

Exercise is more effective than diet control
in preventing high fat diet-induced
 β -amyloid deposition and memory deficit in
amyloid precursor protein transgenic mice

(APP トランスジェニックマウスに
おいて、運動は食事改善よりも
高脂肪食による $A\beta$ の沈着および
学習記憶の悪化を改善する)

前迫 真人

Exercise is more effective than diet control in preventing high fat diet-induced β -amyloid deposition and memory deficit in amyloid precursor protein transgenic mice

Masato Maesako¹, Kengo Uemura², Masakazu Kubota¹, Akira Kuzuya², Kazuki Sasaki¹, Naoko Hayashida¹, Megumi Asada-Utsugi¹, Kiwamu Watanabe², Maiko Uemura², Takeshi Kihara¹, Ryosuke Takahashi², Shun Shimohama³ and Ayae Kinoshita^{1*}

¹From the School of Human Health Sciences, Kyoto University Graduate School of Medicine, Kyoto 606-8507, ²Department of Neurology, Kyoto University Graduate School of Medicine, Kyoto 606-8507, ³Department of Neurology, Sapporo Medical University, Sapporo 060-8556

Running Title: Exercise is more effective than diet control in APP-HFD mice

Address correspondence to: Ayae Kinoshita,
School of Human Health Sciences, Kyoto University Graduate School of Medicine, 53,
Shogoinkawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.
Tel. / Fax: 81-75-751-3969; E-mail: akinoshita@hs.med.kyoto-u.ac.jp

Keywords: Alzheimer disease, β -amyloid, high fat diet, exercise, diet control, neprilysin

Background: Exercise and diet control are fundamental approaches to metabolic conditions caused by high fat diet (HFD).

Results: HFD-induced memory deficit and A β deposition was more ameliorated in the exercise- than in the diet control-induced mice.

Conclusion: Exercise was more effective than diet control in preventing HFD-induced AD development.

Significance: Exercise has the highest priority in the prevention of AD.

Summary

Accumulating evidence suggests that some dietary patterns, specifically high fat diet (HFD), increase the risk of developing sporadic Alzheimer disease (AD). Thus, interventions targeting HFD-induced metabolic dysfunctions may be effective in preventing the development of AD. We previously demonstrated that amyloid precursor protein (APP)-overexpressing transgenic mice fed HFD showed worsening of cognitive function compared to control APP mice on normal diet. Moreover, we reported that voluntary exercise ameliorates HFD-induced memory impairment and β -amyloid (A β) deposition. In the present study, we conducted diet control to ameliorate the metabolic abnormality caused by HFD on APP transgenic mice and compared the effect of diet control on cognitive function to that of voluntary exercise as well as that of combined (diet control plus exercise) treatment.

Surprisingly, we found that exercise was more effective than diet control, although both exercise and diet control ameliorated HFD-induced memory deficit and A β deposition. The production of A β was not different between the exercise- and the diet control-treated mice. On the other hand, exercise specifically strengthened the activity of neprilysin, the A β degrading enzyme, the level of which was significantly correlated with that of deposited A β in our mice. Notably, the effect of the combination treatment (exercise and diet control) on memory and amyloid pathology was not significantly different from that of exercise alone. These studies provide solid evidence that exercise is a useful intervention to rescue HFD-induced aggravation of cognitive decline in transgenic model mice of AD.

Alzheimer disease (AD), the most common cause of dementia, is poised to be a significant public health crisis. The occurrence of AD is largely sporadic, typically affecting individuals aged over 65 years. Amyloid plaque is one of the pathological hallmarks of AD. Amyloid plaques are composed of β -amyloid (A β), which are derived from the amyloid precursor protein (APP) via proteolytic cleavages by β - and γ -secretases. A widely accepted hypothesis about AD pathogenesis is the amyloid cascade hypothesis in which A β plays a crucial role in neurodegeneration [1]. Importantly,

recent studies have implied that soluble A β oligomers may be the main culprit of AD pathogenesis [2-4].

High fat diet (HFD) is prevalent in modern society and HFD-induced metabolic condition is becoming a worldwide issue since it leads to obesity, type 2 diabetes mellitus (T2DM) and hypercholesterolemia. More importantly, accumulating evidence suggests that some dietary patterns, specifically HFD, increase the risk of developing sporadic AD [5]. Experimental studies also support this idea. For example, application of HFD for APP transgenic mice exacerbated pathological alterations in the brain and their memory deficits [6, 7]. In contrast, it is widely known that composite dietary patterns such as the Mediterranean diet, characterized by high intake of vegetables, unsaturated fatty acids and wine, are related to lower risk for AD [8]. These reports clearly indicate that there is an association between metabolic conditions and a higher risk of sporadic AD.

Exercise and diet control are fundamental approaches in the treatment of metabolic conditions. They might also become useful ways to prevent the development of AD. For example, a prospective study revealed that physical activity is protective against the development of cognitive impairment in AD and that the highest activity group showed a lowered incidence of AD [9]. Recently, we also demonstrated that voluntary exercise ameliorates HFD-induced memory deficit and A β deposition in APP transgenic mice [7], indicating that interventions to reduce metabolic conditions can become the preventive method for AD. However, it still remains unknown what kind of intervention targeting metabolic conditions is more effective.

In the present study, we used the AD model mice with metabolic conditions through HFD (APP-HFD mice), which we had previously established [7]. In addition to voluntary exercise, we preventively conducted diet control or the combination of diet control with exercise using APP-HFD mice, followed by comparing the effect of these interventions on cognitive function and amyloid pathology. Here, we show that diet control improved metabolic conditions including hyperinsulinemia and hypercholesterolemia of APP-HFD mice better than exercise. However, exercise more effectively ameliorated HFD-induced memory deficit and A β deposition

than diet control (in spite of higher serum insulin/cholesterol levels). Exercise specifically enhanced the activity of neprilysin, which we speculate may be responsible for the reduction of the A β level. These results clearly indicated that exercise, affecting the process of A β degradation, could be a more effective way to ameliorate the AD progression caused by metabolic dysfunctions than diet control.

Experimental Procedure

Animals and dietary conditions

Human APP transgenic mice overexpressing the familial AD-linked mutations bearing both Swedish (K670N/M671L) and Indiana (V717F) mutation (APPSwe/Ind) [10], which have been imported from the Jackson Laboratory (USA) were used in the present study. They were maintained as heterozygotes and male and female mice were housed separately. Age- and sex-matched (1:1, male: female) mice were exposed to either a standard diet (10% fat, 70% carbohydrate, and 20% protein, Oriental Yeast Co., Ltd., Japan) or an established high fat diet (HFD) (caloric composition, 60% fat, 20% carbohydrate, and 20% protein, Research Diet, Inc., Canada) for 20 weeks, from 2-3 to 7-8 months age. To examine the effect of voluntary exercise (Ex) on APP transgenic mice fed HFD (APP-HFD mice), the cage of the mice was changed to a 2.4 times larger one equipped with a running wheel as well as objects after 10 weeks of HFD (APP-HFD+Ex mice) [7]. The mice spent 10 weeks in the exercise condition in the presence of HFD. To examine the effect of diet control (Dc) on APP-HFD mice, HFD was replaced to standard diet after 10 weeks of HFD and fed with standard diet for another 10 weeks (APP-HFD+Dc mice). To examine the effect of the combinatory intervention of Ex and Dc, the mice were spent 10 weeks in the exercise condition in the presence of standard diet after 10 weeks of HFD (APP-HFD+Ex+Dc mice). After the dietary manipulation, metabolic changes in these mice were analyzed, followed by the assessment of memory function through the Morris water maze test, as described below. After the analysis of memory function, the brains were extracted and were cut sagittally into left and right hemispheres. The left hemisphere was fixed in 4% paraformaldehyde for histological analysis. After removing the

olfactory lobe and cerebellum, the right hemisphere was rapidly frozen in liquid nitrogen for biochemical analysis. All animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals of the Kyoto University.

Assessment of metabolic changes

Blood glucose content was measured by using LabAssay Glucose (Wako, Japan). To assess glucose intolerance in these mice, we conducted the intra-peritoneal glucose tolerance test (IGTT). Mice were given a single dose of intra-peritoneal injection of glucose (2 g/kg body weight) after 14 hours fasting, and blood was collected from the tail-vein periodically over 2 hours (fasting, 30 min, 60 min and 120 min). Plasma insulin concentration was measured by enzyme-linked immunosorbent assay (ELISA) kit specific to insulin (Morinaga Seikagaku, Japan). Plasma concentrations of total cholesterol, high density lipoprotein (HDL)-cholesterol and triglyceride were measured by using cholesterol E-Wako, HDL-cholesterol E-Wako and triglyceride E-Wako (Wako, Japan).

Morris water maze test

In order to assess spatial navigation learning and memory retention, the Morris water maze test was conducted. Initially, animals received a habituation trial during which the animals were allowed to explore the pool of water (diameter 120 cm, height 25 cm, temperature $21 \pm 1^\circ\text{C}$) without the platform present.

Visual cue phase. Following habituation, visible platform training was performed to measure motivation and swimming speed of mice to find a platform. Briefly, distal cues were removed from around the pool, and the platform was labeled with a flag and placed 1 cm above the surface of the water in the center of a quadrant. Mice were placed in the maze and allowed to explore the maze for 60 sec, and if they reached the visible platform, they were allowed to remain there for 20 sec before being returned to their cages. If they did not find the platform within 60 sec, the experimenter led them to the platform and let them remain there for 20 sec. Animals were trained in groups of five, and training was completed once each animal received six trials. This training was performed for 1 day.

Acquisition phase. We measured the ability of mice to understand the spatial relationship between a safe, but invisible platform of 10 cm in diameter (submerged 1 cm below the water level), and visual cues surrounding the maze. The platform was located in the center of one of the four quadrants, and several extramaze cues were distributed across the walls surrounding the pool. During the acquisition phase of training, each mouse received four daily hidden platform training trials with 10-12 min intervals for 5 consecutive days. Animals were allowed 60 sec to locate the platform and 20 sec to rest on it. Mice that failed to find the platform were led there by the experimenter and allowed to rest there for 20 sec.

Probe trial phase. 24 hours following the last acquisition trial, a single 60 sec probe trial was administered to assess spatial memory retention. For the probe trial, animals were returned to the pool without the platform present, and parameters were recorded to assess the ability of the mouse to remember the previous location of the platform.

Performance was recorded with an automated tracking system (TARGET series/2, Japan) during all phases of training. During the visual cue phase of training, speed and latency to the platform were used to compare the activity of the performance between each group. During the acquisition phase, acquisition time (latency to reach the platform) and path length (total distance travelled) were subsequently used to analyze and compare the performance between different treatment groups. The time to the platform quadrant, and the number of entries into the target quadrant were recorded and analyzed during the probe trials.

Immunoblotting and filter trap assay

For immunoblotting analysis, the brain was taken and rapidly frozen using liquid nitrogen. The brain samples from the cerebrum of the male mice were extracted in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X100, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, pH 8.0) with protease inhibitor cocktail (Roche) and sufficiently homogenized on ice. Then the samples were incubated for one night at 4°C and centrifuged at 14,000 g for 20 min. The

supernatants were directly used for Western blot analysis. The detailed protocol has been described previously [11]. 5-20 % polyacrylamide gradient gels (Atto) were used to detect full length APP, β -actin, neprilysin and insulin-degrading enzyme. Both 5-20 % polyacrylamide gradient gels (Atto) and 4-12% NuPAGE Bis-Tris gel (Invitrogen) were used to detect APP CTFs. Mouse monoclonal anti- β -actin and rabbit polyclonal anti-APP C-terminal antibodies were from SIGMA. Rabbit polyclonal anti-neprilysin and mouse monoclonal anti-insulin degrading enzyme antibodies were from Abcam.

Filter trap assay was conducted as described previously [7, 12]. Briefly, the protein concentration of the cerebrum samples in Tris-buffered saline (TBS)-extracted fraction was measured and an equal amount of protein was subjected to vacuum filtration through a 96-well dot blot apparatus (Bio-Rad Laboratories) containing 200 nm pore-sized nitrocellulose membrane. The resultant membrane was then incubated with primary antibody at 4°C overnight. The membrane was then blocked by TBS containing 4% skim milk, and incubated with HRP-linked secondary antibody (GE Healthcare; diluted 1:2000) for 1 hour. The membrane was developed using the ECL Western Blotting Analysis System (GE Healthcare). Anti-A β oligomer antibody (A11, Invitrogen) was used for the detection of A β oligomer in TBS soluble fraction.

Immunohistochemistry

The paraformaldehyde-fixed and paraffin-embedded tissue sections of male mice were incubated with primary antibodies. The sections were then incubated with biotinylated anti-second IgG antibody (1:2,000; Vector Laboratories), followed by the incubation with avidin peroxidase (ABC Elite kit; 1:4,000; Vector Laboratories). Subsequently, the labeling was visualized by incubation with 50 mM Tris-HCl buffer (pH 7.6) containing 0.02% 3,3-diaminobenzidine and 0.0045% hydrogen peroxide. All images were visually analyzed using a microscope, ECLIPSE 80i (Nikon Corporation). For each animal, the sections of the hippocampus were captured then were imported into Image J, and an intensity threshold level was set that allowed for discrimination between the antigen and background labeling. Anti-A β (6E10) antibody (1:1,000; SIGMA) was

used for the detection of A β plaque.

Neprilysin activity assay

The proteolytic activity of neprilysin was measured as described previously with minor modifications [13]. Briefly, the brain samples from the cerebrum of the male mice were extracted in RIPA buffer and protein concentrations were analyzed. 100 μ g of brain lysates were incubated with 50 μ M the substrate 3-dansyl-D-Ala-Gly-p-(nitro)-Phe-Gly (DAGNPG) (SIGMA) and 1 μ M captopril, angiotensin converting enzyme (ACE) inhibitor, in 200 μ l of 50 mM Tris-HCl buffer (pH 7.6) for 1 hr at 37°C. Reactions were stopped by heating samples to 100°C for 5 min, followed by 5000 g x 5 min centrifugation. The 180 μ l of supernatant was diluted into 400 μ l of 50 mM Tris-HCl buffer (pH 7.6) and fluorescence was determined using Infinite 200 PRO (Tecan Japan Co., Ltd.) (excitation 342 nm, emission 562 nm).

Statistical analysis

All values are given in means $\pm$ SE. Comparisons were performed using an unpaired Student's t-test. For comparison of multiparametric analysis, one-way factorial ANOVA, followed by the post hoc analysis by Fisher's PLSD was used. Statistical significance of differences between mean scores during acquisition phase of training in the Morris water maze test was assessed with two-way repeated-measures ANOVA (general linear model/RM-ANOVA) and Fisher's post hoc analysis for multiple comparisons. $p < 0.05$ was considered to indicate a significant difference.

Results

Effects of diet control on the metabolic conditions of APP-HFD mice

In our recent literature, voluntary exercise is shown to be a useful tool for preventing the progress of cognitive dysfunction and amyloid pathology in APP-HFD mice [7]. Notably, voluntary exercise does not ameliorate HFD-induced hyperinsulinemia or hypercholesterolemia but improves glucose intolerance as well as cognitive impairment. Diet therapy is another way to control metabolic dysfunctions. Thus, in the present study, we aimed to elucidate which environmental factor contributes more to metabolic and cognitive functions in APP-HFD mice by controlling the diet (Dc; diet control, APP-HFD+Dc) or

combined diet control with exercise (Ex; exercise, APP-HFD+Ex+Dc) after mice were fed HFD for 10 weeks. Then, we compared the effects of these interventions on cognitive function and A β pathology (Fig. 1A).

As reported previously, APP-HFD mice gained significantly more body weight than the control APP mice and after the introduction of voluntary exercise APP-HFD+Ex mice gained less body weight than APP-HFD mice [7]. On the other hand, the body weight decrease of APP-HFD+Dc and APP-HFD+Ex+Dc mice was much more drastic than that of APP-HFD+Ex mice (Fig. 1B), showing that diet control was more effective in body weight reduction than voluntary exercise. Weekly monitoring of food intake showed that the food intake of the APP-HFD+Dc and APP-HFD+Ex+Dc mice did not change or mildly increased (supplemental Fig. 1), indicating that the diet control-mediated attenuation of body weight was not caused by the reduction of food intake. Fasting serum glucose level was not different among APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice, being significantly lower in the three groups than that in APP-HFD mice (Fig. 1C. pre). In addition, the result of IGTT indicated that the impaired glucose tolerance was improved in the APP-HFD+Ex as well as APP-HFD+Dc and APP-HFD+Ex+Dc mice (Fig. 1C). To examine whether diet control reverses or prevents the development of glucose intolerance, we conducted IGTT 10 weeks after HFD introduction (at the time point when exercise or diet control was started). The fasting glucose level and glucose tolerance of APP-HFD+Dc and APP-HFD+Ex+Dc mice were better than those of APP mice 10 weeks after HFD introduction (supplemental Fig. 2). Therefore, diet control reversed glucose intolerance as well as exercise did [7]. The plasma insulin level of APP-HFD+Dc and APP-HFD+Ex+Dc mice was significantly decreased compared with that of APP-HFD mice (Fig. 1D). The plasma lipid analyses indicated that both total and HDL-cholesterol were significantly decreased in APP-HFD+Dc and APP-HFD+Ex+Dc mice (Fig. 2A and B), suggesting that diet control ameliorated HFD-induced hypercholesterolemia. The level of plasma triglycerides was not different among them (Fig. 2C).

Exercise was more effective in ameliorating memory deficit of APP-HFD mice than diet control

To compare the effect of diet control on HFD-induced memory deficit with that of exercise, we conducted the Morris water maze test. The locomotor activity of APP mice was not affected by HFD, HFD+Ex, HFD+Dc or HFD+Ex+Dc as exemplified by swimming speed (supplemental Fig. 3). During the acquisition phase, APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice showed a daily improvement in their acquisition time. However, APP-HFD+Dc mice took longer time to reach the goal than APP-HFD+Ex mice did (Fig. 3A). In the probe trial phase, the time to get to the platform quadrant in APP-HFD+Dc mice was significantly longer than that in APP-HFD+Ex mice (Fig. 3B). Moreover, the number to cross the previous location of the platform in APP-HFD+Dc mice was significantly smaller than that in APP-HFD+Ex mice (Fig. 3C). These results demonstrated that APP-HFD+Dc mice took a longer time to get to the platform quadrant and failed to cross the previous location of the platform compared to APP-HFD+Ex mice. From these results, we concluded that exercise was the more effective way of ameliorating HFD-induced memory dysfunction than diet control in APP-HFD mice.

The A β pathology of APP-HFD mice was ameliorated better by exercise than by diet control

Since ample A β deposition is a critical hallmark of AD, we compared the effect of diet control on HFD-induced A β accumulation with that of exercise. For this, we conducted immunohistochemical analysis using anti-A β (6E10) antibody to quantitatively examine A β deposition. As shown in Fig. 4A, A β deposition was aggravated by feeding HFD [7], whereas marked reduction of HFD-induced A β deposition was observed in APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice. Interestingly, the level of deposited A β in APP-HFD+Ex was significantly lower than that in APP-HFD+Dc mice (Fig. 4B). Therefore, exercise was more effective in reducing A β accumulation than diet control.

Increasing reports show that the level of TBS-soluble A β oligomers correlate with memory deficits in AD model mice [2-4]. We showed that HFD increases the level of soluble A β oligomers in APP mice, which is reduced by voluntary exercise [7]. We further extended this result by comparing the amount of A β oligomers in the above three conditions. The filter trap analysis indicated that the levels of A β oligomers in the APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice were all significantly decreased, compared with the level in the APP-HFD mice (Fig. 4C and D). Remarkably, the level of A β oligomers in the APP-HFD+Ex mice was statistically lower than that in APP-HFD+Dc mice. Thus, exercise plays a much more significant role than diet control in APP-HFD mice in modulating not only A β deposition but also the level of A β oligomers. This is in line with the result of behavioral experiments.

HFD-enhanced APP processing was equally inhibited by exercise and by diet control

The above results led us to wonder whether the level of A β in our mice was regulated by APP processing or by A β degradation in exercise/diet control conditions. In order to examine how these environmental conditions affected the HFD-induced A β pathology, we first investigated the effect on APP processing in both conditions and compared the result of exercise with that of diet control. For this, we analyzed the level of APP C-terminus fragments through immunoblotting assay using anti-APP C-terminal antibody. APP is cleaved by α - and β -secretases at the extramembrane domain, which produce APP-CTF α and CTF β respectively. APP-CTF α and CTF β were further cleaved by γ -secretase at the intramembrane domain, producing p3 and A β respectively. In the present study, the level of full length APP was not different among control-APP, APP-HFD, APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice (Fig. 5A and B). Moreover, the change of fully glycosylated form was not observed among them. On the other hand, the level of APP-CTF β in the APP-HFD mice was higher than that in the control APP, APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice. Notably, the level of APP-CTF β in APP-HFD+Ex was the same as that in APP-HFD+Dc mice (Fig. 5A

and C), indicating that both exercise and diet control inhibited β -secretases-mediated APP cleavage. This tendency was also observed in the level of APP-CTF α (Fig. 5A and D).

Neprilysin activity was up-regulated by exercise

Next, we examined the effect of environmental intervention on A β degradation process in APP-HFD mice. A β -degrading proteases, including neprilysin, were reported to degrade A β both in vitro and in vivo [14, 15]. To compare the effect of exercise on the degradation of A β with that of diet control, we analyzed the enzymatic neprilysin activity. As shown in Fig. 6A, the activity of neprilysin had a tendency to be suppressed by HFD although it did not reach statistical significance. More importantly, the enzymatic activity of neprilysin in APP-HFD+Ex mice was significantly higher than that in APP-HFD+Dc mice. On the other hand, the neprilysin activity in APP-HFD+Dc mice was the same as that in APP-HFD mice. These results indicated that exercise could up-regulate the enzymatic activity of neprilysin. To clarify whether the up-regulation of neprilysin depended on its expression level, we conducted immunoblotting assay. As shown in supplemental Fig. 4, the levels of neprilysin were not different among APP-HFD, APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice, although those were slightly larger than that in control APP mice. In addition, we examined the expression levels of insulin-degrading enzyme, another A β -degrading protease, and indicated that its levels were also not different among APP-HFD, APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice. In order to confirm the effect of neprilysin activity on the A β deposition in our mice, we conducted correlation analysis between neprilysin activity and the level of A β plaque. The activity of neprilysin was significantly correlated with the level of A β deposition (Fig. 6B).

Discussion

HFD is prevalent in modern society and HFD-induced metabolic conditions are becoming a worldwide issue. Notably, increasing reports have suggested that diet and nutrition are important epigenetic factors for the development of sporadic AD [16-18]. Several epidemiological studies have reported that people with a higher body mass index (BMI) during midlife are at greater risk for developing AD [19-21] and that obesity is

associated with lower brain volumes in patients with mild cognitive impairment (MCI) or AD [22]. Obesity promotes a cascade of pathological conditions including T2DM and dyslipidemia. Remarkably, several drugs targeting T2DM and hypercholesterolemia have been shown to improve cognitive performance in mouse models of AD as well as in patients with early AD [23-26]. In our experiment, serum glucose, serum insulin and serum cholesterol levels were positively correlated with the result of Morris test in APP-HFD mice, confirming that both T2DM and hypercholesterolemia could deteriorate memory deficit in APP mice (supplemental Fig. 5). However, serum glucose level was correlated with memory function in APP-HFD mice better than serum cholesterol and serum insulin levels. In addition, we previously demonstrated that exercise in our experimental condition does not improve HFD-induced hyperinsulinemia and hypercholesterolemia but ameliorates glucose intolerance [7]. Therefore, the intervention to hyperglycemia and glucose intolerance might be important for the prevention of AD.

Magkos et al. reviewed that first-line intervention to metabolic dysfunctions involves lifestyle modifications including diet control and physical activity, and that metabolic dysfunctions may be reversible if addressed early on. They proposed that long-term engagement in lifestyle changes may result in the resolution of such dysfunctions [27]. However, there has been no solid evidence on whether diet control or exercise is more effective in the prevention of AD. Therefore, in the present study, we aimed to compare the effect of diet control on metabolic dysfunctions as well as AD pathology with that of exercise using APP-HFD mice. Our present study clearly revealed that exercise ameliorated HFD-induced memory impairment better than diet control (Fig. 3). Exercise is reported to enhance neurogenesis and results in increased numbers of synapses per neuron [28]. In addition, exercise increases the expression of the brain-derived neurotrophic factor (BDNF), which regulates neuronal development as well as plasticity [29]. In this sense, exercise might specifically induce 'cognitive reserve' which increases cognitive function and enhances complex mental activity as protective factors against dementia [reviewed in 30]. On the other hand, we showed that exercise decreased

the level of soluble A β oligomers as well as deposited A β more than diet control (Fig. 4). Thus, the exercise-induced inhibition of A β oligomers might be involved in better cognitive performance in APP-HFD mice, since the level of soluble A β oligomers is known to correlate with memory deficits due to their synaptotoxicity [2-4]. In line with our present result, Hu et al. previously showed that exercise reduces oligomeric A β levels in the cortex and hippocampus of AD model mice [28]. However, there are a couple of differences between our experimental conditions and theirs. Our mice were fed HFD but their mice were given a standard diet. Moreover, we analyzed oligomeric A β levels after onset of A β deposition but they examined them before A β accumulation. Therefore, our results clearly supported that exercise reduced oligomeric A β levels after onset of A β deposition even if the mice were fed with HFD.

The level of A β within the brain is determined by the balance between its production and its degradation. Since A β is generated by the proteolytic processing of APP, we first examined whether exercise or diet control was acting directly on A β production. Our detail immunoblotting analysis showed that the level of APP CTF β in APP-HFD+Ex mice was almost the same as that in APP-HFD+Dc mice (Fig. 5), indicating that the production of A β might not be different between APP-HFD mice treated with exercise and those treated with diet control. On the contrary, the enzymatic activity of neprilysin, the A β degrading enzyme, was up-regulated in APP-HFD+Ex mice more than in APP-HFD+Dc mice (Fig. 6). Lazarov et al. have reported that voluntary exercise elevates neprilysin activity in the brain of APP transgenic mice, contributing to the lowering of A β levels [31], which is in line with our findings. However, their result is different from ours in that their APP mice which were allowed voluntary exercise, were fed a standard diet. Intriguingly, we found that feeding HFD itself reduced the activity of neprilysin. We further observed that exercise strengthened neprilysin activity even if the mice were fed with HFD. In the present study, the activity of neprilysin was negatively correlated with the level of deposited A β (Fig. 6). Therefore, we assumed that exercise-mediated up-regulation of neprilysin may critically reduce HFD-induced A β

deposition. Importantly, the expression level of neprilysin did not change between the exercise- and the diet control-treated APP-HFD mice (supplemental Fig. 4). Since the activity of neprilysin was clearly up-regulated by exercise only, we concluded that exercise could modulate its activity in an expression level independent manner. We speculate that exercise may specifically modulate the up-stream molecules of neprilysin or may regulate posttranslational modification of neprilysin. The mechanism of neprilysin up-regulation by exercise should be clarified in the future studies.

As classified in Fig. 7A, our result, for the first time, clarified the differential effects of diet control and exercise on metabolic and cognitive dysfunctions. According to our data, diet control significantly improved HFD-induced metabolic conditions, including obesity, hyperinsulinemia and hypercholesterolemia (Fig. 1 and 2), compared with exercise. However, exercise decreased A β oligomers as well as deposited A β (Fig. 4) and ameliorated memory impairment (Fig. 3) compared with diet control. From these results, we conclude that exercise was more effective than diet control in the prevention of HFD-induced amyloid pathology. In Fig. 7B, we present our hypothesis on how diet control and exercise differently ameliorate HFD-induced A β deposition and memory deficit in APP transgenic mice. As described in our previous literature, HFD leads to glucose intolerance and hyperglycemia, which may lead to the up-regulation of β -secretase activity. This up-regulation increases soluble A β oligomers as well as deposited A β levels, followed by memory

deficit [7]. The up-regulation of β -secretase was also reported in HFD-feeding mice from another laboratory [32], and consistently reported in AD cases by several previous reports [33-35]. Thus, this phenomenon might represent the actual pathology of sporadic AD. On the other hand, both diet control and exercise ameliorate HFD-induced glucose intolerance and hyperglycemia, thereby decreasing A β load by inhibiting A β production. However, exercise specifically strengthens the enzymatic activity of neprilysin, which decreases the level of A β in the brain. Surprisingly, the effect of the combination treatment (exercise and diet control) on cognitive function and amyloid pathology was not significantly different from that of exercise only, indicating that exercise is an effective behavioral intervention sufficient to inhibit A β pathology. We suppose this is because exercise affects both the production and the degradation of A β , but diet control modifies only the production of A β . Therefore, for the introduction of intervening metabolic functions targeting the prevention of AD, we provide the first evidence-based comparison of effective interventions, concluding that exercise has the highest priority. Although the beneficial effect of exercise was obtained even under HFD, the magnitude and the nature (i.e. voluntary vs. forced, aerobic vs. anaerobic) of the exercise required to prevent HFD-induced AD progression should be elucidated in future studies. Since metabolic dysfunctions are epidemiologically considered to be risk factors of sporadic AD, evidence-based interventions for metabolic dysfunctions should be carried out to prevent AD.

Footnotes: We appreciate to Dr. S. Oka (Kyoto University, Kyoto, Japan), Mr. J. Morise (Kyoto University, Kyoto, Japan), Dr. Y. Kitamura (Kyoto Pharmaceutical University, Kyoto, Japan) and Dr. K. Takata (Kyoto Pharmaceutical University, Kyoto, Japan) for technical assistance of the brain sample preparation. We give our thanks to Dr. K. Saito (Kyoto University, Kyoto, Japan) for kind support of the Morris water maze test. We greatly appreciate the advice for metabolic analysis to Dr. M. Okuda (PHARMAEIGHT, Kyoto, Japan). We would like to give our thanks to Dr. Y. Tashiro (Kyoto University, Kyoto, Japan) for kind discussion. The work was financially supported by Core Backup Stage Grant from Kyoto University (AK), Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (KU, 23591243) and the Research Grant from Takeda Science Foundation (KU).

¹To whom correspondence should be addressed: School of Human Health Sciences, Kyoto University Graduate School of Medicine, 53, Shogoinkawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. Tel./Fax: 81-75-751-3969; E-mail: akinoshita@hs.med.kyoto-u.ac.jp

²The abbreviations used are: AD, Alzheimer Disease; HFD, high fat diet; APP, amyloid precursor protein; A β , β -amyloid; Ex, exercise; Dc, diet control; CTF, C-terminus fragments

References

1. FINDER, V. H. (2010) *J Alzheimers Dis* **22 Suppl 3**, 5-19
2. WALSH, D. M., KLYUBIN, I., FADEEVA, J. V., CULLEN, W. K., ANWYL, R., WOLFE, M. S., ROWAN, M. J., and SELKOE, D. J. (2002) *Nature* **416**, 535-539
3. LESNE, S., KOH, M. T., KOTILINEK, L., KAYED, R., GLABE, C. G., YANG, A., GALLAGHER, M., and ASHE, K. H. (2006) *Nature* **440**, 352-357
4. SHANKAR, G. M., LI, S., MEHTA, T. H., GARCIA-MUNOZ, A., SHEPARDSON, N. E., SMITH, I., BRETT, F. M., FARRELL, M. A., ROWAN, M. J., LEMERE, C. A., REGAN, C. M., WALSH, D. M., SABATINI, B. L., and SELKOE, D. J. (2008) *Nat Med* **14**, 837-842
5. LUCHSINGER, J. A., TANG, M. X., SHEA, S., and MAYEUX, R. (2002) *Arch Neurol* **59**, 1258-1263
6. HO, L., QIN, W., POMPL, P. N., XIANG, Z., WANG, J., ZHAO, Z., PENG, Y., CAMBARERI, G., ROCHER, A., MOBBS, C. V., HOF, P. R., and PASINETTI, G. M. (2004) *FASEB J* **18**, 902-904
7. MAESAKO, M., UEMURA, K., KUBOTA, M., KUZUYA, A., SASAKI, K., ASADA, M., WATANABE, K., HAYASHIDA, N., IHARA, M., ITO, H., SHIMOHAMA, S., KIHARA, T., and KINOSHITA, A. (2012) *Neurobiol Aging* **33**, 1011.e11-23
8. SCARMEAS, N., STERN, Y., TANG, M. X., MAYEUX, R., and LUCHSINGER, J. A. (2006) *Ann Neurol* **59**, 912-921
9. LAURIN, D., VERREAU, R., LINDSAY, J., MACPHERSON, K., and ROCKWOOD, K. (2001) *Arch Neurol* **58**, 498-504
10. MUCKE, L., MASLIAH, E., YU, G. Q., MALLORY, M., ROCKENSTEIN, E. M., TATSUNO, G., HU, K., KHOLODENKO, D., JOHNSON-WOOD, K., and MCCONLOGUE, L. (2000) *J Neurosci* **20**, 4050-4058
11. MAESAKO, M., UEMURA, K., KUZUYA, A., SASAKI, K., ASADA, M., WATANABE, K., ANDO, K., KUBOTA, M., KIHARA, T., and KINOSHITA, A. (2011) *J Biol Chem* **286**, 25309-25316
12. KITAGUCHI, H., TOMIMOTO, H., IHARA, M., SHIBATA, M., UEMURA, K., KALARIA, R. N., KIHARA, T., ASADA-UTSUGI, M., KINOSHITA, A., and TAKAHASHI, R. (2009) *Brain res* **1294**, 202-210
13. ROSE, J. B., CREWS, L., ROCKENSTEIN, E., ADAME, A., MANTE, M., HERSH, L. B., GAGE, F. H., SPENCER, B., POTKAR, R., MARR, R. A., and MASLIAH, E. (2009) *J Neurosci* **29**, 1115-1125
14. IWATA, N., TSUBUKI, S., TAKAKI, Y., WATANABE, K., SEKIGUCHI, M., HOSOKI, E., KAWASHIMA-MORISHIMA, M., LEE, H. J., HAMA, E., SEKINE-AIZAWA, Y., and SAIDO, T. C. (2000) *Nat Med* **6**, 143-150
15. SHIROTANI, K., TSUBUKI, S., IWATA, N., TAKAKI, Y., HARIGAYA, W., MARUYAMA, K., KIRYU-SEO, S., KIYAMA, H., IWATA, H., TOMITA, T., IWATSUBO, T., and SAIDO, T. C. (2001) *J Biol Chem* **276**, 21895-21901
16. SOLFRIZZI, V., PANZA, F., and CAPURSO, A. (2003) *J Neural Transm* **110**, 95-110
17. PANZA, F., CAPURSO, C., D'INTRONO, A., COLACICCO, A. M., DEL PARIGI, A., SERIPA, D., PILOTTO, A., CAPURSO, A., and SOLFRIZZI, V. (2006) *J Am Geriatr Soc* **54**, 1963-1965
18. SCARMEAS, N., LUCHSINGER, J. A., MAYEUX, R., and STERN, Y. (2007) *Neurology* **69**, 1084-1093
19. KIVIPELTO, M., NGANDU, T., FRATIGLIONI, L., VIITANEN, M., KAREHOLT, I., WINBLAD, B., HELKALA, E. L., TUOMILEHTO, J., SOININEN, H., and NISSINEN, A. (2005) *Arch Neurol* **62**, 1556-1560
20. WHITMER, R. A., GUNDERSON, E. P., QUESENBERRY, C. P., JR., ZHOU, J., and YAFFE, K. (2007) *Curr Alzheimer Res* **4**, 103-109
21. FITZPATRICK, A. L., KULLER, L. H., LOPEZ, O. L., DIEHR, P., O'MEARA, E. S., LONGSTRETH, W. T., JR., and LUCHSINGER, J. A. (2009) *Arch Neurol* **66**, 336-342
22. HO, A. J., RAJI, C. A., BECKER, J. T., LOPEZ, O. L., KULLER, L. H., HUA, X., LEE, S., HIBAR, D., DINOV, I. D., STEIN, J. L., JACK, C. R., JR., WEINER, M. W., TOGA, A. W., and THOMPSON, P. M. (2010) *Neurobiol Aging* **31**, 1326-1339
23. WATSON, G. S., CHOLERTON, B. A., REGER, M. A., BAKER, L. D., PLYMATE, S. R., ASTHANA, S., FISHEL, M. A., KULSTAD, J. J., GREEN, P. S., COOK, D. G., KAHN, S. E., KEELING, M. L., and CRAFT, S. (2005) *Am J Geriatr Psychiatry* **13**, 950-958
24. PEDERSEN, W. A., MCMILLAN, P. J., KULSTAD, J. J., LEVERENZ, J. B., CRAFT, S., and HAYNATZKI, G. R. (2006) *Exp Neurol* **199**, 265-273

25. Reger, M. A., Watson, G. S., Green, P. S., Wilkinson, C. W., Baker, L. D., Cholerton, B., Fishel, M. A., Plymate, S. R., Breitner, J. C., DeGroodt, W., Mehta, P., and Craft, S. (2008) *Neurology* **70**, 440-448
26. Shepardson, N. E., Shankar, G. M., and Selkoe, D. J. (2011) *Arch Neurol* **68**, 1239-1244
27. Magkos, F., Yannakoulia, M., Chan, J. L., and Mantzoros, C. S. (2009) *Annu Rev Nutr* **29**, 223-256
28. Hu, Y. S., Xu, P., Pigino, G., Brady, S. T., Larson, J., and Lazarov, O. (2010) *FASEB J* **24**, 1667-1681
29. Neeper, S. A., Gomez-Pinilla, F., Choi, J., and Cotman, C. (1995) *Nature* **373**, 109
30. Tucker, A. M., and Stern, Y. (2011) *Curr Alzheimer Res* **8**, 354-360
31. Lazarov, O., Robinson, J., Tang, Y. P., Hairston, I. S., Korade-Mirnics, Z., Lee, V. M., Hersh, L. B., Sapolsky, R. M., Mirnics, K., and Sisodia, S. S. (2005) *Cell* **120**, 701-713
32. Thirumangalakudi, L., Prakasam, A., Zhang, R., Bimonte-Nelson, H., Sambamurti, K., Kindy, M. S., and Bhat, N. R. (2008) *J Neurochem* **106**, 475-485
33. Fukumoto, H., Cheung, B. S., Hyman, B. T., and Irizarry, M. C. (2002) *Arch Neurol* **59**, 1381-1389
34. Yang, L. B., Lindholm, K., Yan, R., Citron, M., Xia, W., Yang, X. L., Beach, T., Sue, L., Wong, P., Price, D., Li, R., and Shen, Y. (2003) *Nat Med* **9**, 3-4
35. Li, R., Lindholm, K., Yang, L. B., Yue, X., Citron, M., Yan, R., Beach, T., Sue, L., Sabbagh, M., Cai, H., Wong, P., Price, D., and Shen, Y. (2004) *Proc Natl Acad Sci U S A* **101**, 3632-3637

Figure legends

Fig. 1. Diet control ameliorated HFD-induced obesity and diabetic conditions compared with exercise

(A) Schematic presentation of the interventions targeting metabolic conditions. As described previously [7], APPSwe/Ind mice were maintained with a standard diet in standard laboratory cages until 2–3 months old. Then, age- and sex-matched mice were separated into 5 groups. In the control group, the mice were induced with a standard diet in standard laboratory cages for 20 weeks (control APP mice) (top row, n = 9). In the HFD-induced group, mice were fed HFD in standard laboratory cages for 20 weeks (APP-HFD mice) (2nd row, n = 10). In the HFD with exercise-induced group, mice spent 10 weeks in standard laboratory cages, and then spent 10 weeks in enrichment cages with HFD (Ex: exercise; APP-HFD+Ex mice) (3rd row, n = 8). As a novel intervention, in the diet-control-induced group, after 10 weeks of HFD, we used a standard diet for another 10 weeks (Dc: diet control; APP-HFD+Dc mice) (4th row, n = 7). In the combination group with exercise plus diet control, mice spent 10 weeks in standard laboratory cages with HFD and then spent 10 weeks in enrichment cages with a standard diet (APP-HFD+Ex+Dc mice) (bottom row, n = 7). After 20 weeks, metabolic conditions of these mice were analyzed, followed by ethological, histochemical and biochemical analyses targeting AD pathophysiology.

(B) Relative body weight changes over 20 weeks. The body weight 2 weeks before each diet was regarded as the baseline (100%). Diet control and its combination with exercise significantly inhibited the HFD-induced increase of body weight.

(C) Blood glucose levels during glucose tolerance test after an intra-peritoneal injection of glucose (2 g/kg body weight). The fasting glucose level and glucose tolerance in APP-HFD+Dc mice ($F_{(4,159)} = 26.49$, $p < 0.001$) and APP-HFD+Ex+Dc mice ($p < 0.001$) were clearly improved.

(D) Serum insulin levels during fasting. The serum insulin levels in APP-HFD+Dc mice ($F_{(4,36)} = 9.3$, $p = 0.003$) and APP-HFD+Ex+Dc mice ($p < 0.001$) were significantly decreased compared with that in APP-HFD mice.

Fig. 2. Diet control ameliorated HFD-induced lipid dysfunction compared with exercise

(A) Plasma total cholesterol levels. The total cholesterol levels in APP-HFD+Dc mice ($F_{(4,36)} = 30.29$, $p < 0.001$) and APP-HFD+Ex+Dc mice ($p < 0.001$) were significantly decreased compared with the level in APP-HFD mice.

(B) Plasma HDL cholesterol levels. The HDL cholesterol levels in APP-HFD+Dc mice ($F_{(4, 36)} = 30.96$, $p < 0.001$) and APP-HFD+Ex+Dc mice ($p < 0.001$) were significantly decreased compared with the level in APP-HFD mice.

(C) Plasma triglyceride levels. There was no difference among control, APP-HFD, APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice ($F_{(4, 36)} = 1.65$, n.s.).

Fig. 3. Exercise ameliorated HFD-induced memory deficit compared with diet control

(A) Escape latency in the acquisition phase. APP-HFD+Ex mice took shorter time to the platform than APP-HFD+Dc mice.

(B) The time to the target quadrant in the probe trial phase. APP-HFD+Ex mice took shorter time to the platform than APP-HFD+Dc mice ($F_{(4, 36)} = 23.03$, $p = 0.041$).

(C) The number of entries into the target quadrant in the probe trial phase. APP-HFD+Dc mice were significantly impaired in the number of times they crossed the platform compared with APP-HFD+Ex mice ($F_{(4, 36)} = 13.59$, $p = 0.013$).

Fig. 4. Exercise ameliorated HFD-induced A β accumulation compared with diet control

(A) Immunohistochemical analysis using anti-A β (6E10) antibody. Representative images of A β -immunostained hippocampus sections from control APP, APP-HFD, APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc induced mice, respectively. Scale bar, 2 mm

(B) Cerebral A β loads determined by immunohistochemical and morphometric analyses. The cerebral A β deposition was significantly decreased in APP-HFD+Ex mice compared with that in APP-HFD+Dc mice ($F_{(4, 15)} = 18.35$, $p = 0.039$).

(C) The amount of A β oligomers in the TBS-soluble fractions of control-APP, APP-HFD, APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice analyzed by filter trap assay using anti-A β oligomer (A11) antibody. As described in [12], the A β monomer and low weight oligomers passed through the membrane pore (200 nm pore-sized) and high weight oligomers were detected in this assay.

(D) Statistical analysis of dot density. The average band density of the control APP samples was regarded as 100% and that of other groups was relatively indicated. The relative density of APP-HFD+Ex mice was significantly decreased compared with that of APP-HFD+Dc mice ($F_{(4, 10)} = 47.42$, $p = 0.011$).

Fig. 5. Both diet control and exercise reduced APP CTF β accumulation

(A) Immunoblotting analysis of APP full length, APP CTF α and CTF β . APP full length and APP CTFs (CTF α and CTF β) were detected by anti-APP C-terminus antibody. β -actin was detected as loading control. Long exposure indicated that a same film was exposed longer time. The mark of * indicates glycosylated APP full length. To analyze APP CTFs in detail, two kinds of gels (5-20 % polyacrylamide gradient gels and 4-12% NuPAGE Bis-Tris gels) were used. Unfortunately, we could not clarify the mobility of CTFs bands caused by phosphorylation presumably because of the gel conditions of our experiment.

(B) Statistical analysis of APP full length. The band of APP full length was normalized by that of β -actin. The band density of the control was regarded as 100% and that of other groups was relatively indicated. There was no statistically significant difference among control APP, APP-HFD, APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc mice ($F_{(4, 10)} = 0.47$, n.s.).

(C) Statistical analysis of APP CTF β . The band of APP CTF β was normalized by that of APP full length. The band density of APP CTF β in APP-HFD mice was increased compared with that in control APP mice. However, the band density of APP CTF β in APP-HFD+Ex ($F_{(4, 10)} = 4.27$, $p = 0.003$), APP-HFD+Dc ($p = 0.021$) and APP-HFD+Ex+Dc ($p = 0.023$) mice were significantly decreased compared with that in APP-HFD mice. There was no difference between APP CTF β in APP-HFD+Ex mice and that in APP-HFD+Dc mice.

(D) Statistical analysis of APP CTF α . The band of APP CTF α was normalized by that of APP full length. The band density of APP CTF α in APP-HFD mice was increased compared with that in control APP mice. The band density of APP CTF α in APP-HFD+Ex ($F_{(4, 10)} = 4.36$, $p = 0.034$), APP-HFD+Dc ($p = 0.014$) and APP-HFD+Ex+Dc ($p = 0.024$) mice were significantly decreased

compared with that in APP-HFD mice. There was no difference between APP CTF α in APP-HFD+Ex mice and that in APP-HFD+Dc mice.

Fig. 6. Exercise specifically rescued the HFD-induced deterioration of neprilysin activity

(A) In vitro enzyme activity assay of neprilysin using the fluorescence substrate. The activity of neprilysin in APP-HFD mice tends to be decreased compared with that in control APP mice ($F_{(4, 15)} = 5.58$, $p = 0.061$). On the other hand, neprilysin activity in APP-HFD+Ex mice was significantly higher than that in APP-HFD ($p = 0.023$) or that in APP-HFD+Dc mice ($p = 0.032$).

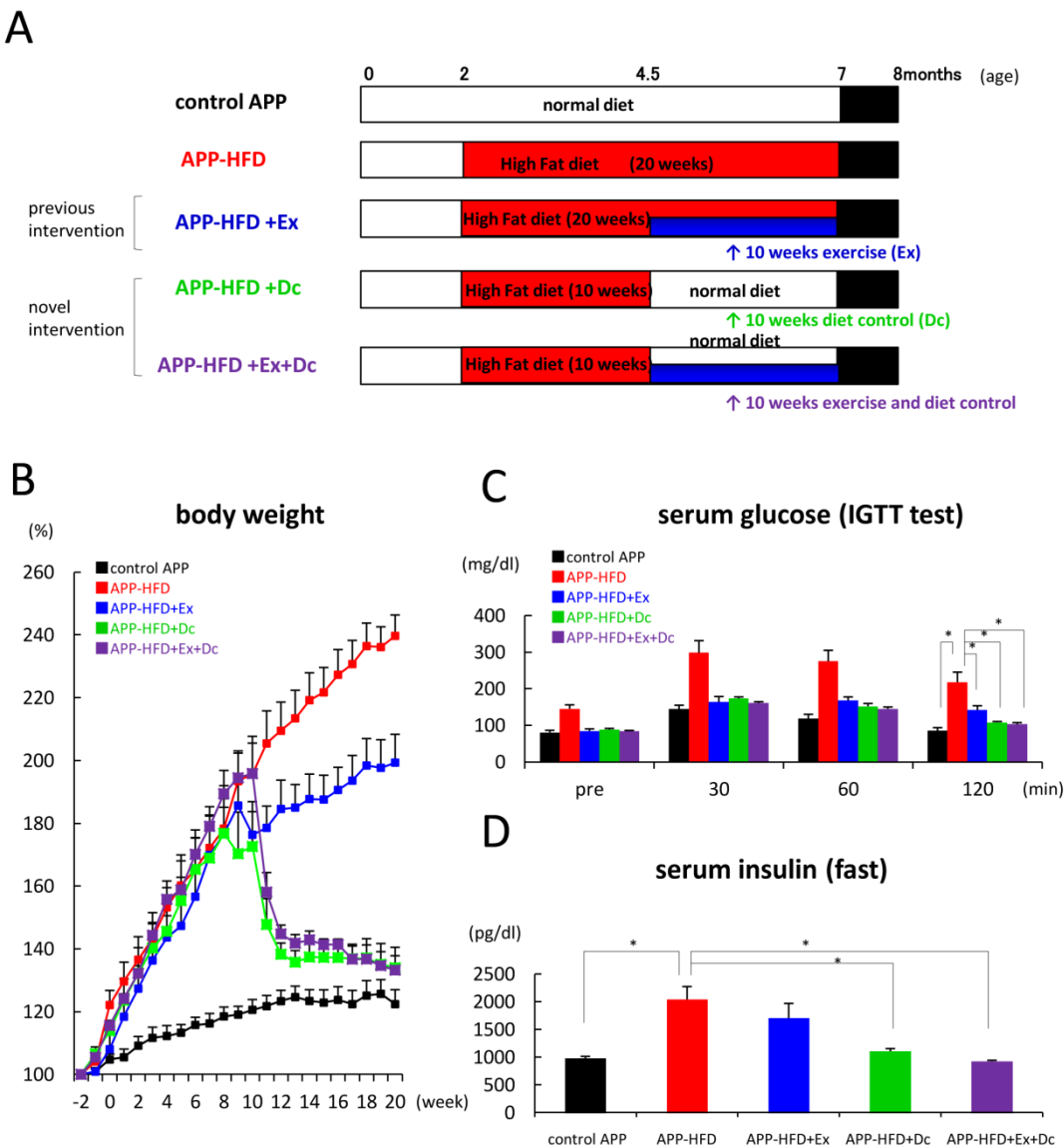
(B) Significant correlation was established by comparing the activity of neprilysin and the level of cerebral A β deposition, using Pearson's correlation coefficients. The activity of neprilysin was negatively correlated with the level of accumulated A β ($r = -0.782$, $p = 0.00003$).

Fig. 7. Schematic presentation of our study

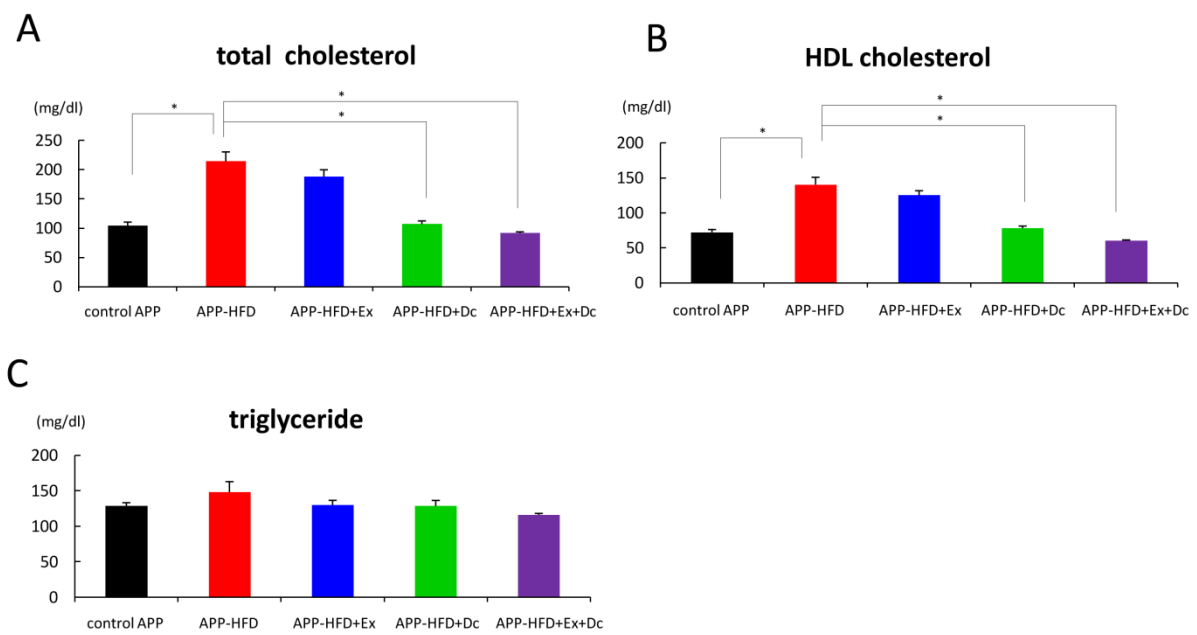
(A) The classification of the results in the present study. The items we analyzed in this report are included in the left lane. On the other hand, the effect of amelioration is showed in the right lane. As shown in this chart, diet control > exercise indicated that diet control ameliorated better than exercise. Diet control significantly improved HFD-induced metabolic conditions, including obesity, hyperinsulinemia and hypercholesterolemia, better than exercise. However, exercise decreased soluble A β oligomers as well as deposited A β and ameliorated memory impairment better than diet control.

(B) Schematic presentation of our hypothesis – how diet control or exercise ameliorated HFD-induced memory deficits and A β accumulation. HFD leads to glucose intolerance and hyperglycemia, which may lead to the up-regulation of β -secretase activity. This up-regulation increases soluble A β oligomers as well as deposited A β levels, followed by memory deficit [7]. On the other hand, both diet control and exercise ameliorate HFD-induced glucose intolerance and hyperglycemia, thereby decreasing soluble A β oligomer and fibrillar A β levels by inhibiting A β production. However, exercise specifically strengthens the enzymatic activity of neprilysin, which degrades A β in the brain.

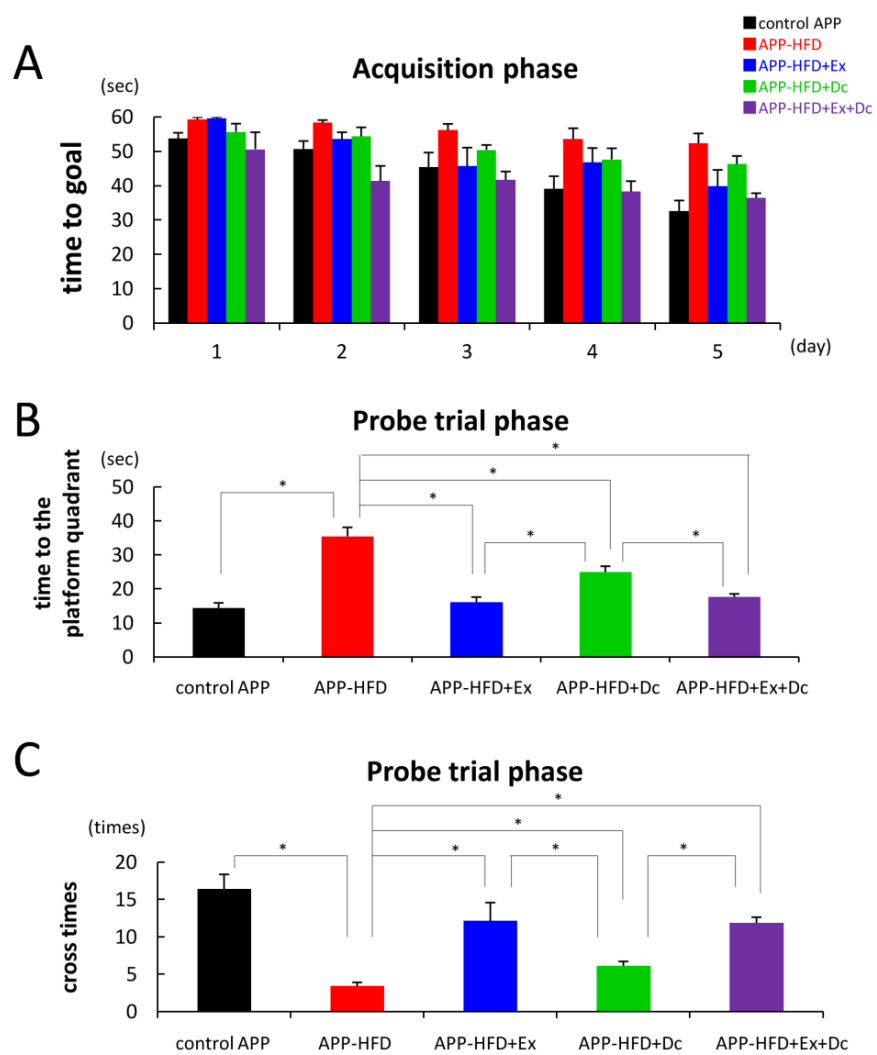
Maesako et al. Fig. 1

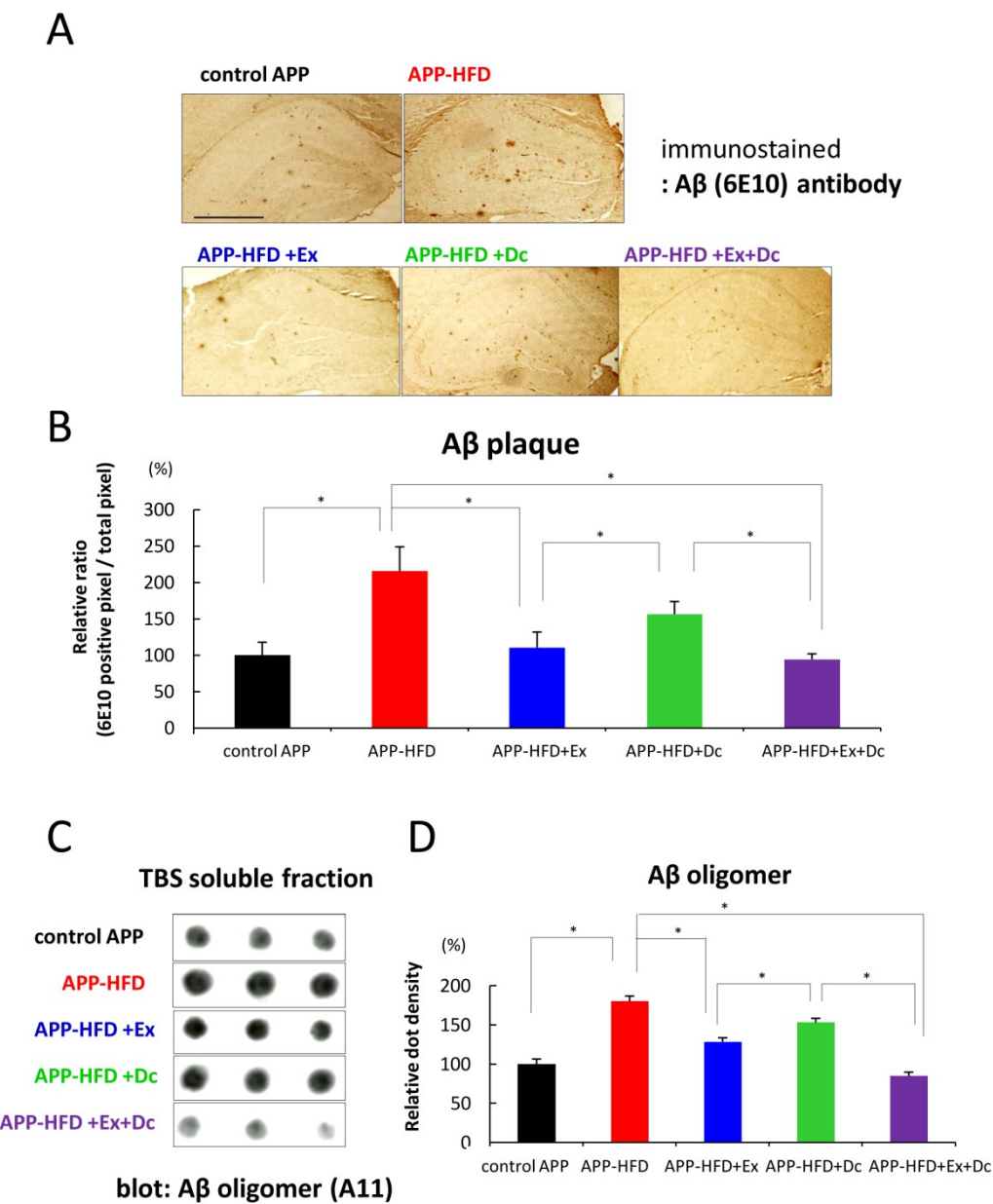


Maesako et al. Fig. 2

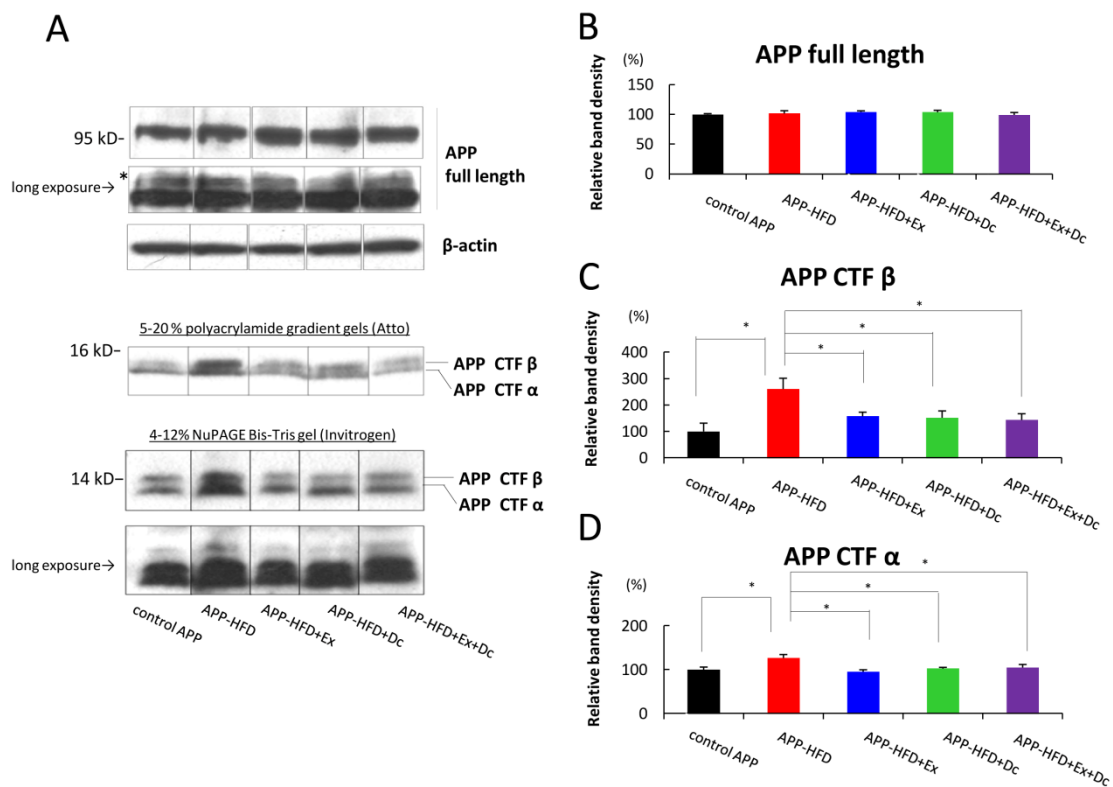


Maesako et al. Fig. 3

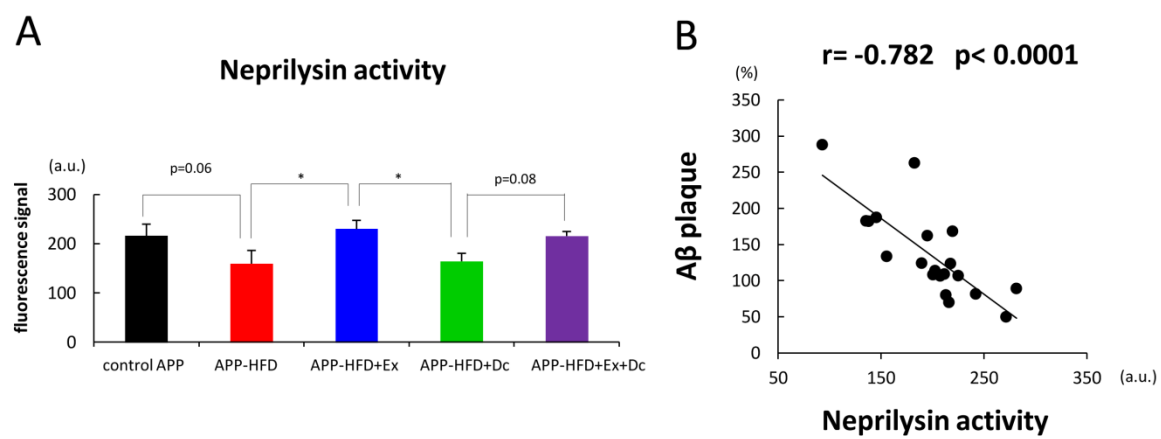




Maesako et al. Fig. 5



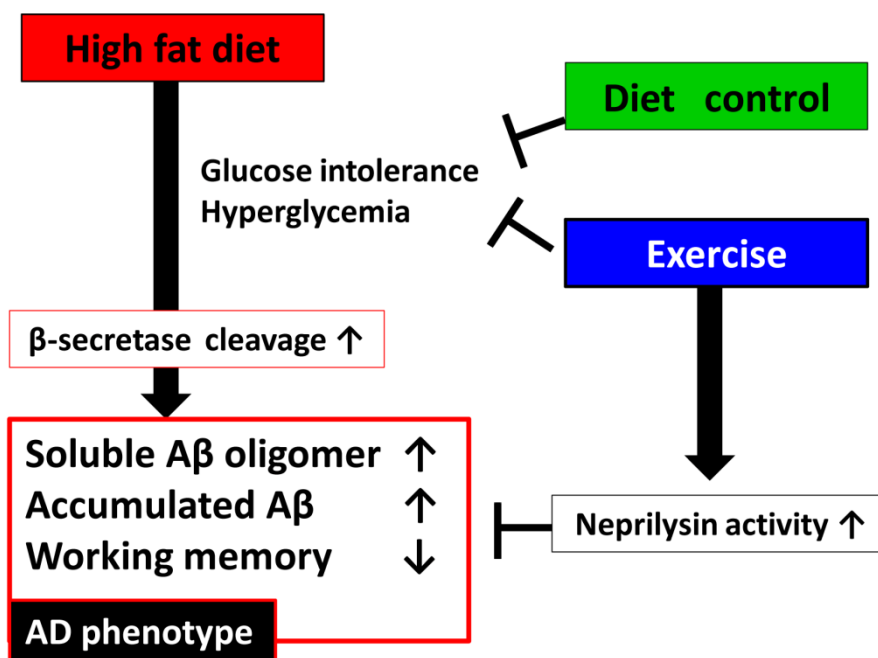
Maesako et al. Fig. 6



A

metabolic conditions	effect of amelioration
obesity	Diet control > Exercise
glucose intolerance/hyperglycemia	Diet control = Exercise
hyperinsulinemia	Diet control > Exercise
hypercholesterolemia	Diet control > Exercise
AD phenotype	effect of amelioration
memory deficit	Diet control < Exercise
A β accumulation	Diet control < Exercise

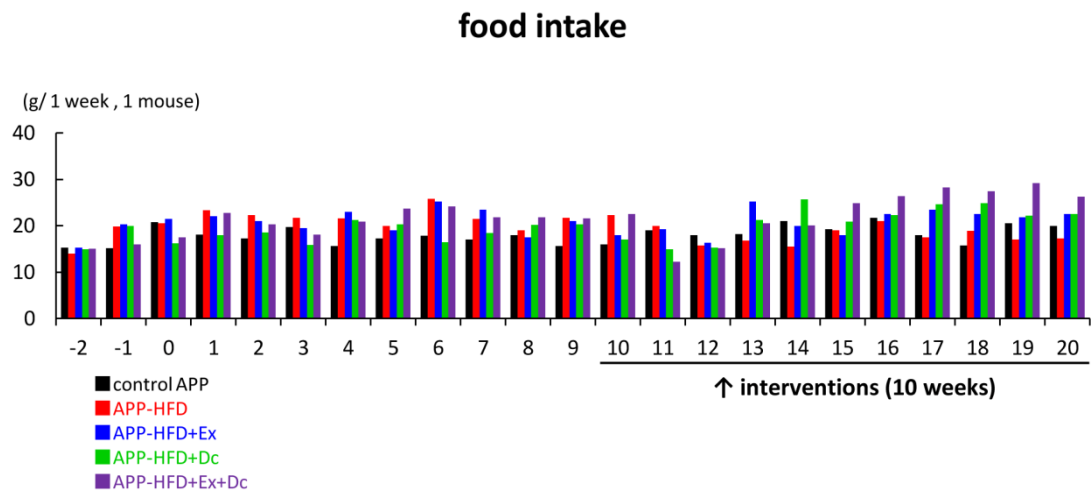
B



Maesako et al., Supplemental Figure

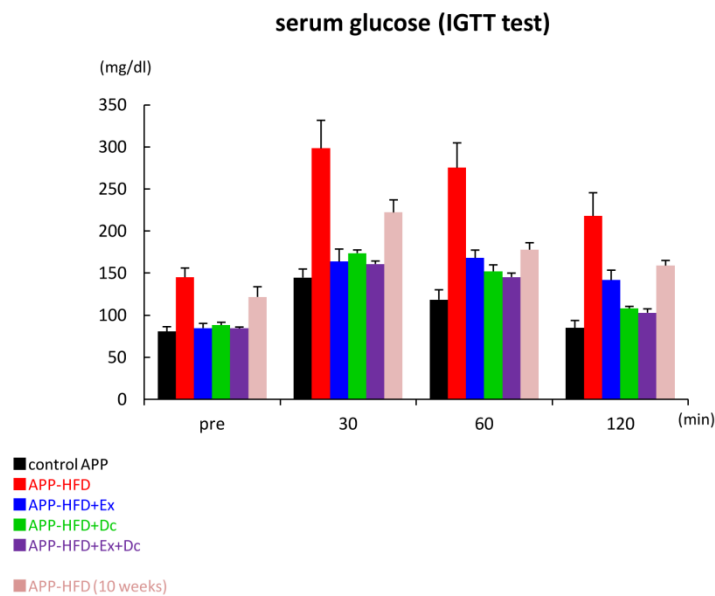
Supplemental Fig. 1. Every week monitoring of the amount of food intake.

The amount of food intake in APP-HFD+Dc and APP-HFD+Ex+Dc mice were monitored every week. After the induction of exercise or diet control, APP-HFD+Dc and APP-HFD+Ex+Dc mice tended to have more food than APP-HFD mice did.



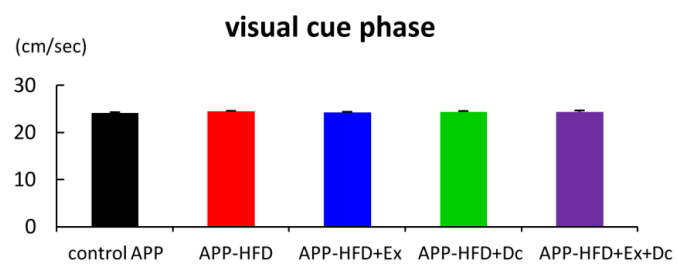
Supplemental Fig. 2. Comparison of glucose tolerance activities of APP-HFD+Dc and APP-HFD+Ex +Dc mice with 10 weeks HFD-induced APP mice by IGTT.

After 10 weeks induction of HFD (at the time of the switch from the poor environment to the EE), the mice were given a single dose of intra-peritoneal injection of glucose (2 g/kg body weight) after 14 hours fasting, and blood was collected from the tail-vein periodically over 2 hours. Blood glucose content was measured by using LabAssay Glucose (Wako, Japan). The glucose tolerance of the mice at the time of the switch from the normal to the exercise condition was worse than that of APP-HFD+Dc and APP-HFD+Ex+Dc mice.



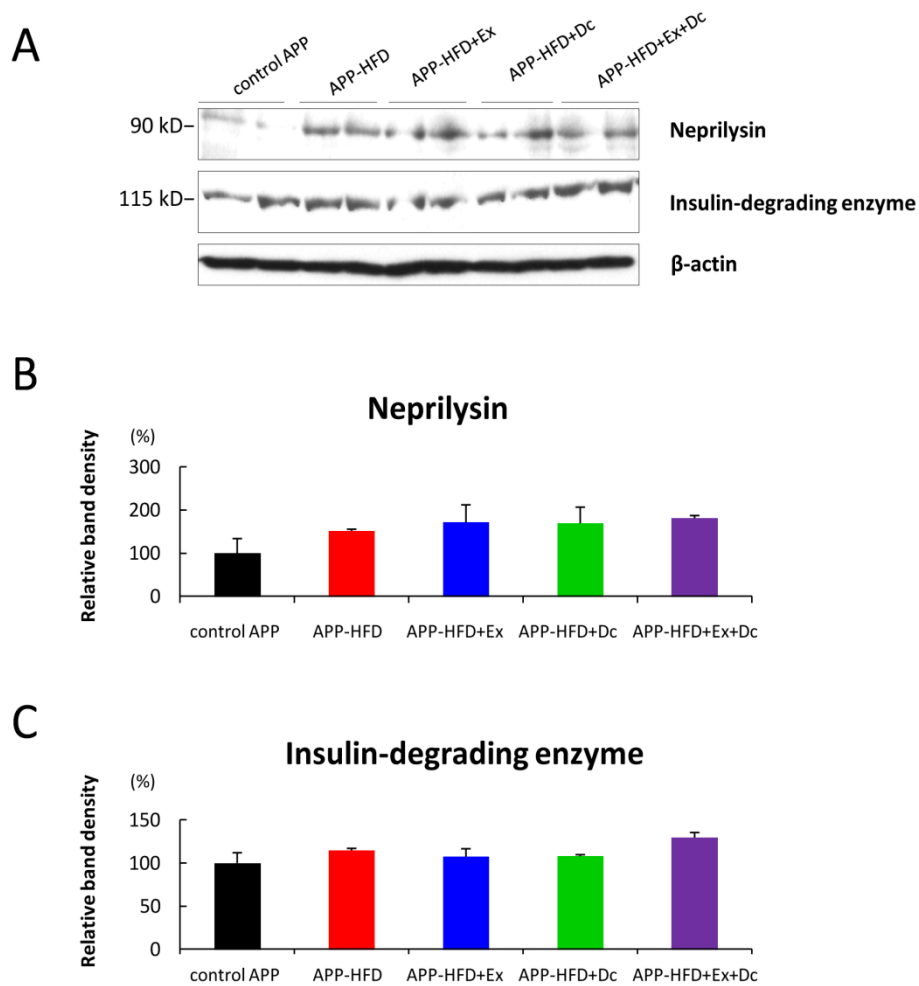
Supplemental Fig. 3. Locomotor activities in Morris water maze test.

Locomotor activity in the visual cue phase of Morris water maze test. There was no statistical significance among control APP, APP-HFD, APP-HFD+Ex, APP-HFD+Dc and APP-HFD+Ex+Dc induced mice.



Supplemental Fig. 4. **The expression levels of neprilysin and insulin-degrading enzyme.**

Immunoblotting analysis for the contents of neprilysin and insulin-degrading enzyme. The expression levels of neprilysin and insulin-degrading enzyme were not different between APP-HFD+Ex and APP-HFD+Dc mice.



Supplemental Fig. 5. Correlation analysis between memory function and metabolic conditions in control APP and APP-HFD mice.

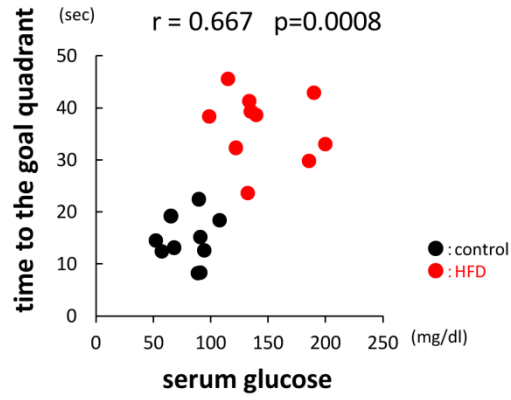
Memory function was assessed by the score of the probe trial phase of Morris water maze test (time to the goal quadrant). The longer time to the goal quadrant indicates that the memory function of mice is disrupted.

A. Significant correlation was established by comparing the scores of the probe trial phase of Morris water maze test (time to the goal quadrant) and the blood glucose levels, using Pearson's correlation coefficients. The time to the goal quadrant was positively correlated with the blood glucose level ($r = 0.667$, $p = 0.0008$).

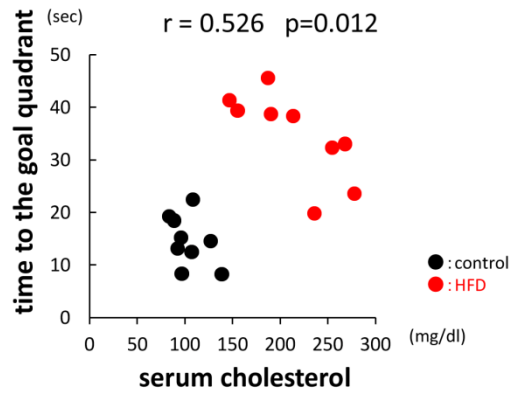
B. Significant correlation was established by comparing the scores of the probe trial phase of Morris water maze test (time to the goal quadrant) and the blood cholesterol levels, using Pearson's correlation coefficients. The time to the goal quadrant was positively correlated with the blood cholesterol level ($r = 0.526$, $p = 0.012$).

C. Significant correlation was established by comparing the scores of the probe trial phase of Morris water maze test (time to the goal quadrant) and the blood insulin levels, using Pearson's correlation coefficients. The time to the goal quadrant was positively correlated with the blood insulin level ($r = 0.433$, $p = 0.045$).

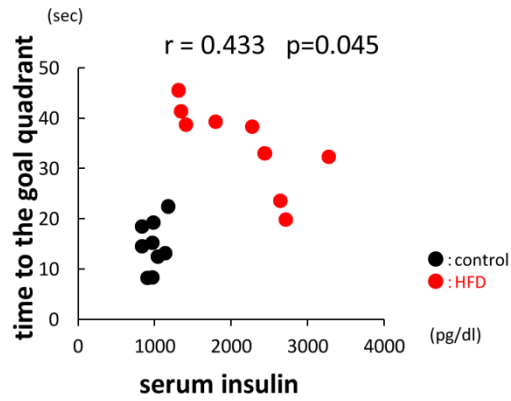
A



B



C



**Environmental enrichment ameliorated high fat diet-induced A β deposition
and memory deficit in APP transgenic mice.**

Masato Maesako¹, Kengo Uemura², Masakazu Kubota¹, Akira Kuzuya², Kazuki Sasaki¹, Megumi Asada¹, Kiwamu Watanabe², Naoko Hayashida¹, Masafumi Ihara², Hidefumi Ito², Shun Shimohama³, Takeshi Kihara¹ and Ayae Kinoshita^{1*}

1. School of Human Health Sciences, Kyoto University Graduate School of Medicine, Kyoto 606-8507
2. Department of Neurology, Kyoto University Graduate School of Medicine, Kyoto 606-8507
3. Department of Neurology, Sapporo Medical University, Sapporo 060-8556

*Address correspondence to: Ayae Kinoshita,

School of Human Health Sciences, Kyoto University Graduate School of Medicine, 53,
Shogoinkawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.

Tel. / Fax: 81-75-751-3969; E-mail: akinoshita@hs.med.kyoto-u.ac.jp

Abstract

The pathogenesis of Alzheimer's disease (AD) is tightly associated with metabolic dysfunctions. In particular, a potential link between type 2 diabetes (T2DM) and AD has been suggested epidemiologically, clinically and experimentally, and some studies have suggested that exercise or dietary intervention reduces risk of cognitive decline. However, there is little solid molecular evidence for the effective intervention of metabolic dysfunctions for prevention of AD. In the present study, we established the AD model mice with diabetic conditions through high fat diet (HFD) in order to examine the effect of environmental enrichment (EE) on HFD-induced AD pathophysiology. Here, we demonstrated that HFD markedly deteriorated memory impairment and increased β -amyloid ($A\beta$) oligomers as well as $A\beta$ deposition in amyloid precursor protein (APP) transgenic mice, which was reversed by exposure to an enriched environment for 10 weeks, in spite of the continuation of HFD. These studies provide solid evidence that EE is a useful intervention to ameliorate behavioral changes and AD pathology in HFD-induced aggravation of AD symptoms in APP transgenic mice.

Keywords

Alzheimer's disease, type 2 diabetes, high fat diet, environmental enrichment, β -amyloid

The abbreviations

AD, Alzheimer's disease; T2DM, type 2 diabetes; HFD, high fat diet; $A\beta$, β -amyloid; APP, amyloid precursor protein; Tg, transgenic; WT, wild type; EE, environmental enrichment; IGTT, intra-peritoneal glucose tolerance test; CTF, C-terminal fragment

1. Introduction

Alzheimer's disease (AD), the most common cause of dementia, is poised to become a significant public health crisis. The occurrence of AD is largely sporadic, typically affecting individuals over 65 years, but a minority of the cases (5%) display familial inheritance with early onset. One of the pathological hallmarks of AD is amyloid plaques. Amyloid plaques are composed of 40–42 residue-peptides, called β -amyloid ($A\beta$) (designated

as A β 40, A β 42), which are derived from the amyloid precursor protein (APP) via proteolytic cleavages by β - and γ -secretases. Presenilin 1 and Presenilin 2 (PS1 and PS2) are known to be the catalytic core of γ -secretase (De Strooper *et al.* 1998). A widely accepted hypothesis about AD pathogenesis is that A β production plays a crucial role in neurodegeneration (Finder 2010). This hypothesis is supported by the discovery of causative mutations in the genes encoding APP, PS1, and PS2, in early onset familial AD (Tandon *et al.* 2000, Bertram *et al.* 2008). Moreover, recent studies have implied small soluble A β oligomers, such as dimers, trimers, and dodecamers, formed during A β aggregation, as being the main culprits of A β toxicity and AD pathogenesis (Hartley *et al.* 1999, Walsh *et al.* 2002, Lesne *et al.* 2006, Shankar *et al.* 2008).

A potential link between type 2 diabetes (T2DM) and AD has been suggested by epidemiological and clinical studies (Ott *et al.* 1999, Biessels *et al.* 2006). Recent experimental studies support this linkage. For example, APP-ob/ob mice, produced by crossing APP transgenic (Tg) mice with diabetic model mice, manifested earlier onset of cognitive dysfunction than APP Tg mice (Takeda *et al.* 2010). Moreover, using dietary interventions such as high fat diet (HFD) or sucrose water for APP Tg mice exacerbated their memory deficits and pathological alterations in the brain (Ho *et al.* 2004, Cao *et al.* 2007). On the other hand, insulin and the insulin-sensitizing drug have been shown to improve cognitive performance in mouse models of AD, as well as in patients with early AD (Watson *et al.* 2005, Pedersen *et al.* 2006, Reger *et al.* 2008). These reports clearly indicate that there is an association of diabetes with a higher risk of sporadic AD. However, the impact of non-pharmacological or preventive intervention targeting AD with diabetes has not been clearly demonstrated so far.

Exercise is beneficial in the prevention and treatment of T2DM, both in human and rodent models (Keller *et al.* 1993, Cotman and Berchtold 2007, Sanz *et al.* 2010). In the environmental enrichment (EE) condition, mice are allowed the freedom to move and exercise voluntarily in the larger cage, with accessibility to complex stimuli (e.g., toys, running wheels), thus being provided with more physical and intellectual stimulation than mice housed in standard laboratory conditions. In the AD research fields, some reports demonstrated that EE applied to AD model mice reduced A β deposition, enhanced synaptic plasticity, and ameliorated cognitive deficits (Lazarov *et al.* 2005, Jankowsky *et al.* 2005, Hu *et al.* 2010). On the other hand, other studies suggested that EE enhanced A β accumulation and failed to improve memory deficits in APP Tg mice with a regular diet

(Jankowsky *et al.* 2003, Cotel *et al.* 2010). Thus, the effect of EE on AD pathophysiology has been controversial.

In the present study, in order to determine whether regular exercise affects cognitive decline, we established the AD model mice with diabetic conditions through HFD (APP-HFD mice), which were subsequently subjected to EE. To test the effect of EE, we conducted ethological, histochemical and biochemical analyses. Here, with the use of established animal models with both conditions, we observed that the APP-HFD mice exhibited even more impaired cognitive function than control APP Tg mice fed with normal diet (control APP mice). Additionally, we demonstrated that EE not only ameliorated obesity and glucose intolerance of the APP-HFD mice but also significantly improved their cognitive function. Notably, histochemical and biochemical analyses suggested that EE ameliorated the A β accumulation in the brains accelerated by HFD. Also, the amount of A β oligomers was elevated in the cerebrum of the APP-HFD mice, which was significantly reduced by EE settings. These results clearly indicated that EE could be an effective way to ameliorate the AD progression caused by metabolic dysfunctions.

2. Material and methods

2.1. Animals and dietary conditions

We used human APP Tg mice overexpressing the familial AD-linked mutations bearing both Swedish (K670N/M671L) and Indiana (V717F) mutation (APP^{Swe/Ind}) (Mucke *et al.* 2000), which have been imported from the Jackson Laboratory (USA). APP^{Swe/Ind} mice were maintained as heterozygotes and male and female mice were housed separately. Age- and sex-matched (1:1, male: female) mice were exposed to either an established high fat diet (HFD) (caloric composition, 60% fat, 20% carbohydrate, and 20% protein, Research Diet, Inc., Canada) or a standard diet (10% fat, 70% carbohydrate, and 20% protein, Oriental Yeast Co., Ltd., Japan) for 20 weeks, from 2-3 to 7-8 months age. To examine the effect of environmental enrichment (EE) on APP Tg mice fed with HFD (APP-HFD mice), the cage of the mice was changed to a 2.4 times larger one equipped with a running wheel as well as objects like stands and toys after 10 weeks of HFD (APP-HFD + EE mice). The mice spent 10 weeks in the EE condition in the presence of HFD. After the dietary manipulation, metabolic changes in these mice were analyzed, followed by the assessment of memory function through the Morris water maze test, as described below. After the analysis

of memory function, the brains were extracted and were cut sagittally into left and right hemispheres. The left hemisphere was fixed in 4% paraformaldehyde for histological analysis. After removing the olfactory lobe and cerebellum, the right hemisphere was rapidly frozen in liquid nitrogen for biochemical analysis. All animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals of the Kyoto University.

2.2 Assessment of metabolic changes

To assess glucose intolerance in these mice, we assessed changes in circulating glucose levels, as a function of time in response to the intra-peritoneal glucose tolerance test (IGTT). Mice were given a single dose of intra-peritoneal injection of glucose (2 g/kg body weight) after 14 hours fasting, and blood was collected from the tail-vein periodically over 2 hours. Blood glucose content was measured by using LabAssay Glucose (Wako, Japan). Plasma insulin concentration was measured by ELISA kit specific to insulin (Morinaga Seikagaku, Japan). Plasma concentrations of total cholesterol, High density lipoprotein (HDL)-cholesterol and triglyceride were measured by using cholesterol E-Wako, HDL-cholesterol E-Wako and triglyceride E-Wako (Wako, Japan).

2.3 Morris water maze test

Behavioral test was performed with a modified version of the Morris water maze test in order to assess spatial navigation learning and memory retention, as previously reported (Fitz *et al.* 2010), with minor modifications. Initially, animals received a habituation trial during which the animals were allowed to explore the pool of water (diameter 120 cm, height 25 cm, temperature $21 \pm ^\circ\text{C}$) without the platform present.

Visual cue phase. Following habituation, visible platform training was performed to measure motivation of the mice to find a platform, visual acuity of the mice, and the ability of mice to use local cues. Briefly, distal cues were removed from around the pool, and the platform was labeled with a flag and placed 1 cm above the surface of the water in the center of a quadrant. Mice were placed in the maze and allowed to explore the maze for 60 sec, and if they reached the visible platform, they were allowed to remain there for 20 sec before being returned to their cages. If they did not find the platform within 60 sec, the experimenter led them to the platform and let them remain there for 20 sec. Animals were trained in

groups of five, and training was completed once each animal received six trials. This training was performed for 1 day.

Acquisition phase. We measured the ability of mice to form a representation of the spatial relationship between a safe, but invisible (submerged 1 cm below the water level), platform (10 cm in diameter) and visual cues surrounding the maze. The platform was located in the center of one of the four quadrants, and several extramaze cues were distributed across the walls surrounding the pool. During the acquisition phase of training, each mouse received four daily hidden platform training trials with 10-12 min intervals for 5 consecutive days. Animals were allowed 60 sec to locate the platform and 20 sec to rest on it. Mice that failed to find the platform were led there by the experimenter and allowed to rest there for 20 sec.

Probe trial phase. 24 hours following the last acquisition trial, a single 60 sec probe trial was administered to assess spatial memory retention. For the probe trial, animals were returned to the pool without the platform present, and parameters were recorded to assess the ability of the mouse to remember the previous location of the platform.

Performance was recorded with an automated tracking system (TARGET series/2, Japan) during all phases of training. During the visual cue phase of training, speed and latency to the platform were used to compare the activity of the performance between each group. During the acquisition phase, acquisition time (latency to reach the platform) and path length (swum distance) were subsequently used to analyze and compare the performance between different treatment groups. The time to the platform quadrant, and the number of entries into the target quadrant were recorded and analyzed during the probe trials.

2.4 Immunoblotting and filter trap assay

For immunoblotting analysis, the brain was extracted and rapidly frozen using liquid nitrogen. The brain samples from the cerebrum of the male mice were extracted in Radio-Immunoprecipitation Assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X100, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, pH 8.0) with protease inhibitor cocktail (Roche, Switzerland) and sufficiently homogenized on ice. Then the samples were incubated for one night at 4°C and centrifuged at 14,000 g for 20 min. The supernatants were directly used for Western blot analysis. The detailed protocol has been described previously (Maesako *et al.* 2011). Mouse monoclonal anti-A β (6E10), β -actin, and rabbit polyclonal anti-APP C-terminal antibodies were from SIGMA (USA).

Filter trap assay was conducted as described previously (Kitaguchi *et al.* 2009). Briefly, the protein concentration of the samples in Tris-buffered saline (TBS)-extracted fraction was measured and an equal amount of protein was subjected to vacuum filtration through a 96-well dot blot apparatus (Bio-Rad Laboratories) containing 200 nm pore-sized nitrocellulose membrane. The resultant membrane was then incubated with primary A β oligomer antibody (A11, Invitrogen; diluted 1:1000) at 4°C overnight. The membrane was then blocked by TBS containing 4% skim milk, and incubated with HRP-linked anti-mouse IgG secondary antibody (GE Healthcare; diluted 1:2000) for 1 hour. The membrane was developed using the ECL Western Blotting Analysis System (GE Healthcare). A β 42 peptides (BACHEM) incubated for 60 min at 37°C was used as a positive control (Maesako *et al.* 2010) and monomeric A β was used as a negative control.

2.5. Immunohistochemistry

The paraformaldehyde-fixed and paraffin-embedded tissue sections of male mice were incubated with anti-A β (6E10) antibody (1:1,000). The sections were then incubated with biotinylated anti-mouse IgG antibody (1:2,000; Vector Laboratories, USA), followed by the incubation with avidin peroxidase (ABC Elite kit; 1:4,000; Vector Laboratories). Subsequently, the labeling was visualized by incubation with 50 mM Tris-HCl buffer (pH 7.6) containing 0.02% 3,3-diaminobenzidine and 0.0045% hydrogen peroxide. All images were visually analyzed using a microscope, ECLIPSE 80i (Nikon Corporation, Japan). On the other hand, for the fluorescent analysis, the tissue sections were incubated with anti-A β (6E10) antibody, followed by incubation with Alexa Fluor 488 anti mouse IgG (Invitrogen, USA). All images were visually analyzed, using a laser confocal scanning microscope, FV10i-LIV (Olympus Corporation, Japan). The A β immunoreactivity was quantified with Image J. For each animal, the sections were captured in the cortex and the hippocampus. Captured images then were imported into Image J, and an intensity threshold level was set that allowed for discrimination between plaque and background labeling. The total number of A β plaque associated pixels (6E10 antibody positive pixels) was calculated in each section, and then the A β load was calculated.

2.6. Measurement of A β by ELISA

The levels of A β 40, A β 42, or A β oligomers were measured by Enzyme-Linked ImmunoSorbent

Assay (ELISA) kits specific to A β 40, A β 42, or A β oligomers (82E1-specific) (IBL, Japan), according to the manufacturer's instructions. We used a standard format for measuring monomeric A β species with the use of C-terminal capturing antibodies and N-terminal or mid-region detecting antibodies. On the other hand, in order to detect A β oligomer species, the same N-terminal antibody, 82E1 (to A β residues 1–16, Immuno-Biological Laboratories, Inc, Minnesota, USA), was used for both capture and detection. In order to prepare the samples, the brain samples from the cerebrum of the male mice were homogenized with TBS. The homogenate was centrifuged at 100,000 g for 1 hour, and the supernatant was collected as the TBS-extracted fraction. Seventy percent formic acid (FA) was added to the pellet, which was homogenized again. The homogenate was incubated for 1 hour at 4°C and then centrifuged at 100,000 g for 1 hour at 4°C. The resultant supernatant was collected as the FA-extracted fraction, which was neutralized with a 20-fold volume of 1 M Tris buffer (pH 11.0).

2.7. Statistical analysis

All values are given in means $\pm$ SE. Comparisons were performed using an unpaired Student's t-test. For comparison of multiparametric analysis, one-way factorial ANOVA, followed by the post hoc analysis by Fisher's PLSD was used. Statistical significance of differences between mean scores during acquisition phase of training in the Morris water maze test was assessed with two-way repeated-measures ANOVA (general linear model/RM-ANOVA) and Fisher's post hoc analysis for multiple comparisons. $p < 0.05$ was considered to indicate a significant difference.

3. Results

3.1. Environmental enrichment ameliorated HFD-induced metabolic dysfunctions

Recent literature has demonstrated that HFD disrupts the metabolic conditions of APP Tg mice (Ho *et al.* 2004). To determine the effect of EE on HFD-induced metabolic dysfunctions, the cage of APP-HFD mice was changed into a larger one with a running wheel and objects like stands and toys. The mice were then fed with HFD for subsequent 10 weeks (Fig. 1). T2DM is characterized by obesity, glucose intolerance, and hyperinsulinemia (DeFronzo 2009). According to our metabolic analysis using weekly monitoring of body weight, an intraperitoneal glucose tolerance test (IGTT) and the ELISA of serum insulin, obesity,

glucose intolerance, and hyperinsulinemia were observed in the APP-HFD mice. Thus, we conclude that the APP-HFD mice, which we generated, exhibited severe T2DM conditions.

Although the APP-HFD mice gained significantly more body weight than the control APP mice (standard diet), the APP-HFD with EE (APP-HFD+EE) mice gained less body weight than the APP-HFD mice after the transfer to the EE setting (Fig. 2A). In spite of being fed with HFD, the APP-HFD+EE mice maintained an even body weight for 10 weeks. Weekly monitoring of food intake showed that the amount of food intake by the APP-HFD+EE mice was larger than that of the APP-HFD mice (supplemental Fig. 1), which indicated that the EE-mediated attenuation of body weight was not caused by the reduction of food intake. Moreover, we monitored the number of running wheel rotation and estimated that the APP-HFD+EE mice ran 1040 ± 49 m per day in the EE setting. The fasting glucose level of the APP-HFD mice was increased, compared with that of the control APP mice, whereas that of the APP-HFD+EE mice was significantly decreased, compared with that of the APP-HFD mice (Fig. 2B). Further, the IGTT results indicated that the impaired glucose tolerance response of the APP-HFD mice was improved in the APP-HFD+EE group (Fig. 2C). To examine whether EE could reverse or prevent glucose tolerance abnormality, we conducted IGTT at the time of the switch from the standard environment to enriched one (10 weeks after HFD introduction). The fasting glucose level and glucose tolerance of APP-HFD+EE mice were better than those of the APP mice at the time of the switch (supplemental Fig. 2). Therefore, EE could reverse glucose tolerance abnormality. The ELISA results indicated that the level of plasma insulin was significantly increased in the APP-HFD mice. However, in contrast to the glucose level, plasma insulin level tended to decrease to some extent, but was not significantly different between the APP-HFD and APP-HFD+EE mice (Fig. 2D).

Next, we conducted plasma lipid analyses. The level of plasma total cholesterol in the APP-HFD mice was significantly increased, compared with that of the control APP mice. On the other hand, the total cholesterol level of the APP-HFD+EE mice was not different from that of the APP-HFD mice (Fig. 3A). Similarly, the level of plasma HDL cholesterol in APP-HFD mice was significantly increased, compared with that in the control APP mice. The plasma HDL level of the APP-HFD+EE mice was comparable with that of the APP-HFD mice (Fig. 3B). On the contrary, the level of plasma triglycerides was not different among the three conditions (Fig. 3C). Taken together, these results indicated that HFD disrupted the metabolic

conditions including body weight, glucose tolerance, plasma insulin, and cholesterol levels of APP Tg mice, among which EE ameliorated body weight and glucose tolerance.

3.2 Environmental enrichment improved HFD-induced memory deficit

Recent literature also demonstrated that HFD leads to the worsening of memory deficit in APP Tg mice (Ho *et al.* 2004). To determine the effect of EE on HFD-induced memory deficit, we conducted the Morris water maze test. In our study, we analyzed 7- to 8-month-old APP Tg mice since they present with visible A β plaques and cognitive impairment sufficient for quantitative evaluations (Mucke *et al.* 2000). Neither HFD nor HFD+EE increased mortality of the mice, nor did they affect the motivation during the visual cue phase of the test (data not shown). In addition, neither HFD nor HFD+EE affected the locomotor activity of the mice, as exemplified by swimming speed (supplemental Fig. 3). During the acquisition phase, the control APP mice showed a daily improvement in their performance, such as acquisition time (Fig. 4A) and path length to the platform (Fig. 4B), whereas the APP-HFD mice did not show any improvement. On the other hand, the APP-HFD+EE mice showed better performance than the APP-HFD mice did (Fig. 4A, B). Moreover, the probe trial demonstrated that the APP-HFD mice took a longer time to get to the platform quadrant (Fig. 4C) and failed to cross the previous location of the platform (Fig. 4D), compared to control mice. Once again, the APP-HFD+EE mice showed better performance than the APP-HFD mice, in this probe trial phase as well (Fig. 4C, D). From these results, we concluded that EE ameliorated HFD-induced memory dysfunction, despite continuing HFD in the AD model mice.

In order to determine whether these events were attributable to metabolic consequences of the diet or to an interaction between the diet and neuropathology in AD model mice, wild type (WT) mice were tested for learning ability, using the Morris water maze test. Metabolic analyses indicated that WT mice also exhibited T2DM conditions (supplemental Fig. 4A, B). After the 4th day, in the acquisition phase of Morris water maze test, acquisition time of the HFD-induced WT (WT-HFD) mice was not different from that of the control WT or the WT-HFD+EE mice, although that of the WT-HFD mice was longer than that of the control WT and the WT-HFD+EE mice from 1st to 3rd day (supplemental Fig. 4C). This tendency was different from the case of the APP mice, since APP-HFD mice consistently took longer time to get to the platform quadrant in the acquisition phase (Fig. 4A). These results suggested that memory dysfunction could be attributable to an interaction between the diet and neuropathology in the AD model mice.

3.3 HFD-induced A β deposition was ameliorated in environmental enrichment condition

HFD is reported to lead to A β accumulation in the brain of APP Tg mice (Ho *et al.* 2004). We considered the possibility that the memory impairment in APP-HFD mice was due to ample A β deposition, and wanted to see the effect of environmental change on HFD-induced A β accumulation. Therefore, we conducted immunohistochemical analysis using anti-A β (6E10) antibody to quantitatively examine A β deposition. As seen in Figure 5A–C, A β deposition in the hippocampus was aggravated in the APP-HFD mice, whereas EE introduction resulted in a marked reduction of HFD-induced A β deposition in the APP-HFD+EE mice.

We next quantified A β contents in the TBS-soluble and -insoluble (FA soluble) fractions using ELISA. In the TBS-soluble fraction, the levels of A β 40, A β 42, and total A β in the APP-HFD mice were comparable to that in the control APP mice. However, the levels of A β 40 and total A β in the APP-HFD+EE mice were significantly decreased, compared with that in the APP-HFD mice (Fig. 5D–F). On the other hand, in FA fraction, the level of A β 40 in the APP-HFD mice was significantly increased, compared with that in the control APP mice. However, the levels of A β 40 in the APP-HFD+EE mice were significantly decreased, compared with that in the APP-HFD mice (supplemental Fig. 5A). A similar tendency was shown in the case of A β 42 and total A β amount in FA fraction, although there was no statistical significance (supplemental Fig. 5B, C). Based on these histochemical and biochemical analyses, we concluded that EE ameliorated HFD-induced A β accumulation in the brain.

Recent reports suggest that the level of soluble A β oligomers correlate with memory deficits in APP Tg mice (Hartley *et al.* 1999, Walsh *et al.* 2002, Lesne *et al.* 2006, Shankar *et al.* 2009). To determine a correlation between A β oligomers and memory impairment in standard housing APP-HFD mice and APP-HFD+EE mice, we performed the ELISA analysis using A β oligomer-specific ELISA kit (Xia *et al.* 2009). The level of TBS-soluble A β oligomers in the APP-HFD mice was significantly increased, compared with that in the control APP mice. This result was consistent with that of the test for memory assessment described above. Remarkably, the levels of A β oligomers in the APP-HFD+EE mice were significantly decreased, compared with that in the APP-HFD mice (Fig. 5G). In addition, we confirmed this result through Filter trap assay, using anti-A β oligomer antibody (supplemental Fig. 6). Thus, at least in HFD-induced conditions, EE appears to play a significant role in modulating the level of A β oligomers.

3.4 Alteration of HFD-induced APP processing by environmental enrichment

To elucidate the mechanism of how EE ameliorated HFD-induced A β accumulation, we analyzed the APP processing through detecting APP C-terminal fragments (CTFs: CTF α , β) through immunoblotting assay. α - and β -Secretases are known to cleave APP at the extramembrane domain, which produce APP-CTF α and CTF β respectively. γ -Secretase cleaves APP-CTF α and CTF β at the intramembrane domain, producing p3 and A β respectively. As shown in the top row of Figure 6A, the level of full length APP was not different among the control, APP-HFD, and APP-HFD+EE mice (Fig. 6B). In this experiment, we used anti-APP C-terminal antibody to detect both APP-CTF α and CTF β . Notably, APP CTFs were more accumulated in the brains of the APP-HFD mice than those of the control APP mice. However, the level of APP CTFs in the APP-HFD+EE mice was significantly decreased, compared with that in the APP-HFD mice in standard housing (Fig. 6C). Next, we examined the amount of APP-CTF β by anti-A β (6E10) antibody which detects 1–17 amino acid residues of A β . The analysis using 6E10 antibody showed that the level of APP-CTF β in the APP-HFD mice was higher than that in the control APP mice, suggesting that the level of APP-CTF β in the APP-HFD+EE mice was significantly decreased, compared with that in the APP-HFD mice (Fig. 6D).

4. Discussion

HFD is prevalent in modern society and HFD-induced metabolic condition is becoming a worldwide issue, since it leads to obesity, T2DM, and hypercholesterolemia. More importantly, recent studies have shown that diet and nutrition have been recognized as important epigenetic factors for the development of sporadic AD (Solfrizzi *et al.* 2003, Panza *et al.* 2006, Scarmeas *et al.* 2007). We and others have previously proposed the causal molecular link between T2DM and AD (Qiu *et al.* 2006, Maesako *et al.* 2010, Maesako *et al.* 2011). However, the effective prevention for AD has not been fully investigated yet. A recent report by McClean *et al.* showed compelling evidence that the diabetes drug liraglutide prevents neuronal degeneration in a mouse model of AD (McClean *et al.* 2011), which suggests that there should be a clinical association of diabetic change with a higher risk of neuronal loss. This further led us to consider a development of an effective prevention

in the early phase of AD. To address this issue, we established the AD model mice with diabetic conditions in the present study, by HFD feeding in APP Tg mice.

In order to search for an effective intervention, we chose a paradigm of environmental enrichment (EE) and examined the effect of EE on both the metabolic conditions and the AD pathology of the mice (Fig. 1). A recent retrospective case control study demonstrated that AD patients were less active (both intellectually and physically) in midlife and that inactivity was associated with a 2.5 fold higher risk of developing AD (Friedland *et al.* 2001). Similarly, a prospective study revealed that physical activity was protective against the development of cognitive impairment in AD and that the highest activity group showed the incidence of AD lowered by 60% (Laurin *et al.* 2001). Since EE is regarded as a useful tool for exercise in mice, we chose this paradigm in the present study to see the impact of exercise on AD pathophysiology. Importantly, Adlard *et al.* have demonstrated that voluntary exercise shows beneficial effects on a Tg mice model of AD (Adlard *et al.* 2005). Since EE condition contains physical and intellectual stimulation, the extent to which intellectual stimulation contributes to the positive outcome still remains controversial. Faherty *et al.* suggested that EE is more effective for facilitating neural changes than exercise alone (Faherty *et al.* 2003), while Lambert *et al.* suggested that exercise, but not cognitive stimulation, improves spatial memory (Lambert *et al.* 2005). In the setting of the present study, metabolic conditions of the APP-HFD+EE mice were clearly ameliorated, compared to those of the APP-HFD mice. Moreover, cognitive stimulation of our setting was smaller than that in previous reports, since we wanted to focus on the effect of exercise on APP-HFD mice. Therefore, we speculated that physical stimulation might play a more important role in our study. The purpose of this experiment was to obtain a deep insight into developing strategies for the prevention of AD; therefore, EE was started at the age before the appearance of visible A β plaques in the brain of APP *Swe/Ind* mice.

Although previous reports examined the effect of EE on AD model mice (Jankowsky *et al.* 2003, Lazarov *et al.* 2005, Jankowsky *et al.* 2005, Hu *et al.* 2010, Cotel *et al.* 2010), the effect of EE on AD mice with diabetic conditions had not yet been examined. Notably, our results indicated that EE ameliorated HFD-induced memory deficit, in spite of continuing high-fat feeding (Fig. 4). EE is known to enhance hippocampal neurogenesis and result in increased numbers of synapses per neuron (Hu *et al.* 2010). We assumed that EE might have improved cognitive dysfunctions of the mice through strengthening of the synaptic activity of the mice. Further, our results demonstrated that EE decreased oligomers and fibrillar A β , indicating that EE also

ameliorated HFD-induced A β accumulation (Fig. 5). An increasing number of reports have suggested that the level of soluble A β oligomers correlates with memory deficits due to their synaptotoxicity (Hartley *et al.* 1999, Walsh *et al.* 2002, Lesne *et al.* 2006, Shankar *et al.* 2009, Jin *et al.* 2011). Therefore, we hypothesized that EE also might have improved memory deficit of the mice through the decrease of soluble A β oligomers, followed by the improvement of A β plaque depositions. Notably, Cotel *et al.* have reported that EE failed to rescue working memory deficits and neuronal loss in APP/PS1 knock-in (KI) mice (Cotel *et al.* 2010). Their result is different from ours in that enriched housing did not show any beneficial effects in terms of working memory and amyloid burden. Our result was obtained from high-fat feeding of APP Tg mice, whereas Cotel *et al.* used conventional diet for APP/PS1 KI mice; however, housing conditions seem similar. We suppose that the combination of physical activity and cognitive stimulus in EE condition may be more beneficial in the reversal of cognitive decline and A β load, which was caused by metabolic dysfunctions due to high-fat feeding.

To clarify the effect of EE on HFD-induced AD pathology, we first investigated the mechanisms of how HFD aggravated A β depositions. Recent literature has suggested that HFD down-regulates the activity of Insulin degrading enzyme (IDE), one of the A β degrading enzymes (Ho *et al.* 2004). In addition, we demonstrated that HFD increased the level of APP CTF β without a change in full-length APP levels (Fig. 6). Unexpectedly, we could not detect the difference of BACE1/ β -secretase level among control APP, APP-HFD, and APP-HFD+EE mice's brains (supplemental Fig. 7A, B). Although emerging evidence has consistently detected significant increases in β -secretase enzyme activity in the sporadic AD brains, the BACE1 enzyme activity in AD is not necessarily reflected by its protein levels (Stockley and Neill 2008), presumably because BACE1 enzyme activity might be regulated by other factors such as trafficking, and subcellular and membrane microdomain localization. Moreover, recent literature has demonstrated that BACE1 enzyme activity is modulated by sphingosine-1-phosphate (S1P), a pluripotent lipophilic mediator (Takasugi *et al.* 2011), suggesting that BACE1 interacting proteins can also control its activity. We compared the BACE1 interacting proteins of the APP-HFD mice brain samples with that of the control-APP samples by immunoprecipitation assay using BACE1 antibody and determined the different profiles in interacting proteins between them (unpublished observation). We speculate that HFD might have changed the interacting state of these proteins with BACE1, without changing the BACE1 protein level. Consequently, we conclude that

HFD could have aggravated A β accumulation via several pathways, including the activation of BACE1/ β -secretase enzyme and the inhibition of A β degradation.

Next, to reveal the mechanism of beneficial effect of EE, we employed ELISA experiments. The ELISA results in TBS-soluble fraction indicated that HFD might not simply increase the production of A β , but that HFD affects the aggregation and deposition of A β (Fig. 5D-F). On the other hand, considering that EE decreased the level of APP CTF β (Fig. 6), we assume that EE could have inhibited HFD-induced BACE1/ β -secretase up-regulation. We speculate that EE could also have changed the state of interacting proteins with BACE1. However, EE also might have improved A β accumulation via the up-regulation of A β clearance, since EE activates Neprilysin, one of the A β degrading enzymes (Lazarov *et al.* 2005). Interestingly, our metabolic analyses suggested that EE did not affect either HFD-induced hyperinsulinemia or hypercholesterolemia, but improved body weight as well as glucose tolerance (Fig. 2, 3), indicating that EE might have ameliorated HFD-induced A β accumulation through the improvements in obesity and glucose tolerance. According to a recent review by Misra, the EE-mediated improvement of glucose tolerance via insulin independent pathway may be caused by the role of adenosine monophosphate (AMP)-activated protein kinase (AMPK) since it is considered a master switch to regulate glucose level without an effect on insulin in exercise-related effects (Misra 2008). To develop an effective intervention, it is important to elucidate the relationship between obesity and glucose intolerance and HFD-induced A β accumulation as well as memory deficit.

In conclusion, we provide convincing evidence that EE ameliorated HFD-induced metabolic dysfunctions, A β deposition, and memory deficit. We showed that EE improved metabolic conditions like obesity and glucose intolerance in APP-HFD mice without rectifying the level of serum insulin. Our result is clinically intriguing in that a rather mild intervention like EE for only 10 weeks prevented further HFD-induced cognitive decline in the AD mouse model. However, the detailed mechanism of how EE ameliorated HFD-induced A β deposition and memory deficit was not clarified in the present study. Although the exact pathogenesis of sporadic AD remains still largely unknown, our results clearly indicate that the intervention for the metabolic condition could be the most effective and practicable way to prevent AD in T2DM patients. Considering that the beneficial effect was obtained even with the continuation of HFD, the detailed mechanism of continuous exercise and its practical application to AD patients should be further verified in future studies.

5. Acknowledgement

We would like to give our thanks to Dr. S.Oka (Kyoto University, Kyoto, Japan), Mr. J.Morise (Kyoto University, Kyoto, Japan), Dr. Y. Kitamura (Kyoto Pharmaceutical University, Kyoto, Japan) and Dr. K. Takata (Kyoto Pharmaceutical University, Kyoto, Japan) for technical assistance of the brain sample preparation. We would like to give our thanks to Dr. K.Saito (Kyoto University, Kyoto, Japan) for kind support of the Morris water maze test. We greatly appreciate the advice for metabolic analysis to Dr. M. Okuda (PHARMAEIGHT, Kyoto, Japan). We would like to give our thanks to Dr. Y. Tashiro (Kyoto University, Kyoto, Japan) for kind discussion. The work was financially supported by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (20300124), the Research Grant from Takeda Science Foundation, the Research Grant from the Kanae Foundation for the Promotion of Medical Science and the Research Grant from the Nakatomi Foundation.

Disclosure statement: The authors declare that they have no actual or potential conflict of interest.

6. References

- Adlard PA, Perreau VM, Pop V, Cotman CW. Voluntary exercise decreases amyloid load in a transgenic model of Alzheimer's disease. *J Neurosci*. 2005;25(17):4217-21.
- Bertram L, Tanzi RE. Thirty years of Alzheimer's disease genetics: the implications of systematic meta-analyses. *Nat Rev Neurosci* 2008;9(10):768-78.
- Biessels GJ, Staekenborg S, Brunner E, Brayne C, Scheltens P. Risk of dementia in diabetes mellitus: a systematic review. *Lancet Neurol* 2006;5(1):64-74.
- Cao D, Lu H, Lewis TL, Li L. Intake of sucrose-sweetened water induces insulin resistance and exacerbates memory deficits and amyloidosis in a transgenic mouse model of Alzheimer disease. *J Biol Chem* 2007;282(50):36275-82.
- Cotel MC, Jawhar S, Christensen DZ, Bayer TA, Wirths O. Environmental enrichment fails to rescue

- working memory deficits, neuron loss, and neurogenesis in APP/PS1KI mice. *Neurobiol Aging*. in press
- Cotman CW, Berchtold NC. Physical activity and the maintenance of cognition: learning from animal models. *Alzheimers Dement*. 2007 ;3:S30-7.
- De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F. Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* 1998;391(6665):387-90.
- DeFronzo RA. Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* 2009;58(4):773-95.
- Faherty CJ, Kerley D, Smeyne RJ. A Golgi-Cox morphological analysis of neuronal changes induced by environmental enrichment. *Brain Res Dev Brain Res*. 2003;141(1-2):55-61.
- Finder VH. Alzheimer's disease: a general introduction and pathomechanism. *J Alzheimers Dis*;22 Suppl 3:5-19.
- Fitz NF, Cronican A, Pham T, Fogg A, Fauq AH, Chapman R, Lefterov I, Koldamova R. Liver X receptor agonist treatment ameliorates amyloid pathology and memory deficits caused by high-fat diet in APP23 mice. *J Neurosci* 2010;30(20):6862-72.
- Friedland RP, Fritsch T, Smyth KA, Koss E, Lerner AJ, Chen CH, Petot GJ, Debanne SM. Patients with Alzheimer's disease have reduced activities in midlife compared with healthy control-group members. *Proc Natl Acad Sci U S A* 2001;98(6):3440-5.
- Hartley DM, Walsh DM, Ye CP, Diehl T, Vasquez S, Vassilev PM, Teplow DB, Selkoe DJ. Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J Neurosci* 1999;19(20):8876-84.
- Ho L, Qin W, Pompl PN, Xiang Z, Wang J, Zhao Z, Peng Y, Cambareri G, Rocher A, Mobbs CV, Hof PR, Pasinetti GM. Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. *FASEB J* 2004;18(7):902-4.
- Hu YS, Xu P, Pigino G, Brady ST, Larson J, Lazarov O. Complex environment experience rescues impaired neurogenesis, enhances synaptic plasticity, and attenuates neuropathology in familial Alzheimer's disease-linked APP^{swe}/PS1^{DeltaE9} mice. *FASEB J* 2010;24(6):1667-81.
- Jankowsky JL, Melnikova T, Fadale DJ, Xu GM, Slunt HH, Gonzales V, Younkin LH, Younkin SG,

- Borchelt DR, Savonenko AV. Environmental enrichment mitigates cognitive deficits in a mouse model of Alzheimer's disease. *J Neurosci* 2005;25(21):5217-24.
- Jankowsky JL, Xu G, Fromholt D, Gonzales V, Borchelt DR. Environmental enrichment exacerbates amyloid plaque formation in a transgenic mouse model of Alzheimer disease. *J Neuropathol Exp Neurol* 2003;62(12):1220-7.
- Jin M, Shepardson N, Yang T, Chen G, Walsh D, Selkoe DJ. Soluble amyloid {beta}-protein dimers isolated from Alzheimer cortex directly induce Tau hyperphosphorylation and neuritic degeneration. *Proc Natl Acad Sci U S A* 2011;108(14):5819-24.
- Keller JB, Bevier WC, Jovanovic-Peterson L, Formby B, Durak EP, Peterson CM. Voluntary exercise improves glycemia in non-obese diabetic (NOD) mice. *Diabetes Res Clin Pract.* 1993;22(1):29-35.
- Kitaguchi H, Tomimoto H, Ihara M, Shibata M, Uemura K, Kalaria RN, Kihara T, Asada-Utsugi M, Kinoshita A, Takahashi R. Chronic cerebral hypoperfusion accelerates amyloid beta deposition in APPSwInd transgenic mice. *Brain Res.* 2009; 1294:202-10.
- Lambert TJ, Fernandez SM, Frick KM. Different types of environmental enrichment have discrepant effects on spatial memory and synaptophysin levels in female mice. *Neurobiol Learn Mem.* 2005;83(3):206-16.
- Laurin D, Verreault R, Lindsay J, MacPherson K, Rockwood K. Physical activity and risk of cognitive impairment and dementia in elderly persons. *Arch Neurol* 2001;58(3):498-504
- Lazarov O, Robinson J, Tang YP, Hairston IS, Korade-Mirnic Z, Lee VM, Hersh LB, Sapolsky RM, Mirnic K, Sisodia SS. Environmental enrichment reduces Abeta levels and amyloid deposition in transgenic mice. *Cell* 2005;120(5):701-13.
- Lesne S, Koh MT, Kotilinek L, Kaye R, Glabe CG, Yang A, Gallagher M, Ashe KH. A specific amyloid-beta protein assembly in the brain impairs memory. *Nature* 2006;440(7082):352-7.
- Maesako M, Uemura K, Kubota M, Ando K, Kuzuya A, Asada M, Kihara T, Kinoshita A. Insulin regulates Presenilin 1 localization via PI3K/Akt signaling. *Neurosci Lett* 2010;483(3):157-61.
- Maesako M, Uemura K, Kubota M, Hiyoshi K, Ando K, Kuzuya A, Kihara T, Asada M, Akiyama H, Kinoshita A. Effect of glycogen synthase kinase 3 beta-mediated presenilin 1 phosphorylation on amyloid beta production is negatively regulated by insulin receptor cleavage. *Neuroscience* 2011;177:298-307.

- McClellan PL, Parthasarathy V, Faivre E, Holscher C. The diabetes drug liraglutide prevents degenerative processes in a mouse model of Alzheimer's disease. *J Neurosci* 2011;31(17):6587-94.
- Misra P. AMP activated protein kinase: a next generation target for total metabolic control. *Expert Opin Ther Targets*. 2008;12(1):91-100.
- Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L. High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J Neurosci* 2000;20(11):4050-8.
- Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM. Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology* 1999;53(9):1937-42.
- Panza F, Capurso C, D'Introno A, Colacicco AM, Del Parigi A, Seripa D, Pilotto A, Capurso A, Solfrizzi V. Diet, cholesterol metabolism, and Alzheimer's disease: Apolipoprotein E as a possible link? *J Am Geriatr Soc* 2006;54(12):1963-5.
- Pedersen WA, McMillan PJ, Kulstad JJ, Leverenz JB, Craft S, Haynatzki GR. Rosiglitazone attenuates learning and memory deficits in Tg2576 Alzheimer mice. *Exp Neurol* 2006;199(2):265-73.
- Qiu WQ, Folstein MF. Insulin, insulin-degrading enzyme and amyloid-beta peptide in Alzheimer's disease: review and hypothesis. *Neurobiol Aging*. 2006 Feb;27(2):190-8.
- Reger MA, Watson GS, Green PS, Wilkinson CW, Baker LD, Cholerton B, Fishel MA, Plymate SR, Breitner JC, DeGroodt W, Mehta P, Craft S. Intranasal insulin improves cognition and modulates beta-amyloid in early AD. *Neurology* 2008;70(6):440-8.
- Sanz C, Gautier JF, Hanaire H. Physical exercise for the prevention and treatment of type 2 diabetes. *Diabetes Metab* 2010;36(5):346-51.
- Scarmeas N, Luchsinger JA, Mayeux R, Stern Y. Mediterranean diet and Alzheimer disease mortality. *Neurology* 2007;69(11):1084-93.
- Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ. Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med* 2008;14(8):837-42.
- Solfrizzi V, Panza F, Capurso A. The role of diet in cognitive decline. *J Neural Transm*

2003;110(1):95-110.

Stockley JH, O'Neill C. Understanding BACE1: essential protease for amyloid-beta production in Alzheimer's disease. *Cell Mol Life Sci* 2008;65(20):3265-89.

Takasugi N, Sasaki T, Suzuki K, Osawa S, Isshiki H, Hori Y, Shimada N, Higo T, Yokoshima S, Fukuyama T, Lee VM, Trojanowski JQ, Tomita T, Iwatsubo T. BACE1 activity is modulated by cell-associated sphingosine-1-phosphate. *J Neurosci* 2011;31(18):6850-7.

Takeda S, Sato N, Uchio-Yamada K, Sawada K, Kunieda T, Takeuchi D, Kurinami H, Shinohara M, Rakugi H, Morishita R. Diabetes-accelerated memory dysfunction via cerebrovascular inflammation and Aβ deposition in an Alzheimer mouse model with diabetes. *Proc Natl Acad Sci U S A*;107(15):7036-41.

Tandon A, Rogaeva E, Mullan M, St George-Hyslop PH. Molecular genetics of Alzheimer's disease: the role of beta-amyloid and the presenilins. *Curr Opin Neurol* 2000;13(4):377-84.

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 2002;416(6880):535-9.

Watson GS, Cholerton BA, Reger MA, Baker LD, Plymate SR, Asthana S, Fishel MA, Kulstad JJ, Green PS, Cook DG, Kahn SE, Keeling ML, Craft S. Preserved cognition in patients with early Alzheimer disease and amnesic mild cognitive impairment during treatment with rosiglitazone: a preliminary study. *Am J Geriatr Psychiatry* 2005;13(11):950-8.

Xia W, Yang T, Shankar G, Smith IM, Shen Y, Walsh DM, Selkoe DJ. A specific enzyme-linked immunosorbent assay for measuring beta-amyloid protein oligomers in human plasma and brain tissue of patients with Alzheimer disease. *Arch Neurol* 2009;66(2):190-9.

7. Figure legends

Fig. 1. Schematic presentation of our experimental design

APP^{Swe/Ind} mice were maintained with standard diet in the standard laboratory cages until 2-3 months age. Then, age- and sex-matched mice were separated into 3 groups. In the control group, the mice were induced with standard diet in the standard laboratory cages for 20 weeks (control APP mice) (top row, n =

9). In the high fat diet (HFD) induced group, the mice were fed with HFD in the standard laboratory cages for 20 weeks (APP-HFD mice) (middle row, n = 10). In the HFD with environmental enrichment (EE) induced group, the mice spent 10 weeks in the standard laboratory cages, then spent 10 weeks in the enrichment cages with HFD (APP-HFD+EE mice) (bottom row, n = 8). After 20 weeks, metabolic conditions of these mice were analyzed, followed by ethological, histochemical and biochemical analyses targeting AD pathophysiology.

Fig. 2. Environmental enrichment ameliorated HFD-induced diabetic conditions

(A) Relative body weight changes over 20 weeks. The body weight of 2 weeks before each diet was regarded as the baseline (100 %). The bodyweight of APP-HFD mice was significantly increased compared with that of control APP mice ($F_{(2, 528)} = 136.81, p < 0.05$). On the other hand, that of APP-HFD +EE mice was significantly decreased compared with that of APP-HFD mice ($p < 0.05$).

(B) Fasting glucose levels. The fasting glucose level of APP-HFD mice was significantly increased compared with that of control APP mice ($F_{(2, 24)} = 19.38, p = 0.02$). On the other hand, the fasting glucose level of APP-HFD+EE mice was significantly decreased compared with that of APP-HFD mice ($p = 0.03$).

(C) Blood glucose levels during glucose tolerance test after an intra-peritoneal injection of glucose (2 g/kg body weight). APP-HFD mice showed impaired glucose tolerance compared with control mice ($F_{(2, 72)} = 35.00, p < 0.05$). On the other hand, APP-HFD+EE had ameliorated HFD-induced glucose intolerance ($p < 0.05$).

(D) Serum insulin levels during fasting or 60 min after glucose injection. At both time points, the serum insulin level of APP-HFD mice was significantly increased compared with that of control APP mice ($F_{(2, 24)} = 8.08, p = 0.003$). The serum insulin level of APP-HFD+EE mice was not significantly decreased compared with that of APP-HFD mice (n.s., $p = 0.27$). n.s. indicated not significantly.

Fig. 3. Environmental enrichment could not ameliorate HFD-induced lipid dysfunction

(A) Plasma total cholesterol levels. The total cholesterol level of APP-HFD mice was significantly increased compared with that of control APP mice ($F_{(2, 24)} = 24.28, p = 0.0003$). That of APP-HFD+EE mice was not significantly decreased compared with that of APP-HFD mice (n.s., $p = 0.14$). n.s. indicated not significantly.

(B) Plasma HDL cholesterol levels. The HDL cholesterol level of APP-HFD mice was significantly increased compared with that of control APP mice ($F_{(2, 24)} = 17.37$, $p = 0.0003$). The HDL cholesterol level of APP-HFD+EE mice was not significantly decreased compared with that of APP-HFD mice (n.s., $p = 0.17$). n.s. indicated not significantly.

(C) Plasma triglyceride levels. There was no difference among control, APP-HFD and APP-HFD+EE mice. ($F_{(2, 24)} = 1.33$, n.s.)

Fig. 4. Environmental enrichment ameliorated HFD-induced memory deficit

(A) Escape latency in the acquisition phase. APP-HFD mice significantly took longer time to the platform compared with control APP mice ($F_{(2, 96)} = 17.33$, $p = 0.012$). On the other hand, APP-HFD+EE mice took less time than APP-HFD mice ($p = 0.34$).

(B) Swimming length in the acquisition phase. APP-HFD mice swam significantly longer than control APP mice ($F_{(2, 96)} = 11.92$, $p = 0.025$). On the other hand, APP-HFD+EE mice swam shorter than APP-HFD mice ($p = 0.37$).

(C) The time to the target quadrant in the probe trial phase. APP-HFD mice significantly took longer time to the platform quadrant compared with control APP mice ($F_{(2, 24)} = 33.02$, $p = 0.002$). On the other hand, APP-HFD+EE mice took less time than APP-HFD mice ($p = 0.002$).

(D) The number of entries into the target quadrant in the probe trial phase. APP-HFD mice were significantly impaired in the number of times they crossed the platform compared with control APP mice ($F_{(2, 24)} = 15.75$, $p = 0.0014$). On the other hand, APP-HFD+EE mice increased the number of times they crossed the platform compared with APP-HFD mice ($p = 0.003$).

Fig. 5. Environmental enrichment ameliorated HFD-induced A β accumulation

(A) Immunohistochemical analysis using anti-A β (6E10) antibody. Representative images of A β -immunostained hippocampus sections from control APP, APP-HFD and APP-HFD+EE induced mice, respectively. Scale bar, 2 mm

(B) High-magnification images of the hippocampus including CA1 and Dentate Gyrus (DG) regions by immunostained analysis using anti-A β (6E10) antibody and Alexa Fluor 488 2nd antibody. The

immunostained signal was much enhanced in APP-HFD mice compared with that in control APP and APP-HFD+EE mice. Scale bar, 0.5 mm

(C) Cerebral A β loads determined by immunohistochemical and morphometric analyses. The cerebral A β deposition was significantly increased in APP-HFD mice compared with that in control APP mice ($F_{(2, 10)} = 5.62$, $p = 0.012$). On the other hand, that in APP-HFD+EE mice was significantly decreased compared with that in APP-HFD mice ($p = 0.023$).

(D) ELISA of A β 40 in TBS-soluble fraction. The level of TBS-soluble A β 40 in APP-HFD mice was the same as that in control APP mice. On the other hand, that in APP-HFD+EE was significantly decreased compared with that in APP-HFD mice ($F_{(2, 10)} = 5.16$, $p = 0.015$).

(E) ELISA of A β 42 in TBS-soluble fraction. There was no statistical significance among control APP, APP-HFD and APP-HFD+EE induced mice ($F_{(2, 10)} = 1.05$, n.s.).

(F) ELISA of total A β (A β 40 + A β 42) in TBS-soluble fraction. The level of TBS-soluble total A β in APP-HFD mice was the same as that in control APP mice. On the other hand, that in APP-HFD+EE was significantly decreased compared with that in APP-HFD mice ($F_{(2, 10)} = 6.35$, $p = 0.037$).

(G) ELISA of A β oligomer in TBS-soluble fraction. The cerebral A β oligomer was significantly increased in APP-HFD mice compared with that in control APP mice ($F_{(2, 10)} = 5.19$, $p = 0.01$). On the other hand, that in APP-HFD+EE was significantly decreased compared with that in APP-HFD mice ($p = 0.049$).

Fig. 6. Environmental enrichment reduced APP CTF β accumulation

(A) Immunoblotting analysis of APP full length, APP CTFs (CTF α , β) and APP CTF β . APP full length and APP CTFs were detected by anti-APP c-terminus antibody. APP CTF β was detected by anti-A β (6E10) antibody. Two different samples from each group were shown. β -actin was detected as loading control.

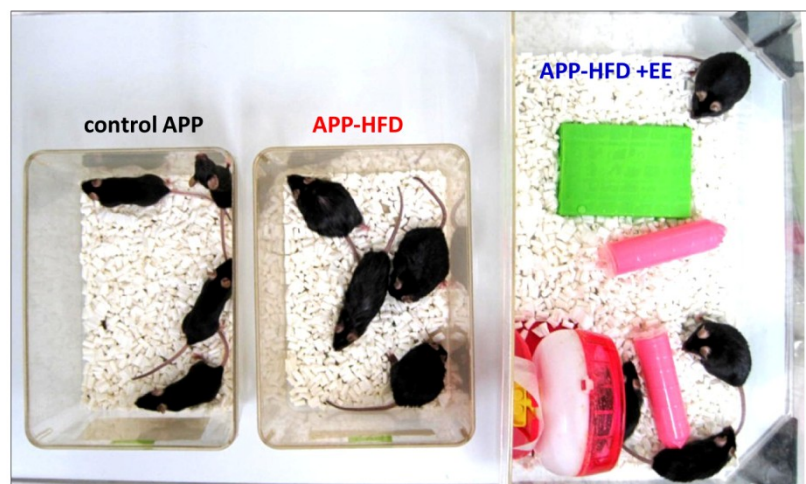
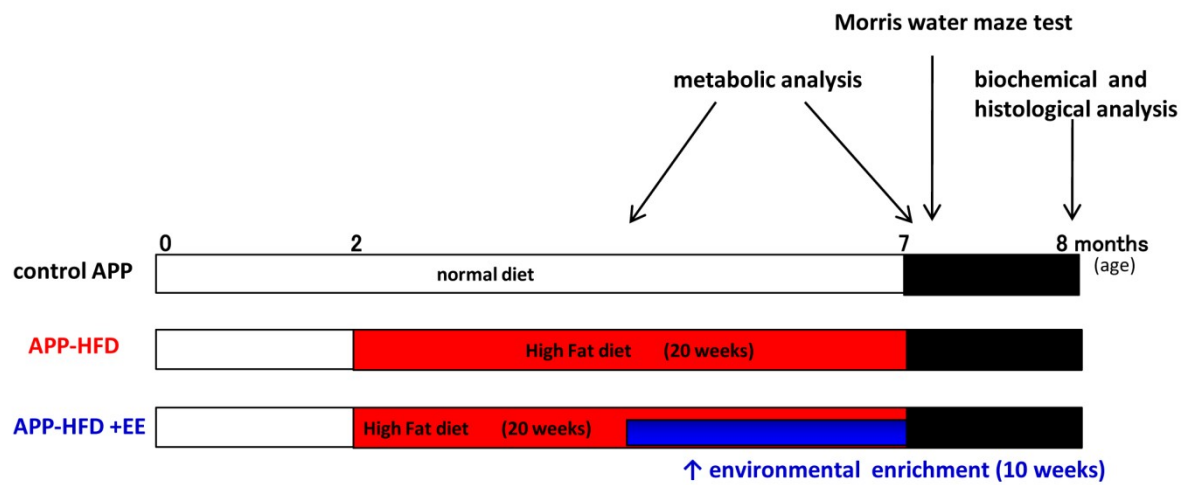
(B) Statistical analysis of APP full length. The band of APP full length was normalized by that of β -actin. The band density of the control was regarded as 100 % and that of other groups was relatively indicated. There was no statistical significance among control APP, APP-HFD and APP-HFD+EE mice ($F_{(2, 10)} = 2.36$, n.s.).

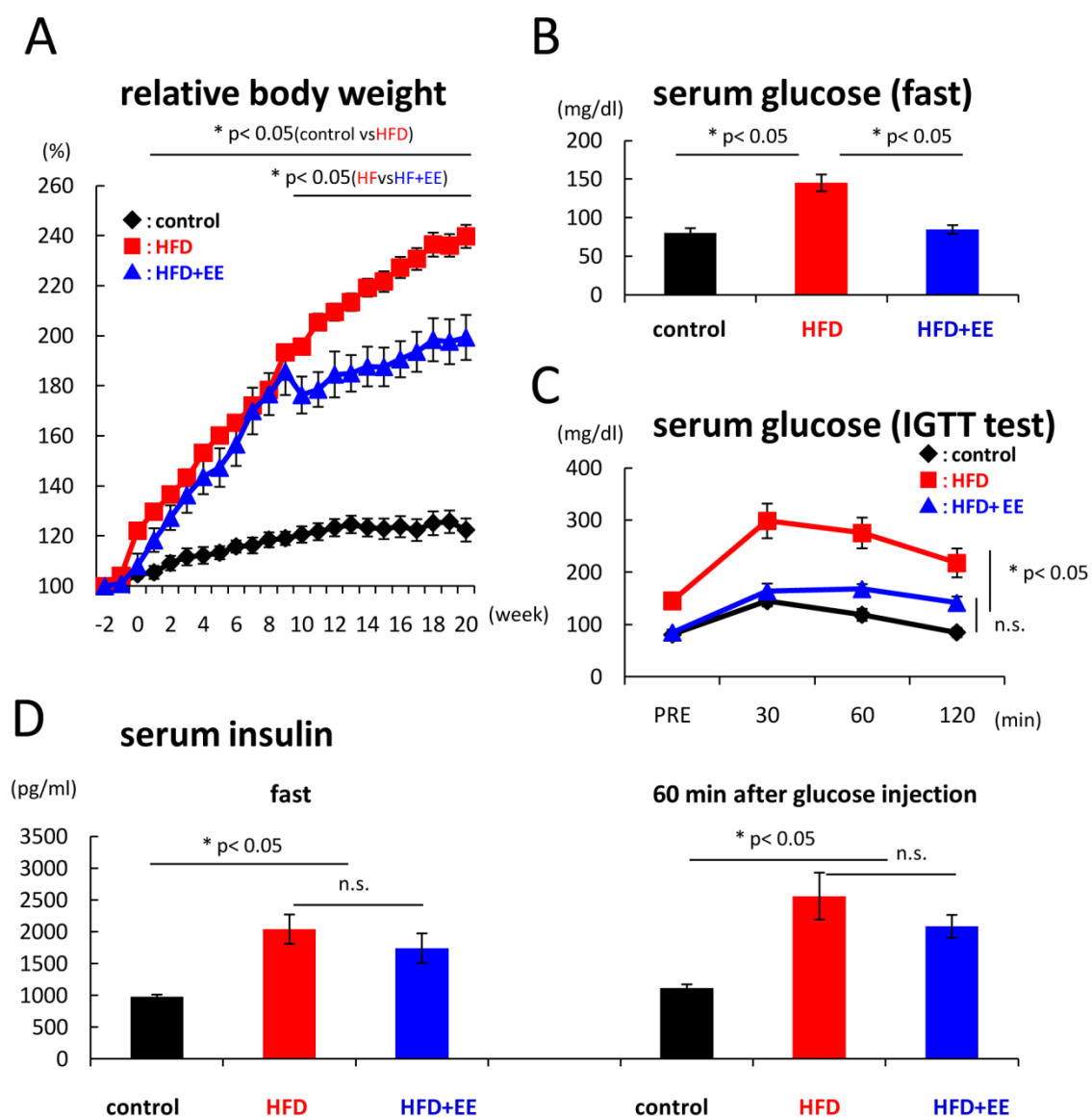
(C) Statistical analysis of APP CTFs. The band of APP CTFs was normalized by that of APP full length. The band density of APP CTFs in APP-HFD mice was increased compared with that in control APP mice

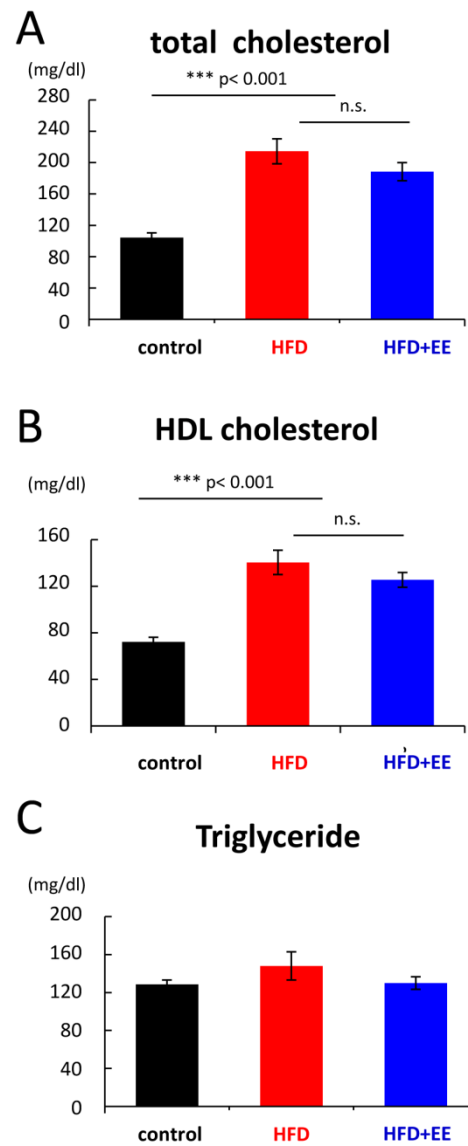
($F_{(2, 10)} = 4.73$, $p = 0.013$). On the other hand, that in APP-HFD+EE mice was significantly decreased compared with that in APP-HFD mice ($p = 0.015$).

(D) Statistical analysis of APP CTF β . The band of APP CTF β was normalized by that of APP full length. The band density of APP CTF β in APP-HFD mice was increased compared with that in control APP mice ($F_{(2, 10)} = 5.67$, $p = 0.011$). On the other hand, that in APP-HFD+EE mice was significantly decreased compared with that in APP-HFD mice ($p = 0.032$).

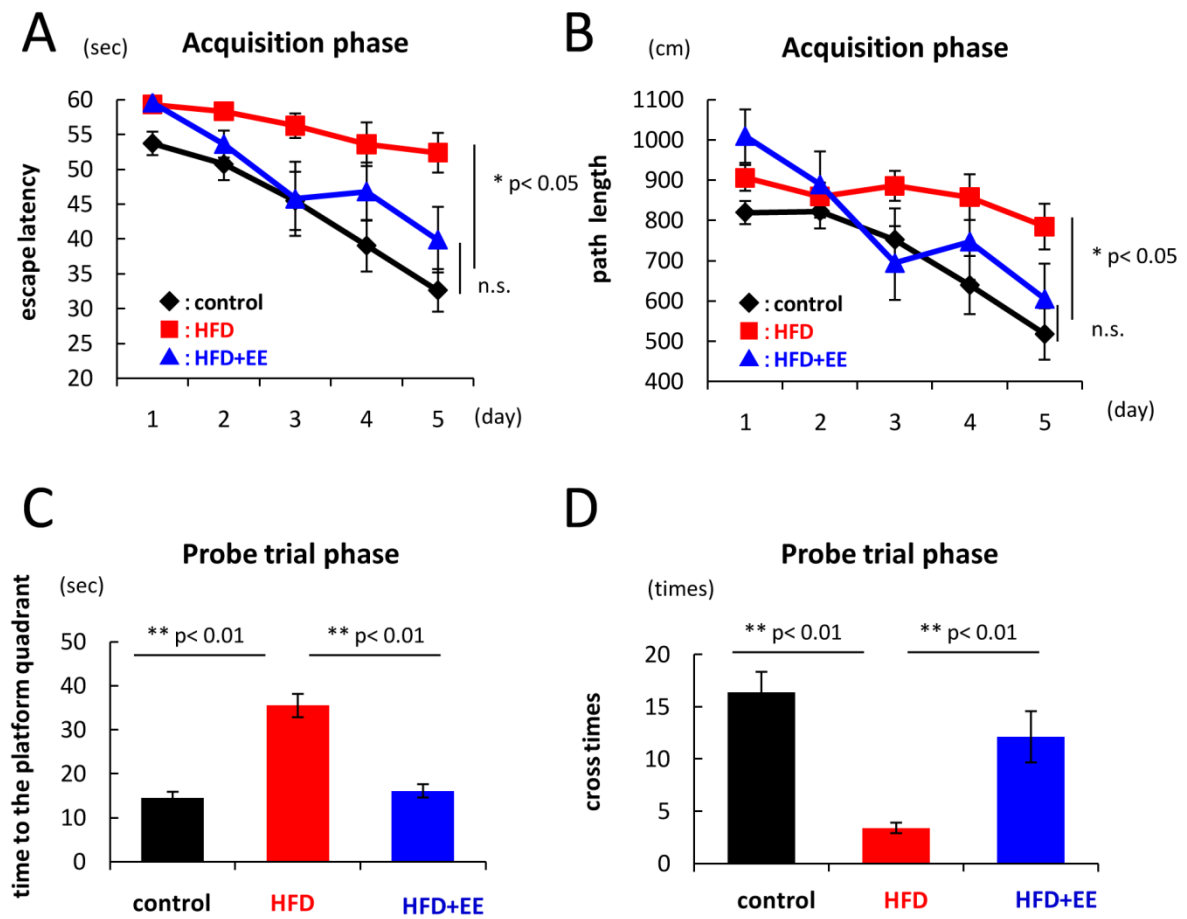
Maesako et al. Fig. 1

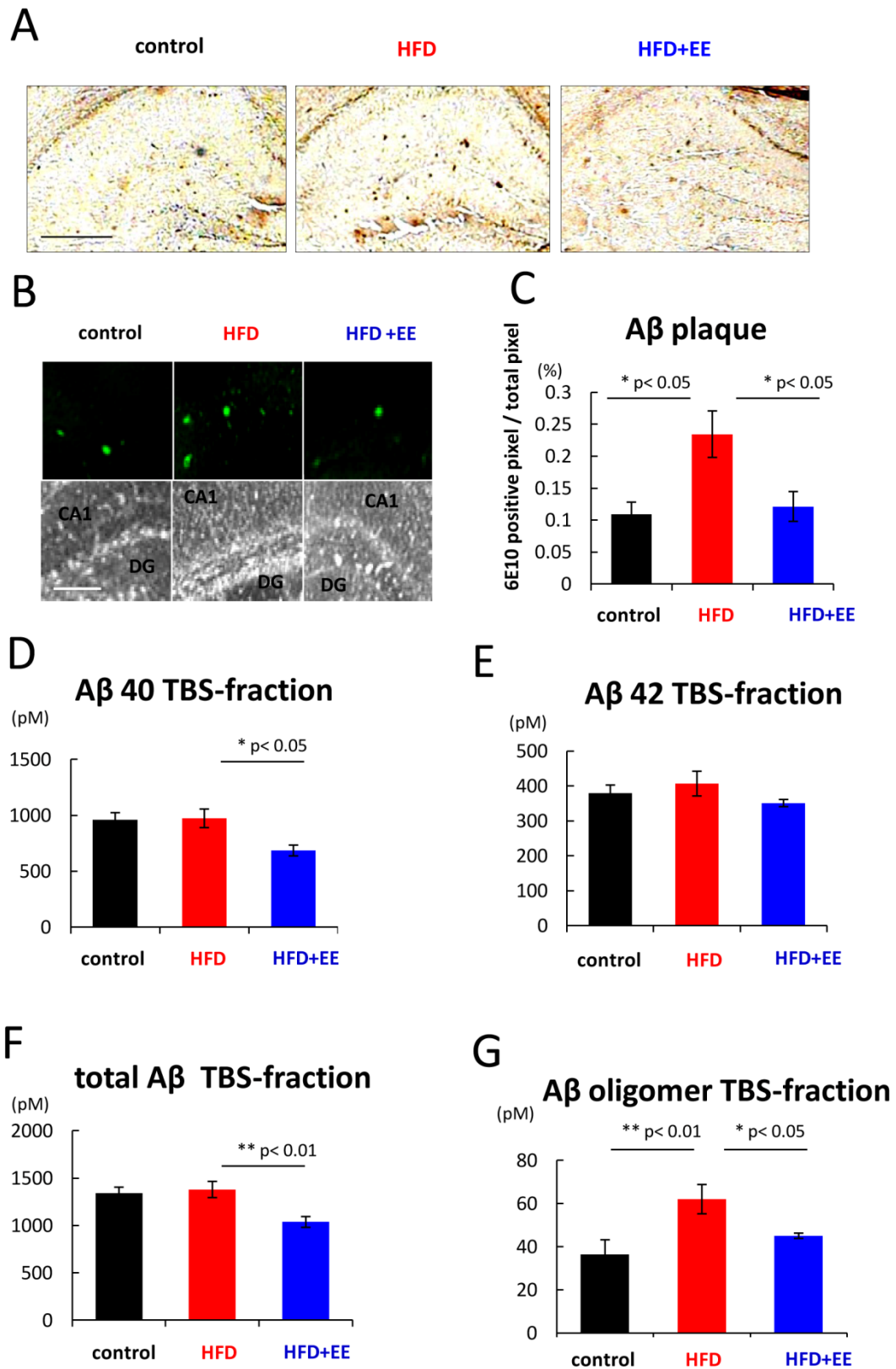




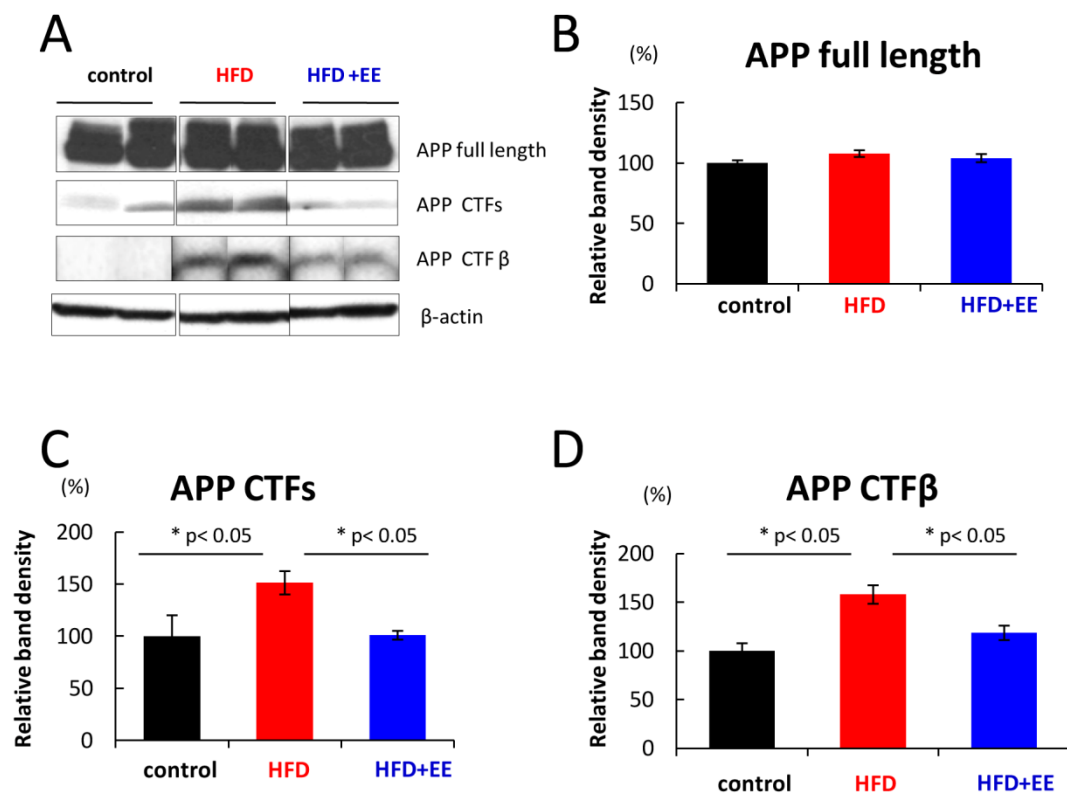


Maesako et al. Fig. 4





Maesako et al. Fig. 6



Maesako et al., Supplemental Figure legends

Supplemental Fig. 1. Every week monitoring of the amount of food intake.

The amount of food intake in control-APP, APP-HFD and APP-HFD+EE mice were monitored every week. After the induction of EE, APP-HFD+EE mice tended to have more food than APP-HFD mice did.

Supplemental Fig. 2. Analysis of glucose tolerance activity of 10 weeks HFD-induced APP mice by IGTT.

After 10 weeks induction of HFD (at the time of the switch from the poor environment to the EE), the mice were given a single dose of intra-peritoneal injection of glucose (2 g/kg body weight) after 14 hours fasting, and blood was collected from the tail-vein periodically over 2 hours. Blood glucose content was measured by using LabAssay Glucose (Wako, Japan). The glucose tolerance of the mice at the time of the switch from the poor environment to the EE was worse than that of APP-HFD mice, which went through EE session.

Supplemental Fig. 3. Locomotor activity was not different in Morris water maze test.

Locomotor activity in the visual cue phase of Morris water maze test. There was no statistical significance among control APP, APP-HFD and APP-HFD+EE induced mice ($F_{(2, 24)} = 1.44$, n.s.).

Supplemental Fig. 4. Analysis of glucose tolerance activity and memory function of wild type mice.

(A) Wild Type (WT) mice were exposed to either HFD or a standard diet for 20 weeks, from 2-3 to 7-8 months age. To examine the effect of EE on WT mice fed with HFD (WT-HFD mice), the cage of the mice was changed to a 2.4 times larger one equipped with a running wheel as well as objects like stands and toys after 10 weeks of HFD (WT-HFD + EE mice). The mice spent 10 weeks in the EE condition in the presence of HFD. After the dietary manipulation, glucose tolerance activity was

examined by IGTT. As same as in APP mice, HFD led to glucose intolerance ($F_{(2, 30)} = 24.06$, $p < 0.001$) but EE ameliorated it in WT mice ($p = 0.043$).

(B) Serum insulin levels during fasting. The serum insulin level of WT-HFD mice was significantly increased compared with that of control WT mice ($F_{(2, 9)} = 12.13$, $p = 0.03$). The serum insulin level of WT-HFD+EE mice was not significantly decreased compared with that of WT-HFD mice. n.s. indicated not significantly.

(C) Escape latency in the acquisition phase of Morris water maze test. After 4th day, acquisition time of HFD-induced WT (WT-HFD) mice was the same as that of control WT or WT-HFD+EE mice, although acquisition time of WT-HFD mice was longer than that of control WT or WT-HFD+EE mice from 1st to 3rd day.

Supplemental Fig. 5 ELISA of A β 40, 42 in Formic Acid (FA)-soluble fraction.

(A) ELISA of A β 40 in FA-soluble fraction. The level of FA-soluble A β 40 in APP-HFD mice significantly increased compared with that in control APP mice ($F_{(2, 10)} = 3.67$, $p = 0.03$). On the other hand, that in APP-HFD+EE mice was significantly decreased it compared with that in APP-HFD mice ($p = 0.045$). (B) ELISA of A β 42 in FA-soluble fraction. There was no statistical significance among control APP, APP-HFD and APP-HFD+EE induced mice ($F_{(2, 10)} = 1.19$, n.s.).

(C) ELISA of total A β (A β 40 + A β 42) in FA-soluble fraction. There was no statistical significance among control APP, APP-HFD and APP-HFD+EE induced mice ($F_{(2, 10)} = 1.81$, n.s.).

Supplemental Fig. 6. Filter trap assay for examination of oligomeric A β level.

(A) The amount of A β oligomers in the TBS-soluble fractions of control-APP, APP-HFD and APP-HFD+EE mice was analyzed by Filter trap assay using anti-A β oligomer antibody.

(B) Dot density was statistically analyzed. The average band density of the control APP samples was regard as 100 % and that of other groups was relatively indicated. The relative density of APP-HFD mice was significantly increased compared with that in control APP mice ($F_{(2, 9)} = 15.31$, $p = 0.004$). On the other hand, that in APP-HFD+EE mice was significantly decreased it compared with that in

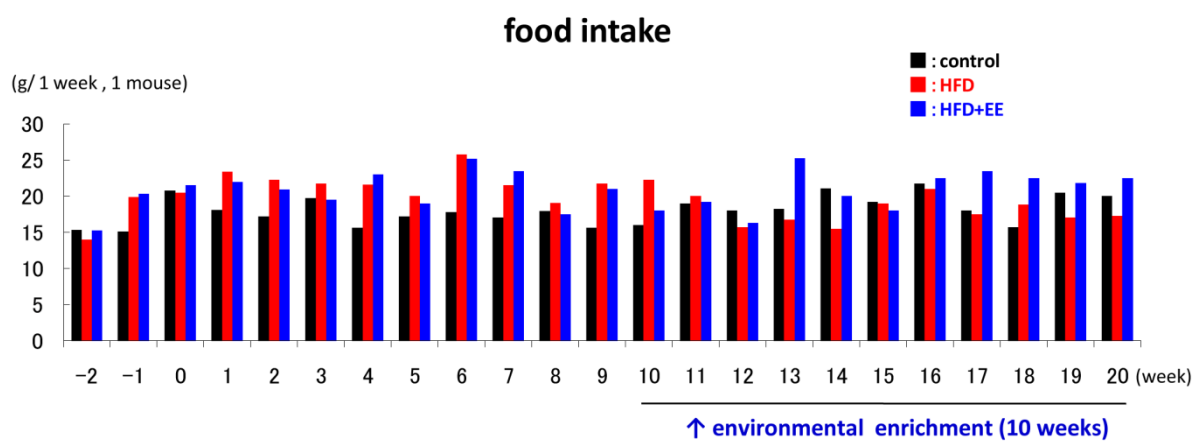
APP-HFD mice ($p = 0.012$).

Supplemental Fig. 7. The expression level of BACE1.

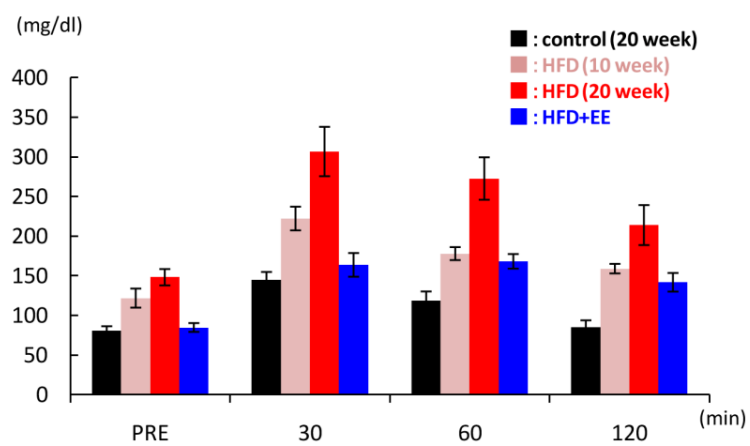
(A) Immunoblotting analysis of BACE1 level. BACE1 was detected by anti-BACE1 c-terminus antibody (Chalbiochem).

(B) Statistical analysis of BACE1. The band of BACE1 was normalized by that of β -actin. The band density of the control APP was regarded as 100 % and that of other groups was relatively indicated. There was no statistical significance among control APP, APP-HFD and APP-HFD+EE mice ($F_{(2,9)} = 0.42$, n.s.).

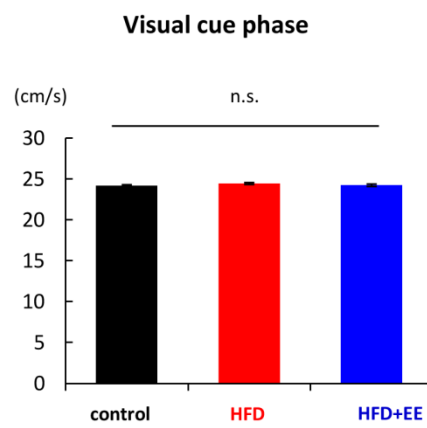
Maesako et al. Supplemental Fig. 1



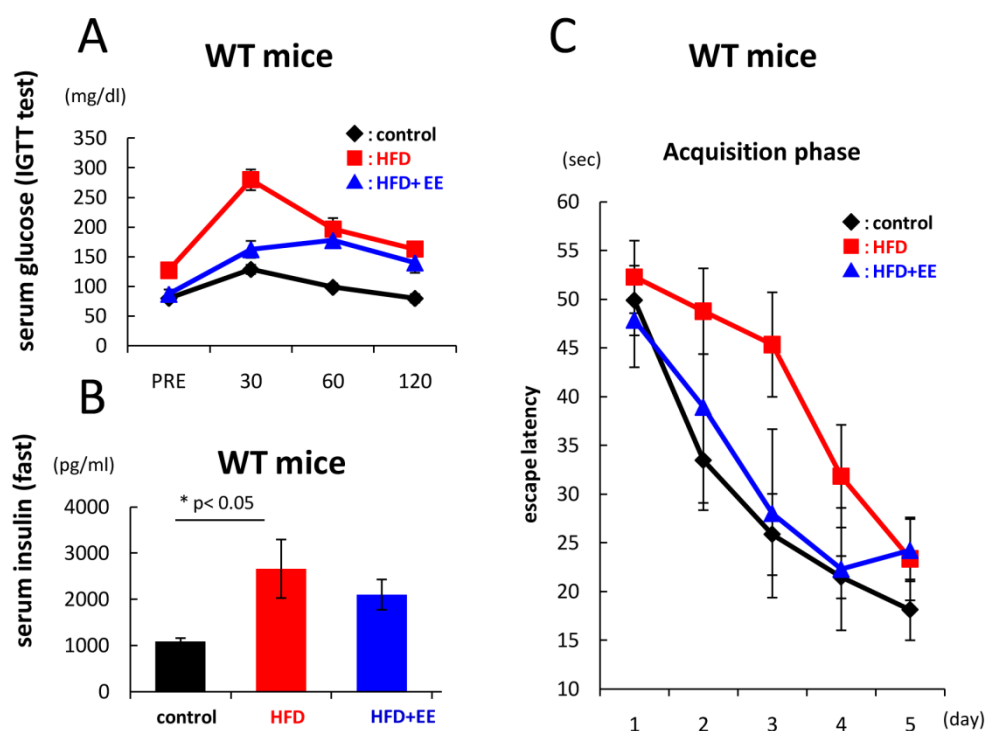
Maesako et al. Supplemental Fig. 2

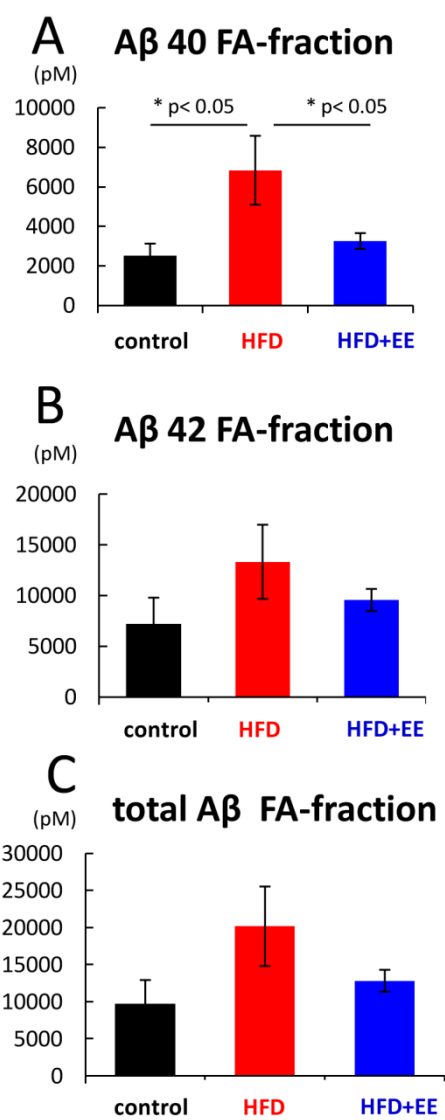


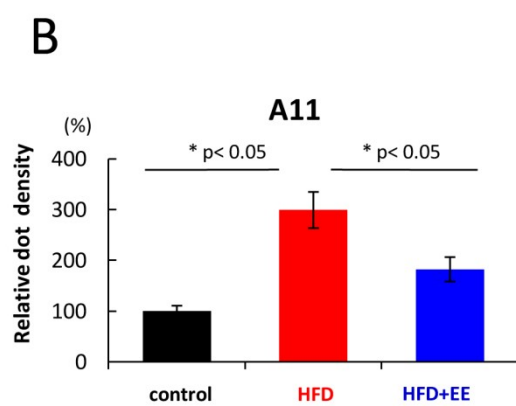
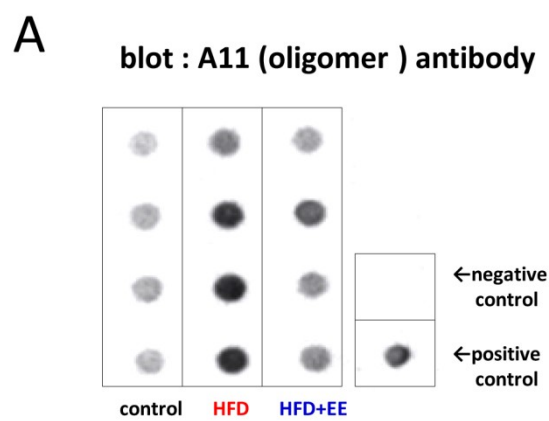
Maesako et al. Supplemental Fig. 3

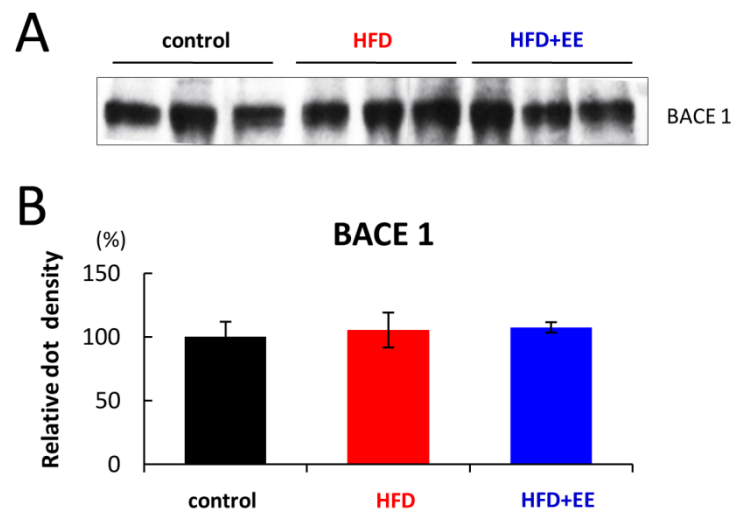


Maesako et al. Supplemental Fig. 4









Continuation of Exercise Is Necessary to Inhibit High Fat Diet-Induced β -Amyloid Deposition and Memory Deficit in Amyloid Precursor Protein Transgenic Mice

Masato Maesako¹, Kengo Uemura^{2,3*}, Ayana Iwata¹, Masakazu Kubota¹, Kiwamu Watanabe², Maiko Uemura², Yasuha Noda¹, Megumi Asada-Utsugi¹, Takeshi Kihara¹, Ryosuke Takahashi², Shun Shimohama⁴, Ayae Kinoshita¹

¹ School of Human Health Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan, ² Department of Neurology, Kyoto University Graduate School of Medicine, Kyoto, Japan, ³ Ishiki Hospital, Kagoshima, Japan, ⁴ Department of Neurology, Sapporo Medical University, Sapporo, Japan

Abstract

High fat diet (HFD) is prevalent in many modern societies and HFD-induced metabolic condition is a growing concern worldwide. It has been previously reported that HFD clearly worsens cognitive function in amyloid precursor protein (APP) transgenic mice. On the other hand, we have demonstrated that voluntary exercise in an enriched environment is an effective intervention to rescue HFD-induced β -amyloid ($A\beta$) deposition and memory deficit. However, it had been unclear whether consumption of HFD after exercising abolished the beneficial effect of exercise on the inhibition of Alzheimer's disease (AD) pathology. To examine this question, we exposed wild type (WT) and APP mice fed with HFD to exercise conditions at different time periods. In our previous experiment, we gave HFD to mice for 20 weeks and subjected them to exercise during weeks 10–20. In the present study, mice were subjected to exercise conditions during weeks 0–10 or weeks 5–15 while being on HFD. Interestingly, we found that the effect of exercise during weeks 0–10 or weeks 5–15 on memory function was not abolished in WT mice even if they kept having HFD after finishing exercise. However, in APP transgenic mice, HFD clearly disrupted the effect of exercise during weeks 0–10 or weeks 5–15 on memory function. Importantly, we observed that the level of $A\beta$ oligomer was significantly elevated in the APP mice that exercised during weeks 0–10: this might have been caused by the up-regulation of $A\beta$ production. These results provide solid evidence that continuation of exercise is necessary to rescue HFD-induced aggravation of cognitive decline in the pathological setting of AD.

Citation: Maesako M, Uemura K, Iwata A, Kubota M, Watanabe K, et al. (2013) Continuation of Exercise Is Necessary to Inhibit High Fat Diet-Induced β -Amyloid Deposition and Memory Deficit in Amyloid Precursor Protein Transgenic Mice. PLoS ONE 8(9): e72796. doi:10.1371/journal.pone.0072796

Editor: Mark P. Mattson, National Institute on Aging Intramural Research Program, United States of America

Received: May 29, 2013; **Accepted:** July 12, 2013; **Published:** September 4, 2013

Copyright: © 2013 Maesako et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The work was financially supported by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (24111524(AK), 23591243(KU)) and the Research Grant from Takeda Science Foundation (KU). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ueken@kuhp.kyoto-u.ac.jp

Introduction

Alzheimer's disease (AD), the occurrence of which is largely sporadic, is characterized by deficits in memory and other cognitive functions. Amyloid plaque is one of the pathological hallmarks of AD and it is composed of β -amyloid ($A\beta$). $A\beta$ is derived from the amyloid precursor protein (APP) via proteolytic cleavages by β - and γ -secretases. $A\beta$, in turn, is degraded by several $A\beta$ -degrading enzymes including neprilysin or insulin-degrading enzyme. A widely accepted hypothesis about AD pathogenesis is the amyloid cascade hypothesis, in which $A\beta$ plays a crucial role as an upstream molecule in neurodegeneration [1]. Considering that $A\beta$ accumulation is clearly observed almost a decade before cognitive impairment is seen in AD patients [2,3], there is growing consensus that prevention of AD at the earliest stage is desirable.

Metabolic conditions including obesity and diabetes mellitus are becoming severe problems. In modern society, the spread of high caloric diet including high fat diet (HFD) may be the critical cause

of metabolic abnormalities. Of course, metabolic conditions are known risk factors of vascular dementia but they could also risk factors of sporadic AD [4–6]. It has been consistently seen that feeding HFD to APP transgenic mice worsens the pathological alterations of $A\beta$ metabolism and memory impairment [7]. On the other hand, the paradigm of environmental enrichment has been frequently used in experiments wherein mice can conduct exercise voluntarily in a larger cage, with complex stimuli (e.g., running wheels, toys), thus being provided with more physical and intellectual stimulation than mice housed in standard conditions [8,9]. Recently, we set up an enriched condition focused on physical stimulation and demonstrated that voluntary exercise inhibits HFD-induced $A\beta$ deposition and memory deficit in APP transgenic mice [10]. More importantly, we have shown that voluntary exercise is more effective than diet control in our experimental setting [11]. Consistent with our results, many reports support the theory that physical activity is closely associated with a decreasing risk of cognitive impairment and

that exercise is an effective strategy to prevent development of AD [12–15].

Considering that metabolic condition is a growing issue worldwide, we wondered whether continuing to have HFD after exercising would abolish the beneficial effect of exercise on memory function. In the present study, we demonstrated that the effect of exercise on memory function was not abolished in WT mice even if they kept having HFD after finishing exercise. However, the exercise-induced improvement of memory was clearly disrupted in APP transgenic mice. We showed that consumption of HFD after exercising increased the level of A β oligomer in APP transgenic mice. Besides, we speculated that the development of A β pathology might have been caused by increase in A β production due to HFD. The results indicated that HFD after finishing exercise might immediately worsen AD pathology, and later disrupt the effect of exercise on memory function. Therefore, continuation of exercise is necessary to inhibit HFD-induced cognitive impairment in the pathological setting of AD.

Methods

Ethics statement

All animal experiments in this study were performed with the approval of the Animal Experiment Committees of Kyoto University, Graduate School of Medicine (Permit Number: 09597). All experiments were conducted in strict adherence to the relevant international guidelines. Every effort was made to minimize suffering of the animals.

Animals, dietary and exercise conditions

WT (C57BL/6 J) mice were obtained from Charles River Laboratories Japan, Inc. (Japan). Human APP transgenic mice (J20 mice) overexpressing familial AD-linked mutations bearing both Swedish (K670N/M671L) and Indiana (V717F) mutation were imported from the Jackson Laboratory (USA) [16]. They were maintained as heterozygotes. Since HFD worsened memory deficit and A β deposition in female APP transgenic mice better than in male (unpublished observation), female mice were used in these experiments. To establish APP transgenic mice fed with HFD (APP-HFD mice), age matched female were exposed to an established HFD (caloric composition, 60% fat, 20% carbohydrate, and 20% protein, Research Diet, Inc., Canada) for 20 weeks, from 2–3 to 7–8 months of age. As a control diet, female APP transgenic mice were exposed to a standard diet (10% fat, 70% carbohydrate, and 20% protein, Oriental Yeast Co., Ltd., Japan). In voluntary exercise (Ex) condition, a cage was changed to an enriched environment which was a 2.4 times larger than the standard cage, and was equipped with a running wheel (12 cm in diameter), toys and a stand. To examine the effects of exercise at different periods on APP-HFD mice, female APP-HFD mice spent weeks 0–10 (APP-HFD+Ex 0–10 mice, $n=8$) or weeks 5–15 (APP-HFD+Ex 5–15 mice, $n=7$) in exercise conditions in the presence of HFD. As a control, we used female APP-HFD mice which spent weeks 10–20 (APP-HFD+Ex 10–20 mice, $n=6$) in exercise conditions which we had previously established [10]. In the experiments of metabolic analyses and memory test, we examined the effect of exercise at different periods on WT mice fed HFD (WT-HFD mice), using the same paradigm ($n=5$, each group). In order to examine the running length per day, we monitored the number of running wheel rotations and estimated it as follows:

$$n \text{ (the number of running wheel rotations)} \times 12 \text{ (cm/diameter)} \times 3.14.$$

After dietary and motile manipulations, metabolic changes in these mice were analyzed, which was followed by assessment of memory function through Morris water maze test, as described below. After the analysis of memory function, brains were extracted and cut sagittally into left and right hemispheres. Tribromoethanol was used for anesthesia in the surgical procedures. The left hemisphere was fixed in 4% paraformaldehyde for histological analysis. After removing the olfactory lobe and the cerebellum, the right hemisphere was rapidly frozen in liquid nitrogen for biochemical analysis.

Assessment of metabolic changes

Blood was collected from the tail-vein. To assess glucose intolerance in these mice, we conducted intra-peritoneal glucose tolerance test (IGTT). Mice were given a single intra-peritoneal injection of glucose (2 g/kg body weight) after 14 hours fasting, and blood was collected periodically over 2 hours (fasting, 30 min, 60 min and 120 min). Plasma glucose content was measured by using LabAssay Glucose (Wako, Japan). Plasma insulin concentration was measured by enzyme-linked immunosorbent assay (ELISA) kit specific to insulin (Morinaga Seikagaku, Japan).

Morris water maze test

In order to assess spatial navigation learning and memory retention, Morris water maze test was conducted using a pool of water (diameter 120 cm, height 25 cm, temperature $21 \pm 1^\circ\text{C}$).

Visual cue phase. Visible platform training was performed to measure the motivation and swimming speed of mice to find a platform. Distal cues were removed from around the pool, and the platform was labeled with a flag and placed 1 cm above the surface of the water in the center of a quadrant. Mice were placed in the maze and allowed to explore the maze for 60 sec; if they reached the visible platform, they were allowed to remain there for 20 sec before being returned to their cages. If they did not find the platform within 60 sec, the experimenter led them to the platform and let them remain there for 20 sec. The training was completed once each animal received six trials. This training was performed for 1 day.

Acquisition phase. We measured the ability of the mice to understand the spatial relationship between a safe, but invisible platform of 10 cm diameter (submerged 1 cm below water level), and visual cues surrounding the maze. The platform was located in the center of one of the four quadrants, and several extramaze cues were distributed across the walls surrounding the pool. During the acquisition phase of training, each mouse received four daily hidden platform training trials with 12 min intervals for 5 consecutive days. The animals were allowed 60 sec to locate the platform and 20 sec to rest on it. The mice that failed to find the platform were led there by the experimenter and allowed to rest there for 20 sec.

Probe trial phase. A day after the last acquisition trial, a single 60 sec probe trial was administered to assess spatial memory retention. For the probe trial, the animals were returned to the pool without the platform present, and the parameters were recorded to assess the ability of the mouse to remember the previous location of the platform.

Performance was recorded with an automated tracking system (ANY-maze, Brain Science. Idea. Co., Ltd., Japan) during all the phases of training. During the visual cue phase of training, the speeds at which the mice reached to the platform were used to compare the activity of the performance among each group. During the acquisition phase, time to goal (latency to reach the platform) was subsequently used to analyze and compare the performance between the different treatment groups. The time

taken to get to the position where platform presented and the time spent in goal quadrant were analyzed during the probe trials.

Measurement of A β by ELISA

In order to prepare the samples for detection of A β , mice cerebrums were homogenized with Tris buffer saline (TBS) with protease inhibitor cocktail (Roche, Germany). The homogenate was centrifuged at 100,000 g for 1 hour, and the supernatant was collected as the TBS-extracted fraction. The pellet was washed in TBS and centrifuged at 100,000 g for 1 hour. Seventy percent formic acid (FA) was added to the pellet, which was homogenized again. The homogenate was incubated for 1 hour at 4°C and then centrifuged at 100,000 g for 1 hour at 4°C. The resultant supernatant was collected as the FA-extracted fraction, which was neutralized with a 20-fold volume of 1 M Tris buffer (pH 11.0).

The levels of A β 40 and A β 42 in FA fraction, or A β oligomer in TBS fraction were measured using ELISA kits specific to A β 40, A β 42, or A β oligomer (82E1-specific) (Immuno-Biological Laboratories Co., Ltd., Japan), and in accordance with the manufacturer's instructions. We used a standard format for measuring monomeric A β species with the use of C-terminal capturing antibodies and N-terminal or mid-region detecting antibodies. To detect A β oligomer species, the same N-terminal antibody, 82E1 (to A β residues 1–16, Immuno-Biological Laboratories, Inc, USA), was used for both capture and detection.

Immunoblotting and filter trap assay

For immunoblotting analysis, mice cerebrums were extracted in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X100, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, pH 8.0) with protease inhibitor cocktail and sufficiently homogenized on ice. The samples were incubated for one night at 4°C and centrifuged at 14,000 g for 20 min. The supernatants were directly used for Western blot analysis. The detailed protocol has been described previously [10,11]. In the previous experiments, we had used two types of gels (5–20% polyacrylamide gradient gels (Atto, Japan) and 4–12% NuPAGE Bis-Tris gel (Invitrogen, USA)) and two types of antibodies (rabbit polyclonal anti-APP C-terminal antibody and mouse monoclonal 6E10 antibody). Since the experiment using 4–12% NuPAGE Bis-Tris gel and rabbit polyclonal anti-APP C-terminal antibody was the most quantitative, the same as used in the present study. Rabbit polyclonal anti-APP C-terminal antibody was obtained from SIGMA.

Filter trap assay was conducted as described previously [11]. Briefly, the protein concentration of the cerebrum samples in TBS-extracted fraction was measured and an equal amount of protein was subjected to vacuum filtration through a 96-well dot blot apparatus (Bio-Rad Laboratories, USA) containing 200 nm pore-sized nitrocellulose membrane. The membrane was then incubated with the primary antibody at 4°C overnight. It was then blocked by TBS containing 4% skimmed milk, and incubated with HRP-linked secondary antibody (GE Healthcare, UK; diluted 1:2000) for 1 hour. Next, it was developed using the ECL Western Blotting Analysis System (GE Healthcare). Anti-oligomer antibody (A11, Invitrogen) was used for the detection of A β oligomer in the TBS soluble fraction.

Immunohistochemistry

The paraformaldehyde-fixed and paraffin-embedded tissue sections of mice were deparaffinized and rehydrated. The antigens of tissue sections were activated by autoclave for 10 minutes in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity

was suppressed by 0.6% hydrogen peroxide. Thereafter, tissue sections were blocked by horse serum and incubated with primary antibodies for 1 hour. The sections were then incubated with Histofine Simple Stain MAX PO (Nichirei Corporation, Japan) for 1 hour. Subsequently, the labeling was visualized by incubation with a solution of 3,3'-diaminobenzidine (Merck & Co., Inc, USA) with hydrogen peroxide. All images were visually analyzed using a microscope, ECLIPSE 80i (Nikon Corporation). Anti-A β (6E10) antibody (1:200; SIGMA) was used for the detection of A β plaque.

Neprilysin activity assay

The proteolytic activity of neprilysin was measured as described previously but with minor modifications [17]. Briefly, mice cerebrums were extracted in RIPA buffer and protein concentrations were analyzed. 25 μ g of extracted samples were incubated with 50 μ M substrate 3-dansyl-D-Ala-Gly-p-(nitro)-Phe-Gly (DAGNPG) (SIGMA) and 1 μ M captopril- angiotensin converting enzyme (ACE) inhibitor- in 200 μ l of 50 mM Tris-HCl buffer (pH 7.6) for 1 hour at 37°C. Reactions were stopped by heating samples to 100°C for 5 min, followed by 5,000 g $\times$ 5 min centrifugation. The 180 μ l of supernatant was diluted into 400 μ l of 50 mM Tris-HCl buffer (pH 7.6) and fluorescence was determined using Infinite 200 PRO (Tecan Japan Co., Ltd., Japan) (excitation 342 nm, emission 562 nm).

Statistical analysis

All values are given in means $\pm$ SE. Comparisons were performed using an unpaired Student's t-test. For comparison of multiparametric analysis, we used one-way factorial ANOVA, followed by a post-hoc analysis using Bonferroni post-hoc test. Statistical significance of differences between mean scores during IGTT and acquisition phase of Morris test were assessed with two-way repeated-measures ANOVA (general linear model/RM-ANOVA) and Bonferroni post-hoc analysis for multiple comparisons. The value $p < 0.05$ was considered to indicate a significant difference.

Results

Effects of exercise at different periods on the metabolic conditions in APP-HFD mice

In our previous experiment, we fed APP transgenic mice with HFD for 20 weeks and the mice were subjected to exercise conditions during weeks 10–20 [10]. However, in the present study, we exposed APP-HFD mice to exercise conditions at different periods: weeks 0–10 (APP-HFD+Ex 0–10 mice) and weeks 5–15 (APP-HFD+Ex 5–15 mice) (Figure 1). The increase of body weight was suppressed during exercising in APP-HFD+Ex 0–10 mice and APP-HFD+Ex 5–15 mice, although it was not as evident as that in APP-HFD+Ex 10–20 mice (Figure 2A). The food intake of APP-HFD+Ex 10–20 mice was the same or mildly increased compared with that of APP-HFD+Ex 0–10 mice and APP-HFD+Ex 5–15 mice (Figure S1), indicating that the attenuation of body weight in APP-HFD+Ex 10–20 mice was not caused by reduction of food intake. Fasting glucose levels in APP-HFD+Ex 0–10 mice and APP-HFD+Ex 5–15 mice were not different from those in APP-HFD mice, which were higher than that in APP-HFD+Ex 10–20 mice (Figure 2B, pre). Glucose tolerance ability was clearly deteriorated in APP-HFD+Ex 0–10 mice and APP-HFD+Ex 5–15 mice compared with that in APP-HFD+Ex 10–20 mice (Figure 2B). Insulin levels were not different among APP-HFD mice, APP-HFD+Ex 0–10 mice, APP-HFD+Ex 5–15 mice and APP-HFD+Ex 10–20 mice (Figure 2C). Importantly, running distances in APP-HFD+Ex 0–10 mice and APP-

HFD+Ex 5–15 mice were longer than those in APP-HFD+Ex 10–20 mice (Figure 2D), indicating that the deterioration of glucose metabolism in APP-HFD+Ex 0–10 mice and APP-HFD+Ex 5–15 mice was not caused by reduction of physical activity. We also examined HFD-induced metabolic changes in WT mice that were subjected to exercise. As in APP transgenic mice, HFD after finishing exercise increased body weight (Figure 2E) and reduced glucose tolerance (Figure 2F and G) in WT-HFD+Ex 0–10 mice and WT-HFD+Ex 5–15 mice. Collectively, these results indicated that HFD after exercising might abolish the effects of exercise in maintaining reduced body weight and improving glucose intolerance.

Effect of exercise on memory function was not abolished by HFD in WT but in APP transgenic mice

To examine whether HFD after exercising abolished the positive effect of exercise on memory function, we conducted Morris water maze test on the mice 20 weeks after having HFD. During the acquisition phase, the acquisition time was equally improved in WT-HFD+Ex 0–10 mice, WT-HFD+Ex 5–15 mice and WT-HFD+Ex 10–20 mice (Figure 3A, upper). In the probe trial phase, the time to get to goal position in WT-HFD+Ex 0–10 mice and that in WT-HFD+Ex 5–15 mice was the same as that in WT-HFD+Ex 10–20 mice, but it was shorter than that in WT-HFD mice (Figure 3B, left). Also, the time in goal quadrant in WT-HFD+Ex 0–10 mice and that in WT-HFD+Ex 5–15 mice was the same as that in WT-HFD+Ex 10–20 mice, but it was longer than that in WT-HFD mice (Figure 3C, left). These results suggested that WT mice might remain the effect of exercise on memory function even if they kept having HFD after exercising. In contrast, the time to get to goal position in APP-HFD+Ex 0–10 mice and that in APP-HFD+Ex 5–15 mice was significantly longer than that in APP-HFD+Ex 10–20 mice, but it was the same as that in APP-HFD mice (Figure 3B, right). Besides, the time in goal

quadrant in APP-HFD+Ex 0–10 mice and that in APP-HFD+Ex 5–15 mice was shorter than that in APP-HFD+Ex 10–20 mice, but it was the same as that in APP-HFD mice (Figure 3C, right). Notably, locomotor activities were not affected by different periods of exercise as exhibited by swimming speeds (Figure S2). Additionally, to examine whether exercise during weeks 0–10 and 5–15 actually improved memory function, we randomly selected two mice from each group- APP-HFD+Ex 0–10 mice, APP-HFD+Ex 5–15 mice and APP-HFD+Ex 10–20 mice- and examined their memory function at the time points 10 or 15 weeks after having HFD (Figure S3). Exercise during weeks 0–10 and 5–15 might be able to enhance memory function in APP-HFD mice. These results collectively indicated that consumption of HFD after finishing exercise could disrupt the positive effect of exercise on memory function in APP transgenic mice, but not in WT mice.

Improvement of A β pathology by exercise was abolished by HFD in APP transgenic mice

As shown in our previous report, HFD aggravated the oligomerization and accumulation of A β , whereas a marked reduction of the same was observed in APP-HFD+Ex 10–20 mice [10]. Immunohistochemical analysis showed that the accumulated A β level in APP-HFD+Ex 0–10 mice was higher than that in APP-HFD+Ex 10–20 mice (Figure 4A). ELISA also showed that the A β 40 level in FA fraction of APP-HFD+Ex 0–10 mice was higher than that of APP-HFD+Ex 10–20 mice (Figure 4B). There was no difference between the levels of A β 42 in APP-HFD+Ex 0–10 and in APP-HFD+Ex 10–20 mice (Figure 4C). Both A β 40 and A β 42 levels in APP-HFD+Ex 0–10 were lower than those in APP-HFD mice (Figure 4B and C). TBS-soluble A β oligomers are known as the most toxic form of A β correlating with memory deficits in AD model mice [18–20]. ELISA showed that the level of A β oligomer in APP-HFD+Ex 0–10 was significantly higher than that in APP-HFD+Ex 10–20 mice (Figure 4D). Filter trap analysis using anti-

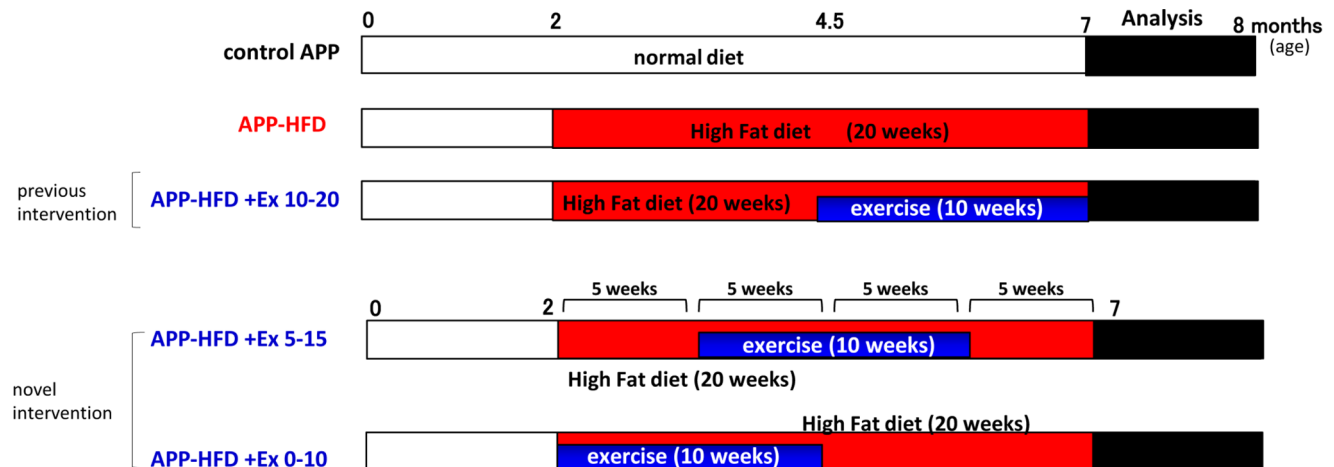


Figure 1. Exercise treatment given to APP-HFD mice at different time periods. Schematic presentation of the exercise treatment given to APP-HFD mice. Female APP transgenic mice were maintained on standard diet in standard laboratory cages until they were 2–3 months old. Then, the mice were separated into 5 groups. In the control group, the mice were fed with a standard diet in standard laboratory cages for 20 weeks (control APP mice) (top row, $n=5$). In the HFD-induced group, the mice were fed HFD in standard laboratory cages for 20 weeks (APP-HFD mice) (2nd row, $n=5$). In the exercise-induced group during weeks 10 to 20 of HFD, the mice spent 10 weeks in standard laboratory cages, and then spent 10 weeks in enrichment cages in the presence of HFD (APP-HFD+Ex 10–20 mice) (3rd row, $n=6$). As novel interventions, in the exercise-induced group during 5 to 15 weeks of HFD, the mice spent 5 weeks in standard laboratory cages, 10 weeks in enrichment cages, and then 5 weeks in standard laboratory cages in the presence of HFD (APP-HFD+Ex 5–15 mice) (4th row, $n=7$). In the exercise-induced group on 0 to 10 weeks of HFD, the mice spent 10 weeks in enrichment cages, and then spent 10 weeks in standard laboratory cages in the presence of HFD (APP-HFD+Ex 0–10 mice) (5th row, $n=8$). After 20 weeks, metabolic conditions of these mice were analyzed, followed by ethological, histochemical and biochemical analyses targeting AD pathophysiology. doi:10.1371/journal.pone.0072796.g001

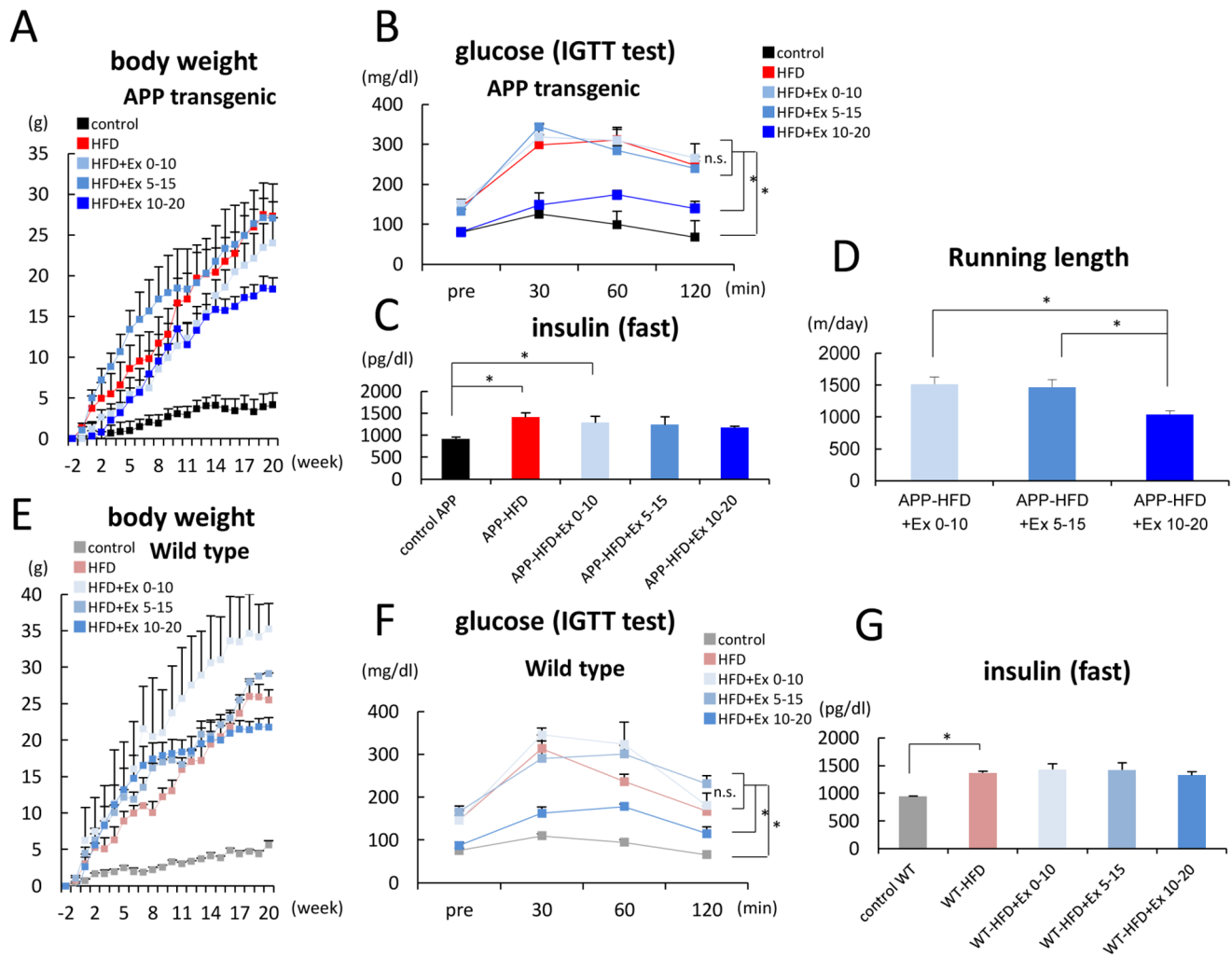


Figure 2. HFD after finishing exercise deteriorated glucose tolerance in APP-HFD mice. (A) Relative body weight changes over 20 weeks in control APP, APP-HFD, APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice. The body weight 2 weeks before each diet was regarded as the baseline (0 g). (B) Blood glucose levels during glucose tolerance test after an intra-peritoneal injection of glucose (2 g/kg body weight). Fasting glucose levels in APP-HFD+Ex 0–10 mice ($F_{(4, 20)} = 9.03$, $p < 0.001$) and in APP-HFD+Ex 5–15 mice ($p = 0.006$) were higher than those in APP-HFD+Ex 10–20 mice. Glucose tolerance abilities in APP-HFD+Ex 0–10 mice ($F_{(4, 60)} = 16.17$, $p < 0.001$) and in APP-HFD+Ex 5–15 mice ($p < 0.001$) were worse than those in APP-HFD+Ex 10–20 mice. The glucose tolerance in APP-HFD mice was the same as those in APP-HFD+Ex 0–10 mice and APP-HFD+Ex 5–15 mice. n.s. indicated not significant. * indicated $p < 0.05$. (C) Blood insulin levels during fasting. Plasma insulin levels in APP-HFD+Ex 0–10 mice and in APP-HFD+Ex 5–15 mice were not different from those in APP-HFD+Ex 10–20 mice ($F_{(4, 20)} = 2.22$). * indicated $p < 0.05$. (D) Average running distance using a running wheel per day (m/day). Running distance was estimated from the number of running wheel rotations. The lengths in APP-HFD+Ex 0–10 mice ($F_{(2, 12)} = 7.61$, $p = 0.003$) and in APP-HFD+Ex 5–15 mice ($p = 0.008$) were significantly longer than those in APP-HFD+Ex 10–20 mice. * indicated $p < 0.05$. (E) Relative body weight changes over 20 weeks in control WT, WT-HFD, WT-HFD+Ex 0–10, WT-HFD+Ex 5–15 and WT-HFD+Ex 10–20 mice. The body weight 2 weeks before each diet was regarded as the baseline (0 g). (F) Blood glucose levels during glucose tolerance test after an intra-peritoneal injection of glucose (2 g/kg body weight). Fasting glucose levels in WT-HFD+Ex 0–10 mice ($F_{(4, 10)} = 12.72$, $p = 0.006$) and in WT-HFD+Ex 5–15 mice ($p < 0.001$) were higher than that in WT-HFD+Ex 10–20 mice. Glucose tolerance abilities in WT-HFD+Ex 0–10 mice ($F_{(4, 30)} = 29.98$, $p < 0.001$) and in WT-HFD+Ex 5–15 mice ($p < 0.001$) were lower than that in WT-HFD+Ex 10–20 mice. n.s. indicated not significant. * indicated $p < 0.05$. (G) Insulin levels during fasting. Insulin levels in WT-HFD+Ex 0–10 mice and in WT-HFD+Ex 5–15 mice were not different from that in WT-HFD+Ex 10–20 mice ($F_{(4, 10)} = 7.24$). doi:10.1371/journal.pone.0072796.g002

oligomer antibody to detect oligomeric A β species also showed the same tendency in the amount of A β oligomer by ELISA assay (Figure 4E). Interestingly, the level of A β oligomer in APP-HFD+Ex 0–10 was the same as that in APP-HFD mice (Figure 4D and E). The amount of A β in FA fraction as well as A β oligomer in APP-HFD+Ex 5–15 mice were at intermediate levels between those in APP-HFD+Ex 0–10 mice and in APP-HFD+Ex 10–20 mice. These results collectively indicated that HFD after finishing exercise might revive the level of soluble A β oligomers.

APP-CTF β was accumulated in the APP mice having HFD after finishing exercise

From the above results, we wondered whether the level of A β oligomers in our mice was regulated by APP processing or by A β degradation. In order to investigate the effect of HFD after exercising on APP processing, we analyzed the level of APP C-terminus fragment (CTF) β through immunoblotting assay. α - and β -secretases cleave APP at the extramembrane domain, producing APP-CTF α and APP-CTF β respectively. γ -Secretase then cleaves

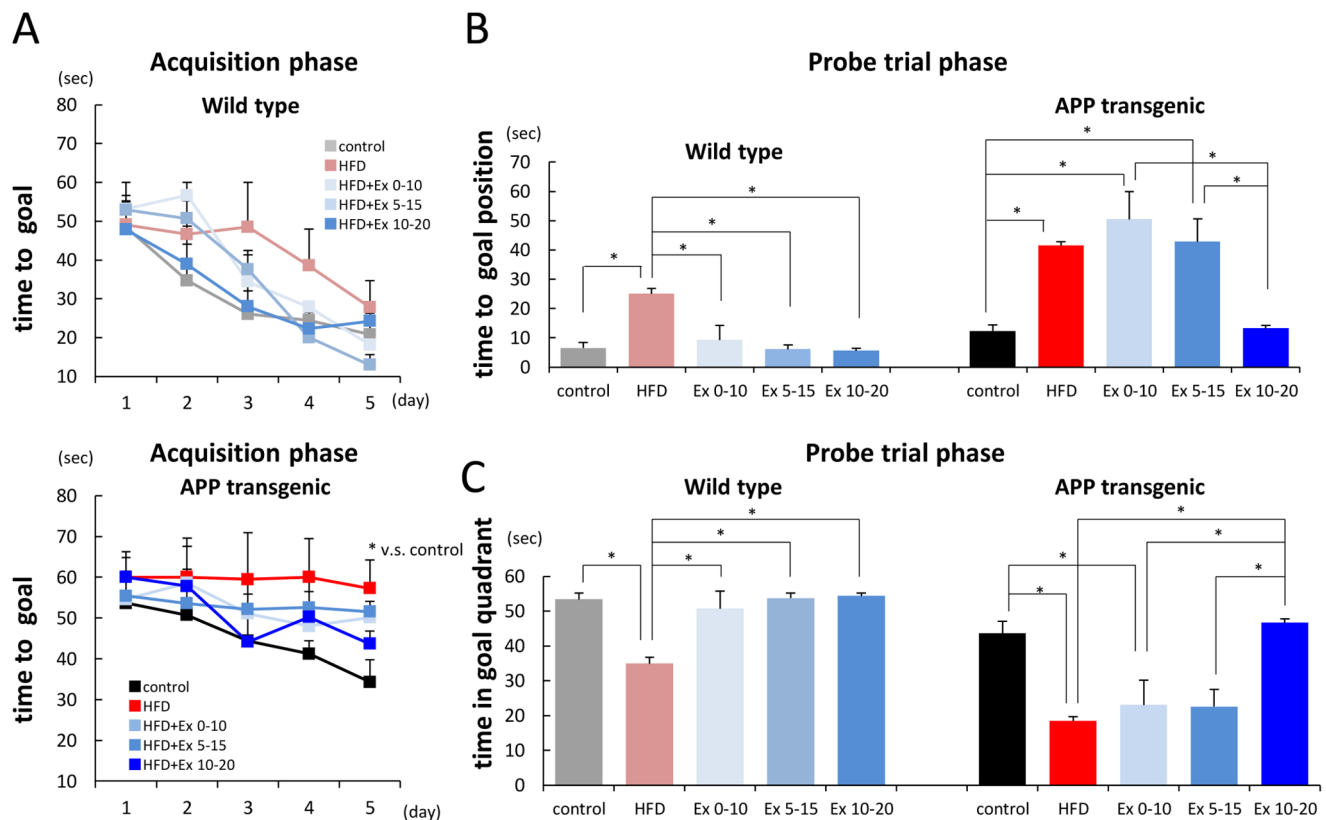


Figure 3. Effect of exercise on memory function was abolished by HFD in APP transgenic mice. (A) The time to get to goal platform of exercise-treated WT-HFD mice (upper) and APP-HFD mice (lower) in the acquisition phase of Morris water maze test, 20 weeks after having HFD. WT-HFD+Ex 0–10 mice and WT-HFD+Ex 5–15 mice took the same time to get to the platform as WT-HFD+Ex 10–20 mice. Also, APP-HFD+Ex 0–10 mice and APP-HFD+Ex 5–15 mice tended to take longer than APP-HFD+Ex 10–20 mice to get to the platform; however, this was statistically insignificant. (B) Time taken to get to goal position of exercise-treated WT-HFD mice (left) and APP-HFD mice (right) in the probe trial phase of Morris water maze test, 20 weeks after having HFD. WT-HFD+Ex 0–10 mice ($F_{(4, 10)} = 18.63$, $p < 0.001$) and WT-HFD+Ex 5–15 mice ($p < 0.001$) took less time to reach the platform position than WT-HFD mice, but they took the same time as WT-HFD+Ex 10–20 mice. On the other hand, APP-HFD+Ex 0–10 mice ($F_{(4, 20)} = 7.89$, $p < 0.001$) and APP-HFD+Ex 5–15 mice ($p = 0.006$) took longer to get to platform position than APP-HFD+Ex 10–20 mice. The time taken by APP-HFD+Ex 0–10 mice and APP-HFD+Ex 5–15 mice was the same as that by APP-HFD mice. * indicated $p < 0.05$. (C) Time in goal quadrant of exercise-treated WT-HFD mice (left) and APP-HFD mice (right) in the probe trial phase of Morris water maze test, 20 weeks after having HFD. WT-HFD+Ex 0–10 mice ($F_{(4, 10)} = 46.97$, $p < 0.001$) and WT-HFD+Ex 5–15 mice ($p < 0.001$) spent more time in goal quadrant than WT-HFD mice, but spent the same time in goal quadrant as WT-HFD+Ex 10–20 mice. On the other hand, APP-HFD+Ex 0–10 mice ($F_{(4, 20)} = 7.09$, $p = 0.003$) and APP-HFD+Ex 5–15 mice ($p = 0.003$) spent less time in goal quadrant than APP-HFD+Ex 10–20 mice. The time for APP-HFD+Ex 0–10 mice and APP-HFD+Ex 5–15 mice was the same as that for APP-HFD mice. * indicated $p < 0.05$. doi:10.1371/journal.pone.0072796.g003

APP-CTF α and APP-CTF β at the intramembrane domain, producing p3 and A β respectively. The level of APP-CTF β in APP-HFD+Ex 0–10 mice was higher than that in APP-HFD+Ex 10–20 mice, although it did not reach the level where it could be considered statistically significant (Figure 5A). The level of APP-CTF β in APP-HFD+Ex 5–15 mice was at intermediate level between that in APP-HFD+Ex 0–10 mice and in APP-HFD+Ex 10–20 mice. This result indicated that HFD after finishing exercise might promote the cleavage of APP by β -secretase, followed by the accumulation of APP-CTF β . Next, we examined the effect of HFD after exercising on A β degradation. Since we have previously reported that exercise strengthens the enzymatic activity of neprilysin, an A β -degrading protease, in APP-HFD mice [11], we conducted *in vitro* neprilysin activity assay. Notably, the enzymatic activity of neprilysin in APP-HFD+Ex 0–10 mice or in APP-HFD+Ex 5–15 mice was the same as that in APP-HFD+Ex 10–20 mice (Figure 5B). This result indicated that HFD for 10 weeks was not sufficient to abolish the exercise-induced up-regulation of neprilysin activity.

Discussion

Many reports have focused on the pathological roles of familial genes as a contributory factor in AD and their functions have been clarified gradually. On the other hand, increasing reports have recently examined the effects of environmental factors on AD pathology [21–29]. Importantly, epidemiological and experimental reports have shown that metabolic conditions caused by high caloric intake constitute an important risk factor in the development of sporadic AD [21,22,25]. Consistent with these backgrounds, it has been demonstrated that feeding HFD to APP mice shows significant deterioration of memory function [7]. It has also been shown that voluntary exercise is effective in the improvement of HFD-induced A β deposition and memory deficit [10]. In the present study, we showed the novel finding that the beneficial effect of exercise on memory function could last for a long time in WT mice, whereas it was immediately abolished in APP transgenic mice if they continued to take HFD after finishing exercise.

It has been proposed that hyperglycemia causes damage to neurons due to increase in the reactive oxygen species [30]. Also,

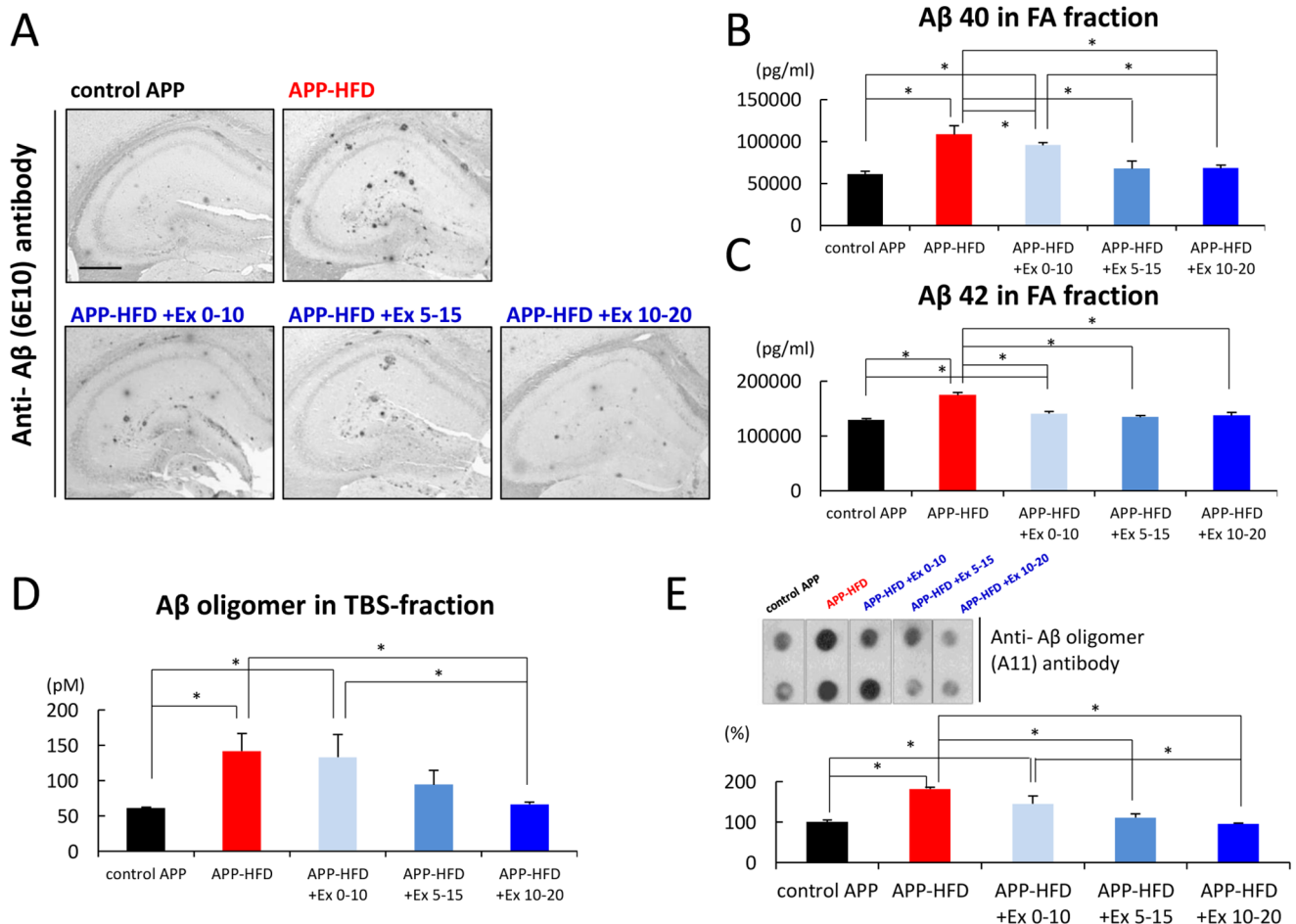


Figure 4. HFD after exercising increased Aβ oligomer as well as deposited Aβ levels in APP-HFD mice. (A) Immunohistochemical analysis using anti-Aβ (6E10) antibody. Representative images of Aβ-immunostained hippocampus sections from control APP, APP-HFD, APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice, respectively. Scale bar, 0.5 mm. Increase of Aβ deposition was observed in APP-HFD+Ex 0–10 and APP-HFD+Ex 5–15 mice compared with that in APP-HFD+Ex 10–20 mice. However, the amount of Aβ deposition in APP-HFD+Ex 0–10 mice and APP-HFD+Ex 5–15 mice was less than that in APP-HFD mice. (B) The amount of Aβ 40 in FA fraction of control APP, APP-HFD, APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice was analyzed by ELISA. Aβ 40 level in FA fraction of APP-HFD+Ex 0–10 mice was higher than that of APP-HFD+Ex 10–20 mice ($F_{(4, 15)} = 10.40$, $p = 0.009$). However, the amount of Aβ 40 in APP-HFD+Ex 0–10 mice ($p = 0.028$) and APP-HFD+Ex 5–15 mice ($p = 0.004$) was less than that in APP-HFD mice. * indicated $p < 0.05$. (C) The amount of Aβ 42 in FA fraction of control APP, APP-HFD, APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice was analyzed by ELISA. There was no statistically significant difference in the level of Aβ 42 among APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice ($F_{(4, 15)} = 24.4$). However, the amount of Aβ 42 in APP-HFD+Ex 0–10 mice ($p < 0.001$) and APP-HFD+Ex 5–15 mice ($p < 0.001$) was lower than that in APP-HFD mice. * indicated $p < 0.05$. (D) The amount of Aβ oligomer in the TBS-soluble fraction of control APP, APP-HFD, APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice was analyzed by ELISA. The level of Aβ oligomer in TBS fraction of APP-HFD+Ex 0–10 mice was higher than that of APP-HFD+Ex 10–20 mice ($F_{(4, 15)} = 3.33$, $p = 0.035$). The amount of Aβ oligomer in APP-HFD+Ex 0–10 mice was the same as that in APP-HFD mice. * indicated $p < 0.05$. (E) The amount of Aβ oligomer in the TBS-soluble fraction of control APP, APP-HFD, APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice was analyzed by filter trap assay using anti-oligomer (A11) antibody to detect oligomeric Aβ. Representative images of dot are shown in upper panel. Statistical analysis of dot density is described at the bottom. The average dot density of the control APP samples was regarded as 100% and that of other groups was relatively indicated. The relative density of APP-HFD+Ex 0–10 mice was higher than that of APP-HFD+Ex 10–20 mice ($F_{(4, 10)} = 12.69$, $p = 0.007$). The dot density of Aβ oligomer in APP-HFD+Ex 0–10 mice was the same as that in APP-HFD mice. * indicated $p < 0.05$. doi:10.1371/journal.pone.0072796.g004

HFD may lead to chronic cerebral hypoperfusion, which induces impairment of working memory [31]. HFD may also lead to a breakdown of the blood-brain barrier, resulting in the leakage of serum-derived components into the brain parenchyma, leading to neuronal dysfunction [32]. They might lead to memory impairment in WT-HFD mice. Actually, obesity and glucose intolerance were clearly observed in WT-HFD+Ex 0–10 and WT-HFD+Ex 5–15 mice (Figure 2), indicating that HFD after finishing exercise disrupted metabolic conditions, which might also damage neuronal functions in these mice. However, in the present study,

we showed that the beneficial effect of exercise on memory function was maintained in WT-HFD mice even though they stopped exercising (Figure 3). As a mechanism, we estimated that the beneficial roles of exercise might keep counteracting HFD-induced neuronal damages (i.e. from reactive oxygen species) in WT-HFD mice. According to previous reports, exercise enhances neurogenesis and increases the number of synapses [9]. Besides, exercise regulates neuronal development as well as plasticity [33]. Therefore, the pathways up-regulated by exercise might be different from those down-regulated by HFD.

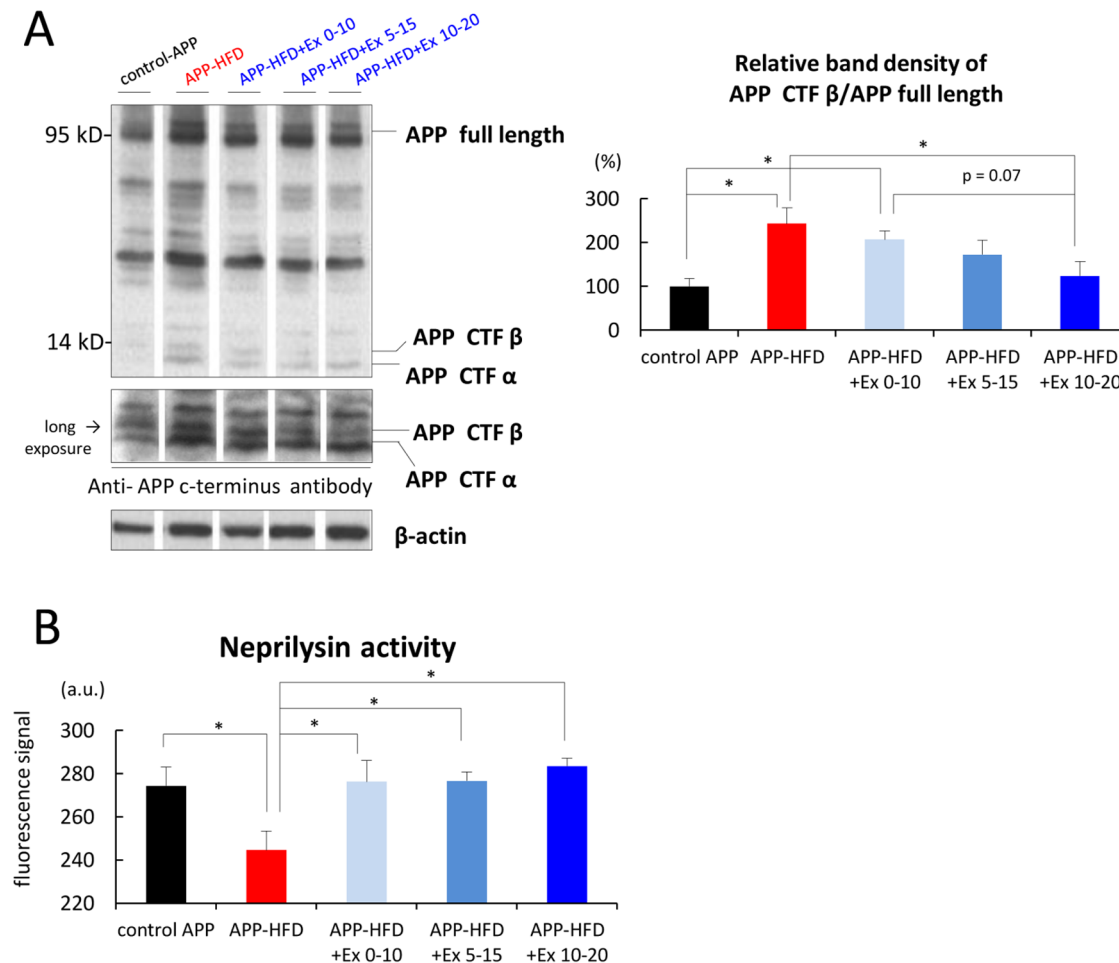


Figure 5. HFD after finishing exercise promoted APP CTFβ accumulation in APP-HFD mice. (A) Immunoblotting analysis of APP full length and APP-CTFβ using APP C-terminus antibody. The number of animals used for immunoblotting analysis is 3–4 per group. Statistical analysis is shown in the right panel. The band of APP CTFβ was normalized by that of APP full length. The band density of the control APP mice was regarded as 100% and that of other groups was relatively indicated. The band density of APP CTFβ in APP-HFD+Ex 0–10 mice tended to be higher compared with that in APP-HFD+Ex 10–20 mice ($F_{(4, 13)} = 3.70$, $p = 0.070$). * indicated $p < 0.05$. (B) *In vitro* enzyme activity assay of neprilysin using fluorescent substrate. The activity of neprilysin in APP-HFD+Ex 0–10 or APP-HFD+Ex 5–15 mice was not different from that in APP-HFD+Ex 10–20 mice ($F_{(4, 15)} = 4.10$). * indicated $p < 0.05$. doi:10.1371/journal.pone.0072796.g005

On the other hand, APP-HFD mice show more significant memory impairment than WT-HFD mice [10], suggesting that the memory impairment in APP-HFD mice was attributable to an interaction between HFD and APP metabolism. In the present study, exercise during early periods was able to inhibit HFD-induced memory impairment in APP-HFD mice (Figure S3). However, the beneficial effect of exercise on memory function was immediately abolished in APP-HFD mice when they kept having HFD after exercising (Figure 3). We observed that toxic Aβ oligomer level in APP-HFD+Ex0–10 mice was the same as that in APP-HFD mice. We also observed that the level of deposited Aβ in APP-HFD+Ex0–10 mice was lower than that in APP-HFD mice (Figure 4). Since the degree of memory impairment in APP-HFD+Ex0–10 mice was the same as that in APP-HFD mice, we speculated that the increase of soluble Aβ oligomer by HFD after finishing exercise might be sufficient to lead to memory loss. Then we examined the molecular mechanisms, based on which HFD after exercising increased Aβ oligomer level. In our previous report, we demonstrated that HFD may promote the cleavage of

APP by β-secretase leading to the production of Aβ, but exercise inhibited it. As a mechanism, we have demonstrated that HFD leads to metabolic conditions such as obesity and glucose abnormalities, followed by up-regulating β-secretase enzyme activity. But exercise can down-regulate β-secretase enzyme activity since it improves HFD-induced metabolic condition [10]. In the present study, we showed that HFD might increase the level of APP-CTFβ after finishing exercise (Figure 5). Thus, once exercise is finished, HFD might rapidly re-increase APP processing. In this sense, the effect of exercise on the inhibition of β-secretase activity might be temporary. We had recently shown that exercise strengthens the enzymatic activity of neprilysin, which may promote the degradation of Aβ [11]. However, in the present study, we showed that HFD after exercising did not lower neprilysin activity (Figure 5). According to these results, we speculated that HFD-promoted Aβ production might be the reason why HFD after finishing exercise increased Aβ oligomer as well as deposited Aβ. However, previous literature has reported that HFD suppresses the activity of insulin-degrading enzyme [7].

Thus, the effects of another A β -degrading enzyme on our results should be clarified in future studies.

Verret et al. have reported that in WT mice, an enriched environment during earlier period has the same effect on memory function as later one under a standard diet condition [34]. Although there is a discrepancy between their experimental setting and ours, we consistently showed that the effect of exercise on memory was maintained even under a HFD condition in WT mice (Figure 3). Verret et al. have also shown that an enriched environment during earlier period is more effective in reserving memory function than that during later period in APP transgenic mice having standard diet [34]. However, we showed that the effect of exercise during earlier period on memory function was clearly reduced 20 weeks after having HFD (Figure 3). In this sense, whether the effect of exercise is maintained might depend on the metabolic conditions in APP transgenic mice. However, there was a limitation in the experiment using APP transgenic mice. In this report, we showed that only 10 weeks of HFD after finishing exercise was sufficient to increase A β oligomer level and subsequently, to disrupt exercise-protected memory function. This rapid alteration might be due to the experimental strategy using transgenic model mice overexpressing APP and producing excess amount of A β . In fact, a previous study using *in vivo* multiphoton microscopy has reported that A β plaques form extraordinarily quickly, over 24 hours, in model mice of AD [35]. Such a rapid alteration of A β deposition may not be relevant in sporadic human AD cases. Considering this discrepancy between APP transgenic mice and human AD cases, it is not clear whether HFD abolishes the beneficial effect of exercise on memory function in human cases as rapidly as in APP transgenic mice. Nevertheless, our findings clearly indicate that continuation of exercise is necessary to rescue HFD-induced aggravation of cognitive decline in APP transgenic mice. Given that the effect of exercise depends on metabolic conditions, one's dietary pattern should be considered a very important factor in the prevention of AD.

Supporting Information

Figure S1 Every week monitoring of the amount of food intake. Every week monitoring showed that average amount of food intake in control APP, APP-HFD, APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice. During the induction of exercise, APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice tended to take more food than APP-HFD mice did. (PDF)

Figure S2 Swimming speeds in Morris water maze test. Locomotor activities of control APP, APP-HFD, APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice were analyzed by swimming speeds in the visual cue phase of Morris water maze tests 10, 15 (A) and 20 weeks (B) after having HFD. There were no statistical differences among control APP, APP-

HFD, APP-HFD+Ex 0–10, APP-HFD+Ex 5–15 and APP-HFD+Ex 10–20 mice.

(PDF)

Figure S3 Exercise at different periods were able to strengthen memory function in APP-HFD mice. 10 weeks after having HFD, the acquisition time was clearly shortened in APP-HFD+Ex 0–10 mice (Figure S3B, left). Furthermore, 15 weeks after having HFD, the acquisition time was also ameliorated in APP-HFD+Ex 5–15 mice (Figure S3B, right). These results indicated that exercise during weeks 0–10 and weeks 5–15 could strengthen memory function in APP-HFD mice. However, at 10 weeks after having HFD, the improvement in the acquisition time was not observed in APP-HFD+Ex 10–20 mice, indicating that HFD for 10 weeks was sufficient to induce memory loss in APP transgenic mice (Figure S3B, left). We also conducted the pilot study in WT-HFD mice using the same strategy in APP-HFD mice. Although HFD for 10 weeks was sufficient to lead to memory deficit in APP transgenic mice, HFD for 10 weeks did not induce memory impairment in WT mice (Figure S3C). (A) Schematic presentation of the pilot study in Morris water maze test. Morris water maze test was conducted 10 weeks and 15 weeks after having HFD. (B) The time to get to the goal platform of exercise-treated APP-HFD mice in the acquisition phase, 10 weeks (left) and 15 weeks (right) after having HFD. 10 weeks after having HFD, APP transgenic mice having HFD (APP-HFD+Ex 10–20 mice) took significant longer time to the platform. On the other hand, APP-HFD+Ex 0–10 mice clearly took shorter time to the platform than APP-HFD+Ex 10–20 mice. (C) The time to get to the goal platform of exercise-treated WT-HFD mice in the acquisition phase, 10 weeks (left) and 15 weeks (right) after having HFD. 10 weeks after having HFD, WT mice having HFD (WT-HFD+Ex 10–20 mice) did not take longer time to the platform. WT-HFD+Ex 0–10 mice took the same time to the platform as WT-HFD+Ex 10–20 mice.

(PDF)

Acknowledgments

We thank to Dr. S. Oka (Kyoto University, Kyoto, Japan), Dr. K. Takata (Kyoto Pharmaceutical University, Kyoto, Japan) and Dr. Y. Kitamura (Kyoto Pharmaceutical University, Kyoto, Japan) for technical assistance of the brain sample preparation. We give our thanks to Dr. K. Saito (Kyoto University, Kyoto, Japan) for kind support of the Morris water maze test. We appreciate to Dr. M. Okuda (PHARMAEIGHT, Kyoto, Japan) for the advice in metabolic analysis. We would like to give our thanks to Dr. Y. Tashiro (Kyoto University, Kyoto, Japan) for kind discussion.

Author Contributions

Conceived and designed the experiments: MM KU AK. Performed the experiments: MM AI MK KW MU YN MA. Analyzed the data: MM KU AI AK. Contributed reagents/materials/analysis tools: RT SS. Wrote the paper: MM KU TK AK.

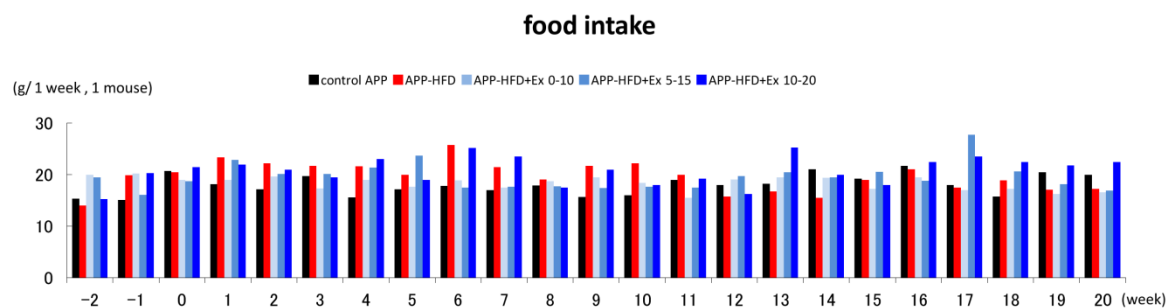
References

- Hardy J, Selkoe D (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 297: 353–3562.
- Sperling R, Aisen P, Beckett L, Bennett D, Craft S, et al. (2011) Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*. 7: 280–292.
- Bateman R, Xiong C, Benzinger T, Fagan A, Goate A, et al. (2012) Clinical and biomarker changes in dominantly inherited Alzheimer's disease. *New Engl J Med*. 367: 795–804.
- Luchsinger J, Reitz C, Honig L, Tang M, Shea S, et al. (2005) Aggregation of vascular risk factors and risk of incident Alzheimer disease. *Neurology*. 65: 545–551.
- Whitmer R (2007) The epidemiology of adiposity and dementia. *Cur Alzheimer Res*. 4: 117–122.
- Craft S (2009) The role of metabolic disorders in Alzheimer disease and vascular dementia: two roads converged. *Arch Neurol*. 66: 300–305.
- Ho L, Qin W, Pompl P, Xiang Z, Wang J, et al. (2004) Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. *FASEB J*. 18: 902–904.
- Lazarov O, Robinson J, Tang Y, Hairston I, Korade-Mirmics Z, et al. (2005) Environmental enrichment reduces A β levels and amyloid deposition in transgenic mice. *Cell* 120: 701–713.
- Hu Y, Xu P, Pigino G, Brady S, Larson J, et al. (2010) Complex environment experience rescues impaired neurogenesis, enhances synaptic plasticity, and

- attenuates neuropathology in familial Alzheimer's disease-linked APP^{swe}/PS1^{ΔE9} mice. *FASEB J*. 24: 1667–1681.
10. Maesako M, Uemura K, Kubota M, Kuzuya A, Sasaki K, et al. (2012) Environmental enrichment ameliorated high-fat diet-induced A β deposition and memory deficit in APP transgenic mice. *Neurobiol Aging*. 33: 1011 e11–23.
 11. Maesako M, Uemura K, Kubota M, Kuzuya A, Sasaki K, et al. (2012) Exercise is more effective than diet control in preventing high fat diet-induced β -amyloid deposition and memory deficit in amyloid precursor protein transgenic mice. *J Biol Chem*. 287: 23024–23033.
 12. Friedland R, Fritsch T, Smyth K, Koss E, Lerner A, et al. (2001) Patients with Alzheimer's disease have reduced activities in midlife compared with healthy control-group members. *Proc Natl Acad Sci U S A*. 98: 3440–3445.
 13. Laurin D, Verreault R, Lindsay J, MacPherson K, Rockwood K (2001) Physical activity and risk of cognitive impairment and dementia in elderly persons. *Arch Neurol*. 58: 498–504.
 14. Buchman A, Boyle P, Yu L, Shah R, Wilson R, et al. (2012) Total daily physical activity and the risk of AD and cognitive decline in older adults. *Neurology*. 78: 1323–1329.
 15. Mattson M (2012) Energy intake and exercise as determinants of brain health and vulnerability to injury and disease. *Cell Metab*. 16: 706–722.
 16. Mucke L, Masliah E, Yu G, Mallory M, Rockenstein E, et al. (2000) High-level neuronal expression of A β 1–42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J Neurosci*. 20: 4050–4058.
 17. Rose J, Crews L, Rockenstein E, Adame A, Mante M (2009) Neuropeptide Y fragments derived from neprilysin processing are neuroprotective in a transgenic model of Alzheimer's disease. *J Neurosci*. 29: 1115–1125.
 18. Walsh D, Klyubin I, Fadeeva J, Cullen W, Anwyl R, et al. (2002) Naturally secreted oligomers of amyloid- β protein potently inhibit hippocampal long-term potentiation in vivo. *Nature*. 416: 535–539.
 19. Lesne S, Koh M, Kotilinek L, Kaye R, Glabe C, et al. (2006) A specific amyloid- β protein assembly in the brain impairs memory. *Nature*. 440, 352–357.
 20. Shankar G, Li S, Mehta T, Garcia-Munoz A, Shepardson N, et al. (2008) Amyloid- β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med*. 14: 837–842.
 21. Luchsinger J, Tang M, Shea S, Mayeux R (2002) Caloric intake and the risk of Alzheimer disease. *Arch Neurol*. 59: 1258–1263.
 22. Morris M, Evans D, Bienias J, Tangney C, Bennett D, et al. (2003) Dietary fats and the risk of incident Alzheimer disease. *Arch Neurol*. 60: 194–200.
 23. Cao D, Lu H, Lewis T, Li L (2007) Intake of sucrose-sweetened water induces insulin resistance and exacerbates memory deficits and amyloidosis in a transgenic mouse model of Alzheimer disease. *J Biol Chem*. 282: 36275–36282.
 24. Julien C, Tremblay C, Phivilay A, Berthiaume L, Emond V, et al. (2010) High-fat diet aggravates amyloid- β and tau pathologies in the 3xTg-AD mouse model. *Neurobiol Aging*. 31: 1516–1531.
 25. Xu W, Atti A, Gatz M, Pedersen N, Johansson B, et al. (2011) Midlife overweight and obesity increase late-life dementia risk: a population-based twin study. *Neurology*. 76: 1568–1574.
 26. Hiltunen M, Khandelwal V, Yaluri N, Tiilikainen T, Tusa M, et al. (2012) Contribution of genetic and dietary insulin resistance to Alzheimer phenotype in APP/PS1 transgenic mice. *J Cell Mol Med*. 16: 1206–1222.
 27. De Felice F (2013) Alzheimer's disease and insulin resistance: translating basic science into clinical applications. *J Clin Invest*. 123: 531–539.
 28. Li S, Jin M, Zhang D, Yang T, Koeglspurger T, et al. (2013) Environmental novelty activates β 2-adrenergic signaling to prevent the impairment of hippocampal LTP by A β oligomers. *Neuron*. 77: 929–941.
 29. Moreno-Gonzalez I, Estrada L, Sanchez-Mejias E, Soto C (2013) Smoking exacerbates amyloid pathology in a mouse model of Alzheimer's disease. *Nat Commun*. 4: 1495.
 30. Sharma R, Buras E, Terashima T, Serrano F, Massaad C, et al. (2010) Hyperglycemia induces oxidative stress and impairs axonal transport rates in mice. *PLoS ONE*. 5: e13463.
 31. Shibata M, Yamasaki N, Miyakawa T, Kalaria R, Fujita Y, et al. (2007) Selective impairment of working memory in a mouse model of chronic cerebral hypoperfusion. *Stroke*. 38: 2826–2832.
 32. Serlin Y, Levy J, Shalev H (2011) Vascular pathology and blood-brain barrier disruption in cognitive and psychiatric complications of type 2 diabetes mellitus. *Cardiovasc Psychiatry Neurol*. 2011: 609202.
 33. Neeper S, Gomez-Pinilla F, Choi J, Cotman C (1995) Exercise and brain neurotrophins. *Nature*. 373: 109.
 34. Verret L, Krezyon A, Halley H, Trouche S, Zerwas M, et al. (2013) Transient enriched housing before amyloidosis onset sustains cognitive improvement in Tg2576 mice. *Neurobiol Aging*. 34: 211–225.
 35. Meyer-Luehmann M, Spire-Jones T, Prada C, Garcia-Alloza M, de Calignon A, et al. (2008) Rapid appearance and local toxicity of amyloid- β plaques in a mouse model of Alzheimer's disease. *Nature*. 451: 720–724.

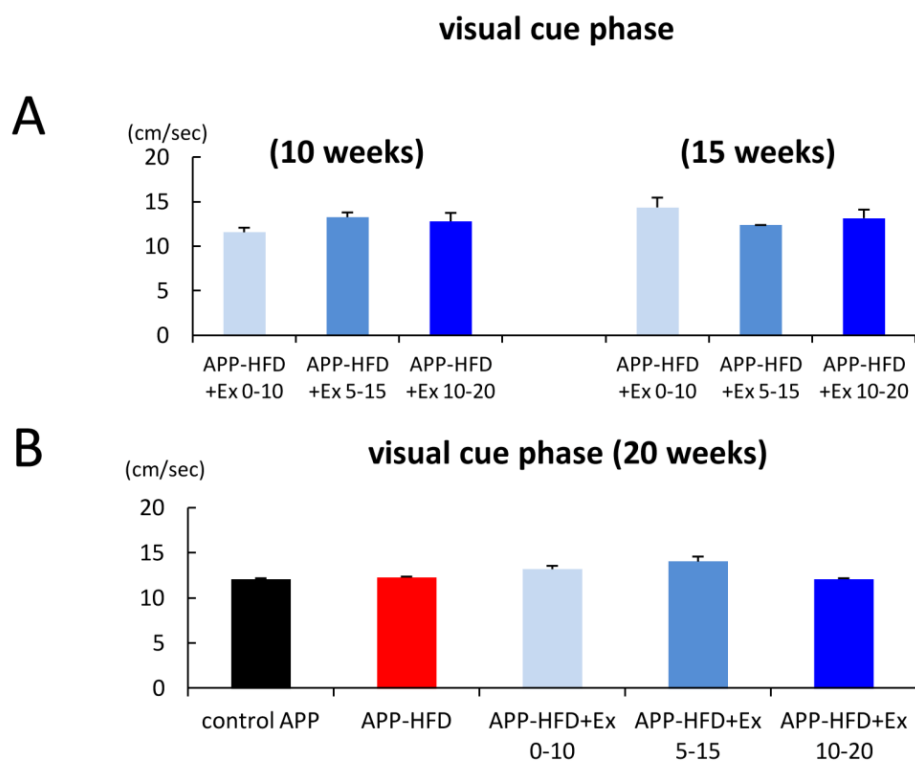
Supplemental Fig. 1. Every week monitoring of the amount of food intake

Every week monitoring showed that average amount of food intake in control APP, APP-HFD, APP-HFD+Ex 0-10, APP-HFD+Ex 5-15 and APP-HFD+Ex 10-20 mice. During the induction of exercise, APP-HFD+Ex 0-10, APP-HFD+Ex 5-15 and APP-HFD+Ex 10-20 mice tended to take more food than APP-HFD mice did.



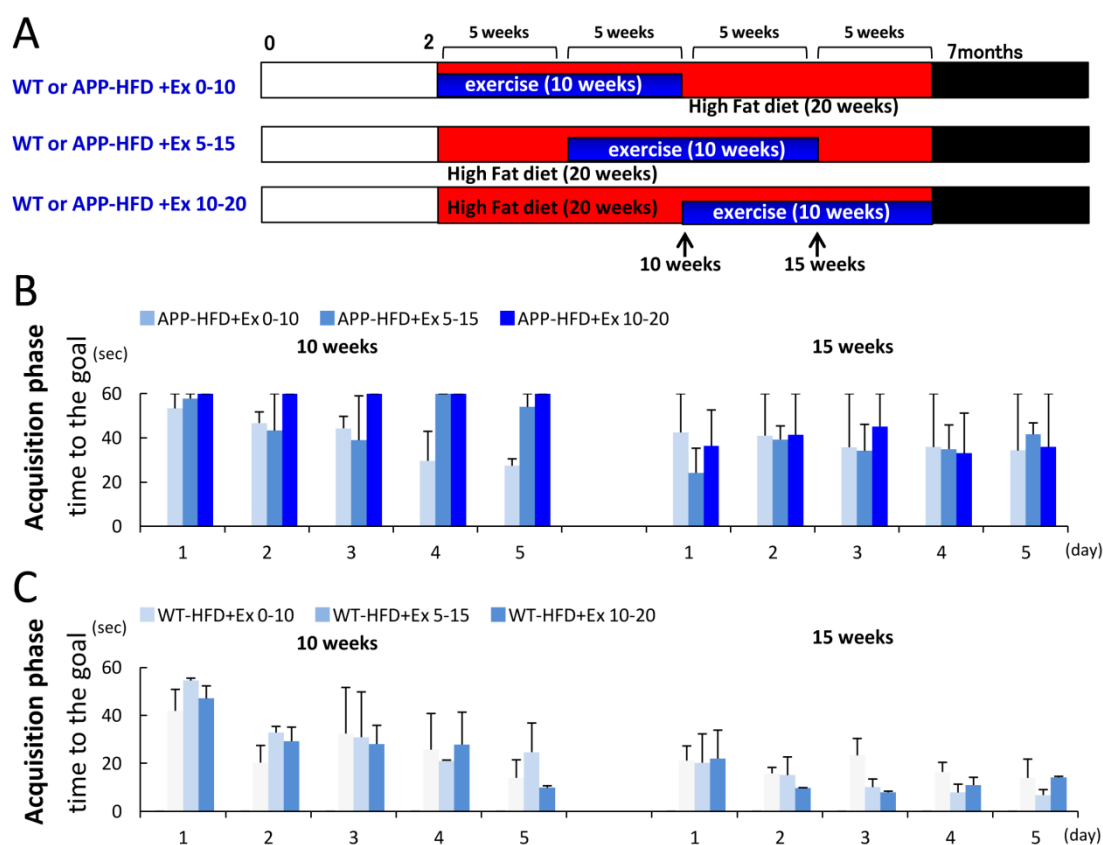
Supplemental Fig. 2. Swimming speeds in Morris water maze test

Locomotor activities of control APP, APP-HFD, APP-HFD+Ex 0-10, APP-HFD+Ex 5-15 and APP-HFD+Ex 10-20 mice were analyzed by swimming speeds in the visual cue phase of Morris water maze tests (A) 10, 15 and (B) 20 weeks after having HFD. There were no statistical differences among control APP, APP-HFD, APP-HFD+Ex 0-10, APP-HFD+Ex 5-15 and APP-HFD+Ex 10-20 mice.



Supplemental Fig. 3. Exercise at different periods were able to strengthen memory function in APP-HFD mice

10 weeks after having HFD, the acquisition time was clearly shortened in APP-HFD+Ex 0-10 mice (Supplemental Fig. 3B, left). Furthermore, 15 weeks after having HFD, the acquisition time was also ameliorated in APP-HFD+Ex 5-15 mice (Supplemental Fig. 3B, right). These results indicated that exercise during weeks 0-10 and weeks 5-15 could strengthen memory function in APP-HFD mice. However, at 10 weeks after having HFD, the improvement in the acquisition time was not observed in APP-HFD+Ex 10-20 mice, indicating that HFD for 10 weeks was sufficient to induce memory loss in APP transgenic mice (Supplemental Fig. 2B, left). We also conducted the pilot study in WT-HFD mice using the same strategy in APP-HFD mice. Although HFD for 10 weeks was sufficient to lead to memory deficit in APP transgenic mice, HFD for 10 weeks did not induce memory impairment in WT mice (Supplemental Fig. 3C).



Supplemental Fig. 3 legends

(A) Schematic presentation of the pilot study in Morris water maze test. Morris water maze test was conducted 10 weeks and 15 weeks after having HFD.

(B) The time to get to the goal platform of exercise-treated APP-HFD mice in the acquisition phase, 10 weeks (left) and 15 weeks (right) after having HFD. 10 weeks after having HFD, APP transgenic mice having HFD (APP-HFD+Ex 10-20 mice) took significant longer time to the platform. On the other hand, APP-HFD+Ex 0-10 mice clearly took shorter time to the platform than APP-HFD+Ex 10-20 mice.

(C) The time to get to the goal platform of exercise-treated WT-HFD mice in the acquisition phase, 10 weeks (left) and 15 weeks (right) after having HFD. 10 weeks after having HFD, WT mice having HFD (WT-HFD+Ex 10-20 mice) did not take longer time to the platform. WT-HFD+Ex 0-10 mice took the same time to the platform as WT-HFD+Ex 10-20 mice.