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ORIGINAL ARTICLE

Physical exercise increases GFAP expression and induces morphological changes in hippocampal astrocytes

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Abstract Physical exercise has an important influence on brain plasticity, which affects the neuron–glia interaction. Astrocytes are susceptible to plasticity, and induce and stabilize synapses, regulate the concentration of various molecules, and support neuronal energy metabolism. The aim of our study was to investigate whether physical exercise is capable of altering the morphology, density and expression of glial fibrillary acidic protein (GFAP) in astrocytes from the CA1 region of rat hippocampus. Thirteen male rats were divided in two groups: sedentary ($n = 6$) and exercise ($n = 7$). The animals in the exercise group were submitted to a protocol of daily physical exercise on a treadmill for four consecutive weeks. GFAP immunoreactivity was evaluated using optical densitometry and the morphological analyses were an adaptation of Sholl's concentric circles method. Our results show that physical exercise is capable of increasing the density of GFAP-positive astrocytes as well as the regional and cellular GFAP expression. In addition, physical exercise altered astrocytic morphology as shown by the increase observed in the degree of ramification in the lateral quadrants and in the length of the longest astrocytic processes in the central quadrants. Our data demonstrate important changes in astrocytes promoted by physical exercise, supporting the idea that these cells are involved in regulating neural activity and plasticity.

Keywords Physical exercise · Astrocytes · GFAP · Hippocampus · Sholl

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Introduction

There is a considerable evidence to show that physical exercise has a positive effect on brain function in both humans (Hillman et al. 2008; Laurin et al. 2001) and animals (Albeck et al. 2006; Farmer et al. 2004; Stranahan et al. 2010). Physical exercise improves cognitive functions (Kashihara et al. 2009; Kramer et al. 2006) and memory (Alaei et al. 2008; de Senna et al. 2011), reduces anxiety and depression (Martinsen 2008), and has protective properties on a wide variety of neurological diseases, such as Parkinson's disease (Smith and Zigmond 2003), Alzheimer's disease (Mirochnic et al. 2009) and ischemic stroke (Stummer et al. 1994).

Studies designed to shed light on the neurobiological bases of these benefits have demonstrated that physical exercise is involved in cerebral plasticity. Exercise can induce neurogenesis (Kim et al. 2003; van Praag et al. 1999a, b, 2005) and increase the release of neurotrophic factors such as: brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), fibroblast growth factor (FGF) and their mRNAs (Berchtold et al. 2010; Gómez-Pinilla et al. 1997; Neeper et al. 1996). Physical exercise can also induce long-term potentiation (LTP) (van Praag et al. 1999a)

and angiogenesis (van der Borght et al. 2009). In addition, physical activity can increase the most common markers of hippocampal synaptic and structural plasticity, such as synapsin I, neurofilaments, microtubule-associated protein 2 (Ferreira et al. 2011).

Nevertheless, there are only a few studies that have investigated the effects of exercise in astrocytes in animals and humans. Some of these studies report that physical exercise was able to increase the glial fibrillary acidic protein (GFAP) expression as well as the number of GFAP-positive astrocytes in the frontoparietal cortex and striatum (Li et al. 2005), and stimulate the proliferation of the astrocytes in the subgranular zone of the hippocampus of rodents (Uda et al. 2006).

Thus, the goal of our study was to analyze the effects of physical exercise in the morphology of GFAP-positive hippocampal astrocytes, more specifically, in the *stratum radiatum* within the CA1 (Interaural 6.70 mm/Bregma −2.30 mm to Interaural 4.70 mm/Bregma −4.30 mm), a region that contains numerous astrocytes and is involved in important functions including learning and memory (Catalani et al. 2002; Squire et al. 2004).

Materials and methods

Animals

For this study, 13 male Wistar rats, aged approximately 3 months and weighing about 200–300 g were obtained from the Instituto de Ciências Básicas da Saúde (ICBS), UFRGS. They were maintained in a controlled environment with food and water ad libitum, at a 12:12 h dark:-light schedule. The animals were divided into two groups: 1-Sedentary (Sed; 6 animals), 2-Exercise (Exe; 7 animals). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA). All efforts were made to minimize animal suffering and reduce the number of animals needed.

Exercise program

Considering that for humans the guidelines recommend practicing physical activity for at least 30 min most days of the week (Hillman et al. 2008), our investigation adopted a protocol of light intensity exercise adapted from a previous study (Yoon et al. 2007). In summary, the animals in the exercise group walked on an adapted motorized treadmill for 30 min, 5 days a week for four consecutive weeks. In all the exercise sessions, the treadmill was maintained at a speed of 4 m/min for the first 5 min, and then 6 m/min for the remaining 25 min.

GFAP immunohistochemistry

For the immunohistochemical study, all rats were deeply anesthetized with ketamine (90 mg/kg) and xylazine (15 mg/kg) (i.p.) and injected with heparin (1,000 IU; Cristalia, Brazil). Using a peristaltic pump (Milan, Brazil, 50 mL/min), the animals were perfused through the left cardiac ventricle with 200 mL of saline solution followed by 200 mL of fixative solution of 4 % paraformaldehyde (Reagen, Brazil) diluted in 0.1 M phosphate buffer (PB), pH 7.4. Brains were extracted from the skull, post-fixed for 4 h in the same fixative solution at room temperature, cryoprotected in 15 % sucrose solution in PB at 4 °C until they sank (about 24 h) and then transferred to a solution of 30 % sucrose (Synth, Brazil) in PB at 4 °C until they sank (also about 24 h), and then frozen in liquid nitrogen (Nitrovet, Brazil). After these procedures, the brains were kept in a freezer (−70 °C) for further analyses. Coronal brain sections (50 µm) were obtained using a cryostat (Leica, Germany) and one in every five sections was collected for analysis. Brain sections were collected in phosphate-buffered saline (PBS) and processed for GFAP immunohistochemistry (Dutra et al. 2012). Free floating sections were washed and blocked with 2 % bovine serum albumin (BSA) in PBS containing 0.4 % Triton X-100 (PBS-Tx, Sigma Chemical Co., USA) for 30 min. They were then incubated with polyclonal GFAP antiserum raised in rabbit (Dako, UK) diluted 1:500 in 0.3 % of PBS-Tx for 48 h at 4 °C. After being washed with PBS-Tx twice, sections were incubated in anti-rabbit IgG whole molecule peroxidase-conjugated antibody produced in goat (Sigma, USA) diluted 1:150 in PBS-Tx at room temperature for 2 h. The reaction was developed by incubating the sections in a medium containing 0.06 % 3,3′-diaminobenzidine (DAB, Sigma-Chemical Co., USA) dissolved in PBS for 10 min and in the same solution containing 1 µL of 3 % H_2O_2 per mL of DAB medium for an additional 10 min. Immediately after the DAB + H_2O_2 revelation, the sections were rinsed in PBS, dehydrated in series of increasing ethanol concentrations (70, 90 and 100 %, 2 min each) cleared with xylene and covered with Permount and coverslips. As a control to rule out unspecific binding, in a few sections the primary antibody was omitted and replaced by PBS. In order to minimize differences in the staining of astrocytes and in background levels, the brains in both experimental groups were fixed and post-fixed in identical solutions for the same length of time, processed at the same time and incubated in the same immunostaining medium for the same period of time.

Astrocytic density estimation

The number of GFAP-immunoreactive astrocytes per mm^2 in the *stratum radiatum* of the CA1 was estimated using an

Olympus BX 50 microscope coupled to a Motic Images Plus 2.0 camera and Image Pro Plus (Image Pro-Plus 6.1, Media Cybernetics, Silver Spring, EUA) software.

For this analysis, three digitized images (20×) from selected areas were obtained from each section. Altogether, five sections from each animal were analyzed. Thus, 15 images were analyzed per animal. Three randomized squares measuring 5,828 μm^2 and named areas of interest (AOIs) were overlaid on each image. The astrocytes located inside this square or intersected by the upper and/or right edges of the square were counted. Astrocytes intersected by the lower and/or left edges of the square were not counted.

GFAP immunoreactivity evaluation

The intensity of GFAP immunoreactivity was measured using semi-quantitative densitometric analysis (Ferraz et al. 2003; Xavier et al. 2005; Martinez et al. 2006) with the same software employed to estimate the astrocytic density. The same images used to estimate astrocytic density were used in the analysis of regional optical density (OD). The images were converted to an 8-bit gray scale (256 gray levels) and three AOIs (5,828 μm^2) were overlaid on each image.

For the analysis of cellular OD, three digitized images (40×) were obtained from each section. Altogether, five sections from each animal were analyzed. Thus, fifteen images were analyzed in each animal. The images were converted to gray scale and one AOI measuring 10.37 μm^2 was placed over the astrocytic soma in each image. Cellular GFAP expression was only measured in the glial soma, immunoreactivity in the processes was not measured.

All lighting conditions and magnifications were kept constant during the process of capturing the images. Blood vessels and other artifacts were avoided and the background correction was performed according to the formula previously described in Xavier et al. (2005).

Morphological analysis of astrocytes

The morphological analysis was done using the same images employed to measure cellular optical density. For the analysis of astrocytic ramification, an adaptation of Sholl's concentric circles technique was used (Sholl 1953; Dall'Oglio et al. 2008). Briefly, seven virtual circles with 3.91 μm intervals were drawn around each astrocyte.

The degree of ramification of the astrocytes was measured by counting the number of times the astrocytic processes intersected with each virtual circle in both the lateral (i.e. right/left) and central (i.e. superior/inferior) quadrants around the astrocytes.

Primary process quantification was performed by counting the processes extending directly from the soma in both the lateral and central quadrants of astrocytes in the same sections.

The longest primary process in each quadrant was measured by tracing the process with a manual measurement tool found in the Image Pro Plus software.

Statistical analysis

An unpaired (Student's) t test was used to compare the groups ($p < 0.05$), using Graph Pad 4.0 software. Data are expressed as mean ± standard deviation.

Results

In our study, astrocytes from the *stratum radiatum* within the CA1 region of the hippocampus were analyzed (Fig. 1). In both groups, it was possible to observe the soma and processes of GFAP-positive astrocytes. Some of these processes were long and thin, extending from the soma (primary process) and giving rise to many fine ramifications (Fig. 1). These astrocytes were seen to connect to neighboring astrocytes by slightly touching their distal processes (Fig. 1).

In the qualitative analysis, we observed an increased number of GFAP-positive astrocytes, and a stronger immunoreaction in the exercise group when compared to sedentary animals (Fig. 1).

In order to confirm our qualitative morphological findings, quantitative and semi-quantitative evaluations were performed, respectively, involving an estimation of astrocytic density and measurements of regional and cellular optical density.

Astrocytic density, regional and cellular optical density

Daily physical exercise was able to increase astrocytic density (Fig. 2a; $p < 0.001$). Increases in regional (Fig. 2b; $p < 0.05$) and cellular (Fig. 2c; $p < 0.01$) GFAP immunoreactions were also observed in the exercise group.

Analysis of astrocytic ramification

As shown in Fig. 3b, physical exercise induced an increase in the number of total ramifications ($p < 0.05$). This increase in the total number of intersections is due to the fact that the number of intersections increased in the lateral quadrants in the exercise group (Fig. 3d; $p < 0.05$). No difference was observed in the number of intersections counted in the central quadrants (Fig. 3c).

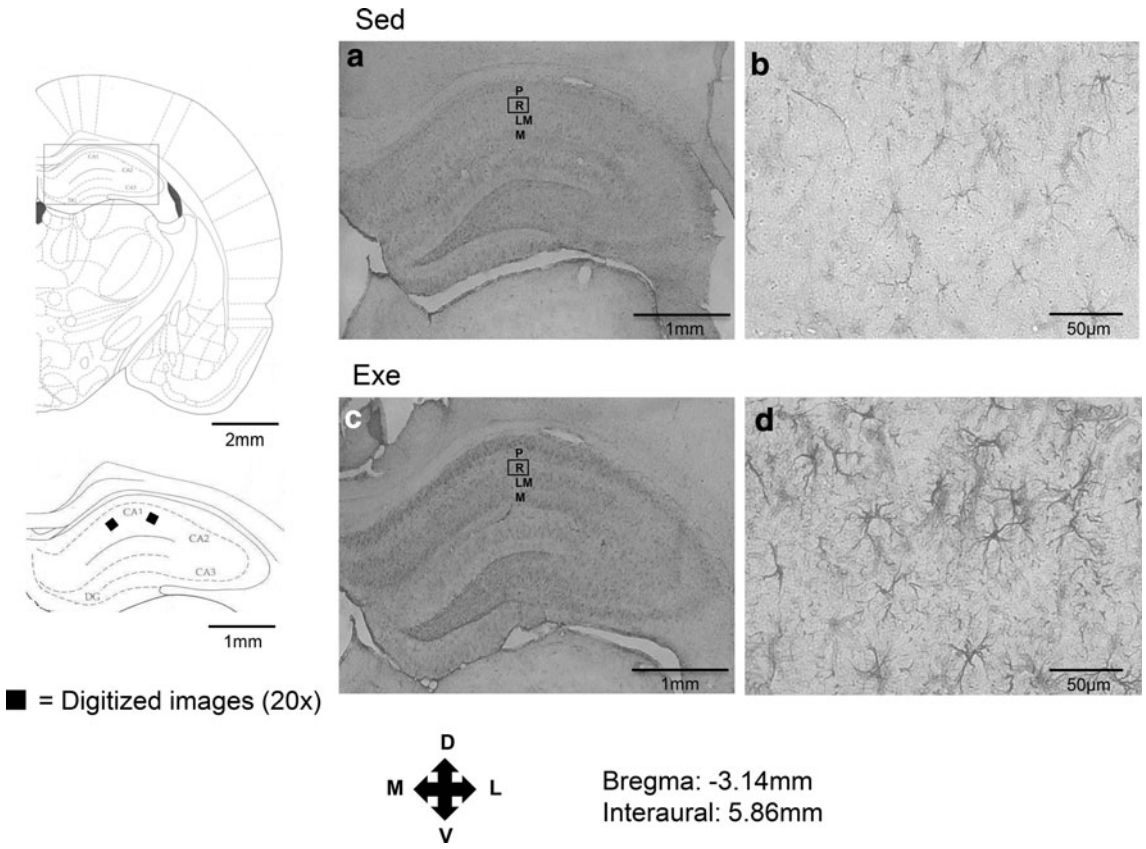

Fig. 1 Digitized images of the hippocampus after GFAP immunohistochemistry showing the CA1 region. **a**, **b** sedentary, **c**, **d** exercise. Note the astrocytic soma and processes stained for GFAP and the increase in astrocytic density and GFAP expression in the exercise group. *Filled square* areas of capture at 20×. *P* stratum pyramidale, *R* stratum radiatum, *LM* stratum lacunosum moleculare and *M* stratum moleculare. Adapted from Paxinos and Watson (1998)

Analysis of the primary processes

There were no significant differences in the number of central, lateral and total primary processes (Fig. 4a–c).

Length of the primary processes

The analysis of the length of the longest primary central and lateral processes demonstrated that physical exercise was able to increase the length of the astrocytic processes in the central quadrant (Fig. 5a; $p < 0.05$) when compared to the sedentary group. No differences were observed in the astrocytic process length in the lateral quadrant (Fig. 5b).

Discussion

In our study, we used GFAP as an astrocytic marker. Other markers including, S100 protein, vimentin and glutamine synthetase are used to analyze alteration in the proprieties of glial cells (Catalani et al. 2002). However, our choice to use GFAP is based on the fact that other markers present some serious disadvantages when compared to GFAP.

For example, in the study by Wu et al. (2005), while antibodies for glutamine synthetase and S100β were found to clearly stain the nuclei of astrocytes, the cytoplasm and processes were only poorly stained. It has also been noted that with S100 immunohistochemistry the astrocytic processes appear to be smaller when compared to GFAP immunostained astrocytes (Björklund et al. 1983). Moreover, although glutamine synthetase was first thought to be specific for astrocytes, later studies revealed that this enzyme is also detectable in oligodendrocytes (Tansey et al. 1991).

Like GFAP, vimentin is also a good marker of astrocytic morphology, but it is predominantly expressed in immature glial cells (Dahl et al. 1981; Pixley and Vellis 1984), and in our study, we only focused on the effects of exercise on the structure and function of fully developed astrocytes.

Other glial markers that could have been used are members of the glutamate transporters family. Some studies have used immunoreaction to detect these transporters (i.e. GLAST and GLT). However, these markers are

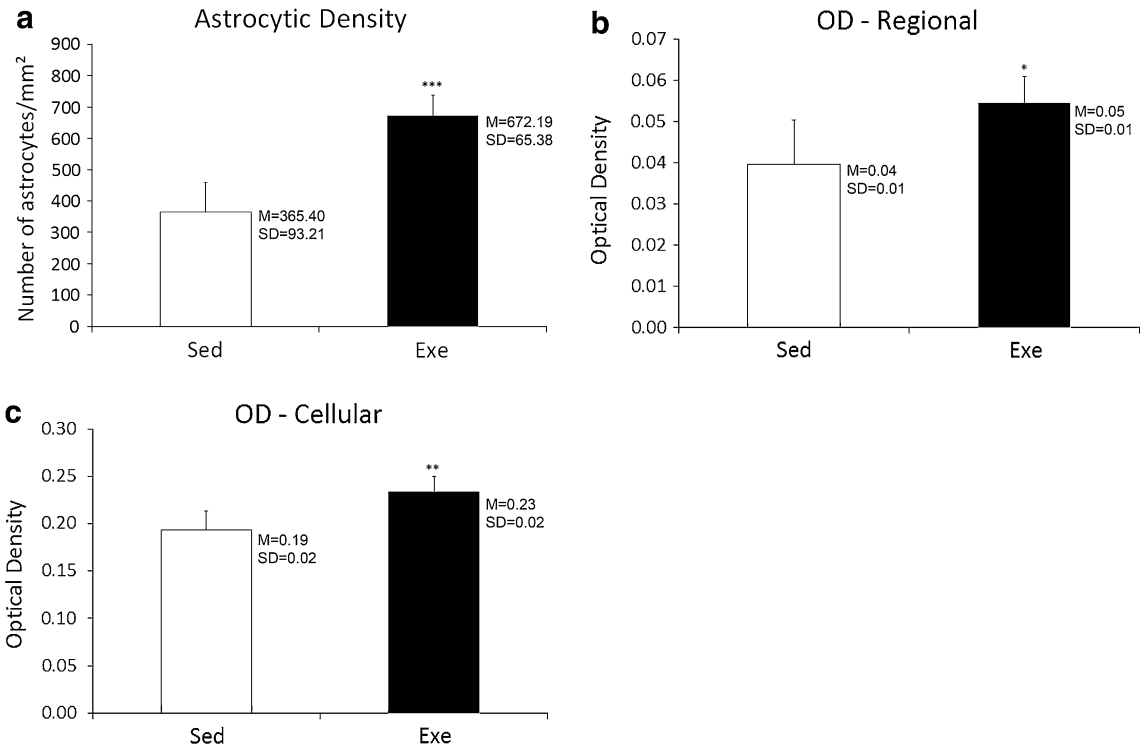

Fig. 2 Effects of physical exercise on the astrocytic density and GFAP expression. **a** Astrocytic density, **b** regional optical density ($p = 0.015$) and **c** cellular optical density ($p = 0.007$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (mean ± SD). *Sed* sedentary, *Exe* exercise, *OD* optical density

not good tracers for morphological analyses, because they usually produce unclear images (Coleman et al. 2004; Zhang et al. 2011).

Therefore, GFAP immunolabeling is still generally considered a reliable means of identifying astrocytes (Theodosis et al. 2008). GFAP has been used as the main marker for astrocytical reactivity in several different areas such as: obesity (Buckman et al. 2012), schizophrenia (Williams et al. 2012), neuroinflammation (Schäfer et al. 2012), the effects of vitamin C (Hashem et al. 2012), and the effects of resveratrol (Yuan et al. 2012). Furthermore, GFAP is a cytoskeletal protein required for the formation of stable astrocytic processes (Weinstein et al. 1991), which is ideal for morphological analyses.

In our study, we have demonstrated that physical exercise can morphologically alter GFAP-positive astrocytes in the *stratum radiatum* within the CA1 region of the hippocampus in healthy rats. We found an increase in the density of astrocytes in the exercise group (Fig. 2a). This result is supported by previous studies which demonstrated an increase in astrocytic density (Uda et al. 2006) and in the proliferation of GFAP-positive cells, in the subgranular zone of the hippocampus, in exercised rats (Komitova et al. 2005). One explanation for this increase in astrocytic density is that physical exercise is capable of increasing FGF and NGF (Neeper et al. 1996; Gómez-Pinilla et al. 1997), since previous studies have demonstrated that FGF and NGF are able to induce astrocytic proliferation (Gómez-Pinilla et al. 1995; Lewis et al. 1992; Yokoyama et al. 1993).

We observed an increase in regional GFAP expression brought on by physical exercise (Fig. 2b). Our finding is in accordance with previous studies which have demonstrated similar alterations in the hippocampus (Ferreira et al. 2011; Rodrigues et al. 2010). Another study also found exercise evoked an increase in regional GFAP expression in different brain regions (Li et al. 2005). However, using a more intense exercise protocol, de Senna et al. (2011) reported no changes in GFAP regional optical density, in contrast to our study. This difference is probably a result of the different exercise protocols used, indicating that regional optical density generated by GFAP could differ according to the protocol used. Unfortunately, the study by de Senna et al. (2011) did not include an analysis using Sholl method, which could have provided some very interesting information.

The increase in GFAP expression has been widely described in neurologic dysfunctions such as depression (Kraig et al. 1991), electrically induced seizures (Steward et al. 1991, 1997) and augmented cerebral activity brought on by an increase in extracellular potassium concentration (Canady et al. 1990). But in many instances, these alterations observed in astrocytes may reflect a substantial increase in astroglial metabolism and protein synthesis,

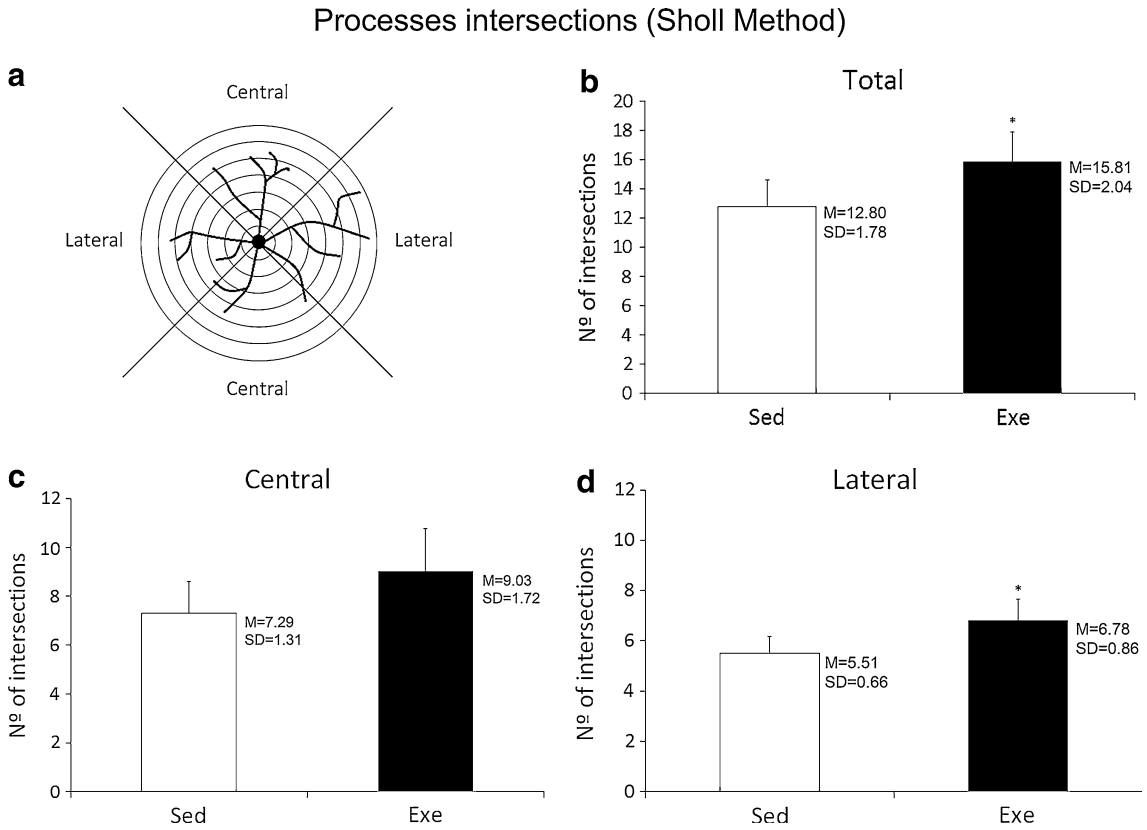

Fig. 3 Effects of physical exercise on the ramification in GFAP-positive astrocytes. **a** A schematic representation of Sholl's concentric circles method, **b** total intersections of the processes with the *circles* ($p = 0.026$), **c** number of intersections of astrocytic processes in the central quadrants ($p = 0.089$) and **d** number of intersections in the lateral quadrants ($p = 0.020$). *$p < 0.05$ (mean ± SD). *Sed* sedentary, *Exe* exercise, *Central* central quadrants, *Lateral* lateral quadrants

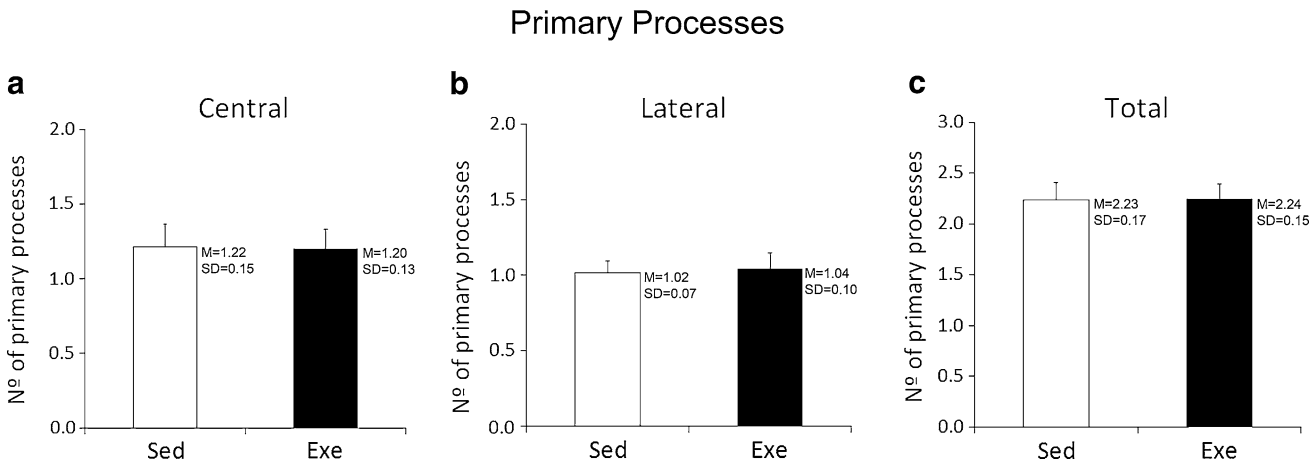

Fig. 4 Effects of physical exercise on the number of primary processes in GFAP-positive astrocytes. **a** Primary processes in the central quadrants ($p = 0.711$). **b** Primary processes in the lateral quadrants ($p = 0.640$) and **c** total primary processes, $p = 0.925$ (mean ± SD). *Sed* sedentary, *Exe* exercise

consistent with a healthy cellular hypertrophy in response to increased physiologic demands (Eddleston and Mucke 1993).

Furthermore, the astrocytes activation can play an important role in neural plasticity in healthy animals as shown by the increase in the density and expression of GFAP immunoreactive astrocytes following behavioral and environmental manipulations (Jones et al. 1996; Sirevaag and Greenough 1991; Matsutani and Leon 1993; Gómez-Pinilla et al. 1998). In addition, some studies have observed

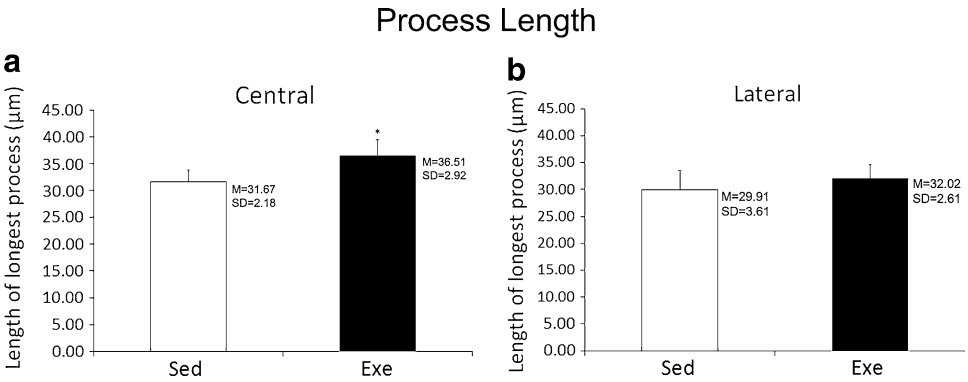

Fig. 5 Effects of physical exercise on the length of the longest primary processes in GFAP-positive astrocytes. **a** Length of the longest primary process in the central quadrants, $p = 0.011$ and **b** length of the longest primary process in the lateral quadrants ($p = 0.287$). $^*p < 0.05$ (mean ± SD). *Sed* sedentary, *Exe* exercise

that the decrease in GFAP expression leads to abnormal astrocyte–neuronal interaction, neuronal physiology and abnormal behavior in rodents (McCall et al. 1996; Shibuki et al. 1996).

Another finding of our study is the increase in cellular GFAP expression (Fig. 2c). In a very similar study, in which the researchers analyzed the effects of environmental enrichment (EE) on astrocytes, no alteration in this parameter was observed (Viola et al. 2009). EE is a housing condition that facilitates enhanced sensory, cognitive and also motor stimulation by placing various objects/tasks inside the home cages in which the animal is free to interact at will (Nithianantharajah and Hannan 2006). The differences between the results of the present study and that of Viola et al. (2009) might be related to the motor stimulus employed. EE is very broad, whereas physical exercise is specific and uniform. Thus, the increase in the cellular GFAP expression found in the present study could be associated to the regular motor stimulus provided by our protocol.

In this study, we also observed morphological alterations analyzed by the Sholl method in GFAP-positive astrocytes. Most astrocytes in the *stratum radiatum* region have a fusiform shape almost perpendicular to *stratum pyramidale* with the long axis oriented parallel to the descending apical dendrites of the CA1 pyramidal cells (Nixdorf-Bergweiler et al. 1994; Bushong et al. 2002). The longitudinal arrangement of astrocytes in relation to apical CA1 dendrites suggests that a structural arrangement between astrocytes and neurons exists in this region (Bushong et al. 2002). Our results show an increase in the degree of ramification of astrocytes in lateral quadrants, but not in central quadrants in the exercised group (Fig. 3d). Therefore, the increase observed in the ramification indicates that physical exercise induced a slight change in the morphology of the astrocytes in this region, with them adopting more stellate shape. Similar data have been described by Viola et al. (2009), in which they demonstrated that housing in an enriched environment was capable of changing the same parameter in the *stratum radiatum*.

We also observed an increase in the length of the longest primary process in the central quadrant in the exercised animals (Fig. 5a). This is in accordance with a recent study that reported that reaching skills training can induce an increase in the length of astrocytic processes, which was observed in association with enhanced sensorimotor recovery after intracerebral hemorrhage (Mestriner et al. 2011). Astrocyte processes contain cytoskeletal GFAP molecule, in which the assembly of the cytoskeleton facilitates such changes in astrocyte morphology (Rodnight et al. 1997) and some studies have observed that decreased GFAP expression leads to a reduced capacity to form stable astrocytic processes in rodents (Chen and Liem 1994; Weinstein et al. 1991).

As previously mentioned, physical exercise is capable of increasing BDNF expression (Neeper et al. 1996) and a related study demonstrated that the astrocytes from layer I within the motor cortex treated with BDNF presented similar alterations in astrocytic morphology to those observed in our study (Ohira et al. 2007). Thus, BDNF could be one of the molecular mechanisms responsible for the alterations that we observed in the astrocytes from exercised animals in our study. Moreover, physical exercise is capable of inducing LTP (van Praag et al. 1999a), which has been shown to produce alterations in the morphology of astrocytes around potentiated synapses to accompany neuronal plasticity in the *dentate gyrus* (Wenzel et al. 1991).

In the same way, the dendritic spines are adaptable and respond to changes in activity by altering their structure, astrocytic processes dynamically alter their morphology and interact with synapses in response to environmental cues (Allen and Barres 2005). Physical exercise is able to increase the length, density and complexity of the dendritic spines in the hippocampus (Dietrich et al. 2008; Eadie et al. 2005; Lin et al. 2012) and changes in the dendritic spines are typically coordinated with changes in the astrocytic processes (Haber et al. 2006).

Evidence clearly shows that remodeling of astrocytic processes is closely linked to neuronal activity and often

occurs in synchrony with morphological changes in neighboring neurons and synaptic inputs, a kind of astrocytic-neuronal plasticity that highlights the brain's capacity for activity-dependent modulation (Theodosis et al. 2008). The "tripartite" synapse, in which information flows not only between the traditional pre- and postsynaptic neuronal partners but, in addition, between astrocytic processes (Perea et al. 2009), suggests astrocytic processes directly influence synaptic activity, which reflects the cooperation between neurons and astrocytes. Therefore, the alterations seen in the astrocytes in our study could reflect neuronal and synaptic changes in response to physical exercise, suggesting that structural changes in both neurons and glia contribute to synaptic plasticity in the hippocampus.

An interesting hypothesis is that astrocytes could present some degree of polarization, as found in neurons and epithelial cells (Alberts et al. 2008). Thus, the "apical", "basal" and "lateral" portions of astrocytes could present different types of proteins, receptors, etc. The concept of polarity in astrocytes hypothesized in our study has been previously suggested in other studies (Nixdorf-Bergweiler et al. 1994; Derouiche et al. 2012).

The polarization of astrocytes is an exciting idea because, in the *stratum radiatum* region of the CA1, the apical dendrites of the pyramidal cells are heavily innervated by Schaffer collaterals that produce an excitatory glutamatergic input at a distal site and also by interneurons that generate inhibitory GABAergic inputs, at a proximal shaft (Andersen et al. 2007; Freund and Buzsáki 1996; Verkhratsky and Butt 2007). These differences in the synaptic configurations could explain the anisotropic nature found using Sholl analysis.

The involvement of astrocytes in angiogenesis has long been recognized (Penfold et al. 1990; Suárez et al. 1994), suggesting astrocytes have a functional role in the vascularization of neural tissue. These studies also indicate that astrocytes might participate directly and actively in the regulation of capillary formation. Another study also showed that exercise induces astroglial proliferation in the same areas that exhibit angiogenesis (Li et al. 2005). This association of angiogenesis and astroglial proliferation during exercise suggests that both astrocytes and endothelial cells could participate in the formation of new blood vessels in the brain.

Various studies have reported important neuronal and molecular alterations related to physical exercise, however, there is little is clear evidence about the relation between physical exercise and astrocytes. Thus, the main contribution of our study was to demonstrate that physical exercise is able to increase regional and cellular GFAP, as well as the number of GFAP-positive astrocytes in the hippocampus. Furthermore, these alterations were accompanied by important morphological changes in the degree of ramification and the length of the astrocytic processes.

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Conflict of interest The authors declare that they have no conflicts of interest.

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