

Original Article

A β mediates Sigma receptor degradation via CaN/NFAT pathway

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Abstract: Sigma receptor is an endoplasmic reticulum protein and belongs to non-opioid receptor. Increasing evidence shows that Sigma receptor activation can significantly attenuate AD induced neurological dysfunction and the functional deficiency of Sigma receptor plays an important role in the A β induced neuronal loss. This study aimed to investigate the influence of extracellular accumulation of A β on the Sigma receptor expression. Our results showed the increase in extracellular A β had little influence on the mRNA expression of Sigma receptor, but gradually reduced its protein expression. Co-immunoprecipitation was employed to evaluate the interaction of Sigma receptor with other proteins. Results showed BIP could bind to Sigma receptor to affect the ubiquitination of Sigma receptor. Further investigation showed there was a NFAT binding site at the promoter of BIP. Then, Western blot assay was performed to detect NFAT expression. Results showed extracellular A β affected the nuclear translocation of NFAT and the CaN activity of NFAT also increased with the accumulation of extracellular A β . In this study, NFAT-BIP luciferase reporter gene system was constructed. Results showed NFAT was able to regulate the transcription of BIP. Thus, we speculate that extracellular A β accumulation may activate CaN/NFAT signaling pathway to induce chaperone BIP expression, which results in Sigma receptor ubiquitination and its degradation.

Keywords: Alzheimer disease, β -amyloid, sigma receptor, calcineurin, nuclear factor of activated T cells

Introduction

Alzheimer disease (AD) is a common neurodegenerative disease in old people [1-3]. Progressive cognition impairment may cause the loss of social function and life skills in AD patients [4, 5]. Extracellular A β accumulation has been found as a major molecular mechanism underlying the pathogenesis of AD [6-8]. The abnormal A β accumulation may disrupt the intracellular Calcium homeostasis of neurons, resulting in neurotoxicity. The calcium influx may increase the calcineurin (CaN) activity [9-12]. Then, CaN may increase the intracellular calcium concentration via homocysteine dependent way. Our results showed CaN activity increased significantly, and inhibition of CaN activity could significantly reduce the homocysteine induced cell injury [13]. In clinical trials, studies have shown that CaN expression increases dramatically in the brain of AD patients. Our in vitro experiment also reveals

that A β is able to significantly increase CaN activity of hippocampal neurons.

Sigma receptor is an endoplasmic reticulum protein and belongs to non-opioid receptor family. Neurons, astrocytes and microglia have sigma receptor expression. In recent years, increasing evidence shows that sigma receptor activation is able to markedly attenuate the neurological dysfunction in AD patients, and patients with early AD have reduced expression of sigma receptor [14, 15]. Under normal condition, sigma receptor may bind to BiP, which is essential for its location on the endoplasmic reticulum. Sigma receptor can regulate multiple cellular processes and the target proteins of sigma protein include ion channels (such as potassium, sodium and calcium ion channels on cell membrane), neurotransmitter receptors and protein kinases [16]. Available studies have confirmed that one of important functions of BiP is to recruit E3 ubiquitin ligase, leading to

A β mediates CaN/NFAT pathway

Table 1. The primers of qPCR

qPCR gene	Primer	Sequence
CaN	Forward Primer	TTCGCGGAAACCATGAATGTA
	Reverse Primer	GGCAGTCGAAGGCATCCAT
NFAT	Forward Primer	GGAGAGTCCGAGAATCGAGAT
	Reverse Primer	TTGCAGCTAGGAAGTACGTCT
BIP	Forward Primer	ACTTGGGGACCACCTATTCT
	Reverse Primer	GTTGCCCTGATCGTTGGCTA
SigmaR	Forward Primer	CTGGGCACTCAAACCTCGTC
	Reverse Primer	CTCCACGATCAGCCGAGAGA
GAPDH	Forward Primer	TGACCTCACTACATGGTCTACA
	Reverse Primer	CTTCCCATTCTCGGCCTTG

the ubiquitination and subsequent degradation of BiP, which is crucial for the maintenance of normal cell function. Whether A β induced degradation of sigma receptor is also dependent on this pathway has never been reported.

Further investigations of our group indicated the nuclear factor of activated T cells (NFAT) binding site at the transcription factor binding site of promoter of BIP gene. NFAT is a transcription factor with multiple regulatory activities. Some studies have shown that the pathogenesis of acute and chronic diseases such as AD, bronchial asthma, inflammatory bowel disease, diabetes, osteoporosis, arthritis and myocarditis is closely related to the abnormal activation of NFAT. The NFAT activity is regulated by the calcium/calmodulin-dependent protein phosphatase C. The abnormal A β accumulation induced calcium influx may cause abno.

Taken together, we hypothesize that A β accumulation may activate CaN/NFAT signaling pathway to induce chaperone BIP expression and then E3 ubiquitin ligase is recruited to induce the ubiquitination of sigma receptor, leading to its degradation, which may compromise the neuroprotection of sigma receptor. Our findings may provide new evidence for the degradation of sigma receptor in AD.

Materials and methods

Cell culture and treatment

Mouse neuroblastoma N2A cells (obtained from ATCC) were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) at

37°C in an atmosphere containing 5% CO₂. N2A cells (5×10⁵/mL) were cultured for 48 h in either 5 μmol/L or 10 μmol/L A β ₂₅₋₃₅ (Sigma); N2A cells not cultured in A β ₂₅₋₃₅ were used as controls. The 293T cell line (ATCC), from human embryo kidney cells, was cultured in DMEM (Gibco) containing 10% FCS at 37°C in 5% CO₂.

FK506 (CaN inhibitor; 10 μM; Selleck, USA) was used to investigate the effect of CaN on the protein expression of sigma receptor. To investigate the ubiquitination of BiP/Sigma-1R complex, 20 μM MG132 (a ubiquitination inhibitor; Selleck, USA) and siRNA (5'-GCUCCAGGCAAUGGAUAAUUU-3'; 30 ng) targeting HRD1 gene were used.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using Trizol reagent (Invitrogen), and reverse transcription of the RNA was carried out using an M-MLV1 kit (Promega), in accordance with the manufacturers' instructions. The primers used for BiP, Sigma-1R and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; used as the reference gene) were designed using Primer 5.0 (Premier Biosoft), and their sequences are detailed in **Table 1**. Fluorogenic qPCR was carried out using a ABI system with SYBR Green incorporation (Fast Start Universal SYBR Green Master, Rox; Roche). The amplification conditions were: 94°C for 10 min; 94°C for 15 s; 60°C for 1 min; 40 cycles.

Detection of CaN activity

The activity of CaN was determined using a chemiluminescence assay kit (BioVision) according to the manufacturer's instructions.

Western blot assay

The whole cell protein was extracted using the M-PER Protein Extraction Kit (Pierce), while the nuclear and cytoplasmic proteins were independently extracted using the NE-PER Protein Extraction Kit (Pierce) according to the manufacturer's instructions. 20 μg of protein was transferred onto a polyvinylidene difluoride (PVDF) membrane after SDS-PAGE electrophoresis, and labeled using monoclonal primary antibodies against Sigma-1R (Cell Signaling Technology) (1:1,000), SigmaR (Cell Signaling

A β mediates CaN/NFAT pathway

Technology) (1:1,000), BIP (Abcam) (1:800), and NFAT (Cell Signaling Technology) (1:1,000). The secondary antibody (Amersham Life Sciences) was labeled with horseradish peroxidase, and electrochemiluminescence (ECL) used for visualization of bands. A bio-red fluoro analyzer was used to determine the molecular weight and net optical density (OD) of the target band.

Co-immunoprecipitation (Co-IP)

N2H cells were independently treated with 5 μ mol/L A β_{25-35} and 10 μ mol/L A β_{25-35} for 48 h. The supernatant was removed, and cells were washed in PBS twice. After addition of 30 μ L of IP lysis buffer (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 5 mM β -ME, 0.1 mM PMSF, protease inhibitor cocktail [aprotinin, pepstain A, Leupeptin, Antipain, TPCK and Benzamidine]), cells were incubated at 4°C for 30 min and then centrifuged at 12000 g for 30 min. The supernatant was harvested. 1 μ g of BIP antibody and 30 μ L of protein A/G-beads were added to the cell lysate, followed by incubation at 4°C overnight. After centrifugation at 4°C for 5 min at 3000 g, the supernatant was removed, and the sediment (protein A/G-beads) was washed with 1 ml of lysis buffer 5 times. After addition of 15 μ L of 2 \times SDS loading buffer, the mixture was boiled for 10 min, followed by Western blot assay.

ChIP-PCR

N2H cells were treated with 10 μ mol/L A β_{25-35} for 48 h and the supernatant was removed. Cells were washed in PBS twice and then fixed in 4% paraformaldehyde (2 ml) for 10 min. After addition of 2.5 M glycine, incubation was done for 5 min and cells were then washed in PBS. The cells were transferred into EP tubes and re-suspended in series of sucrose extraction buffer (0.25 M, 0.4 M and 1.5 M). Cells were centrifuged and 300 μ L of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, PMSF and protease inhibitor cocktail) was added. Cells were sonicated on ice. Then, the lysate was centrifuged at 12000 g and the supernatant was collected. After dilution, the lysate was divided into two parts which were mixed with anti-NFAT1 (abcam, MA, USA) and isotype IgG, respectively. Then, antibody binding beads were independently added to them, followed by incubation overnight with continuous shaking.

Cells were washed with dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, PMSF and protease inhibitor cocktail [aprotinin, pepstain A, Leupeptin, Antipain, TPCK and Benzamidine]), hypotonic solution (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0), hypertonic solution (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl and pH 8.0), lithium chloride solution (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 2 mM EDTA, 20 mM Tris-HCl and pH 8.0) and TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) 7 times. The products were eluted with elution buffer at 65°C overnight. The elution products were purified with DNA purification kit (BeckmanCoulter, CA, USA) and 50 μ L of purified products were obtained for ChIP. The primers for detection of NFAT1 binding site of BIP promoter were as follows: BIP1 forward primer: 5'-GTTGTAAGCATTTCCTCCAT-3', reverse primer: 5'-GAACTCAGGACCTTTGGA-3', BIP2 forward primer: 5'-GATTCATAGGCTCAACAT-3', reverse primer: 5'-CTACTTGGGCCATTTATT-3', BIP3 forward primer: 5'-TCTTTAGCATTATCAGCCCTAT-3', reverse primer: 5'-ACCACCTTTCACGAGCAA-3'.

Vector constructs and luciferase activity assay

The 2100-bp sequence covering -2000/+100 in the promoter of BIP gene was cloned with following primers: forward: 5'-GACAGGATGAACTGAAATTTGA-3' and reverse: 5'-AAGCGGCCTCCTCCGCAACTCA-3'. The following oligonucleotides were used as the sense primers for construction of the following vectors: F2 (BIP-2152), 5'-AGTGACTGTGCGTGTGATG-3'; F3 (BIP-1687), 5'-AGTGACTGTGCGTGTGATG-3'; F4 (BIP-1005), 5'-ACATAATAAGCTCAAGTAGTAAC-3'. F5 (BIP-518), 5'-AGCTGAGAAGCACCAGGATTCTCA-3'. The anti-sense primer in all cases was 5'-TCCGATTGGTGAAGTCGCTACTCGTT-3'. The PCR products were cloned into the corresponding sites of pGL3-basic vector (Promega Corp., Madison, WI). All luciferase constructs were verified by sequencing.

In addition, the NFAT binding site of BIP promoter was mutated and the above sequences with different deletion mutations were constructed. Then, these promoter sequences and pGL3-basic vector were used to construct luciferase transcript which was co-transfected with NFAT1 gene. The luciferase activity was adjusted at

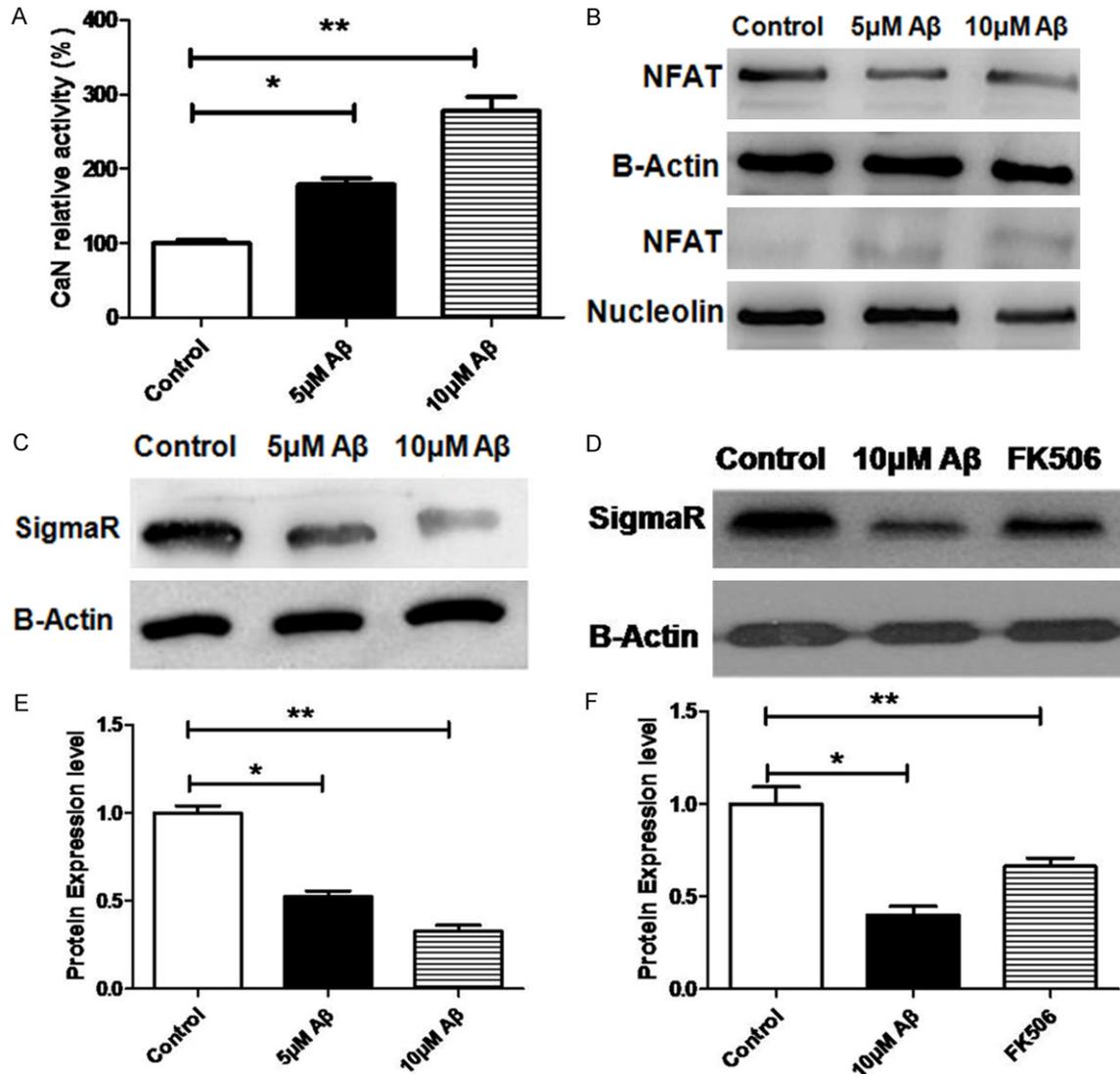


Figure 1. A β_{25-35} activates CaN/NFAT pathway and reduces Sigma-1R/BIP expression in N2A cells. A: CaN activity after treatment with A β at different concentrations. B: NFAT protein expression in the cytoplasm and nucleus after treatment with A β at different concentrations. C: SigmaR protein expression after treatment with A β at different concentrations. D: Influence of CaN inhibitor FK506 on SigmaR expression in N2A cells after treatment with 10 μ M A β . C, E: Optical density in Western blot assay; D, F: Optical density in Western blot assay. All data are expressed as means \pm SD (n=5/each group). *P<0.05, **P<0.01.

different states. Cells in Transwell chamber were incubated with 50 ng of pRL-SV40 plasmid and the renilla luciferase served as an internal reference.

Silencing of CaN with siRNA

N2A cells were seeded into 24-well plate at a density of 5×10^4 cells/well. Cells were maintained in DMEM with 10% FBS and antibiotics. When the cell confluence reached 70-90%, cells were maintained in serum free DMEM and treated with 20 pmol CaN specific siRNA

(5'-AGGCATAGGACTACAAGGGAAGCTTC-3') and LipofectamineTM2000 (Invitrogen Corporation, Carlsbad, CA) at room temperature for 20 min. 100 μ l of siRNA/lipofectamin compound was added to each well, followed by incubation at 37°C for 24 h in an environment with 5% CO₂.

Cell immunofluorescence staining

Cells in plates were washed in PBS thrice (3 min for each) and then fixed in 4% paraformaldehyde for 15 min, followed by washing in PBS thrice (3 min for each). Cells were treated with

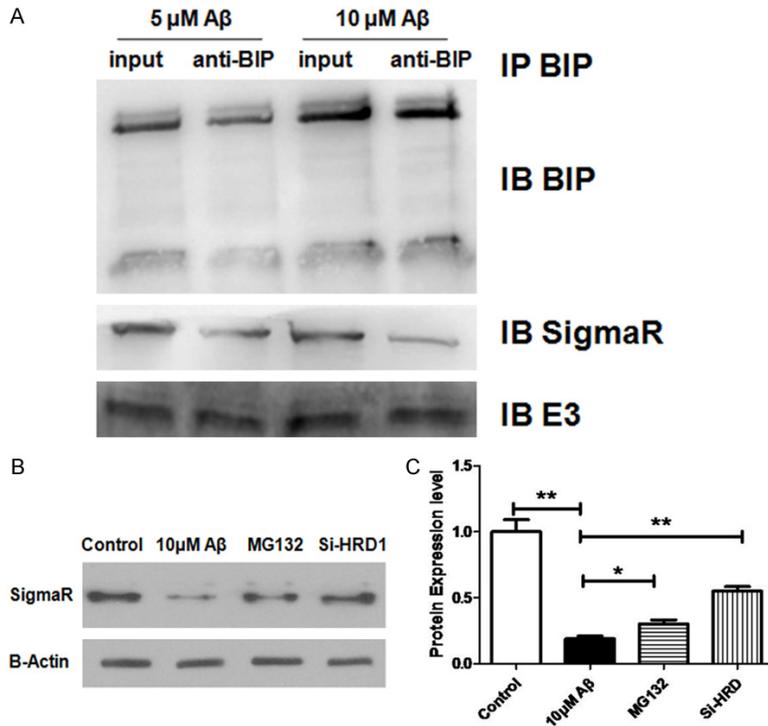


Figure 2. BiP/Sigma-1R complex influences Sigma-1R ubiquitination and degradation in N2A cells. A: BiP/Sigma-1R complex co-immunoprecipitation after treatment with A β at different concentrations. Input: no beads and antibody after A β treatment; anti-BiP: binding protein in the presence of BiP antibody. Immunoprecipitation was performed with anti-BiP antibody and then Western blotting to detect the expression of BiP, SigmaR and E3 ligase in the complex. B: Effect of E3 ligase on SigmaR protein expression: E3 ligase inhibitor MG132 and E3 ligase specific siRNA were used to treat N2A cells after treatment with 10 μ M A β , and then Western blotting was performed to investigate the influence of E3 ligase on SigmaR expression. B, C: Optical density in Western blotting. All data are expressed as means \pm SD (n=5/each group). *P<0.05, **P<0.01.

0.5% Triton X-100 in PBS at room temperature for 20 min, followed by washing in PBS thrice (3 min for each). The residual PBS was removed and cells were treated with normal goat serum at room temperature for 30 min and then with primary antibody (anti-SigmaR; 1:200) in a humidified environment at 4°C over night. Cells were incubated with goat anti-rabbit IgG H&L (FITC, 1:2000) (Abcam, MA, USA) for 1 h and then with DAPI in dark for 5 min for nuclear staining. After mounting, cells were observed under a fluorescence microscope.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (IBM, USA) software, and data are expressed as mean \pm standard deviation (SD). Com-

parisons between groups were done using the t-test, and a value of P<0.05 was considered statistically significant.

Results

A β ₂₅₋₃₅ activates CaN/NFAT pathway and reduces sigma-1R/BiP expression in N2A cells

Western blot assay of whole, nuclear and cytoplasmic proteins was used to investigate whether A β ₂₅₋₃₅ treatment resulted in the activation of CaN/NFAT signaling and inhibition of Sigma-1R expression in N2A cells. Exposure of N2A cells to 5 μ mol/L or 10 μ mol/L A β ₂₅₋₃₅ for 48 h increased CaN activity in a concentration-dependent manner, as compared to untreated N2A cells (Figure 1A). A β ₂₅₋₃₅ (5 μ mol/L or 10 μ mol/L) also caused a concentration-dependent reduction in the cytoplasmic NFAT protein expression and a concentration-dependent increase in the nuclear NFAT protein expression (Figure 1B). This suggests that A β ₂₅₋

₃₅ increases CaN activity, leading to the translocation of NFAT from the cytoplasm to the nucleus. Interestingly, the sigma-1R expression in N2A cells was reduced by A β ₂₅₋₃₅ in a concentration-dependent manner (Figure 1C) and FK506 (a CaN inhibitor) was able to alleviate the inhibitory effect of A β ₂₅₋₃₅ on sigma-1R expression (Figure 1D). These indicate that the A β ₂₅₋₃₅ induced inhibition of sigma-1R expression in N2A cells is mediated, at least in part, by the CaN/NFAT pathway. Target prediction showed the BiP promoter sequence was a potential transcription regulation target of NFAT. Consistent with this prediction, exposure of N2A cells to 5 μ mol/L or 10 μ mol/L A β ₂₅₋₃₅ for 48 h resulted in a concentration-dependent increase in both mRNA (Figure 3A) and protein expression (Figure 3B) of BiP. This implies that

A β mediates CaN/NFAT pathway

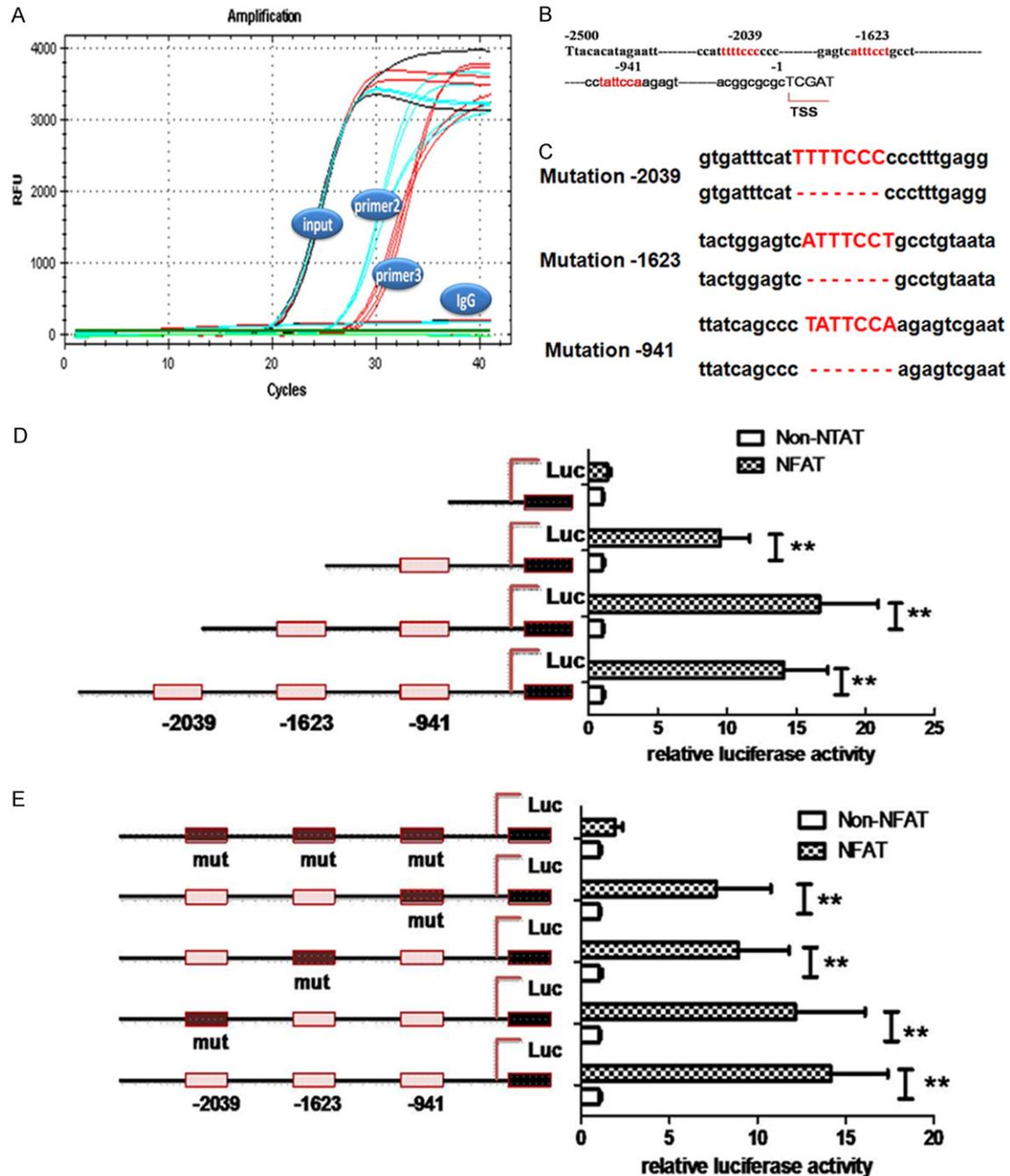


Figure 3. Transcriptional regulation of BIP by NFAT. (A) ChIP-PCR was used to detect the binding site of NFAT in BIP promoter. NFAT1 specific antibody was used to precipitate the corresponding genomic DNA segments and then real-time PCR was done to amplify the binding segments of NFAT1. Input: DNA segments that were broken by ultrasound and not immunoprecipitated, but amplified with 3 pairs of primers. IgG: IgG was used to immunoprecipitate DNA segments that were broken by ultrasound, followed by amplification with 3 pairs of primers. Primer2 and primer3: after immunoprecipitation with anti-NFAT1 antibody, the binding segments were amplified with 3 pairs of primers. Primer 2 and primer 3 were designed for the amplification of binding site at -1623 bp and -941 bp in (B), respectively. (B) Prediction of NFAT binding site in the BIP promoter. (C) Mutation binding sites of luciferase expression vector. In the synthesis of BIP promoter, deletion mutation was designed at 3 binding sites. (D) Effect of NFAT on the transcriptional activity of BIP promoters. BIP promoters with different length were cloned and contained different binding sites of NFAT. In 293T cells, the BIP promoter was cotransfected with NFAT1 gene, and then luciferase activity was detected. (E) Effect of NFAT on the transcriptional activity of BIP promoters with mutation at different sites. In the synthesis of BIP promoter, the deletion mutation was designed at the NFAT binding site. In 293T cells, the BIP promoter was cotransfected with NFAT1 gene, and then luciferase activity was detected. All data are expressed as means \pm SD (n=5/each group). *P<0.05, **P<0.01.

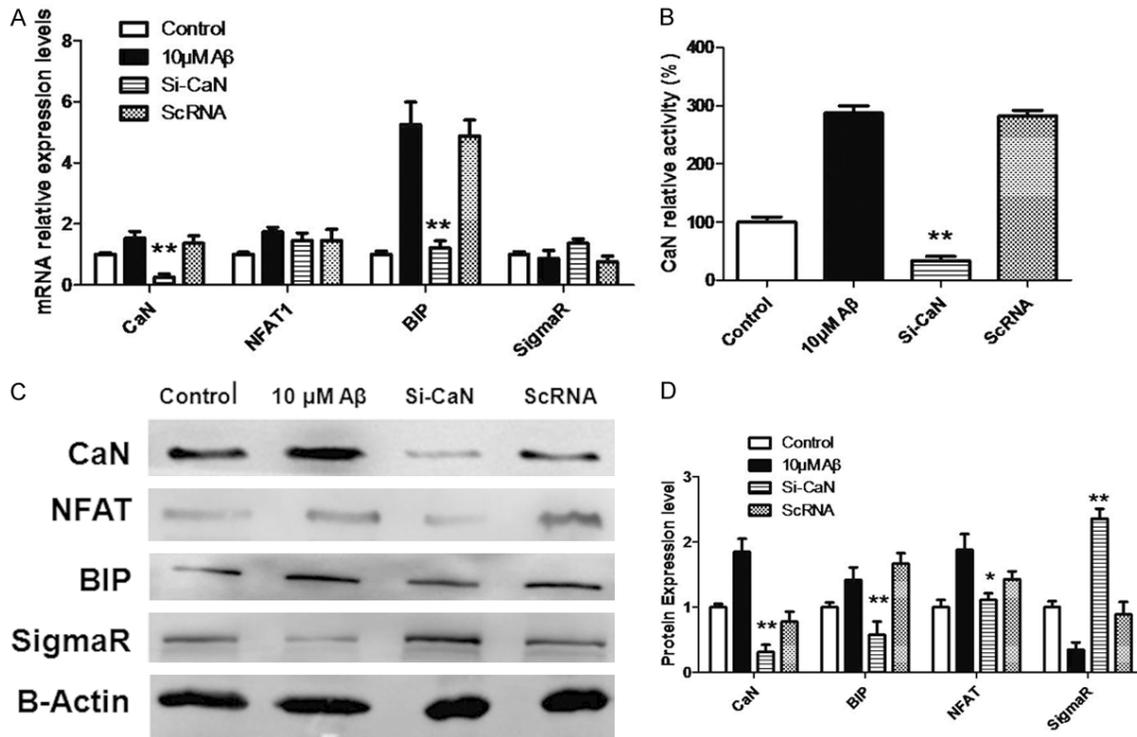


Figure 4. Effects of CaN/NFAT pathway on Sigma and BIP expression in N2A cells after A β_{25-35} treatment. A: Detection of mRNA expression. N2A cells were treated with both 10 μ M A β and CaN specific siRNA. Normal cells served as a control and scramble ScRNA as a negative control. B: Detection of CaN activity. Specific siRNA was used to down-regulate CaN mRNA expression and then CaN activity was detected with corresponding kit. C: Western blotting was done to detect protein expression. After silencing of CaN expression, Western blotting was done to detect the protein expression of CaN, NFAT, BIP and SigmaR. C, D: Optical density was detected after Western blotting. All data are expressed as means \pm SD (n=5/each group). *P<0.05, **P<0.01.

A β_{25-35} may induce the translocation of NFAT from the cytoplasm to the nucleus (**Figure 1B**) and then it binds to BiP promoter to induce BIP expression.

BiP/sigma-1R complex influences sigma-1R ubiquitination and degradation in N2A cells

Co-IP was employed to investigate the interaction between BiP and Sigma-1R, and its possible effect on the Sigma-1R ubiquitination. In N2A cells treated with A β_{25-35} (5 or 10 μ mol/L) for 48 h, sigma-1R and ubiquitin were both found to exist in the form of a complex that contained BiP (**Figure 2A**). To explore whether sigma-1R ubiquitination influenced sigma-1R expression, degradation of ubiquitin-conjugated proteins was inhibited using MG132 (a ubiquitin-proteasome inhibitor) or HRD-1 siRNA (silencing of E3 ubiquitin-protein ligase). Both MG132 and HRD-1 siRNA partially decreased the inhibitory effect of A β_{25-35} on sigma-1R protein expression in N2A cells (**Figure 2B**). This suggests that the A β_{25-35} induced inhibition of

sigma-1R protein expression involves, at least in part, the ubiquitination and degradation of sigma-1R, and that this may be mediated by BiP.

NFAT regulate BIP expression at transcription level in N2A cells

After A β treatment, genomic DNA was extracted from N2A cells and sonicated. Then, precipitation was done with NFAT specific antibody. According to the NFAT binding site determined by bioinformatics analysis, 3 pairs of primers were designed with input as a positive control and IgG as a negative control, followed by fluorescence PCR. As shown in **Figure 3A**, the target segments could be expanded in input group with 3 pairs of primers, but no signal was observed in IgG group. The second pair and third pair of primers could be used to expand target segment in input group, suggesting that there is a transcriptional binding site in the promoter of BIP (Hspa5) and its binding may initiate the BIP (Hspa5) transcription. We further

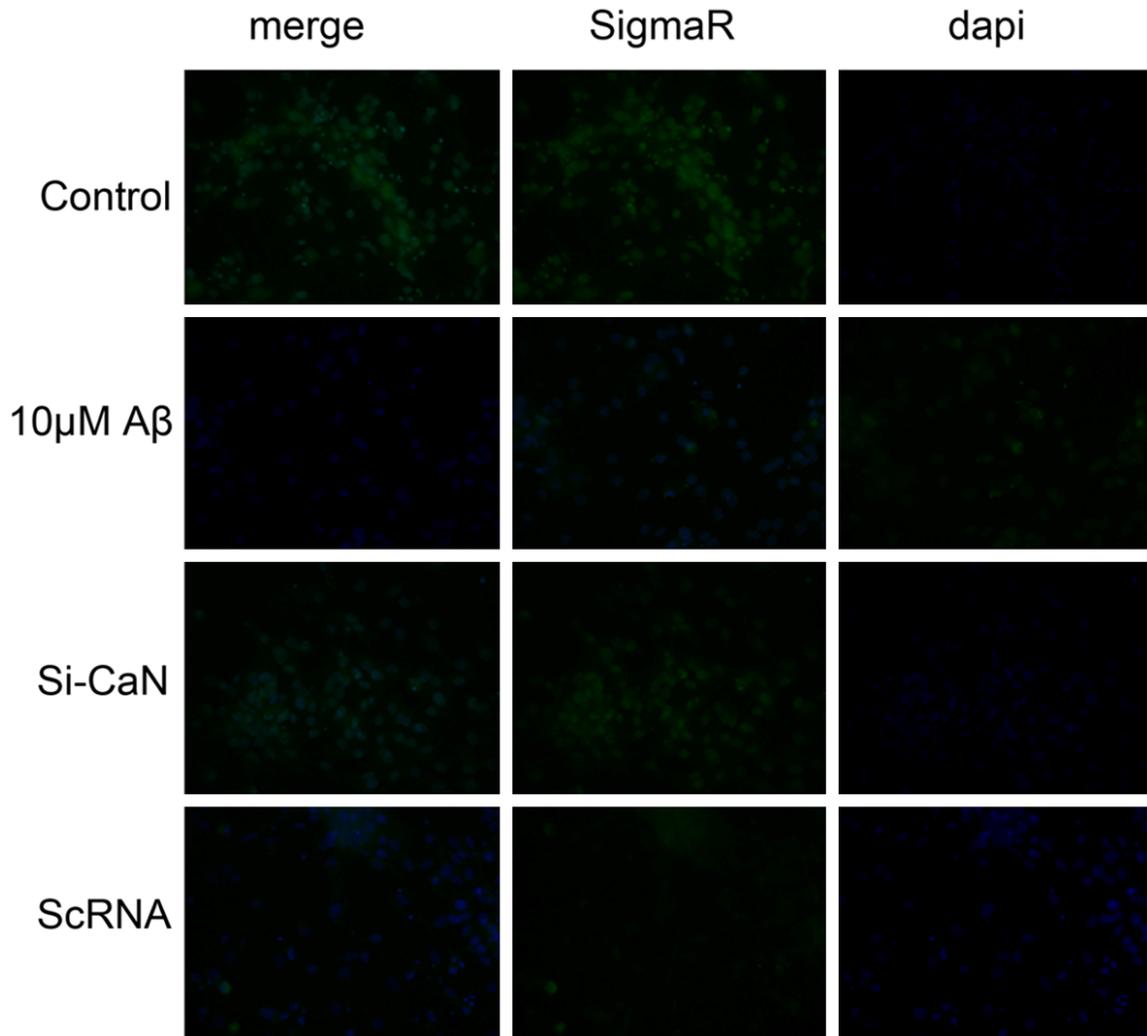


Figure 5. Effects of CaN/NFAT pathway interfering on SigmaR expression in N2A cells. N2A cells were treated with both 10 μ M A β and specific CaN siRNA. Normal cells served as a control and scramble ScRNA as a negative control. After 48-h treatment, fluorescence intensity was measured. Blue: Dapi positive nucleus; Green: SigmaR.

constructed fluorescent reporter system of NFAT induced BIP transcription. As shown in **Figure 3B**, in the presence of complete BIP promoter, 1500 bp BIP promoter and 1000 bp BIP promoter, NFAT was able to induce the transcription of luciferase, but luciferase transcription was not induced in the presence of 500 bp BIP promoter, suggesting that there is no NFAT binding site in the 500 bp BIP promoter.

CaN/NFAT pathway is essential for the effects of A β ₂₅₋₃₅ on sigmaR/BIP expression

In A β ₂₅₋₃₅ treated N2A cells siRNA was used to silence CaN expression and then the CaN/NFAT pathway, sigma receptor and BIP expression were detected. Results showed CaN silencing significantly reduced the CaN expression

and activity (**Figure 4A** and **4B**) and also decreased the nuclear translocation of NAFT (**Figure 4A**). This indicates that siRNA mediated silencing of CaN is effective to block CaN/NFAT pathway. We further detected the mRNA and protein expression of BIP and sigma receptor. Results indicated that siRNA mediated silencing of CaN dramatically reduced the mRNA and protein expression of BIP, but sigma receptor expression increased markedly. This confirms that CaN/NFAT pathway is essential for the A β ₂₅₋₃₅ induced degradation of sigma receptor.

Blocking of CaN/NFAT pathway affects sigmaR expression in N2A expression

Immunofluorescence staining was done to detect the sigmaR expression in A β ₂₅₋₃₅ treated N2A in which the nucleus was blue (DAPI) and

sigmaR was green. As shown in **Figure 5**, more blue fluorescence was observed in normal cells. After treatment with 10 μ mol/L A β ₂₅₋₃₅, SigmaR expression reduced significantly, suggesting extracellular A β accumulation may affect sigmaR expression. After blocking CaN in CaN/NFAT pathway, sigmaR expression increased dramatically as compared to A β treated cells, but the sigmaR expression remained unchanged in random scRNA group as compared to A β treated cells. This indicates that extracellular A β accumulation may affect the sigmaR protein expression via CaN/NFAT pathway.

Discussion

AD is a neurodegenerative disease characterized by progressive cognition impairment. Currently, there are no measures for the early prevention, diagnosis and effective treatment of AD, and it is estimated that there are more than 90 million people affected by AD worldwide in 2050 [17]. AD is pathologically characterized by neurofibrillary tangles, senile plaque formation and neuron loss. A β is a major component of senile plaque. It may selectively increase N-methyl-D-aspartate (NMDA) receptor activity and induce the calcium influx via the NMDA receptor channel, resulting in calcium overload and subsequent neuronal death [18].

Sigma-1 (σ 1) receptor is a G protein-coupled receptor containing 223 amino acid residues and has a high expression in the limbic system including the hippocampus. Recent studies indicate that Sigma receptor play important roles in the neuroprotection, improvement of learning disability and psychiatry treatment [19]. Some investigators employ PET for the detection of Sigma-1 (σ 1) receptor, and results showed the Sigma-1 (σ 1) receptor density and activity in the hippocampus and cortex of AD patients are significantly lower than in subjects without cognition impairment. Under normal condition, Sigma-1 (σ 1) receptor localizes the membrane of mitochondrion related endoplasmic reticulum and forms calcium sensitive chaperone structure with BIP. In the presence of specific activator (such as cocaine and pentazocine), Sigma-1 (σ 1) receptor separates from BIP, translocates to other compartments in cells and then regulates IP3 receptor, NMDA receptor or dopamine receptor on endoplasmic reticulum or sodium, potassium and calcium

ion channels [20-22]. In addition, it may also affect intracellular TCA cycle and ATP production, regulate oxidative stress and mitochondrial function, and modify the secretion of some neurotransmitters (such as glutamate, dopamine, norepinephrine, acetylcholine, γ -aminobutyric acid). Thus, it is generally accepted that Sigma-1 (σ 1) receptor is crucial for the pathogenesis and development of AD. A variety of studies confirm that Sigma-1 (σ 1) receptor is able to regulate PI3K-Akt-mTOR signaling pathway to attenuate A β toxicity, promote neuronal growth in the hippocampus and regulate the expression of apoptosis related genes (such as Bax, Bcl-2 and caspase-3) to inhibit neuronal apoptosis [23, 24].

BIP is a member of HSP70 family in the endoplasmic reticulum and may maintain the normal protein conformation, regulate protein spatial localization and recruit E3 ubiquitin ligase for ubiquitination. BIP on the endoplasmic reticulum may bind to sigma σ -1 receptor, which is important for the maintenance of normal function of sigma σ -1 receptor [25, 26]. In our study, real time PCR and Western blot assay were employed to detect BIP expression. Results showed the mRNA and protein expression of BIP reduced significantly. In addition, Western blot assay was employed to detect the protein expression of BIP and its binding protein. Results confirmed the binding between BIP and sigma σ -1 receptor, and ubiquitin group was also detectable in the complex. This phenomenon was reversed after treatment with ubiquitin ligase inhibitor and siRNA. Thus, we speculate that, after A β treatment, BIP recruits ubiquitin ligase E3, leading to the ubiquitination and degradation of sigma σ -1 receptor.

To further explore the mechanism underlying the A β induced BIP expression, bioinformatics was employed to analyze the promoter sequence of BIP gene. Results showed there were NFAT binding sites in the promoter of BIP gene. NFAT is a transcription factor family and plays important role in the gene transcription during immune response. The NFAT activation is regulated by calcium/calmodulin-dependent protein phosphatase C. NFAT is a factor that may regulate multiple cell processes such as T cell activation, differentiation and auto-tolerance. Several studies have confirmed that the pathogenesis of some acute and chronic diseases (such as bronchial asthma, Alzheimer's

disease and diabetes) is closely related to the NFAT activation. In our study, results showed A β induced CaN activation was able to induce the nuclear translocation of NFAT, leading to the increase in transcriptional activity of NFAT. Bioinformatics analysis was employed to predict BIP (HSPA5) as a potential target of NFAT. Detection of BIP expression indicated that BIP expression was related to the nuclear translocation of NFAT. To further confirm whether NFAT is able to initiate BIP expression, NFAT-BIP, luciferase reporter gene expression system was constructed. In the presence of full length BIP promoter, the NFAT1 induced transcription of luciferase reduced. When the promoter was only 500 bp in length, NFAT failed to induce the transcription of luciferase. Experiment on the mutation of NFAT binding site in BIP promoter further confirmed the deficiency of NFAT binding site in the BIP promoter, which displayed a reduced transcription of BIP. When the three binding sites became mutant, NFAT failed to induce the transcription of luciferase. These findings indicate that NFAT is able to bind to the promoter of BIP gene to regulate BIP expression.

To investigate the role of CaN/NFAT pathway in the A β induced degradation of SigmaR, CaN was inhibited *in vitro*. In the presence of A β at a high concentration, siRNA target CaN was able to inhibit CaN and block CaN/NFAT signaling pathway. After inhibition of CaN mRNA expression, the CaN activity reduced in N2A cells, accompanied by reductions in NFAT expression and BIP expression and increase in sigmaR expression. This was further confirmed by Western blot assay and immunofluorescence. This suggests that CaN/NFAT is important for the A β induced SigmaR degradation. These findings provide evidence for the therapy of AD targeting SigmaR.

Taken together, our findings indicate extracellular A β accumulation may induce calcium influx in neurons and activate CaN/NFAT signaling pathway to initiate BIP expression, which recruits ubiquitin ligase E3, leading to the ubiquitination and degradation of sigma σ -1 receptor. Thus, the neuroprotection of SigmaR is compromised, resulting in neuronal apoptosis.

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Disclosure of conflict of interest

None.

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