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Original Research

Moderate-Intensity Treadmill Exercise Regulates GSK3α/β Activity in the Cortex and Hippocampus of APP/PS1 Transgenic Mice

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Abstract

Background: Physical exercise has been shown to be beneficial for individuals with Alzheimer's disease (AD), although the underlying mechanisms are not fully understood. **Methods**: Six-month-old Amyloid precursor protein/Presenilin 1 (APP/PS1) transgenic (Tg) mice and wild-type (Wt) mice were randomly assigned to either a sedentary group (Tg-Sed, Wt-Sed) or an exercise group (Tg-Ex, Wt-Ex) undertaking a 12-week, moderate-intensity treadmill running program. Consequently, all mice were tested for memory function and amyloid β (Aβ) levels and phosphorylation of tau and protein kinase B (Akt)/glycogen synthase kinase-3 (GSK3) were examined in tissues of both the cortex and hippocampus. **Results**: Tg-Sed mice had severely impaired memory, higher levels of Aβ, and increased phosphorylation of tau, GSK3α tyrosine279, and GSK3β tyrosine216, but less phosphorylation of GSK3α serine21, GSK3β serine9, and Akt serine473 in both tissues than Wt-Sed mice in respective tissues. Tg-Ex mice showed significant improvement in memory function along with lower levels of Aβ and less phosphorylation of tau (both tissues), GSK3α tyrosine279 (both tissues), and GSK3β tyrosine216 (hippocampus only), but increased phosphorylation of GSK3α serine21 (both tissues), GSK3β serine9 (hippocampus only), and Akt serine473 (both tissues) compared with Tg-Sed mice in respective tissues. **Conclusions**: Moderate-intensity aerobic exercise is highly effective in improving memory function in 9-month-old APP/PS1 mice, most likely through differential modulation of GSK3α/β phosphorylation in the cortex and hippocampus.

Keywords: APP/PS1 mouse; aerobic exercise; memory function; amyloid-β; Akt/GSK3 pathways

1. Introduction

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disease. Extensive distribution of β-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs) in the brain are the major histopathological hallmarks of AD [1,2]. One of the hypotheses of AD pathogenesis suggests that amyloid plaques or Aβ-peptides act as the direct cause and initiate cascade effects, leading to a series of reactions such as tau pathology, inflammation, synaptic dysfunction, neuronal loss, and ultimately dementia [2].

AD neuropathology initiated by Aβ deposition is believed to begin decades prior to the onset of clinic symptoms, and Aβ deposition has been observed in the cortex at 6 weeks of age and in the hippocampus at 3–4 months of age in Amyloid precursor protein/Presenilin 1 (APP/PS1) AD mouse models [2,3]. Hyperactivity of glycogen synthase kinase-3 (GSK3) is believed to be critically involved in the enhancement of Aβ from β-amyloid precursor protein (APP) and in tau hyperphosphorylation [4,5]. GSK3 is a highly conserved protein kinase for glycogen synthase and is encoded by two highly related genes: GSK3α (51 kDa) and GSK3β (47 kDa). Under physiological conditions, GSK3 is activated via phosphorylation at tyrosine residue 279 of GSK3α (p-GSK3α tyr279) and tyrosine residue 216 of GSK3β (p-GSK3β tyr216) and inhibited via phosphorylation at serine residue 21 of GSK3α (p-GSK3α ser21) and serine residue 9 of GSK3β (p-GSK3β ser9) [6]. The neuronal reaction to Aβ and tau proteopathic stress is complex, and whether the GSK3 biochemical response is similar in the cortex and hippocampus is largely unknown.

Given that GSK3 is relevant to the pathophysiological processes of AD, inhibition of GSK3 activity has been deemed to be an optional strategy in AD prevention and treatment. Physical inactivity is generally believed to be one of the risk factors associated with AD [7,8], whereas physical exercise has been proven to reduce both the progression of AD-like neuropathology in the TgCRND8 mouse model [9,10] and the risk of AD [11], and improve cognitive function in AD patients [12]. Many studies have been performed in both AD animal models and AD patients to explore the molecular mechanisms underlying exercise-induced improvement in memory or cognition. In several studies on AD animal models, exercise induced significant reductions in Aβ levels and tau phosphorylation at multiple sites in both the cortex and hippocampus [13–17], which was believed to be associated

with inhibition of GSK3 activity via enhancement in levels of p-GSK3α/β ser21/9 [17–19]. However, in a study on 6-month-old APP/PS1 transgenic (Tg) mice, housing mice in an enriched environment for 4 weeks was unable to modulate GSK3β (ser9) activity in either the hippocampus or the cortex [20]. The discrepancies in these results might be due to variations in the forms of stimulation/exercise, intervention times in the AD animals/patients (AD stage), and duration of the interventions. In a previous study, 3-week voluntary wheel running in 15–19-month-old mice did not significantly effect Aβ levels [21]. However, in another study, 5-month treadmill exercise in 3- month and 12-month-old mice [15] significantly reduced soluble Aβ40/42 in mice of both ages and significantly reduced Aβ plaque burden in the 3-month-old mice but not the 12-month-old mice. Therefore, intervention type and duration as well as AD stage seem to play important roles in the outcomes.

To our knowledge, only a few studies have investigated the underlying molecular mechanisms of memory function improvement in AD mouse models induced by aerobic exercise with respect to regulation in protein kinase B (Akt)/GSK3 activity in both the cortex and hippocampus. More importantly, no study has yet investigated the mechanisms in term of GSK3α/β activity in both the cortex and hippocampus of moderate AD stage APP/PS1 mice. To fill these knowledge gaps, 6-month-old APPSwe/PS1M146V double Tg AD mice and wild-type (Wt) mice were subjected to 12-week, moderate-intensity treadmill running. After the training, spatial learning and memory were tested, and levels of Aβ, phosphorylation of tau, phosphorylation of Akt at ser473 (a major upstream modulator of GSK3), and phosphorylation of GSK3α and GSK3β at tyr279/216 and ser21/9 were examined in tissues of both the cortex and hippocampus from both Tg mice and Wt mice. By examining the effects of aerobic exercise on memory function in Tg mice, and levels of Aβ, phosphorylation of tau, phosphorylation of Akt at ser473, and phosphorylation of GSK3α and GSK3β at tyr279/216 and ser21/9 in both the cortex and hippocampus, we aimed to explore the molecular mechanisms underlying the beneficial effects of physical exercise on AD development.

2. Materials and Methods

2.1 Experimental Design

Male APPSwe/PS1M146V double-Tg mice and Wt mice were randomly assigned to a sedentary group (Tg-Sed, Wt-Sed) or an aerobic exercise group (Tg-Ex, Wt-Ex). After the physical training, all mice were subjected to an eight-arm radial maze test for spatial learning and memory evaluation, then the mice were sacrificed and brain tissues of the cortex and hippocampus were extracted for further analysis.

2.2 Animals and Ethic Approval

Six-month-old male Tg mice (n = 20) and Wt male littermates (n = 20) were purchased from Beijing HFK Bioscience Co., Ltd (Beijing, China). The mice were kept under standard laboratory conditions (12:12 h light-dark cycle, at 22 $\pm$ 2 °C and 45%–55% relative humidity) and provided with food and water *ad libitum*. Both Tg mice and Wt mice were randomly and evenly assigned to an exercise group (Tg-Ex, n = 10; Wt-Ex, n = 10) or a sedentary group (Tg-Sed, n = 10; Wt-Sed, n = 10). Evidence has shown variations in β-amyloid pathological phenotype [22]; thus, to avoid sex-induced variations in results, the study used only male APPSwe/PS1M146V double-Tg mice.

Ethics approval was obtained from the Ethics Committee of the Beijing Sport University (2015015) and the Guiding Principles for Care and Use of Animals was followed.

2.3 Aerobic Exercise Training

Before the 12-week exercise training program, both Wt-Ex and Tg-Ex mice were familiarized with the treadmill training by running on a treadmill for 3 consecutive days at a speed of 10 m/min with a 0° inclination. After the familiarization, each training session started with a standard warm-up by running on the treadmill for 10 min at a speed of 12 m/min, followed by running for 50 min at a speed of 15 m/min, which was defined here as moderate intensity on the basis of our previous studies where mice running at a speed of 12–15 meters/min corresponded to an oxygen consumption of around 65–75% of maximal oxygen consumption [23–25]. The training was carried out once per day, 5 days per week for 12 consecutive weeks. Wt-Sed and Tg-Sed mice were placed on the treadmill for 10 min with the same time schedule as Wt-Ex and Tg-Ex mice but undertook no exercise training.

2.4 Eight-Arm Radial Maze Test

On the next day following the last training session, all mice took an eight-arm radial maze test in a quiet environment with weak light to facilitate the assessment of spatial memory. The eight-arm radial maze apparatus (JLBehv-8ARMM, Shanghai Jiliang, Shanghai, China) is comprised of eight arms, spaced equidistantly, and visual reference cues were hung 1 m above the maze apparatus. The test process has been previously described in detail [26]. Briefly, the test includes 3 days of adaption and thereafter 10 consecutive days of testing. To motivate the mice to seek the chocolate crumbs in the maze arms, the food supply to the mice was reduced 3 days before the adaption so that the body weights of the mice were reduced to 80–85% of their initial weights. Thereafter, the food supply was maintained at such a level as to keep the body weights relatively stable throughout the experiment [27,28]. Body weights of the mice were measured every other day to avoid >20% reduction. During the adaptation, the mice had 3 days to familiarize themselves with the test set by freely exploring the maze for 10 min with all eight of the arms baited with chocolate crumbs (0.08 g).

Before the test, chocolate crumbs were set in four randomly selected arms, and the test began with putting the mice in the center of the platform heading towards arm number one and ended when the mice had visited all the four baited arms within a maximum time of 10 min. If the mice failed to visit all the baited arms within 10 min, the mice would be excluded from group value calculations. The test was conducted on each individual mouse for 10 consecutive days. Working memory errors, reference memory errors, and time needed to complete each trial were recorded. Evaluation of working memory error was performed by counting the number of re-entries into a baited or non-baited arm, and similarly, evaluation of reference memory error was performed by counting the number of entries into a non-baited arm. For a specific mouse, the chocolate crumbs were placed in the same four arms during the 10 trials. By the end of the tests, two mice in each group failed to finish the test within the time limit, resulting in only eight mice left in each group for statistical analysis.

2.5 Brain Tissue Processing and Immunostaining

The mice were anesthetized with isoflurane, then perfused with 4% paraformaldehyde (P1110, Solarbio, Beijing, China) in 0.1M sodium phosphate buffer (PBS) pH7.4 through the left ventricle of the heart. The brains were removed from the skulls and postfixed by immersion in the same fixative for 48 h at 4 °C. The cortical and hippocampal tissues were dissected and dehydrated with 20% and 30% sucrose in 0.1 M PBS at 4 °C. Subsequently, both tissues were frozen rapidly in liquid nitrogen and stored at –80 °C until use. For immunostaining, cross sections (35 µm) were cut using a cryostat (CM1850, Leica, Wetzlar, Hessian, Germany) and the sections were collected on glass slides. The sections from the left hemisphere were used for immunohistochemistry, and the right hemisphere for immunofluorescence. The immunofluorescence and immunohistochemistry staining procedures have been previously described in detail [23,29,30]. The primary and secondary staining antibodies are listed in Table 1.

The staining was examined under a microscope (IX71-F22PH, OLYMPUS, Tokyo, Japan) connected to a computer equipped with a digital camera (modelDP71, OLYMPUS, Tokyo, Japan). Image J software (National Institutes of Health, Bethesda, MD, USA) was used for image analysis. Aβ plaque levels were quantified on six randomly selected areas of each section/mouse stained with immunofluorescence by calculating the mean fluorescence intensity, which is the ratio of integrated density to the area, using the same position on the section as a reference.

2.6 Western Blotting

The mice were anesthetized with isoflurane (26675-46-7, Sigma-Aldrich, Saint Louis, MO, USA) then decapitated. After dissection of the cortex and hippocampus on ice, the tissues were homogenized with phosphatase inhibitors and radio immunoprecipitation assay lysis buffer (R0278, Sigma-Aldrich, St. Louis, MO, USA). The protein extraction, protein content determination, and electrophoresis procedures were the same as those described in our previous studies [23,29,30]. The primary and secondary antibodies are listed in Table 1.

Protein quantification was performed using Bio-Rad, ChemiDoc XRS+ System Image Lab software (version 6.0.1, Bio-Rad, Hercules, CA, USA). The protein amounts were expressed as semiquantitative ratios between the value of a specific protein and the value of the reference protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Levels of microtubule-associated protein tau (p-tau) ser262 and p-tau ser396 were expressed as relative values of total tau, levels of p-GSK3α ser21 and p-GSK3α tyr279 were expressed as relative values of total GSK3α, and levels of p-GSK3β ser9 and p-GSK3β tyr216 were expressed as relative values of total GSK3β.

2.7 Statistical Analysis

Statistical analysis was performed using SPSS software (version 22.0, SPSS Inc., Chicago, IL, USA). Group values of a specific variable were calculated based on measurements of 10 consecutive days for all mice in the group. Repeated measurement analysis of variance (ANOVA) was performed to analyze over-time changes in the eight-arm radial maze test performance among groups (i.e., Wt-Sed, Wt-Ex, Tg-Sed, and Tg-Ex), followed by Bonferroni post-hoc analysis when group, time, and group-time interaction were significant. Two-way ANOVA (with genotype [Wt or Tg] and exercise [Sed or Ex] as two factors) was used to analyze the results of western blotting, and a simple main effect (multiple comparison adjusted by the Bonferroni approach) was reported when genotype-exercise interaction was significant. The student's *t*-test was applied for the analysis of immunofluorescence staining between Tg-Sed and Tg-Ex mice. A probability of less than 0.05 was used to reject the null hypothesis and deemed to indicate a statistically significant difference. All data are expressed as mean (standard deviation) (mean (SD)), except for the Aβ plaque level data, which was expressed as mean $\pm$ standard error of the mean (mean $\pm$ SEM).

3. Results

3.1 Eight-Arm Radial Maze Test

Repeated-measures ANOVA revealed that test days and groups had significant effects on the working memory errors (Fig. 1A) and reference memory errors (Fig. 1B). Both working memory errors (test days: $F = 6.317$, $p < 0.001$) and reference memory errors (test days: $F = 2.144$, $p < 0.001$) were significantly decreased following the progress of the test in all the four groups, and significantly different between groups (groups: working memory errors, $F = 8.783$, $p < 0.001$; reference memory errors, $F = 13.543$, $p < 0.001$). Interaction between groups and test

Table 1. Antibodies used in Western Blotting, Immunohistochemistry and Immunofluorescence staining.

Antibody	Primary antibody				Secondary antibody	Dilution	Application
	Antigen	Dilution	Host	Isotype			
ab131354[a]	Tau phospho S262	1:5000	Rabbit	IgG	Goat Anti- Rabbit IgG (H+L)	1:20,000	Western Blot
ab109390[a]	Tau phospho S396	1:5000	Rabbit	IgG	Goat Anti- Rabbit IgG (H+L)	1:20,000	Western Blot
ab32057[a]	Tau	1:5000	Rabbit	IgG	Goat Anti- Rabbit IgG (H+L)	1:20,000	Western Blot
ab28808[a]	GSK3α phospho S21	1:5000	Rabbit	IgG	Goat Anti- Rabbit IgG (H+L)	1:20,000	Western Blot
ab131097[a]	GSK3β phospho S9	1:10,000	Rabbit	IgG	Goat Anti- Rabbit IgG (H+L)	1:20,000	Western Blot
ab68476[a]	GSK3(α+β) phospho (Y279+Y216)	1:3000	Rabbit	IgG	Goat Anti- Rabbit IgG (H+L)	1:20,000	Western Blot
ab131344[a]	GSK3α	1:5000	Rabbit	IgG	Goat Anti- Rabbit IgG (H+L)	1:20,000	Western Blot
ab93926[a]	GSK3β	1:5000	Mouse	IgG2a	Goat Anti- Mouse IgG (H+L)	1:20,000	Western Blot
ab81283[a]	Akt phospho S473	1:10,000	Rabbit	IgG	Goat Anti- Rabbit IgG (H+L)	1:20,000	Western Blot
ab8805[a]	Akt	1:5000	Rabbit	IgG	Goat Anti- Rabbit IgG (H+L)	1:20,000	Western Blot
60004 -1-Ig[b]	GAPDH	1:5000	Mouse	IgG2b	Goat Anti- Mouse IgG (H+L)	1:20,000	Western Blot
SIG-39320	Aβ	1:500	Mouse	IgG	Goat anti-rabbit IgG-horseradish peroxidase	1:500	Immunofluorescence
SIG-39320	Aβ	1:500	Mouse	IgG	Biotinylated goat anti-mouse IgG	1:500	Immunohistochemistry

[a]Purchased from Abcam, Cambridge, UK; [b]purchased from Proteintech Group, Chicago, IL, USA; All the secondary antibodies were purchased from Jackson Immuno Research Laboratories, West Grove, PA, USA. The primary and secondary antibodies for western blotting were diluted in 5% bovine serum albumin in Tris-buffered saline. GSK3, glycogen synthase kinase-3; Aβ, β-amyloid; Akt, protein kinase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IgG, immunoglobulin G.

days had no significant effect on either working memory errors or reference memory errors (groups $\times$ test days: working memory errors, $F = 1.095$, $p = 0.361$; reference memory errors, $F = 0.489$, $p = 0.957$). The main effect of test days and groups revealed that Tg-Sed mice showed significantly higher working memory errors ($p < 0.001$) and reference memory errors ($p = 0.003$) than Wt-Sed mice, and Tg-Ex mice showed significantly lower working memory errors ($p = 0.018$) and reference memory errors ($p = 0.042$) than Tg-Sed mice (Fig. 1A,B).

The time needed to complete each repetition of the test is shown in Fig. 1C. The time decreased significantly following the progress of the test for all four groups (test days: $F = 64.724$, $p < 0.001$), and was significantly different between groups (groups: $F = 103.1$, $p < 0.001$). Significant interaction between groups and test days was also found (groups $\times$ test days: $F = 14.746$, $p < 0.001$). The Bonferroni post-hoc analysis revealed that Tg-Sed mice needed significantly longer time to complete the test on day 1 ($p < 0.01$), day 2 ($p < 0.01$), day 3 ($p < 0.01$), day 4 ($p < 0.01$), day 6 ($p < 0.01$), day 7 ($p < 0.01$), and day 9 ($p < 0.01$) than Wt-Sed mice on the corresponding days. In contrast, Tg-Ex mice needed significantly shorter time to complete the test on day 2 ($p < 0.05$), day 3 ($p < 0.01$), day 4 ($p < 0.05$), day 6 ($p < 0.01$), and day 7 ($p < 0.05$) than Tg-Sed mice on the corresponding days.

3.2 Aβ Plaques

Aβ plaques were not observed in either the cortex or hippocampus of Wt-Sed or Wt-Ex mice, but were in both tissues of both Tg-Sed and Tg-Ex mice (Fig. 2A,B). Statistical analysis revealed significantly lower levels of Aβ plaques in both tissues of Tg-Ex mice than in respective tissues of Tg-Sed mice (Fig. 2C).

3.3 Tau Phosphorylation

Western blot analysis revealed four different protein bands with different molecular weights, representing phosphorylation of total tau, p-tau ser262, p-tau ser396, and reference protein GAPDH, respectively, in the cortex (Fig. 3

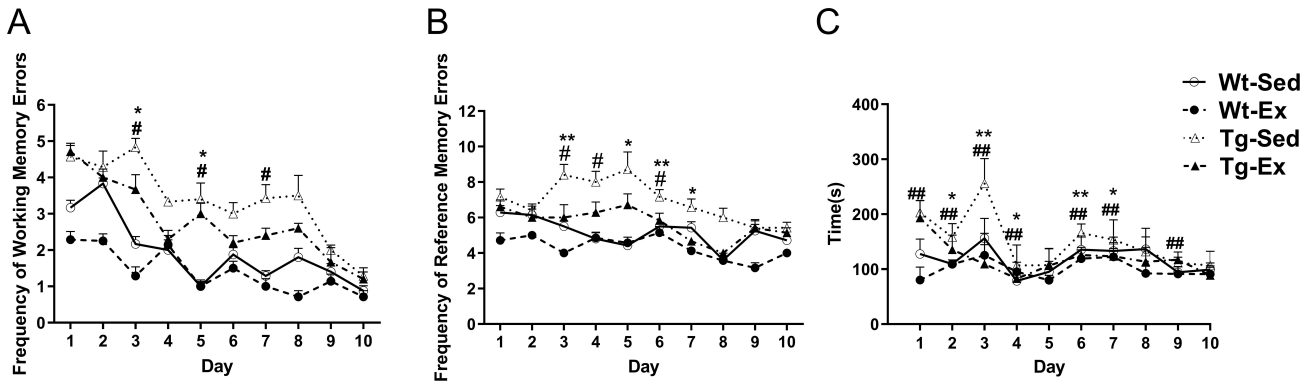

Fig. 1. Results of the eight-arm radial maze test. Both working memory errors (A) and reference memory errors (B) were significantly decreased following the progress of the test in all four groups (n = 10/group; test days: working memory errors, $F = 6.317$, $p < 0.001$; reference memory errors, $F = 2.144$, $p < 0.001$) and were significantly different between groups (groups: working memory errors, $F = 8.783$, $p < 0.001$; reference memory errors, $F = 13.543$, $p < 0.001$). Tg-Sed mice (n = 8) showed significantly higher working memory errors and reference memory errors than Wt-Sed mice (n = 8) on three test occasions. Tg-Ex mice (n = 8) had significantly lower working memory errors on two test occasions, and reference memory errors than Tg-Sed mice (n = 8) on four test occasions. Time needed to complete the test was significantly decreased following the progress of the test for all four groups (n = 10/group; test days: $F = 64.724$, $p < 0.001$) and significantly different between groups (C; groups: $F = 103.1$, $p < 0.001$). Tg-Sed mice needed significantly longer time to complete the test than Wt-Sed mice on seven test occasions, whereas Tg-Ex mice needed significantly shorter time to complete the test than Tg-Sed mice on five test occasions (C). Data are expressed as mean (SD). Note: Wt, wild-type; Tg, transgenic; Sed, sedentary; Ex, exercise; SD, standard deviation. $^{*}p < 0.05$, Tg-Ex vs Tg-Sed; $^{**}p < 0.01$, Tg-Ex vs Tg-Sed; $^{\#}p < 0.05$, Tg-Sed vs Wt-Sed; $^{\#\#}p < 0.01$, Tg-Sed vs Wt-Sed.

upper panel, left) and hippocampus (Fig. 3 upper panel, right; all original figures of Western Blot can be found in the **Supplementary Material**) of Wt-Sed, Wt-Ex, Tg-Sed, and Tg-Ex mice.

Tau phosphorylation levels and their comparisons between different groups are shown in Fig. 3A–F.

Two-way ANOVA revealed that genotype had significant effects on total tau in both the cortex (genotype: $F = 72.203$, $p < 0.001$) and hippocampus (genotype: $F = 98.128$, $p < 0.001$), but there were no significant exercise effects or interaction between genotype and exercise on total tau in both the cortex (exercise: $F = 5.012$, $p = 0.061$; genotype × exercise: $F = 0.007$, $p = 0.935$) and hippocampus (exercise: $F = 0.885$, $p = 0.361$; genotype × exercise: $F = 0.572$, $p = 0.460$). The main effect of genotype revealed that Tg groups had significantly higher levels of total tau in both the cortex and hippocampus than in respective tissues of Wt groups (Fig. 3A,D).

Two-way ANOVA revealed that genotype and exercise had significant effects on the levels of p-tau ser262 in both the cortex (genotype: $F = 8.348$, $p = 0.012$; exercise: $F = 8.820$, $p = 0.009$) and hippocampus (genotype: $F = 6.981$, $p = 0.021$; exercise: $F = 7.098$, $p = 0.017$), but there was no significant interaction between genotype and exercise on the levels of p-tau ser262 in both the cortex (genotype × exercise: $F = 0.028$, $p = 0.870$) and hippocampus (genotype × exercise: $F = 3.967$, $p = 0.070$). The main effect of genotype and exercise revealed that Tg groups had significantly higher levels of p-tau ser262 in both the cortex and hippocampus than in respective tissues of Wt groups. Exercise groups had significantly lower levels of p-tau ser262 in both the cortex and hippocampus than in respective tissues of sedentary groups (Fig. 3B,E).

Two-way ANOVA revealed that genotype and exercise had significant effects on the levels of p-tau ser396 in both the cortex (genotype: $F = 5.821$, $p = 0.031$; exercise: $F = 19.320$, $p = 0.001$) and hippocampus (genotype: $F = 46.984$, $p = 0.001$; exercise: $F = 15.278$, $p = 0.002$), but there was no significant interaction between genotype and exercise on the levels of p-tau ser396 in both the cortex (genotype × exercise: $F = 0.981$, $p = 0.335$) and hippocampus (genotype × exercise: $F = 3.078$, $p = 0.103$). The main effect of genotype and exercise revealed that Tg groups had significantly higher levels of p-tau ser396 in both the cortex and hippocampus than in respective tissues of Wt groups. Exercise groups had significantly lower levels of p-tau ser396 in both the cortex and hippocampus than in respective tissues of sedentary groups (Fig. 3C,F).

3.4 GSK3 Kinase Activity in the Cortex and Hippocampus

Western blotting revealed three different protein bands with different molecular weights, representing total GSK3α, p-GSK3α ser21/p-GSK3α tyr279, and reference protein GAPDH, respectively, in both tissues of each group (Fig. 4 upper panel). Quantification of the bands and comparisons of both p-GSK3α ser21 and p-GSK3α tyr279 levels in both tissues between different groups are shown in the histogram (Fig. 4A–D).

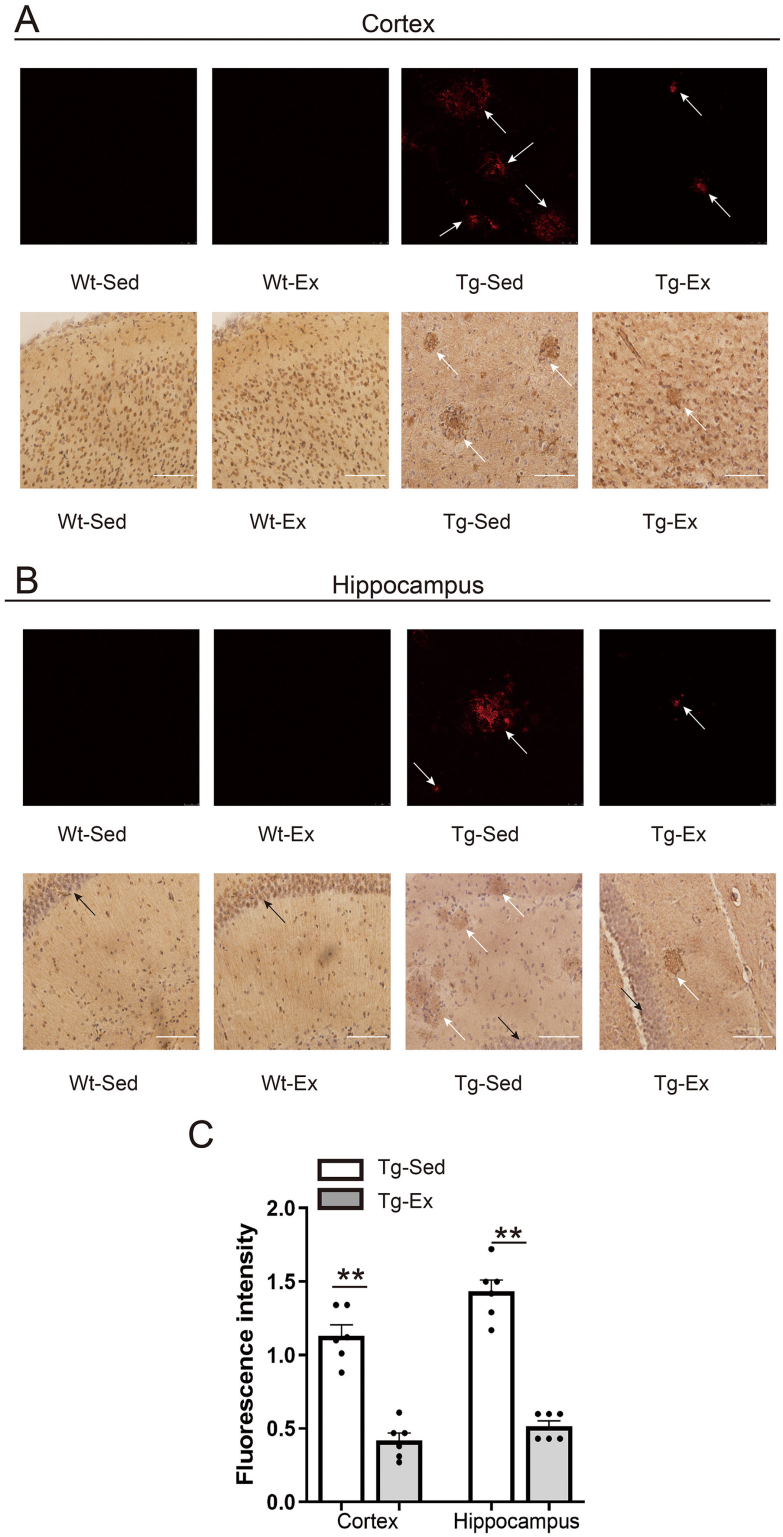

Fig. 2. Aβ plaque levels in the cortex and hippocampus. Representative images of immunofluorescence staining (upper panel) and immunohistochemical staining (lower panel) in the cortex (A) and hippocampus (B), revealing Aβ plaques (white arrows) in the cortex and hippocampus, and pyramidal layer in the hippocampus (black arrows). Quantification of the fluorescence staining for Aβ plaques and comparisons of the results between Tg-Sed and Tg-Ex mice in respective tissues of the cortex and hippocampus are shown in the histogram (C). In both the cortex and hippocampus, Tg-Ex mice had significantly lower levels of Aβ plaques than in the respective tissues of Tg-Sed mice (n = 6/group). Data are expressed as mean ± SEM. Note: Wt, wild-type; Tg, transgenic; Sed, sedentary; Ex, exercise; SEM, standard error of the mean.$^{**}p < 0.01$ (Tg-Ex vs Tg-Sed). Scale bar = 25 µm.

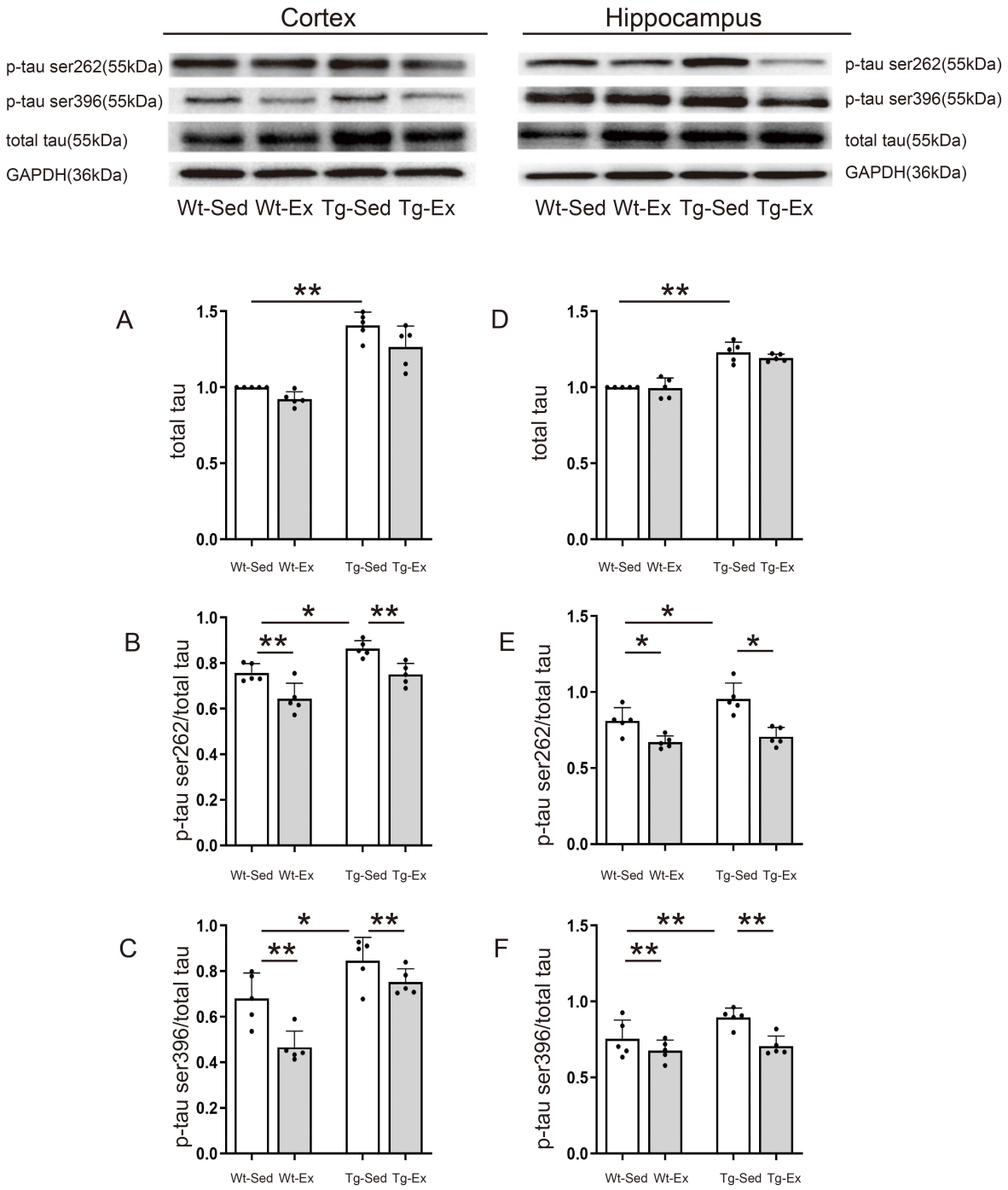

Fig. 3. Tau phosphorylation levels in the cortex and hippocampus. Representative images of western blots for tau (A,D), p-tau ser262 (B,E), and p-tau ser396 (C,F) in the cortex (upper panel, left) and hippocampus (upper panel, right) (n = 5/group). Tau in the cortex (A) and hippocampus (D) was significantly increased in Tg-Sed mice compared with Wt-Sed mice and this increase was not blocked by the treadmill exercise. P-tau ser262/tau (B) and p-tau ser396/tau (C) in the cortex were significantly increased in Tg-Sed mice compared with Wt-Sed mice but significantly decreased in Tg-Ex mice compared with Tg-Sed mice and Wt-Ex mice compared with Wt-Sed mice. P-tau ser262/tau in the hippocampus (E) was significantly increased in Tg-Sed mice compared with Wt-Sed mice but significantly decreased in Tg-Ex mice compared with Tg-Sed mice and Wt-Ex mice compared with Wt-Sed mice. P-tau ser396/tau in the hippocampus (F) was significantly increased in Tg-Sed mice compared with Wt-Sed mice but significantly decreased in Tg-Ex mice compared with Tg-Sed mice and Wt-Ex mice compared with Wt-Sed mice. Data are expressed as mean (SD). Note: Wt, wild-type; Tg, transgenic; Sed, sedentary; Ex, exercise; SD, standard deviation. *$p < 0.05$; **$p < 0.01$.

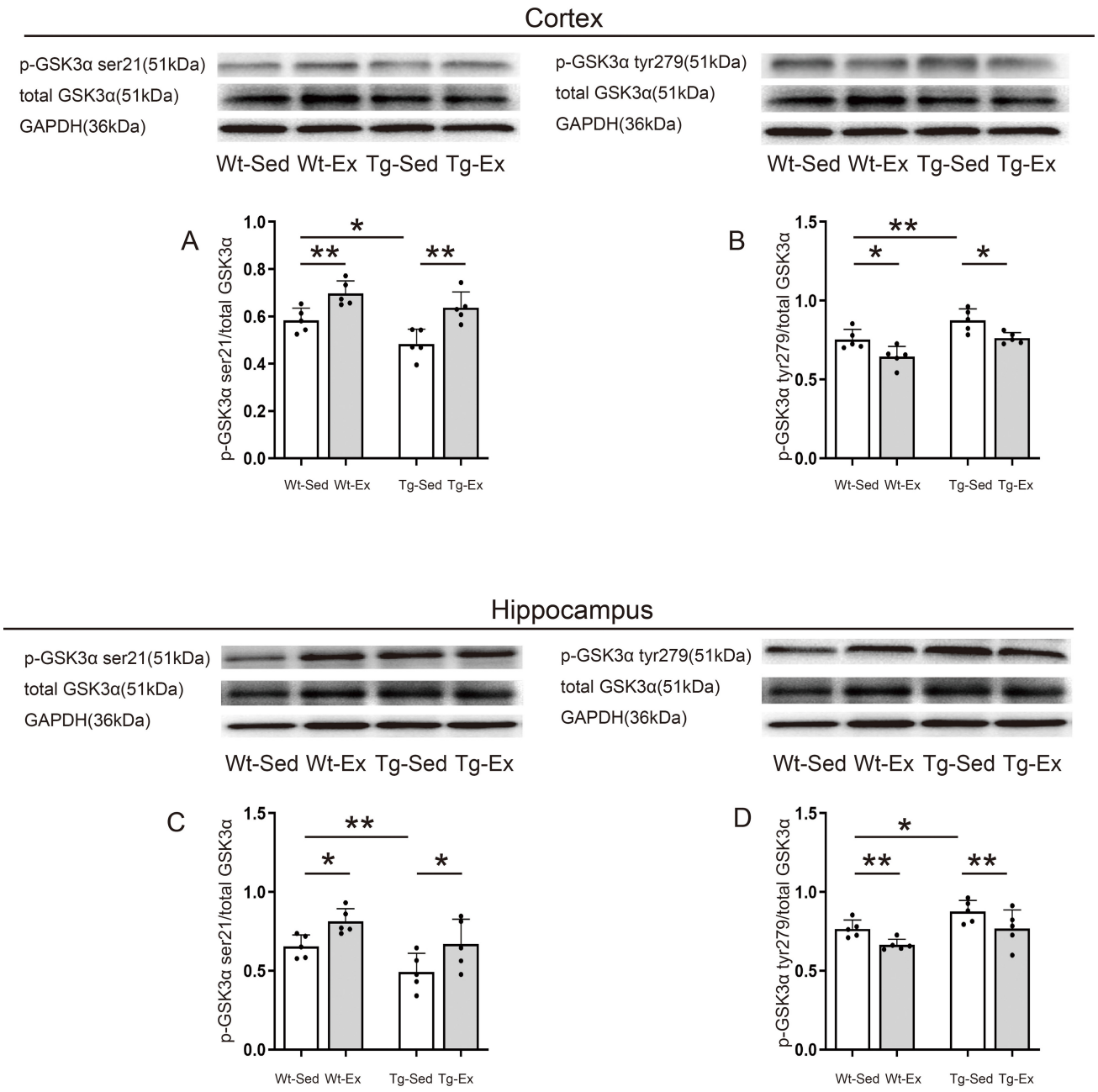

Fig. 4. GSK3α kinase activity in the cortex and hippocampus. Representative images of western blots for p-GSK3α ser21 (A,C), p-GSK3α tyr279 (B,D), and GSK3α (A–D) in the cortex and hippocampus (n = 5/group). In the cortex of Tg-Sed mice, p-GSK3α ser21/GSK3α was significantly decreased compared with that of Wt-Sed mice but significantly increased in Tg-Ex mice compared with Tg-Sed mice and Wt-Ex mice compared with Wt-Sed mice (A). P-GSK3α tyr279/GSK3α in the cortex was significantly increased in Tg-Sed compared with Wt-Sed but significantly decreased in Tg-Ex compared with Tg-Sed mice and Wt-Ex mice compared with Wt-Sed mice (B). P-GSK3α ser21/GSK3α in the hippocampus was significantly decreased in Tg-Sed mice compared with Wt-Sed mice but significantly increased in Tg-Ex mice compared with Tg-Sed mice and Wt-Ex mice compared with Wt-Sed mice (C). P-GSK3α tyr279/GSK3α in the hippocampus was significantly increased in Tg-Sed mice compared with Wt-Sed mice but significantly decreased in Tg-Ex mice compared with Tg-Sed mice and Wt-Ex mice compared with Wt-Sed mice (D). Data are expressed as mean (SD). Note: Wt, wild-type; Tg, transgenic; Sed, sedentary; Ex, exercise; SD, standard deviation. *$p < 0.05$; **$p < 0.01$.

Two-way ANOVA revealed that genotype and exercise had significant effects on the levels of p-GSK3α ser21 (genotype: $F = 7.654$, $p = 0.017$; exercise: $F = 12.397$, $p = 0.004$) and p-GSK3α tyr279 (genotype: $F = 17.736$, $p = 0.001$; exercise: $F = 8.980$, $p = 0.011$) in the cortex, but there was no significant interaction between genotype and exercise on the levels of p-GSK3α ser21 (genotype $\times$ exercise: $F = 0.713$, $p = 0.415$) and p-GSK3α tyr279

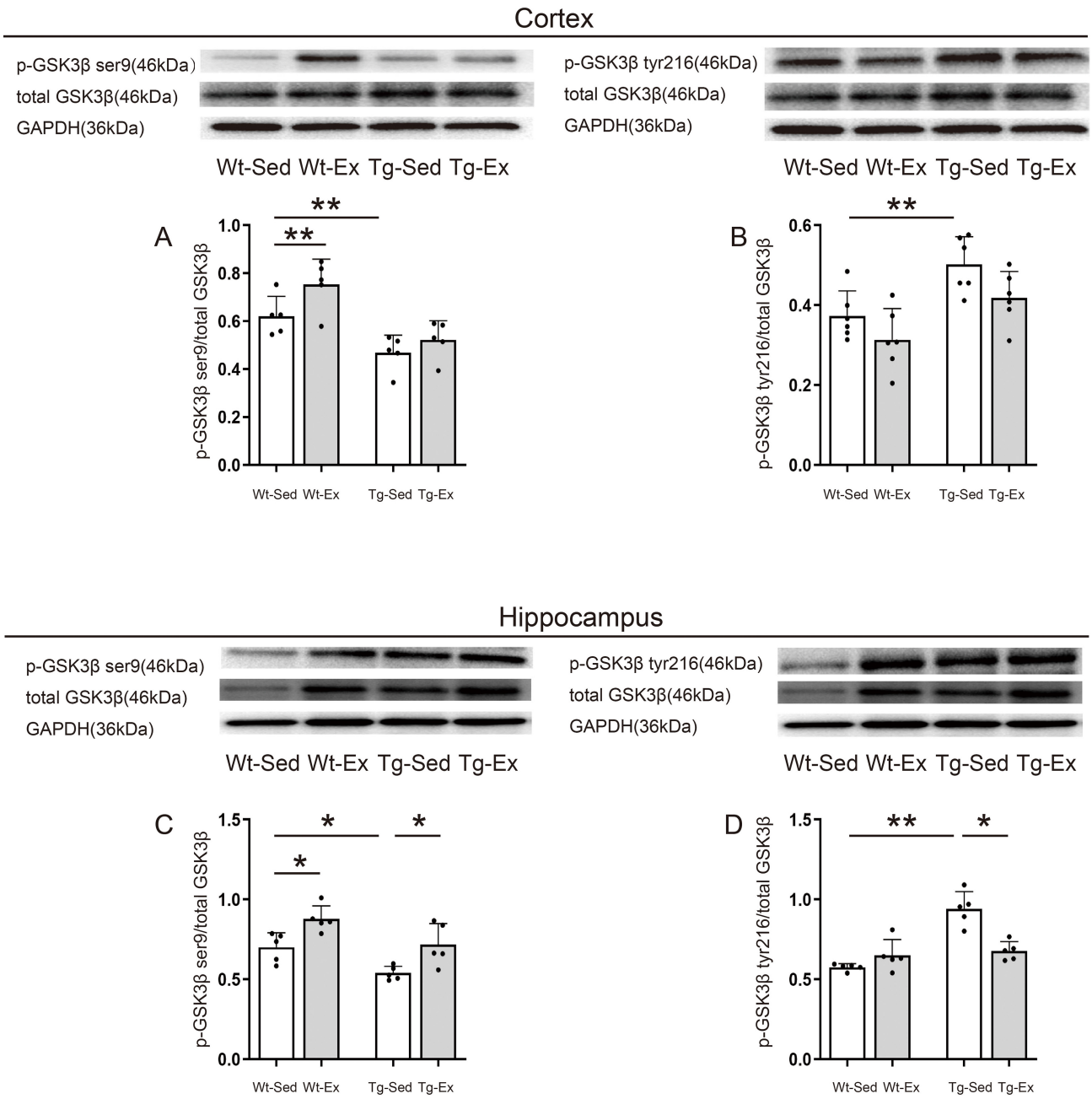

Fig. 5. GSK3β kinase activity in the cortex and hippocampus. Representative images of western blots for p-GSK3β ser9 (A,C), p-GSK3β tyr216 (B,D), and GSK3β (A–D) in the cortex and hippocampus (n = 5/group). P-GSK3β ser9/GSK3β in the cortex was significantly decreased in Tg-Sed mice compared with Wt-Sed mice but only increased in Wt-Ex mice compared with Wt-Sed (A). P-GSK3β tyr216/GSK3β in the cortex was significantly increased in Tg-Sed mice compared with Wt-Sed mice but similar between Tg-Ex and Tg-Sed mice, and between Wt-Ex and Wt-Sed mice (B). P-GSK3β ser9/GSK3β in the hippocampus was significantly decreased in Tg-Sed mice compared with Wt-Sed mice but significantly increased in Tg-Ex mice compared with Tg-Sed mice and Wt-Ex mice compared with Wt-Sed mice (C). P-GSK3β tyr216/GSK3β in the hippocampus was significantly increased in Tg-Sed mice compared with Wt-Sed mice but significantly decreased in Tg-Ex mice compared with Tg-Sed mice, and similar between Wt-Ex and Wt-Sed mice (D). Data are expressed as mean (SD). Note: Wt, wild-type; Tg, transgenic; Sed, sedentary; Ex, exercise; SD, standard deviation. *$p <$ 0.05; **$p < 0.01$.

(genotype $\times$ exercise: $F = 0.003$, $p = 0.955$) in the cortex. The main effect of genotype and exercise revealed that Tg groups had significantly lower levels of p-GSK3α ser21 and higher levels of p-GSK3α tyr279 in the cortex than in respective tissues of Wt groups. Exercise groups had significantly higher levels of p-GSK3α ser21 and lower levels

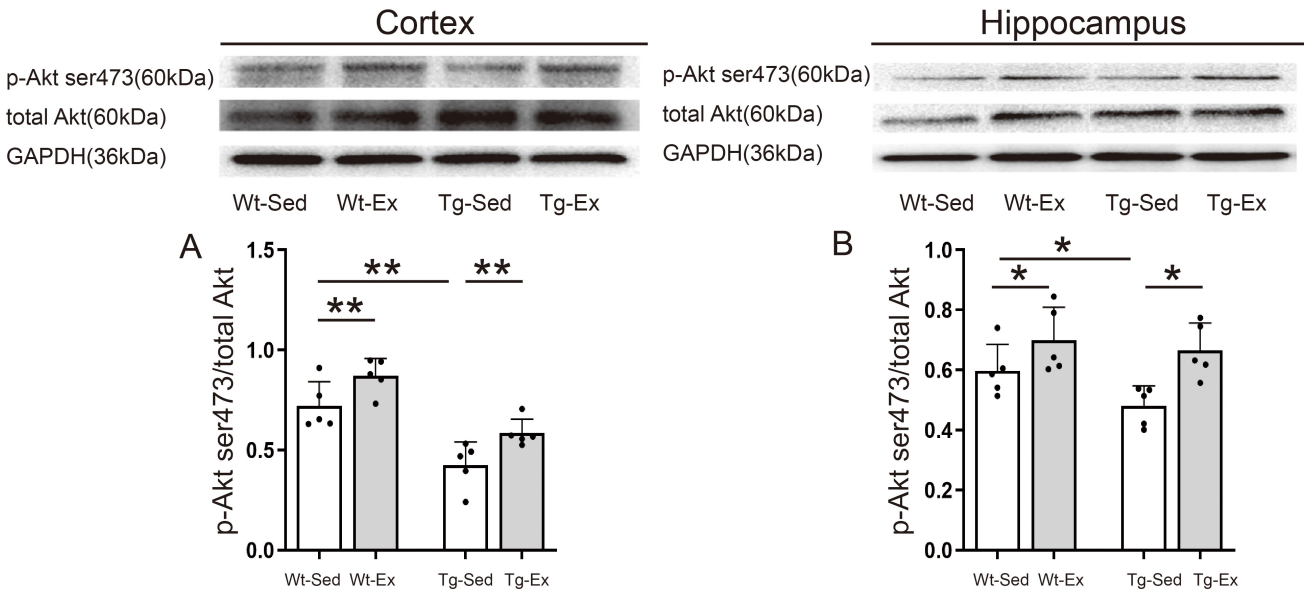

Fig. 6. Akt kinase activity in the cortex and hippocampus. Representative images of western blots of p-Akt ser473, total Akt, and reference protein GAPDH in the cortex (upper panel, left) and hippocampus (upper panel, right; n = 5/group). P-Akt ser473/Akt in the cortex was significantly decreased in Tg-Sed mice compared with Wt-Sed mice but significantly increased in Tg-Ex mice compared with Tg-Sed mice and Wt-Ex mice compared with Wt-Sed mice (A). P-Akt ser473/Akt in the hippocampus was significantly decreased in Tg-Sed mice compared with Wt-Sed mice, and both significantly increased in Tg-Ex and Wt-Ex mice (B). Data are expressed as mean (SD). Note: Wt, wild-type; Tg, transgenic; Sed, sedentary; Ex, exercise; SD, standard deviation. *$p < 0.05$; **$p < 0.01$.

of p-GSK3α tyr279 in the cortex than in respective tissues of sedentary groups (Fig. 4A,B).

Two-way ANOVA revealed that genotype and exercise had significant effects on the levels of p-GSK3α ser21 (genotype: $F = 9.573$, $p = 0.009$; exercise: $F = 6.217$, $p = 0.028$) and p-GSK3α tyr279 (genotype: $F = 5.397$, $p = 0.035$; exercise: $F = 33.209$, $p < 0.001$) in the hippocampus, but there was no significant interaction between genotype and exercise on the levels of p-GSK3α ser21 (genotype $\times$ exercise: $F = 0.684$, $p = 0.424$) and p-GSK3α tyr279 (genotype $\times$ exercise: $F = 0.156$, $p = 0.699$) in the hippocampus. The main effect of genotype and exercise revealed that Tg groups had significantly lower levels of p-GSK3α ser21 and higher levels of p-GSK3α tyr279 in the hippocampus than in respective tissues of Wt groups. Exercise groups had significantly higher levels of p-GSK3α ser21 and lower levels of p-GSK3α tyr279 in the hippocampus than in respective tissues of sedentary groups (Fig. 4C,D).

P-GSK3β ser9 and p-GSK3β tyr216 levels in both tissues are shown in Fig. 5. Western blotting revealed three different protein bands with different molecular weights, representing total GSK3β, p-GSK3β ser9/p-GSK3β tyr216, and reference protein GAPDH, respectively, in both tissues of each individual group (upper panel in Fig. 5A–D). Quantification of the bands and comparisons between different groups are shown in the histograms (Fig. 5A–D).

Two-way ANOVA revealed that genotype had significant effects on the levels of p-GSK3β ser9 (genotype: $F = 34.192$, $p = 0.001$) and p-GSK3β tyr216 (genotype: $F = 15.190$, $p = 0.001$) in the cortex. Significant exercise effects and interaction between genotype and exercise were observed on the levels of p-GSK3β ser9 (exercise: $F = 11.851$, $p = 0.003$; genotype $\times$ exercise: $F = 5.349$, $p = 0.034$) but not on p-GSK3β tyr216 (exercise: $F = 3.952$, $p = 0.064$; genotype $\times$ exercise: $F = 0.095$, $p = 0.762$) in the cortex. The main effect of genotype revealed that Tg groups had significantly lower levels of p-GSK3β ser9 and higher levels of p-GSK3β tyr216 in the cortex than in respective tissues of Wt groups. The multiple comparison test revealed that Wt-Ex mice had significantly higher levels of p-GSK3β ser9 ($p < 0.01$) than Wt-Sed mice (Fig. 5A).

Two-way ANOVA revealed that genotype and exercise had significant effects on the levels of p-GSK3β ser9 (genotype: $F = 5.821$, $p = 0.034$; exercise: $F = 6.871$, $p = 0.024$) and p-GSK3β tyr216 (genotype: $F = 39.914$, $p = 0.001$; exercise: $F = 8.074$, $p = 0.019$; genotype $\times$ exercise: $F = 10.467$, $p = 0.010$) in the hippocampus, but there was no significant interaction between genotype and exercise on the levels of p-GSK3β ser9 (genotype $\times$ exercise: $F = 0.393$, $p = 0.637$) in the hippocampus. The main effect of genotype and exercise revealed that Tg groups had significantly lower levels of p-GSK3β ser9 in the hippocampus than in respective tissues of Wt groups. Exercise groups had significantly higher levels of p-GSK3β ser9 in the hippocampus than in respective tissues of sedentary groups

(Fig. 5C). The multiple comparison test revealed that Tg-Sed mice had significantly higher levels of p-GSK3β tyr216 ($p < 0.01$) than Wt-Sed mice (Fig. 5D). In contrast, Tg-Ex mice ($p = 0.011$) had significantly lower levels of p-GSK3β tyr216 than Tg-Sed mice, and similar levels of p-GSK3β tyr216 in Wt-Ex and Wt-Sed mice ($p = 0.785$) (Fig. 5D).

3.5 Akt Phosphorylation in the Cortex and Hippocampus

Western blotting revealed three different protein bands with different molecular weights, representing p-Akt ser473, total Akt, and reference protein GAPDH, respectively, in both tissues of each group (Fig. 6 upper panel). Quantification of the bands and comparisons between different groups are shown in Fig. 6.

Two-way ANOVA revealed that genotype and exercise had significant effects on the levels of p-Akt ser473 in the cortex (genotype: $F = 38.969$, $p = 0.001$; exercise: $F = 12.950$, $p = 0.004$) and hippocampus (genotype: $F = 5.484$, $p = 0.044$; exercise: $F = 5.202$, $p = 0.048$), but there was no significant interaction between genotype and exercise on the levels of p-Akt ser473 in the cortex (genotype $\times$ exercise: $F = 0.221$, $p = 0.648$) and hippocampus (genotype $\times$ exercise: $F = 1.433$, $p = 0.262$). The main effect of genotype and exercise revealed that Tg groups had significantly lower levels of p-Akt ser473 in both the cortex and hippocampus than in respective tissues of Wt groups. Exercise groups had significantly higher levels of p-Akt ser473 in both the cortex and hippocampus than in respective tissues of sedentary groups (Fig. 6A,B).

4. Discussion

The study revealed that moderate-stage AD APP/PS1 mice had significantly impaired memory function, along with significantly higher levels of Aβ plaques, total tau, p-tau ser262, and p-tau ser396 in both the cortex and hippocampus than Wt mice, whereas 12-week, moderate-intensity treadmill exercise in AD mice induced significant improvements in memory function together with a significant reduction in Aβ plaques and all the three tau molecules in both tissues. Importantly, the moderate-stage AD APP/PS1 mice had significantly lower levels of both p-GSK3α ser21 and p-GSK3β ser9, but higher levels of both p-GSK3α tyr279 and p-GSK3β tyr216 in both tissues than Wt sedentary mice. However, after the 12-week exercise training, the hippocampus had significantly higher levels of both p-GSK3α ser21 and p-GSK3β ser9 but lower levels of both p-GSK3α tyr279 and p-GSK3β tyr216, whereas the cortex had significantly higher levels of p-GSK3α ser21 but only lower levels of p-GSK3α tyr279, in comparison with sedentary AD mice. The results strongly suggest the effectiveness of the training in improving memory function in the AD mice, which was believe occurred most likely through differential regulation of the Akt/GSK3 signaling pathways in the cortex and hippocampus.

The double-Tg APPSwe/PS1M146V mouse strain is currently widely used in studying the pathogenesis of AD, and impaired memory in these mice has been reported previously [31–33]. In our study, we observed significantly impaired memory function in the moderate-stage AD APP/PS1 mice. However, the impaired memory was rescued mice after the 12-week aerobic exercise training program. This is consistent with previous studies where regular exercise led to cognitive improvement/reservation, as reflected by lower working memory errors and reference memory errors [34,35], and shorter time to complete the Maze test in other AD mouse models such as the triple-Tg and TgCRND8 lines. Regular exercise also decreased completion time of the Floor Maze test in AD patients [36,37]. However, contradictory results showing no improvement in cognitive performance after regular exercise have also been observed in 10-month-old APP/PS1 mice, 9-month-old triple-Tg AD female mice, and AD patients [38–41]. The reasons for this are attributed to many different factors such as exercise type and intensity, health status, and wide range of Tg animal strains and AD stages [34,36,37,42]. The present observation of significant improvement in memory function in the moderate-stage AD stage Tg-Ex mice suggests both the efficiency of the training protocol and the increased susceptibility of the mice to the training, i.e., memory function can be improved in moderate-stage AD APP/PS1 mice.

The double-Tg APPSwe/PS1M146V mouse strain is characterized by initial and progressive enhancement of Aβ plaques [31–33]. Indeed, clusters of Aβ plaques were clearly observed in both the cortex and hippocampus of the 6-month-old AD mice, similar to the observations in double-Tg APPSwe/PS1M146V mice [31–33]. However, the 12-week moderate-intensity exercise training program induced a significant reduction in Aβ plaques in both tissues, suggesting that moderate-intensity aerobic exercise is effective in regulating Aβ plaque production in moderate-stage AD mice. Similar results have been observed in other AD mouse models where exercise training in Tg2576 AD mice resulted in lower Aβ plaques in both the cortex and hippocampus [43], and enriched housing of TgCRND8 AD mice induced the reduction of Aβ plaque burden [44].

Intracellular accumulation of phosphorylated tau is another hallmark lesion in AD [45]. The present study extensively studied tau by examining total tau, p-tau ser262, and p-tau ser396 in both the cortex and hippocampus. The results revealed that while the AD mice had significantly higher levels of total of tau, p-tau ser262, and p-tau ser396 in both the cortex and hippocampus than in that of Wt mice, moderate-intensity exercise training induced significant reductions in all three tau molecules in both tissues (see Fig. 3). The results are consistent with the observations on Aβ plaques in AD-exercised mice, confirming the effectiveness of the exercise in preventing or delaying deterioration of AD development in moderate-stage AD

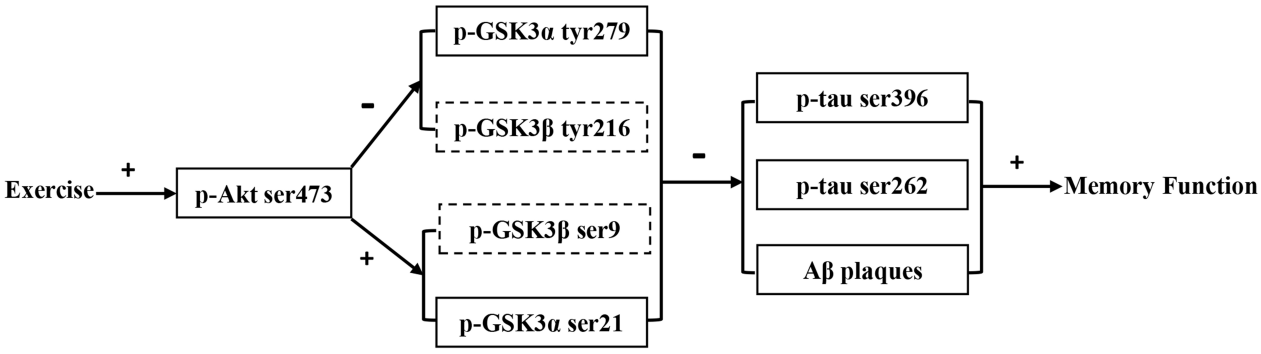

Fig. 7. Schematic illustration of molecular reactions in the cortex and hippocampus. Exercise induced the increased levels of p-Akt ser473 in both the cortex and hippocampus, which subsequently led to increased inhibition of both GSK3α and GSK3β in the two regions by increasing both p-GSK3α ser21 and p-GSK3β ser9 but decreasing both p-GSK3α tyr279 and p-GSK3β tyr216 in the hippocampus, as well as by increasing p-GSK3α ser21 but decreasing p-GSK3α tyr279 in the cortex. The inhibition of GSK3α and GSK3β in both regions resulted in the reduction in both p-tau ser396 and p-tau ser262, as well as in Aβ plaques, and eventually improved memory function or delayed memory deterioration in Tg-Ex mice. Note: solid lines indicate that the molecule was present in both the cortex and hippocampus; dashed lines indicate that the molecule was only present in the hippocampus. +, activation or improvement; –, inhibition.

mice. Interestingly, the study revealed that the exercise also induced a significant reduction in p-tau ser262 and p-tau ser396 in both the cortex and hippocampus of Wt mice compared with Wt sedentary mice, indicating the significance of physical exercise in preventing the occurrence of AD. Similar to the present observations, exercise-induced reduction in Aβ plaques and tau phosphorylation have been observed previously [13,16], along with improvement in cognitive function and behaviors [16,17,46]. Considering the observations in the improvement in memory function, and the reductions in Aβ plaques and tau molecules in the AD-exercised mice, we assume that the reductions are closely associated with the improvement in memory function. Nonetheless, it is worth noting that a previous study on another AD mouse model revealed that exercise had no significant effect on 12-month TgCRND8 mice in abnormal APP metabolism, tau pathology, or angiogenesis [12].

To explore the molecular mechanisms underlying the improvement in memory function and the significant reduction in Aβ plaques in both tissues of AD mice following a 12-week exercise training program, the study further examined Akt/GSK3 signaling pathways. GSK3 deregulation has been proven to account for memory impairment, increased Aβ production, hyper-phosphorylation of tau, and inflammatory responses in AD [47]. Indeed, our study revealed that the AD sedentary mice had significantly lower levels of both p-GSK3α ser21 and p-GSK3β ser9, but higher levels of both p-GSK3α tyr279 and p-GSK3β tyr216 (see Figs. 4,5) in both tissues, along with significantly lower memory function than Wt sedentary mice. As p-GSK3α tyr279 and p-GSK3β tyr216 function to activate GSK3, whereas p-GSK3α ser21 and p-GSK3β ser9 inhibit GSK3 [48], the reduced levels of p-GSK3α ser21 and p-GSK3β ser9 together with the enhanced levels of p-GSK3α tyr279 and p-GSK3β tyr216 in the AD sedentary mice are believed to work coordinately to activate GSK3α and GSK3β, leading to Aβ plaque formation and resulting in enhanced Aβ plaque levels (see Fig. 3). These findings suggest that GSK3α and GSK3β phosphorylation were similar in both tissues, although Aβ pathology occurred earlier in the cortex than the hippocampus.

After the 12-week exercise training program in the AD mice, the hippocampus had significantly higher levels of both p-GSK3α ser21 and p-GSK3β ser9 but lower levels of both p-GSK3α tyr279 and p-GSK3β tyr216, whereas the cortex had significantly higher levels of p-GSK3α ser21 but lower levels of p-GSK3α tyr279 in comparison to respective tissues of AD sedentary mice (see Figs. 4,5). The activity of GSK3α/β is believed to be maintained through phosphorylation at both tyr279/216 sites (activation) and ser21/9 sites (inhibition) [49], though the activity of GSK3 has also been suggested to be regulated through phosphorylation at Ser9/21, rather than Tyr 216/279 [50]. Thus, in both tissues of the exercised-AD mice, the enhanced levels of both p-GSK3α ser21 and p-GSK3β ser9 together with the reduced levels of both p-GSK3α tyr279 and p-GSK3β tyr216 are believed to work coordinately to result in the reduction in GSK3 activity, which subsequently led to the decrease in Aβ plaques and tau phosphorylation. However, the exercise training had significant impact on both GSK3β and GSK3α in the hippocampus, but not on GSK3β in the cortex of the AD mice, indicating that the aerobic exercise is sufficient to suppress the GSK3 signal in the hippocampus, but not the cortex of moderate-stage AD mice.

To further explore the molecular signaling pathways of GSK3 activation, we examined the activity of the GSK3

upstream regulator Akt. The results revealed that both tissues of AD sedentary mice had significantly lower p-Akt ser473 levels than respective tissues of Wt sedentary mice (see Fig. 6). However, after the 12-week exercise training program, both tissues of the AD mice showed significantly higher levels of p-Akt ser473 than respective tissues of AD sedentary mice. As GSK3 could be inactivated by Akt ser473 phosphorylation [51,52], the increased p-Akt ser473 levels indicated enhanced inhibition of GSK3 activity in both tissues of AD exercised-mice.

Considering all the results, we propose a scenario of molecular reactions in the two regions of the brain of the APP/PS1 mice in response to the exercise training as shown in Fig. 7: the exercise induced enhancement in p-Akt ser473 in both the cortex and hippocampus, which subsequently led to enhancement in inhibition of both GSK3α and GSK3β by increasing both p-GSK3α ser21 and p-GSK3β ser9 but decreasing both p-GSK3α tyr279 and p-GSK3β tyr216 in the hippocampus, as well as by increasing p-GSK3α ser21 but decreasing p-GSK3α tyr279 in the cortex. The inhibition of GSK3α and GSK3β activity in the cortex and hippocampus resulted in the reduction in both p-tau ser396 and p-tau ser262, as well as in Aβ plaques in both regions, and eventually improved memory function or delayed memory deterioration in the AD-exercised mice.

5. Conclusions

In conclusion, moderate-intensity aerobic training regulates GSK3α/β activity in the cortex and hippocampus of 9-month-old APP/PS1 mice. The effects of exercise are more prominent in the hippocampus, but similar in the cortex. As such, it is never too late to initiate an exercise program to delay the progression of AD, which serves as a promising preventive intervention.

6. Limitations

The present study used APP/PS1 Tg mice, which overexpress a chimeric mouse/human APP (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9), both of which exist in the central nervous system and are associated with early-onset familial AD. Because this mouse model displays very similar biochemical aspects to that of human AD, particularly regarding amyloid pathology, it is widely used in studies of AD. However, it has inherent limitations with respect to the human AD condition, as it represents only a small percentage of all AD patients. In most AD patients, the disease is sporadic with a multifactorial etiology, making it challenging to generalize findings obtained from this model to all AD patients. Sporadic AD, the most common form, has not yet been faithfully modeled. Nevertheless, the APP/PS1 model is a powerful tool in AD research.

Abbreviations

AD, Alzheimer's disease; Aβ, β-amyloid; APP, β-amyloid precursor protein; GSK3, glycogen synthase kinase-3; NFTs, neurofibrillary tangles.

Availability of Data and Materials

The datasets generated for this study are available on request to the corresponding authors.

Author Contributions

PH and BG have contributed equally to this work. LZ and JY designed the research study. PH, BG and LM performed the research. PH, BG and LM analyzed the data. PH and BG wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was approved by the local ethics committee of Beijing Sport University (2015015) and carried out according to the requirements of The Guiding Principles for Care and Use of Animals.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2307136.

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