



Research Article

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Scutellaria Barbata Flavonoids Regulate Protein Phosphorylation of Glycogen Synthase Kinase-3 β and Tau in N2a Cells with A β_{25-35} -Induced Neurotoxicity Alzheimers

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease caused by chronic or progressive structural damage of the brain. Neurofibrillary tangles (NFTs) induced by excessive phosphorylation of tau protein have toxicity to neurons and to be one of the main pathogenesis of AD. Then, inhibiting the excessive phosphorylation of Tau protein is the key point to treat AD. While glycogen synthase kinase-3 β (GSK-3 β) plays an important role in causing abnormal hyperphosphorylation of AD-like Tau protein. It has been confirmed that GSK-3 β can catalyze the hyperphosphorylation of Tau protein at several sites. Therefore, we research the regulatory effects of Scutellaria barbata flavonoids (SBF) on β -amyloid₂₅₋₃₅ (A β_{25-35}) induced Tau phosphorylation at Ser202, Ser199, Ser404, Ser214, Thr231 sites and regulatory mechanism by GSK-3 β in mouse brain neuroblastoma cells(N2a). Our results showed that compared with control group, the protein expressive level of p-Tau (Ser202), p-Tau (Ser199), p-Tau (Ser404), p-Tau (Ser214) and p-GSK-3 β (Tyr216) in model group were higher than those of control group ($p < 0.05$, $p < 0.01$). The expression of p-GSK-3 β (Ser9) were markedly decreased ($p < 0.01$) and p-Tau (Thr231) has no obvious change ($p > 0.05$) in model group, as compared with control group. Concerning to the inhibitor TWS119 group, p-Tau (Ser202), p-Tau (Ser199), p-Tau (Thr231) and p-GSK-3 β (Ser9) protein expressive level increased ($p < 0.01$), p-Tau (Ser404), p-Tau (Ser214) protein expression decreased ($p < 0.01$) and p-GSK-3 β (Tyr216) protein expressive level was not obviously, as compared with control group. Compared with model group, p-Tau (Ser202), p-Tau (Ser404), p-Tau (Ser214) and p-GSK-3 β (Tyr216) protein expression level in A β_{25-35} +TWS119 group significantly decreased ($p < 0.01$), p-Tau (Ser199), p-Tau (Thr231), p-GSK-3 β (Ser9) A β_{25-35} + TWS119 set of protein expression levels or have no obvious change. Compared with A β_{25-35} + TWS119 group, each point of protein expression levels the SBF treatment group were decreased ($p < 0.01$ or $p < 0.05$), in addition to p-GSK-3 β (Ser9) protein content increase. Our results indicate that SBF can inhibit the abnormal phosphorylation of Tau protein by regulating the activity of GSK-3 β (Ser9) and decreasing the contents of Ser214 and Ser404 in Tau protein phosphorylation sites. Therefore, inhibiting the activity of GSK-3 β may become one of the feasible and effective methods for the treatment of AD for achieving the goal of neuron protection.

Keywords: Scutellaria barbata flavonoids; A β_{25-35} ; N2a cells; Phosphorylation; Tau protein; GSK-3 β

Abbreviations: SBF: ScutellariaBarbata Flavonoids; N2a: Mouse Neuroblastoma Cells; Sk-3 β : Glycogen Synthase Kinase-3 β ; Ws119: 4,6-Disubstituted Pyrrolopyrimidine; Ad: Alzheimer's Disease; A β : Amyloid Beta; Sp: Senile Plaques; Nfts: Neurofibrillary Tangles; Dmem: Dubecco's Modified Eagle Medium; Tyr216: Tyrosine 216; Ser9: Serine9

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease commonly found in the elderly that has insidious onset and gradually worsens with age [1]. Its clinical symptoms

mainly include memory loss and cognitive dysfunction. AD has increasingly gained attention from medical and pharmaceutical professionals internationally. Research has shown that incidence of AD is mainly caused by extracellular amyloid beta (A β) deposition

in the hippocampus, cerebral cortex, and subcortical tissues to form senile plaques (SP) and by neurofibrillary tangles (NFTs) due to hyperphosphorylation of intracellular Tau protein. SP and NFT pathology are closely connected to each other. SP formation induced by A β deposition is an initiating factor of AD. It also induces or accelerates the intracellular phosphorylation of Tau protein [2], which has attracted more and more attention from researchers. Overexpression of glycogen synthase kinase-3 beta (GSK-3 β) is closely related to the development and progression of AD and significantly increases the expression of phosphorylated Tau protein in cells and animals [3]. A β leads to a loss of dendritic spines in neurons, leading to an increased activity of GSK-3 β ; the change in Tau protein is the secondary effect of A β deposition. Researchers believe that GSK-3 β may be one of the intracellular connections between A β and Tau protein phosphorylation [4]. Experimental verification of this hypothesis may provide a solid theoretical basis for AD prevention and treatment by inhibiting neurotoxicity in the brain caused by Tau protein hyperphosphorylation.

A series of studies in recent years have shown that flavonoids have a good preventive or therapeutic effect on neurodegenerative diseases, and this effect is mostly multi-target, multi-channel, and multi-system with few side effects [5,6]. Scutellaria barbata flavonoids (SBF) are a flavonoid mixture extracted from the aerial part of Scutellaria barbata L. that has multiple effects, such as an anti-inflammatory effect, anti-tumor effect, anti-oxidation effect, and improvement of memory impairment [7]. Previous studies from our group have shown that SBF improves learning and memory impairments, immunity, and endocrine abnormality in ovariectomized rats [8]. It also has a significant protective effect on A β -induced astrocyte damage in vitro [9], significantly inhibits A β aggregate-induced apoptosis of cortical cells [10], improves A β aggregate-induced mitochondrial apoptosis pathway abnormalities in nerve cells [11], and reduces Tau protein phosphorylation by

regulating the activity of related enzymes to inhibit A β aggregate-induced NFT deposition in rat brains [12]. However, the effects of SBF on A β_{25-35} -induced Tau protein phosphorylation and GSK3 β in mouse neuroblastoma N2a cells have not been reported.

N2a cells are mouse neural crest-derived cells from neuroblastoma in the mouse brain. Their biological activities are close to those of nerve cells. They have morphological and biological characteristics of neural stem cells and, thus, have often been used as a substitute for nerve cells for basic research on drugs. In this study, A β_{25-35} was used to induce Tau protein hyperphosphorylation in N2a cells, which were used as an in vitro model to evaluate the effects of SBF on the expression of phosphorylated (p)-Tau (Ser202), p-Tau (Ser199) p-Tau (Ser404), p-Tau (Ser214), p-Tau (Thr231), p-GSK-3 β (Ser9), and p-GSK-3 β (Tyr216) proteins in different groups by Western blot analysis. A GSK-3 β inhibitor, TWS119, was used to confirm that SBF reversed A β_{25-35} -induced Tau protein phosphorylation by affecting GSK-3 β activity. This study provides essential experimental and theoretical bases for the application of SBF in AD prevention and treatment.

Results

Effects of SBF on p-Tau (Ser202) protein expression in N2a cells with A β_{25-35} -induced neurotoxicity

As shown in Figure 1, the p-Tau (Ser202) protein expression of N2a cells was 1.06 times higher in the model group and 1.03 times higher in the TWS119 group ($P < 0.01$) than in the blank control group. Compared with the model group, the p-Tau (Ser202) protein expression was 10.20% lower in the A β_{25-35} +TWS119 group. In addition, compared with the A β_{25-35} +TWS119 group, the p-Tau (Ser202) protein expression of N2a cells was 57.52%, 50.02%, and 65.07% lower in the SBF+A β_{25-35} +TWS119 groups treated with 1.125 mg/L, 2.25 mg/L, and 4.5 mg/L SBF, respectively ($P < 0.01$).

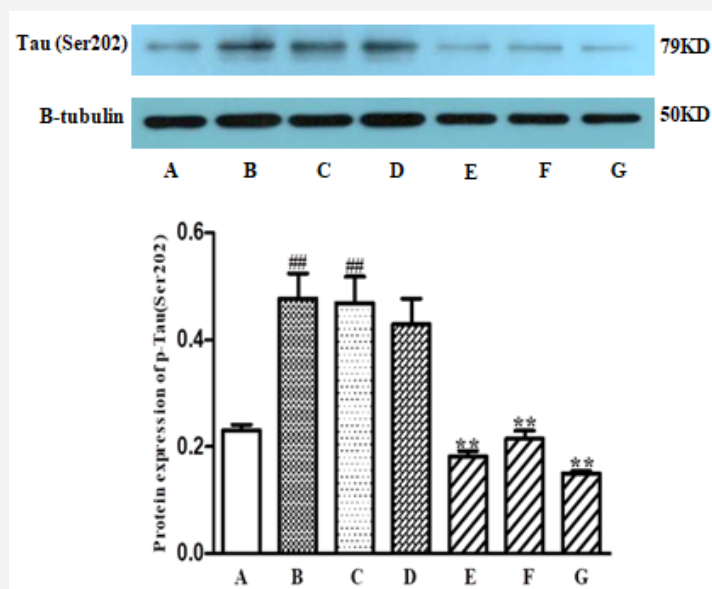


Figure 1: The effect of SBF on the protein expression of p-Tau (Ser202) in the N2a cell by composited A β_{25-35} . A: control group; B: model group; C: WTS119 group; D: A β_{25-35} + TWS119 group; E: SBF (1.125mg/L) + A β_{25-35} + TWS119 group; F: SBF (2.25mg/L) + A β_{25-35} + TWS119 group; G: SBF (4.5mg/L) + A β_{25-35} + TWS119 group. A β_{25-35} . Mean \pm SD. n=3. ### $p < 0.01$ vs control group; ** $p < 0.05$ vs A β_{25-35} + TWS119 group.

Effects of SBF on p-Tau (Ser199) protein expression in N2a cells with Aβ₂₅₋₃₅-induced neurotoxicity

As shown in Figure 2, compared with the blank control group, the p-Tau (Ser199) protein expression of N2a cells was 97.78% higher in the model group ($P < 0.01$) and 1.19 times higher in the TWS119 group ($P < 0.01$). Compared with the model group, the p-Tau

(Ser199) protein expression of N2a cells was 12.10% higher in the Aβ₂₅₋₃₅+TWS119 group. Compared with the Aβ₂₅₋₃₅+TWS119 group, different concentrations of SBF treatment (1.125 mg/L, 2.25 mg/L, and 4.5 mg/L) reduced the p-Tau (Ser199) protein expression of N2a cells by 49.41%, 74.68%, and 92.34%, respectively, in different SBF+Aβ₂₅₋₃₅+TWS119 groups, with a dose-dependent significant difference ($P < 0.01$).

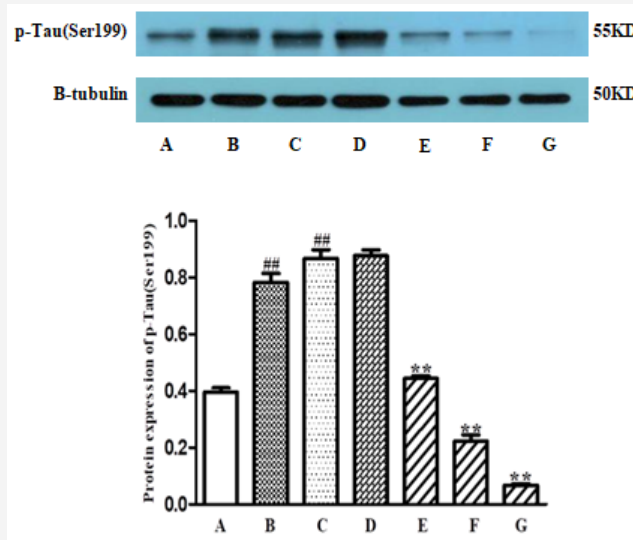


Figure 2: The effect of SBF on the protein expression of p-Tau (Ser199) in the N2a cell by composed. A: control group; B: model group; C: WTS119 group; D: Aβ₂₅₋₃₅ + TWS119 group; E: SBF (1.125mg/L) + Aβ₂₅₋₃₅ + TWS119 group; F: SBF (2.25mg/L) + Aβ₂₅₋₃₅ + TWS119 group; G: SBF (4.5mg/L) + Aβ₂₅₋₃₅ + TWS119 group. Aβ₂₅₋₃₅ Mean ± SD. n =3. ##p<0. 01 vs control group; **p<0. 01 vs Aβ₂₅₋₃₅ + TWS119 group.

Effects of SBF on p-Tau (Ser404) protein expression in N2a cells with Aβ₂₅₋₃₅-induced neurotoxicity

As shown in Figure 3, compared with the blank control group, the p-Tau (Ser404) protein expression of N2a cells was 30.74% higher in the model group ($P < 0.01$) and was 17.14% lower in the TWS119 group ($P < 0.01$). Compared with the model group, the

p-Tau (Ser404) protein expression of N2a cells was 37.22% lower in the Aβ₂₅₋₃₅+TWS119 group. Compared with the Aβ₂₅₋₃₅+TWS119 group, 1.125 mg/L, 2.25 mg/L, and 4.5 mg/L SBF treatments significantly reduced the p-Tau (Ser404) protein expression of N2a cells by 67.54%, 60.22%, and 87.32%, respectively, in different SBF+Aβ₂₅₋₃₅+TWS119 groups ($P < 0.01$).

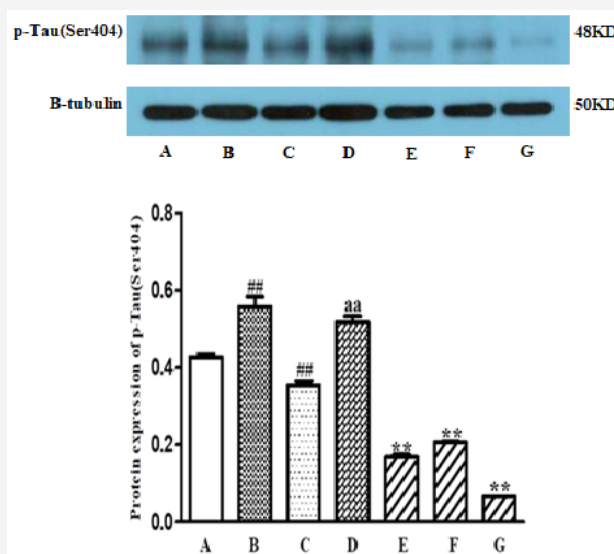


Figure 3: The effect of SBF on the protein expression of p-Tau(Ser404)in the N2a cell by composed Aβ₂₅₋₃₅. A: control group; B: model group; C: WTS119 group; D: Aβ₂₅₋₃₅ + TWS119 group; E: SBF (1.125mg/L) + Aβ₂₅₋₃₅ + TWS119 group; F: SBF(2.25mg/L)+ Aβ₂₅₋₃₅ + TWS119 group; G: SBF(4.5mg/L)+ Aβ₂₅₋₃₅ + TWS119 group. Mean ± SD. n =3. ##p<0.01 vs control group; ##p<0.01 vs model group; **p<0. 01 vs Aβ₂₅₋₃₅ + TWS119 group.

Effects of SBF on p-Tau (Ser214) protein expression in N2a cells with A β_{25-35} -induced neurotoxicity

As shown in Figure 4, compared with the blank control, the p-Tau (Ser214) protein expression of the N2a cells was 8.35% higher ($P < 0.01$) in the model group and was 28.31% lower ($P < 0.01$) in the TWS119 group. Compared with the model group, the p-Tau (Ser214)

protein expression of N2a cells was 6.36% lower in the A β_{25-35} +TWS119 group ($P < 0.01$). Compared with the A β_{25-35} +TWS119 group, 1.125 mg/L, 2.25 mg/L, and 4.5 mg/L SBF treatments significantly reduced the p-Tau (Ser214) protein expression of N2a cells by 39.50%, 40.83%, and 40.44%, respectively, in different SBF+A β_{25-35} +TWS119 groups ($P < 0.01$).

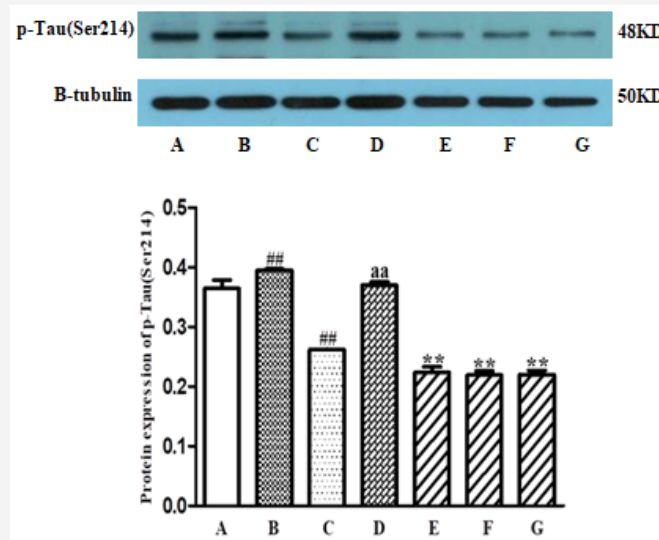


Figure 4: The effect of SBF on the protein expression of p-Tau (Ser214) in the N2a cell by composited A β_{25-35} . A: control group; B: model group; C: WTS119 group; D: A β_{25-35} + TWS119 group; E: SBF (1.125mg/L) + A β_{25-35} + TWS119 group; F: SBF(2.25mg/L)+ A β_{25-35} + TWS119 group; G: SBF(4.5mg/L)+ A β_{25-35} + TWS119 group. Mean \pm SD. n=3. ## $p < 0.01$ vs control group; aa $p < 0.01$ vs model group; ** $p < 0.01$ vs A β_{25-35} + TWS119 group.

Effects of SBF on p-Tau (Thr231) protein expression in N2a cells with A β_{25-35} -induced neurotoxicity

As shown in Figure 5, the p-Tau (Thr231) protein expression of the N2a cells showed no significant difference between the blank control and the model groups but was 37.10% higher in the TWS119 group than in the blank control group ($P < 0.01$). Compared with

the model group, the p-Tau (Thr231) protein expression of the N2a cells was significantly higher in the A β_{25-35} +TWS119 group ($P < 0.01$). Compared with the A β_{25-35} +TWS119 group, 1.125 mg/L, 2.25 mg/L, and 4.5 mg/L SBF treatments significantly reduced the p-Tau (Thr231) protein expression of N2a cells by 67.78%, 42.63%, and 71.86%, respectively, in different SBF+A β_{25-35} +TWS119 groups ($P < 0.01$).

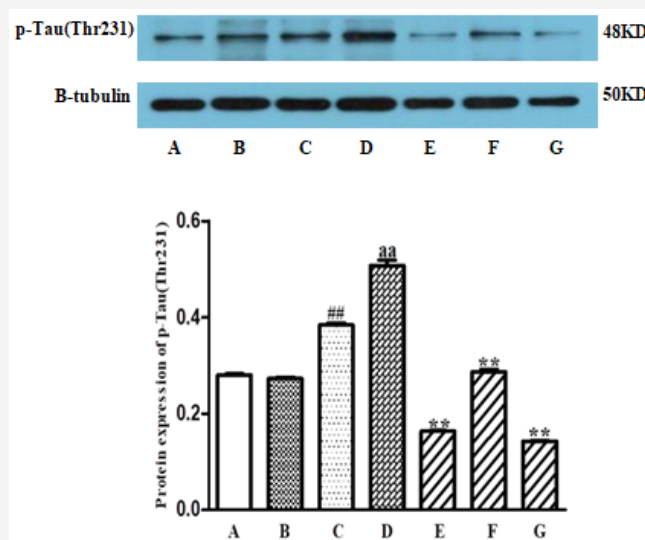


Figure 5: The effect of SBF on the protein expression of p-Tau(Thr231) in the N2a cell by composited A β_{25-35} . A: control group; B: model group; C: WTS119 group; D: A β_{25-35} + TWS119 group; E: SBF (1.125mg/L) + A β_{25-35} + TWS119 group; F: SBF(2.25mg/L)+ A β_{25-35} + TWS119 group; G: SBF(4.5mg/L)+ A β_{25-35} + TWS119 group. Mean \pm SD. n=3. ## $p < 0.01$ vs control group; aa $p < 0.01$ vs model group; ** $p < 0.01$ vs A β_{25-35} + TWS119 group.

Effects of SBF on p-GSK-3β (Ser9) protein expression in N2a cells with Aβ₂₅₋₃₅-induced neurotoxicity

As shown in Figure 6, compared with the blank control group, the p-GSK-3β (Ser9) protein expression of N2a cells was 43.29% lower in the model group (P < 0.01). GSK-3β (Ser9) negatively regulated GSK-3β, which was activated to increase the phosphorylation of Tau protein. In addition, the p-GSK-3β (Ser9) protein expression

in N2a cells was 28.31% higher in the TWS119 group, suggesting that GSK-3β was significantly inhibited. Compared with the model group, the p-GSK-3β (Ser9) protein expression of the N2a cells was 44.25% higher in the Aβ₂₅₋₃₅+TWS119 group (P < 0.01). Compared with the Aβ₂₅₋₃₅+TWS119 group, 1.125 mg/L, 2.25 mg/L, and 4.5 mg/L SBF treatments significantly reduced the p-GSK-3β (Ser9) protein expression of N2a cells by 40.31%, 46.61%, and 25.09%, respectively, in different SBF+Aβ₂₅₋₃₅+TWS119 groups (P < 0.01).

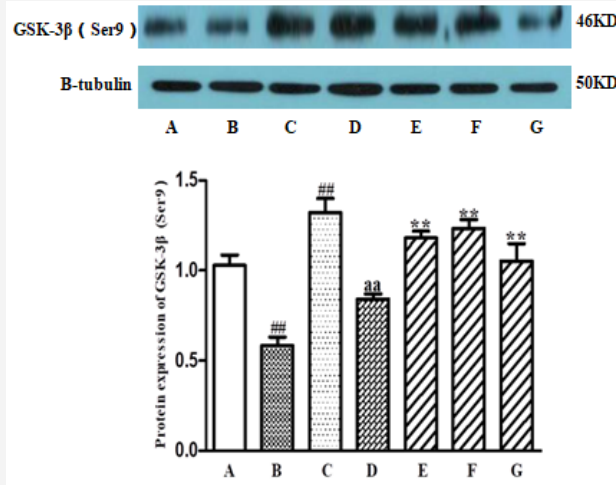


Figure 6: The effect of SBF on the protein expression of GSK-3β(Ser9) in the N2a cell by composited Aβ₂₅₋₃₅. A: control group; B: model group; C: WTS119 group; D: Aβ₂₅₋₃₅+ TWS119 group; E: SBF (1.125mg/L)+ Aβ₂₅₋₃₅+ TWS119 group; F: SBF(2.25mg/L)+ Aβ₂₅₋₃₅+ TWS119 group; G: SBF(4.5mg/L)+ Aβ₂₅₋₃₅+ TWS119 group. Mean ± SD. n=3. ##p<0.01 vs control group; ^{aa}p<0.01 vs model group; **p<0.01 vs Aβ₂₅₋₃₅+ TWS119 group.

Effects of SBF on p-GSK-3β (Tyr216) protein expression in N2a cells with Aβ₂₅₋₃₅-induced neurotoxicity

As shown in Figure 7, compared with the blank control group, the p-GSK-3β (Tyr216) protein expression of N2a cells was 35.02% higher in the model group (P < 0.05). GSK-3β (Tyr216) positively regulated GSK-3β, which was activated in the model group and increased the phosphorylation of the Tau protein. However, the p-GSK-3β (Tyr216) protein expression in N2a cells showed no

significant change in the TWS119 group. Compared with the model group, the p-GSK-3β (Tyr216) protein expression in N2a cells was 22.41% lower in the Aβ₂₅₋₃₅+TWS119 group (P < 0.01). Compared with the Aβ₂₅₋₃₅+TWS119 group, 1.125 mg/L, 2.25 mg/L, and 4.5 mg/L SBF treatments significantly reduced the p-GSK-3β (Tyr216) protein expression of N2a cells by 28.78%, 40.26%, and 33.50%, respectively, in different SBF+Aβ₂₅₋₃₅+TWS119 groups (P < 0.01, P < 0.05).

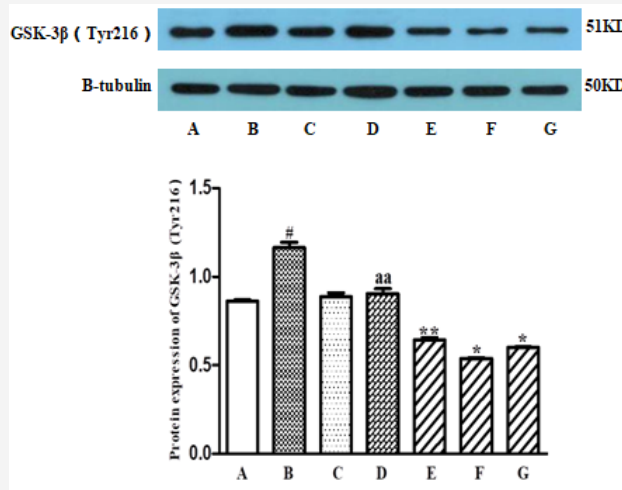


Figure 7: The effect of SBF on the protein expression of GSK-3β(Tyr216) in the N2a cell by composited Aβ₂₅₋₃₅. A: control group; B: model group; C: WTS119 group; D: Aβ₂₅₋₃₅+ TWS119 group; E: SBF (1.125mg/L)+ Aβ₂₅₋₃₅+ TWS119 group; F: SBF(2.25mg/L)+ Aβ₂₅₋₃₅+ TWS119 group; G: SBF(4.5mg/L)+ Aβ₂₅₋₃₅+ TWS119 group. Mean ± SD. n=3. #p<0.05 vs control group; ^{aap}p<0.01 vs model group; **p<0.01, *p<0.05 vs Aβ₂₅₋₃₅+ TWS119 group.

Discussion

Aging is a major risk factor for the onset of AD. With the progression of global aging, the number of AD patients has increased in recent years. AD has become one of the most disabling and economically burdensome diseases in the world [13]. A recent study has shown that abnormally phosphorylated Tau protein acts as a mediator of A β toxicity, playing a key role in neuron damage and leading to neuronal death and dysfunction [14]. Some clinical studies have shown that the severity of AD is positively correlated with the number of NFTs in the brain of the patient. Abnormal phosphorylation of Tau protein is the leading cause of neuronal death and degeneration [15]. Tau protein is the most highly distributed microtubule-associated protein in neuronal axons and plays an important role in microtubule assembly, microtubule stabilization, and apoptosis inhibition [16]. A study has shown that when Tau protein is abnormally phosphorylated, its binding affinity is reduced, resulting in microtubule instability, disrupting the axonal transport system, and ultimately leading to the formation of intracellular NFTs, which spread to different brain regions, affecting neurotrophic substance transport in brain cells, causing toxicity to neuronal synapses and spinous processes, and promoting neuronal death [17].

A β deposition is a predisposing factor for abnormal phosphorylation of Tau protein. Numerous studies have shown that A β_{25-35} aggregation significantly increases the phosphorylation of Tau protein in Ser202, Ser199, Ser404, Ser214, Thr231, Ser396, and Ser262 sites [18,19]. A β can activate three protein kinases, GSK-3 β , mitogen-activated protein kinase, and cyclin-dependent kinase 5 (CDK5) to cause hyperphosphorylation of the downstream substrate, Tau protein [20]. GSK3 β is a highly conserved multifunctional protein serine/threonine phosphokinase and the strongest protein kinase that phosphorylates Tau protein [21]. A β attenuates the regulation of the N-methyl-D-aspartate (NMDA) receptor by GSK-3 β through enhancing GSK-3 β activity, thereby producing NMDA excitotoxicity and accelerating the progression of AD [22]. GSK-3 β activation is the basis of Tau protein phosphorylation. Activated GSK-3 β can phosphorylate more than 40 Tau protein sites, of which Ser199, Thr231, Ser413, Thr181, Ser202, Thr205, Thr212, Tyr216, and Ser404 are the main phosphorylation sites [17,23]. The activation of GSK-3 β is regulated by phosphorylation of its own sites of Tyr216 and Ser9. High GSK-3 β (Tyr216) increases the activity of GSK-3 β to promote Tau protein phosphorylation. In contrast, high GSK-3 β (Ser9) reduces the activity of GSK-3 β to inhibit Tau protein phosphorylation [24].

TWS119, obtained from high-throughput screening, is an inhibitor of GSK-3 β that tightly binds to GSK-3 β to induce neuronal production and nerve cell differentiation, as indicated by surface plasmon resonance quantification, with an IC₅₀ of 30 nM. This process aids in biological research on stem cells [25]. A study has shown that TWS119 activates the Wnt/ β -catenin signaling pathway to play a key role in regulating the self-renewal and pluripotency of stem cells [26]. In the early asymptomatic stage of AD, GSK-3 β activity is increased, and TWS119 significantly inhibits GSK-3 β activity to prevent myelin destruction and mis localization of myelin

basic protein in subcellular structures caused by presenilin PS1^{M146V} and A β_{1-42} , leading to dementia [27]. Therefore, TWS119 was used as a tool for inhibiting GSK-3 β in this study to evaluate if GSK-3 β is associated with Tau phosphorylation. This study used A β_{25-35} to simulate AD with hyperphosphorylation of Tau protein in N2a cells. GSK-3 β inhibitor TWS119 was used to inhibit GSK-3 β activity. Our results showed that the expression of p-Tau (Ser202), p-Tau (Ser199), p-Tau (Ser404), p-Tau (Ser 214), p-Tau (Thr231), and p-GSK-3 β (Tyr216) protein was upregulated, while the expression of p-GSK-3 β (Ser9) protein was downregulated, in the N2a cells of the A β model group, suggesting that the phosphorylation levels of Tau protein in the N2a cells of the model group were increased, and different concentrations of SBF significantly reduced the expression of phosphorylated Tau protein ($P < 0.05$, $P < 0.01$).

Figures 3&4 demonstrate the protein expression of p-Tau (Ser404) and p-Tau (Ser214), respectively, in different groups. The protein expression of p-Tau (Ser404) and p-Tau (Ser214) of the A β model group was significantly higher than that of the blank control group ($P < 0.01$), indicating that A β_{25-35} enhanced GSK-3 β activity and increased the phosphorylation of Tau protein at the Ser404 and Ser214 sites, while GSK-3 β inhibitor TWS119 significantly reduced the protein expression of p-Tau (Ser404) and p-Tau (Ser214), indicating that TWS119 inhibited GSK-3 β activity to lower the phosphorylation of Tau protein at the Ser404 and Ser214 sites. Compared with the model group, the protein expression of p-Tau (Ser404) and p-Tau (Ser214) was significantly lower in the A β_{25-35} +TWS119 group, indicating that TWS119 inhibited GSK-3 β activity. Compared with the A β_{25-35} +TWS119 group, different concentrations of SBF significantly reduced the protein expression of p-Tau (Ser404) and p-Tau (Ser214) in the N2a cells ($P < 0.01$), confirming that SBF significantly inhibited the phosphorylation of Tau protein. SBF may reduce the hyperphosphorylation of Tau (Ser404) and Tau (Ser214) sites through inhibiting the activity of GSK-3 β . Figures 1,2&5 illustrate the protein expression of p-Tau (Ser202), p-Tau (Ser199), and p-Tau (Thr231), respectively, in different groups. The protein expression of p-Tau (Ser202), p-Tau (Ser199), and p-Tau (Thr231) was significantly higher in the TWS119 group than in the blank control group. Compared with the model group, the protein expression of p-Tau (Ser202), p-Tau (Ser199), and p-Tau (Thr231) remained at high levels in the A β_{25-35} +TWS119 group. Different concentrations of SBF significantly reduced the protein expression of p-Tau (Ser202), p-Tau (Ser199), and p-Tau (Thr231) in different SBF+A β_{25-35} +TWS119 groups ($P < 0.01$), indicating that GSK-3 β inhibitor TWS119 did not exert an inhibitory effect on these phosphorylation sites, and SBF showed an obvious effect against the hyperphosphorylation. SBF reduced the protein expression of p-Tau (Ser202), p-Tau (Ser199), and p-Tau (Thr231), which may be achieved not by inhibiting GSK-3 β activity but by affecting other protein kinases, such as CDK5 and PKA.

Figure 6 shows the protein expression of p-GSK-3 β (Ser9) in the N2a cells of different groups. The protein expression of p-GSK-3 β (Ser9) in the N2a cells was significantly lower in the model group and higher in the WTS119 group than in the blank control group ($P < 0.01$). Theoretically, GSK-3 β (Ser9) negatively regulates GSK-

3 β . Thus, hyperphosphorylation of Tau protein increases GSK-3 β activity, thereby reducing the expression of p-GSK-3 β (Ser9) protein, which is consistent with our current findings. TWS119 inhibited GSK-3 β activity and led to higher expression of p-GSK-3 β (Ser9) protein, suggesting that TWS119 inhibited GSK-3 β by affecting the activity of the GSK-3 β (Ser9) site phosphorylation. The protein expression of p-GSK-3 β (Ser9) in the N2a cells of the A β_{25-35} +TWS119 group was higher than that of the model group, suggesting a significant inhibitory effect of TWS119. Different concentrations of SBF increased the protein expression of p-GSK-3 β (Ser9) in the N2a cells compared with the A β_{25-35} +TWS119 group, suggesting that SBF reversed the reduction of protein expression of p-GSK-3 β (Ser9) in the N2a cells caused by A β_{25-35} . SBF reduced the hyperphosphorylation of Tau protein, most likely by regulating GSK-3 β (Ser9).

As shown in Figure 7, compared with the blank control group, the protein expression of p-GSK-3 β (Tyr216) was significantly higher, and Tau protein phosphorylation was higher in the N2a cells of the model group. However, no significant difference of the p-GSK-3 β (Tyr216) protein expression was found between the TWS119 and control groups, suggesting that TWS119 had no inhibitory effect on GSK-3 β (Tyr216). However, compared with the model group, the protein expression of p-GSK-3 β (Tyr216) in the N2a cells was significantly lower in the A β_{25-35} +TWS119 group, indicating that reduction of p-GSK-3 β (Tyr216) protein expression in N2a cells occurred even when the inhibitor had no effects. This phenomenon may be consistent with a recent finding that Tau protein phosphorylation initially protects neurons, while A β attacks this protective effect until the protective effect gradually disappears [27]. The results of this study suggested that the neurotoxicity of A β_{25-35} may increase GSK-3 β (Tyr216) activity and enhance Tau protein phosphorylation in N2a cells, possibly representing an early stage of AD state, in which the first step of Tau protein phosphorylation is to reduce A β toxicity. However, in later stages, Tau protein hyperphosphorylation leads to a complete reversal of effects and enhances neurotoxicity. Another possibility is that TWS119 had an inhibitory effect on the abnormally enhanced GSK-3 β (Tyr216) activity but no inhibitory effect on normal GSK-3 β (Tyr216). Compared with the A β_{25-35} +TWS119 group, different concentrations of SBF reduced the expression of phosphorylated Tau proteins in different sites, suggesting that SBF reversed the elevation of p-GSK-3 β (Tyr216) in the N2a cells caused by A β_{25-35} . This also suggested that SBF reduced Tau protein phosphorylation by regulating GSK-3 β (Tyr216).

Materials and methods

Experimental materials and instruments

N2a cells were purchased from Beijing DingguoChangsheng Biotechnology Co., Ltd. (Beijing, China). SBF was provided by the Institute of Traditional Chinese Medicine of Chengde Medical University (Hebei Province, China), with a total flavonoid content of 93.1%. A β_{25-35} was purchased from Dalian Meilun Biotechnology Co., Ltd. (Liaoning Province, China, batch number: S0901A). High-glucose Dulbecco's Modified Eagle Medium (DMEM) was purchased

from Gibco (Thermo Fisher Scientific, Waltham, MA, batch number: 8115403). Fetal bovine serum was purchased from BI (batch number: 1616316). Dimethyl sulfoxide was purchased from Tianjin Oubokai Chemical Co., Ltd. (Hebei Province, China). Trypsin was purchased from Wuhan Huamei Biological Engineering Co., Ltd. (Hebei Province, China). GSK-3 β inhibitor 4,6-disubstituted pyrrolopyrimidine (TWS119) was purchased from Selleckchem (catalog No.S1590). The lysate, protein marker, stripping buffer, and bicinchoninic acid (BCA) protein quantification kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Anti-GSK-3 β (Ser9) and anti-GSK-3 β (Tyr216) antibodies were purchased from SAB (serial numbers 1002-1 and 11301, respectively). Mouse monoclonal beta-tubulin (β -tubulin) antibody was purchased from Immunone. Anti-p-Tau (Ser202), p-Tau (Ser199), p-Tau (Ser404), p-Tau (Thr214), and p-Tau (Thr231) antibodies were purchased from Abcam. Goat anti-rabbit IgG (H+L) was purchased from CWBio (item number: CW0103S). Horseradish peroxidase (HRP) goat anti-mouse (H+L) was purchased from Solarbio (item number: SE131). An enhanced chemiluminescence (ECL) kit was purchased from Applygen Technologies Inc. (Beijing, China). An ultra-clean bench was purchased from SP-DJ Pudong Optical Instrument Factory (Shanghai, China). The cell incubator was purchased from Taibai (Japan, LNA-122D). An inverted microscope was purchased from Olympus Corporation (Japan). A cryogenic refrigerated centrifuge was purchased from Eppendorf. A constant-temperature double-layer shaking incubator was purchased from Changzhou Noki Instrument (Jiangsu Province, China). A pure water machine was purchased from Beijing Oriental Science & Technology Development Ltd. (Beijing, China). The electrophoresis apparatus was purchased from Bio-Rad.

Experimental methods

Culture and passage of N2a cells: N2a cells were cultured in high-glucose DMEM containing 10% fetal bovine serum and 100 U/mL penicillin at 37°C in an incubator with 5% CO₂. The medium was changed every other day until it reached 80-90% confluency. The cells were subcultured at 1:2-1:3 and digested by 0.25% trypsin for 20-30 s, followed by discarding the trypsin solution for further digestion in dry conditions. Morphologies of the N2a cells were observed under a microscope. The cells changed into spherical shapes with obvious gaps between cells and were detached. Complete culture medium was added to stop the trypsinization, followed by pipetting evenly to resuspend the cells and transferring the cell suspension into a new flask. After adding culture medium, the cell suspension was incubated at 37°C in an incubator with 5% CO₂ until it reached the logarithmic growth phase. The cells were then ready for later experiments.

Experimental grouping and processing: N2a cells in the logarithmic growth phase were inoculated into T25 sterile cell culture flasks at a cell density of 1 × 10⁵ cells/mL per well, 6 mL per flask, and incubated at 37°C in an incubator with 5% CO₂ for 12 h. All cells in the wells were randomly divided into control, model, TWS119, A β_{25-35} +TWS119, and different concentrations of SBF+A β_{25-35} +TWS119 groups. Cells in the TWS119 and A β_{25-35} +TWS119 groups contained 45 nmol/L (final concentration) of

TWS119. Cells in the different SBF+A β_{25-35} +TWS119 groups were incubated with different concentrations of SBF (i.e., 1.125 mg/L, 2.25 mg/L, and 4.5 mg/L) together with TWS119 (i.e., 45 nmol/L final concentration) for 12 h. The model, A β_{25-35} +TWS119, 1.125 mg/L SBF+A β_{25-35} +TWS119, 2.25 mg/L SBF+A β_{25-35} +TWS119, and 4.5 mg/L SBF+A β_{25-35} +TWS119 groups had 35 μ mol/L-A β_{25-35} (final concentration) added to each of them separately to react for 12 h.

Cell harvesting: The supernatant of each group of cells was discarded. After washing the cells with pre-cooled PBS twice, 3 mL serum-free medium was added to each group of cells, followed by scraping the cells with a cell scraper into a suspension, pipetting the cells into an Eppendorf tube for centrifugation at 4,000 rpm for 5 min, discarding the supernatant and keeping the cell pellet at -80°C for later use.

Immunoblotting: Western blot analysis was used to detect the expression of phosphorylated Tau protein in Ser202, Ser199, Ser404, Ser214, and Thr231 and protein expression in GSK-3 β (Ser9) and GSK3 β (Tyr216) phosphorylation sites in different groups of N2a cells. Different groups of cells preserved in a -80°C freezer was collected and placed on ice, followed by adding 200 μ L protein lysate (RIPA:PMSF = 100:1) to pipette up and down few times to allow thorough contact between the cells and protein lysate and lysing on ice for 5-10min. After lysing, samples were centrifuged at 12,000 rpm for 5 min to collect the supernatant, i.e., total proteins, which were quantified by the BCA method to determine the protein concentrations in different groups. The remaining total proteins in the samples had 5 \times loading buffer added to them and were denatured in 100°C water for 5min, followed by 12% SDS-polyacrylamide gel electrophoresis (100 V, 90min). The separated proteins in the SDS-PAGE gels were transferred to wet PVDF membranes in 300 mA constant current for 2h. Subsequently, the protein membranes were completely immersed in 5% skim milk-TBST solution to block for 30min and then incubated with primary antibodies diluted in 5% skim milk-TBST solution at room temperature for 10min and overnight at 4°C. The next day, the protein membrane was incubated at room temperature for 30min and washed in TBST 5 times (3 min each). The protein membrane was incubated with secondary antibody diluted in 5% skim milk-TBST solution at room temperature on a shaker for 40 min. After washing the membrane in TBST 6 times (3 min each), the protein membrane was reacted with ECL reagent for 5 min, followed by exposing the film for 10 s to 5min (exposure time was adjusted with different light intensities) and developing the image for 2min and fixing. β -tubulin was used as internal reference to determine the relative protein expression of p-Tau (Ser202), p-Tau (Ser199), p-Tau (Ser404), p-Tau (Ser 214), p-Tau (Thr231), p-GSK-3 β (Ser9), and p-GSK-3 β (Tyr216). Each experiment was repeated 3 times. After scanning the film, the developed protein bands were analyzed by Quantity One 4.6.2 software to determine the relative expression of target proteins using the target protein-to- β -tubulin ratios of grayscale.

Statistical analyses: SPSS 19.0 (IBM SPSS, Chicago, IL) software was used for statistical analysis. Data were presented as mean \pm standard deviation (SD). Mean values from multiple groups were

compared by one-way ANOVA. Pairwise comparison between means was performed using the least significant difference (LSD) method. $P < 0.05$ was considered a statistically significant difference.

Conclusion

This study confirmed that SBF reversed the damage of N2a cells induced by A β_{25-35} . Through regulating the activity of the GSK-3 β (Ser9) site phosphorylation and reducing the phosphorylation of Tau protein Ser214 and Ser404 sites, SBF inhibited the abnormal phosphorylation of Tau proteins. Our findings showed that SBF significantly inhibited the abnormal phosphorylation of Tau protein, which may involve other mechanisms and require further verification. Nevertheless, it is undeniable that SBF has a significant therapeutic effect in AD by targeting Tau protein. This finding brings a new hope for the prevention and treatment of AD in the future.

Authors contributions

Shang Yazhen and Cao Qinying conceived and designed the experiment. Cai Hongling, Cheng Jianjun and Li Caixia conducted the test and wrote the manuscript.

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Competing Interests

All authors declare that they have no competing interests regarding this manuscript.

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