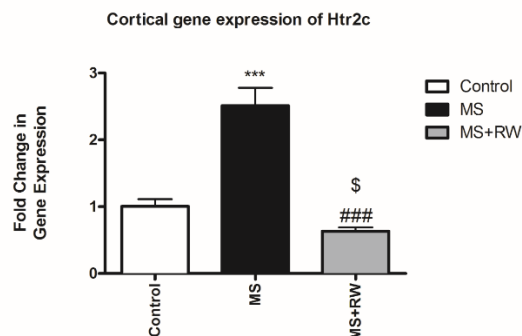
Real-time PCR program includes: 95 for 10 minutes - 95 for 15 seconds, 60 for 1 minute (repetition of 40 cycles). GAPDH is used for the control gene, and the expression level combined with Each gene is measured. The expression level of the desired genes is measured by the CT-ΔΔ2 methods. All materials used in RNA extraction and Real-time PCR are from Qiagen company (made in Germany), and cDNA synthesis is from Roche.

**2.3-Statistical Analysis**

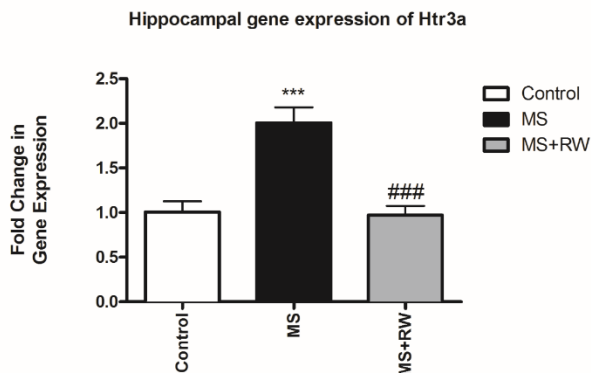
A comparison was made (t-test) between the control and stress groups regarding the expression of the relevant genes in the hippocampus and cortex. The results showed that MS significantly increased Htr3a gene expression in the hippocampus of adult rats (P<0.001, Fig. 2a). Similar results were obtained in the case of the cortex (P<0.001, Fig. 2b). The investigation by qRT-PCR showed that childhood stress caused significant changes in the expression of the 5htr2c gene in the hippocampus compared to the control group (P<0.001, Fig. 2c). Similar results were obtained in the cortex (P<0.001, Fig. 2d). Meanwhile, MS was not able to change the expression of the control gene that was considered in this study, Hprt1 (P>0.05). These results are shown in Figure 1.



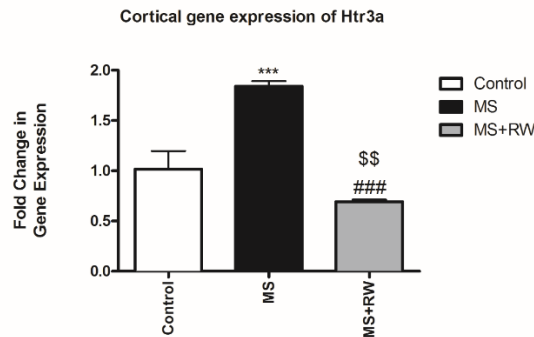
**Fig1.a**



59 **Fig1.b**



**Fig1.C**



**Fig1.d**

**Figure 1 shows the expression of 5htr3a and 5htr2c genes in control and maternal separation stress groups.**

In this study, the effect of voluntary RW or two-wheel rodent exercise on the impact of childhood stress on the expression of relevant genes in the hippocampus and cortex of adult rats was investigated. A significant difference between the experimental groups was observed using the ONE-WAY ANOVA statistical test. These significant changes were seen in the case of Htr2c in the hippocampus ( $P < 0.001$ , Fig. 1a) and in the cortex ( $P < 0.001$ , Fig. 1b). Tukey's supplementary statistical analysis revealed that voluntary exercise RW+MS significantly decreased the expression of the 5htr2c gene ( $P < 0.001$ , Fig. 1a) in the hippocampus and in the cortex ( $P < 0.001$ , Fig. 1b) compared to the MS group. At the same time, Tukey's test showed that voluntary exercise RW+MS compared to the MS group was able to reduce the expression of the 5htr3a gene in the hippocampus ( $P < 0.001$ , Fig.1c) and cortex ( $P < 0.001$ , Fig.1d) of rats.

**3. Results**

**Conclusions**

The results of this research determined that childhood stress in the form of separation from the mother is capable of causing anxiety behaviors in adult male rats. In this study, it was found that the effects of exercise can be related to the mentioned serotonin receptors because voluntary RW exercise caused a decrease in the expression of these genes in the hippocampus and cortex. It has been reported in many studies that the serotonin system is related to mood and anxiety disorders. Meanwhile, many studies believe that 5HT-3 and 5HT-2c receptors play a significant role in anxiety disorders. The results of this research revealed that these two genes' expression increased in the stress group's hippocampus and cortex. Hippocampus and cortex are both essential structures of the brain that are related to the occurrence of depression and anxiety, and cognitive behaviors. The increase in the expression of these receptors in previous studies has shown the event of depression and anxiety in humans and animals. This is a confirmation of the fact that childhood stress can cause tension in adulthood through disturbances in the expression of these genes. This article, written based on the master's thesis in the second person of the article, showed for the first time that voluntary exercise during adolescence could reduce the expression of these genes in the brain and has a protective role against stress. On the other hand, the results of this research determined that adolescence is of particular importance, and treatment at this stage can have positive effects.

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