

# **Twendee X ameliorates phosphorylated tau, $\alpha$ -synuclein and neurovascular dysfunction in Alzheimer's disease transgenic mice with chronic cerebral hypoperfusion**

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**Running headline:** Neuroprotective effect of Twendee X.

**Keywords:** Alzheimer's disease, APP23 mice, chronic cerebral hypoperfusion, phosphorylated tau,  $\alpha$ -synuclein, neurovascular dysfunction.

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**Abbreviations used:** AD, Alzheimer's disease; A $\beta$ , amyloid- $\beta$ ; BCCAs, bilateral common carotid arteries; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CCH, chronic cerebral hypoperfusion; CTX, cortex; DG, dentate gyrus; GFAP, glial fibrillary acidic protein; HI, hippocampus; LEL, Lycopersicon esculentum lectin; M, months; NAGO, N-acetylglucosamine oligomers; NVU, neurovascular unit; PBS, phosphate-buffered saline; PDGFR $\beta$ , platelet-derived growth factor receptor  $\beta$ ; PFA, paraformaldehyde; TH, thalamus; TrkB, tropomyosin receptor kinase B; TwX, Twendee X; WT, wild type.

## Abstract

**Background:** The pathological impact of chronic cerebral hypoperfusion (CCH) on Alzheimer's disease (AD) is still poorly understood. In the present study, we investigated the role of CCH on an AD mouse model in phosphorylated tau and  $\alpha$ -synuclein pathology, neurovascular unit, cerebrovascular remodeling, and neurovascular trophic coupling. Moreover, examined protective effect of a new antioxidant Twendee X (TwX).

**Methods:** APP23 mice were implanted to bilateral common carotid arteries stenosis with ameroid constrictors to gradually decrease the cerebral blood flow. The effects of the administration of TwX were evaluated by immunohistochemical analysis and Immunofluorescent histochemistry.

**Results:** The present study revealed that the expressions of phospho-tau and phospho- $\alpha$ -synuclein were significantly increased in the APP23 + CCH mice group as compared with wild type and APP23 mice groups ( $*p < 0.05$  and  $**p < 0.01$  vs WT;  $\# p < 0.05$  and  $\#\# p < 0.01$  vs APP23). In addition, CCH significantly exacerbated MMP-9 activation relating to blood-brain barrier destruction ( $**p < 0.01$  vs WT;  $\# p < 0.05$  and  $\#\# p < 0.01$  vs APP23), enhanced neurovascular remodeling, and impaired a neurovascular trophic coupling in the vascular endothelial BDNF expression of the APP23 + CCH group. TwX treatment (20 mg/kg/d, from 4.5 to 12 months) significantly reduced tau and  $\alpha$ -synuclein pathologies, ameliorated neurovascular dysfunction compared with APP23 + CCH group.

**Conclusions:** Our findings indicate that administration of a new anti-oxidative mixture TwX substantially reduced the above neuropathologic abnormalities, suggesting a potential therapeutic benefit of TwX for AD with CCH.

## **Introduction**

Alzheimer's disease (AD) represents approximately 69% of all dementia among the people older than 75 years, <sup>1</sup> which is associated with amyloid- $\beta$  (A $\beta$ ) plaques, neurofibrillary tangles, and white matter abnormality. <sup>2-3</sup> A $\beta$  plaques and neurofibrillary tangles consist mostly of A $\beta$  and tau proteins. <sup>4</sup> In addition, up to 50% of AD patients exhibit significant Lewy bodies pathology, <sup>5-7</sup> in which  $\alpha$ -synuclein is the primary component of Lewy bodies. <sup>8</sup>

The dysfunction of the neurovascular unit (NVU) is regarded as an early event in AD, <sup>9</sup> and chronic cerebral hypoperfusion (CCH) is defined as one of the major mechanisms of cerebral vascular disorders. <sup>3,10</sup> Our recent studies showed CCH strongly enhanced AD pathology in mice. <sup>11-12</sup>

Twendee X (TwX) is a new multiple antioxidant mixture that contains ingredients such as coenzyme Q10, ascorbic acid, L-glutamine and cystine. Our previous study showed the neuroprotective effects of TwX in an acute cerebral ischemia mice model by reducing ischemic infarct and attenuating both oxidative stress and inflammation. <sup>13</sup>

Therefore, the present study aimed to investigate a possible therapeutic effect of TwX on tau and  $\alpha$ -synuclein pathology, damage of NVU and neurovascular trophic coupling in an AD mouse model with CCH.

## **Materials and methods**

### **Animals**

All animal experimental procedures were approved by the guidelines of the Animal Committee of the Graduate School of Medicine and Dentistry of Okayama University (OKU-

2014-095). All experiments were carried out on APP23 mice which overexpress human APP751 isoform carrying the Swedish double mutation (KM670/671NL)<sup>14</sup> and wild type (non-transgenic) littermates. APP23 male mice were obtained from Dr. Takashi Saito (RIKEN Brain Science Institute, Saitama, Japan) and maintained as hemizygotes by mating APP23 male mice with C57BL/6J female mice (CLEA Japan, Tokyo, Japan). At 4 months (M) of age (before surgery), the body weights of male mice were 29.2 grams (g) to 32.1 g, the body weights of female mice were 20.5 g to 22.9 g, and there was no significant difference between APP23 mice and wild type littermates. The offspring were genotyped using a PCR assay with DNA obtained from tail tissue samples. All mice were housed in 12:12-hour light-dark cycle with controlled temperature and free access to food and water.

### **Experimental Groups and Drug Treatments**

Four experimental groups of mice were designed in the present study: wild type mice (WT + sham surgery, male n = 5, female n = 5), APP23 group (APP23 + sham surgery, male n = 5, female n = 5), CCH group (APP23 + CCH, male n = 7, female n = 5) and TwX group (APP23 + CCH + TwX, male n = 6, female n = 7). For the mice in CCH and TwX groups, ameriod constrictors with an inner diameter of 0.75mm and an open gap of 0.5mm (Research Instruments NW, Lebanon, OR, USA) were applied to the bilateral common carotid arteries (BCCAs) at 4 M of age as described previously.<sup>11-12</sup>

TwX is a mixture antioxidants,<sup>13</sup> which contains ingredients as follows: coenzyme Q10 (3.6 wt%; AQUA Q10 P40-NF, Nissin Pharmaceutical, Tokyo, Japan), niacin amid (0.7 wt%), L-cystine (18.2 wt%), ascorbic acid (34.2 wt%), succinic acid (3.6 wt%), fumaric acid (3.6

wt%), L-glutamine (34.6 wt%), and riboflavin (1.5 wt%; Bislase inj, Toa Eiyo, Tokyo, Japan).

The mice in TwX group began to receive TwX (20 mg/kg per day) by oral gavage once daily from 4.5 M of age until sacrifice. This dose of TwX was decided according to a previous study which showing its neuroprotective effectiveness in transient middle cerebral artery occlusion mice.<sup>13</sup> The present study is a part of whole project in this mice model mainly focusing on abnormal protein deposition, NVU and neurovascular trophic coupling.

### **Tissue preparation**

The mice aged 12 M were deeply anesthetized with pentobarbital (40 mg/kg) and perfused transcardially with 20ml chilled phosphate-buffered saline (PBS, pH 7.4), followed by cold 4% paraformaldehyde (PFA). The brains were removed and post-fixed in 4% PFA overnight, then transferred into 10, 20 and 30% (wt/vol) sucrose in PBS for 24h, respectively. They were then sectioned in to coronal brain sections (20  $\mu$ m) by using a cryostat at -20°C and mounted on silane-coated glass slides.

### **Immunohistochemical analysis**

After incubation in 0.3% hydrogen peroxide/methanol followed by 5% bovine serum albumin (BSA), the sections were incubated overnight at 4°C with the following primary antibody: mouse anti-tau antibody (1:500, Cell Signaling Technology, Beverly, MA, USA), mouse anti-phospho-tau antibody (1:100, Thermo Fisher Scientific, Rockford, IL, USA), mouse anti-phospho- $\alpha$ -synuclein antibody (1:500, Wako, Osaka, Japan) and rabbit anti-MMP-9 antibody (1:100, Millipore Corporation, Billerica, MA, USA). Sections were washed with

PBS, then incubated with suitable biotinylated secondary antibodies (1:500; Vector Laboratories) at room temperature for 2 h. After washed with PBS, sections were treated with the avidin–biotin–peroxidase complex (VECTASTAIN Elite ABC Kit; Vector Laboratories) for 30 min and visualized with 3, 3'-diaminobenzidine (DAB). For semiquantitative analysis of tau, phospho-tau, phospho- $\alpha$ -synuclein and MMP-9 staining, 3 separated sections per brain from 3 levels (-1.3, -1.8 and -2.3 mm posterior to the bregma), and 5 random selected regions from cerebral cortex (CTX), hippocampus (HI), and thalamus (TH) were captured at  $\times 200$  magnification with a light microscope (Olympus BX-51, Tokyo, Japan). The pixel intensity was measured using ImageJ software by 2 individuals who were blind to experimental group, and then calculated as a percentage of WT group.

### **Immunofluorescent histochemistry**

To determine the remodeling of NVU and changes of neurovascular trophic coupling, double immunofluorescence histochemistry were performed for collagen IV plus glial fibrillary acidic protein (GFAP), brain-derived neurotrophic factor (BDNF) plus N-acetylglucosamine oligomers (NAGO), BDNF plus tropomyosin receptor kinase B (TrkB), BDNF plus GFAP and BDNF plus platelet-derived growth factor receptor  $\beta$  (PDGFR $\beta$ ). Lycopersicon esculentum lectin (LEL) is a glycoprotein with specific affinity for NAGO, which is expressed in mature vascular endothelial cells.<sup>15</sup> Primary antibodies were used as follows: rabbit anti-collagen IV antibody (1:500, Abcam, Cambridge, MA, USA), goat anti-GFAP antibody (1:1000, R&D Systems, Minneapolis, MN, USA), sheep anti-BDNF antibody (1:100, Abcam), biotinylated LEL (1:200, Vector Laboratories, Burlingame, CA, USA), rabbit anti-TrkB antibody (1:200,

Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-PDGFR $\beta$  antibody (1:100, Cell Signaling Technology). After washing, sections were incubated with fluorescent secondary antibody, and then scanned with a confocal microscope equipped with an argon and HeNe1 laser (LSM-780; Zeiss, Jena, Germany).

### Statistical analysis

All data were expressed as mean  $\pm$  SD. Statistical comparison was performed using one-way ANOVA analysis followed by Tukey-Kramer test as a post hoc test. Statistically significance was defined as  $p < 0.05$ .

## Results

### Changes of tau pathology and $\alpha$ -synuclein phosphorylation after CCH

As shown in Fig. 1, there was no significant difference between 4 groups in total-tau expressions (Fig. 1A and 1B). In contrast, 3 APP23 groups displayed significant increases of phospho-tau (AT8) immunoreactivity than WT group in the CTX with the highest expression in CCH group, which was recovered by TwX treatment (Fig. 1A and 1C, \* $p < 0.05$  and \*\* $p < 0.01$  vs WT; # $p < 0.05$  vs APP23; \$ $p < 0.05$  vs CCH). Similar tendencies were observed in 3 APP23 groups in phospho-tau of CA1 (Fig. 1A and 1C).

The expression of phospho- $\alpha$ -synuclein significantly increased in 3 APP23 groups in CTX, CA1, dentate gyrus (DG) and TH, especially in CCH group (Fig. 2A and 2B). Compared with CCH group, the administration of TwX greatly decreased such accumulation of phospho- $\alpha$ -synuclein in both CTX and TH (Fig. 2A and 2B, \* $p < 0.05$  and \*\* $p < 0.01$  vs WT; # $p < 0.05$

and <sup>##</sup>*p* < 0.01 vs APP23; <sup>§</sup>*p* < 0.05 and <sup>§§</sup>*p* < 0.01 vs CCH).

### **MMP-9 and NVU remodeling after CCH**

The MMP-9 was only slightly detected in neurons of CTX and blood vessel wall of TH (Fig. 3A) in WT mice, which was significantly upregulated in 3 APP23 groups especially in CCH group and was greatly recovered by TwX treatment (Fig. 2A and 2B, <sup>\*\*</sup>*p* < 0.01 vs WT; <sup>#</sup>*p* < 0.05 and <sup>##</sup>*p* < 0.01 vs APP23; <sup>§</sup>*p* < 0.05 and <sup>§§</sup>*p* < 0.01 vs CCH).

Double immunofluorescent analysis of collagen IV/GFAP showed morphological changes of collagen IV and astrocytes in HI and TH of 3 APP23 groups (Fig. 4A and 4B). The changes were much enhanced in TH of APP23 + CCH groups, which was ameliorated by TwX treatments (Fig. 4B).

### **Changes of neurovascular trophic coupling after CCH**

BDNF was immunopositive in vascular endothelial cells (Fig. 5A), neurons (Fig. 5B), astrocytes (Fig. 6A) and pericytes (Fig. 6B), but which was significantly decreased in 3 APP23 groups compared with WT group with a remarkable decline in CCH group (Fig. 5 and 6). TwX treatment showed a significant restoration of BDNF expression in all cell types compared with CCH group (Fig. 5 and 6).

## **Discussion**

In present study, we demonstrated that the administration of antioxidative mixture TwX attenuated tau hyperphosphorylation (Fig. 1) and phospho- $\alpha$ -synuclein deposition (Fig. 2),

decreased MMP-9 activation (Fig. 3), alleviated NVU remodeling (Fig. 4), and ameliorated neurovascular trophic coupling damage (Figs. 5-6) in the CCH model of APP23 mice. Although numerous studies indicated that A $\beta$ , tau, and  $\alpha$ -synuclein play synergistical roles in AD pathogenesis,<sup>4, 16-17</sup> a pathological impact of CCH is still poorly understood on these protein accumulations. Here our results newly found that CCH significantly promoted tau hyperphosphorylation and  $\alpha$ -synuclein accumulation in AD mice, which were ameliorated by the administration of TwX (Figs. 1-2).

APP23 mice are characterized by increasing A $\beta$  deposition in the brain from 6 M of age, tau hyperphosphorylation could also be detected at 6 M.<sup>14</sup> In our previous studies, cognitive deficit, A $\beta$  pathology, and neurovascular dysfunction had become remarkable at 12 M of age.<sup>11-12</sup> Thus we studied the effect of TwX at 12 M of age in this model in the present study.

After implanting the ameriod constrictors, the mice cerebral blood flow, which was demonstrated by a laser-Doppler flowmeter, showed a gradually decreased and reached a steady status until 28 d.<sup>12, 30</sup> In another paper, we showed the effect of TwX on improving cognitive deficit, reducing primary AD pathology with inhibiting inflammatory and oxidative stresses.<sup>30</sup>

NVU is structurally composed of vascular endothelial cells, vascular smooth muscle cells, astrocytes, and perivascular neurons,<sup>9</sup> which functionally serves the basis of neurovascular coupling.<sup>18-20</sup> The dysfunction of NVU impaired CBF regulation and BBB transport, leading to the impairment of A $\beta$  clearance to enhance AD pathology.<sup>21-22</sup> The present APP23 + CCH group showed the higher MMP-9 expression than the simple APP23 group, and exacerbated BBB leakage and abnormal neurovascular remodeling, which were suppressed by TwX treatment (Figs. 3-4).

BDNF is a member of the neurotrophin family,<sup>23</sup> which plays important role in neuronal growth and survival as well as synaptic plasticity in both animal models and AD patients.<sup>24-25</sup> Therefore, the reduction of BDNF expression of AD and MCI patients is related to cognitive impairment.<sup>26-29</sup> The present results indicated that CCH decreased the BDNF expression in endothelium cells, neuronal cells, astrocytes and pericytes (Figs. 5-6), which was recovered by TwX to protect against CCH injury on neurovascular coupling in APP23 mice (Figs. 5-6).

In conclusion, the present study newly revealed that CCH enhanced tau hyperphosphorylation and phospho- $\alpha$ -synuclein levels, exacerbated the NVU dysfunction and neurovascular coupling in APP23 mice, and TwX treatment remarkably ameliorated such neuropathologic abnormalities in APP23 + CCH model mice commonly found in elderly societies.

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## Conflict of interest

The authors disclose no potential conflict of interests.

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## Figure Legends

Fig. 1) (A) Single immunohistochemistry of total-tau and phospho-tau (p-tau, AT8) changes in cerebral cortex (CTX) and hippocampal CA1 of AD mice model with chronic cerebral hypoperfusion (CCH). (B) Quantitative analysis of total-tau and (C) p-tau ( $*p < 0.05$  and  $**p < 0.01$  vs WT;  $\#p < 0.05$  vs APP23;  $\$p < 0.05$  vs CCH. Scale bar = 50  $\mu\text{m}$ ).

Fig. 2) (A) Single immunohistochemistry of phosphorylated  $\alpha$ -synuclein expression in CTX, hippocampal CA1 and dentate gyrus (DG), and thalamus (TH) of AD mice model with CCH. (B) Quantitative analysis of phosphorylated  $\alpha$ -synuclein ( $*p < 0.05$  and  $**p < 0.01$  vs WT;  $\#p < 0.05$  and  $###p < 0.01$  vs APP23;  $\$p < 0.05$  and  $$$p < 0.01$  vs CCH. Scale bar = 50  $\mu\text{m}$ ).

Fig. 3) (A) Single immunohistochemistry of MMP-9 in the parenchyma and blood vessels of CTX and TH of AD mice with CCH. (B) Quantitative analysis of MMP-9 ( $**p < 0.01$  vs WT;  $\#p < 0.05$  and  $###p < 0.01$  vs APP23;  $\$p < 0.05$  and  $$$p < 0.01$  vs CCH. Scale bar = 50  $\mu\text{m}$ ).

Fig. 4) Double immunofluorescent analysis of collagen IV plus GFAP in the hippocampus (HI) (A) and TH (B). Note the significant enlarged vessels with morphological changes of the collagen IV and astrocyte in APP23+CCH group, especially in TH (Scale bar = 50  $\mu\text{m}$ ).

Fig. 5) Double immunofluorescent analysis of BDNF plus NAGO (A) or TrkB (B) in CTX. Note the significantly decreased BDNF immunoreactivity in endothelium cells and neurons of 3 APP23 groups, especially in APP23+CCH group (Scale bar = 50  $\mu\text{m}$ ).

Fig. 6) Double immunofluorescent analysis of BDNF plus GFAP (A) or PDGFR $\beta$  (B) in CTX.

Note the significantly decreased BDNF immunoreactivity in astrocytes and pericytes of 3

APP23 groups, especially in APP23+CCH group (Scale bar = 50  $\mu$ m).