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Differential effects of swimming training on neuronal calcium sensor-1 expression in rat hippocampus/cortex and in object recognition memory tasks

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ABSTRACT

Physical activity has been proposed as a behavioral intervention that improves learning and memory; nevertheless, the mechanisms underlying these health benefits are still not well understood. Neuronal Calcium Sensor-1 (NCS-1) is a member of a superfamily of proteins that respond to local Ca^{2+} changes shown to have an important role in learning and memory. The aim of the present study was to investigate the effects of swimming training on NCS-1 levels in the rat brain after accessing cognitive performance. Wistar rats were randomly assigned to sedentary (SG) or exercised groups (EG). The EG was subject to forced swimming activity, 30 min/day, 5 days/week, during 8 weeks. Progressive load trials were performed in the first and last week in order to access the efficiency of the training. After the 8 week training protocol, memory performance was evaluated by the novel object preference and object location tasks. NCS-1 levels were measured in the cortex and hippocampus using immunoblotting. The EG performed statistically better for the spatial short-term memory (0.73 ± 0.01) when compared to the SG (0.63 ± 0.02; $P < 0.05$). No statistically significant exercise-effect was observed in the novel object preference task (SG 0.65 ± 0.02 and EG 0.68 ± 0.02; $p > 0.05$). In addition, chronic exercise promoted a significant increase in hippocampal NCS-1 levels (1.8 ± 0.1) when compared to SG (1.17 ± 0.08; $P < 0{,}05$), but had no effect on cortical NCS-1 levels (SG 1.6 ± 0.1 and EG 1.5 ± 0.1; $p > 0.05$). Results suggest that physical exercise would modulate the state of the neural network regarding its potential for plastic changes: physical exercise could be modulating NCS-1 in an activity dependent manner, for specific neural substrates, thus enhancing the cellular/neuronal capability for plastic changes in these areas; which, in turn, would differentially effect ORM task performance for object recognition and displacement.

1. Introduction

Regular physical activity is an essential component of a healthy lifestyle, with positive effects in human cognitive performance [15,23,28]. Physical activity has been shown not only to activate [19] but also to cause plastic changes within the central nervous system in many of the same areas involved in memory processing [9,18]. In fact, in animals performing voluntary wheel running, the hippocampal expression of BDNF is increased, along with others genes involved with synaptic plasticity, such as synapsin I, synaptotagmin and syntaxin [44,66]. Furthermore, animal studies have demonstrated that exercise protects neurons from a variety of brain insults [13,65] and promotes neurogenesis [68].

Nevertheless, physical exercise may have contradictory effects on modulating cognitive function depending on the type and duration of the physical activity as well as the type of memory evaluated. Forced treadmill running can improve [32,35,47] but also impair [43] object recognition memory (ORM), though having no effect on the Morris water maze memory evaluation [43,47]. Interestingly, voluntary exercise enhances object recognition performance in animals categorized as low-runners, but compromises performance in high and very-high runners [27]. The apparent paradox regarding the dual-effect of exercise in ORM can be explained by exploring in further detail the type of memory being evaluated and the brain areas primarily involved.

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Object recognition memory is a declarative-like memory that may be commonly divided into two distinct components: the novelty and the spatial location of the objects [8,20]. The before mentioned components have been shown to differentially recruit brain regions involved in ORM, such as the hippocampus and other cortical structures, specially the perirhinal cortex [21,72]. For example, using a spontaneous object recognition test designed to minimize spatial and contextual factors, Forwood et al. [26] showed that rats with complete excitotoxic lesions of the hippocampus had compromised performance in spatial memory tasks while the performance in the novelty object task was unaltered.

The distinct components of ORM, besides the anatomical considerations mentioned above, also have important neurochemical particularities that further differentiate them. Although there is an overwhelming amount of data describing the biochemical cascades involved in ORM [37,55,64,73], this section will concentrate on the processes that are also affected by physical exercise. For example, the enhanced expression of neurotrophins, from both glia and neuron alike, not only play a critical role in memory formation [35,39], but are also modulated by moderate physical exercise [25,58]. It has been shown that physical exercise up-regulates BDNF (Brain Derive Neurotrophic Factor) and GDNF (Glial Derived Neurotrophic Factor) expression, having a direct consequence in memory performance tasks [63]. However, the association between memory improvement and exercise, as mentioned before, is greatly dependent on the type of exercise used and the kind of memory being tested [27,36,48]. One possible hypothesis is that exercise enhances the potential to plastic changes in an activity dependent manner; thus, in other words, different kinds of exercise would recruit different neural substrates and specifically modulate, in those substrates, the potential to induce plastic changes. Within the biochemical cascade of BDNF and GDNF [70], the Neuronal Calcium Sensor (NCS-1) protein has a transcriptional response associated with an activity-dependent neuronal plasticity [29]. NCS-1 is a mammalian orthologue of frequenin, member of an important family of proteins able to detect and transmit calcium signals across several cellular responses [34]; which widely distributed in the nervous system, particularly expressed in the hippocampus [29,41,50]. NCS-1 enhances the number of functional synapses [14], acts in short-term plasticity in rat hippocampal cell cultures [62] and is expressed in several neural networks commonly known to play an essential role in associative learning and memory [31]. The overexpression of this protein has been shown to enhance evoked neurotransmitter release and exocytosis [51,54] as well as to promote synaptic plasticity, exploratory behavior, and rapid acquisition of spatial memory [59].

Furthermore, NCS-1 differentially modulates the distinct components of ORM, enhancing displaced object recognition performance while having no effect on novel object recognition tasks [59]. Altogether, these data suggest that NCS-1 is a promising candidate to intermediate the effects of exercise in specific cognitive processes. Our hypothesis is that swimming training exercise modulates NCS-1 expression in the neural substrates involved with one, but not the other, ORM components.

The present study was designed to assess whether physical exercise in rats enhances NCS-1 expression. Considering that NCS-1 expression is particularly high in the hippocampus, exercise paradigms that modulate NCS-1 will have greater impact on memory tasks that are primarily related to this neural substrate (i.e. the spatial memory task). Animals were subject to a chronic swimming exercise paradigm and tested for both novelty object and spatial memory tasks. NCS-1 expression in the cortex and hippocampus was quantified by immunoblotting optical densitometry.

2. Material and methods

2.1. Animals

The CEBIO-ICB-UFMG vivarium supplied sixty ($n = 60$) Male Wistar rats weighing between 150–200 g. The animals were allowed free access to food and water and were housed four animals/cage, in a temperature controlled environment, under a 12 h light–dark cycle. Efforts were made to avoid any unnecessary distress to the animals and the lowest possible number of animals was used. All experimental procedures were conducted in accordance with NIH guidelines for the care and use of animals and with approved animal protocols from the Institutional Animal Care and Use Committees at the Universidade Federal de Minas Gerais (Protocol no. 21/2009).

2.2. Swimming exercise protocol

The swimming apparatus consisted of four independent glass pools with dimensions 20 cm × 20 cm × 70 cm, with a closed loop of circulating water maintained at 32 ± 1 °C. Animals were placed and removed from the apparatus using a "fish" net. In the first week, all animals were submitted to an adaptation protocol consisting of four daily swimming episodes of different durations (10, 15, 20 and 25 min). In the fifth day all animals were subject to a progressive load test in order to determine the maximal supported load (MSL) per rat (load(g)/b.w.(g) × 100). The MSL was determined by placing each animal in the water while a step increase of weight, corresponding to 1% of the rats' body weight, was attached to the tail every 3 min until exhaustion (determined by 10 continuous seconds submerged). At this point, animals were randomly divided in two groups: exercise group (EG, $n = 30$) and sedentary group (SG, $n = 30$). The EG was subject, in the following 8 weeks, for 5 days/week, to 30 min of continuous swimming training exercise with 60% of the MSL workload obtained in the progressive load test; adapted from Manchado et al. [40].

The maximal weight carried by the animal in the progressive load test was converted to percentage of the animal body weight. Thus, every week animals were weighed and, using the previously calculated percentage value, a new maximal load was obtained and the 60% workload was determined [6]. At the end of training protocol a new progressive load test was performed to compare the physical capacity of both groups.

The SG animals were exposed to shallow water, instead of the swimming protocol of the EG, for 30 min/5 days/week during the 8 weeks of the experiment. An acrylic platform placed 5 cm under water surface allowed SG animals to stand normally with their head above the water without need to swim.

The SG and EG groups were further divided in three groups each: novel object preference task ($n = 10$), object location task ($n = 15$) and immunoblotting NCS-1 labeling ($n = 5$).

2.3. Behavioral test

Exploration occurred in an open-topped arena (50 cm × 50 cm × 40 cm) made of acrylic. The walls inside the arena were surrounded with a black paper so that no external stimuli could be seen during the experiment. The stimuli presented were copies of objects composed of plastic pieces (Gulliver, São Caetano do Sul, SP, Brazil) that varied in shape, color, and size. Objects were fixed in the base of the arena to avoid displacement during tasks. To control the odor cues, the open field arena and the objects were thoroughly cleaned with water, dried and ventilated for a few minutes between experiments. Twenty-four hours before sample protocol all animals were habituated to the experimental arena in the absence of any specific behavioral stimulus for 20 min.

Two object recognition tasks were performed: novel object preference task and object location task. Different sets of animals were used for each learning task. The procedures comprised an acquisition or sample phase, followed by a preference test after a delay of 60 min to test short-term memory (STM).

2.3.1. Novel object preference task

In this object recognition task rats were placed in an open box and allowed to explore two identical objects (sample phase) for 5 min and then returned to their home cage. After a delay of 60 min, the rats were returned to the open box where they were exposed to two different objects (test phase), one identical to the one previously encountered in the sample phase, therefore now familiar, and the other is novel. The rats were allowed to explore both objects for more 5 min [1].

2.3.2. Object location task

In another version of the task, recognition memory of object location is measured. In this task, rats are exposed in the choice phase to two identical objects. These objects are identical to those previously shown in the sample phase except that one of these objects is moved to a new location in the arena of the box. Time exploring each object was assessed for 5 min [22].

The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals. Exploratory behavior was defined as sniffing or touching the object with the nose and/or forepaws. Any other behavior, such as sitting on or turning around the object was not considered as exploration. A blind observer to the treatment recorded the time spent to explore each object and

the discrimination index was calculated as the time exploring the novel or displaced object divided by the total time spent exploring both objects. Therefore, discrimination indexes significantly above chance performance (grater to the hypothesized population mean equal to 0.5; using one-sample *t*-test significance, $p < 0.05$) represent a preference for the novel or displaced object as opposed to the familiar one [22].

2.4. Immunoblotting

Animals were killed by decapitation twenty four hours after the last progressive load test has been performed to compare the physical capacity of both groups and their brain rapidly (<1 min) removed and submerged in cold (4 °C) artificial cerebrospinal fluid (ACSF) containing (in mMol/L): 127 NaCl, 2 KCl, 10 glucose, 1.2 KH_2PO_4, 26 $NaHCO_3$, 2 $MgSO_4$, 2 $CaCl_2$, 10 HEPES bubbled with 95% O_2/5% CO_2. Both hippocampi and cortices were dissected and separately sonicated in lysis buffer containing (in mMol/L): 125 NaCl, 20 TRIS-HCl, 10 EGTA, 5 EDTA, protease inhibitor cocktail (40 μL/mL, Sigma–Aldrich, USA) and centrifugation at 13,000 × *g* for 20 min at 4 °C. Supernatants were transferred to plastic tubes and stored (−80 °C). Protein was quantified by Bradfordís method [11].

Equal amounts of protein (35 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE 12%) and transferred to PVDF membranes. The membranes were sequentially immunoblotted using antibodies raised against NCS-1 (FL-190, 1:2000 dilution, Santa Cruz Biotechnology, USA) and actin (1:2000 dilution, Chemical International, USA) for 2 h at room temperature. Antibody binding was revealed by incubation with a goat anti-rabbit horseradish peroxidase-linked IgG (1:20,000 dilution, Molecular Probes, USA) or a goat anti-mouse horseradish peroxidase-linked IgG (1:7000 dilution, Molecular Probes, USA) for 1 h at room temperature, in the presence of Immobilon immunoblotting detection system (Millipore corporation, USA). Chemiluminescense was detected by autoradiography using Kodak film and apparent bands of NCS-1 and actin were quantified by densitometry using the software Image J (http://rsbweb.nih.gov/ij/). For each experiment, values obtained for NCS-1 were corrected having actin values as reference.

2.5. Statistics

Data are presented as means ± S.E.M. Statistical comparisons were made using Student's *t*-test for progressive load test, behavioral memory test and NCS-1 Immunoblotting. In behavioral test, first we performed one-sample *t*-test, against a 0.5 value expected population mean, to evaluate if animals explore the new object or displaced object over the familiar one during the test session. The comparison between groups SG v.s. EG, was performed by means of two-way ANOVA *pos hoc* Tukey for discrimination index. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Exercise training

Before the 8-week training procedure, animals were evaluated according to the maximum supported load (MSL–17.0 ± 0.4 g). After being randomly assigned to either SG or EG animals, the MSL values were not statistically different between groups (16.6 ± 0.6 g and 17.5 ± 0.7 g, respectively). However, at the end of the training protocol, the ability to support load by the EG (7.3 ± 0.4% body mass) was significantly higher than the SG (5.6 ± 0.2% body mass, $t(48) = 3.5$, $p < 0.001$), Fig. 1.

3.2. Object location memory

There was no overall exercise-effect on total time of exploration during the sample phase of the object location task (SG = 12 ± 7 s; EG = 17 ± 10 s; $P > 0.05$). Also, the one-sample *t* test confirmed that the performance in the test phase of both groups [EG, $t(14) = 13.57$, $P < 0.0001$, and SG, $t(14) = 5.85$, $P < 0.0001$] was statistically different from chance level. However, as showed in the Fig. 2A, there was a statistical difference between SG (0.63 ± 0.02) and EG (0.73 ± 0.01) ($t(28) = 3.47$, $P = 0.001$) in the test phase. Taken together our results demonstrated that the swimming training protocol did not affect the exploratory activity of the rats but did improve performance in the object location memory.

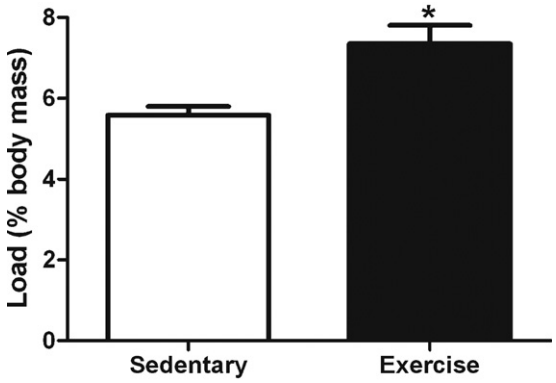

Fig. 1. Effects of chronic swimming training on progressive load test. The maximal supported load (load(g)/b.w.(g) × 100) was determined by placing each animal in the water while a step increase of weight, corresponding to 1% of the rats' body weight, was attached to the tail every 3 min until exhaustion (determined by 10 continuous seconds submerged). Data are shown as means ± SEM ($n = 25$) of the maximal % body mass supported (g), in the end of 8 week. *$P < 0.05$ in Student's *t*-test.

3.3. Novel object preference memory

The swimming exercise also did not alter the exploratory activity of the rats in the sample phase of the novel object preference task (SG = 9 ± 1 s; EG = 7 ± 0.6 s; $P > 0.05$). One-sample *t* test confirmed that the performance in the test phase, of both groups [EG, $t(9) = 7.00$, $P < 0.0001$, and SG, $t(9) = 7.10$, $P < 0.0001$], was statistically different from chance level. In contrast to the object location task, Student's *t* test showed no significant difference between the SG (0.65 ± 0.02) and EG (0.68 ± 0.02) ($t(18) = 1.78$, $P = 0.09$) (Fig. 2B). In summary, our results do not show an effect of chronic swimming exercise in the novel object preference memory.

Furthermore, there were no differences on total time of exploration during the test phase of the object location task (SG = 8.6 ± 1.4 s; and EG = 7.3 ± 1.0 s; $P > 0.05$) or of the novel object preference task (SG = 8.8 ± 2.0 s; and EG = 6.9 ± 0.5 s; $P > 0.05$), as observed in the Fig. 2C.

3.4. NCS-1 expression

The Fig. 3 illustrates the effects of exercise on NCS-1 protein levels in the cortex and hippocampus. Exercise (1.8 ± 0.1) increased NCS-1 expression in the hippocampus of rats when compared to the SG(1.2 ± 0.08) ($t(8) = 3.8$, $P = 0.005$). In contrast, EG did not change NCS-1 expression in the cortex (SG = 1.6 ± 0.1 and EG = 1.5 ± 0.1) ($t(8) = 0.73$, $P = 0.48$). Our results show that swimming training increases NCS-1 expression specifically in the hippocampus.

4. Discussion

As proof of its efficiency, the 8 week training protocol (using 60% of MSL during swimming) significantly increased the ability of the EG animals to support higher loads when compared to the SG. Such known adaptations to exercise are a result of a coordinated response of multiple organ systems, including pulmonary, cardiovascular, endocrine-metabolic and skeletal muscle [45,61]. In order to avoid undesirable effects of intense physical exercise, such as muscle lesions and incomplete substrate levels restoration between one exercise-session and the next, our training sessions used a load that corresponded to less than 6% of the animal's body weight, reference value based on other studies found throughout literature [30,40]. The MSL/b.w. values shown in Fig. 1 are similar to these found in other report, Gobatto et al. [30] shows that after physical training, the animals elevated the maximal load to 8% of body mass.

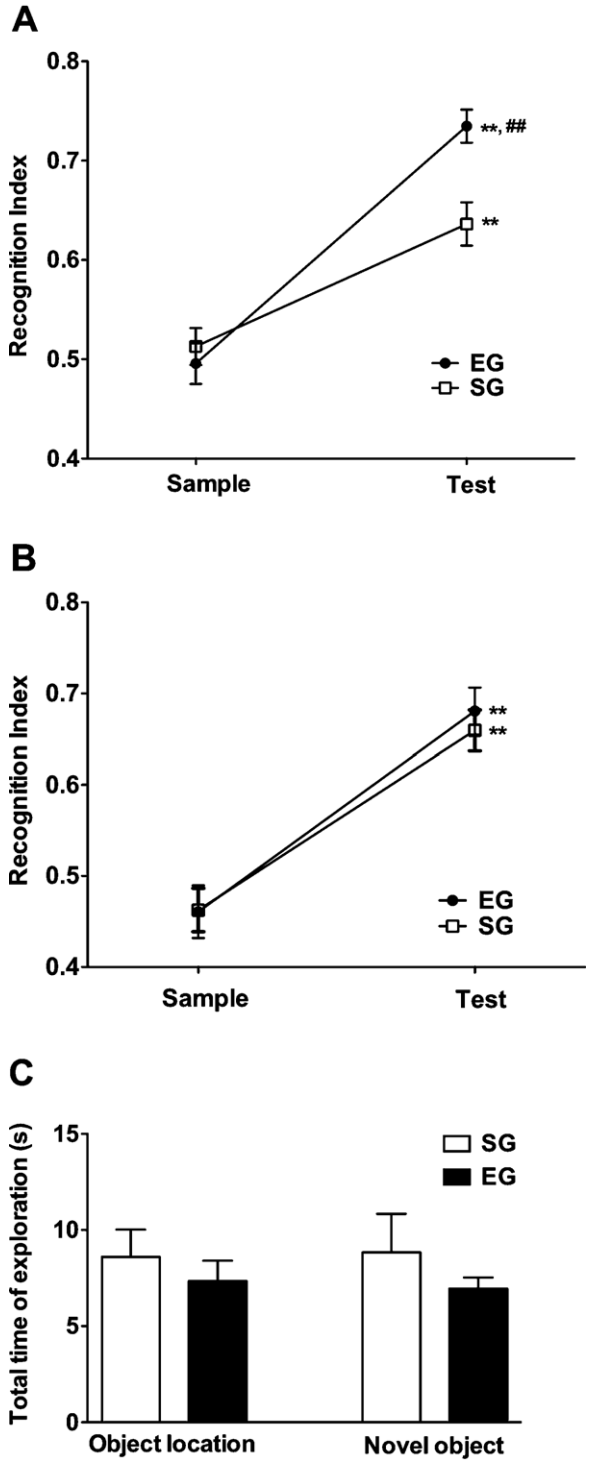

Fig. 2. Effects of chronic swimming training on behavioral test. (A) Object location task. (B) Novel object preference task. Rats were exposed to two equal objects for 5 min in the sample session. (C) Total time of exploration during test phase. One hour later a short-term memory (STM) test was carried out: animals were exposed to a stationary object and a displaced object again for 5 min in the object location task ($n = 15$) or the animals were exposed to a familiar object and a novel object again for 5 min in the object preference task ($n = 10$). Data are presented as means ± SEM of the displaced object and novel object discrimination index respectively. $^{**}P < 0.01$ vs. before sample, $\#\#P < 0.01$ Exercise vs. Sedentary in Two-way ANOVA, *post hoc* Tukey. Total time of exploration are presented as means ± SEM of the time in seconds, the statistical test used was the unpaired *t* test. SG: sedentary group, EG: exercised group.

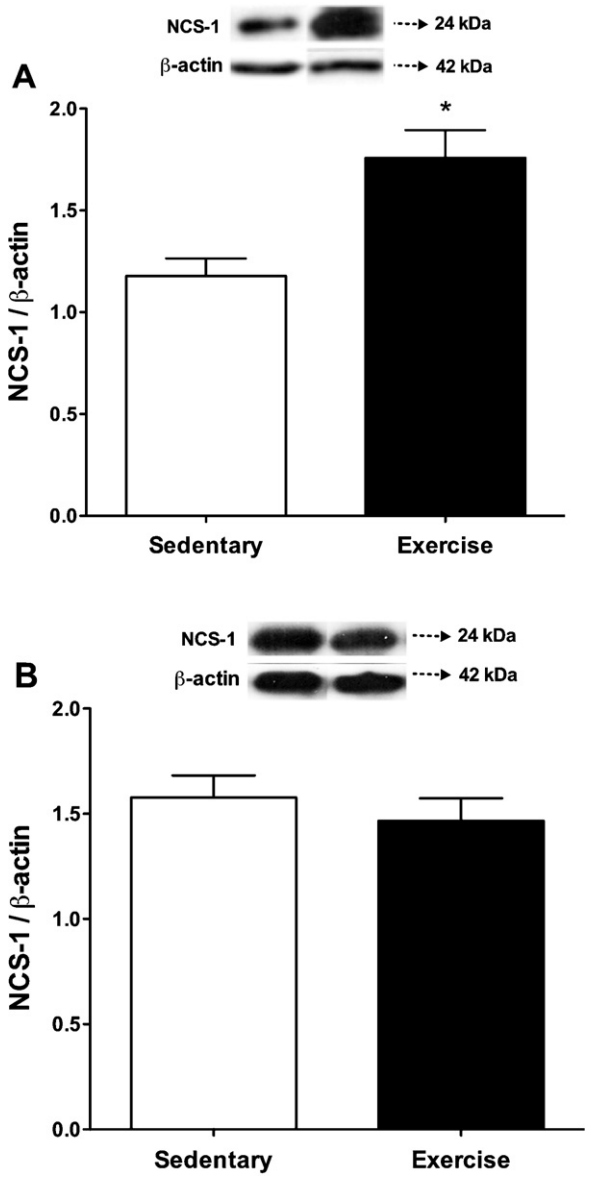

Fig. 3. Effects of chronic swimming training on NCS-1 expression in hippocampal and cortical rats. Representative immunoblotting scanned images and quantification by optic densitometry from (A) hippocampus ($n = 4$) and (B) cortex ($n = 5$). Data are presented as means ± SEM. $^{*}P < 0.05$ in Student's *t*-test; normalized against β-actin levels.

In the behavioral test, during the sample phase, sedentary and exercise groups spent equivalent time investigating the objects and no differences between groups were observed in ambulation, anxiety, grooming or risk-assessing behaviors (data not shown). Altogether, this data suggest that swimming exercise does not result in an enhanced stress response. In fact, previous research showed that although acute forced exercise elevates corticosterone [52,53], hypothalamic-pituitary-adrenal axis seems to adapt to chronic exercise. In effect after several weeks of training, the exercise no longer elevates corticosterone levels [24].

Regarding the memory results, both groups were able to discriminate between displaced and stationary object, however, the exercise group showed stronger preference for the displaced object, indicating enhancement of short-term object recognition memory. In the novel object task, although the groups were able to discriminate between novel and familiar objects, there was no statistical

difference in the discrimination of objects between the sedentary and the exercise group during the test phase. This difference could be explained by the distinct recruitment of the cortex and hippocampus in these two kinds of memory [71]. In fact, perirhinal cortex lesion affects object recognition but not spatial memories [3]. Moreover, gene expression studies provide exactly the same pattern of differential involvement of these neural substrates. The presentation of novel, individual visual stimuli evokes increased c-Fos levels in the perirhinal cortex but not in the hippocampus. In contrast, performing spatial tasks or being exposed to novel spatial arrays of visual stimuli does not increase perirhinal c-Fos levels but does alter c-Fos levels throughout much of the hippocampal formation [2].

Although there is no doubt that the hippocampus participates either directly or indirectly in all memory tasks used in this study; there are some controversies about the hippocampus degree of importance on the distinct components of ORM. For example, using a spontaneous object recognition test designed to minimize spatial and contextual factors, Forwood et al. [26] showed that rats with complete excitotoxic lesions of the hippocampus have the recognition memory intact at all delays tested, showing memory deficits only for spatial memory performance. In contrast, other authors, using a protocol similar to ours, showed that rats and mice with lesion in the hippocampus recognize a new object shortly after training but not after 1–24 h [46,16,33]. Also, pharmacological manipulation of the dorsal hippocampus affects consolidation and reconsolidation of long-term novel object recognition memory without affecting short-term memory [57]. In addition, based on c-Fos changes in the hippocampus after training, Albasser et al. [5] suggest a complex pattern of hippocampal activity potentially involved in the detection of novel objects; and Clarke et al. [17] showed that the object recognition memory consolidation is accompanied by transient potentiation in the hippocampal CA3-CA1 synapses. Nevertheless, our results are confined to short-term plastic changes (Fig. 2 – only evaluates performance 60 min after training) that do not involve protein synthesis. Furthermore, the referred short-term change must involve a mechanism that has been intrinsically modified by exercise; such as, in accordance with our hypothesis, the synaptic facilitation induced by activity-dependent NCS-1 up-regulation [60]. Altogether, these results may explain why swimming exercise had little or no effect in the novel object preference paradigm. In accordance, one very similar finding, in a study conducted in mice, showed that NCS-1 dentate gyrus expression was necessary to promote ORM spatial location performance enhancement; but had no effect on novel object recognition performance [59].

The interpretation of the behavioral data presented here is furthermore strengthened by the immunoblotting results showing that the exercise paradigm significantly increased NCS-1 expression in the hippocampus (related to spatial memory) but not in cortical areas.

Our results indicate that probably the chronic swimming training used in this experiment significantly affected the region of the hippocampus, improving the spatial memory, as suggested by an increase in the hippocampal NCS-1 expression for the EG. On the other hand, the exercise protocol had no effect on object recognition memory and similarly on cortical NCS-1 expression. Ours results are in accordance with others authors that have reported favorable effects of physical exercise on memory [4,7,10,49,56,67,69].

Interestingly, Saab et al. [59], demonstrated that mice with inducible overexpression of NCS-1 in dentate gyrus had better ability to discriminate between displaced and stationary objects when compared to wild type group, but no difference was observed for the familiar and novel objects in short and long-terms object recognition memory tasks. These data suggest a participation of NCS-1 in mechanisms involving synaptic plasticity and acquisition of spatial memory.

The cause-effect relation of hippocampal NCS-1 expression and its functional role in memory is not straightforward. Nevertheless, there are some hypotheses concerning its role in the modulation of several target proteins. NCS-1 can substitute or potentiate calmodulin (CaM) functions [60]. Thus, its regulated transcription is likely to cause a functional change in synapse sensitivity to Ca^{2+} and Ca^{2+}-signaling pathways [12,42], via the activation of proteins as cyclic nucleotide phosphodiesterase (PDE), calcineurin and nitric oxide synthase [60]. Another hypothesis is that NCS-1 binds dopamine type-2 receptor, (D2R), regulates D2R phosphorylation through an interaction with G protein-coupled receptor kinase 2 (GRK2), and controls DR2 surface expression [38]. In an important report, Saab et al. [59] showed that direct application of a cell permeant peptide (DNIP) designed to interfere with NCS-1 binding to the DR2 reversed the improvement in the acquisition of spatial memory in genetically modified mice that overexpress NCS-1.

In conclusion, the present study reinforces the promnesic exercise-effect and shows for the first time that swimming exercise up regulates NCS-1 expression in the hippocampus. A negative result regarding NCS-1 expression in the hippocampus would have compromised the rational of a possible cause-effect relation with the improved performance in spatial-memory. Nevertheless, although the precise role of NCS-1 in the hippocampus of training animals remains not understood, our results contribute to a possible role of Ca^{2+} signaling through NCS-1 in the pathway involved in the enhancement of spatial memory acquisition during regular swimming training.

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