Effects of low-intensity motor balance and coordination exercise on cognitive functions, hippocampal Aβ deposition, neuronal loss, neuroinflammation, and oxidative stress in a mouse model of Alzheimer's disease

Running title: Effects of low intensity coordination exercise in SAMP8

Kazuki Nakanishi[1], Harutoshi Sakakima[1], Kosuke Norimatsu[1], Shotaro Otsuka[2], Seiya Takada[2], Akira Tani[1], Kiyoshi Kikuchi[3]

1. Department of Physical Therapy, School of Health Sciences, Faculty of Medicine, Kagoshima University, Kagoshima, Japan
2. Department of Systems Biology in Thromboregulation, Kagoshima University Graduate School of Medical and Dental Science, Kagoshima, Japan
3. Division of Brain Science, Department of Physiology, Kurume University School of Medicine, Kurume, Japan

Address correspondence to: Harutoshi Sakakima, PhD

Department of Physical Therapy, School of Health Sciences, Faculty of Medicine, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima, 890-8544 Japan

Tel +99-275-6778 Fax +99-275-6801

E-mail sakaki@health.nop.kagoshima-u.ac.jp

Highlights

- Exercise prevents age-related behavioral and cognitive decline in the early stages of Alzheimer's disease progression.
- Hippocampal Aβ depositions, neuronal loss, and neuroinflammation were suppressed by exercise.
- Exercise modulate pro-inflammatory M1 phenotype microglia and A1 phenotype astrocyte.
- Exercise suppresses iNOS and nNOS activities.

ABSTRACT

It is well known that physical exercise reduces the risk of Alzheimer's disease (AD) and age-related cognitive decline. However, its mechanisms are still not fully understood. This study aimed to investigate the effect of aging and rotarod exercise (Ex) on cognitive function and AD pathogenesis in the hippocampus using senescence-accelerated prone mice prone 8 (SAMP8) mice. Cognitive functions clearly declined at 9-months of age. Amyloid-beta (Aβ) deposition, neuronal loss, and glia activation-induced neuroinflammation increased with aging. The rotarod Ex prevented the decline of cognitive functions corresponding to the suppression of Aβ deposition, neuroinflammation, neuronal loss, inducible nitric oxide synthase (NOS) activities, and neuronal NOS activities. In addition, the rotarod Ex suppressed pro-inflammatory M1 phenotype microglia and A1 phenotype astrocytes. Our findings suggest that low-intensity motor balance and coordination exercise prevented age-related cognitive decline in the early stage of AD progression, possibly through the suppression of hippocampal Aβ deposition, neuronal loss, oxidative stress, and neuroinflammation, including reduced M1 and A1 phenotypes microglia and astrocytes.

Key words: SAMP8, rotarod exercise, neuroinflammation, M1 phenotype microglia, A1 phenotype astrocyte, NOS activity

1. Introduction

A number of physical and mental health conditions are associated with the aging process. The decline of physical and cognitive functions is a common health problem in older people. Reduced physical function is related to a decline in daily physical activity and a higher risk of falling in older adults (Best et al., 2015). Furthermore, the risk of cognitive decline and dementia is increased with age. Alzheimer's disease (AD) is the most common neurodegenerative disorder that causes dementia (Barnes et al., 2011). Its main pathological changes including extracellular amyloid-beta (Aβ) aggregation and intracellular neurofibrillary tangles composed of hyperphosphorylated tau are well known hallmarks of AD (Lu et al., 2017). In addition, the pathogenesis of AD is believed to be associated with a series of neurodegenerative events in the hippocampus, including microglia and astroglia activation, neuroinflammation, oxidative damage, metabolic energy failure, and consequent neuronal apoptosis (Smith et al., 2000; Liu et al., 2014; Tang et al., 2016; Liddelow et al., 2017; Grimaldi et al., 2019; Zhang et al., 2019). Therefore, the hippocampus is one of the major brain sites of neuroplasticity and memory function.

Previous studies have indicated that neuroinflammation is associated with the onset and progression of AD and is characterized by glia activation (e.g., microglia and astroglia) and release of inflammatory mediators (e.g., IL-1β, TNFα) (Lynch. 2010; Cherry et al., 2015; Olmos-Alonso et al., 2016). Microglial cells are primary mediators of the innate immune response and are rapidly mobilized to the site of injury in the central nervous system. Microglia cells can be polarized into either a detrimental (M1) or a beneficial (M2) phenotype in neurodegenerative disease. Notably, the

proinflammatory cytokines released by M1-like microglia are believed to contribute to neuronal cell damage (Zhou et al., 2014; Loane and Kumar. 2016; Tang et al., 2016; Takada et al., 2020). Microglia-mediated neuroinflammatory responses play an important role in Aβ deposition and AD-related cognitive impairment (Feng et al., 2019; Zhang et al., 2019). Furthermore, microglia-mediated inflammatory cytokines activate astrocytes to highly neurotoxic A1 phenotype cells. These are characterized by increased complement component 3 (C3) that induce neuronal cell death (Liddelow et al., 2017. Clark et al., 2019). A1 astrocytes are present in various neurodegenerative diseases, including AD (Liddelow et al., 2017). A1-like astrocytes might be destructive to synapses or neuron loss and therefore have a 'harmful' function (Liddelow and Barres. 2017).

In addition, oxidative stress also plays an important pathogenic role in AD (Moreira et al., 2009; Ali et al., 2009). It is a proximal event in the pathogenesis of AD and occurs prior to the onset of symptoms in AD (Ali et al., 2009). Oxidative damage occurs when the oxidative balance is disturbed, such that reactive oxygen production exceeds cellular anti-oxidant defenses (Smith et al., 2000). Oxidative damage can be mediated through the release of nitric oxide (NO), which is produced by three nitric oxide synthase (NOS) isoenzymes: inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS) (Ali et al., 2009). NO produced in response to Aβ triggers mitochondrial fission, synaptic loss, and neuronal cell death (Cho et al., 2009). Taken together, the onset and processes that underlie the pathogenesis of AD involve several factors. To date, no effective treatments for age-related neurodegenerative diseases, including AD, have been discovered. AD tends to progress irreversibly and is associated with high socioeconomic and personal costs (Hou et al.,

2019). Although pharmacological therapy has been utilized for AD treatment, they have significant side effects.

It was recently found that regular physical exercise and a modifiable lifestyle can reverse cognitive impairment by modifying several noxious factors, reduce the risk of AD, and slow the progression of early AD (Zhang et al., 2019; Valenzuela et al., 2020). Furthermore, aerobic exercise may be useful for preventing age-related hippocampal deterioration and maintaining neuronal health in humans (Firth et al., 2018). In the animal model of AD, physical exercise has been found to decrease Aβ deposition, neuroinflammation, and oxidative stress in the hippocampus, contributing to improved cognitive function (Ryan et al., 2016; Lu et al., 2017; Zhang et al., 2018). Furthermore, physical exercise shifts activated microglia from the M1 to M2 phenotype in the hippocampus of amyloid precursor protein (APP)/PS1 mice (Zhang et al., 2019). However, the effect of physical exercise on the phenotype of reactive astrocytes, such as the A1 phenotype, in AD is still unclear.

In addition, exercise increases the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus, a neurotrophin that promotes neuronal survival and synaptic integrity (Li et al., 2019). Increased BDNF levels may induce hippocampal neurogenesis, and beneficially contribute to cognitive function in AD (Choi et al., 2018). Physical exercise results in many beneficial mechanisms that might affect AD through different pathways. Therefore, the mechanism underlying the beneficial effects of physical exercise on AD remains unclear. Furthermore, Aβ stimulates NOS activity through a nuclear factor-kB pathway in astrocytes (Akama et al., 2000), and Aβ has been assumed to cause

elevated NOS levels in AD (Ali et al., 2009). However, the effect of physical exercise on NOS activity in the hippocampus of AD is unknown.

Motor balance exercise programs, such as rod motor exercises, improve functional outcomes in animal models of stroke more than treadmill exercise regimens (Ding et al., 2004). Furthermore, motor balance exercise programs increase the expression of BDNF and its receptors in the motor cortex after stroke (Sakakima et al., 2012). If physical exercise is initiated at the early stage of AD, not when neurological health is compromised, it can be beneficial for preventing or delaying the progression of AD (Zhang et al., 2019). Therefore, the present study investigated the effects of low-intensity rod motor exercise, which requires motor balance and coordination activities, on AD pathogenesis in the early stages of AD progression.

Senescence-accelerated mouse prone 8 (SAMP8) mice have drawn considerable attention in AD research (Morley. 2002; Butterfield et al., 2005; Gang et al., 2011; Li et al., 2019; Zhang et al., 2019). SAMP8 mice are a non-genetically modified strain of mice with an accelerated aging process. They share similar characteristics with aged humans, such as reduced lifespan, lordosis, hair loss, and physical activity (Takeda et al., 1997). Furthermore, SAMP8 mice spontaneously show cognitive impairment, Aβ aggregation in the cortex or hippocampus, and tau hyperphosphorylation (Del Valle et al., 2010; Manich et al., 2011; Akiguchi et al., 2017). Therefore, SAMP8 mice are a suitable animal model of AD to investigate the fundamental mechanisms of age-related decline in physical activity and cognitive deficits at gene or protein levels.

In the present study, we investigated the onset of age-related decline in physical and cognitive functions of SAMP8 mice. We also determined the neurodegenerative events in the hippocampus, including Aβ deposition, loss of neurons, microglia, and astrocyte activation-induced neuroinflammation with age. Furthermore, we investigated the effects of low-intensity motor balance and coordination exercise on hippocampal Aβ deposition, neuron loss, neuroinflammation, and NOS activity in the early stage of AD progression in SAMP8 mice.

2. Methods

2.1. Animals and experimental design

A total of 63 one-month-old male SAMP8 mice (body weight: 23.8 ± 1.7 g, mean ± SD) and a total of 26 age-matched SAM resistant 1 (SAMP1) mice (22.3 ± 0.8 g) were obtained from Japan SLC animal supply (Hamamatsu Japan). SAMR1 mice were used as controls. SAMP8 and SAMR1 mice showed an increase in weight with age (at 9-months-old: 28.4 ± 2.2 g and 37.9 ± 2.2 g, respectively). However, the mean body weight of SAMP8 mice was significantly smaller than that of SAMR1 at 9-months-old ($p < 0.01$). Mice were housed in a temperature-controlled (23.0 ± 1.0 °C) cage with a 12 h light/dark cycle and free access to food and water.

The present study investigated the behavioral and cognitive functions and neurodegenerative events in the hippocampus of SAMP8 mice aged 3, 5, 7, or 9-months. Subsequently, we examined the effects of exercise interventions from the early stage of AD progression using

immunohistochemical analyses and western blotting. The experimental protocol was approved by the ethics board of the Institute of Experimental Animal Science of Kagoshima University.

2.2. Exercise protocols

Exercise was performed using a motorized rotarod treadmill (MK-670, Muromachi Kikai CO, LTD, Japan). The rod was 3 cm in diameter and covered with smooth rubber. Seven-month-old SAMP8 mice were randomly divided into an exercise group (n = 20, Ex group) and no-exercise group (n = 25, No-Ex group). The mice in the Ex group were required to run at a constant speed of 25 rpm (2.4 m/min) for 5 days per week, 15 min/day, for 10 weeks (from 7 to 9-months). If the mice fell during the exercise session, they were placed back on the rotating rod. The mice in the No-Ex group were allowed to move freely in their cages. As the SAMP8 mice were exercised from 7 to 9-months in this study, the 9-month-old mice were equal to those in the No-Ex group. Exercise was performed at room temperature and during the daytime (10:00 AM to 4:00 PM). Bodyweight was periodically measured to monitor the stress induced by the rotarod treadmill exercise.

2.3. Behavioral test

The locomotive activity of both mice was measured using an open-field test at 3-months (SAMP8; n =12, SAMR1; n = 15), 5-months (n =18, n = 14), 7-months (n =13, n = 14), and 9-months-old (n =10, n = 7). In addition, the SAMP8 mice of the Ex group (n = 13) underwent an open-field test after the exercise intervention to evaluate their locomotive activity. An open-field apparatus (55 cm × 60 cm × 40 cm) was placed in a quiet environment and wiped clean with a 70% ethanol solution between tests. The mice were placed and familiarized in the center of the open-field for 5 min, and

spontaneous activities were recorded for 1 h using a video camera (Logicool HD Pro Webcam C920r) mounted above the open-field. The locomotive distance and the ratio of high-speed waking (higher than 15 cm/sec) were measured using SMART version 3.0 (video camera system, Panlab, Barcelona, Spain).

In addition, the exercise durability of both mice was examined using a motorized rotarod task (MK-670, MUROMACHI KIKAI CO, LTD, Japan) at 3-months (SAMP8; n = 8, SAMR1; n = 14), 5-months (n = 6, n = 14), 7-months (n = 18, n = 14), and 9-months-old (n = 8, n = 14). In addition, the physical function of SAMP8 mice in the Ex group (n = 10) was evaluated after the exercise intervention. Each mouse was placed on the rotarod cylinder, and the duration that the mouse remained on the cylinder was measured. The rotation speed was increased from 0 to 40 rpm in increments of 4.0 rpm every 6 sec. The trial ended if the animal fell into the cylinder. Each animal was given two trials, and the average latency (seconds) was used in the analysis.

2.4. Recognition memory test

The novel-objective recognition (NOR) test was used to assess recognition memory function at 3-months (SAMP8; n =10, SAMR1; n = 16), 5-months (n = 7, n = 16), 7-months (n = 6, n = 16), and 9-months-old (n =5, n = 8). In addition, the SAMP8 mice of the Ex group (n = 5) were evaluated after the exercise intervention using the NOR test. The experimental apparatus consisted of an open plastic box (33.5 cm × 23 cm × 26 cm). The test included two trials. After habituation to the test area for 10 min, two identical cylinder blocks were placed at opposite corners of the area. The mouse was allowed to explore for 10 min for the first trial. Sixty min after the first trial, one of the cylinder blocks

(familiar object) was exchanged for a novel object. The novel object was different in shape and color but was consistent in height. In the second trial, the mouse was returned to the test area with the familiar and novel object for 10 min. The discrimination index was calculated as the time spent exploring the familiar object - the time spent exploring the novel object/ the time spent exploring the familiar object + the time spent exploring the novel object.

2.5. Histology and immunohistochemistry

The SAMP8 mice were sacrificed at 3 (n = 3), 7 (n = 3), and 9 (n = 8) months-old. Then, the Ex group (n = 8) underwent histological and immunohistochemical analyses. The SAMP1 mice were sacrificed at 9-months-old (n = 7). Mice were deeply anesthetized with sodium pentobarbital and perfused through the heart with heparinized physiological saline. The brain was carefully removed and cut into two 3 mm thick coronal sections from the frontal tip using a brain slicer. The slices were immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C overnight. After fixation, the tissue was histologically and immunohistochemically processed. Paraffin-embedded coronal brain sections (4-μm thick) were stained with hematoxylin and eosin staining and Nissl staining for morphological evaluation of the hippocampus neurons.

The coronal sections were stained with the following antibodies: mouse anti-β-amyloid (Aβ: a marker of Aβ deposition) antibody (Biolegend Inc.; 4G8), rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1, a marker of resting microglia) (Wako, Osaka, Japan; #019-19741), rabbit anti-glial fibrillary acidic protein (GFAP; a marker of activated astrocytes) (Cosmo Bio Co., Japan;

RO1003), rabbit anti-neuronal nuclei (NeuN; a marker of neurons) (Cell Signaling Technology, Inc.; #24307), and rabbit anti-BDNF antibody (Santa Cruz, CA, USA, sc20981).

Following deparaffinization and rehydration, endogenous peroxidase was blocked with methanol containing 3.0% hydrogen peroxide for 10 min. If necessary, antigen activation was performed according to the manufacturer's protocol. The sections were then rinsed 3 times (5 min each) with phosphate-buffered saline (PBS, pH 7.6) and blocked with 10% skimmed milk in PBS for 20 min. All sections were individually incubated at 4 °C overnight with the following antibodies: mouse anti-Aβ (1:1000), rabbit anti-Iba1 (1:2000), rabbit anti-GFAP (1:1000), rabbit anti-NeuN (1: 400), and rabbit anti-BDNF (1:50). The sections were then washed in PBS 3 times for 5 min each and incubated for 60 min with goat anti-rabbit IgG conjugated to a peroxidase-labeled dextran polymer (EnVision; Dako, CA, USA). Finally, the sections were rinsed with PBS, and their immunoreactivity was visualized by diaminobenzidine staining.

The co-localization of the rabbit anti-C3 (1: 200) and mouse anti-GFAP (1: 500) immunoreactivities were examined by immunofluorescence staining. After incubation with 2 primary antibodies and PBS wash, the sections were incubated for 60 min with both Alexa Fluor 488-conjugated goat anti-rabbit IgG (1: 100) and Alexa Fluor 555-conjugated goat anti-mouse IgG antibodies (1: 100). The sections were washed with PBS and counterstained with 4', 6-diamino-2-phenylindole for 10 min. Finally, they were mounted with an aqueous mounting medium, and immunofluorescent staining was observed with a fluorescence microscope (EVOS f1; AMG, Mill Creek, WA, US).

2.6. Quantitative analysis of the immunostained sections

The unilateral hippocampus in each immunostained section was imaged at 10× magnification using a microscope and camera. The ratios of Iba1- and GFAP-positive areas were quantitatively measured in the hippocampus. The stratum radiatum of the unilateral hippocampus in the Aβ immunostained section was imaged at 20× magnification, and the ratios of the Aβ-positive areas were quantitatively measured in the hippocampus. In addition, NeuN- and BDNF-positive neurons in the CA1 and CA3 regions in the unilateral hippocampus were imaged at 20× magnification. The number of NeuN-positive neurons and the ratio of BDNF-positive neurons in the CA1 and CA3 regions were quantitatively measured. The quantitative analysis of Aβ-, Iba1-, GFAP-, NeuN-, and BDNF-immunostained areas was performed in 3, 7, and 9-month-old mice. All quantitative analyses were performed using Image J version 1.46r (NIH, USA), and a quantitative analysis of each immunolabeled area was performed by 2 individuals.

2.7. Western blotting

Western blotting was performed to detect the protein levels in the right and left hippocampus of SAMP8 mice aged 3 and 9-months-old, the Ex group, and SAMR1 mice aged 9-months-old (n=3 for each group). The right and left hippocampi were surgically excised on ice and homogenized in T-Per reagent (Pierce Protein Research Products, 78510). Approximately 10 μg of protein in each sample was loaded in a 4-20% mini-protean precast gel (Bio-Rad) and transferred to a polyvinylidene fluoride (PVDF) or nitrocellulose membrane. After blocking with Tris-buffered saline/Tween 20 buffer (0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 5% skim milk for

1 h at room temperature, the membrane was incubated with primary antibody overnight at 4 °C, and then with the secondary horseradish peroxidase-labeled antibody for 1 h at room temperature. Detection was performed using the immobilon chemiluminescent HRP substrate (Millipore). The following antibodies were used: rabbit anti-Aβ antibody (1: 500; Abcam plc, Cambridge, UK; ab2539), rabbit anti-BDNF (1: 500; Abcam plc, Cambridge, UK; ab108319), rabbit anti-Iba-1 (1: 1000; Wako, Osaka, Japan; 016-20001), rabbit anti-GFAP (1: 1000; Cosmo Bio Co., Japan; RO1003), rabbit anti-tumor necrosis factor-α (TNF-α; a marker of proinflammatory and M1 microglia) (1: 500; Abcam plc, Cambridge, UK; ab6671), rabbit anti-arginase-1 (a marker of M2 microglia) (1: 1000; Cell Signaling Technology, Inc, US; #93668), rabbit anti-iNOS (1: 1000; Abcam plc, Cambridge, UK; ab15323), rabbit anti-phospho-eNOS (p-eNOS; 1: 1000; Cell Signaling Technology, Inc, US; #9571), rabbit anti-nNOS (1: 1000; Abcam plc, Cambridge, UK; ab76067), rabbit anti-complement component 3 (C3; a marker of astrocytic A1 phenotype) (1: 100; Cell Signaling Technology, Inc, US; #11887), and anti-mouse α-tubulin (1:2000; Proteintech Group, Inc; 66031-1-lg). The protein bands were visualized with a chemical luminescence system (WSE-6100 Lumino Graph I, ATTO, Tokyo, Japan) and measured using Image J version 1.46r. The intensities were normalized to the corresponding band intensities of α-tubulin from the same samples.

2.8. Statistical analysis

Statistical analyses were performed with both parametric and non-parametric tests after the Shapiro-Wilk test. Either the Mann-Whitney U test or the independent student's t-test were applied for between-group analyses. The time course for the percentages of immunostained areas or

quantified protein levels were analyzed using either a one-way analysis of variance or the Kruskal-Wallis test, followed by Bonferroni's posthoc tests for multiple comparisons. A p-value of < 0.05 was considered statistically significant. Data are expressed as the mean ± standard error (SE). All data were analyzed using SPSS version 26 (IBM, Chicago, IL, USA).

3. Results

3.1. Effects of aging and exercise on the age-related decline of behavioral and cognitive functions in SAMP8 mice.

First, we investigated the effects of aging on locomotive activities and physical and cognitive functions in SAMP8 and SAMR1 mice (Fig. 1). The locomotive (locomotion distance and the ratio of high-speed waking time) and physical (walking time of the rotarod task) functions of the SAMP8 mice were decreased compared with those of age-matched SAMR1 mice at each time point. In the SAMP8 mice, the locomotive and physical functions significantly declined with age ($p < 0.001$, Fig. 1A-C). At 9-months-old, the locomotive activities and physical functions were significantly decreased compared with those of 5 or 7-month-old and age-matched SAMR1 mice ($p < 0.01$). These findings suggest that SAMP8 mice displayed age-related decline of locomotive and physical functions from approximately 7-months-old. In contrast, there were no significant differences in the locomotive and physical functions at each time point in the SAMR1 mice.

Recognition memory was significantly impaired in 9-month-old SAMP8 mice compared to 7 and 9-month-old SAMR1 mice, which exhibited a decreased performance in exploring the novel

object ($p < 0.01$, Fig. 1D). Our results indicate that the recognition memory function of SAMP8 mice showed a clear decline at 9-months of age.

To determine if exercise can prevent the age-related decline of physical and cognitive functions of SAMP8 mice, we performed an exercise intervention for ten weeks from 7-months of age. The Ex group showed significantly improved locomotive activity and physical functions compared to the No-Ex group ($p < 0.01$, Fig. 1E-G). In addition, animals in the Ex group exhibited remarkable cognitive improvement compared to the No-Ex group ($p < 0.01$, Fig. 1H).

3.2. Effects of aging and exercise on Aβ deposition and loss of neuronal cells in the hippocampus of SAMP8 mice.

We investigated the effects of aging on Aβ deposition and the expression of NeuN-positive neurons in the hippocampus using immunohistochemistry and a western blotting analysis (Fig. 2, 3). Aβ deposition was mainly observed as granules in the stratum radiatum of the hippocampus at 9-months-old. Therefore, we examined the ratio of the Aβ immunoreactive area in the stratum radiatum of the hippocampus (Fig. 2A). Our immunohistochemical analysis showed that Aβ deposition in the hippocampus of SAMP8 mice was significantly increased with aging ($p < 0.01$, Fig. 2B, C, E, F). The ratio of the Aβ immunoreactive area was significantly increased at 9-months-old compared to 3 and 7-month-old mice ($p < 0.05$, Fig. 2D). Unexpectedly, the western blotting analysis did not show a significant difference in the Aβ protein levels in the whole hippocampus between three-month and 9-month-old SAMP8 mice (Fig. 2G, H). The ratio of the Aβ immunoreactive area and Aβ protein levels in the 9-month-old SAMP8 mice were significantly increased compared to the age-matched SAMR1

mice ($p < 0.01$, Fig. 2D, H). These findings suggest that the increase in Aβ deposition in the hippocampus was observed from 7 to 9-months, which may be the early stage of AD progression in SAMP8 mice.

In addition, we examined the effect of aging on the loss of neurons in the hippocampal CA1 and CA3 regions in SAMP8 mice (Fig. 3). The number of NeuN-positive neurons in the CA1 and CA3 regions was significantly decreased with aging ($p < 0.05$, Fig. 3A-C). The NeuN-positive neurons in both regions in 9-month-old mice were significantly decreased compared to 3 or 7-month-old mice. This shows that the loss of age-related hippocampus neurons was clearly observed at 9-months-old ($p < 0.01$, Fig. 3B, C).

Therefore, we investigated the effects of physical exercise on hippocampal Aβ deposition, the loss of neurons, and the expression of BDNF in the early stage of AD progression. The animals in the Ex group had a significantly decreased ratio of the Aβ immunoreactive area in the hippocampus compared to the No-Ex group (Fig. 2I). However, there was no difference in the protein level of hippocampal Aβ between the Ex and No-Ex groups (Fig. 2J). The number of NeuN-positive neurons in the CA1 region was significantly increased in the Ex group compared to the No-Ex group ($p < 0.05$, Fig. 3A, E). However, the number of NeuN-positive neurons in the CA3 region was not significantly different between groups (Fig. 3F). In addition, the ratio of BDNF-positive neurons in the CA1 region was significantly increased in the Ex group compared to the No-Ex group ($p < 0.05$, Fig. 3D, I). However, the ratio of BDNF-positive neurons in the CA3 region was not significantly different between the groups (Fig. 3J). The protein level of hippocampal BDNF was increased in the

Ex group compared to the No-Ex group, but a quantitative analysis of the BDNF protein levels did not show significant differences between the groups (Fig. 3G, H).

3.3. Effects of aging and exercise on age-related inflammatory responses and M1 microglia and A1 astrocyte phenotype in the hippocampus of SAMP8 mice.

We investigated the effects of aging on inflammatory responses, including microglia and astrocyte activation, in the hippocampus of SAMP8 mice (Fig. 4). The Iba-1-immunoreactive microglia were significantly increased in the hippocampus of 9-months-old mice compared to that in 7-months-old and age-matched SAMR1 mice ($p < 0.05$, Fig. 4A, B). Similarly, the GFAP-immunoreactive astrocytes in the hippocampus significantly increased with aging ($p < 0.05$, Fig. 4A). The ratios of GFAP immunoreactivity were significantly increased at 9-months-old compared to those of 3-months-old, 7-months-old, and age-matched SAMR1 mice ($p < 0.05$, Fig. 4C).

Considering that Iba-1 positive microglia and GFAP-positive astrocyte cells were significantly increased in the hippocampus of 9-months-old SAMP8 mice, we examined the effects of physical exercise on microglia and astrocyte activation through the M1 and M2 microglia phenotype and A1 astrocyte phenotype. The ratio of Iba-1 positive microglia and the protein level of Iba-1 were significantly decreased in the Ex group compared to the No-Ex group ($p < 0.01$, Fig. 4D, F, H). In addition, the protein levels of M1 phenotype markers (TNF-α and iNOS) were significantly decreased in the Ex group compared to the No-Ex group ($p < 0.01$, Fig. 5A, B, D). In contrast, the protein level of the M2 phenotype marker arginase-1 was increased in the Ex group, but not significantly increased in the Ex group compared to the No-Ex group (Fig. 5A, C).

Furthermore, the ratio of GFAP-positive astrocytes and the protein level of GFAP were significantly decreased in the Ex group compared to the No-Ex group ($p < 0.05$, Fig. 4E, G, I). Previous studies have demonstrated that activated microglia induce neurotoxic A1 phenotype astrocytes. Therefore, we examined whether exercise could decrease the neurotoxic A1 phenotype activated astrocytes in the early stages of AD progression. Interestingly, the protein level of A1 phenotype markers (C3) was significantly decreased in the Ex group compared to the No-Ex group ($p < 0.001$, Fig. 6A, B). Immunofluorescence staining showed that the C3 positive cells co-localized with some GFAP-positive astrocytes (Fig. 5C-E). These findings suggest that exercise decreased the proinflammatory phenotype activated microglia and astrocytes, thereby attenuating hippocampal neuroinflammation in the early stage of AD progression.

3.4. Effects of exercise on oxidative stress in the hippocampus of SAMP8 mice.

Oxidative stress leads to oxidative damage in many cellular components. Therefore, we investigated the effects of physical exercise on oxidative stress using NOS activity in the hippocampus of SAMP8 mice (Fig. 5A). The Ex group showed significantly decreased protein levels of nNOS activity and iNOS activity compared to the No-Ex group ($p < 0.01$, Fig. 5D, F). However, the protein level of p-eNOS activity was not significantly different between groups (Fig. 5E), suggesting that exercise altered nNOS and iNOS activities in the hippocampus during the early stage of AD progression.

5. Discussion

It is well known that physical exercise is benefit for overall brain health, and is to prevent the risk of AD as well as cognitive decline with aging (Valenzuela et al., 2020). However, its mechanisms are still not fully understood. The present study indicated that SAMP8 showed AD pathogenesis, including an increase in Aβ deposition, loss of hippocampal neurons, microglia, and astrocyte activation mediated neuroinflammation, thereby decreasing behavioral and cognitive performance with aging. Furthermore, we found that low-intensity motor balance and coordination exercise improved AD pathogenesis in the hippocampus. Notably, exercise modulated not only the suppression of M1 phenotype microglia but also the suppression of A1 phenotype astrocytes in the hippocampus. These beneficial effects may be associated with the inhibition of Aβ accumulation and counteract the decline of age-related behavioral and cognitive performance in the early stages of AD.

Immunohistochemical studies have shown that SAMP8 mice have an age-related increase in Aβ deposition in the hippocampus (Takemura et al., 1993; Fukunari et al., 1994; Del Valle et al., 2010). In addition, microglia activation and induction of neuroinflammation was associated with the accumulated Aβ in the hippocampus in AD (Sarlus et al., 2017). Our immunohistochemical analysis showed that the increase in Aβ deposits was associated with an inflammatory response in the hippocampus with aging. Neuroinflammation promotes amyloid precursor protein (APP) expression and Aβ deposition (Lian et al., 2018). In addition, chronic neuroinflammation including microglia and astrocyte activations is a cause of AD progression and emerged in the brain before the Aβ deposits (Heneka et al., 2013; Olmos-Alonso et al., 2016). Our results showed that exercise reduced neuroinflammation and Aβ deposition in the hippocampus. Therefore, our findings suggest that

exercise produced significant neuroprotection of hippocampus and preserved behavioral and cognitive functions in the early stage of AD progression. However, the Aβ protein level results did not clearly indicate a difference between the Ex and No-Ex groups. The immunohistochemical analysis primarily examined the stratum radiatum of the hippocampus, whereas the western blot analysis assessed the overall hippocampus. Therefore, the contrasting results may be due to differences in the methods employed to examine protein expression.

Cognitive decline of AD is a consequence of loss of neurons in the hippocampus in AD model mice (Jiang et al., 2018). The hippocampal neuronal density in the CA1 and CA3 regions, especially in CA1 region, was obviously decreased in AD patients (Padurariu et al., 2012). Our results showed that the decrease in neurons in the CA1 and CA3 regions corresponded with behavioral alterations and cognitive dysfunction in SAMP8 mice. In addition, exercise improved these behavioral alterations and cognitive dysfunctions and suppressed neuronal loss in the CA1 and CA3 regions. Running exercise improved spatial learning and memory functions, reduced Aβ plaque in the hippocampus, delayed the loss of neurons, induced neurogenesis, and promoted the survival of newborn neurons in a mouse AD model (Chao et al., 2018; Valenzuela et al., 2020). Our results showed that exercise increased the number of BDNF-positive neurons in the CA1 and CA3 regions, especially CA1, which was significantly increased. Furthermore, exercise may help to facilitate BDNF expression and may play in the moderation of the relationship between BDNF and memory function (Loprinzi et al., 2019). Therefore, the exercise-induced increase in BDNF expression and suppression of neuronal loss in the hippocampus, especially in CA1, may be part of the important

structural basis of the improved behavioral and cognitive functions. However, neurons of the motor cortex are associated with behavioral and motor functions. Therefore, further studies are needed to investigate the pathological changes in the motor cortex.

The age-related activation of microglia and proinflammatory cytokine are reduced hippocampal neurogenesis (Ryan et al., 2016). In AD pathogenesis, microglial activation may play a dual role. Acute microglial activation leads to decreased Aβ accumulation by increasing phagocyte or clearance, and chronic microglial activation contributes to neurotoxicity and synapse loss by triggering several proinflammatory cascades (Sarlus et al., 2017). In addition, endogenous stimuli including Aβ and tau oligomers exit in the milieu that may persistently activate M1 pro-inflammatory responses and finally lead to irreversible neuron loss (Tang et al., 2016). Therefore, the shift of microglia from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype may have therapeutic potential targeting neuroinflammation in AD. Our results show that exercise suppressed the age-related activation of M1 phenotype microglia in the hippocampus. Exercise shifts activated microglia polarization to M2 phenotype anti-inflammatory responses in early AD progression (Zhang et al., 2019). Therefore, our results suggest that decreased M1 phenotype microglia contributed to the suppression of inflammatory damaged neurons. These anti-inflammatory responses may partially contribute to the overall beneficial effects of physical exercise on age-related neurodegeneration and cognitive dysfunction.

Astrocytes as well as microglia are secondary player in AD process and contribute to synaptic and neuronal loss (Grimaldi et al., 2019). Reactive astrocytes and activated microglia exist

surrounding Aβ plaques, implicating their role in AD pathogenesis (Cai et al., 2017). Neuroinflammation induces two different types of reactive astrocytes, termed 'A1' and 'A2', respectively (Liddelow et al., 2017). A1 reactive astrocytes might have 'harmful' functions, which promote proinflammatory, contrary, A2 reactive astrocytes might have 'helpful' functions, which promote survival and growth neurons through upregulated many neurotrophic factors (Liddelow et al., 2017). Microglial activations through pro-inflammatory cytokines such as TNF-α and IL1β can convert astrocytes into a neurotoxic A1 phenotype in AD (Grimadi et al 2019; Clark et al., 2019). Our findings showed that exercise reduced the protein level of complement C3, a marker of neurotoxic A1 phenotype astrocytes, which may be associated with reduced protein levels of TNF-α after exercise. Taken together, the present study demonstrated that exercise reduced hippocampal neuroinflammation by modulating M1 phenotype microglia and A1 phenotype astrocytes, which may contribute to the suppression of Aβ accumulation and hippocampal neuronal loss, thereby improving behavioral and cognitive functions.

The M1 phenotype pro-inflammatory state, characterized by production of inflammatory mediators and NO via upregulation of iNOS (Sarlus et al., 2017). In addition, reactive astrocytes release inflammatory mediators and induced oxidative stress, both of which are known to contribute to Aβ production and accumulation (Cai et al., 2017). Therefore, oxidative stress is a hallmark of AD pathogenesis. An increase in NOS activities with aging is believed to be responsible for increased astrogliosis in the hippocampus of SAMP8 mice (Han et al., 2010). Our findings suggest that exercise suppresses neuroinflammation in the hippocampus, which may be associated with attenuated NO

levels thorough the reduce of iNOS and nNOS, but not eNOS activity. As exercise training could improve age-induced microvascular changes with the upregulation of VEGF and eNOS (Viboolvorakul et al., 2014), the eNOS activity might not decrease by exercise in the hippocampus. Neuroinflammation and oxidative stress are key processes involved pathogenesis of AD and the process of aging (Smith et al., 2000; d'Avila et al., 2018). Therefore, the anti-oxidative effects of exercise likely contribute to exercise-induced neuroprotection and cognitive improvement.

In the exercise regimens, moderate and intense exercise programs induce benefits of exercise against AD (Valenzuela et al., 2020). On the other hand, acute exercise increased production of pro-inflammatory markers and apoptotic proteins in hippocampus of older mice (Packer et al., 2015). Strong evidence supports the benefits of endurance exercise for improving cognitive in patients with AD (Herold et al., 2019). However, it may be difficult to continuously moderate and intense exercise programs for older, frail adults. In addition, the training continuously at high intensity has been reported to results in pro-inflammatory status in human (Abkenar et al., 2019). Therefore, we selected a low-intensity exercise program. We chose the rotating rod motor exercise based on reports that it enhanced synaptic plasticity after stroke (Ding et al., 2004; Sakakima et al., 2012). Our findings suggest that low-intensity motor balance and coordination exercise as well as moderate-intensity exercise may be effective in suppressing Aβ deposition, neuroinflammation, neuron loss, and oxidative stress in the hippocampus during the early stage of AD progression. Therefore, low-intensity motor balance and coordination exercises may help improve behavioral and cognitive function in the early stages of AD.

A limitation of our study is that failure to consider helpful astrocytes (A2 phenotype astrocyte). It has been reported that A2 phenotypic astrocytes are expressed by ischemia, not inflammation (Liddelow et al., 2017). In addition, it remains unclear whether the A2 phenotypic astrocytes change with aging. However, this study focused on the effects of exercise on hippocampal inflammation in the early stages of AD. Our findings provide novel insights for further investigation on the effect of physical exercise in treatment of AD.

5. Conclusion

Our results demonstrate that regular low-intensity coordination exercise might prevent age-related behavioral alterations and cognitive dysfunction in early AD progression by suppressing Aβ deposition, neuroinflammation, neuronal loss, and NOS activities in the hippocampus. In addition, the present study demonstrated that exercise moderated not only the proinflammatory suppression of M1 phenotype microglia but also A1 phenotype astrocytes in hippocampal neuroinflammation. our findings provide novel insights for further investigation on the effect of physical exercise in treatment of AD.

Disclosure statements

The authors report no conflicts of interest in this work.

Acknowledgements

The authors greatly appreciate Ms. Hui Gao for providing technical assistance. This work was supported by a grant from the JSPS KAKENHI (grant no: JP20H04039) to Harutoshi Sakakima and JSPS Fellows (grant no: 20J14080) to Kazuki Nakanishi.

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Figure legends

Figure. 1 Effects of aging and exercise on the age-related behavioral decline and cognitive dysfunctions evaluated by the open-field test, rotarod task, and novel object recognition test in SAMP8 mice. The locomotion distance (A), the ratio of high-speed waking time (B), and the walking time in the rotarod task (C) significantly declined with age. Recognition memory was impaired in 9-months-old SAMP8 mice (D). The SAMP8 mice in the Ex group had significantly improved behavioral and cognitive function compared to the No-Ex group (E-H). Data are shown as the mean ± SE. **$p < 0.01$

Figure. 2 Effects of aging and exercise on Aβ depositions and protein levels in the hippocampus of SAMP8 mice. The unilateral hippocampus was quantitatively measured (A). Immunohistochemical staining showed that Aβ deposition was mainly observed as granules in the stratum radiatum of the hippocampus and significantly increased with aging. (B, C, E, F). Therefore, we examined the ratio of the Aβ immunoreactive area in the stratum radiatum of the hippocampus (square). The ratio of the Aβ immunoreactive area was significantly increased in 9-months-old mice (D). The ratio of the Aβ

immunoreactive area and the Aβ protein levels of the 9-months-old were significantly increased compared to that of age-matched SAMR1 mice (D, G, H). The Ex group had a significantly decreased ratio of the Aβ immunoreactive area in the hippocampus compared to the No-Ex group (I), but no significant difference was observed in the protein level (J). Py: pyramidal layer, Rad: stratum radiatum, LMol: lacunosum moleculare, DG: dentate gyrus. Data are shown as the mean ± SE. *p < 0.05, **p < 0.01. Scale bar = 500 μm (A) and 50 μm (B - F).

Figure. 3 Effects of aging and exercise on the hippocampal neurons and the expression of BDNF in the CA1 and CA3 regions of SAMP8 mice. The number of NeuN-positive neurons in the CA1 and CA3 regions were significantly decreased with aging (A, B, C). The number of NeuN-positive neurons in the CA1 regions of the Ex group was significantly increased compared to those of the No-Ex group (E). The ratios of BDNF-positive neurons in the CA1 region were significantly increased in the Ex group compared to the No-Ex group (D, I). However, the number of NeuN-positive neurons and BDNF-positive neurons in the CA3 region were not significantly different between the groups (F, J). The Ex group had increased protein levels of hippocampus BDNF compared to the No-Ex group, but no significant difference was observed (G, H). Data are shown as the mean ± SE. **p < 0.01, *p < 0.05. Scale bar = 200 μm (A) and 50 μm (D).

Figure. 4 Effects of aging and exercise on microglia response and astrocytosis in the hippocampus of SAMP8 mice. The Iba-1-immunoreactive microglia (A, B) and the GFAP-immunoreactive

astrocytes (A, C) in the hippocampus were significantly increased in 9-months-old SAMP8 mice. In the Ex group, the ratio of Iba-1 positive microglia (D) and the protein level of Iba-1 (F, H) were significantly decreased compared to the No-Ex group. Furthermore, the ratio of GFAP positive astrocyte (E) and the protein level of GFAP (G, I) were significantly decreased in the Ex group compared to the No-Ex group. Data are shown as the mean ± SE. **$p < 0.01$, *$p < 0.05$. Scale bar = 50 μm (A).

Figure. 5 Effects of exercise on the M1 and M2 microglia phenotype markers and oxidative stress in the hippocampus of SAMP8 mice. The protein levels of M1 phenotype markers (TNF-α, iNOS) were significantly decreased in the Ex group compared to the No-Ex group (A, B, D). In contrast, the protein level of the M1 phenotype marker (arginase-1) was increased in the Ex group, but no significant difference was observed (A, C). The Ex group displayed significantly decreased protein levels of nNOS and iNOS activity compared to the No-Ex group (A, D, F). However, the protein level of the p-eNOS activity was not significantly different between the groups (A, E). Data are shown as the mean ± SE. **$p < 0.01$

Figure. 6 Effects of exercise on A1 phenotype astrocytes in the hippocampus of SAMP8 mice. The protein level of A1 phenotype markers (C3) was significantly decreased in the Ex group compared to the No-Ex group (A, B). Immunofluorescence staining showed that the C3 positive cells were co-

localized with some GFAP-positive astrocytes (C-D). Data are shown as the mean ± SE. **p < 0.01. Scale bar = 25 μm (all panels).