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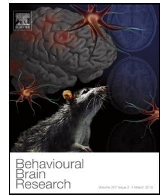

Short communication

# Treadmill exercise induces age and protocol-dependent epigenetic changes in prefrontal cortex of Wistar rats

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## HIGHLIGHTS

- Daily moderate and single session protocols impact differently cortical epigenetic marks.
- Daily moderate exercise increase H4 acetylation levels in cortices from aged animals.
- DNMT3b content changes in aged cortex is related to exercise-induced effects.

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## ABSTRACT

Some studies have linked age-related beneficial effects of exercise and epigenetic mechanisms. Although, the impact of treadmill exercise on histone acetylation, histone and DNA methylation marks in aged cortices yet remains poorly understood. Considering the role of frontal cortex on brain functions, we investigated the potential of different exercise protocols, single session and daily exercise, to modulate epigenetic marks, namely global H4 acetylation, histone methyltransferase activity (HMT H3K27) and levels of DNA methyltransferase (DNMT1 and DNMT3b) in prefrontal cortices from 3 and 21-months aged Wistar rats. The animals were submitted to two treadmill exercise protocols, single session (20 min) or daily moderate (20 min/day during 14 days). The daily exercise protocol induced an increased in histone H4 acetylation levels in prefrontal cortices of 21-months-old rats, without any effects in young adult group. DNMT3b levels were increased in aged cortices of animals submitted to single session of exercise. These results indicate that prefrontal cortex is susceptible to epigenetic changes in a protocol dependent-manner and that H4 acetylation levels and DNMT3b content changes might be linked at least in part to exercise-induced effects on brain functions.

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Epigenetic mechanisms have been associated with age-related modifications [1]. Histone acetylation, histone and DNA methylation have been suggested as critical regulators of transcriptional activity. Attachment of acetyl groups to lysine residues of the amino-terminal tails of histones (H), namely histone acetylation, have been widely associated with enhanced transcriptional activity [2,3]. Regarding, Lovatel and colleagues [4] described lower levels of global histone H4 acetylation in hippocampi of aged rats. It is interesting to note that the impairment in aversive memory was related to hippocampal H4 acetylation level, indicating the role of histone hypoacetylation status in hippocampus aging process. Although normal aging process is associated with decline in processing speed, memory, and executive function [5,6], the frontal cortex has a central role in high-order cognitive functions such as attention, decision-making and working memory [7]. In addition, tissue alterations in frontal, temporal, and parietal cortices have been described [8,9], the impact of aging process on histone modifications in rodent cortices are rarely exploited [10,11].

Interestingly, it has been described that methylation of histone H3 at lysine 27 (H3K27) is frequently found in transcriptionally silent regions [12]. This epigenetic mechanism is catalyzed by histone methyltransferases. Some pathological process such as cancer progression has been linked to increased H3K27 methylation levels and activity of its related methyltransferase (HMT) enzyme such as EZH2 (enhancer of zeste homolog 2) [13]. Although EZH2

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hyperactivity is considered as a key factor in ataxia-telangiectasia neurodegeneration, disease characterized by premature aging [14], there are no studies, to our knowledge, reporting the activity of histone methyltransferases in the healthy aging process.

Moreover, DNA methylation is an essential epigenetic mark for normal function of the brain and its disruption has been related to impairments of brain functions [15]. This mark occurs on cytosine specially located in CG dinucleotides (CpG) catalyzed by a family of enzymes called DNA methyltransferases (DNMT). It is interesting to note that there are several evidences showing differences in methylation status of different genomic sites in aging process, since DNA methylation is lost globally and increased at promoter-associated CpG islands [16,17]. Several tissues, including peripheral leukocytes and organs, such as brain and lung, have demonstrated this profile [16–20] According Wu and Zhang [21] changes in methylation with age could reflect the accumulation of stochastic methylation events over time and/or DNA methyltransferases alterations, although this hypothesis are rarely exploited.

Hippocampal epigenetic modifications seem to be involved with exercise effects in brain functions. Previous data have shown that moderate daily exercise protocol (20 min/day during 2 weeks) improved global H4 acetylation concomitantly to better aversive memory performance in hippocampi of 20-month-old rats, without any effect in the young adult group [4]. Besides, Elsner described an age-dependent effect of exercise on epigenetic parameters and colleagues [22] since a single exercise session decreased both DNMT3b and DNMT1 levels in hippocampi of young adult rats, without any effect in the aged group [22]. The impact of exercise on epigenetic marks in frontal cortex of healthy aged rodents has received little attention.

Therefore, our aim was to investigate the effects of single session or moderate daily treadmill protocol on epigenetic markers, namely histone H4 acetylation, HMT H3K27 activity and DNMT3b, DNMT1 levels, in frontal cortices from young adult and aged Wistar rats.

All experimental procedures were carried out following the NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996). The Local Ethics Committee (Comitê de Ética em Pesquisa-UFRGS) approved all handling and experimental conditions (number. 21449). Male Wistar rats at different ages were used, 3 months and 21 months old. The animals were maintained under standard conditions (12 h light/dark, $22 \pm 2$ °C) with food and water *ad libitum* and housed 3 per cage.

Rats were randomly divided into sedentary (SED) or exercised (EXE) groups. EXE were submitted to exercise protocol, which consisted of running sessions on an adapted motorized rodent treadmill (INBRAMED TK 01, Porto Alegre, Brazil), with individual Plexiglas lanes. SED was handled exactly as the experimental animals, and left on the treadmill for 5 min without any stimulus to run. Animals run at 60% of their maximal oxygen uptake [22–24]. Peak oxygen uptake ($VO_2$) was measured indirectly in all animals before training. Two exercise protocols were employed: a single session of treadmill exercise (20 min) where animals run at 7.36 m/min (young adult) and 5.08 m/min (aged) for the first 4 min, 11.04 m/min (young adult) and 7.62 m/min (aged) for 12 min, and 7.36 m/min (young adult) and 5.08 m/min (aged) for the last 4 min and daily moderate exercise protocol (20 min running session each day for 2 weeks). Rats of 21-months-old were adapted to the treadmill by running, in the first few sessions at 5.17 m/min for the first 2 min, 6.46 m/min for the next 4 min, 7.75 m/min for 8 min, 6.46 m/min for 4 min and 5.17 m/min for the last 2 min. Thereafter, animals ran at 5.17 m/min for the first 4 min, 7.75 m/min for 12 min and 5.17 m/min for the last 4 min. Young adult animals also experience the first two days of adaption; they run at 6.85 m/min for the first 2 min, 8.56 m/min for the next 4 min, 10.28 m/min for 8 min, 8.56 m/min for 4 min, and 6.85 m/min for the last 2 min. After adaptation animals ran 4 min at 6.85 m/min, the next 12 min at 10.28 m/min for 12 min, and the last 4 min at 6.85 m/min [25]. Animals that initially refused to run were encouraged by gently tapping their backs. Neither electric shock nor physical prodding were used in this study.

The procedures took place between 14:00 and 17:00 h. Rats were decapitated 1 h after the single session or after the last training session of daily moderated protocol. Brains were excised quickly, rinsed in saline, placed on ice. The frontal cortices were quickly dissected out, aliquoted, immediately snap-frozen in liquid nitrogen and stored at −80 °C until the biochemical assays. One aliquot was homogenized with specific lyses buffer in order to obtain histone extract to determine global H4 acetylation levels. Nuclear extract was obtained from another aliquot to evaluate both DNMT1/3b content and HMT H3K27 activity. The protein concentration was measured by the Coomassie Blue method using bovine serum albumin as standard [26].

Global histone H4 acetylation levels were determined using the Global Histone H4 Acetylation Assay Kit (Catalog number P-4009, EpiQuik USA) according to the manufacturer's instructions. The frontal cortices were homogenized with specific kit lyses buffer for nuclear extraction followed by the histone extraction. After incubations with TCA, HCl and acetone and few centrifugations, pellet was used for the H4 acetylation detection. Samples were incubated with capture antibody followed by detection antibody. After, samples were incubated with developing solution. Stop solution was added and the absorbance was measured on a microplate reader (450 nm).

Prefrontal cortex was homogenized in three volumes of ice-cold lysis buffer pH 7.4 containing (in mM): 250 sucrose; 20 Tris–HCl; 1 EDTA; 1 EGTA; 10 KCl; 1 DTT; 0.1 PMSF; 0.0001 okadaic acid. The lysates were centrifugated (16,000 *g* for 5 min) at 4 °C in a microcentrifuge tube, and supernatant was removed for analysis. HMT H3K27 enzyme activity, as well as the content of DNMT1 and DNMT3b were determined using Histone Methyltransferase Activity/Inhibition (H3K27) and DNMT1, DNMT3b Assay Kits (Catalog #P-3005, #P-3011 and #P-3013 respectively, *EpiQuik*®). All the procedures were done according to the manufacturer's instructions. The supernatant and standards curve was incubated at 37 °C for 60 min, with the capture antibody followed by detection antibody. The plate was incubated away from the light with Developing Solution at room temperature for 10 min. The Stop Solution was added and the absorbance was measured on a microplate reader (450 nm).

Results were expressed as percentage of control, where young adult SED group was taken as 100%. Kolmogorov–Smirnov was used as a normality test. Kruskal–Wallis followed by Dunn test was employed to analyze Global H4 acetylation levels and HMT H3K27 enzyme activity; the results were expressed as medians (25th/75th of percentiles). Content of DNMT3b and DNMT1 were analyzed by two-way Analysis of Variance (ANOVA) with exercise and age as independent variables, followed by post hoc Duncan multiple range tests when appropriate. Data were expressed by mean ± standard deviation. Statistical analysis was performed using the GraphPad-Prism 6.0 (GraphPad Software, La Jolla, CA) and Statistical Package for the Social Sciences (SPSS) software. In all tests, $p < 0.05$ was considered to indicate statistical significance.

Single session of exercise was not able to induce alterations in global H4 acetylation levels in both tested ages (young adult and aged) (Fig. 1a). Nonetheless, the daily moderate exercise protocol increased global H4 acetylation levels only in aged frontal cortices ($KW = 13.51$ $p = 0.0037$), without any effect on young adult group (Fig. 1b).

Single session and moderate exercise protocol did not affect cortical HMT H3K27 enzyme activity levels in both tested age groups (Fig. 2). In addition, neither single session nor moderate exercise protocol altered cortical DNMT1 levels in young adult or aged rats (Fig. 3).

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