

# High-Fat-Diet Intake Enhances Cerebral Amyloid Angiopathy and Cognitive Impairment in a Mouse Model of Alzheimer's Disease, Independently of Metabolic Disorders

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**Background**—The high-fat Western diet is postulated to be associated with the onset and progression of Alzheimer's disease (AD). However, the role of high-fat-diet consumption in AD pathology is unknown. This study was undertaken to examine the role of high-fat-diet intake in AD.

**Methods and Results**—5XFAD mice, a useful mouse model of AD, and control wild-type mice were fed (1) high-fat diet or (2) control diet for 10 weeks. The effects on cerebral AD pathology, cognitive function, and metabolic parameters were compared between each group of mice. High-fat diet significantly enhanced cerebrovascular  $\beta$ -amyloid ( $A\beta$ ) deposition ( $P<0.05$ ) and impaired cognitive function ( $P<0.05$ ) in 5XFAD mice, but not in wild-type mice. High-fat diet enhanced hippocampal oxidative stress ( $P<0.05$ ) and NADPH oxidase subunits, gp91<sup>phox</sup> ( $P<0.01$ ) and p22<sup>phox</sup> ( $P<0.01$ ) in 5XFAD mice, but not in wild-type mice. Furthermore, high-fat diet reduced cerebral occludin ( $P<0.05$ ) in 5XFAD mice, but not in wild-type mice. Thus, 5XFAD mice exhibited greater susceptibility to high-fat diet than wild-type mice regarding cerebrovascular injury and cognitive impairment. On the other hand, 5XFAD mice fed high-fat diet exhibited much less increase in body weight, white adipose tissue weight, and adipocyte size than their wild-type counterparts. High-fat diet significantly impaired glucose tolerance in wild-type mice but not in 5XFAD mice. Thus, 5XFAD mice had much less susceptibility to high-fat-diet-induced metabolic disorders than wild-type mice.

**Conclusions**—High-fat diet, independently of metabolic disorders, significantly promotes the progression of AD-like pathology through enhancement of cerebral amyloid angiopathy and oxidative stress. (*J Am Heart Assoc.* 2016;5:e003154 doi: 10.1161/JAHA.115.003154)

**Key Words:** brain • cerebrovascular disorders • nutrition • obesity

There has been solid evidence that long-term high-fat Western diet consumption causes an increased incidence of metabolic diseases such as obesity, diabetes, hyperinsulinemia, etc, and cardiovascular diseases. Furthermore, epidemiological and clinical data have suggested that high-fat diet may contribute to the development of

Alzheimer's disease (AD) or cognitive decline.<sup>1–3</sup> Cross-sectional study<sup>4</sup> and longitudinal population-based study<sup>5</sup> provided the findings that intake of saturated fatty acid is correlated with impaired cognitive function. Population-based prospective studies<sup>6,7</sup> have indicated that high intake of saturated fatty acid over a 4- to 6-year period caused a greater risk for the development of AD and mild cognitive impairment for subjects aged 65 years or more. Although the underlying mechanisms of high-fat diet-induced metabolic disorders and cardiovascular diseases have been extensively studied, little is known regarding the effects of the high-fat Western diet on brain diseases such as AD or cognitive impairment.

As people live longer, the burden of cognitive impairment becomes increasingly important. AD is the most commonly diagnosed cause of cognitive dysfunction among the elderly.<sup>8,9</sup> AD is a chronic neurodegenerative disorder characterized by the development of cortical extracellular amyloid plaques and the intracellular neurofibrillary tangles formed by the aggregated tau protein.<sup>8,9</sup> A growing body of evidence indicates that cerebrovascular injury or dysfunction is

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frequently observed in patients with AD and could significantly influence clinical manifestation and severity of AD and contributes to cognitive decline.<sup>10,11</sup> Particularly, cerebral amyloid angiopathy (CAA), the accumulation of amyloid- $\beta$  (A $\beta$ ) in cerebral vessel walls, is most commonly associated with AD, and contributes to cerebrovascular injury or dysfunction.<sup>10–14</sup> Counteracting cerebrovascular impairment becomes an increasingly critical therapeutic strategy for AD as well as stroke. However, the exact role of high-fat Western diet intake on cerebrovascular injury and cognitive dysfunction in AD remains to be defined.

In the present study, to elucidate the potential contribution of high-fat diet to AD pathology, we investigated the effect of high-fat-diet consumption on AD-related pathology and cognitive function in a mouse model of AD. We obtained the first evidence that high-fat diet accelerated CAA and cognitive impairment in the AD mouse model, independently of metabolic disorders.

## Methods

### Animals

All experiments were approved by the Kumamoto University Committee for Laboratory Animal Care and Use. Cryopreserved embryos, which were purchased from Jackson Laboratory (Bar Harbor, ME), were implanted into pseudopregnant foster mice to make 5XFAD mice. 5XFAD mice overexpress human amyloid precursor protein (APP695) with Swedish (K670N, M671L), Florida (I716V), and London (V717I) familial AD mutations, also with mutant human presenilin-1 (M146L, L286V) controlled by murine Thy-1 promoter.<sup>15</sup> 5XFAD mice generate A $\beta$ <sub>1–42</sub> almost exclusively, rapidly accumulate amyloid plaque formation, and also recapitulate major pathologic and behavioral characteristics of AD.<sup>15–17</sup> 5XFAD mice were backcrossed into the C57BL/6J background at least 10 generations to reduce genetic variation. The mice C57BL/6J (male) were purchased from SLC Japan (Shizuoka, Japan). All mice were treated with a 12-hour light–darkness cycle in an animal facility, and were given standard chow and water ad libitum.

### Study Protocol

In cohorts 1 and 2, 13-month-old male 5XFAD mice (n=12) and control C57BL/6J wild-type mice (n=12) were divided into 2 groups (n=6 in each group), and were fed (1) high-fat diet or (2) control diet for 10 weeks (Figure 1). The control diet was D12450B (3.85 kcal/g; 10 kcal% fat [4.4 kcal% lard], 20 kcal% protein, and 70 kcal% carbohydrate [35 kcal% sucrose and 31 kcal% cornstarch]) (Research Diets, Inc., New Brunswick, NJ, USA).<sup>18,19</sup> High-fat diet was D12492 (5.24 kcal/g; 60 kcal

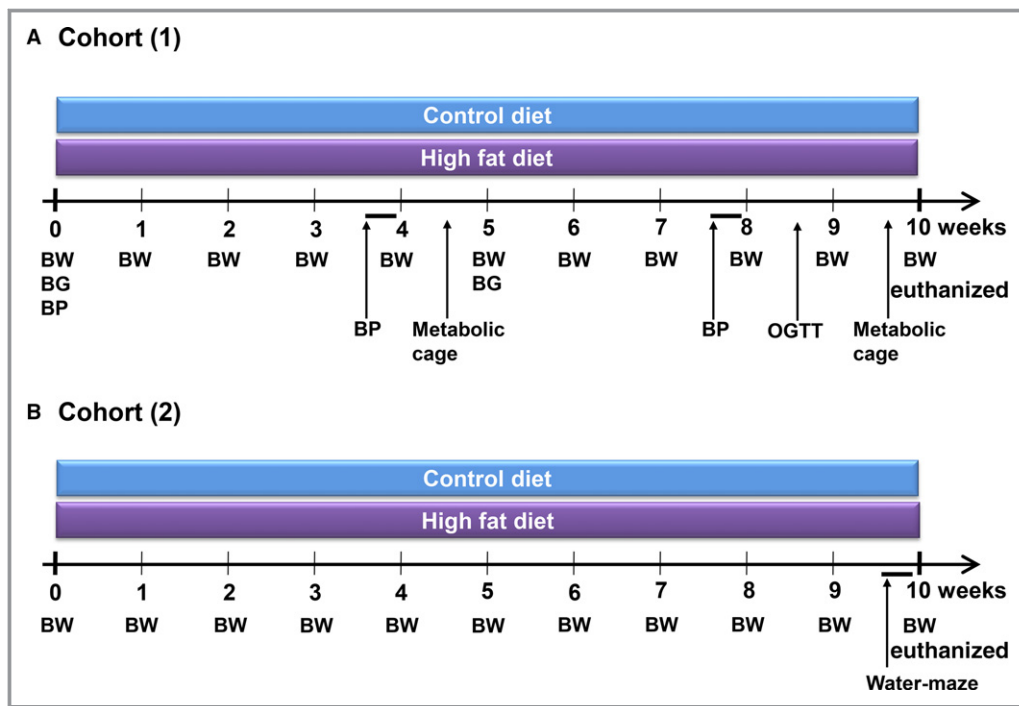
% fat [54 kcal% lard], 20 kcal% protein, and 20 kcal% carbohydrate) (Research Diets, Inc.). The content of cholesterol in lard in both diets was 0.72 mg/g. In cohort 1, body weight was measured every week throughout the treatment. Individual mice were housed in a metabolic cage at 0, 5, and 10 weeks after start of the treatment, to evaluate food intake, water intake, and urine volume. Nonfasting blood glucose was measured at 5 weeks after start of the treatment, and oral glucose tolerance test was performed at 8 weeks. Blood pressure was measured at 0, 4, and 8 weeks. In cohort 2, all groups of mice were subjected to the Morris water-maze test at 10 weeks of the treatment (Figure 1B). In both cohorts 1 and 2, after 10 weeks of the treatment, mice were anesthetized with isoflurane, perfused by phosphate-buffered saline, and brain, heart, white adipose tissue, and tibia were rapidly excised for the measurement of various parameters as described below.

### Morris Water Maze Test

Morris water maze test was performed to evaluate learning and reference/working memory, and groups were blinded to the examiners, as previously described.<sup>20</sup> Briefly, swimming paths were video-tracked with a camera fixed on the ceiling of the room and analyzed by the software (Muromachi Kikai, Tokyo, Japan). A training session was carried out before the hidden platform test sessions. Mice were given 60 s free swimming before being guided to climb onto the hidden platform, followed by remaining there for 30 s, and then returning to their cages. On the hidden platform test, the mice had 4 sessions per day on the following 4 consecutive days (days 1–4). During each session, mice were released from 3 randomly assigned starting points and swam for 100 s. Escape latency is defined as the time (s) taken to find and escape onto the submerged platform.<sup>21</sup> On the probe test at day 5, the hidden platform was moved away and the mice swam freely for 100 s. The time that mice spent in the target quadrant was recorded. On the visible platform test, the platform was elevated above the water surface and placed in a different position, which was performed after the probe test on day 5. The mice were given 4 sessions of a visible trial with an intersession interval of 20 minutes.

### Oral Glucose Tolerance Test

The oral glucose tolerance test was performed as previously described.<sup>22</sup> Mice were deprived of food overnight and then orally given glucose (1 mg/g body weight). Tail vein bloods were taken at 0, 30, 60, and 120 minutes after glucose administration to measure blood glucose concentrations. Blood glucose concentrations were measured by a portable glucose meter (Sanwa Kagaku Kenkyusho Co., Ltd, Nagoya, Japan).



**Figure 1.** Experimental design of cohort 1 (A) and cohort 2 (B). In cohort 1, cerebrovascular and cerebral parenchymal amyloid deposition, cerebral oxidative stress, and various cerebral protein levels by Western blot analysis were analyzed after 10 weeks of high-fat-diet feeding. In cohort 2, the main purpose of the experiment was to perform the water maze test. Body weight was measured every week, and tibia length, brain weight, left ventricular weight, and white adipose weight were measured after animals were euthanized (after 10 weeks of high-fat-diet feeding). The data on body weight, tibia length, and various tissue weights were obtained from both cohort 1 and cohort 2. BG indicates blood glucose; BP, blood pressure; BW, body weight; OGTT, oral glucose tolerance test.

## Measurement of Blood Pressure

Blood pressure of mice was measured at 0, 4, and 8 weeks after start of the treatment using the tail cuff method (BP-98A; Softron Co, Tokyo, Japan), as described.<sup>23</sup>

## Measurement of Brain Superoxide

At the end of the experiment, the brains were removed and divided at the point of bregma, and the caudal side of each brain was immediately frozen in Tissue-Tek OCT embedding medium (Sakura Finetek, Tokyo, Japan). Dihydroethidium (DHE) was used to evaluate tissue superoxide levels in situ as described.<sup>24</sup> DHE fluorescence of each tissue section in the 2 fields of the hippocampal CA1 region and somatosensory cortex on both sides for each animal at  $\times 200$  magnification was quantified by Win ROOF version 5.8 analysis software (Mitani Corporation, Tokyo, Japan). Mean fluorescence was quantified and expressed relative to values obtained from wild-type mice fed control diet. To confirm that DHE fluorescence was derived from superoxide, brain

sections were preincubated with 250 U/mL polyethylene-glycol superoxide dismutase (SOD) (Sigma-Aldrich, Saint Louis, MO, USA) for 30 minutes followed by DHE staining, according to our previous method.<sup>25–27</sup>

## Immunohistochemical and Immunofluorescence Analysis

To determine the deposition of  $A\beta_{1-40}$  and  $A\beta_{1-42}$ , brain sections (8- $\mu$ m thickness) were incubated in 90% formic acid for 5 minutes followed by 0.3%  $H_2O_2$ /phosphate-buffered saline for 30 minutes, and then stained by  $A\beta_{1-40}$  antibody (1:500, Code No. 18580; Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) and  $A\beta_{1-42}$  antibody (1:500, Code No. 18582; Immuno-Biological Laboratories Co., Ltd.) overnight at 4°C, reacted with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody for 1 hour at room temperature, and visualized with 3, 3'-diaminobenzidine (Dako Cytomation, Glostrup, Denmark), according to our previous method.<sup>23</sup>

Accumulated areas of A $\beta_{1-42}$  and A $\beta_{1-40}$  in the 2 fields of hippocampal CA1 region and somatosensory cortex on both sides for each animal at  $\times 200$  magnification were calculated by WinRoof version 5.8 analysis software and was calculated as a percentage of the positive area per region of interest.

For assessment of CAA, brain sections were incubated with an antibody against the basement membrane marker collagen IV (Col IV; 1:200, rabbit; Abcam, Cambridge, MA) and A $\beta_{1-40}$  (1:1000, mouse; Abcam) followed by Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse secondary IgG (1:200; Invitrogen, Carlsbad, CA), imaged using the appropriate filters.

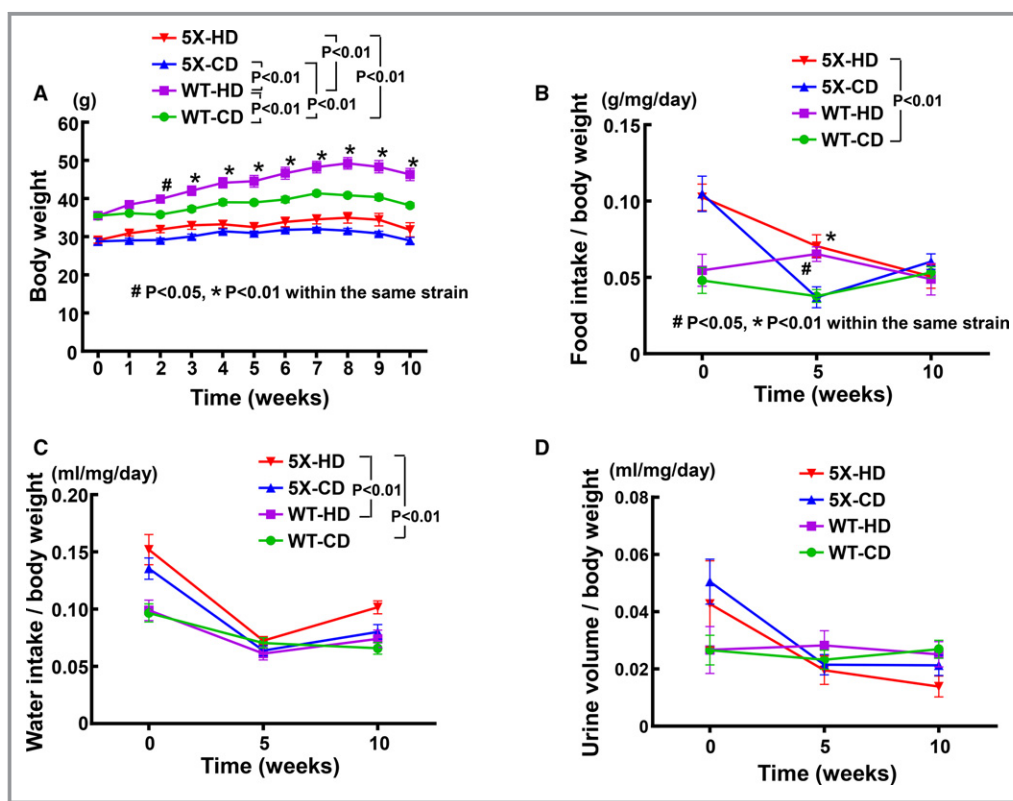
### Quantitative Analysis of Regional A $\beta$ Deposition and Microvascular CAA

Collagen IV and A $\beta_{1-40}$  positive cerebral microvessels in the regions of the hippocampus were quantified on the same set of systematically sampled immunofluorescence stain. To

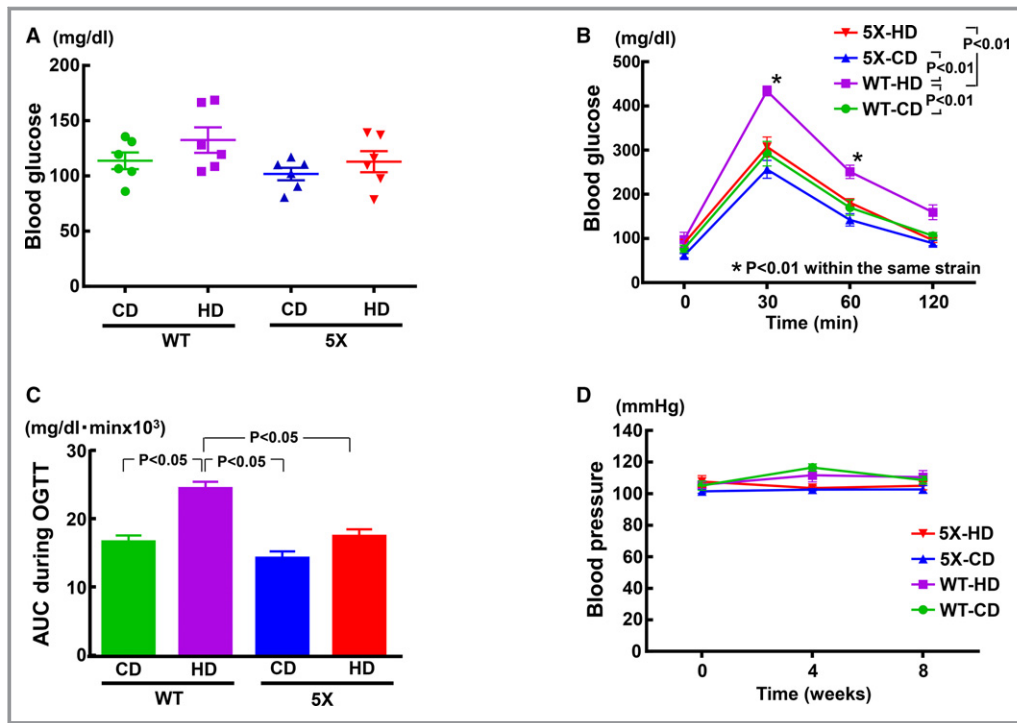
determine CAA capillary density, photos were taken in bilateral hippocampal CA1 regions at  $\times 200$  magnification. Capillary density was calculated as a percentage of collagen IV positive area per region of interest. Cerebral microvessels ( $n=20$  per animal) positive for both A $\beta_{1-40}$  and collagen IV, ranging in diameter from 20 to 100  $\mu\text{m}$ , were randomly imaged by fluorescence microscopy.<sup>28</sup> Next, to quantify the A $\beta_{1-40}$  positive cortical arteries, photos were taken at  $\times 400$  magnification on the bilateral cortical surface. The percentage of amyloid plaques that colocalized with collagen IV immunostaining was calculated using Win ROOF version 5.8 analysis software (Mitani Corporation).

### Assessment of White Adipose Tissue Size

White adipose tissue was fixed in paraffin, cut into 4- $\mu\text{m}$ -thick sections, and were stained with hematoxylin–eosin. In individual mice, at least 30 adipocytes were measured for size under a microscope, and the average was used for the value of each sample.



**Figure 2.** Time course of body weight (A), food intake (B), water intake (C), and urine volume (D). In (A), body weight data from cohort 1 and cohort 2 were combined for analysis. 5X indicates 5XFAD mice; CD, control diet; HD, high-fat diet; WT, wild-type mice. Values are means  $\pm$  SEM. In (A),  $n=12$  in WT-CD,  $n=12$  in WT-HD,  $n=12$  in 5X-CD,  $n=11$  in 5X-HD. In (B through D),  $n=6$  in WT-CD,  $n=6$  in WT-HD,  $n=6$  in 5X-CD,  $n=6$  in 5X-HD. Statistical analysis was performed by 2-factor ANOVA with repeated measures followed by post hoc Bonferroni's multiple comparisons test.



**Figure 3.** Blood glucose (A), oral glucose tolerance test (B and C), and blood pressure (D). 5X indicates 5XFAD mice; AUC, area under curve; CD, control diet; HD, high-fat diet; OGTT, oral glucose tolerance test; WT, wild-type mice. Values are means±SEM.  $n=6$  in WT-CD,  $n=6$  in WT-HD,  $n=6$  in 5X-CD,  $n=6$  in 5X-HD. In (A), statistical significance was tested with 1-way ANOVA followed by the Tukey's multiple comparison post hoc test between each group. In (B and D), statistical analysis was performed by 2-factor ANOVA with repeated measures followed by post hoc Bonferroni's multiple comparisons test. In (C), statistical significance was tested with Kruskal–Wallis test followed by Steel–Dwass post hoc test.

## Western Blot Analysis

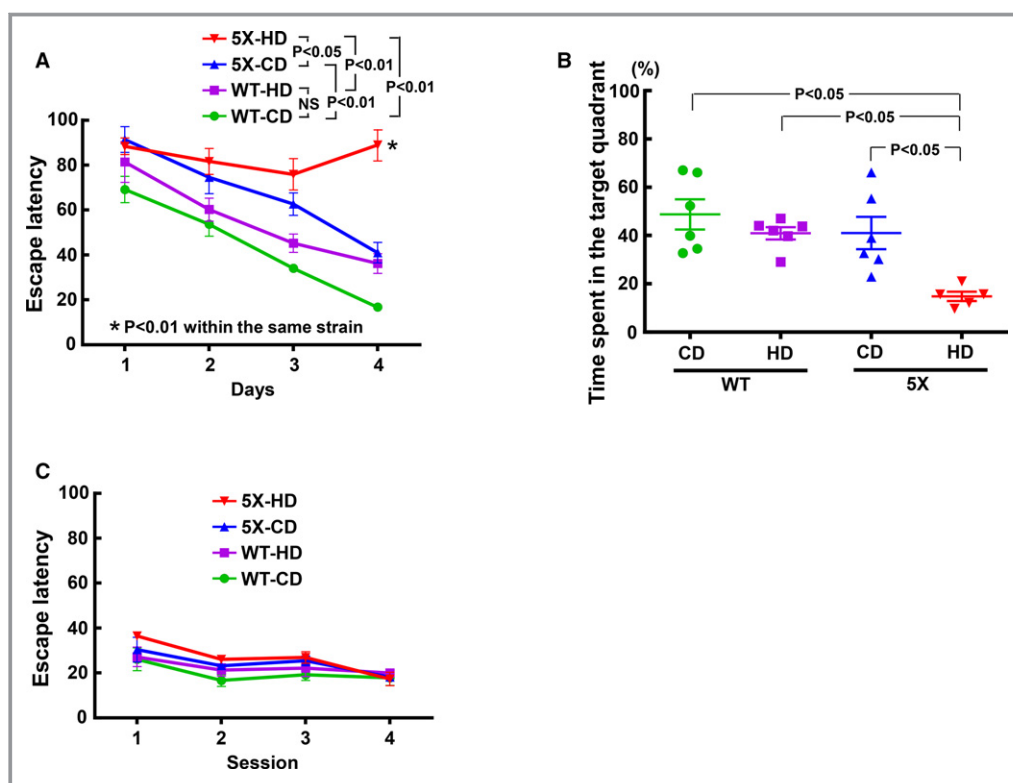
Western blot analysis using the rostral side of each brain was performed according to our previous method.<sup>29</sup> Primary antibodies used were as follows: anti-gp91<sup>phox</sup> (91 kDa) ( $\times 2000$ , Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-p22<sup>phox</sup> (22 kDa) ( $\times 1000$ , Santa Cruz Biotechnology, Inc.), anti-p67<sup>phox</sup> (67 kDa) ( $\times 5000$ , BD Biosciences, San Jose, CA), anti-occludin (65 kDa) ( $\times 30\,000$ , Invitrogen, Carlsbad, CA), anti-brain-derived neurotrophic factor (BDNF) (14 kDa) ( $\times 2000$ , Santa Cruz Biotechnology, Inc.), anti-glyceraldehyde-3-phosphate dehydrogenase (37 kDa) ( $\times 5000$ , Santa Cruz Biotechnology, Inc.), anti-cyclooxygenase-2 (COX-2) (72 kDa) ( $\times 5000$ ; Acris, Herford, Germany), anti-extracellular-superoxide dismutase (EC-SOD) (45 kDa) ( $\times 5000$ , Upstate, Charlottesville, VA), anti-copper-zinc superoxide dismutase (Cu/Zn-SOD) (19 kDa) ( $\times 5000$ ; Stressgen Bioreagents, Victoria, Canada), anti-manganese superoxide dismutase (Mn-SOD) (25 kDa) ( $\times 5000$ ; Assay Designs, Farmingdale, NY), and anti-catalase (60 kDa) ( $\times 5000$ ; Cell Signaling Technology, Danvers, MA). Intensity of the bands was quantified by using analysis software (Image J; National Institute of Health, Bethesda, MD). In

individual samples, each value was corrected for glyceraldehyde-3-phosphate dehydrogenase.

## Statistical Analysis

Statistical analysis was performed by GraphPad Prism (version 6.01) for Windows (GraphPad Software Inc., San Diego, CA) and Ekuseru-Tokei 2012 statistical software (Social Survey Research Information Co., Ltd., Tokyo, Japan). All data were presented as mean±SEM. Data on time course experiments were analyzed by 2-way ANOVA with repeated measures followed by Bonferroni post hoc test for multiple comparisons. Statistical significance was tested with 1-way ANOVA followed by the Tukey's post hoc test between each group. When similar variances were not obtained among comparison groups or a normal distribution was not confirmed among comparison groups, data were analyzed with Kruskal–Wallis test followed by Steel–Dwass post hoc test. Unpaired Student *t* test was used for comparison between 2 groups. The correlation between body weight and white adipose weight was analyzed by Spearman correlation test. Differences were considered statistically significant at a value of  $P<0.05$  in all





**Figure 4.** Escape latency of the hidden platform test (A), time spent in the target quadrant of probe test (B), and escape latency of visible test (C) estimated by Morris water maze test. High-fat diet significantly increased escape latency of the hidden platform test in 5XFAD mice but not in wild-type mice (A). High-fat diet significantly decreased time spent in the target quadrant of probe test in 5XFAD mice but not in wild-type mice (B). 5X indicates 5XFAD mice; CD, control diet; HD, high-fat diet; WT, wild-type mice. Values are means $\pm$ SEM.  $n=6$  in WT-CD,  $n=6$  in WT-HD,  $n=6$  in 5X-CD,  $n=5$  in 5X-HD. In (A and C), statistical analysis was performed by 2-factor ANOVA with repeated measures followed by post hoc Bonferroni's multiple comparisons test. In (B), statistical significance was tested with Kruskal–Wallis test followed by Steel–Dwass post hoc test.

the tests. The method of statistical analysis is described in all figure legends.

## Results

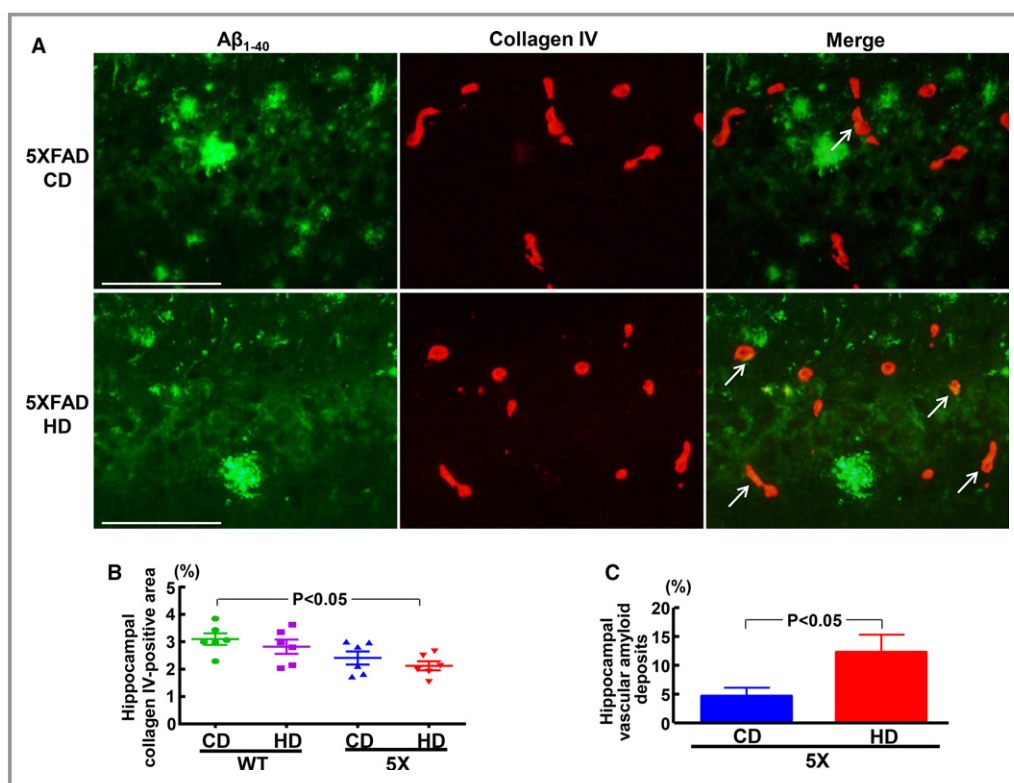
### Body Weight, Food Intake, Water Intake, and Urine Volume

As shown in Figure 2A, body weight of wild-type mice was significantly and progressively increased by high-fat-diet feeding more than by control diet feeding ( $P<0.01$ ). On the other hand, there was no significant increase in body weight of 5XFAD mice by high-fat diet compared with control diet. As shown in Figure 2B, there was no significant difference in food intake at 5 and 10 weeks between high-fat-diet-fed groups of 5XFAD and wild-type mice, and between control-diet-fed groups of both strains. As shown in Figure 2C and 2D, there was no significant difference in water intake or urine volume at 5 and

10 weeks between 5XFAD and wild-type mice, regardless of high-fat diet or control diet.

### Blood Glucose, Oral Glucose Tolerance Test, and Blood Pressure

As shown in Figure 3A, there was no significant difference in blood glucose levels among 4 groups at 5 weeks of the treatment. However, as shown by oral glucose tolerance test in Figure 3B and 3C, in wild-type mice, area under curve during oral glucose tolerance test was significantly greater in the high-fat-diet-fed group than in control-diet-fed group. On the other hand, there was no significant difference in blood glucose levels and in area under curve during oral glucose tolerance test between high-fat-diet-fed and control-diet-fed groups of 5XFAD mice. As shown in Figure 3D, there was no significant difference in blood pressure among 4 groups of mice throughout the experiment.



**Figure 5.** Quantification of hippocampal collagen IV-positive area (B) and hippocampal vascular amyloid deposition (C). A, Representative photomicrographs of  $A\beta_{1-40}$  (green) and collagen IV (red) double-immunostaining in the hippocampus. B, Quantification of percent area of collagen IV immunostaining, an established marker of the vessel basal lamina, in the hippocampus of each group of mice. Hippocampal collagen IV-positive area was significantly less in 5XFAD mice fed high-fat diet than in wild-type mice fed control diet. C, Quantification of percent area of amyloid deposits colocalized with microvessels in the hippocampus. Hippocampal vascular amyloid deposits were greater in 5XFAD mice fed high-fat diet than in those fed control diet. 5X indicates 5XFAD mice; CD, control diet; HD, high-fat diet; WT, wild-type mice. Values are means $\pm$ SEM.  $n=6$  in WT-CD,  $n=6$  in WT-HD,  $n=6$  in 5X-CD,  $n=6$  in 5X-HD. In (B), statistical significance was tested with 1-way ANOVA followed by the Tukey's multiple comparison post hoc test between each group. In (C), statistical analysis was performed by unpaired Student  $t$  test. Scale bar=50  $\mu$ m.

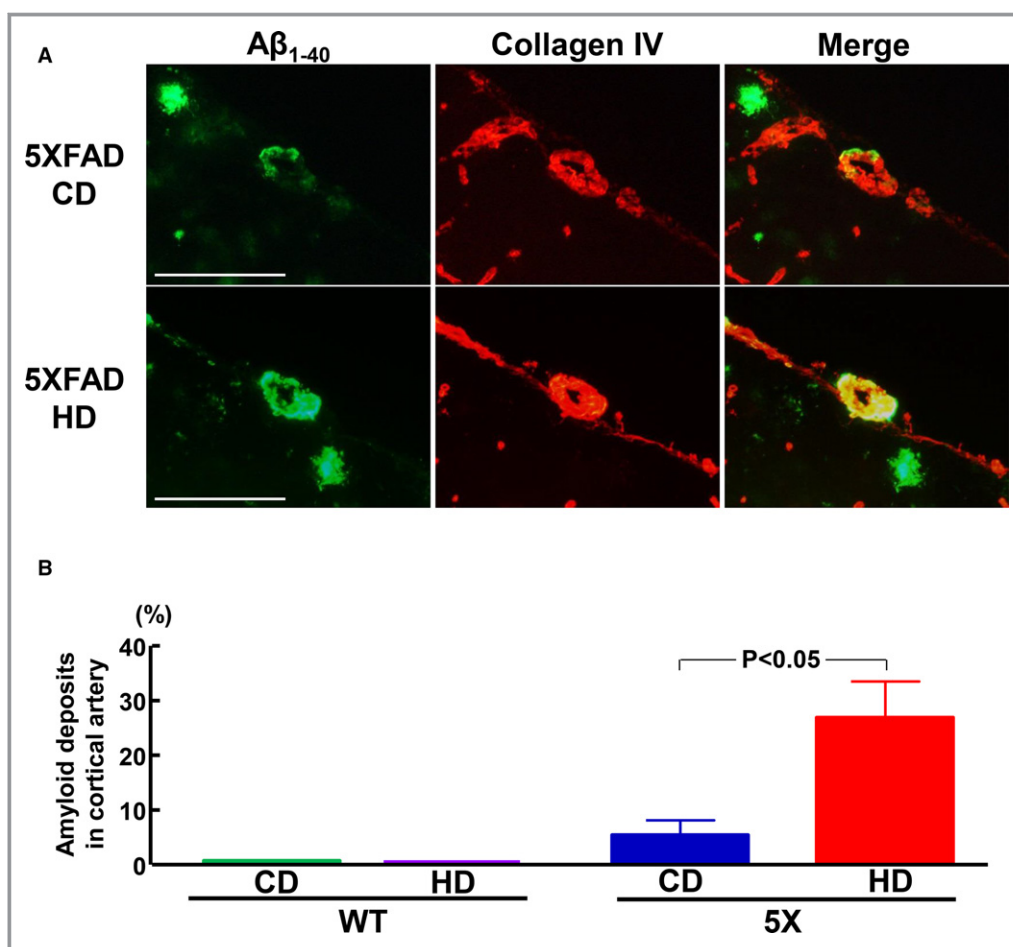
## Cognitive Function by Morris Water Maze Test

As shown by the hidden platform test in Figure 4A, escape latency of 5XFAD mice fed high-fat diet was significantly greater than those fed control diet ( $P<0.05$ ). On the other hand, there was no significant difference in escape latency between high-fat diet and control-diet groups of wild-type mice. As shown by the probe test in Figure 4B, 5XFAD fed high-fat diet exhibited a smaller percent time spent in the target quadrant than those fed control diet ( $P<0.05$ ), while time spent in the target quadrant was comparable between both groups of wild-type mice. As shown in Figure 4C, there was no significant difference in escape latency of the visible test among 4 groups of mice.

## Cerebrovascular and Cerebral Parenchymal Amyloid Deposition

As shown in Figure 5B, hippocampal collagen IV positive area, an established marker of the vessel basal lamina, was significantly less in high-fat diet-fed 5XFAD mice than in control diet-fed wild-type mice ( $P<0.05$ ). As shown by quantification of the collagen IV and  $A\beta_{40}$  double immunostaining area in Figure 5C, 5XFAD mice fed high-fat diet exhibited higher percentage area of amyloid deposit in hippocampal microvessels than those fed control diet ( $P<0.05$ ). Hippocampal microvessel amyloid deposits were negligible in both groups of wild-type mice (data not shown).

As shown in Figure 6, percent area of  $A\beta$  deposition in the cortical branch of the middle cerebral artery was also



**Figure 6.** Quantification of amyloid deposits in cortical branch of middle cerebral artery. A, Representative photomicrographs of  $A\beta_{1-40}$  (green) and collagen IV (red) immunostaining in cortical branch of middle cerebral artery. B, Quantification of percent area of amyloid deposits colocalized with cortical artery of each group of mice. High-fat diet significantly increased amyloid deposits in cortical artery of 5XFAD mice but did not in cortical artery of wild-type mice. 5X indicates 5XFAD mice; CD, control diet; HD, high-fat diet; WT, wild-type mice. Values are means $\pm$ SEM.  $n=6$  in WT-CD,  $n=6$  in WT-HD,  $n=6$  in 5X-CD,  $n=6$  in 5X-HD. In (B), statistical analysis was performed by unpaired Student *t* test. Scale bar=50  $\mu$ m.

significantly greater in 5XFAD mice fed high-fat diet than in those fed control diet ( $P<0.05$ ). On the other hand, amyloid deposits were undetectable in both groups of wild-type mice.

Figure 7 shows cerebral hippocampal or parenchymal  $A\beta_{1-40}$  or  $A\beta_{1-42}$  deposits in both groups of 5XFAD mice. There was no significant difference between control group and high-fat diet-fed group of 5XFAD mice regarding percent area of hippocampal or parenchymal  $A\beta_{1-40}$  or  $A\beta_{1-42}$  deposits. In both groups of wild-type mice, hippocampal or parenchymal  $A\beta_{1-40}$  or  $A\beta_{1-42}$  deposits were undetectable (data not shown).

### Cerebral Oxidative Stress

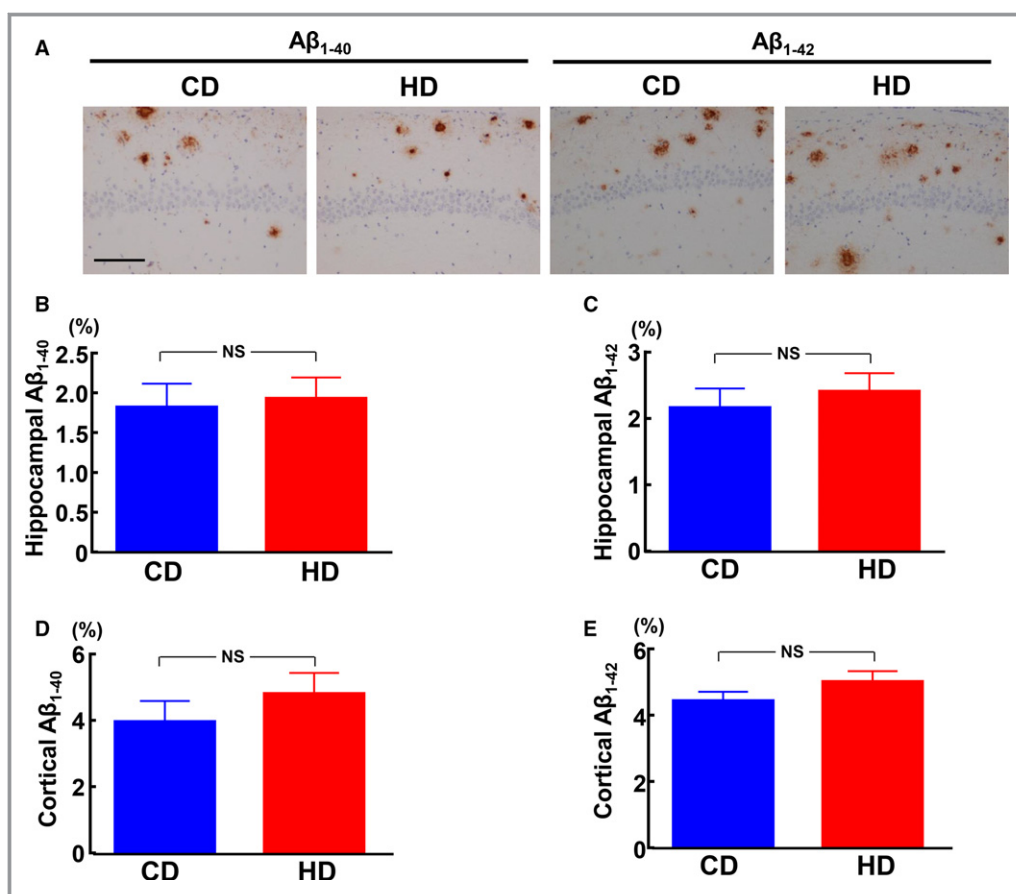
As shown in Figure 8A, in 5XFAD mice, high-fat diet significantly enhanced hippocampal superoxide levels

compared with control diet ( $P<0.05$ ). On the other hand, high-fat diet failed to alter hippocampal superoxide levels compared with control diet in wild-type mice. As shown in Figure 8B, high-fat-diet-fed 5XFAD mice had greater cortical superoxide levels than wild-type mice fed control diet ( $P<0.05$ ) or high-fat diet ( $P<0.05$ ). Pretreatment with SOD significantly reduced the intensity of DHE fluorescence in brain tissue of 5XFAD mice fed a high-fat diet (Figure 8C), which is consistent with our previous reports<sup>25–27</sup> and confirming that DHE fluorescence was derived from superoxide.

### NADPH Oxidase Subunits, Occludin, and BDNF

As shown by Western blot analysis in Figure 9A through 9C, in 5XFAD mice, high-fat diet significantly increased cerebral





**Figure 7.** Quantification of parenchymal Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub> deposits in hippocampus (B and C) and cortex (D and E). A, Representative photomicrographs of Aβ<sub>1-40</sub> or Aβ<sub>1-42</sub> immunostaining in hippocampus of each group of mice. High-fat diet did not significantly alter hippocampal or cortical Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub> levels in 5XFAD mice. CD indicates control diet; HD, high-fat diet. Values are means±SEM. n=6 in 5X-CD, n=6 in 5X-HD. Statistical analysis was performed by unpaired Student *t* test. Scale bar=100 μm. NS indicates not significant.

gp91<sup>phox</sup> ( $P<0.01$ ) and p22<sup>phox</sup> ( $P<0.01$ ) compared with control diet. On the other hand, high-fat diet failed to alter these NADPH oxidase subunits in wild-type mice. Cerebral p67<sup>phox</sup> levels were similar between control diet and high-fat-diet-fed groups of 5XFAD mice or wild-type mice. As shown by Figure 9E and 9F, cerebral occludin levels were lower in high-fat diet group than in control diet group of 5XFAD mice ( $P<0.05$ ), while there was no significant difference in cerebral occludin levels between control- and high-fat-diet-fed groups of wild-type mice. High-fat-diet-fed 5XFAD mice tended to have lower cerebral BDNF levels than those fed control diet (although the difference did not reach statistical significance), and had significantly less BDNF levels than wild-type mice fed control diet ( $P<0.05$ ) and high-fat diet ( $P<0.05$ ) (Figure 9G).

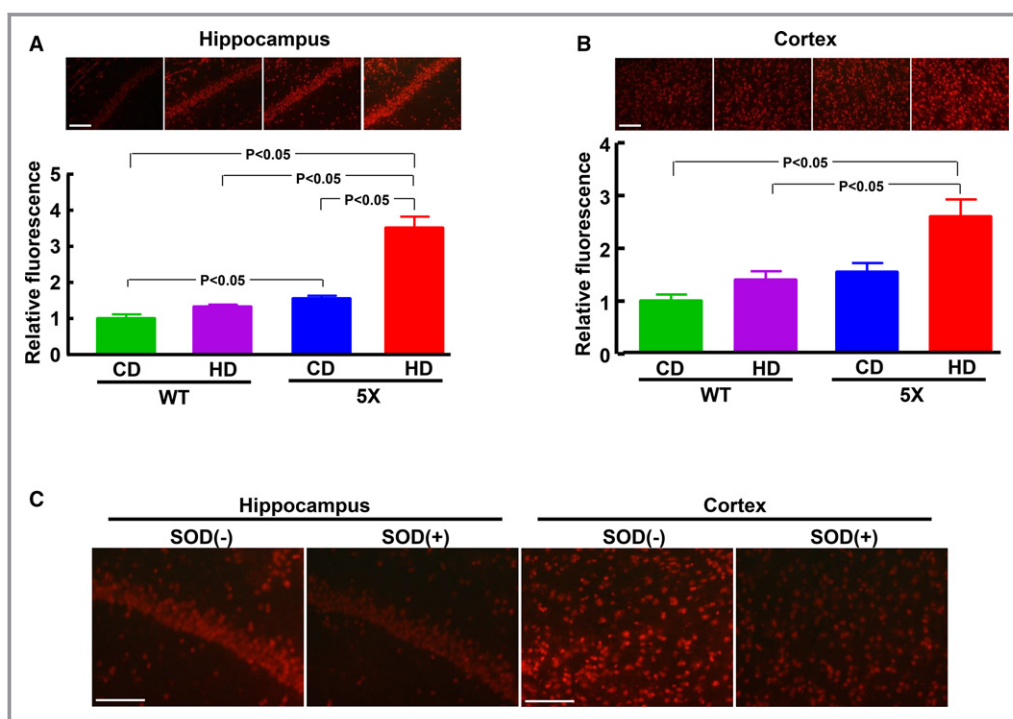
### COX-2, SOD Isoforms, and Catalase

As shown in Figure 10B, cerebral COX-2 levels were greater in 5XFAD mice than in wild-type mice. However, there was

no significant difference between 5XFAD mice fed high-fat diet and control diet regarding COX-2 levels. As shown in Figure 10C through 10F, there was no significant difference between 5XFAD mice and wild-type mice regarding extracellular (EC)-SOD, Cu/Zn-SOD, Mn-SOD, or catalase, regardless of high-fat diet or control diet.

### Tibia Length, Brain Weight, Cardiac Weight, White Adipose Weight, and Adipose Tissue Size

In wild-type mice, body weight at the end (10 weeks) of the treatment was greater in high-fat diet group than control diet group ( $P<0.01$ ) (Figure 11A). On the other hand, body weight did not significantly differ between control-diet and high-fat-diet groups of 5XFAD mice. Tibia length was similar among 4 groups of mice (Figure 11B). Brain weight did not differ among 4 groups of mice (Figure 11C). As shown in Figure 11D, left ventricular weight of high-fat-diet-fed wild-type mice was greater than that of control-diet-fed 5XFAD



**Figure 8.** Cerebral oxidative stress in hippocampus (A), cortex (B), and effect of pretreatment with superoxide dismutase on dihydroethidium fluorescence (C). Upper panels in (A and B) indicate representative photomicrographs of hippocampal and cortical sections, respectively, stained with dihydroethidium (DHE). High-fat diet significantly increased hippocampal superoxide levels in 5XFAD mice but did not in wild-type mice. C, DHE-stained brain section from high-fat-diet-fed 5XFAD mice with or without superoxide dismutase (SOD) pretreatment. 5X indicates 5XFAD mice; CD, control diet; HD, high-fat diet; SOD (–), DHE-stained section without SOD pretreatment; SOD (+), DHE-stained section with SOD pretreatment; WT, wild-type mice. Values are means $\pm$ SEM.  $n=6$  in WT-CD,  $n=6$  in WT-HD,  $n=6$  in 5X-CD,  $n=6$  in 5X-HD. Statistical significance was tested with Kruskal–Wallis test followed by Steel–Dwass post hoc test. Scale bar=100  $\mu$ m.

( $P<0.01$ ) and high-fat diet-fed 5XFAD mice ( $P<0.05$ ). As shown in Figure 11E, 5XFAD mice fed control diet exhibited smaller white adipose weight than wild-type mice fed control diet ( $P<0.01$ ). The increase in white adipose weight by high-fat diet was much less in 5XFAD mice than in wild-type mice ( $P<0.01$ ). Similarly, the increase in adipose cell size induced by high-fat diet was much less in 5XFAD mice than in wild-type mice ( $P<0.05$ ).

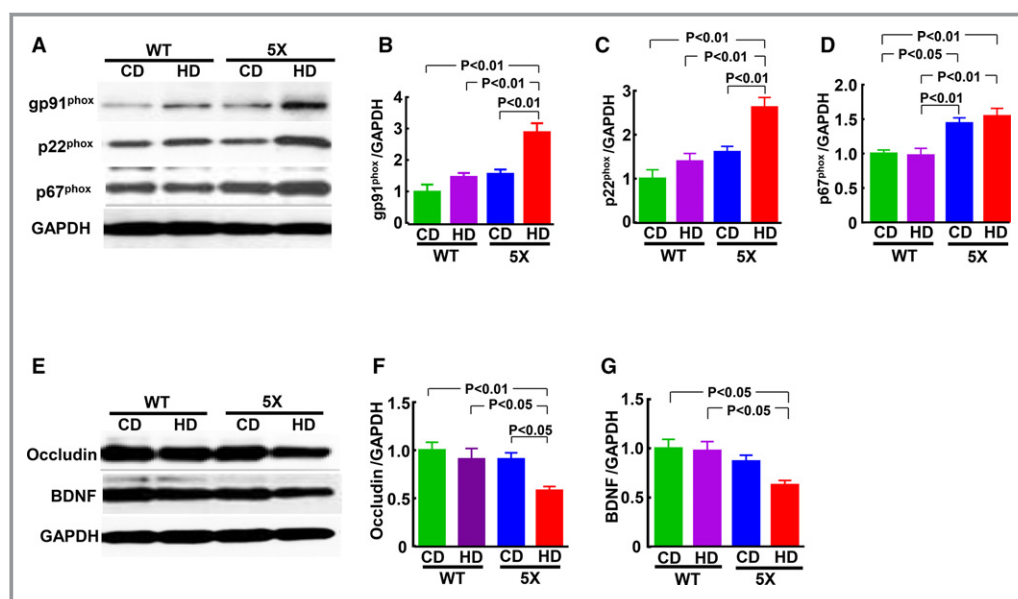
As shown in Figure 12, there was a positive relationship between body weight and white adipose tissue weight at 10 weeks of the treatment in wild-type mice ( $r=0.953$ ;  $P<0.01$ ) and in 5XFAD ( $r=0.545$ ;  $P<0.05$ ).

## Discussion

Emerging epidemiological, clinical, or experimental evidence support the notion that cerebrovascular injury or dysfunction, particularly CAA, is critically involved in the incidence and development of AD. Furthermore, consumption of high-fat Western diet has been suggested to be associated with the development of AD.<sup>1–3,30</sup> However, at present, the potential effect of high-fat-diet intake on AD pathology is

poorly understood. The major findings of the present study were that short-term high-fat-diet intake, independently of metabolic disorders such as obesity and glucose intolerance, accelerates cerebrovascular A $\beta$  deposition, enhances hippocampal oxidative stress, decreases cerebral occludin, and subsequently exacerbates cognitive impairment in a mouse model of AD. On the other hand, in spite of significant induction of obesity and glucose intolerance by high-fat diet, wild-type mice displayed no apparent brain injury and cognitive dysfunction by short-term high-fat-diet feeding. Therefore, our present work provided the experimental novel findings indicating that high-fat-diet consumption worsens AD-like pathology, independently of metabolic disorders.

In the present study, we examined the effect of short-term (10 weeks) high-fat-diet intake on 5XFAD mouse compared with wild-type mouse, because little is known regarding the effect of short-term high-fat-diet intake on the brain. Hippocampus is the site of structural abnormalities closely associated with early stages of AD and other cognitive decline.<sup>31–33</sup>

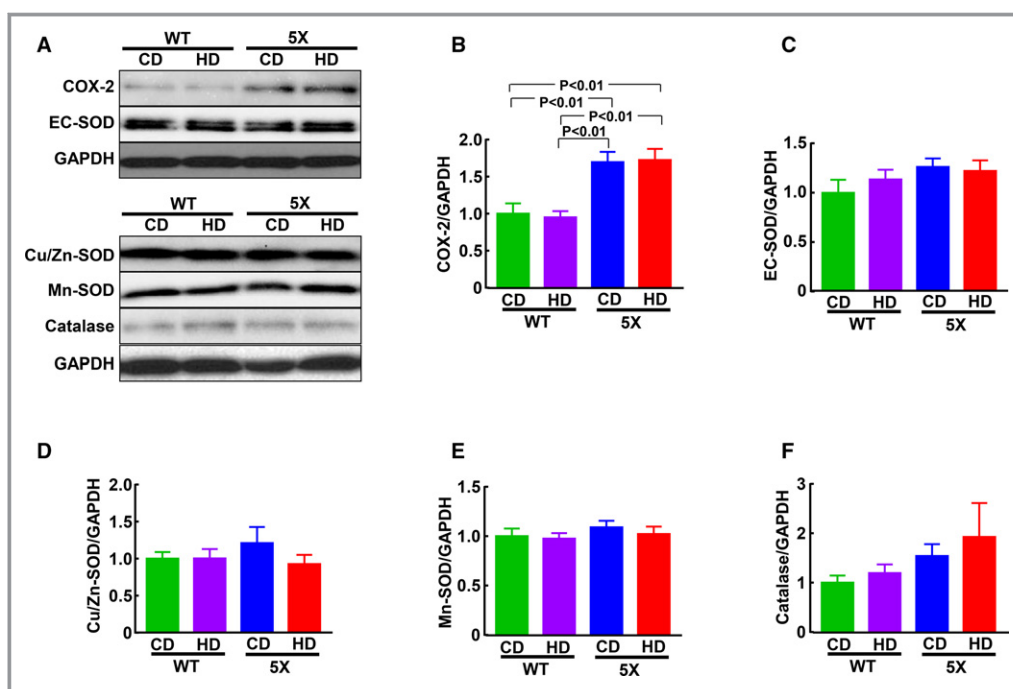


**Figure 9.** Cerebral gp91<sup>phox</sup>, p22<sup>phox</sup>, and p67<sup>phox</sup> levels (B, C, and D, respectively) and cerebral occludin and BDNF levels (F and G, respectively). A, indicates representative Western blot of gp91<sup>phox</sup>, p22<sup>phox</sup>, and p67<sup>phox</sup>, and (E) indicates representative Western blot of occludin and BDNF. High-fat diet significantly increased cerebral gp91<sup>phox</sup> and p22<sup>phox</sup> levels and significantly decreased cerebral occludin and BDNF levels in 5XFAD mice, while high-fat diet did not significantly change these protein levels in wild-type mice. 5X indicates 5XFAD mice; BDNF, brain-derived neurotrophic factor; CD, control diet; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HD, high-fat diet; WT, wild-type mice. Values are means ± SEM. n=6 in WT-CD, n=6 in WT-HD, n=6 in 5X-CD, n=6 in 5X-HD. Statistical significance was tested with 1-way ANOVA followed by Tukey's multiple comparison post hoc test between each group.

Hippocampal dysfunction causes impairment of learning and memory for spatial relations among objects in the environment. In the present study, we used the Morris water maze test for the estimation of cognitive function, because this test has been recognized as a robust and reliable test that is strongly correlated with hippocampal synaptic plasticity and *N*-methyl-D-aspartate receptor function.<sup>34</sup> As is consistent with previous reports by us<sup>23</sup> and others,<sup>35</sup> the present study showed that short-term high-fat-diet intake did not significantly cause cognitive impairment and brain injury in wild-type mice. On the other hand, the same short-term consumption of high-fat diet significantly enhanced cognitive impairment in 5XFAD mice, as shown by the Morris water maze test. These findings on cognitive function demonstrate that 5XFAD mouse brain was more vulnerable to high-fat-diet-induced cognitive dysfunction than control mouse brain. Cerebrovascular A $\beta$  accumulation, namely CAA, is well known to be critically involved in the incidence and development of AD and cognitive impairment.<sup>10–14</sup> To elucidate the potential mechanism underlying cognitive impairment by high-fat diet in 5XFAD, we examined the effect of high-fat diet on cerebrovascular A $\beta$  deposition. Of note are the observations that high-fat-diet intake significantly increased hippocampal microvessel A $\beta$  deposition and cortical arterial A $\beta$  deposition in 5XFAD mice, while A $\beta$  deposition was not detectable in

either group of wild-type mice. Furthermore, we also examined the effect of high-fat diet on cerebral parenchymal A $\beta$  deposition. In contrast to the significant increase in cerebrovascular A $\beta$  deposition by high-fat diet in 5XFAD mice, high-fat diet failed to increase cerebral parenchymal A $\beta$  deposition in 5XFAD mice. Thus, high-fat diet preferentially increased the deposition of A $\beta$  in cerebral vascular walls but not in cerebral parenchyma of 5XFAD mice. Taken together with the fact that CAA accelerates cognitive impairment of AD and hippocampus plays a critical role in cognitive impairment of AD, our present findings provided the evidence that exacerbation of cognitive impairment by high-fat diet consumption in 5XFAD mouse is at least in part mediated by the exacerbation of CAA rather than parenchymal A $\beta$ . Furthermore, we also found that occludin, which is the cerebral endothelial tight junction protein involved in blood–brain barrier function, was significantly reduced by high-fat diet in 5XFAD mice but not in wild-type mice. This reduction of occludin also supports the notion that high-fat diet preferentially causes cerebrovascular injury in 5XFAD mice.

Accumulating evidence indicates that cerebral oxidative stress, particularly hippocampal oxidative stress, is involved in the progression of cognitive impairment in AD.<sup>36–38</sup> Furthermore, cerebrovascular A $\beta$  deposition is known to enhance local oxidative stress.<sup>36–38</sup> Therefore, in the present study, we

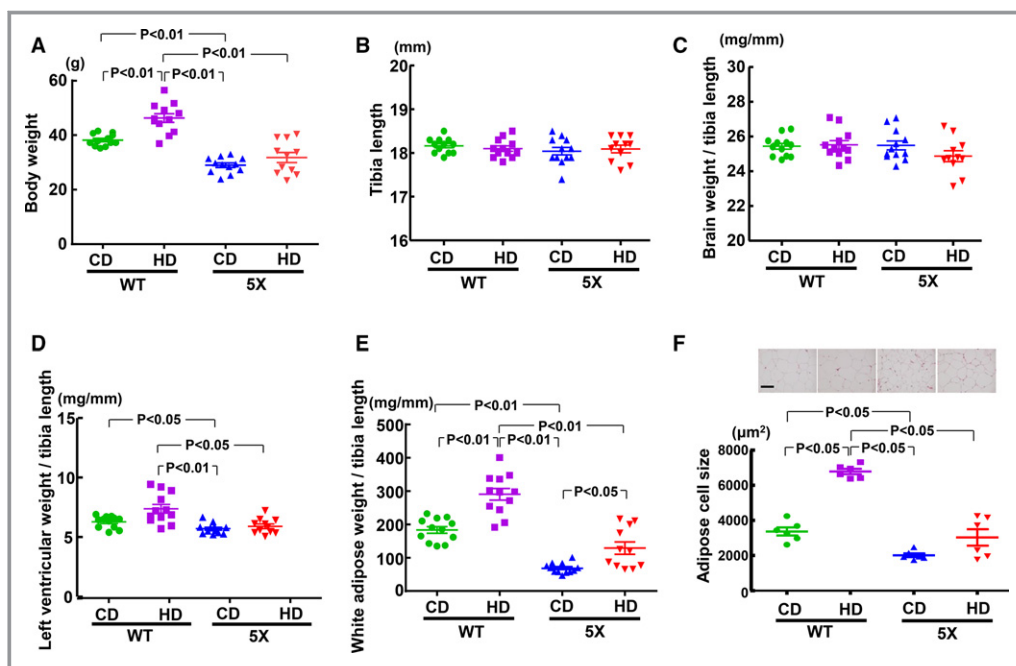


**Figure 10.** Cerebral cyclooxygenase-2, extracellular-superoxide dismutase, copper-zinc superoxide dismutase, manganese superoxide dismutase, and catalase. A, Representative Western blot of cyclooxygenase-2, extracellular-superoxide dismutase, copper-zinc superoxide dismutase, manganese superoxide dismutase, and catalase. 5X indicates 5XFAD mice; CD, control diet; COX-2, cyclooxygenase-2; Cu/Zn-SOD, copper-zinc superoxide dismutase; EC-SOD, extracellular-superoxide dismutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HD, high-fat diet; Mn-SOD, manganese superoxide dismutase; WT, wild-type mice. Values are means $\pm$ SEM.  $n=6$  in WT-CD,  $n=6$  in WT-HD,  $n=6$  in 5X-CD,  $n=6$  in 5X-HD. In (B through D), statistical significance was tested with 1-way ANOVA followed by Tukey's multiple comparison post hoc test between each group. In (E and F), statistical significance was tested with Kruskal–Wallis test followed by Steel–Dwass post hoc test.

also examined the effect of high-fat diet on cerebral oxidative stress in 5XFAD mice compared with wild-type mice. Interestingly, high-fat-diet intake significantly enhanced hippocampal oxidative stress in 5XFAD mice, while it failed to increase hippocampal oxidative stress in wild-type mice. NADPH oxidase is believed to play a major role in the generation of oxidative stress in various tissues including the brain. Therefore, we examined the effect of high-fat diet on NADPH oxidase subunits to elucidate the potential role of NADPH oxidase. In the present study, we found that high-fat-diet intake in 5XFAD mice significantly increased cerebral gp91<sup>phox</sup> and p22<sup>phox</sup> compared with control diet, while high-fat diet did not significantly alter these NADPH oxidase subunits in wild-type mice. Taken together with the fact that both gp91<sup>phox</sup> and p22<sup>phox</sup> are key subunits of NADPH oxidase, our present findings suggest that the enhancement of oxidative stress by high-fat diet in 5XFAD mice might be at least in part attributed to the increase in gp91<sup>phox</sup> and p22<sup>phox</sup>. Therefore, cognitive impairment by high-fat diet in 5XFAD mice seems to be attributed to the enhancement of hippocampal oxidative stress through the increase of NADPH

oxidase subunits as well as the exacerbation of CAA. Besides NADPH oxidase, various superoxide-regulating enzymes, such as SOD isoforms, catalase, and COX-2, are known to be involved in brain oxidative stress. Therefore, in the present work, we also measured these enzymes. In contrast to the significant increase in cerebral gp91<sup>phox</sup> and p22<sup>phox</sup>, high-fat-diet intake did not significantly affect 3 SOD isoforms, catalase, or COX-2 in 5XFAD mice, thereby supporting the critical role of NADPH oxidase in brain oxidative stress of 5XFAD mice fed a high-fat diet. However, future study is required to determine the detailed mechanism underlying brain oxidative stress in 5XFAD mice fed a high-fat diet.

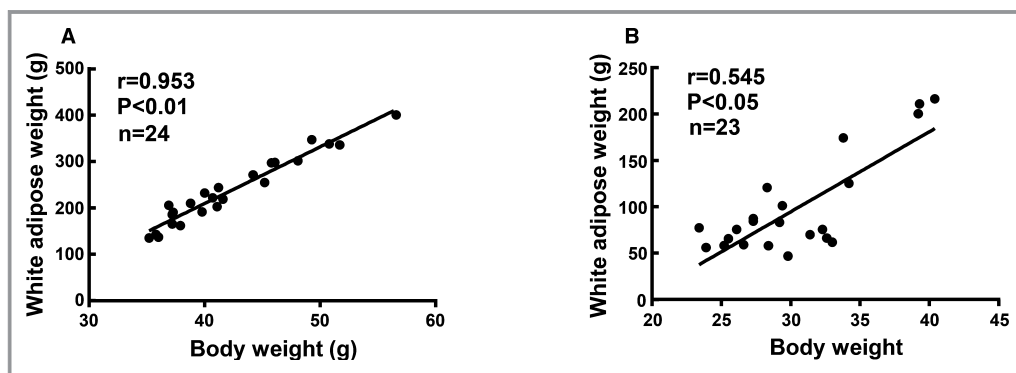
Besides oxidative stress, BDNF has been postulated to participate in the pathophysiology of AD.<sup>39–41</sup> BDNF is a neurotrophin involved in the survival and growth of neurons and abundantly expressed in the hippocampus and cortex.<sup>42,43</sup> Inhibition of the BDNF pathway can reduce synaptic plasticity and neurogenesis in the hippocampus, which causes the impairment of hippocampal-dependent learning and memory.<sup>3,44</sup> In the present study, high-fat-diet intake tended to decrease cerebral BDNF levels in 5XFAD mice and BDNF



**Figure 11.** Body weight (A), tibia length (B), brain weight (C), cardiac weight (D), white adipose weight (E), and adipose cell size (F) at the end of the treatment (10 weeks of the treatment). In (A through E), the data from cohort 1 and cohort 2 were combined for analysis. Upper panels in (F) indicate representative photomicrographs of hematoxylin-eosin-stained white adipose tissue sections. High-fat diet significantly increased body weight in wild-type mice but did not in 5XFAD mice (A). White adipose weight/tibia length (E) and adipose cell size (F) were significantly greater in high-fat-fed wild-type mice than in high-fat-fed 5XFAD mice. 5X indicates 5XFAD mice; CD, control diet; HD, high-fat diet; WT, wild-type mice. Values are means $\pm$ SEM.  $n=12$  in WT-CD,  $n=12$  in WT-HD,  $n=12$  in 5X-CD,  $n=11$  in 5X-HD. In (A, D, E, and F), statistical significance was tested with Kruskal–Wallis test followed by Steel–Dwass post hoc test. In (B and C), statistical significance was tested with 1-way ANOVA followed by the Tukey’s multiple comparison post hoc test between each group. Scale bar=100  $\mu\text{m}$ .

levels in 5XFAD mice fed high-fat diet were significantly less than those in both groups of wild-type mice. These results suggest that the downregulation of BDNF may also participate

in cognitive impairment induced by high-fat-diet intake in 5XFAD mice. However, further study is required to elucidate our proposal.



**Figure 12.** Relationship between body weight and white adipose tissue weight in wild-type mice (A) and 5XFAD mice (B). The data on body weight and white adipose tissue weight from cohort 1 and cohort 2 were combined for analysis. The correlation between body weight and white adipose weight was statistically significant in wild-type mice (A) and in 5XFAD mice (B).  $n=24$  in wild-type mice,  $n=23$  in 5XFAD mice. The correlation between body weight and white adipose weight was analyzed by Spearman correlation test. 5X indicates 5XFAD mice.



Another intriguing finding of the present work was less susceptibility of 5XFAD than wild-type mice with regard to high-fat-diet-induced metabolic disorders. Epidemiological studies show that obesity in midlife is a risk factor for developing dementia as well as for cardiovascular diseases.<sup>45</sup> On the other hand, overweight and obesity in late life are associated with lower dementia risk.<sup>46</sup> Furthermore, there is some evidence suggesting that body weight loss may be a risk factor for AD and cognitive impairment.<sup>47–50</sup> Body weight loss in AD is associated with dementia severity and faster clinical progression.<sup>47–50</sup> Thus, epidemiological studies suggest a close relationship between body weight and AD or cognitive impairment. In the present study, as expected, high-fat-diet intake in wild-type mice significantly and progressively increased body weight, white adipose tissue weight, and white adipose size, and significantly impaired glucose tolerance. On the other hand, unexpectedly, the increase in body weight, white adipose tissue weight, and adipose size in 5XFAD by high-fat diet was much less than that in wild-type mice. Differing from wild-type mice fed high-fat diet, 5XFAD mice fed high-fat diet exhibited no apparent impairment of glucose tolerance. Our data obtained by metabolic cage analysis indicated that this significant difference between 5XFAD and wild-type mice regarding obesity and glucose intolerance is not attributed to food intake or water intake, because there was no decrease in these parameters in high-fat-fed 5XFAD mice compared with their wild-type counterparts. Furthermore, in the present study, blood pressure was not altered by high-fat-diet feeding in either 5XFAD or wild-type mice. Collectively, these findings show that the above-mentioned exacerbation of AD-like pathology by high-fat diet in 5XFAD mice is not attributed to metabolic disorders. Therefore, high-fat diet seems to directly accelerate progression of AD-like pathology in 5XFAD independently of metabolic disorders. However, the present work did not allow us to elucidate the reason why 5XFAD mice fed high-fat diet exhibited much less obesity and no glucose intolerance compared with their wild-type counterparts. Future study is required to elucidate the potential significance of less obesity and less glucose intolerance in AD pathology of 5XFAD mice.

### Study Limitations

Fitz et al<sup>51</sup> investigated the effect of high-fat diet for 4 months on cerebral amyloid pathology and cognitive function in 9-month-old APP23 transgenic mice, a different Alzheimer's model mouse from 5XFAD. However, they did not examine the effect of high-fat diet on CAA, cerebral oxidative stress, BDNF, and adipose tissue in AD mouse. This group of investigators has found that 4 months of high-fat-diet feeding increases cerebral parenchymal A $\beta$  levels and exacerbates cognitive impairment in APP23 mice,<sup>51</sup> providing different

findings from our present observations that high-fat diet for 10 weeks did not significantly increase cerebral parenchymal A $\beta$  deposits in 5XFAD mice in spite of the significant increase in cerebrovascular A $\beta$  deposits. These different findings between the previous report<sup>51</sup> and our present work regarding the effect of high-fat diet on cerebral parenchymal A $\beta$  deposition seem to be explained by the differential period of high-fat-diet feeding (4 months versus 10 weeks), the use of different Alzheimer's model mouse (APP23 versus 5XFAD mice) or the use of different age of mice (9-month-old versus 13-month-old). Further study is needed to elucidate the precise role of parenchymal A $\beta$  versus cerebrovascular A $\beta$  in high-fat-diet-induced cognitive impairment in Alzheimer's disease.

Furthermore, another limitation of the present study was that the measurement of brain oxidative stress was performed only by DHE staining, although we verified that DHE fluorescence was derived from superoxide as shown by the significant reduction of DHE fluorescence by SOD pretreatment. Further study investigating the effect of antioxidant on 5XFAD mice is warranted to define the role of oxidative stress in 5XFAD mice fed high-fat diet.

In conclusion, the present study provided the first evidence that high-fat diet, independently of metabolic disorders, accelerated CAA and enhanced hippocampal oxidative stress, thereby promoting cognitive impairment in an AD mouse model. Therefore, our present work provided a novel insight into the significance of high-fat-diet consumption in the pathogenesis of AD.

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