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Letter to the Editor

Banxia Xiexin decoction ameliorated cognition via the regulation of insulin pathways and glucose transporters in the hippocampus of APPswe/PS1dE9 mice

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Abstract

Reduced glucose utilization and deficient energy metabolism that occur in the early stages of Alzheimer's disease correlate with impaired cognition, and this information is evidence that Alzheimer's disease is a metabolic disease that is associated with brain insulin/insulin-like growth factor resistance. This research aimed to investigate the effects of Banxia Xiexin decoction (BXD) on cognitive deficits in APPswe/PS1dE9 double transgenic mice and verify the hypothesis that BXD treatment improves cognitive function via improving insulin signalling, glucose metabolism and synaptic plasticity in the hippocampus of APPswe/PS1dE9 double transgenic mice. We used 3-month-old APPswe/PS1dE9 double transgenic mice as the case groups and wild-type littermates of the double transgenic mice from the same colony as the control group. Forty-five APPswe/PS1dE9 double transgenic mice were randomly divided into the model group, donepezil group and BXD group. The mice in the control and model groups were administered 0.5% carboxymethyl cellulose orally. The Morris water maze and step-down test were conducted to evaluate the cognitive performance of APPswe/PS1dE9 double transgenic mice after BXD treatment. Ultrastructure of synapses was observed in the hippocampal CA1 area. Proteins involved in insulin signalling pathways and glucose transports in the hippocampus were assessed through immunohistochemical staining and western blot. After 3 months intervention, we found that BXD treatment improved cognitive performance and the synaptic quantity and ultrastructure, restored insulin signalling and increased the expression of glucose transporter 1 (GLUT1) and GLUT3 levels. These findings suggest that the beneficial effect of BXD on cognition may be due to the improvement of insulin signalling, glucose metabolism and synaptic plasticity.

Keywords

Alzheimer's disease, cognition, glucose metabolism, insulin signalling, synapse

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Introduction

It has been reported that reduced glucose utilization and deficient energy metabolism occurred in the early stages of alzheimer's disease (AD) and correlated with impaired cognition. These evidence supports that Alzheimer's disease is a metabolic disease which is associated with brain insulin/insulin-like growth factor (IGF) resistance.[1] Deficit in cerebral glucose utilization and insulin and IGF signalling, which correlates with cognitive impairment, were observed in AD patients and models.[2] PI3K/Akt pathway is a primary mediator for the metabolic response to insulin and IGF.[3] Moreover, previous researches have shown that cognitive function was improved by restoring the PI3K/Akt pathway.[4–6] Glucose utilization is not only regulated by insulin signalling but also depends on glucose transporters (GLUTs). GLUT1 and GLUT3, the two major brain GLUTs that are responsible for glucose uptake into the neurons, were decreased in AD brain.[7] It was demonstrated that decreased GLUT1 and GLUT3 contributed to diminished brain glucose uptake and correlated to the cognitive deficit in AD.[8]

However, to date, an effective therapy that prevents or delays progression of AD is not available. Traditional Chinese medicine (TCM) has been proven to be effective and safe for AD treatment. 'Dementia Treated by Resolving Phlegm' was written in Shi Shi Mi Lu during the Qing Dynasty. Banxia Xiexin decoction (BXD), a TCM written in Shang Han Lun, has the effect of resolving phlegm, strengthening spleen, clearing heat and drying dampness. Therefore, BXD has been chosen to treat AD patients in the clinical practice with good effects. However, the underlying mechanism is still unknown. We have found that the individual herbs used in BXD had several neuroprotective effects through the activation of the PI3K/Akt pathway.[9–11] Therefore, we hypothesized that BXD might play a neuroprotective role through the activation of the PI3K/Akt pathway. In addition, synapses are the structural basis of neural connection and cognitive function.[12] This study aimed to investigate the therapeutic effects of BXD in APPswe/PS1dE9 double transgenic mice and illustrate its underlying mechanism via improving insulin signalling, glucose metabolism and synaptic plasticity.

Materials and methods

Animals

Three-month-old male APPswe/PS1dE9 double transgenic mice and wild-type (WT) littermates of the double transgenic mice from the same colony were included in the experiment. These mice were purchased from the Institute of Laboratory Animal Science of the Chinese Academy of Medical Sciences (SCXK (Beijing) 2009-0004) and raised in the Barrier Environment Animal Lab of the Key Laboratory of Pharmacology of Dongzhimen Hospital, affiliated with Beijing University of Chinese Medicine (SYXK (Beijing) 2009-0028). APPswe/PS1dE9 double transgenic mice were randomly divided into the model group (TG), donepezil (DNP) group and BXD group. Fifteen APPswe/PS1dE9 double transgenic mice were included in each group and 15 WT littermates in the control group (WT). The experiment was performed according to P.R. China legislation for the use and care of laboratory animals. The protocol was approved by the Ethics Committee of Dongzhimen Hospital affiliated to Beijing University of Chinese Medicine (BUCM), Beijing, China.

Drug preparation and treatment

Donepezil was obtained from Health Materials Pharmaceutical Co., Ltd., China (Cat. No. 140635). Donepezil, was used as a positive control group in our research, has been shown to improve the cognitive function associated with AD, which can also regulate the PI3K/Akt pathway. BXD is composed of *Pinellia ternata* (9g), *Radix scutellariae* (6g), Rhizoma coptidis (3g), Rhizoma zingiberis (6g), ginseng (6g), Radix Glycyrrhizae Preparata (6g) and Fructus jujubae (9g). BXD was processed into boil-free granules by Beijing Dongzhimen Hospital. All herbs were obtained from the original sources and extracted according to the standards listed in the National Pharmacopoeia of China. All materials were identified by Beijing Dongzhimen Hospital, the First Affiliated Hospital of Beijing University of Chinese Medicine (BUCM). Voucher specimens were deposited in the Beijing Dongzhimen Hospital. The decoction was made by boiling the mixture twice in distilled water at 100°C for 30 min. The drug solution was then cooled and dried into granules. The granule was

dissolved in 0.5% sodium carboxymethyl cellulose (CMC) and gavaged to the mice at 650 mg/kg/day body weight for 3 months. Donepezil was dissolved in 0.5% CMC and gavaged to the mice at 0.92 mg/kg/day body weight for 3 months. An equivalent amount of 0.5% CMC was gavaged to the mice in the control and model groups.

Morris water maze test

Morris water maze (MWM) test was performed to assess spatial learning and memory. It includes a water pool, platform and recording system. The circular pool was 120 cm in diameter and a transparent platform was hidden under water. A video tracking system (Shanghai Frameshift Digital Co., Ltd, China) was placed above the centre of the pool. The pool was filled with water made opaque with milk. The depth of the water was 1 cm above the platform. The pool was divided into four equal quadrants, and each quadrant was marked by a different visual cue. The platform was placed in the same location during the experiment. All animals were tested for five consecutive days. Each mouse was placed in the water by hand facing the pool wall at one of the four quadrants and was given 120 s to find the submerged platform. They were allowed to rest for 10 s until they reached the platform. If the mice failed to reach the platform within 120 s, they were gently guided to the platform and kept rested for 10 s to reinforce their memory. The escape latency (time needed to locate the submerged platform) was recorded for each trial. If a mouse was not able to locate the platform within 120 s, the escape latency in these cases was recorded as 120 s. On the sixth day, a post-training probe trial was conducted with the platform removed. Each mouse was released into the water from one of the four quadrants and allowed to swim for 120 s. The time spent in the platform quadrant was recorded.

Step-down test

The training apparatus is a plastic box divided into five rooms (each room is 120 × 120 × 180 mm in size), and the floor of which was made of parallel 0.1-cm-calibre stainless steel bars, which is spaced 0.5 cm apart. An insulated platform (diameter: 5 cm; height: 6 cm) was placed in each room. On the first day of training, mice were allowed to have a 3-min learning course, during which they were permitted to move freely through the chamber and then were placed on the platform. If the animals stepped down from the platform, they were punished through an electric foot shock (36 V, AC). After 24 h, the mice were placed on the platform, and the latency to step down was recorded. This method was used to measure memory retention.

Transmission electron microscopy

Three mice from each group were used as specimens for electron microscopy. The hippocampus was immediately dissociated in an ice bath, and the hippocampal CA1 area was quickly removed and fixed in 2.5% glutaraldehyde liquid at 4°C for 2 h. Next, the specimens were fixed with 1% osmium tetroxide for 2 h. After the specimens were washed with buffer and dehydrated in a graded series of acetone (50%, 70%, 90% and 100%), samples were embedded in Epon812 resin at room temperature for 24 h. The polymerization was carried out at 60°C for 48 h. Samples were sectioned (50 nm) for electron microscopy. Some of the ultra-thin sections were stained with saturated uranyl acetate and lead citrate using standard procedures. Samples were viewed and photographed using a HITACHI H-7650 transmission electron microscope (HITACHI, Japan).

Immunohistochemical staining and quantification

Six mice from each group were anaesthetized with 4% chloral hydrate. After cutting the left ventricular apex, we inserted a catheter and cut the right auricle at the same time. Normal saline (NS) was injected directly into the heart until a clear fluid flowed from the right ventricle. Subsequently, we injected 4% paraformaldehyde (PFA) into the heart until the liver and limbs were hardened.

Fixed brains were sliced from the coronal section at a thickness of 4 μm when the hippocampus appeared. Briefly, the slices were warmed at 56°C for 2 h and then were dewaxed in dimethylbenzene and hydrated in a graded series of alcohol (100%, 95%, 80% and 70%). Subsequently, sections were incubated in 3% H_2O_2 for 15 min and then were microwave treated for antigen retrieval for 5 min. Tissues were incubated with primary antibodies (the PI3K antibody rabbit anti-mouse, Cat. No. ab74136, diluted 1:100 and the Akt antibody rabbit anti-mouse, Cat. No. ab8805, diluted 1:1000) in

humidified boxes at 4°C overnight. In the next day, slices were incubated with biotin-conjugated secondary antibodies (ZSGQ-BIO, Beijing, China). DAB was performed to detect the expression of PI3K and Akt proteins in the hippocampal CA1 area. Negative controls using rabbit serum instead of primary antibodies were included in each test to verify the specificity of antibodies.

Six sections per mouse were used for analysis. The numbers of positively stained neurons in the hippocampal CA1 area were counted at ×20 magnification. Photographs were taken using Motic Med 6.0 Image software and analysed with Image-pro Plus.

Western blot

Six mice from each group were involved in western blot. We used protease inhibitors, but not phosphatase inhibitors and sonication, in the process of protein extraction. 5× SDS-PAGE loading buffer was used. The samples of protein were denatured by boiling for 5 min and centrifuged. Then, an equal amount 40 μg of total protein was separated through SDS-PAGE at a constant voltage of 90 V of electrophoresis. The constant voltage would be adjusted to 120 V once the samples reached the junction between stacking and separating gel. Next, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After 2 h of transferring at 200 mA current, the samples were blocked with 5% skimmed milk for an hour at room temperature and incubated with the PI3K (rabbit anti-mouse, Cat. No. ab74136, diluted 1:1000), Akt (rabbit anti-mouse, Cat. No. ab8805, diluted 1:500), p^{ser473}-Akt (rabbit anti-mouse, Cat. No. Cst4060s, diluted 1:1000), Glut1 (rabbit anti-mouse, Cat. No. ab652, diluted 1:1000), Glut3 (rabbit anti-mouse, Cat. No. ab41525, diluted 1:6000) and β-actin (mouse anti-mouse, Cat. No. ab6276, diluted 1:5000) antibodies overnight at 4°C. After the membranes were washed three times with PBST, the secondary antibody was added. The samples were then incubated for an hour. Enhanced chemiluminescence was used, and the X-ray films were exposed. The protein bands were analysed using ImageJ software.

Statistical analysis

Analyses were performed with SPSS 20.0. All data were presented as the mean ± standard deviation (SD). A P value of <0.05 was considered statistically significant. If a normality test was performed to show that data were normally distributed ($P > 0.05$) and variances were equal, data from more than two groups were analysed with a one-way analysis of variance (ANOVA) test. The least significant difference (LSD) test was then chosen for post hoc multiple comparisons. If not, non-parametric test Kruskal–Wallis test were performed.

Results

BXD improved the spatial learning and memory and memory retention of APPswe/PS1dE9 double transgenic mice

Based on the MWM test, the escape latency of the model group was longer compared to that of the control group (days 1–2, $P < 0.01$; days 3–5, $P < 0.01$) (Figure 1(a)). The DNP group has a shorter escape latency compared with the model group (days 3–4, $P < 0.01$; day 5, $P < 0.05$) (Figure 1(a)). The escape latency of the BXD group was shorter than that of the model group (days 3–4, $P < 0.05$) (Figure 1(a)).

As shown in Figure 1(b), the swimming distance of the mice in the model group was significantly increased compared with that of the control group (days 1–5, $P < 0.01$). Compared with the model group, the swimming distance of the mice in the BXD group was significantly decreased (days 2–3, $P < 0.05$; day 5, $P < 0.05$) as well as the DNP group (days 2–4, $P < 0.05$).

In the memory retention test, the mice in model group spent significantly less time in the target quadrant than those of the control group ($P < 0.01$, Figure 1(c)). The residence time in the target quadrant of the mice in the BXD group was longer than that of the model group ($P < 0.05$, Figure 1(c)).

In the step-down test, the latency of step-down was significantly shorter in the model group compared to that of the control group ($P < 0.01$, Figure 1(d)). After BXD treatment, a significant improvement in latency time was observed in the BXD group compared to the model group ($P < 0.01$, Figure 1(d)).

BXD improved the ultrastructure of synapses in the APPswe/PS1dE9 double transgenic mice

As shown in Figure 2(a), in the control group, the synapses appeared normal with a vivid presynaptic

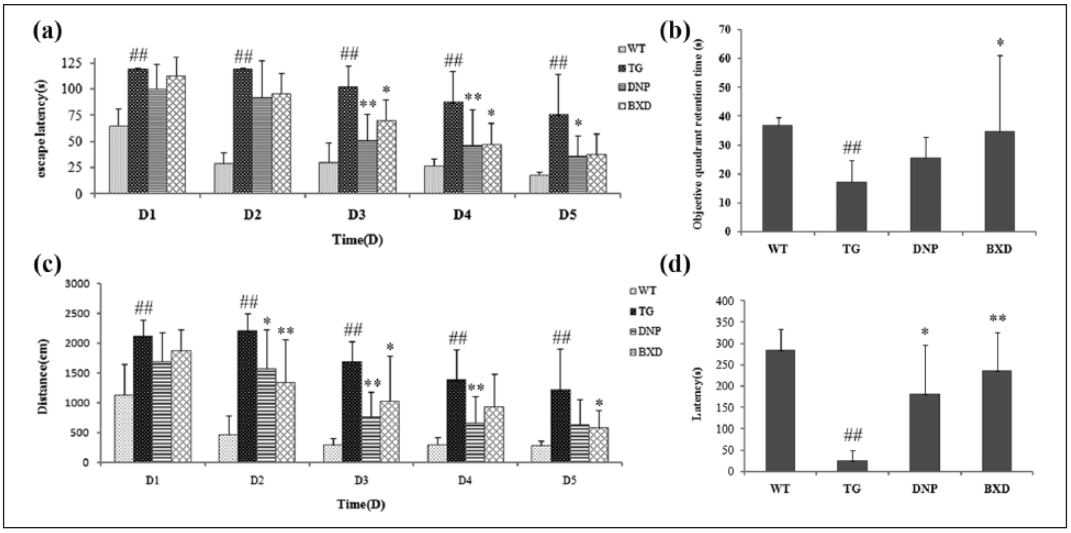

Figure 1. Effect of BXD on APPswe/PS1dE9 double transgenic mice in the Morris water maze test and step-down test. (a) Escape latency of MWM test. Graphs show mean time ± SD taken to reach the platform (escape latency) for days 1 through 5. The escape latency of the BXD group was shorter than that of the model group on day 3 and day 4. (b) Swimming distance. The swimming distance of the mice in the BXD group was significantly decreased on days 2–3 and day 5. (c) Objective quadrant retention time. The residence time in the target quadrant of the mice in the BXD group was longer than that of the model group. (d) Latency of step-down test. The latency of step-down was significantly longer in the BXD group compared to that of the model group. Data are represented as means ± SD. $^{\#}P < 0.05$ versus the WT group, $^{\#\#}P < 0.01$ versus the WT group, $^{*}P < 0.05$ versus the TG group, $^{**}P < 0.01$ versus the TG group.

and postsynaptic membrane and synaptic cleft. Synaptic vesicles were aligned and amassed inside the presynaptic membrane that is close to the synaptic cleft. In the model group, synaptic vesicles with different shapes and sizes could be observed in the presynaptic membrane. The synapse in the DNP group was almost normalized. In the BXD group, synapses were improved. The synaptic cleft was clear, and the synaptic vesicles were in good condition.

We analysed the five fields of the CA1 area in one mouse that was randomly chosen from each group under the electron microscope at ×15,000 magnifications. The number of the synapses in the model group was significantly lower than that of the control group. Compared with the model group, the number of the synapses in the BXD group and DNP group were higher (Table 1).

BXD restored the PI3K/Akt signalling pathway in the hippocampus of APPswe/PS1dE9 double transgenic mice

We performed immunohistochemical staining, and slightly stained and sparse cells were observed in the hippocampus of the mice in the model group. Compared with the model group, positive cells in the DNP group and BXD group were densely distributed (Figure 2(b) and (c)). Quantification showed decreased PI3K-stained cells and Akt-stained cells in the model group compared with the control group ($P < 0.01$, Figure 2(b); $P < 0.01$, Figure 2(c)). PI3K-stained cells and Akt-stained cells in the DNP and BXD groups were significantly more than those in the model group ($P < 0.01$, Figure 2(b); $P < 0.01$, Figure 2(c)).

Insulin activates the insulin receptor (IR) followed by the activation of the insulin receptor substrate (IRS), phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT). The results of western blot analysis showed that a significant down-regulation of PI3K, Akt and p^{ser473}-Akt ($P < 0.05$, Figure 3(a)–(c)) in the hippocampus of the mice in the model group compared with the control group. After BXD treatment, the expression of PI3K, Akt and p^{ser473}-Akt was elevated compared with the model group ($P < 0.05$, Figure 3(a)–(c)).

BXD increased the expression of GLUT 1 and GLUT 3 in the hippocampus of APPswe/PS1dE9 double transgenic mice

Western blot showed that the level of proteins GLUT 1 and GLUT 3 was significantly reduced in the model group compared with that of the control

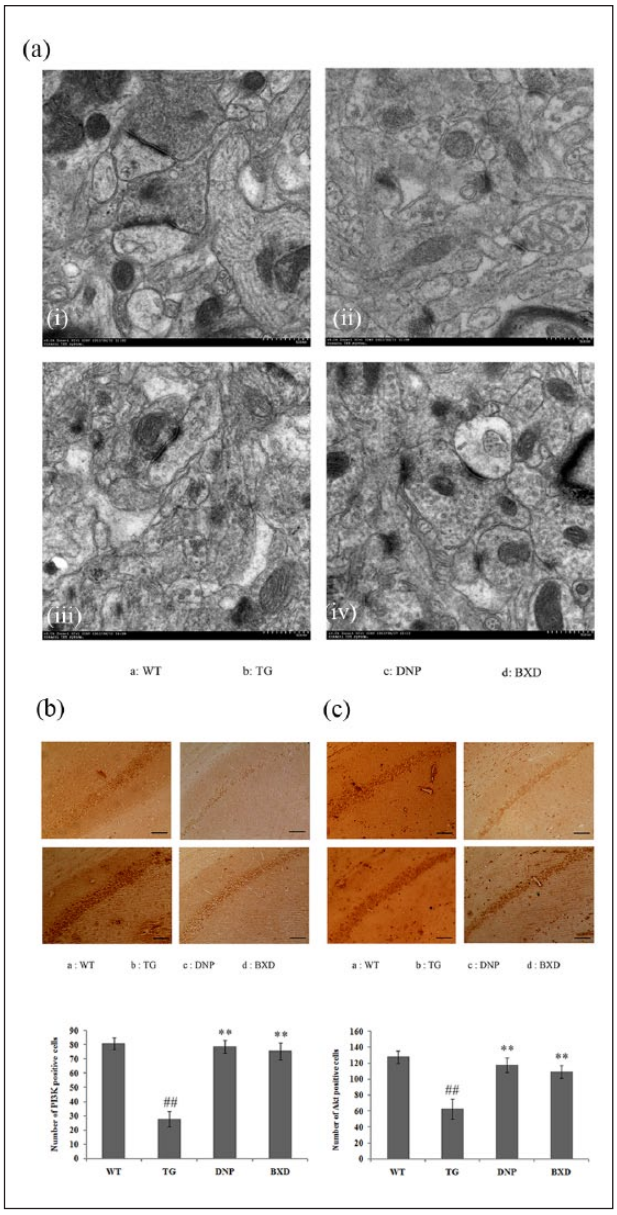

Figure 2. (a) Ultrastructure of synapses in the APPswe/PS1dE9 double transgenic mice: (i) the synapses in the control group, appeared normal with a vivid presynaptic and postsynaptic membrane and synaptic cleft, (ii) in the model group, synaptic vesicles with different shapes and sizes could be observed in the presynaptic membrane, (iii) the synapse in the DNP group was almost normalized and (iv) in the BXD group, synapses were improved. (b) Immunohistochemical staining of PI3K. Compared with the model group, PI3K-stained cells in the DNP and BXD groups were significantly more than those in the model group. Data are represented as means ± SD. #$P < 0.05$ versus the WT group, ##$P < 0.01$ versus the WT group, *$P < 0.05$ versus the TG group, **$P < 0.01$ versus the TG group. (c) Immunohistochemical staining of Akt. Compared with the model group, Akt-stained cells in the DNP and BXD groups were significantly more than those in the model group. Data are represented as means ± SD. #$P < 0.05$ versus the WT group, ##$P < 0.01$ versus the WT group, *$P < 0.05$ versus the TG group, **$P < 0.01$ versus the TG group.

group ($P < 0.01$, Figure 3(d) and (e)). After treatment, the expression of GLUT 1 and GLUT 3 in the BXD group was increased ($P < 0.01$, Figure 3(d) and (e)), as well as the DNP group ($P < 0.01$, Figure 3(d) and (e)).

Discussion

BXD has been used to treat patients with AD, and positive effects were observed in clinical practice. In our research, the mice treated with BXD had a lower latency in reaching the hidden platform and shorter swimming distance, suggesting that BXD improved the spatial learning and memory ability of APPswe/PS1dE9 mice. Furthermore, the step-down latency of the mice was significantly longer in the BXD group than that of the model group. These findings indicate that BXD treatment can improve the spatial learning and memory and memory retention of APPswe/PS1dE9 mice. However, the molecular mechanism of BXD in improving cognition is still unknown.

The impaired insulin signalling in the brain of mice led to brain pathology and cognitive deficits that resemble changes found in patients with sporadic AD.[2] PI3K/Akt pathway, primarily a mediator for the metabolic response to insulin.[3] It was reported that markedly diminished responses to the PI3K/Akt signalling pathway in the hippocampal formation in AD cases.[13] Moreover, previous researches have shown that cognitive function was improved by restoring the PI3K/Akt pathway.[4–6] Treatment with BXD increased the levels of PI3K, Akt and p-Akt. These results suggested that BXD may improve cognitive impairment by promoting the recovery of insulin signal transduction in the mice brain. Moreover, individual herbs used in BXD have neuroprotective effects through the activation of the PI3K/Akt pathway. For example, Ginsenoside Rb1 from ginseng,[10] Berberine in coptis.[11] These compounds may contribute to the effects of BXD in neuroprotection.

Decreased GLUT1 and GLUT3 in the AD brain correlated to the cognitive deficit.[7,8] In our study, the levels of both GLUT1 and GLUT3 were significantly decreased in the model group. The expressions of GLUT1 and GLUT3 were significantly increased after BXD treatment. Our data suggest that the beneficial effect of BXD on cognition may be through the mediation of GLUT1 and GLUT3 expressions.

Table 1. Synapses counting in different groups by transmission electron microscopy (scale bars = 1.5 μm).

Group	Field of vision					Mean
	1	2	3	4	5	
Cont	30	25	24	26	26	26.2
Mod	10	11	9	12	10	10.4
DNP	24	26	22	23	26	24.2
BXD	23	22	22	19	18	20.8

DNP: donepezil; BXD: Banxia Xiexin decoction.

Significant loss of synapses in hippocampus has been observed in AD, which is strongly associated with cognitive impairment.[12] In our study, BXD treatment improved the quantity and ultrastructure of synapses, which contributed to the neuroprotective effect of BXD.

In summary, the present study showed that BXD treatment improved cognitive performance and the synaptic quantity and ultrastructure, restored insulin signalling and increased GLUT1 and GLUT3

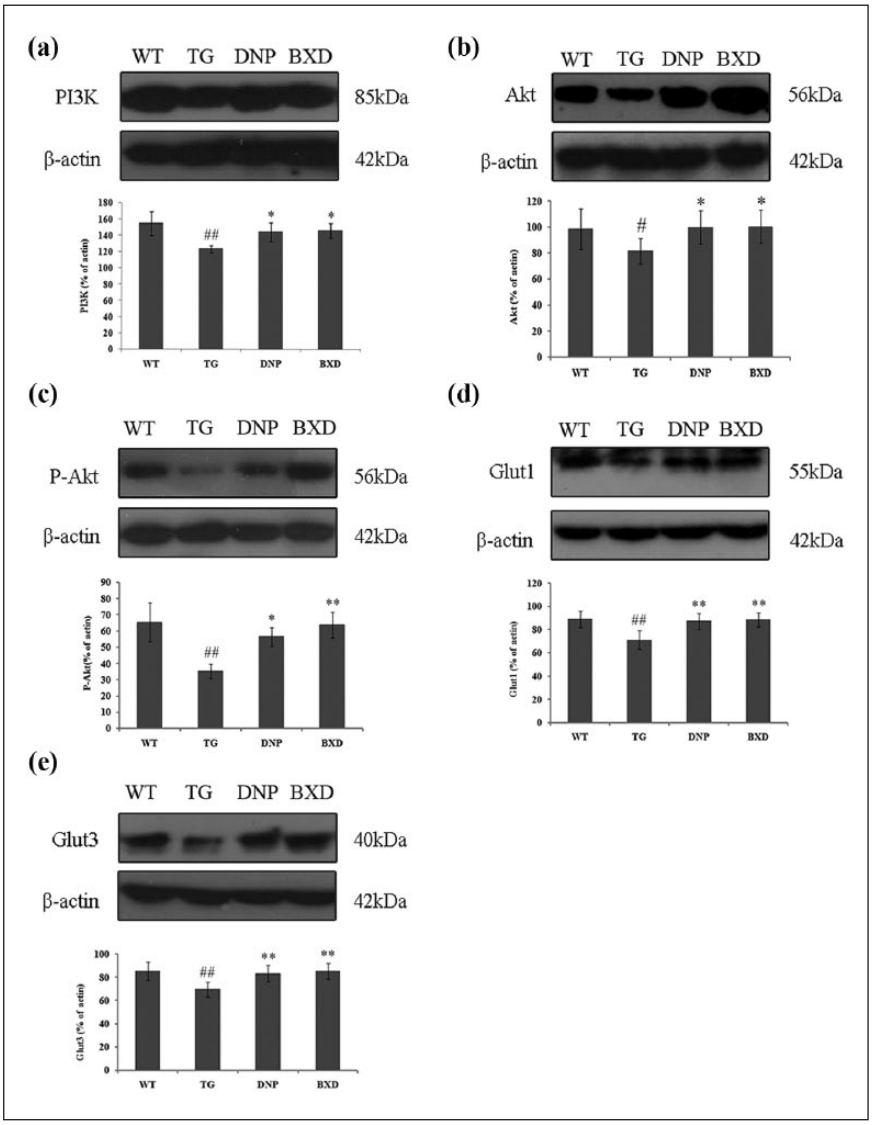

Figure 3. (a)–(c) The expression of PI3K, Akt and p-Akt in the hippocampus of APPswe/PS1dE9 double transgenic mice. Western blot analysis showed that a significant down-regulation of PI3K, Akt and p-Akt in the hippocampus of the mice in the model group compared with the control group. After BXD treatment, the expression of PI3K, Akt and p-Akt was elevated compared with the model group. Data are represented as means ± SD. $^{\#}P < 0.05$ versus the WT group, $^{\#\#}P < 0.01$ versus the WT group, $^{*}P < 0.05$ versus the TG group, $^{**}P < 0.01$ versus the TG group. (d, e) The expression of GLUT 1 and GLUT 3 in the hippocampus of APPswe/PS1dE9 double transgenic mice. Western blot showed that the expression of GLUT 1 and GLUT 3 in the BXD group was increased compared with that of the model group. Data are represented as means ± SD. $^{\#}P < 0.05$ versus the WT group, $^{\#\#}P < 0.01$ versus the WT group, $^{*}P < 0.05$ versus the TG group, $^{**}P < 0.01$ versus the TG group.

levels. These findings suggest that the beneficial effect of BXD on cognition may be due to the improvement of insulin signalling, glucose metabolism and synaptic plasticity.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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