Cell Mol Neurobiol (2016) 36:1151–1159
DOI 10.1007/s10571-015-0311-6

ORIGINAL RESEARCH

Genistein Inhibits Aβ25–35-Induced Synaptic Toxicity and Regulates CaMKII/CREB Pathway in SH-SY5Y Cells

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Received: 7 September 2015 / Accepted: 24 November 2015 / Published online: 11 December 2015

Abstract Genistein (Gen), as a functional food in human diet, has shown many beneficial effects on neurodegenerative diseases such as Alzheimer's disease (AD). But the neuroprotective mechanism of Gen is not clear. Because synaptic failure is considered as the earliest phase in the pathogenesis of AD, we try to validate our hypothesis that synapse may be one target of Gen on protecting neurons. In this study, SH-SY5Y cells were pre-incubated with or without Gen for 2 h followed by the incubation with Aβ25–35 (25 μmol/L) for another 24 h. Flow cytometry, Western Blots, and RT-PCR analysis were used to test the synaptic factors. The data showed that Gen pre-treatment could reverse the Aβ25–35-induced down-regulation of synaptophysin and postsynaptic marker postsynaptic density-95. In addition, the down-regulation of NR1 and NR2B induced by Aβ25-35 which are subunits of *N*-methyl-D-aspartate receptor also could be antagonized by pre-treatment of Gen. Moreover, the factors of CaMKII/CREB signaling pathway were detected. The results showed that mRNA and protein expressions of (Ca^{2+})/calmodulin(CaM), CaMKII/pCaMKII, and CREB/pCREB were significantly down-regulated by Aβ25–35, but they were all restored by the pre-treatment of Gen. Furthermore, Gen also maintained the intracellular Ca^{2+} concentration which was disturbed by Aβ25–35. In conclusion, these results suggested that Gen could protect synaptic dysfunction induced by Aβ, and the mechanism might be associated with the regulation of synaptic markers and Ca^{2+} level through activating CaM/CaMK/CREB signaling pathway.

Keywords Genistein · Synaptic plasticity · Synaptophysin · Calcium · CREB · Neuroprotection

Abbreviations

Aβ	β-Amyloid peptides
AD	Alzheimer's disease
SYN	Synaptophysin
PSD-95	Postsynaptic density-95
NMDAR	*N*-Methyl-D-aspartate receptor
CaM	Calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CREB	cAMP response element binding protein

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by cognitive decline and loss of memory function (Goedert and Spillantini 2006). More than 35 million people worldwide are suffering from AD, and the number of patients will increase to 115 million by the year of 2050 (Querfurth and LaFerla 2010). It is reported that β-amyloid peptide (Aβ)-forming senile plaques, hyperphosphorylated tau-generating neurofibrillary tangles, reduced synaptic density and neuronal loss are important pathological features in AD patients (Querfurth and LaFerla 2010). Evidences suggest that the

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accumulation of Aβ plays a key role in synaptic dysfunction which could cause the cognitive impairment in AD. Moreover, synaptic failure is considered as the earliest changes in the pathogenesis of AD (Ripoli et al. 2013). Therefore, looking for a safe and effective substance which can reverse the synaptic failure induced by Aβ may be an effective way to delay the progression of AD.

Increasing evidence demonstrates that genistein (Gen) has significant protective effects on Aβ-induced neurotoxicity (Liao et al. 2013). It is reported by our previous studies and others that Gen could modulate anti-apoptotic factors activities, control cell survival, inhibit tyrosine kinase activity, regulate the Akt and Mitogen-activated protein kinase (MAPK) signaling pathways (Yu et al. 2009; Alvarez-Florez et al. 2013). It is also shown in our study that Gen could attenuate oxidative stress by maintaining redox balance and stabilizing mitochondrial membrane integrity (Xi et al. 2011). In addition, we also find Gen could suppress Aβ25–35-induced reactive oxygen species (ROS) over-production in synaptosomes in rat brain to inhibit the early phase of the pathogenesis of AD (Ding et al. 2011).

Although the neuroprotective effects of Gen were demonstrated, whether Gen could exert protection on synapse and how its potential mechanisms work are still unclear. In this study, we tried to certify our hypothesis that if Gen could reverse Aβ-induced mRNA and protein disordered expression of synaptic marker synaptophysin (SYN) and postsynaptic marker postsynaptic density-95 (PSD-95), overloaded calcium (Ca^{2+}), disturbed expressions of the factors in Ca^{2+}-related calmodulin (CaM)/Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)/cAMP response element binding protein (CREB) signaling pathway and subunits of *N*-methyl-D-aspartate receptor (NMDAR). The results of this study will further illustrate the role of Gen on protecting synapse and reveal its potential mechanism on preventing cognitive impairment.

Materials and Methods

Materials

Human neuroblastoma cell line (SH-SY5Y) was obtained from Peking Union Medical Centre Laboratory (Beijing, China). Gen, Aβ25–35 was purchased from Sigma-Aldrich (USA), dissolved in deionized water at concentration of 625 μM and kept at −20 °C until use. Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), and penicillin (10,000 units/ml)/streptomycin (10,000 μg/ml) (P/S) were purchased from Gibco Biotechnology Company (USA).

Cell Culture

SH-SY5Y was cultured in DMEM supplemented with FBS (10 %) and penicillin (100 U/ml)/streptomycin (100 U/ml) at 37 °C in the atmosphere of CO_2 (5 %)/air (95 %). SH-SY5Y was seeded at an appropriate density (1×10^6 cells/cm^2) in culture dishes. The culture medium of SH-SY5Y was replaced every 2–3 day. All the experiments were manipulated in the protocol that SH-SY5Y was pre-treated with or without Gen (50 μM) for 2 h followed by treatment with Aβ25–35 (25 μM) for another 24 h, and DMEM was added to the control group correspondingly.

Flow Cytometry

Fluctuation of the intracellular Ca^{2+} level of SH-SY5Y was observed by Flow cytometry. Cell was washed twice with Hank's Balanced Salt Solution (HBSS) which is Ca^{2+} and Mg^{2+} free. Then HBSS was discarded and 0.25 % trypsin was added with the following of the collection of suspension. Fluorescent Ca^{2+}-sensitive dye (Fluo4-AM) was added into cell suspension (Invitrogen-Gibco-Molecular Probes, Karlsruhe, Germany) at the final concentration of 5 μmol/L, and then incubated 30 min in 37 °C and darkness. After the incubation, the cell suspension was centrifuged at 1000 r/min for 5 min and supernatant was discarded, then precipitate was washed with HBSS three times and the final cell suspension was collected. After that, the cell suspension was measured at excitation wavelength 488 nm, emission wavelength 526 nm by FACS (Aria, BD, USA). The fluorescence intensity value of 1×10^4 cells in each group was recorded, which is the reaction of intracellular free Ca^{2+} concentration. Each sample was set up in triplicate within the detection, and at least three independent experiments were performed.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA of SH-SY5Y was purified by Trizol reagent (Invitrogen, Carlsbad, CA, USA). The mRNA expression of SYN, PSD-95, NMDAR subtype NR1 and NR2B, CaM, CaMK, CREB, and β-actin (invariant control) were analyzed by RT-PCR. Reverse-transcription (RT) was carried out by using reverse transcriptase kit (Promega Corporation, Madison, WI, USA). All PCR primers were designed and synthesized by Sangon Biotech, China. The primer sequence is shown in Table 1. PCR was carried out in accordance with our previous study (Xi et al. 2012).

Table 1 Primer sequences and annealing temperature

Primer	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Annealing temperature (°C)
β-Actin	CCTGGGCATGGAGTCCTGTG	TCCTTCTGCATCCTGTCGGC	60
SYN	CTCCACTCCTCCCAACTCTG	CCACACCTCCTCTCCAAGAC	60
PSD-95	GGGAGATGGAATACGAGGAA	CAAACAGGATGCTGTCGTTG	60
NR1	CACCTCCGTCACGCACAAG	AGGGGTGGGAGTGAAGTGG	58
NR2B	GAAGATGGCTACCAGATG	CCAGGGTCACAATGCT	54
CaM	CGGTTCTAGCAAACACCA	GGAGGGCTTCAACTTATG	54
CaMKII	TCTGTCAACGATCCACG	CTGAGAAGTTCCTGGAGAC	52
CREB	AACCAGCAGAGTGGAGAT	CTTGAACTGTCTGCCCAT	54

Western Blot Analysis

The cells were chipped off by cell scrapers with PBS. Then cell suspension was centrifuged at 800×*g* for 5 min. After that, the liquid supernatant was discarded and the RIPA buffer was added into cells. The cells were lysed for 40 min at 4 °C by shaking in RIPA buffer, and then centrifugated at 12,000×*g* for 20 min. The supernatant was collected for protein analysis. The concentration of total protein was detected by BCA protein assay kit (Pierce Biotechnology, USA). Protein samples (50 μg) were loaded and separated by electrophoresis in 12 or 10 % SDS-acrylamide gel, and then transferred to polyvinylidene fluoride blots at 60 V for 2 h. 5 % non-fat dry milk dissolved in Tris-Buffered Saline Tween-20 (TBST) was used to block the transferred membrane at room temperature for 1 h. The primary antibodies for protein analysis include anti-SYN, anti-PSD-95, anti-CaMKII, anti-CREB, and anti-pCREB Ser133 which were purchased from Cell Signaling Technology. And the antibodies for anti-CaM, anti-pCaMKII (Thr-286), anti-NR1, and anti-NR2 B were purchased from Abcam. Primary antibody was individually incubated with membrane at 4 °C overnight. After the primary antibody reaction, membrane was washed with TBST three times and incubated with proper secondary antibody for 1 h. At the end, membrane was washed three times with TBST. The Proto Blot VRII kit (Promega) was used to detect the signal. FluoChem VRFC2 (Alpha Innotech Corporation) was used to photograph and analyze the gray value of the protein expression in each group. Every experiment was repeated at least three times.

Statistical Analysis

All data were expressed as mean ± S.D. Differences between groups were determined by appropriately using one-way ANOVA with SPSS 11.5 (Chicago, America). All statistical tests were two-sided, and a significant value was set as $P < 0.05$.

Results

Gen Regulated the mRNA and Protein Expressions of SYN and PSD-95

The disordered expressions of SYN and PSD-95, impairment markers of synaptic plasticity, have been well testified in early and late stages of AD (Shao et al. 2011). To evaluate the protective effects of Gen on synapse, mRNA and protein expressions of presynaptic marker SYN and postsynaptic maker PSD-95 were detected. Results showed that the mRNA and protein expressions of SYN (Fig. 1) and PSD-95 (Fig. 2) were significantly decreased in Aβ25–35 group compared with that in control group. On the contrary, Gen pre-treatment could reverse the reduction of mRNA and protein expressions of SYN (Fig. 1) and PSD-95 (Fig. 2) induced by Aβ25–35.

Gen Protected the Expression of Synapse-Related NMDAR

The NMDARs, located in the postsynaptic density (PSD), are related in the formation of synaptic plasticity. NR1 and NR2 which are the subunits of NMDARs play the key role on regulating synaptic depression or potentiation which can lead to the neuron death (Ryan and Grant 2009). Our results showed that Aβ25–35 could significantly decrease the mRNA and protein expressions of NR1 (Fig. 3) and NR2B (Fig. 4) in SH-SY5Y. However, Gen pre-treatment could increase the mRNA and protein expressions of NR1 and NR2B compared with the Aβ25–35 treatment only (Figs. 3, 4).

Gen Modulated Fluctuation of the Intracellular Ca^{2+} Level

As we know, Ca^{2+} flux through NMDARs is a critical factor in synaptic plasticity. The fluctuation of Ca^{2+} level is a second messenger to activate specific signaling

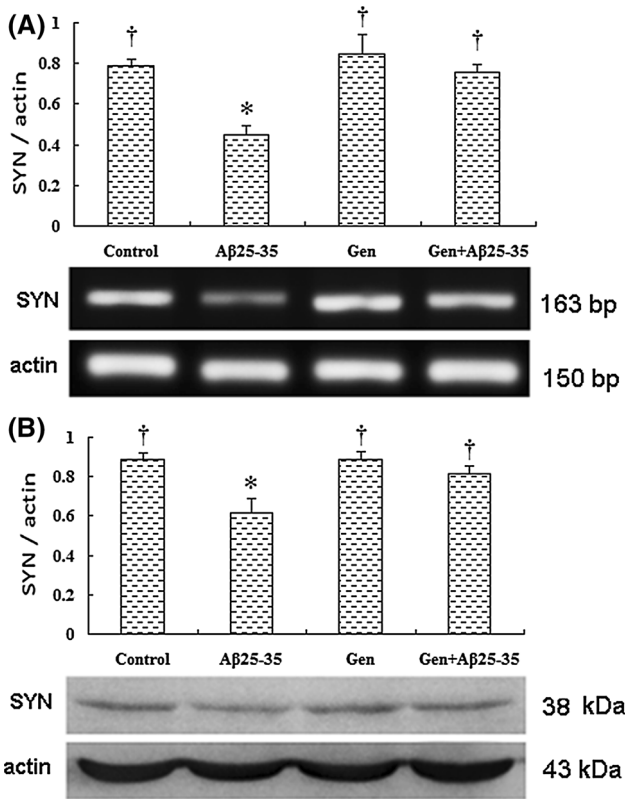

Fig. 1 The mRNA (**a**) and protein (**b**) expressions of Synaptophysin (SYP) in SH-SY5Y cells. They were measured by RT-PCR and Western Blot from untreated cells (Control group); cells exposed to 25 μM Aβ25–35 (Aβ25–35 group); cells exposed to 50 μM genistein alone (Gen group); cells exposed to 50 μM genistein 2 h before 25 μM Aβ25–35 was added (Gen + Aβ25–35 group). All the data were shown as mean ± S.D. $*P < 0.05$ compared with Control group; $\dagger P < 0.05$ compared with Aβ25–35 group

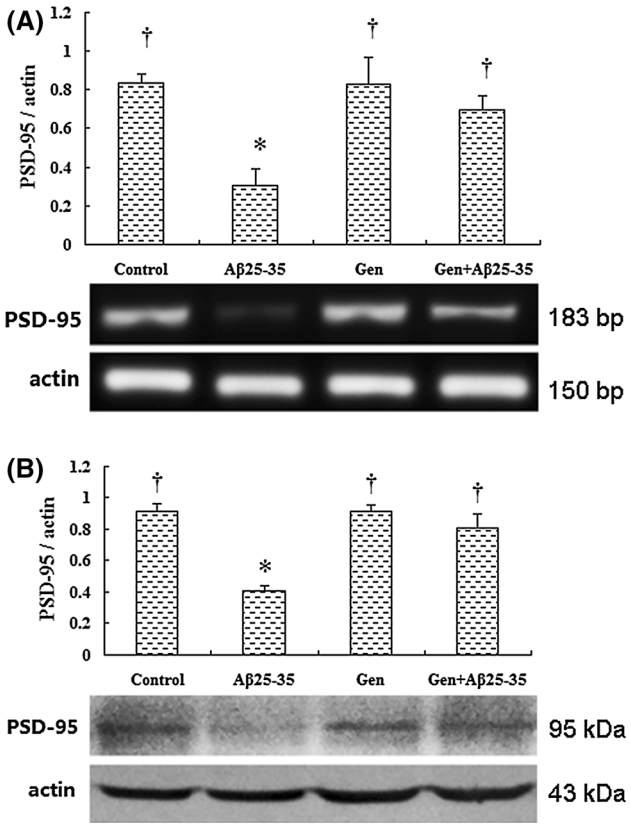

Fig. 2 The mRNA (**a**) and protein (**b**) expression of PSD-95 in SH-SY5Y cells. They were measured by RT-PCR and Western Blot from untreated cells (Control group); cells exposed to 25 μM Aβ25–35 (Aβ25–35 group); cells exposed to 50 μM genistein alone (Gen group); cells exposed to 50 μM genistein 2 h before 25 μM Aβ25–35 was added (Gen + Aβ25–35 group). All the data were shown as mean ± S.D. $*P < 0.05$ compared with Control group; $\dagger P < 0.05$ compared with Aβ25–35 group

pathways related to the structure and function of neuron, and it is also a basic cellular mechanism for learning and memory (Zheng et al. 2011). In our research, compared with control group, intracellular Ca^{2+} level accumulation in the cells of Aβ25–35 group was significantly increased (Fig. 5). However, the elevated accumulation of Ca^{2+} level caused by Aβ25–35 could be restrained after pre-treatment with Gen.

Gen Prevented the Changes of CaMK II/CREB Signaling Pathway

As a crucial intracellular messenger, Ca^{2+} is regulated by CaM/CaMKII signaling pathway which is a key to modulate the excitability of neurons (Bossuyt and Bers 2013). CREB is a unique transducer of long-term memory which can be activated by CaMKII cascade. It is important for the induction of input-specific synaptic plasticity (Nonaka et al. 2014). Our data showed that the mRNA and protein expressions of CaM (Fig. 6), CaMK II (Fig. 7) and CREB (Fig. 8) in the cells of Aβ25–35 group were significantly down-regulated compared with control group. Gen pre-treatment prevented the down-regulation of all above mRNA and protein induced by Aβ. Furthermore, the pCaMK II (Fig. 7) and pCREB (Fig. 8) were significantly declined in Aβ25–35 group, but they were up-regulated in Gen pre-treatment group.

Discussion

Synaptic failure is considered as the earliest phase of AD progression. It can lead to the progressive abnormal of synapses and neuronal circuits (Selkoe 2002). Over the past years, our team has attempted to find the effective phytochemicals which can resist or prevent Aβ-induced neuronal dysfunction. Gen, a main active ingredient of soybean isoflavone, has been proved to have anti-oxidative, anti-apoptosis, anti-inflammatory, and neuronprotective effects in neuron/astrocytes/cerebrovascular/mitochondria dysfunction induced by Aβ (Xi et al. 2012;

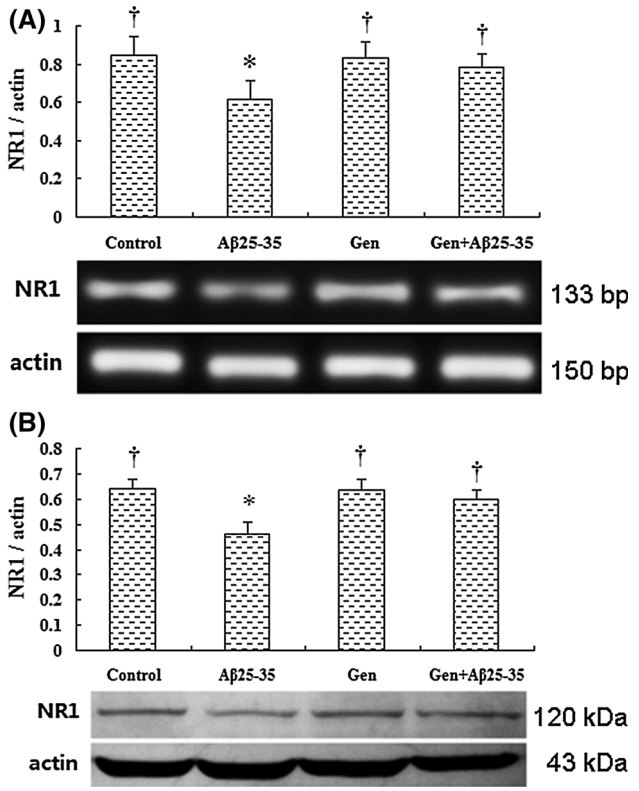

Fig. 3 The mRNA (**a**) and protein (**b**) expression of NR1 in SH-SY5Y cells. They were measured by RT-PCR and Western Blot from untreated cells (Control group); cells exposed to 25 μM Aβ25–35 (Aβ25–35 group); cells exposed to 50 μM genistein alone (Gen group); cells exposed to 50 μM genistein 2 h before 25 μM Aβ25–35 was added (Gen + Aβ25–35 group). All the data were shown as mean ± S.D. $*P < 0.05$ compared with Control group; $\dagger P < 0.05$ compared with Aβ25–35 group

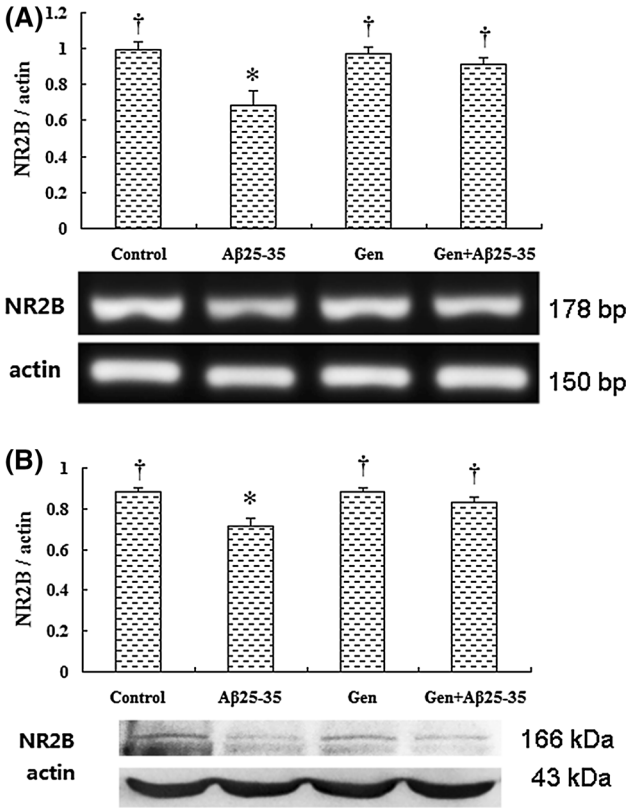

Fig. 4 The mRNA (**a**) and protein (**b**) expression of NR2B in SH-SY5Y cells. They were measured by RT-PCR and Western Blot from untreated cells (Control group); cells exposed to 25 μM Aβ25–35 (Aβ25–35 group); cells exposed to 50 μM genistein alone (Gen group); cells exposed to 50 μM genistein 2 h before 25 μM Aβ25–35 was added (Gen + Aβ25–35 group). All the data were shown as mean ± S.D. $*P < 0.05$ compared with Control group; $\dagger P < 0.05$ compared with Aβ25–35 group

Ma et al. 2013; Feng et al. 2012). Other studies also demonstrated that Gen could ameliorate both memory impairment and Aβ-induced neuronal death (Bagheri et al. 2012). There is evidence (Luo et al. 2012) to suggest that pre-treatment with Gen could significantly increase cell viability and PKC activity, decrease the levels of intracellular calcium, attenuate Hoechst/PI staining and block caspase-3 activity in Aβ25–35-treated PC12 cells. In this study, we find that Gen can modulate the disordered synaptic markers, receptors and the factors of synapse-related signaling pathways, which have been described as the main characters in the pathogenesis of AD.

Decreased synaptic density and neuronal loss are significant pathological features of AD (Querfurth and LaFerla 2010). SYN is an integral membrane protein of small synaptic vesicles, and it has been identified as a target for synaptic structure and connectivity in many studies (Glantz et al. 2007). PSD is composed of postsynaptic scaffold protein which determines structural and functional integrity of excitatory synapses, and it is also composed of postsynaptic excitatory receptors (Chen et al. 2008). Evidence proves that presynaptic SYN and postsynaptic PSD-95 have been down-regulated in the brain of AD patients (Shao et al. 2011). It is reported that curcuminoids could up-regulate PSD-95, synaptophysin and CaMK IV expressions in hippocampus of AD rat model (Ahmed et al. 2010). It is also shown that high dose of soy germ phytoestrogens treatment significantly increased the synaptic formation proteins in the hippocampus of ovariectomized rats, such as synaptophysin, spinophilin, synapsin-1 and PSD-95 (Pan et al. 2010). In fact, our results showed that pre-treatment of Gen could reverse the reduction of mRNA and protein expressions of SYN and PSD-95 which reflect the prevention of early synaptic dysfunctions induced by Aβ25–35.

The NMDARs, which play a critical role in glutamatergic excitatory neurotransmission, are related in the formation of synaptic plasticity (Shao et al. 2011). It is reported soluble Aβ could lead to a loss of synaptic proteins by suppressing NR2A function which could be

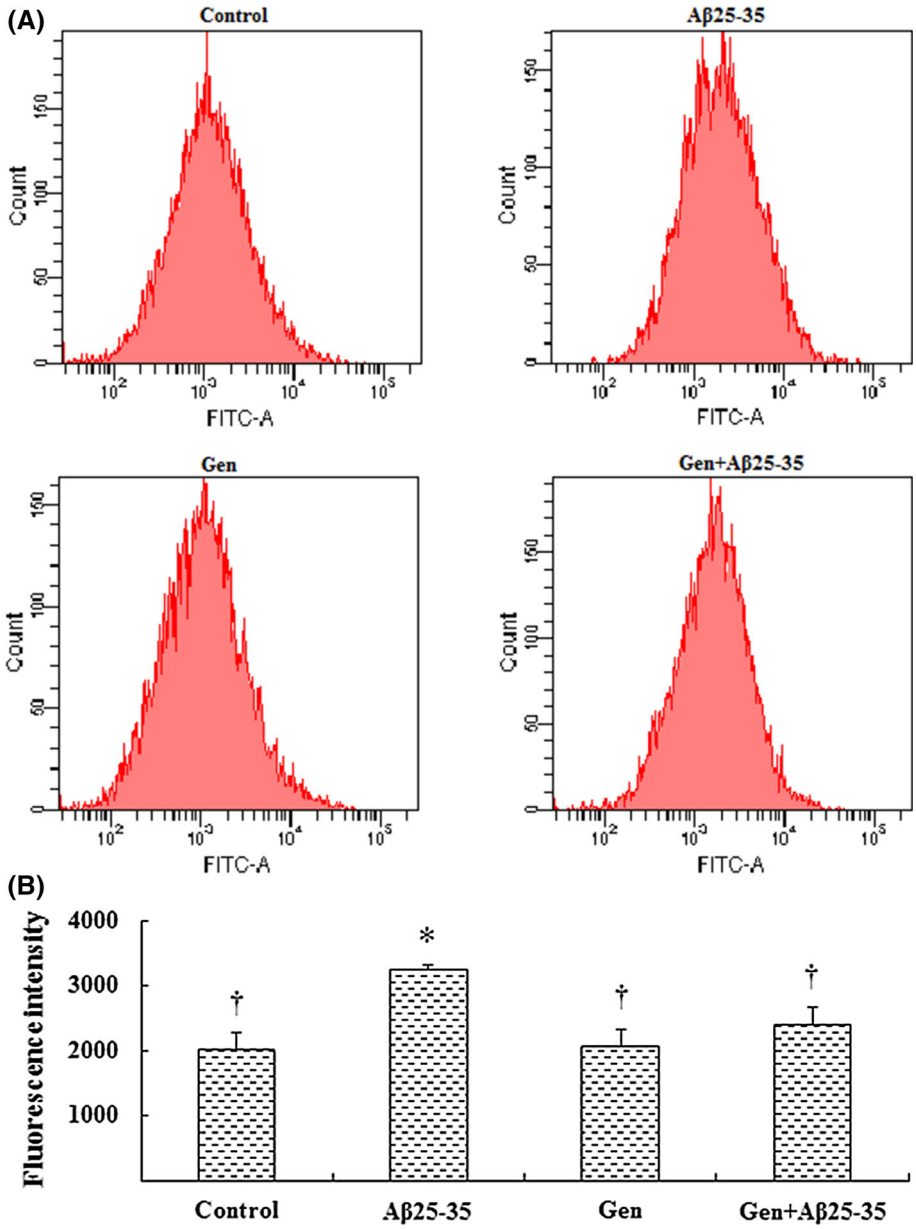

Fig. 5 The effect of Gen on the levels of Ca^{2+} in SH-SY5Y cells. The fluorescence intensity of cells was shown by the peak value from untreated cells (Control group); cells exposed to 25 μM Aβ25–35 (Aβ25–35 group); cells exposed to 50 μM genistein alone (Gen group); cells exposed to 50 μM genistein 2 h before 25 μM Aβ25–35 was added (Gen + Aβ25–35 group). All the data were shown as mean ± S.D. $*P < 0.05$ compared with Control group; † $P < 0.05$ compared with Aβ25–35 group

described as key targets for Aβ-induced neurotoxicity (Liu et al. 2010). In the postsynaptic compartment, Ca^{2+} entering the cell via NMDARs could cause Ca^{2+} release, and this progress could amplify the NMDAR-related signal which will result in the change of synaptic plasticity (Paula-Lima et al. 2013). Aβ oligomers could significantly increase extra-synaptic NMDA response, and the over-activation of NMDAR could cause the disorder of Ca^{2+} flux and impairment of synaptic plasticity (Li et al. 2011). However, it is shown that *Scutellaria baicalensis* extract could conduct neuroprotection through inhibiting the function of NMDAR by interacting with the glycine binding site (Yang et al. 2014). In addition, *Ginkgo biloba* extract could be used for inhibiting NMDA-evoked currents in cultured cortical cells (Szasz et al. 2008). Moreover, theanine, the green tea constituent, could down-regulate NMDA-dependent CA1-long-term potentiation (LTP) and up-regulate NMDA-independent CA1-LTP both in human and animal studies (Lardner 2014). Similarly, in our research, Gen can modulate the NMDAR subtype NR1/NR2B and inhibit over-accumulation of intracellular Ca^{2+}. These results suggested that Gen may protect synaptic dysfunction through regulating the NMDAR and Ca^{2+} flux.

In order to find out the mechanism of Gen on regulating the NMDAR and Ca^{2+} flux, we detected the factors of CaM/CaMKII/CREB signaling pathway. CREB is an

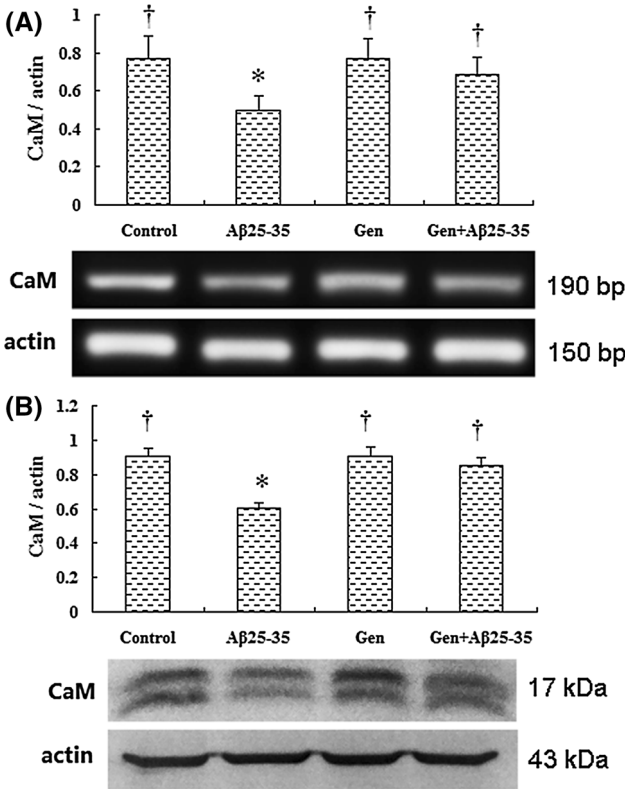

Fig. 6 The mRNA (**a**) and protein (**b**) expression of calmodulin (CaM) in SH-SY5Y cells. They were measured by RT-PCR and Western Blot from untreated cells (Control group); cells exposed to 25 μM Aβ25–35 (Aβ25–35 group); cells exposed to 50 μM genistein alone (Gen group); cells exposed to 50 μM genistein 2 h before 25 μM Aβ25–35 was added (Gen + Aβ25–35 group). All the data were shown as mean ± S.D. $*P < 0.05$ compared with Control group; $\dagger P < 0.05$ compared with Aβ25–35 group

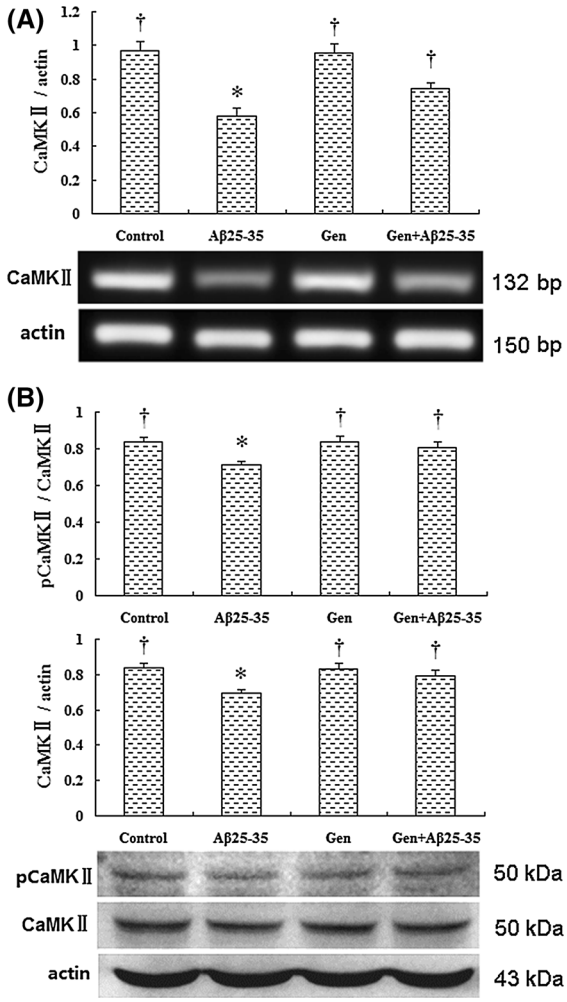

Fig. 7 The mRNA (**a**) and protein (**b**) expression of CaMKIIand pCaMKII(B) in SH-SY5Y cells. They were measured by RT-PCR and Western Blot from untreated cells (Control group); cells exposed to 25 μM Aβ25–35 (Aβ25–35 group); cells exposed to 50 μM genistein alone (Gen group); cells exposed to 50 μM genistein 2 h before 25 μM Aβ25–35 was added (Gen + Aβ25–35 group). All the data were shown as mean ± S.D. $*P < 0.05$ compared with Control group; $\dagger P < 0.05$ compared with Aβ25–35 group

important nuclear transcription factor in neuronal survival, neurogenesis, and synaptic plasticity. It has been shown that CREB signaling pathway has positive regulation effects on synaptic function and cognitive health in AD mice models (Gong et al. 2013). Numerous studies show that Aβ not only cause early synaptic dysfunctions, spine loss, and memory deficits, but also disturb intracellular Ca^{2+} homeostasis (Lazzari et al. 2015), which could consequently lead to a disordered signal transduction of CaM/CaMKII pathway (Liang et al. 2012). In our study, we found the expression of CaM, CaMKII/pCaMKII, and CREB/pCREB in both mRNA and protein levels were down-regulated after Aβ administration. However, Gen could reverse the disordered expressions of these factors induced by Aβ25–35 in this signaling pathway. These findings are consistent with a previous report that natural polyphenol resveratrol could activate CaMKII, which could conduct neuroprotection and modulate cell death (Kim et al. 2010). Our previous research also shows that intragastric administration of SIF could significantly improve impaired neuroplasticity and alleviate the down-regulated expressions of CaM, CaMK II, and CREB induced by the injection of Aβ1–42 into the lateral ventricle (Ding et al. 2013). Additionally, polyphenol pterostilbene could modulate the binding activity of CREB and SP-1 to the MMP-2 promoter by decreasing the protein expressions of CREB and SP-1 (Lin et al. 2014). All these results implied that Gen could play an important role on the expression and activity of CaM/CaMKII/CREB. Moreover, Gen might also affect the expression and activity of downstream molecules in this signaling pathway, and finally play a neuroprotective effect on chronic diseases such as AD.

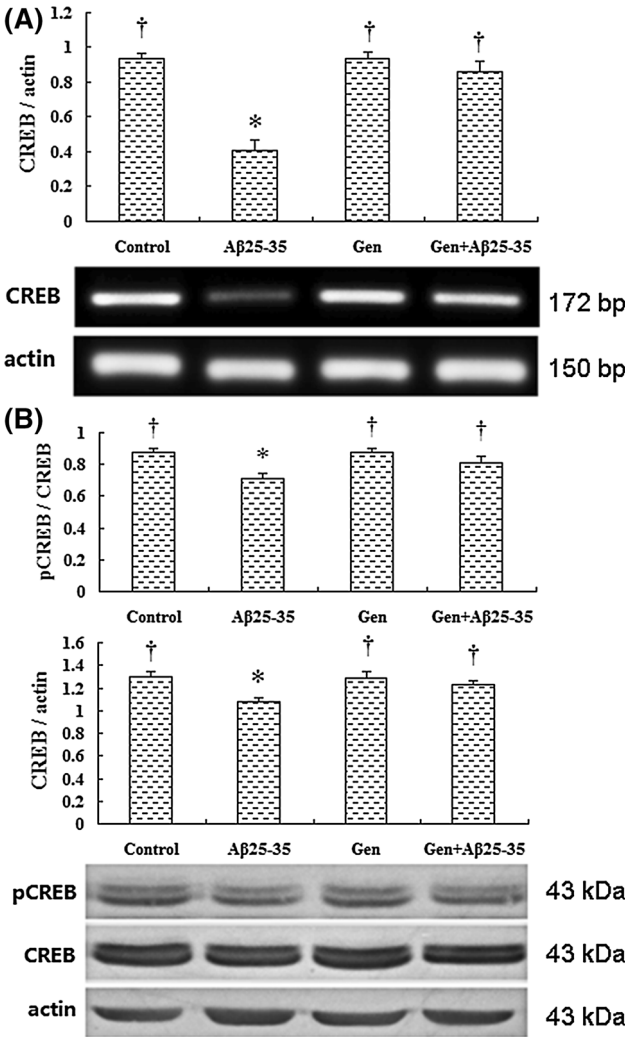

Fig. 8 The mRNA (**a**) and protein (**b**) expression of CREB and pCREB(B) in SH-SY5Y cells. They were measured by RT-PCR and Western Blot from untreated cells (Control group); cells exposed to 25 μM Aβ25–35 (Aβ25–35 group); cells exposed to 50 μM genistein alone (Gen group); cells exposed to 50 μM genistein 2 h before 25 μM Aβ25–35 was added (Gen + Aβ25–35 group). All the data were shown as mean ± S.D. $^{*}P < 0.05$ compared with Control group; †$P < 0.05$ compared with Aβ25–35 group

In conclusion, our results showed that pre-treatment with Gen significantly alleviated Aβ25–35-induced synaptic dysfunctions. Gen could inhibit the down-regulation of the presynaptic marker of SYN and postsynaptic marker of PSD-95 induced by Aβ25–35 through regulating intracellular Ca^{2+} levels and up-regulating the expression of factors in CaM/CaMKII/CREB signaling pathway. These findings suggest that flavonoids could protect synapses of neurons, which will promote the capacity of cell to resist the toxicity of Aβ. Simultaneously, these results could further illustrate that supplementation of Gen might be an effective way on preventing neurodegenerative diseases.

Acknowledgments This work was supported by National Natural Science Foundation of China (Nos. 81172661, 81302427 and 81330065), National High Technology Research and Development Program (863 Program) of China (No. 2010AA023003).

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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