Research report

Long-term treadmill exercise inhibits the progression of Alzheimer's disease-like neuropathology in the hippocampus of APP/PS1 transgenic mice

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HIGHLIGHTS

- Treadmill exercise inhibits the progression of AD-like neuropathology in APP/PS1 mice.
- The regulation of APP processing is involved in exercise-induced decrease in A\(^\beta\) deposition.
- Treadmill exercise attenuates AD-like neuropathology by inhibiting the activity of GSK3.

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ABSTRACT

Previously our study has demonstrated that long-term treadmill exercise improved cognitive deficit in APP/PS1 transgenic mice of Alzheimer's disease (AD) paralleled by enhanced long-term potentiation (LTP). The present study was undertaken to further investigate whether the treadmill running could inhibit the progression of Alzheimer's disease (AD)-like neuropathology in hippocampus of the APP/PS1 mouse models of AD, and to define a potential molecular mechanism underlying the exercise-induced reduction in AD-like neuropathology. Five months of treadmill exercise resulted in a robust reduction in \(\beta\)-amyloid (A\(^\beta\)) deposition and tau phosphorylation in the hippocampus of APP/PS1 mice. This was accompanied by a significant decrease in APP phosphorylation and PS1 expression. We also observed GSK3, rather than CDK5, was inhibited by treadmill exercise. These results indicate that treadmill exercise is sufficient to inhibit the progression of AD-like neuropathology in the hippocampus of APP/PS1 transgenic mouse model, and may mediate APP processing in favor of reduced A\(^\beta\) deposition. In addition, we demonstrate that treadmill exercise attenuates AD-like neuropathology in AD transgenic mice via a GSK3 dependent signaling pathway.

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1. Introduction

Alzheimer's disease (AD), the most common cause of dementia in aged people, is a chronic neurodegenerative disorder characterized by progressive memory loss and severe cognitive decline. A rapidly ageing population makes AD become a major public health problem. Interest has therefore focused on safe and effective therapeutic strategy for the prevention and treatment of AD. Several epidemiological studies have suggested that physical exercise may reduce the risk of AD and be sufficient to slow the onset and progression of AD [1,2,3,4,6,7,8,10,11]. Recently, exercise training is considered to be one of the best candidates for amelioration of the pathological phenotypes of AD [1,80]. However, the effect of exercise on cognitive function and neuropathology in transgenic mouse models of AD remains controversial.

The impact of exercise was found in general, dependent on type, duration, or intensity of physical activity in addition to the time point of the intervention. In rodent studies, wheel running and treadmill running remain the best studied modalities of physical exercise. Of these, a treadmill exercise regime is closer to human physical training. But many studies have found that treadmill exercise with electric stimulant in animals was considered forced, and could have deleterious effects on health by increasing stress levels [61,89,90]. Therefore, in our previous studies, treadmill exercise without electric stimulant was administered in a "voluntary" fashion, and assessed the effects of exercise begun before the onset of pathology in APP/PS1 transgenic mouse models of AD [51]. Although our studies demonstrate that the treadmill exercise modality improved learning and memory function via the Morris water maze tests in APP/PS1 mice paralleled by enhanced...
long-term potentiation (LTP) [51], exercise-induced changes of the neuropathology in transgenic mice remains unknown.

The predominant neuropathological hallmarks of AD include extracellular senile plaques containing insoluble β-amyloid (Aβ) peptide and intracellular neurofibrillary tangles (NFTs) which are composed of hyperphosphorylated microtubule-associated protein tau [32,85]. Aβ peptides are generated by successive proteolysis of β-amyloid precursor protein (APP). Previously, it is believed that the key to plaque formation was an abnormal processing of APP resulting in the Aβ peptide. Thus it is possible that treadmill exercise could interfere with Aβ deposition through the regulation of APP processing. While Aβ has attracted attention of most AD researchers, studies of the neurofibrillary tangle and its constituent, the tau microtubule-associated protein, have progressed to a point where clear therapeutic strategies are emerging. Consequently, it will be of great interest to learn whether treadmill exercise has an impact on tau pathology.

To investigate further whether the treadmill running could inhibit the progression of AD-like neuropathology in the APP/Presenilin (PS) 1 transgenic mouse models of AD, we examined the effects of the treadmill running on amyloid pathology and tau pathology in the hippocampus of APP/PS1 transgenic mice. Furthermore, we have sought to define a number of potential molecular biological mechanisms to account for the observed pathological changes.

2. Materials and methods

2.1. Animals

APP/PS1 double-transgenic mice were originally obtained from the Jackson Laboratory (West Grove, PA, USA). APP/PS1 mice were maintained on a C57BL/6j genetic background and genotyped by PCR analysis of genomic DNA from tail biopsies. All animals were kept in standard plastic cages (4 mice per cage) under conventional laboratory conditions (22–24 °C, 40–60% relative humidity), with ad libitum access to food and water and maintained on a standard 12-h light:12-h dark cycle. All protocols followed the care and use of medical laboratory animals (Ministry of Health, Peoples Republic of China, 1998) and were approved by the laboratory animal ethical standards of China Medical University.

2.2. Experimental paradigm

The experimental design was performed as previously described [51]. At the age of 3 months, female APP/PS1 transgenic mice (n = 24) and wild-type litterman controls (n = 24) were randomly assigned into four groups (n = 12 each): wild-type control group (WTc), wild-type exercise group (WTe), transgenic control group (TgC), transgenic exercise group (TgE). The velocity (m/min) was used to determine the running conditions for the exercise mice. All of the exercise mice were allowed to adapt to treadmill running for 10 min each day on 2 consecutive days (first day at 5 m/min; second day at 8 m/min). After acclimatization, all of the exercise mice were subjected to exercise (5–11 m/min) treatment for 30 min each day, 5 days per week, for 5 months (from the 3rd to the 8th months of age). During each training session, running time and speed were started at 5 m/min for 5 min, increased to 8 m/min for 5 min, and then reached a maximum 11 m/min for 20 min. To avoid stress derived from shock stimulant, a treadmill exercise paradigm without electric stimulant was used in this study. The mice in the non-exercise groups were left on the treadmill, without running, for the same period as the exercise groups. No animal was excluded from the experiment. According to a previous study this long-duration training protocol maintains exercise intensity from 45% to 55% of VO2 max [6]. After 5-month exercise, animals were evaluated for spatial learning and memory using the Morris water maze task at 8 months of age. A week after the end of the water maze task, electrophysiology studies were carried out. Tissue was collected immediately following electrophysiology studies. The experimental timeline is presented in Fig. 1.

2.3. Tissue preparation

At the end of electrophysiology recording, the animals were sacrificed. Six mice of each group were decapitated rapidly. Brains were quickly removed from the skulls. Left hippocampus was immediately frozen in liquid nitrogen and then stored at −80 °C for sandwich enzyme-linked immune sorbent assay (ELISA) and Western blot analyses. Right hippocampus prepared for real-time RT-PCR were left in TRIzol reagent (Invitrogen, USA) and stored at −80 °C until use. The rest mice of each group were dissected for histological analyses (n = 6 per group) were transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were removed, post-fixed for 24 h in 4% paraformaldehyde, and embedded in paraffin. Serial 5-μm-thick coronal brain sections were cut and then stored at room temperature until stained for morphological analysis.

2.4. Immunohistochemistry

As described previously [28], standard avidin-biotinylated complex (ABC) immunohistochemistry (IHC) staining was performed to analyze the distribution of Aβ plaques in the APP/PS1 mouse brain. Briefly, paraffin sections were deparaffined in xylene and rehydrated through graded alcohols, and then treated in 0.1 M Tris–HCl buffer saline (TBS, pH 7.4) containing 3% hydrogen peroxide (H2O2) for 10 min to reduce endogenous peroxidase activity. After washing with TBS, sections were boiled in citric acid buffer for 3 min in a microwave oven. The sections were then rinsed, treated with 5% bovine serum albumin (BSA) for 30 min, and subsequently incubated overnight with mouse anti-Aβ antibody (Sigma: 1:500) at 4 °C. The antibody reacts specifically with amino acid residues 1–12 of β-amyloid protein. It was used to visualize β-amyloid deposits rather than the precursor form. After rinsing, sections were incubated with biotinylated goat anti-mouse IgG (1:200) for 1 h, followed by streptavidin peroxidase incubation for 1 h at room temperature. After rinsing, the sections were stained with 0.0255% diaminobenzidine (DAB) for 1 min. The stained sections were dehydrated through graded alcohols, cleared in xylene, and covered with neutral balsam. All sections were examined with a light microscope equipped with a digital camera (Olympus, Tokyo, Japan). Control sections were treated with identical solutions but without primary antibody followed by all subsequent incubations as described above. On control sections, no staining was observed (data not shown).

A one-in-six series of coronal section was collected [81] and immunohistochemically stained for Aβ plaques. Quantitative image analysis was performed for Aβ IHC by taking micrographs of 5 sections with the same reference position form each mouse brain. The number of Aβ-positive plaques in the hippocampus was counted through a 20× objective, and the comparison between each group was made using Image-Pro plus 6.0 software. Aβ burden was assessed as the percentage of the total area of the hippocampus that contained regions of Aβ deposits through a 10× objective. The data were analyzed with the above-mentioned software.

2.5. Western blot analysis

Total protein was extracted from hippocampus and the Western blots were prepared as described previously [82]. Briefly, samples were homogenized at 1:5 (w:v) in ice-cold lysis buffer containing a mixture of protease inhibitors. The resulting homogenate was centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatants were collected and the total protein levels were measured by a BCA protein assay.

Fig. 1. Experimental timeline of this study. After 2 days of adaptation, mice in exercise groups were subjected to treadmill exercise training for 30 min each day, 5 days per week, for 5 months. Morris water maze task was conducted on 8 consecutive days after 5-month exercise, followed by a 7 days break. Tissue was collected immediately following electrophysiology studies on the last day.
kit (Pierce Biotechnology, USA) using a UV 1700 PharamSpec ultraviolet spectrophotometer (Shimadzu, Japan).

Proteins (50 μg) were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels by electrophoresis. After electrophoretic transfer onto polyvinylidene fluoride membranes (Millipore, USA) and blocking in 5% non-fat milk for 1 h, the membranes were incubated sequentially overnight at 4 °C with the following primary antibodies: rabbit anti-APP695 (1:4000; Millipore, USA), rabbit anti-phospho-APP (Thr668) (1:1000; Cell Signaling Technology, USA), rabbit anti-disintegrin and metalloproteinase-domain-containing protein 10 (ADAM10) (1:1000; Millipore, USA), rabbit anti-β-site APP-cleaving enzyme 1 (BACE1) (1:500; Millipore, USA), rat anti-PS1 (1:500; Millipore, USA), rabbit anti-CDOH-terminal fragments (CTFs) (1:4000; Sigma, USA), mouse anti-anti-APPs (1:500; Immunobiological Laboratories, Japan), mouse anti-sAPPβ (1:500; Immunobio-logical Laboratories, Japan), rabbit anti-tau (1:400; Abcam, USA), mouse anti-phosphor-tau (Thr231) (1:1000; Invitrogen, USA), rabbit anti-phosphor-tau (Thr205) (1:1000; Abcam, USA), rabbit anti-phosphor-tau5 (Ser239) (1:1000; Abcam, USA), rabbit anti-glycogen synthase kinase (GSK) 3α/β (1:1000; Cell Signaling Technology, USA), rabbit anti-phospho-GSK3β (Ser21)/3β (Ser9) (1:1000; Cell Signaling Technology, USA), rabbit anti-cyclin-dependent kinase (CDK) 5 (1:1000; Abcam, USA), rabbit anti-phospho-CDK5 (Tyr15) (1:1000; Abcam, USA), rabbit anti-p35/25 (1:800; Millipore, USA), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000; KangChen, China). After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies (1:5000; Santa Cruz Biotechnology, USA) for 1 h.

The membranes were processed for protein detection using Super Signal West Pico chemiluminescent Substrate (Pierce Biotechnology, USA) and ChemDoc XRS with Quantity One software (Bio-Rad, USA). GAPDH was used as loading control. The relative intensities of protein bands were analyzed using Image-pro plus 6.0 analysis software (Media Cybernetics, USA).

2.6. mRNA extraction and quantitative real-time RT-PCR analysis

mRNA extraction and quantitative real-time RT-PCR were performed as described previously [51]. In brief, total RNA from dissected hippocampus was isolated with Trizol, complementary DNA (cDNA) was obtained from total RNA using PrimeScript™ RT Reagent Kit (TaKaRa Biotech, Dalian, China) according to the manufacturer’s instructions. Real-time quantitative PCR analysis was performed on the ABI 7500 Real-time PCR System (Applied Biosystems, USA), using real-time quantitative PCR SYBR Premix Ex Taq™ kit (TaKaRa Biotech, Dalian, China). Triplicate reactions were run per sample.

Relative quantification of gene expression was determined based on the threshold cycle (Ct) value for each PCR reaction. ΔCt values represent normalized target gene levels with respect to the internal control (β-actin). ΔΔCt values were calculated as the ΔCt of each sample minus the mean ΔCt of the calibrator samples (WT). Relative quantification of gene expression (relative amount of target RNA) was determined by the ΔΔCt method. The fold change in expression was calculated using the equation 2^{-ΔΔCt}. All primer sets had comparable efficiency of amplification. After amplification, the specificity of the PCR products was verified by a melting curve analysis to ensure that a single product of expected melt curve characteristics was obtained.

The primer sequences (designed and synthesized by TaKaRa Biotech, Dalian, China) are presented in Table 1.

2.7. Sandwich ELISA

Hippocampus samples were homogenized in 50.0 M guanidine buffer (diluted in standard dilution) and centrifuged for 30 min at 4 °C. The supernatants were then loaded onto 96-well plates. The detection of Aβ1–40 and Aβ1–42 was carried out with the corresponding detection kits (Invitrogen, USA) according to the instructions of the manufacturers. The absorbance was recorded at 450 nm using a 96-well plate reader.

2.8. Statistics

All values are expressed as means ± S.D. Two-way ANOVA with exercise treatment and genotype was used for evaluation of differences among groups. Comparison of data from two groups was made by unpaired Student’s t-tests. Differences were considered statistically significant at P < 0.05.

3. Results

3.1. Treadmill exercise prevents Aβ plaque accumulation and Aβ generation in the hippocampus of APP/PS1 mice

We investigated whether treadmill exercise could prevent Aβ deposition in hippocampus of wild-type and APP/PS1 mice. Brain sections of mice were subjected to IHC analysis with an antibody against Aβ. IHC staining demonstrated a clear abundance of Aβ plaques in hippocampus of transgenic mice (Fig. 2A), whereas deposits were absent in wild-type mice (data not shown). Importantly, both the number and size of the Aβ-immunoreactive neuritic plaques were markedly reduced in the hippocampus of APP/PS1 mice after 5-month treadmill exercise (Fig. 2A). Quantitative analysis showed that plaque numbers in hippocampus of TgE mice were significantly decreased by 34.96 ± 10.77% compared with TgC mice (P < 0.05, Fig. 2B). Moreover, we also detected the Aβ burden by measuring the areas of Aβ-positive neuritic plaques in hippocampus. In TgE group, the areas of Aβ plaques were strongly reduced by 22.63 ± 9.52% in the hippocampus compared with TgC mice (P < 0.05, Fig. 2C). Next, we measured the soluble Aβ level in the hippocampus of all groups by sandwich ELISA. Our results showed the levels of Aβ1–40 and Aβ1–42 in TgE mice were reduced significantly by 28.62 ± 4.11% and 20.06 ± 8.64%, respectively, compared with TgC mice (P < 0.05, for both; Fig. 2D and E). There was no Aβ1–40 and Aβ1–42 detected in wild-type mice. These data suggest an inhibitory effect of treadmill exercise on Aβ deposition.

3.2. Regulation of APP processing in APP/PS1 mice via treadmill exercise

To verify the effects of treadmill exercise on APP processing in APP/PS1 mice, the levels of APP mRNA and APP protein in hippocampus were measured by quantitative real-time PCR and western blot, respectively. The results showed there were no significant differences in the expression levels of APP mRNA (ΔΔCt: TgE –0.0363 ± 0.5086, TgC 0.1619 ± 0.6857; 0.98-fold relative to TgC group; P > 0.05) and protein (P > 0.05; Fig. 3A and B) between control group and exercise group in APP/PS1 mice, suggesting that treadmill exercise interferes with Aβ deposition, possibly independent of APP expression.

Given the important role of APP phosphorylation at C-terminal Thr668 (p-APP668) in APP processing and neurodegeneration [48], we determined APP phosphorylation in the hippocampus tissues by western blotting using an antibody against p-APP668 (Fig. 3A). A significantly elevation of p-APP668 was detected in TgE mice (P < 0.05; Fig. 3C). In contrast, p-APP668 was significantly reduced by 32.88 ± 7.89% in TgE mice compared with TgC mice (P < 0.05; Fig. 3C). Similarly, treadmill exercise induced a marked decrease by 23.91 ± 12.33% in wild-type mice (P < 0.05; Fig. 3C). The results suggest that treadmill exercise has a modest inhibitory effect on the protein expression of p-APP668.

We then attempted to further determine whether treadmill exercise was involved in APP cleavage. The APP cleavage enzymes (including ADAM10, BACE1, and PS1) in hippocampus samples were detected by western blot analysis (Fig. 3D). Although the expression of ADAM10, a candidate of α-secretase, was markedly

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Table 1

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<thead>
<tr>
<th>Sense primer (5′-3′)</th>
<th>Antisense primer (3′-5′)</th>
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<tr>
<td>APP</td>
<td>5′-GGCTCAGAAAGGCATTGCAATC-3′</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5′-CCGGAGGAAGACCTTATGCAAC-3′</td>
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reduced by 10.32 ± 6.92% (p < 0.05) in the TgC mice compared with WT C mice, no significant difference in the expression levels of ADAM10 was observed between the exercise mice and the respective controls (p > 0.05) (Fig. 3E). Similarly, the results obtained showed no detectable changes for the BACE1, a major β-secretase, in the hippocampus between the groups (Fig. 3F). In contrast, treadmill exercise reduced the level of PS1 by 50.07 ± 5.87% in APP/PS1 mice (p < 0.05; Fig. 3G). These findings suggest that treadmill exercise influences APP processing via a mechanism beyond modulating the expression of ADAM10 and BACE1.

Next, we measured the expression levels of APP cleavage fragments that are important to explain the production of Aβ, including sAPPα, sAPPβ and CTFs, in the hippocampus simples from all the groups (Fig. 3H). Quantitative analysis revealed a slight but non-significant increase (p > 0.05; Fig. 3I) in the expression of sAPPα in TgE mice compared with TgC mice, while a significant 24.48 ± 4.37% decrease (p < 0.05; Fig. 3J) in sAPPβ was observed in the TgE mice. Western blot analysis using an anti-CTFs antibody showed a significant 36.07 ± 9.37% decrease (p < 0.05; Fig. 3K) in the production of CTFs in TgE mice relative to TgC mice.
Fig. 3. Treadmill exercise regulates amyloid precursor protein (APP) procession in the hippocampus of APP/PS1 mice. (A) Immunoblotting showing the expression levels of APP695 and phosphorylation of APP at threonine (Thr) 668 (p-APP668) in the hippocampus of all groups. GAPDH was the internal control. (B and C) Quantitative analysis of the expression levels of APP695 (B) and p-APP668 (C). No significant difference in APP expression levels was observed between TgC and TgE mice, whereas treadmill exercise significantly reduced p-APP668 protein levels in both wild-type mice and APP/PS1 mice. (D) Immunoblotting showing the expression levels of metalloproteinase domain-containing protein 10 (ADAM10), β-secretase 1 (BACE1), and presenilin 1 (PS1) in the hippocampus of all groups. GAPDH was the internal control. (E–G) Quantitative analysis of the expression levels of ADAM10 (E), BACE1 (F), and PS1 (G). Compared with the WtC mice, in TgC mice, the level of ADAM10 was markedly reduced, whereas the level of PS1 was significantly increased. Treadmill exercise resulted in a significant reduction of PS1 protein expression in APP/PS1 mice. But treadmill exercise did not affect the expression level of ADAM10 in wild-type mice or APP/PS1 mice. Similarly, there was no significant difference in the expression level of BACE1 between the groups. (H) Immunoblotting showing the expression levels of sAPPα, sAPPβ and CTFs in the hippocampus of all groups. GAPDH was the internal control. (I–K) Quantitative analysis of the expression levels of sAPPα (I), sAPPβ (J) and CTFs (K). Treadmill exercise resulted in an increased level of sAPPα in the hippocampus of APP/PS1 mice, but the difference was not statistically significant. However, treadmill exercise significantly reduced the levels of sAPPβ and CTFs. *P<0.05, as compared to relative control. *P<0.05, as compared to WtC.
Taken together, these results suggest that treadmill exercise attenuates amyloidogenic processing of APP and promotes non-amyloidogenic processing in the hippocampus of APP/PS1 transgenic mice.

3.3. Treadmill exercise reverses tau hyperphosphorylation in the hippocampus of APP/PS1 mice

Given the beneficial role of treadmill exercise in AD deposition and APP processing, we attempted to determine a possible role of treadmill training in tau pathology in APP/PS1 mice. Although NFTs was not observed in APP/PS1 transgenic mice (date not shown) consistent with previous reports [44,60], accumulating evidence has suggested hyperphosphorylated tau appears in the APP/PS1 mouse brain after the onset of Aβ deposition [19,35,44]. Accordingly, we further investigated a possible role of treadmill exercise on tau hyperphosphorylation in APP/PS1 mice. TAU hyperphosphorylation was assessed by Western blotting using antibodies against different phosphorylation sites on tau, including Thr205, Thr231, Ser396 and Ser404. Western blotting showed that phosphorylation levels of tau at all these sites were significantly increased in the hippocampus of APP/PS1 transgenic mice (Fig. 4A). In contrast, a robust decrease in tau phosphorylation was observed in the hippocampus of the TgE mice (Fig. 4A). Quantitative analysis showed that the levels of tau phosphorylation at Thr205, Thr231, Ser396 and Ser404 were decreased by 30.26±6.34% (P<0.05; Fig. 4C), 21.32±8.61% (P<0.05; Fig. 4D), 55.98±4.36% (P<0.05; Fig. 4E) and 29.18±7.82% (P<0.05; Fig. 4F), respectively, in TgE mice relative to TgC mice. Both of these reductions represented a return to wite-type levels. In addition, no significant difference in the total tau levels was revealed between the groups (P>0.05; Fig. 4B). The results suggest that treadmill exercise exerts a potential therapeutic effect on tau pathology besides amyloid pathology.

3.4. Treadmill exercise alleviates the progressions of Alzheimer-like neuropathology in APP/PS1 mice via GSK3 rather than CDK5

To determine a potential signal molecule involved in exercise-induced changes of pathology in APP/PS1 transgenic mice, we attempted to examine whether treadmill exercise plays a role in regulating GSK3 and/or CDK5, which have been previously shown to be associated closely with amyloid pathology [5,36,62] and tau pathology [37,73].

The activity of CDK5 was detected by measuring the protein levels of CDK5, phosphorylated CDK5 at Tyr15, CDK5 activators p35, and its truncated form p25 (Fig. 5A). Western blot analysis showed that the protein expression levels of CDK5 phosphorylation in the hippocampus of TgE mice were significantly increased by 204.78±19.86% (P<0.05; Fig. 5C) compared with WtE mice, whereas the total CDK5 protein levels remained unchanged between the groups (P>0.05; Fig. 5B). Consequently, TgE mice showed a remarkable increase in the ratio of p-CDK5/CDK5 by 202.58±38.58% (P<0.05; Fig. 5D). Similarly, with respect to WtE mice, a significant increase in p35 levels by 87.1±5.86% (P<0.05; Fig. 5E) was observed in TgE mice, with a concomitant increase in p25 levels by 48.73±12.28% (P<0.05; Fig. 5F). However, these changes were not reversed by treadmill exercise in APP/PS1 mice (P>0.05; Fig. 5C–F).

We also investigated whether treadmill exercise is able to modulate GSK3 activity in the hippocampus of APP/PS1 mice. Western blot analyses were employed to assess the expression levels of both GSK3α/β and phosphorylated GSK3α (ser21)/β (ser9) (Fig. 6A). While we did not observe a difference in the total GSK3α and total GSK3β levels between the groups (P>0.05; Fig. 6B and E), the phosphorylation of GSK3α at ser21 and the phosphorylation of GSK3β at ser9 were markedly decreased by 34.81±10.07% (P<0.05; Fig. 6C) and 48.73±12.28% (P<0.05; Fig. 6F), respectively, in TgE mice compared with WtE mice. Thus, the ratios of p-GSK3α/GSK3α and p-GSK3β/GSK3β were significantly reduced by 48.73±12.28% (P<0.05; Fig. 6D) and 39.61±3.67% (P<0.05; Fig. 6G), respectively, in APP/PS1 mice. As expected, a marked reversal of the decreased phosphorylation of GSK3α at ser21, as well as the phosphorylation of GSK3β at ser9, was observed in TgE mice (P<0.05, for both; Fig. 6C and F). Consequently, treadmill exercise increased the ratios of p-GSK3α/GSK3α and p-GSK3β/GSK3β by 23.42±8.33% (P<0.05; Fig. 6D) and 23.42±8.33% (P<0.05; Fig. 6G), respectively, in APP/PS1 transgenic mice. Similarly, the ratios of p-GSK3α/GSK3α and p-GSK3β/GSK3β were markedly increased in WtE mice compared with WtC mice (P<0.05, for both; Fig. 6D and G).

Taken together, these results suggest that treadmill exercise inactivates both GSK3α and GSK3β in the hippocampus of APP/PS1 transgenic mice, while treadmill exercise has no effect on CDK5 activity. Therefore, it seems that treadmill exercise prevents the progressions of AD-like neuropathology in the hippocampus of APP/PS1 mice not via CDK5 but via GSK3.

4. Discussion

It is well established that physical exercise has benefits for overall brain health, brain plasticity and cognitive function [16]. Consistent with other researches in animal models of AD [1,7,34,41,42,49,80], we have reported previously that treadmill exercise attenuated behavioral deficits in APP/PS1 transgenic mice paralleled by LTP enhancement. Thus, treadmill exercise may be the same benefits as voluntary exercise for improving cognitive impairment in a mouse model of AD. In the present study, we focused on the exercise-induced changes of AD-like neuropathology in the APP/PS1 mouse models of AD. Our findings indicate that 5-month treadmill exercise inhibits accumulation of Aβ deposits and tau hyperphosphorylation in APP/PS1 double-transgenic mice. Importantly, we provided evidence that treadmill exercise may mediate APP processing in favor of reduction of Aβ deposits. Finally, we also demonstrate that the potential signal molecule underlying the protective effects of treadmill exercise is likely GSK3 rather than CDK5.

The aggregated Aβ peptides are believed to play a central role in AD pathology [4,13,27,83]. The deposition of Aβ and plaque load plays an important role in the loss of cognitive function in AD. Consistent with previous studies [8,39,63,78], our results showed a large increase in Aβ production and deposition in the hippocampus of APP/PS1 transgenic mice, whereas we demonstrate that treadmill exercise significantly reduces both Aβ deposition (as assessed by immunohistochemistry) and the levels of soluble Aβ1–40 and Aβ1–42 (as assessed by ELISA). Several recent studies have also demonstrated reduced brain Aβ1–40 and Aβ1–42 levels following exercise [1,42], and further suggested that long-term exercise was enough to reduce Aβ plaque deposition [1]. Other findings are in contrast to these studies on the effects of exercise. Exercise did not influence Aβ pathology nor benefit cognitive performance [66,87]. And even, the study by Richter et al. suggested that wheel-running may be another form of stereotypic behaviour [66]. Recently, the 3×Tg mouse model for AD, which displays both Aβ and tau accumulation, was used to study pathological changes in AD. Though long-term wheel running was shown to have the neuroprotection in 3×Tg-AD mice, the traditional markers of AD neuropathology were not altered [26,56]. Nevertheless, wheel running lasted for 11 months increased neurogenesis at 20 months of age in 3×Tg-AD mice [56], while 6 months of wheel running reduced oxidative stress and improved synaptic function in the 7-month-old 3×Tg-AD mice [26]. Mouse strain differences may play
protein Phosphorylation levels produce months plaque cortex amyloidogenic that lasted exercise of tau effects the cruc... Several p-tau lines of evidence indicate that through the non-amyloidogenic α-secretase pathway, APP protein is cleaved to produce the sAPPα fragment [20], which is beneficial for neuronal survival [58, 82], whereas through the amyloidogenic β-secretase pathway, APP protein is cleaved to form neurotoxic Aβ, which is involved in AD pathogenesis [10, 31, 74]. The inhibitory effect of treadmill exercise on the accumulation of Aβ deposits in APP/PS1 transgenic mice is likely attributable to its modulation of APP processing, because the production of APP-CTFs, the direct precursor of Aβ [21], was decreased by treadmill exercise. In addition, a previous study has shown that long-term voluntary exercise can decrease Aβ load in the TgCRND8 transgenic model of AD accompanied by no significant decrease in the levels of total APP, CTFs, or α-, β-, and γ-secretase activity, while short-term voluntary exercise decreased both the α-CTFs and β-CTFs of APP paralleled by the unchanged levels of total APP and secretase activity [1]. The study demonstrated it is possible that multiple pathways may be activated to regulate APP processing either directly or indirectly. They proposed that increase in neuronal activity as a possible mechanism for the exercise-mediated regulation of APP processing. However, another study has suggested that treadmill...
exercise-mediated neuroprotection likely involve the prevention of Aβ42 expression by up-regulation of ADAM17 mRNA and down-regulation of BACE1 mRNA in ageing rats [91].

APP processing can be modulated by different mechanisms, including but not limited to an altered APP expression as well as expression/activity of secretase involved in APP processing. Here we detected the expression but not activity of secretase. In the current study, we did not observe a significant difference in the expression of APP, ADAM10, or BACE1 between the TgC mice and TgE mice. However, a reduction in the levels of CTFs as well as sAPPβ was observed in exercising APP/PS1 mice. This also was paralleled by a slight but non-significant decrease in sAPPα. Accordingly, we propose that treadmill exercise may modulate APP processing through the changes of α-secretase and β-secretase activity. A previous study showed that Thr668 phosphorylation facilitates BACE10 and increases Aβ generation, suggesting that Thr668 phosphorylation of APP is a molecular mechanism regulating APP cleavage by BACE1 [48]. When considered together, our findings that treadmill exercise reversed the elevation of APP phosphorylation at Thr668 in APP/PS1 mice suggest that exercise-induced inhibition of APP phosphorylation at Thr668 decreases Aβ generation through the repression of BACE1 activity. Furthermore, the competition between α-secretase and β-secretase may account, at least in part, for the increase of sAPPα. In addition, we firstly found treadmill exercise decreased the expression of PS1 in the hippocampus of APP/PS1 mice, which is integral component of the γ-secretase directly responsible for the activity of γ-secretase [75]. Taken together, our results indicate that treadmill exercise may prevent the amyloidogenic pathway and modulate the processing of APP to the non-amyloidogenic pathway. However, additional studies are required to address fully the potential mechanism that exercise can regulate the APP processing.

NFT, consist of intracellular hyperphosphorylated tau, is one of the major pathological hallmarks of AD. Early researches showed that the accumulation of NFTs is associated with the development of the clinical symptoms in AD [29,86]. As the critical roles of hyperactivated tau in the formation of NFTs, highly phosphorylated tau has been regarded as the critical mechanism in the progression of AD. Hyperphosphorylated tau leads to memory deficits and loss of functional synapse in a transgenic mouse model [72]. There is growing evidence that the augmentation of the amount of phosphorylated tau at disease-relevant sites is triggered by Aβ
The hyperphosphorylation of tau would increase its tendency to aggregate and further promote the formation of paired helical filaments (PHFs) which would evolve into NFTs [30]. It is also reported that Aβ can be internalised or generated intracellularly, providing the opportunity for Aβ to directly or indirectly facilitate NFT formation [45]. It is thus likely that the prevention of memory deficits by treadmill exercise in APP/PS1 mice results partly from the reduction in hippocampal Tau pathology. Previously studies have shown that tau phosphorylation is significantly increased in APP/PS1 transgenic mice [19,35,44]. Consistently, we found that the phosphorylation levels of tau at the Thr205, Thr231, Ser396 and at the Ser40 residues were significantly increased in hippocampus of APP/PS1 mice. However, we observed that the levels of tau phosphorylation at all the detected sites were markedly reduced after 5-month treadmill exercise. In line with our data, previous reports have shown that exercise (wheel running or treadmill running) decreases tau hyperphosphorylation levels in both models of tau and amyloid pathologies, resulting in the promoting the memory impairments [7,49,79]. These results, together with the results of the present study, suggest that the suppression of tau
pathology is likely an important mechanism of the preventive effect of exercise on cognition impairment in AD animal models.

Currently, a growing subject of interest involves the effect of tau pathology on Aβ production and amyloid plaque formation in AD. It has been shown that tau pathology can in turn affect the formation of Aβ and subsequently, amyloid plaque formation [8,59]. In line with these studies, it is further reported that the inhibition of tau phosphorylation by blocking tau kinases prevents Aβ-induced cell death and that neurons cultured from tau-depleted mice are resistant to Aβ toxicity [38,43,52,65,67]. Tau kinases may play roles in the pathological accumulation of Aβ by phosphorylating APP and activating γ-secretase [53]. Our above-mentioned results have shown that treadmill exercise inhibits Aβ generation and deposition with the reduction of phosphorylated APP and PS1 expression in APP/PS1 mice. Based on our present studies, it is reasonable to speculate that the prevention of tau pathology by exercise contributes to the impacts of exercise on Aβ.

Furthermore, we attempt to elucidate a potential signaling pathway underlying the exercise-induced reduction in AD-like neuropathology in APP/PS1 mice. Although the mechanistic link between the two pathological hallmarks of AD remains elusive, GSK3 and CDK5, main kinases in AD, have been shown to be associated closely with both amyloid pathology [5,36,62] and tau pathology [37,73]. The role of the kinases in APP phosphorylation may be a key aspect of altered APP processing and the pathological accumulation of Aβ [18,59]. Consistent with a previous research [19], hyperactivity of GSK3β/β and CDK5 was observed in APP/PS1 transgenic mice. Therefore, we could hypothesize that 5-month treadmill exercise prevents AD-like pathological progression by modulating the expression or activity of GSK3 and/or CDK5. In agreement with this hypothesis, some groups have reported that exercise changes the activity of various kinases in the hippocampus [12,14,49]. The activation of GSK3α/β through their autophosphorylation has been implicated in AD pathogenesis [33]. Increased GSK3α activity is mainly associated with the processing of APP and generation of Aβ [62], whereas activation of GSK 3β is predominantly involved in tau phosphorylation and the formation of NFTs [55,64,69]. In contrast, despite the role of GSK3 in modulation of APP processing is still a matter of investigation [22,40]; reduction of GSK3α/β activity was reported to reduce Aβ levels in APP mice [62,76]. In addition, activation of PI3K/AKT signaling pathway represses GSK3α/β activity by phosphorylating GSK3α/β and consequently reduces Aβ and tau hyperphosphorylation [33]. Recently, it was demonstrated that exercise inhibited GSK3α/β activity in Sprague–Dawley rats, as well as in different AD transgenic models [14,49,79]. Similarly, in the current studies, treadmill exercise increased phosphorylation levels of GSK3α/β in the hippocampus of wild-type mice and APP/PS1 mice, indicating that inhibition of GSK3α/β activity might contribute to a reduction of Aβ and tau phosphorylation. On the other hand, CDK5 is also believed to be a key kinase responsible for APP phosphorylation [36,50,84], and tau phosphorylation [3,54]. Interestingly, induction of p25, its activator protein, has been shown to induce production and accumulation of Aβ in vivo [17], probably by transcriptional regulation of BACE1 expression [85]. However, we did not observe the inhibiting of CDK activity and the decreased expression of p25 following exercise training, which is paralleled by stable BACE1 expression. As discussed above, the activity of GSK3α/β, rather than CDK5, was inhibited by treadmill exercise, thereby resulting in the reduction of AD-like neuropathology.

5. Conclusion

In conclusion, our findings demonstrate that the beneficial effect of 5-month treadmill exercise on cognitive improvement in APP/PS1 transgenic mice is likely attributable to the combined effects of decreased levels of toxic Aβ peptides and tau hyper-phosphorylation. The present study also provides evidence that the downregulation of expression in APP phosphorylation and PS1 are partly responsible for a reduction in Aβ levels and Aβ burden induced by treadmill exercise in APP/PS1 mice, indicating that treadmill exercise can regulate the processing of APP. In addition, our findings demonstrate that treadmill exercise attenuates AD-like neuropathology in AD transgenic mice via a GSK3α/β dependent pathway. Because GSK3 is one of the key signaling mediators of the P3K signaling pathway [24] and Wnt signaling pathway [88], further studies will be required to investigate which signaling pathway is involved in exercise-mediated AD neuropathology.

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