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Treadmill Exercise Improved Memory Evocation and Upregulated Alpha7 Nicotinic Receptors Density in Lower Cognitive Performance Rats

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Abstract

Chronic and moderate exercise promotes biochemical and physiological changes in the organism, leading to, among other benefits, improves in cognitive deficits. Formation of long-term memory can be modulated by α7 cholinergic nicotinic receptors (nAChR). However, until now there is no evidence that physical exercise could ameliorate the functioning of the cholinergic system and contribute to memory improvement in animals with learning and memory difficulties. This study evaluated the effects of moderate exercise to the memory and the density of α7 nAChR in less-responsive rats to active avoidance task. Male Wistar rats (300-470 g) were submitted to active avoidance apparatus and those that had a bad performance were classified as less-responsive rats. Part of these animals were submitted to a moderate physical training on treadmill for one hour per day, five days per week, during eleven weeks. The other half was left in cages (sedentary group). On the end of this period, trained animals presented significant reduction on the blood pressure, when compared to their sedentary controls and an improve of 2.95 times in memory. After that, brains were extracted and submitted to autoradiography for α7 nAChR using [^{125}I]-α-bungarotoxin. It was observed a significant increase in the density of this receptor in the hippocampus and in the shell portion of *nucleus accumbens* of trained animals when compared to sedentary ones. In conclusion, moderate physical exercise improved memory evocation of less-responsive animals and increased the density of α7 nAChR. These data reinforce the importance of the moderate exercise to those who present learning and memory difficulties.

Keywords: Moderate exercise; Cholinergic transmission; Cognitive deficits; Long-term memory

Introduction

Physical activity has been widely associated with higher life quality, considering the effects in mental, social and health parameters [1]. Apart from the evident benefits for the cardiovascular and endocrine system [2], nowadays some studies sought to investigate the molecular alterations produced by chronic and moderate physical activity that also improve brain function. Memory processes are modulated by emotions, hormones and neurotransmitters. The cholinergic system is implicated in long-term potentiation (LTP) and is being related to memory formation in several brain areas [3,4]. There is evidence for the involvement of the alpha7 nicotinic acetylcoline receptor (nAChR) in hippocampal activity [5], memory reconsolidation [6] and sustained attention [7-9]. In humans, several studies have shown that physical exercise can modulate cognitive functions, and act as a potential non-pharmacological treatment for patients who have cognitive disorders, such as Alzheimer's disease, although the molecular mechanisms for such benefits are not clear [10]. Also, many studies in animal models have demonstrated beneficial effects of physical activity in cognitive processes using distinct exercise protocols, such as voluntary wheel running [11] and treadmill running [12]. In addition, it is well known that physical exercise increase the density of neurotrophins like the brain-derived neurotrophic factor (BDNF), which are involved in neuroplasticity and that this neurotrophin can stabilize the expression of α7 nicotinic receptors in the hippocampus and in parasympathetic neurons [13,14]. Regular physical activity with submaximal intensity can also upregulate the expression of proteins that are involved in cellular antioxidant mechanisms, as peroxiredoxins that work as scavengers for hydrogen peroxides [15]. Moreover, lower oxidative damage and increase in antioxidant enzymes were also described in both humans and rats that undergo chronic regular physical activity [16,17]. Concerning this scenario, our hypothesis is that in animals that have difficulties to learn and memorize a task, moderate physical exercise may improve the cholinergic system function, increasing the density of the α7 nicotinic cholinergic receptor in brain areas that are important for memory processing, contributing to the improvement in the cognitive performance. Therefore, this work verified the effects of a chronic moderate physical exercise protocol in treadmill running in the memory evocation in lower cognitive performance rats and also investigated the α7 nAChRs density and the oxidative balance in brain regions related with memory.

Materials and Methods

Animals

Male Wistar rats, from our own breeding colony, 3 to 4 months old, weighting 300-470 g, were housed within controlled room temperature (22-24°C) and humidity (55-65%) in a 12:12 h light/dark inverted cycle (lights on at 6 pm). Food and water were supplied *ad libitum*. All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health publication 86-23, Bethesda, MD) and were approved by The Ethics Committee on Experimental Research from Santa Casa de Sao Paulo School of Medical Sciences. All efforts were made to minimize the number of animals used and their suffering.

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Behavioral protocol

Rats were submitted to two-way active avoidance shuttle box (40 × 20 × 22 cm, Ugo Basile, Comerio, Italy) that is divided into two identical compartments, which are accessible to each other by a narrow open passage in the wall. In order to lower the level of contextual freezing, a training session was performed in which the animal was placed in the shuttle box and remained there for 5 min without any stimulus. After this period, the animal was exposed to 50 trials of avoidance conditioning (acquisition test). Each trial consisted of 2 s conditioned stimulus (CS), i.e., a buzzer (70 dB, 760 Hz) and light (40 W), followed by 4 s unconditioned stimulus (UCS), a mild foot shock of 0.7 mA delivered through the grid floor. Each trial was separated by fixed intertrial intervals (20 s). During the sessions the number of conditioned avoidance responses (CAR) were recorded, i.e., when the rat crossed into the other compartment before the UCS [9]. One week later, the same experimental protocol was conducted (test 1) and, another one, immediately after the 11 weeks treadmill exercise protocol (test 2).

Groups

Within one week interval between the acquisition session and the Test 1, approximately half part of the animals show the capacity to remember the task, as the animals have clear learning variability in memory performances in this task. Currently, animals that reach 30-70% of CAR are selected to the following proceedings, as shown earlier by our group [9,18]. In the present work, the CAR in the acquisition session and in the Test 1 were compared and thus, a subtraction between test 1 and acquisition test was performed (delta values). A distribution frequency was calculated and, according to this, animals with delta values above 8 (median value) were considered "responsive", whereas those ones with values below 8 were considered "less responsive". After that, they were submitted to the treadmill exercise training.

Exercise protocols

Adaptation to treadmill and maximal exercise test (MET): Initially all animals were adapted to the treadmill, walking for 10 min at 0.3 km/h for four consecutive days to get familiar with the equipment. The MET consisted in a progressive increase in speed every 3 minutes of 0.3 km/h, starting at 0.3 km/h and progressively up to the maximal speed supported by each rat. Three tolerance tests were performed: after the adaptation period, in order to determine the protocol of training; at the end of 5th week of training, to adapt and adjust the values of speed and time; and at the end of the training period, to compare the efficacy of the training [19].

Treadmill training program: The training program was performed on a treadmill designed for human use (Imbrasport®) and adapted for eight rats simultaneously. The protocol was designed considering 50 to 70% maximal running speed of the MET, therefore consisting in a physical exercise with moderate intensity [19,20]. Animals run once a day, 5 days per week for 11 week. Sessions were performed between 8:30 am and 11:30 am (dark phase). The entire training process was carried out without using any tail shock.

Measurement of systolic arterial blood pressure

A tail cuff sphygmomanometer (W.A. Baum Co.$^{\circ}$, USA) was used to measure the systolic arterial pressure. It was performed three times during the exercise protocol, and the first measurement was obtained before the beginning of treadmill adaptation. The two times left were performed one day after each MET [20,21]. Data are presented in mean of arterial pressure, as follows:

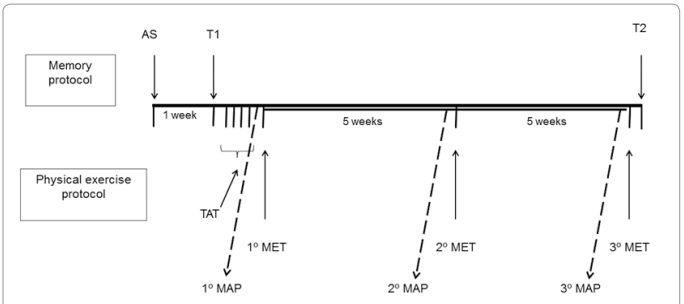

Figure 1: Time-line: Memory protocol – Acquisition session (AS) was the first contact of the animals with the active avoidance apparatus. After one week, all animals were tested again (T1) and, at the end of the experiment after 11 weeks, the protocol was repeated (T2). During this period, the physical exercise protocol was conducted. To familiarize the animals to the treadmill apparatus, all of them were submitted to treadmill adaptation time (TAT), which last for four days. After this period, all animals had their mean arterial pressure (MAP) measured and they were submitted to maximal exercise test (MET).

MAP=DP+1/3(SP-DP) Where MAP is the mean arterial pressure; DP is diastolic pressure and SP is systolic pressure.

Time-line of experimental design

Figure 1 shows the time-line for the experimental design of the present work.

Autoradiography

The method used was adapted from a previously described procedure [9,22]. Briefly, after behavioral observations, animals were killed by decapitation and the brains were extracted, immediately frozen in dimethylbutane and stored at -80°C until use. Serial sections of brains (20 μm) were cut on a cryostat chamber (-20 to -22°C, Microm HM 505N, Francheville, France), thaw-mounted on gelatin coated slides, desiccated for 5 min at room temperature and kept at -80°C until use. For receptor autoradiography, incubations were conducted for 90 min at room temperature using 5 nM [^{125}I]-α-bungarotoxin for identification of α7 nAChR, an irreversible antagonist of the receptor. Specific binding of the toxin for α7 nAChR, in this concentration, accounted for 80.2% of the total binding as stated earlier [9]. This was measured in saturation curves of [^{125}I]-α-bungarotoxin (1 mg protein incubated with 0.1 to 20 nM, at 25°C, for 120 min) that were done by our research team previously, using 2 μM unlabeled α-bungarotoxin. Observed Kd of these curves were 1.2 ± 0.1 nM (n=3), which were similar to those described in the literature (0.91 nM) [23]. Non-specific binding was assessed using 2 μM of the unlabelled toxin. The radioligand was diluted in 50 mM phosphate buffer containing 1 mM ethylenodiaminotetracetic acid and 0.1 mM phenylmethilsulphonyl fluoride, pH 7.4. At the end of the incubation period, slides were transferred sequentially through 4 rinses of 4 min each in 50 mM phosphate buffer at 4°C, and rapidly dipped into cold distilled water to remove salt excess. Sections were air dried and juxtaposed against Hyperfilm-MP (double-coated, 24 cm×30 cm, Amersham Biosciences GE Healthcare, Uppsala, Sweden) for 7 days (room temperature) along with autoradiographic [^{125}I] microscales (20 μm, Amersham Biosciences GE Healthcare). The films were developed in D-19 Kodak developer and fixed in Kodak Ektaflo solution. The autoradiograms were quantified densitometrically using the MCID image analysis system (Imaging Research Inc., Ontario, Canada). For each specimen, α7 nAChR binding sites were measured on 6-12 sections. The specific binding was determined by superposing, and then subtracting the non-specific binding (6.82 fmol/mg) from the total binding from similar adjacent sections.

Drugs

[^{125I}]-α-bungarotoxin (143.2 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA) and non-labeled α-bungarotoxin was purchased from Sigma. All other drugs used were of analytical grade.

Statistical analysis

Results were represented as means ± standard-errors and analyzed using GraphPad Prism Program (GraphPad Software, San Diego, CA, version, 5.0). Differences between CAR in training and test sessions were determined using Student-t test. All other data were analyzed using two-way ANOVA followed by Bonferroni's *post-hoc* test. Differences were considered significant when P<0.05.

Results

Group formation according to conditioned avoidance responses (CAR)

A total of 61 animals were submitted, initially, to the active avoidance apparatus. During the acquisition session in the equipment, the percentage of CAR was 5.7 ± 1.1% (n=61) in a total of 50 tasks. After one week, the tasks were repeated (test 1) and the same animals presented a significant increase of three times in percentage of CAR (17.2 ± 2.2%, P<0.0001) (Figure 2A). In order to verify the individual performance of each rat, the difference between CAR in test session and training session was calculated (delta, Δ). The median value of Δ was 8. According to this, animals were divided into "responsive" (Δ>8) and "less-responsive" (Δ ≤ 8) to the task (Figure 2B). Less-responsive animals accounted to 33 rats from the total (Figure 2B).

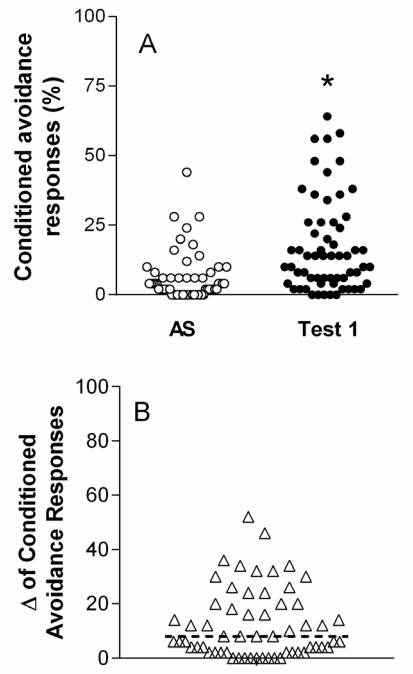

Figure 2: CAR values dispersion of animals (n=61) in acquisition session and test 1, in the active avoidance equipment (panel a) and deltas dispersion (panel b). Symbols in "A" represent individual values for each animal; in "B", symbols represent deltas from the difference between test 1 and training session. Dotted line represents the calculated median of CAR. *: P<0.0001.

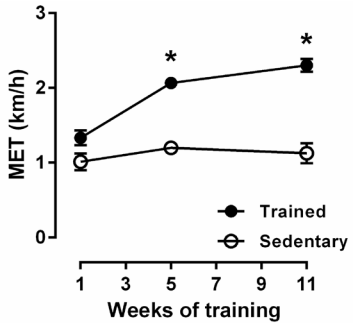

Figure 3: Maximal exercise tests (MET) of sedentary (n=8) and trained (n=10) rats measured in three different times: before the beginning of physical training, after the 5th week and the 11th week of training. Symbols and vertical bars are means ± standard-errors of means. *: P<0.001.

Group formation according to maximal exercise test (MET) in the treadmill and physical training effects

Animals were submitted to four days adaptation in the treadmill (10 min, 0.3 km/h). After that period, physical performance was verified in MET. From the 33 less-responsive animals submitted to the initial tests, 18 were considered able to get in the physical training. According to the performance in the MET, these animals were divided into two groups: sedentary (MET=1.01 ± 0.11 km/h, n=8) and trained (MET=1.33 ± 0.10 km/h, n=10).

Animals were submitted to two other METs, one after the 5th and the other after the 11th training weeks. Animals from sedentary group did not show any improvement in exercise tests. However, a significant and progressive increase in MET of trained rats was observed along the exercise tests [F(1,51)=92.73, P<0.0001]. MET of trained rats was significant higher in 5th week (1.72 times) and in the 11th week (2.04 folds) of training when compared to MET of sedentary group (1.20 ± 0.08 km/h and 1.12 ± 0.12 km/h, P<0.0001, respectively) (Figure 3). Except for the treadmill training, sedentary animals were manipulated similarly to trained animals.

Evaluation of mean arterial pressure (MAP)

Before the treadmill training protocol, MAP of both sedentary (91.25 ± 2.25 mmHg) and trained (89.00 ± 1.83 mmHg) animals was similar. Physical training promoted a significant reduce of MAP of trained animals after 5 weeks (81.33 ± 1.83 mmHg, P<0.01) and 11 weeks (76.33 ± 1.01 mmHg, P<0.001) of training, when compared to the first measurement. There was a statistical interaction [F(1,54)=5.34, P<0.001] between MAP of sedentary and trained animals along the 11 weeks of training, meaning that the effect of physical training on blood pressure depended on the total duration of the protocol. In this way, trained animals showed a significant difference in MAP, when compared to sedentary group (88.75 ± 1.18 mmHg), after the 11th week of moderate physical exercise [F(1,54)=18.04, P<0.001] (Figure 4).

Effects of training exercise in memory performance

After 11 weeks of exercise training, animals were submitted once again to the active avoidance equipment following the same protocol used before the treadmill training protocol. Sedentary animals showed a non-significant increase in CAR evaluated in test 2 (16.5 ± 4.4%) when compared to test 1 (6.7 ± 1.7%, Figure 4), possibly because of the manipulation they were submitted during the same time the other group was trained. However, animals that were submitted to physical training showed a significant improvement in memory evocation in

test 2 (14.0 ± 3.5%, P<0.05), when compared to CAR observed in test 1 (4.7 ± 1.0%) (Figure 5).

Evaluation of α7 nicotinic cholinergic receptor (AChR) density in brains of rats after treadmill exercise

Expression of α7 AChR was verified in many brain areas of both sedentary and trained animals, however with no statistical differences between both groups. Nevertheless, in the stratum oriens of hippocampus, trained animals presented a significant higher density of α7 (13.0 ± 1.3 fmols/mg, P<0.001), when compared to sedentary samples (5.54 ± 0.9 fmols/mg) (Figures 6, 7A and 7B). In the same way, it was observed a rise in density of α7 in the shell portion of *Nucleus accumbens* of trained rats (14.1 ± 2.8 fmols/mg), when compared to the same area in sedentary animals (9.5 ± 1.9 fmols/mg) (Figures 6, 7C and 7D).

Discussion

In the present study, we demonstrate that moderate physical exercise for 11 weeks can reduce arterial pressure of normotensive rats and improve the memory of rats that could not answer adequately to a task. This behavioral improve is followed by an increase in density of cholinergic nicotinic α7 receptors that clearly have a significant role in modulation of memory formation. To achieve the desired behavioral pattern, rats were divided into those who could answer and those who could not answer a task. It was evident that half part of Wistar rats submitted to subsequent trials (50 trials) in the active avoidance equipment did not make the connection between an unconditional stimulus and a conditional one. In this way, this called "less responsive" animals could have developed a fear for the context (as in a fear conditioning context) or simply could not acquire the information. It

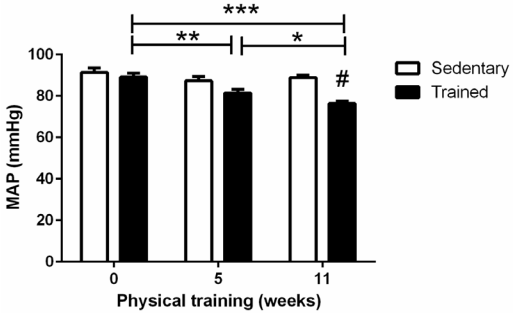

Figure 4: Mean arterial pressure (MAP) of sedentary (n=8) and trained rats (n=10) in different times along the training protocol. Histograms and vertical bars are means ± standard-errors of means. *: P<0.05; **: P<0.01; ***: P<0.001; #: P<0.001.

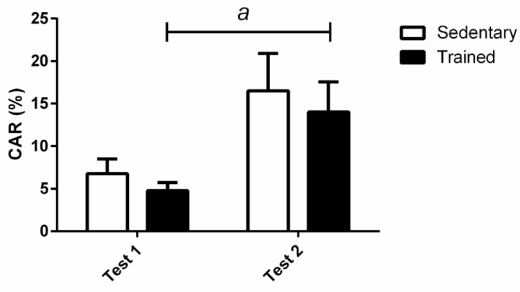

Figure 5: Conditioned avoidance responses (CAR) of less-responsive sedentary and trained animals in tests 1 and 2 obtained in the active avoidance equipment. Histograms and vertical bars are means ± standard-errors of means. *a*: P<0.05.

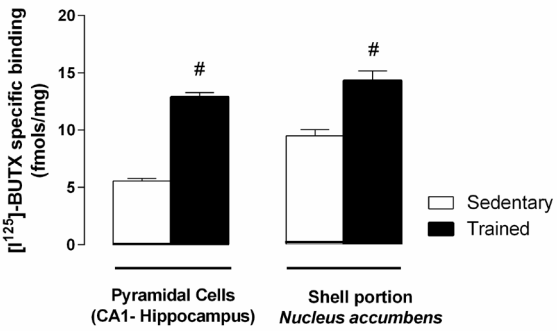

Figure 6: Specific binding of [^{125}I]-α-BUTX to α7 nicotinic cholinergic receptor in the pyramidal cells of CA1 area of hippocampus and shell portion of *nucleus accumbens* of less-responsive animals. Histograms and vertical bars are means ± standard-errors of means. #: P<0.0001.

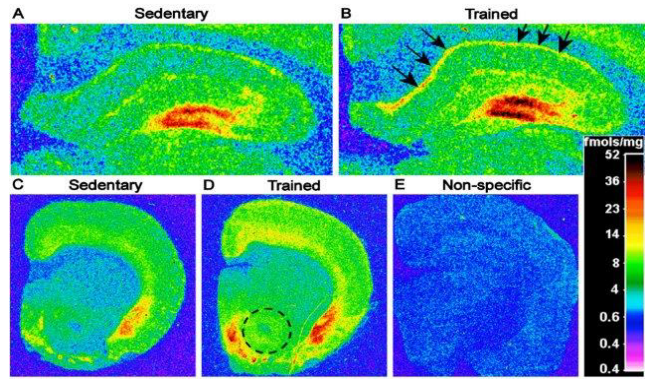

Figure 7: Photomicrographs of autoradiograms representing anatomical distribution of total binding sites for α7 nACh receptor in the stratum oriens of hippocampus (A and B), evidenced by arrows, and in the shell of the *nucleus accumbens* (C and D), evidenced by dotted line. Non-specific binding sites are represented in E.

is well known that learning and memory deficits in humans can appear in many phases along the aging process and can affect the developing of cognitive skills during the childhood or can bring difficulties in diary activities in the elderly. In this way, strategies that are able to modify or delay the losses can minimize the negative consequences for each one. Physical activity is surely one of these strategies that can be viewed as a prophylactic activity for mental health, once it has significant influence in essential mechanisms for memory consolidation and retrieve, besides offering countless social and psychological benefits [24,25]. Here, this non-pharmacological strategy was used as a possibility to improve the mnemonic capabilities of rats that could not respond to the task. For that, animals were submitted to an inverted cycle (lights on at 6 pm), once it is already known that these animals are metabolic active during the dark period. In forced protocols, animals make the physical exercises in higher intensities with higher energetic requirements. In treadmill running, there is the activation of neuroendocrine mechanisms and the stress forces the animal to run in a constant velocity, according to the protocol configuration, which can have variations in velocity, duration, and treadmill inclination.

Moderate exercise improved physical performance

In the present work it was used a chronic and moderate protocol once it is considered as a treatment that positively alters the oxidative homeostasis of tissues and cells, increasing the resistance to oxidative stress [26,27] and bringing benefits to general health. Besides, it was

already shown that the intensity-controlled training in a treadmill leads to consistent effects of physical training [28]. It was observed significant increases in the maximal exercise test as long as significant decreases in the mean arterial pressure, showing that after 11 weeks of diary exercise training, animals increased their capacity to resist to a forced exercise test.

Moderate exercise improved memory of less-responsive rats

In order to verify the benefits of the moderate physical exercise to the mnemonic processes, animals were separated according to their cognitive skills. The active avoidance apparatus was used as it requires the animal's attention to avoid an aversive stimulus [9]. It leads to the secretion of neurotransmitters and hormones as acetylcholine, β-endorphin, norepinephrine, glucocorticoids and vasopressin with actions in the amygdala. These neuro-humoral alterations incorporate to the experiences as components of the fear memory [29,30]. According to the rats' performance in the active avoidance equipment, it was possible to separate them into responsive and less responsive to the task. Once the rat behavior is related to the animal's response to the environment, the deltas between the training session and test 1, performed one week later were calculated and animals were distributed in the groups according to the dispersion. Such conduct valorizes the individual variability, which could be neglected if a limit was imposed. Although a non-significant increase in CAR response was also observed in sedentary animals, after the treadmill training the less responsive trained animals presented a significant increase of 3 times in the response to the active avoidance equipment, when compared to their performance before the beginning of the protocol. These data support the hypothesis that, even those animals that have low capacity of learning and memorize may have their performance improved with a moderate routine of exercises. This improve can be due to alterations in neurotransmitters, growth proteins or hormones levels, that positively affect the central nervous system.

Moderate physical exercise increased α7 nAChR density

A growing body of evidence shows that treadmill exercise increases the expression of the neurotrophin brain-derived neurotrofic factor (BDNF) leading to improves in cognition [31,32]. BDNF is expressed in cortex and hippocampus [33] and can activate multiple pathways, depending on its target receptor, leading to changes in neurotransmitter systems. As the BDNF expression is modified by physical exercise, this mechanism may contribute to changes in brain function and improves in memory [34]. The cholinergic system is one example of those systems that is under influence of BDNF function. BDNF stabilizes newly formed cholinergic synapses of α7 nicotinic receptors in hippocampus interneurons and parasympathetic neurons [13,14,35]. In this way, we showed here that the density of α7 nicotinic cholinergic receptors was increased in the stratum oriens of hippocampus and *Nucleus accumbens* after 11 weeks of moderate physical exercise. Recently, our research team showed that strategies like sustained attention and enriched environment altered the density of this receptor in areas related to memory as hippocampus, frontal cortex and amygdala [12,22]. This receptor is linked to the formation of long-term memory as it can modulate long-term potentiation [36], the most accepted biological phenomenon to explain memory formation. In the stratum oriens, nAChR are located in inhibitory GABAergic interneurons. It has been postulated that this increase in α7 nAChR in inhibitory interneurons might be a way to support hippocampus homeostasis, inhibiting glutamatergic firing. In this way, a balance of excitatory and inhibitory inputs occur leading to "cell assemblies" which form a plausible explanation of how groups of neurons behave during high activity, including learning and memory formation [37].

Conclusions

In this work, it was shown that moderate physical exercise during 11 weeks could improve the cognitive capacity of less responsive rats to remember a task. Animals also showed decreased blood pressure and an increase in physical conditioning evidenced by improve in the maximal exercise test. This is the first work that shows that physical activity can increase the density of α7 nicotinic receptor in some brain areas related to memory. So, it is suggested the involvement of the cholinergic system in this improvement. These data reinforce the importance of the moderate exercise to those who present learning and memory difficulties.

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