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Original article

Physical exercise during the adolescent period of life increases hippocampal parvalbumin expression

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Abstract

In order to investigate whether physical exercise during development would promote changes the calcium-binding protein parvalbumin (PV) expression in the hippocampal formation, we performed an immunostaining study after an aerobic exercise program in rats during adolescent period of life. Wistar rats were submitted to daily exercise program in a treadmill between postnatal day 21 and 60. Running time and speed were gradually increased during the subsequent days until 18 m/min for 60 min. After the aerobic exercise program, animals of all groups were killed and PV immunostaining procedures were performed. The results showed significant increase of protein level in the hippocampal formation and PV-immunoreactive neurons in CA1 and CA2/CA3 regions of rats submitted to exercise when compared with control rats. This finding indicates that aerobic exercise program during adolescent period promotes neuroplastic changes in hippocampal formation.

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1. Introduction

In the last decades, studies have dedicated to the understanding of neurobiological bases of physical exercise to the maintenance and improvement of neural function in humans and animals (for review, see [1]). Studies in adult animals demonstrate that physical exercise modifies the expression of neurotrophic factors [2–5], the growth of neuronal processes [4,6] and neurogenesis in the hippocampal formation [7–9], a highly plastic region of the brain important for memory, learning and emotional processes [10,11].

Although the effects of physical exercise on the central nervous system (CNS) of adult animals have been well documented, little is known of its effects in the developing brain. The development of highly organized structures in the CNS is a complex process and stimuli in this period could determine the functional integrity at adult stage. Experience and learning events can modulate the functional maturation of the brain by neuroplastic processes. These stimuli occurring during early postnatal brain development may result in the development of more complex neural circuitry [12]. Since the capacity for neuroplasticity decreases with increasing age [13,14], it is very important to assess how exercise may beneficially regulate neuroplasticity during early life, and to determine the basic mechanism of such effects. A preliminary study conducted by Uysal et al. [15] demonstrated that exercise during the adolescent

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period performed a better spatial memory in Morris water maze and increased cell density in the hippocampal dentate gyrus. Additionally, a recent investigation demonstrated increased hippocampal neurogenesis and gene expression in juvenile rats [16].

Previous studies have used the expression of the calcium-binding protein parvalbumin (PV) as a useful marker to study hippocampal changes in response to physical exercise [17,18]. PV is a low (12 kDa) molecular weight protein expressed in a population of nonpyramidal cells which form a heterogeneous group of neurons in the neocortex and hippocampal formation, particularly a population of inhibitory interneurons that have a high firing rate and a high oxidative metabolism [19–22]. These inhibitory interneurons are essential for information processing and play a crucial role in controlling excitatory transmission in the hippocampal neurons [23]. In our previous study a higher number of PV-immunoreactive (PV-ir) hippocampal neurons in rats submitted to acute physical exercise (voluntary and forced exercise) were observed [18]. As the interference of physical exercise during brain development has been poorly explored, the present investigation was performed to study the occurrence of structural changes in hippocampal formation after physical exercise during the animal development by means of PV immunostaining approach. To test this idea, we used immunohistochemistry and immunoblot analyses in rats submitted to treadmill exercise during adolescent period.

2. Materials and methods

2.1. Animals and protocol of physical exercise

Male Wistar rats at postnatal day 21 (P21) were divided into two groups: exercise group ($n = 10$), control group ($n = 10$). The colony room was maintained at 21 ± 2 °C with a 12-h light/dark schedule, and ad libitum food and water throughout the experiments. Animals of the exercise group were familiarized with the apparatus for three days by placing them on a treadmill (Columbus instruments) for 5 min/day at speed of 8 m/min at 0% degree incline. Electric shocks were used sparingly to motivate the rats to run. To provide a measure of trainability, we rated each animal's treadmill performance on scale of 1–5 according to the following anchors [1, refused to run, 2, below average runner (sporadic, stop and go, wrong direction), 3, average runner, 4, above average runner (consistent runner occasiolly fell back on the treadmill), 5, good runner (consistently stayed at the front of the treadmill)] [24,25]. Animals with a mean rating of 3 or higher were included to the exercise group. The animals which were excluded from the exercise groups did not form the control group. This procedure was used to exclude possible different levels of stress between animals. Subsequently, selected animals were submitted to a physical exercise program performed between P21 and P60, 7 days per week. Each training session started with a 5 min warm-up at 8–10 m/min. Running time and speed gradually increased during the subsequent days, until reach 18 m/min during 60 min (Table 1). Animals of the control group were transferred to the experimental room and handled in the same way as animals of the exercise group (privation of water and food during treadmill exercise). At P60, all animals were killed and prepared to PV immunostaining procedures. All experimental protocols were approved by the ethics committee of the Universidade Federal de São Paulo (UNIFESP).

2.2. Immunoblotting

For immunoblotting, animals' hippocampi (four animals for each group) were removed immediately after decapitation. The hippocampi were homogenized in lysis buffer [125 mM Tris–HCl buffer pH 6.8, containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 1% mercaptoethanol and 1 mM sodium vanadate] and stored at −80 °C. The analysis for PV expression was performed by mean of immunoblotting. The samples were sonicated, and protein concentration was determined by Bradford's Method [26]. After dilution 40 μg of proteins were applied to Tricine/SDS/Polyacrylamide Gel (7.5 × 5.0 cm; 12% separating gel; 4% stacking gel), according to the methods described by Schägger and Von Jagow [27]. The Gels were blotted in 25 mM Tris, 192 mM glycine, 20% (by vol.) methanol pH 8.3 on 0.2 μm cellulose nitrate sheets (GE). The blots were incubated with antibody against PV (monoclonal 1:1000, Swant) at 4 °C overnight. Peroxidase-conjugated goat anti-mouse IgG (Vectasin) was used according to the manufacturer's instructions. For a revelation was used chemiluminescence detection system (ECL) with exposure to X-ray film (Hyperfirm and ECL Kit, GE). The reprobing of membranes was required for incubation with anti-β-actin immunoglobulins (1:1000, Sigma), it used with internal control. The blots were stripped by incubating with 0.1 M NaOH solution for 5 min at room temperature.

2.3. Immunohistochemistry

Six animals from each group were deeply anesthetized (Tionembutal, 50 mg-kg, i.p.), and perfused transcardially with 0.01 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The brains were removed, briefly postfixed in 4% paraformaldehyde in PBS, cut coronally with a vibratome in 50 μm-thick sections, which were collected in series (one in each 10 cut sections). A sequence of three sections per animal (Bregma, −2.8/−4.3 mm) was selected for immunocyto-

Table 1
Physical exercise protocol in animals during development.

Postnatal day	Velocity (m/min)	Time (min)
21	8	5
22	8	5
23	8	5
24	9	5
25	9	10
26	10	10
27	10	15
28	11	15
29	11	20
30	12	20
31	12	30
32	13	30
33	13	35
34	14	35
35	14	40
36	15	40
37	15	45
38	16	45
39	16	50
40	17	50
41	17	55
42	18	55
43 ~ 60	18	60

chemistry process. The immunoperoxidase procedure was performed on free-floating sections using antibody against PV (monoclonal 1:7000, Swant, Bellinzola, Switzerland). Paired sections of animals from both groups were processed in the same vial in order to minimize the intergroup differences during the immunohistochemical procedure. The sections were pre-treated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity, rinsed in PBS, preincubated for 45 min in 10% normal serum in PBS with 0.2% Triton X-100, and then incubated in primary antibodies overnight at 4 °C. Sections were then rinsed in PBS, incubated in biotinylated anti-mouse IgG (Vector, Burlingame, USA) at a dilution of 1:200 in PBS (1 h at room temperature), rinsed in PBS, incubated in avidin–biotin peroxidase complex (ABC; Vectastain, Vector) for 1 h, washed several times in PBS, and then incubated in 0.075% diaminobenzidine in 0.002% H_2O_2. Sections were finally washed in PBS, mounted on gelatin-coated slides, dehydrated, and coverslipped.

2.4. Quantification and data analysis

The immunoblotting was performed to determine the expression of PV in the hippocampal formation. The molecular weights of proteins PV and β-actin (10–12 KDa and 42–45 KDa, respectively) were determined by running a prestained protein ladder (Rainbow – GE). The band densities on immunoblots were measured using the Densirag software (Biocom, France). All values were reported (means ± SD) of relative expression for β-actin.

The material processed for immunohistochemistry was performed to analyze the spatial distribution of PV-ir hippocampal neurons. The sections were analyzed quantitatively at the microscope, under bright-field illumination, independently by two investigators. Counts of PV-ir neurons were performed using the magnification of 200× in *stratum pyramidalis* of Cornus Ammonis (subfields CA1, CA2/CA3) and in *stratum granule* of dentate gyrus (DG) of the hippocampal formation. For each animal, the average number of PV-ir neurons in a given region was obtained through counts of three sections (both hippocampi of the each section). The values were expressed as means ± SD.

The statistical analyses between exercise and control groups were performed using Student's *t*-test. Values were considered significant when $p < 0.05$.

3. Results

3.1. Hippocampal PV expression

Quantitative immunoblotting analysis showed that the PV density was significantly enhanced in hippocampal formation of rats submitted to aerobic treadmill exercise (~30%, 1.27 ± 0.1, $p < 0.002$) when compared to the control group (1.0 ± 0.001) (Fig. 1). No difference in β-actin immunoreactivity was detected between the studied groups ($p > 0.05$).

3.2. Hippocampal distribution of PV-ir neurons

PV immunoreactivity in this study was similar to those which have been described before [20]. Briefly, PV-ir neurons in control animals were mostly located within or in the vicinity of the pyramidal cell layer of CA1, CA2 and CA3 and in the stratum oriens and stra-

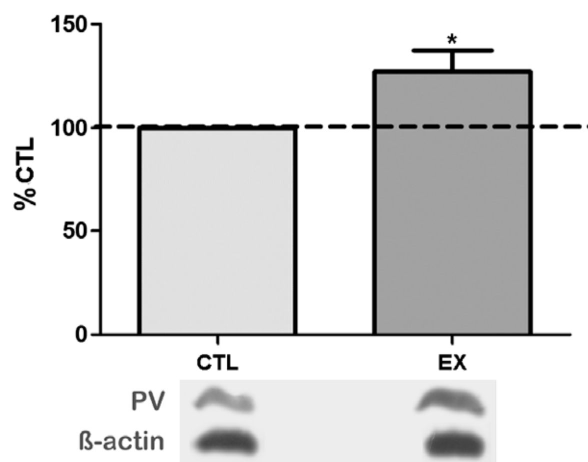

Fig. 1. Immunoblot study of parvabulmin protein (PV) in the hippocampi of rats from exercise group (EX) and control group (CTL). Significant enhance of PV density in the hippocampi of rats submitted to aerobic treadmill exercise during adolescent period ($^{*}p < 0.002$).

tum radiatum of CA1, CA2 and CA3. In the DG, PV-immunoreactivity was observed in the granule-cell layer (Fig. 2). In trained rats, the pattern of PV-immunoreactivity in the studied regions was similar to control rats. However, the statistical analysis revealed that the aerobic treadmill exercise increased significantly the number of PV-ir neurons in the CA1 (61.9 ± 5.5, $p < 0.0001$) and CA2/CA3 (18.2 ± 2.8, $p < 0.05$) when compared to the control group (CA1 $= 42.2 \pm 4.3$; CA2/CA3 $= 14.7 \pm 1.75$) (Fig. 3). No difference was observed in the DG region between the studied groups (EX $= 35.0 \pm 5.6$; CTL $= 34.4 \pm 7.1$, $p > 0.05$).

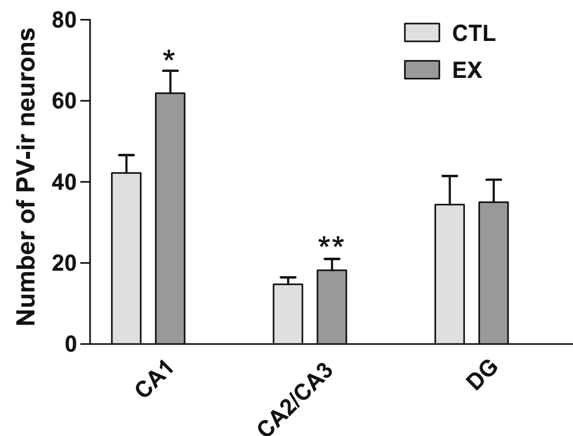

Fig. 3. Number of parvalbumin-immunoreactive (PV-ir) neurons in the hippocampal formation of rats from exercise group (EX) and control group (CTL). An increased of PV-ir neurons in rats trained during adolescent period was detected in the CA1 and CA2/Ca3 regions ($^{*}p < 0.0001$; $^{**}p < 0.05$).

4. Discussion

Although a number of studies have investigated the effect of different experience, such as enriched environment in animals during development [28,29], only a few works have applied a programmed physical exercise in adolescent animals [15,16,30]. Our study investigated the effect of a physical exercise program in animals during the adolescent period of life. Immunostaining analyses showed that the aerobic treadmill exercise performed during adolescent period induced

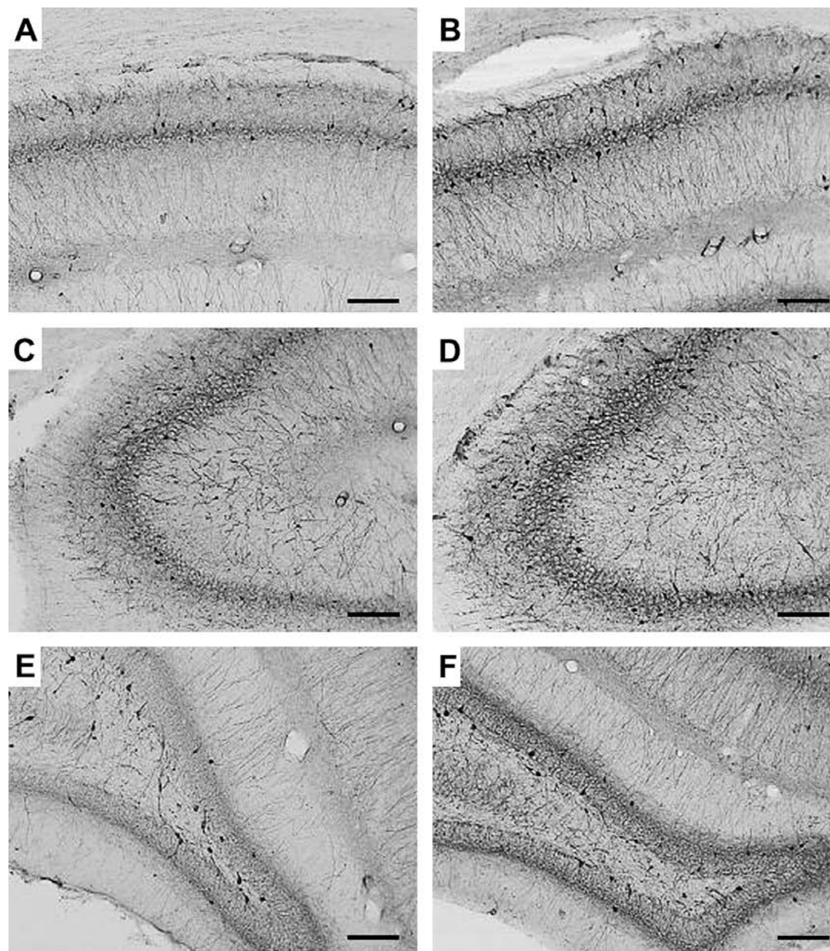

Fig. 2. Photomicrographs of parvalbumin-immunoreactive (PV-ir) neurons in the hippocampal formation of rats from exercise group (B, D and F) and control group (A, C and E) (scale bar = 150 μm). A and B: CA1; C and D: CA2/CA3; E and F: dentate gyrus.

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significant changes the hippocampal PV expression. This finding is in accordance with previous observation that shows an influence of regular aerobic exercise on hippocampal formation of adolescent rats [15]. Additionally, we showed that the exercise protocol used was able to induce morphological alteration in brain of adolescent animals. It is important to point out that different intensities of exercise may induce positive and negative changes in brain plasticity. Low and moderate exercises do not alter apoptosis in brain of adult rats [31]. In Uysal and collaborators work animals ran at speed of 8 m/min, 30 min daily, for five consecutive days a week [15]. In our exercise protocol, animals ran at a superior intensity (until 18 m/min during 60 min, 7 days per week). Although easy [15] and moderate intensity (our study) induced positive changes in hippocampal plasticity of rats examined in the adult life, this is a subject that deserves more attention.

Whereas the general structure of the brain is formed before birth, complete development of the complex neural networks depends on postnatal experience. Researches have demonstrated that juvenile stimuli can affect adult behavior [32,33]. Treadmill running by pregnant rats was shown to produce increased the mRNA expression of brain-derived neurotrophic factor (BDNF), enhanced hippocampal cell survival, and improved the short-term memory ability in the rats' pups [34]. In Uysal et al. [15] study, the exercise during the adolescent period performed a better spatial memory in Morris water maze and increased cell density in the hippocampal dentate gyrus. In another study, exercise influenced neurogenesis and mRNA BDNF expression, *N*-methyl-D-aspartate receptor type 1 (NMDAR1) and vascular endothelial growth factor (VEGF) in the hippocampal formation of 5-weeks-old rats trained for one week [16]. Our experiments do not allow identifying whether the increase in PV-ir occurs in new neurons or in pre-existing ones (CA1 and CA2/CA3 regions). It is important to note that neurogenesis induced by early life exercise may have a significant impact on brain structure and functional development. We speculate that the result of this present study could be attributed to cell proliferation and BDNF. The new cell formation in the hippocampal formation is most prevalent in young rats, and an increase cell proliferation in the dentate gyrus has been observed in 4-weeks-old rats trained for five days [30]. To this point, reductions in PV expression in hippocampal formation of BDNF mutant have been reported [35].

The physiological role of PV at the cellular and network levels is less clear. However, several studies have suggested that many biological processes in the CNS linked with calcium ions are regulate via interaction with intracellular calcium-binding proteins [22]. PV acts in neurons as an endogenous calcium buffer that affects temporal-spatial characteristics of calcium transients. In fact, it has been suggested that calcium-binding proteins protect against Ca^{2+} overload, rendering neurons more resistant against excitotoxicity [22]. In this way, several reports have proposed that alterations of the calcium-binding proteins might be involved in numerous disorders of the brain [21,22]. For instance, it has been observed that PV deficient reduces the threshold of seizures and increases the severity of the seizures induced by the convulsant drug pentylenetetrazol [36]. Similarly, mice with a targeted mutation of the gene-encoding urokinase plasminogen activator receptor showed a higher susceptibility to seizure activity and a reduction in interneurons that expression the PV [37]. These studies indicate that a down-regulation of PV could facilitate the development of epilepsy. Additionally, we recently investigated the effect of this treadmill exercise protocol during development on the amygdala kindling process (a valuable model for the agreement of the basic mechanisms of progressive epileptogenesis) [24]. The results showed that the physical exercise training in rats during the adolescent period of life did not retard the amygdala kindling development (stage 5) in the adulthood, but altered the initial stage of kindling (stage 1) [24].

In conclusion, while it is well documented that exercise has beneficial effects of neurons, further studies are needed to explore the mechanisms of exercise improving hippocampal functions in adolescent period of life. We also would like to point out that the main purpose of the present study was not designed to clarify these mechanisms, but primarily focused on morphological findings induced by exercise during brain development. Researches in this topic are relevant for determining optimum exercise strategies for people, particularly for children and teenagers. Future studies may include the impact of early life exercise on the adult brain, duration of exercise effects, and the effects of exercise intensity on cognition in both adolescents and adults.

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