Twendee X Ameliorates Phosphorylated Tau, α-Synuclein and Neurovascular Dysfunction in Alzheimer's Disease Transgenic Mice With Chronic Cerebral Hypoperfusion

Xia Liu, MS, Toru Yamashita, MD, PhD, Jingwei Shang, MD, PhD, Xiaowen Shi, MS, Ryuta Morihara, MD, PhD, Yong Huang, BSc, Kota Sato, MD, PhD, Mami Takemoto, MD, PhD, Nozomi Hishikawa, MD, PhD, Yasuyuki Ohta, MD, PhD, and Koji Abe, MD, PhD

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 α-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-α-synuclein were significantly increased in the APP23 + CCH mice group as compared with wild type and APP23 mice groups (*$P < .05$ and **$P < .01$ versus WT; $^{\#}P < .05$ and $^{\#\#}P < .01$ versus APP23). In addition, CCH significantly exacerbated MMP-9 activation relating to blood-brain barrier destruction (**$P < .01$ versus WT; $^{\#}P < .05$, and $^{\#\#}P < .01$ versus 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/day, from 4.5 to 12 months) significantly reduced tau and α-synuclein pathologies, ameliorated neurovascular dysfunction compared with APP23 + CCH group. *Conclusions:* Our findings indicate that administration of a new antioxidative mixture TwX substantially reduced the above neuropathologic abnormalities, suggesting a potential therapeutic benefit of TwX for AD with CCH.

Key Words: Alzheimer's disease—APP23 mice—chronic cerebral hypoperfusion—phosphorylated tau—α-synuclein—neurovascular dysfunction

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; 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; PDGFRβ, platelet-derived growth factor receptor β; PFA, paraformaldehyde; TH, thalamus; TrkB, tropomyosin receptor kinase B; TwX, Twendee X; WT, wild type

From the Department of Neurology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan.

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Address correspondence to: Prof. Koji Abe, MD, PhD, Department of Neurology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. E-mail: pggx277x@hotmail.com.

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Introduction

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

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

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.[13]

Therefore, the present study aimed to investigate a possible therapeutic effect of TwX on tau and α-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) [14] and wild type (nontransgenic) 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)-32.1 g, the body weights of female mice were 20.5 g-22.9 g, and there was no significant difference between APP23 mice and wild type littermates. The offspring were genotyped using a polymerase chain reaction (PCR) assay with deoxyribonucleic acid (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 .75 mm and an open gap of .5 mm (Research Instruments NW, Lebanon, OR) were applied to the bilateral common carotid arteries (BCCAs) at 4 M of age as described previously.[11,12]

TwX is a mixture antioxidants,[13] which contains ingredients as follows: coenzyme Q10 (3.6 wt%; AQUA Q10 P40-NF, Nissin Pharmaceutical, Tokyo, Japan), niacin amid (.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.[13] 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 20 mL chilled phosphate-buffered saline (PBS, pH 7.4), followed by cold 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde overnight, then transferred into 10%, 20%, and 30% (wt/vol) sucrose in PBS for 24 hours, respectively. They were then sectioned in to coronal brain sections (20 μm) by using a cryostat at −20°C and mounted on silane-coated glass slides.

Immunohistochemical Analysis

After incubation in .3% hydrogen peroxide/methanol followed by 5% bovine serum albumin, the sections were incubated overnight at 4°C with the following primary antibody: mouse antitau antibody (1:500, Cell Signaling Technology, Beverly, MA), mouse anti−phospho-tau antibody (1:100, Thermo Fisher Scientific, Rockford, IL), mouse anti−phospho-α-synuclein antibody (1:500, Wako, Osaka, Japan), and rabbit anti-MMP-9 antibody (1:100, Millipore Corporation, Billerica, MA). Sections were washed with PBS, then incubated with suitable biotinylated secondary antibodies (1:500; Vector Laboratories) at room temperature for 2 hours. After washed with PBS, sections were treated with the avidin−biotin−peroxidase complex (VECTASTAIN Elite ABC Kit; Vector Laboratories) for 30 minutes and visualized with 3, 3′-diaminobenzidine. For semiquantitative analysis of tau, phospho-tau, phospho-α-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 ×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, BDNF plus tropomyosin receptor kinase B, BDNF plus GFAP and BDNF plus platelet-derived growth factor receptor β. Lycopersicon esculentum lectin is a glycoprotein with specific affinity for N-acetylglucosamine oligomers, which is expressed in mature vascular endothelial cells.[15] 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 Lycopersicon esculentum lectin (1:200, Vector Laboratories, Burlingame, CA, USA), rabbit anti-tropomyosin receptor kinase B antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-platelet-derived growth factor receptor β 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 ± SD. Statistical comparison was performed using one-way ANOVA analysis

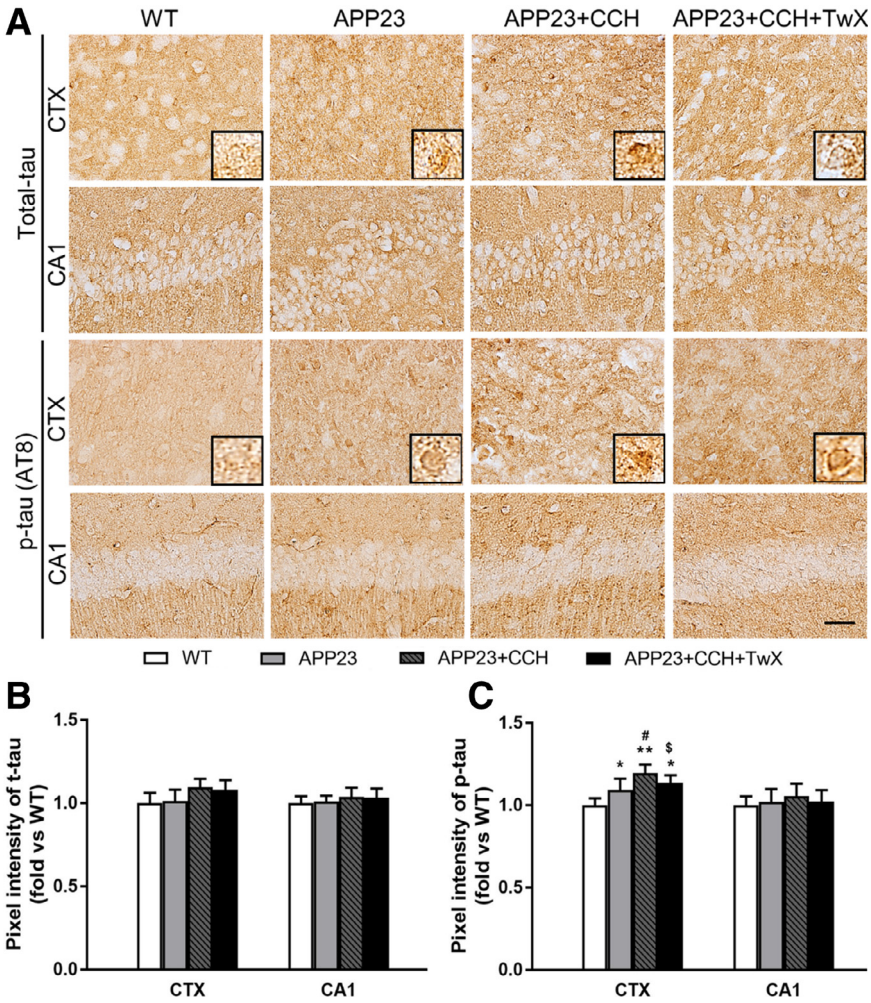

Figure 1. *(A) Single immunohistochemistry of total-tau and phospho-tau (p-tau, AT8) changes in cerebral cortex (CTX) and hippocampal CA1 of AD mice model with chronic cerebral hypoperfusion (CCH). (B) Quantitative analysis of total-tau and (C) p-tau (*P < .05 and **P < .01 versus WT; # P < .05 versus APP23; $P < .05 versus CCH. Scale bar = 50 μm). Color version of figure is available online.*

followed by Tukey-Kramer test as a posthoc test. Statistically significance was defined as *P*less than .05.

Results

Changes of Tau Pathology and α-Synuclein Phosphorylation After CCH

As shown in Figure 1, there was no significant difference between 4 groups in total-tau expressions (Fig 1A, B). 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,C, $^{*}P < .05$ and $^{**}P < .01$ versus WT; $^{\#}P < .05$ versus APP23; $^{\$}P < .05$ versus CCH). Similar tendencies were observed in 3 APP23 groups in phospho-tau of CA1 (Fig 1A,C).

The expression of phospho-α-synuclein significantly increased in 3 APP23 groups in CTX, CA1, dentate gyrus and TH, especially in CCH group (Fig 2A,B). Compared with CCH group, the administration of TwX greatly decreased such accumulation of phospho-α-synuclein in both CTX and TH (Fig 2A,B, $^{*}P < .05$ and $^{**}P < .01$ versus WT; $^{\#}P < .05$ and $^{\#\#}P < .01$ versus APP23; $^{\$}P < .05$ and $^{\$\$}P < .01$ versus CCH).

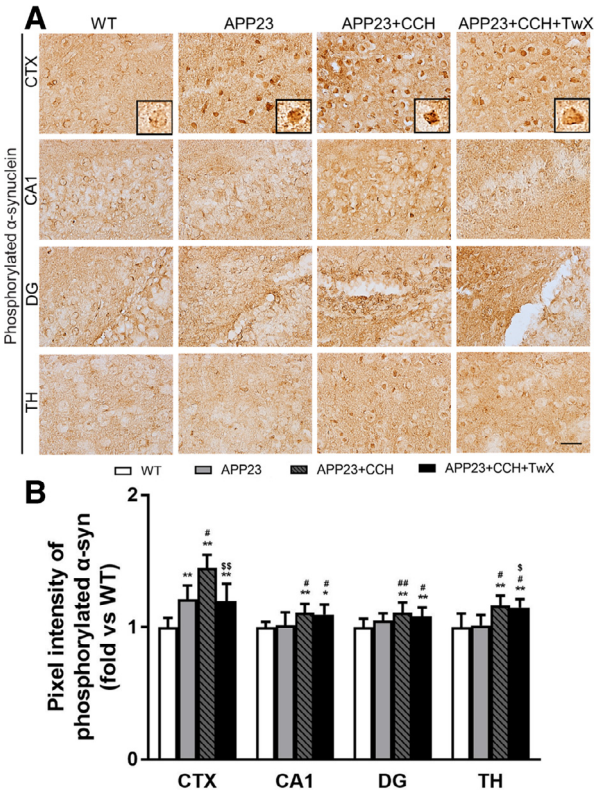

Figure 2. *(A) Single immunohistochemistry of phosphorylated α-synuclein expression in CTX, hippocampal CA1 and dentate gyrus (DG), and thalamus (TH) of AD mice model with CCH. (B) Quantitative analysis of phosphorylated α-synuclein (*P < .05 and **P < .01 versus WT; # P < 0.05 and ## P < 0.01 versus APP23; $P < 0.05 and $$P < .01 versus CCH. Scale bar = 50 μm). Color version of figure is available online.*

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,B, $^{**}P < .01$ versus WT; $^{\#}P < .05$ and $^{\#\#}P < .01$ versus APP23; $^{\$}P < .05$ and $^{\$\$}P < .01$ versus 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,B). 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, 6). TwX treatment showed a significant restoration of BDNF expression in all cell types compared with CCH group (Fig 5 and 6).

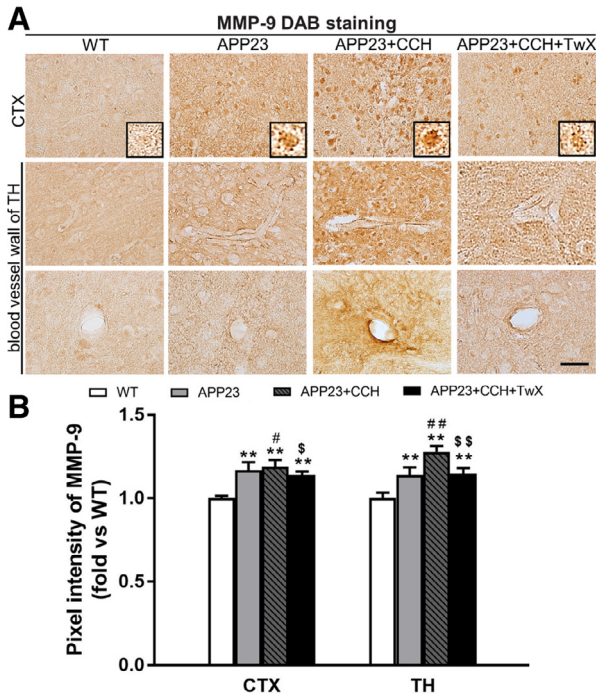

Figure 3. *(A) Single immunohistochemistry of MMP-9 in the parenchyma and blood vessels of CTX and TH of AD mice with CCH. (B) Quantitative analysis of MMP-9 (**P < .01 versus WT; # P < .05 and ## P < .01 versus APP23; $P < .05 and $$P < 0.01 versus CCH. Scale bar = 50 μm).Color version of figure is available online.*

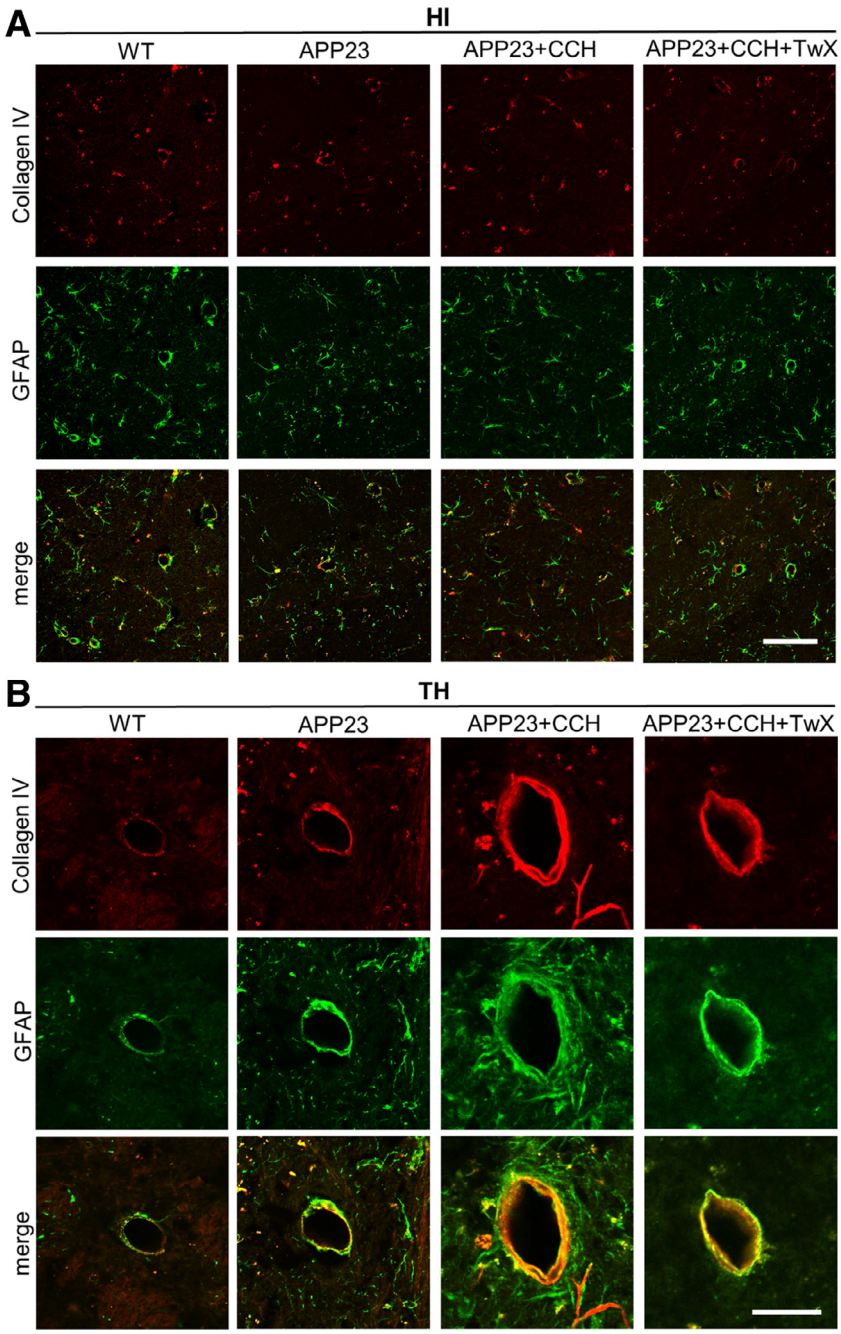

Figure 4. *Double immunofluorescent analysis of collagen IV plus GFAP in the hippocampus (HI) (A) and TH (B). Note the significant enlarged vessels with morphological changes of the collagen IV and astrocyte in APP23 + CCH group, especially in TH (Scale bar = 50 μm). Color version of figure is available online.*

Discussion

In present study, we demonstrated that the administration of antioxidative mixture TwX attenuated tau hyperphosphorylation (Fig 1) and phospho-α-synuclein deposition (Fig 2), decreased MMP-9 activation (Fig 3), alleviated NVU remodeling (Fig 4), and ameliorated neurovascular trophic coupling damage (Fig. 5, 6) in the CCH model of APP23 mice. Although numerous studies indicated that Aβ, tau, and α-synuclein play synergistical roles in AD pathogenesis,[4,16,17] 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 α-synuclein accumulation in AD mice, which were ameliorated by the administration of TwX (Fig. 1, 2).

APP23 mice are characterized by increasing Aβ deposition in the brain from 6 M of age, tau hyperphosphorylation could also be detected at 6 M.[14] In our previous

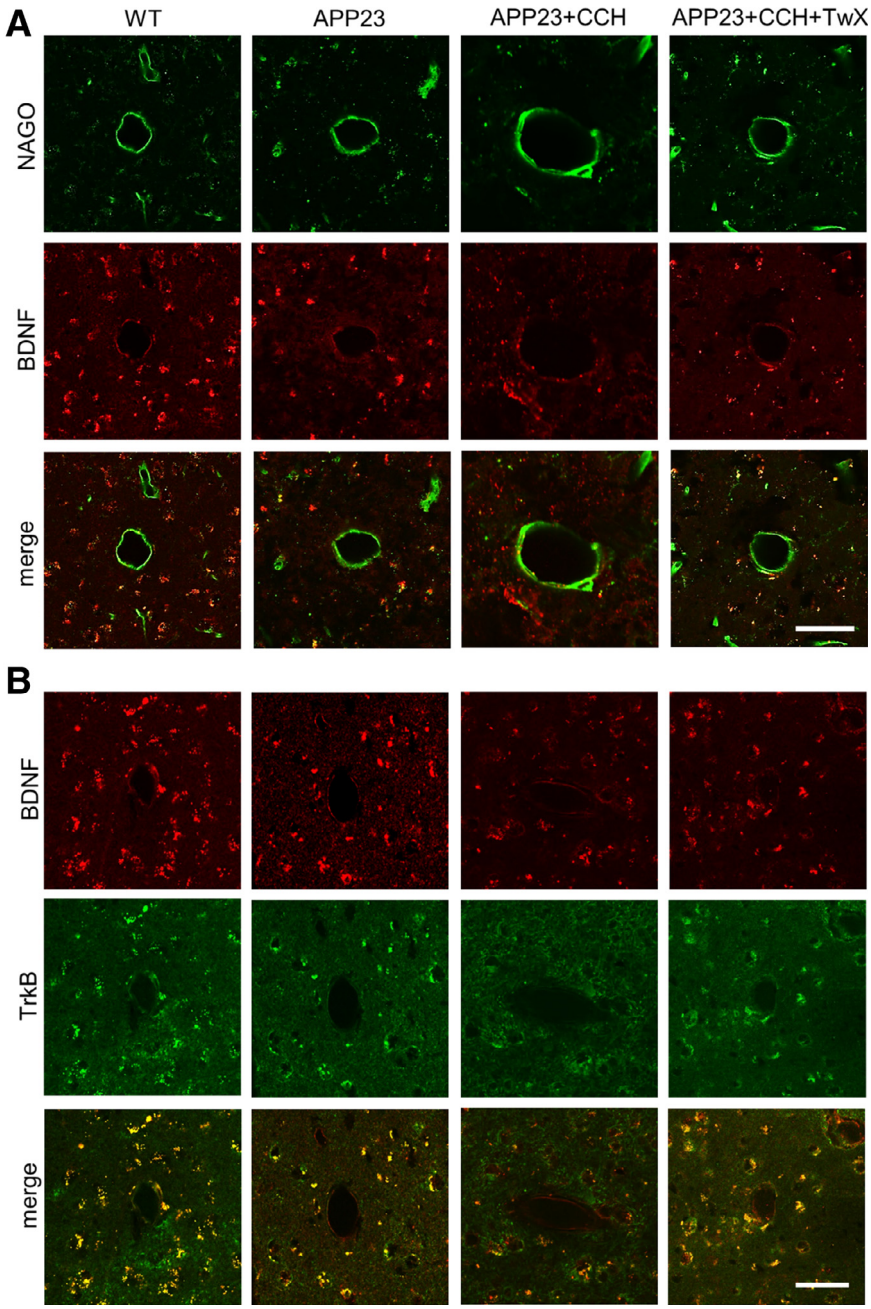

Figure 5. *Double immunofluorescent analysis of BDNF plus NAGO (A) or TrkB (B) in CTX. Note the significantly decreased BDNF immunoreactivity in endothelium cells and neurons of 3 APP23 groups, especially in APP23+CCH group (Scale bar = 50 μm). Color version of figure is available online.*

studies, cognitive deficit, Aβ pathology, and neurovascular dysfunction had become remarkable at 12 M of age.[11,12] 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 days.[12,18] In another paper, we showed the effect of TwX on improving cognitive deficit, reducing primary AD pathology with inhibiting inflammatory and oxidative stresses.[18]

NVU is structurally composed of vascular endothelial cells, vascular smooth muscle cells, astrocytes, and perivascular neurons, [9] which functionally serves the basis of neurovascular coupling.[19-21] The dysfunction of NVU impaired cerebral blood flow (CBF) regulation and blood-brain barrier (BBB) transport, leading to the impairment of Aβ clearance to enhance AD pathology.[22,23] 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 (Fig. 3, 4).

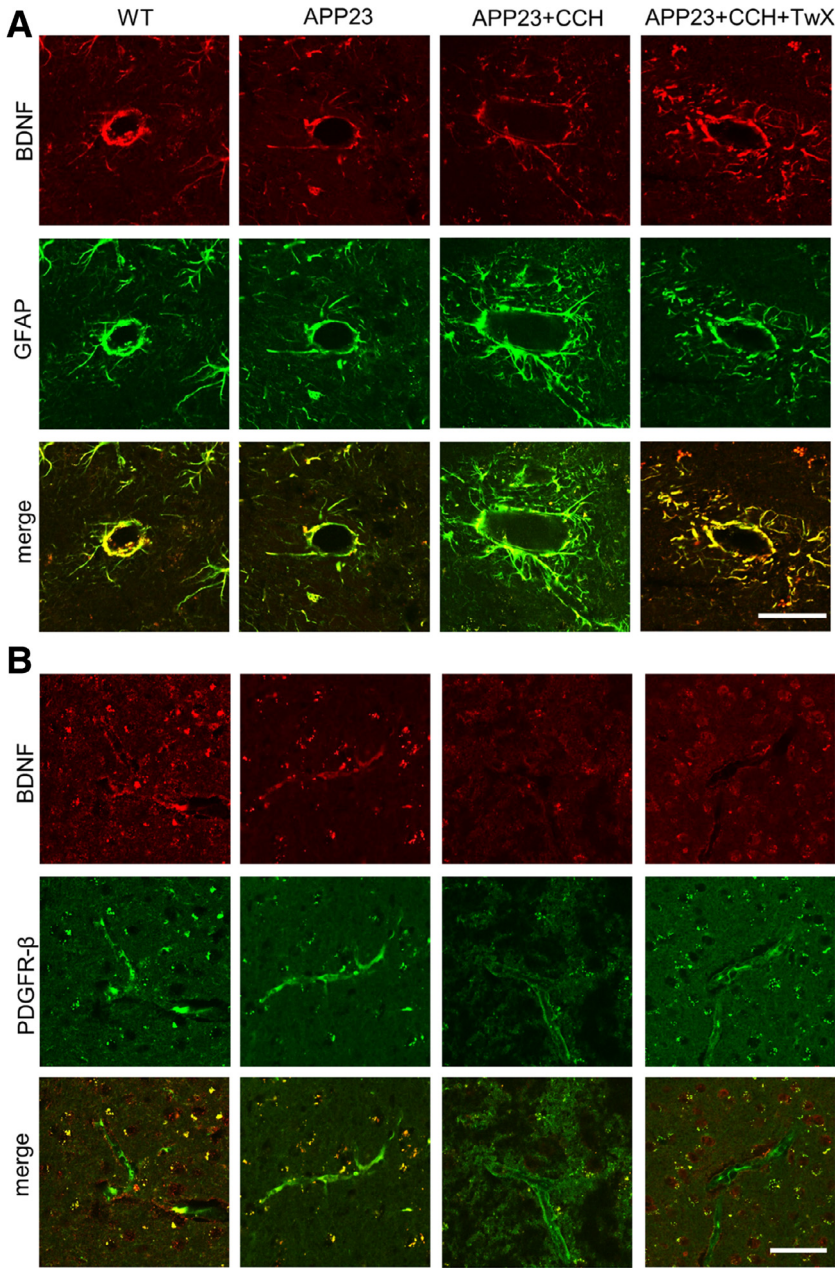

Figure 6. *Double immunofluorescent analysis of BDNF plus GFAP (A) or PDGFRβ (B) in CTX. Note the significantly decreased BDNF immunoreactivity in astrocytes and pericytes of 3 APP23 groups, especially in APP23 + CCH group (Scale bar = 50 μm). Color version of figure is available online.*

BDNF is a member of the neurotrophin family,[24] which plays important role in neuronal growth and survival as well as synaptic plasticity in both animal models and AD patients.[25,26] Therefore, the reduction of BDNF expression of AD and MCI patients is related to cognitive impairment.[27-30] The present results indicated that CCH decreased the BDNF expression in endothelium cells, neuronal cells, astrocytes, and pericytes (Fig. 5, 6), which was recovered by TwX to protect against CCH injury on neurovascular coupling in APP23 mice (Fig. 5, 6).

In conclusion, the present study newly revealed that CCH enhanced tau hyperphosphorylation and phospho-α-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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