Research Article

TRPV1 Modulator Ameliorates Alzheimer-Like Amyloid-β Neuropathology via Akt/Gsk3β-Mediated Nrf2 Activation in the Neuro-2a/APP Cell Model

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Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder for which there is no effective therapeutic strategy. PcActx peptide from the transcriptome of zoantharian *Palythoa caribaeorum* has recently been identified and verified as a novel antagonist of transient receptor potential cation channel subfamily V member 1 (TRPV1). In the present study, we further investigated the neuroprotective potential of PcActx peptide and its underlying mechanism of action, in an N2a/APP cell model of AD. Both Western blot and RT-PCR analysis revealed that PcActx peptide markedly inhibited the production of amyloid-related proteins and the expression of BACE1, PSEN1, and PSEN2. Moreover, PcActx peptide notably attenuated the capsaicin-stimulated calcium response and prevented the phosphorylation of CaMKII and CaMKIV (calcium-mediated proteins) in N2a/APP cells. Further investigation indicated that PcActx peptide significantly suppressed ROS generation through Nrf2 activation, followed by enhanced NQO1 and HO-1 levels. In addition, PcActx peptide remarkably improved Akt phosphorylation at Ser 473 (active) and Gsk3β phosphorylation at Ser 9 (inactive), while pharmacological inhibition of the Akt/Gsk3β pathway significantly attenuated PcActx-induced Nrf2 activation and amyloid downregulation. In conclusion, PcActx peptide functions as a TRPV1 modulator of intercellular calcium homeostasis, prevents AD-like amyloid neuropathology via Akt/Gsk3β-mediated Nrf2 activation, and shows promise as an alternative therapeutic agent for AD.

1. Introduction

Alzheimer's disease (AD), which is one of the most prevalent neurodegenerative disorders, is clinically characterized by a progressive pattern of memory loss coupled with cognitive impairment [1, 2]. Studies have shown that AD contributes to 60-70% of all dementia cases [3, 4]. Senile plaques (SP) formed by extracellular accumulation of amyloid-β (Aβ) peptide and neurofibrillary tangles (NFTs) formed by intracellular deposition of hyperphosphorylated microtubule-associated tau protein are two critical pathological features

of AD [5, 6]. Current pharmacological approaches for treating AD mostly target toxic Aβ and tau proteins; no effective treatments are available for halting or reversing the progression of the disease, although some therapies may be able to alleviate symptoms and improve patient quality of life [7]. Aβ peptides arise from sequential proteolytic cleavage of transmembrane amyloid precursor protein (APP) by β-secretase (BACE1) and the γ-secretase complex, which is called amyloidogenic pathway [8, 9]. Although the precise mechanisms underlying the Aβ-related pathogenesis of AD remain unclear, accumulating evidence suggests that

calcium homeostasis perturbation and oxidative stress could drive the onset and progression of AD [10, 11]. Aβ deposition in the synapse could prompt the generation of reactive oxygen species (ROS) and disruption of calcium homeostasis, thereby triggering cell death and neurodegeneration [12]. Meanwhile, excessive intercellular calcium in turn enhances ROS generation, which leads to oxidative stress, mitochondrial dysfunction, and calcium signaling dysregulation [11]. Therefore, oxidative stress is both a cause and consequence of calcium homeostasis disruption. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key modulator of the endogenous antioxidative defense against oxidative stress. Under oxidative stress conditions, Nrf2 dissociates with Kelch-like ECH-associated protein 1 (Keap1), translocates to the nucleus, and then dimerizes with small Maf family proteins and binds to antioxidant response element (ARE) [13]. The interaction of Nrf2 and ARE promotes the transcription of downstream genes encoding phase II response antioxidative enzymes, such as heme oxygenase 1 (HO-1) and NAD(P)H quinone oxidoreductase (NQO1), to counteract oxidative stress [14]. Meanwhile, the protein kinase B (PKB or Akt)/glycogen synthase kinase 3β (Gsk3β) pathway is involved in the activation of Nrf2 by phosphorylation [15]. Since multiple reports have confirmed that activation of Nrf2 may prompt neuroprotective activity in neurodegenerative models, we assume that Akt/Gsk3β-mediated Nrf2 activation could be a target for preventing AD pathology [16–18].

Multiple protein and peptide drugs have been identified as having therapeutic potential for various neurological diseases [19–22]. Kunitz-type peptides can cross the blood-brain barrier (BBB) [23–25] and exhibit neuroprotective activity *in vivo* and *in vitro* [26–28]. PcActx peptide, a Kunitz-like peptide derived from zoantharian *Palythoa caribaeorum*, was the first transient receptor potential cation channel subfamily V member 1 (TRPV1) antagonist of zoantharian species discovered in our previous study [29]. PcActx peptide prevented pentylenetetrazol- (PTZ-) induced seizure-related behavior and inhibited ROS overproduction through regulating calcium and GABAergic-glutamatergic signaling [29]. TRPV1 is a calcium-permeable channel, and its activation impairs calcium homeostasis and induces the oxidative stress; this may contribute to cell death and neurodegeneration [30–32]. Kim *et al.* reported that the TRPV1 agonist capsaicin elicits cell death in neurons and microglia. TRPV1 activation increases the intercellular calcium concentration, triggers mitochondrial damage, and induces cytochrome c release and caspase-3 cleavage, which are all involved in AD pathogenesis. However, these effects are attenuated by the TRPV1 antagonist capsazepine and iodoresiniferatoxin (IRTX) [31, 32]. Furthermore, IRTX, a TRPV1 antagonist, can inhibit Aβ-induced ROS production and microglia priming, suggesting that the TRPV1 channel could be implicated in microglia-induced oxidative stress in AD [33]. In contrast to the inhibitory effect of TRPV1 antagonism on Aβ production, capsaicinoids (capsaicin and dihydrocapsaicin), as TRPV1 agonists, increase Aβ production by enhancing the expression of β and γ-secretase and inhibiting the Aβ

degradation [34]. Calcium influx via the TRPV1 channel triggers Gsk3β activation, which stimulates the phosphorylation and ubiquitination of Nrf2 [35]. Consequently, modulation of TRPV1-mediated calcium homeostasis may be a novel target for therapeutic strategies for AD. In the present study, we aimed to investigate the neuroprotective potential and mechanism of action of PcActx peptide in the Neuro-2a cells stably transfected with the APP gene (N2a/APP cell), as an *in vitro* experimental model of AD.

2. Materials and Methods

2.1. Chemicals and Reagents. PcActx peptide was produced via an oxidative folding method after synthesizing the linear form, as described in our previous research [29]. Dulbecco's modified Eagle's medium (DMEM, Cat. No.: 12800017), fetal bovine serum (FBS, Cat. No.: 1805387), penicillin-streptomycin (PS, Cat. No.: 15140163), phosphate-buffered saline (PBS, Cat. No.: 21600010), and trypsin (Cat. No.: 25200072) were purchased from Gibco (Carlsbad, CA, USA). G418 (Cat. No.: A1720), DMSO (Cat. No.: D2650), thiazolyl bluet tetrazolium bromide (MTT, Cat. No.: M5655), PMSF protease inhibitor (Cat. No.: P7626), and capsaicin (Cat. No.: M2028) were purchased from Sigma (St. Louis, MO, USA). LY294002 (Cat. No.: S1737), RIPA lysis buffer (Cat. No.: P0013C), nuclear and cytoplasmic protein extraction kit (Cat. No.: P0028), HRP-linked anti-rabbit/mouse secondary antibody (Cat. No.: A0208/A0216), DCFH-DA (Cat. No.: S0033), and DAPI (Cat. No.: C1005) were obtained from Beyotime Company (Shanghai, China). The lactate dehydrogenase (LDH) kit (Cat. No.: 11644793001) and Transcriptor First Strand cDNA Synthesis Kit (Cat. No.: 04379012001) were purchased from Roche Applied Science (Penzberg, Germany). Capsazepine (Cat. No.: HY15640) was purchased from MCE (NJ, USA). Fluo-4/AM (Cat. No.: F14201) was purchased from Invitrogen (San Diego, CA, USA). SDS-polyacrylamide gel (Cat. No.: 1610172) and PVDF membrane (Cat. No.: 1620177) were purchased from Bio-Rad (Hercules, CA, USA). ECL Select Western Blotting Detection Reagent kit (Cat. No.: 12644055) was purchased from GE Healthcare (Chicago, IL, USA). Antibodies against Aβ 1-40 (Cat. No.: 12990), Aβ 1-42 (Cat. No.: 14974), PSEN1 (Cat. No.: 5643), PSEN2 (Cat. No.: 9979), p-CaMKII (Thr 286, Cat. No.: 12716), CaMKII (Cat. No.: 11945), CaMKIV (Cat. No.: 4032), Nrf2 (Cat. No.: 12721), Keap1 (Cat. No.: 8047), NQO1 (Cat. No.: 62262), HO-1 (Cat. No.: 43966), p-Akt (Ser 473, Cat. No.: 4060), Akt (Cat. No.: 4691), p-Gsk3β (Ser 9, Cat. No.: 5558), Gsk3β (Cat. No.: 9315), GAPDH (Cat. No.: 2118), and lamin B (Cat. No.: 13435) and Alexa-Fluor-488 conjugated anti-rabbit IgG secondary antibody (Cat. No.: 4412) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against APP (Cat. No.: ab32136), BACE1 (Cat. No.: ab183612), and TRPV1 (Cat. No.: ab6166) were purchased from Abcam (Cambridge, UK). Antibody against p-CaMKIV (Thr 196, Cat. No.: PA5-37833), protease inhibitor cocktail (Cat. No.: 78446), and BCA Protein Assay Reagent (Cat. No.: 23225) were purchased from Thermo Fisher Scientific (Waltham, MA,

USA). Antibody against Nrf2 (for immunofluorescence assay; Cat. No.: 66504) was purchased from Proteintech (Rosemont, IL, USA). RNAprep Pure Cell/Bacteria Kit (Cat. No.: 4992235) was purchased from TIANGEN (Beijing, China). SYBR® Premix Ex Taq™ II Kit (Cat. No.: RR82LR) was purchased from TaKaRa (Shiga, Japan).

2.2. Cell Culture and Treatment. Wild-type N2a (N2a/WT) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). N2a cells stably expressing human Swedish mutant APP695 gene (N2a/APP, also called N2S) were gifted from Prof. Jia-Hong Lu (University of Macau, Macao, China) [36]. Cells were maintained in DMEM medium containing 10% (v/v) FBS and 1% (v/v) PS and incubated in a humidified atmosphere of 5% CO_2 at 37°C. G418 (1%, v/v) was dissolved in culture medium to select transfected N2a/APP cells.

N2a/WT cells were used as a control, and N2a/APP cells were used as a cell model of AD. After seeding for 24 h, cells were treated with DMSO or PcActx peptide at different concentrations (5 or 10 μm) for 24 h. In the following experiments, cells were exposed to LY294002 (10 μm) for 2 h prior to 24 h PcActx peptide incubation. PcActx peptide was dissolved in dd-H_2O, and LY294002 was dissolved in DMSO, as a stock solution (10 mM). Both solutions were stored at -20°C for bioactive assay.

2.3. Cell Viability and Cytotoxicity Assay. Cell viability and cytotoxicity were measured by MTT and LDH assay, respectively. Briefly, N2a/WT and N2a/APP cells were placed in 96-well culture plates (8×10^3 cells/well) for 24 h and subjected to the indicated treatments. The medium was collected for LDH detection according to the manufacturer's protocol, while the remaining cells were incubated with MTT to assess viability. The absorbance at 490 nm for LDH assay and 570 nm for MTT assay were measured using the FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Calcium Measurement. A calcium marker (Fluo-4/AM) was employed to evaluate the calcium response of the cultured cells. Cells were seeded on a 12-well plate and pretreated with capsazepine (2.5 μM, TRPV1 antagonist) or PcActx peptide (5 and 10 μM) for 4 h. Cells were then stained with 2 μM Fluo-4/AM in the dark at 37°C for 30 min in Tyrode's solution, and excess Fluo-4/AM probe was washed three times with Tyrode's solution. Subsequently, the cells were challenged with 250 μM capsaicin (TRPV1 agonist), and the calcium response (FITC) was immediately estimated using the Cell^R imaging system of an IX81 florescence microscope (Olympus Corp., Tokyo, Japan).

2.5. Western Blot Analysis. The whole-cell proteins were extracted by ice-cold RIPA lysis buffer using protease and phosphatase inhibitor cocktail. Subcellular fractionations were prepared using nuclear and cytoplasmic protein extraction kits. The total protein concentration was measured by BCA protein assay reagent. Aliquot protein samples (30 μg) were electrophoresed in 10% or 12% SDS-polyacrylamide gel and transferred onto PVDF membranes. Membranes were blocked by 5% nonfat milk for 1 h and then incubated with specific primary antibodies (1 : 1000 dilution) overnight at 4°C. The blots were subsequently incubated with HRP-conjugated anti-rabbit/mouse secondary antibodies (1 : 2000 dilution) and visualized with an ECL kit using the Bio-Rad ChemiDoc XRS Imaging System. The densitometry of the bands was quantified via the ImageJ software (NIH, Bethesda, MD, USA).

2.6. Quantitative Real-Time PCR. Total RNA and cDNA from N2a/WT and N2a/APP cells were prepared using the RNAprep Pure Cell/Bacteria Kit and Transcriptor First Strand cDNA Synthesis Kit, respectively, following the standard protocol. RT-PCR was conducted using SYBR® Premix Ex Taq™ II on the Vii7 Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturer's instructions. The mRNA level was calculated through the $2^{-\Delta\Delta CT}$ method after normalization to GAPDH. The primer sequences used are listed in Table 1.

2.7. ROS Detection. Intracellular ROS levels were estimated by the fluorescent probe, DCFH-DA. After treating the cells with PcActx peptide for 24 h, they were incubated with DCFH-DA (10 μM, diluted in DMEM) for 30 min at 37°C in the dark. Excess DCFH-DA was washed off by PBS. The fluorescence intensity (FITC-A) of each group was monitored using the BD Accuri C6 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For each sample, at least 10,000 events were recorded, and the obtained data were quantitatively analyzed by the FlowJo software (FlowJo LLC, Ashland, OR, USA).

2.8. Immunofluorescence Assay. Cells (5×10^4 cells/well) were seeded in a 24-well plate for 24 h and treated with PcActx peptide for another 24 h. After that, they were fixed by 3.7% PFA for 15 min and permeabilized by 0.3% Triton X-100 (diluted in PBS) for 20 min. Then, the cells were blocked with 2% BSA for 1 h and subsequently incubated with anti-Nrf2 primary antibody (1 : 500) overnight at 4°C. Cells from all group were further incubated with Alexa-Fluor-488 conjugated anti-rabbit IgG secondary antibody (1 : 1000) for 1 h in the dark at room temperature. DAPI staining was applied for nuclei observation. The samples were imaged using the cellSens imaging system of an IX73 florescence microscope (Olympus Corp.).

2.9. Statistical Analysis. All statistical analyses were performed using the GraphPad Prism software (ver. 8.0; GraphPad Software, Inc., San Diego, CA, United States). Results are presented as means ± standard deviation (SD). The normality and homogeneity of variance of the data were assessed using the D'Agostino-Pearson and Bartlett' tests, respectively. All data were found to be normally distributed and homogeneous, and one-way ANOVA was used for the analysis, followed by Dunnett's test for multiple comparisons. $p < 0.05$ was considered as statistically significant.

TABLE 1: Primers for RT-PCR.

Gene	Primer sequence 5'-3'
APP	Forward - TTTGGCACTGCTCCTGCT
	Reverse - CCACAGAACATGGCAATCTG
APLP1	Forward - ACACCTGGGTCCCAGTGAAT
	Reverse - GAGAGGACGATGAGGGAGCC
APLP2	Forward - CGACGGCACCATGTCAGAC
	Reverse - CAACGAGGCATCACGGC
BACE1	Forward - GCAGGGCTACTACGTGGAGA
	Reverse - CAGCACCCACTGCAAAGTTA
PSEN1	Forward - TACAAGTACCGCTGCTACAAGTTC
	Reverse - GCACTTCCCCAAGGTAGATATAGG
PSEN2	Forward - TACAAGTACCGCTGCTACAAGTTC
	Reverse - GCACTTCCCCAAGGTAGATATAGG
GAPDH	Forward - TGCACCACCAACTGCTTAGC
	Reverse - GGCATGGACTGTGGTCATGAG

3. Results

3.1. PcActx Peptide Showed No Obvious Cytotoxicity in N2a/WT or N2a/APP Cells.
The viability and cytotoxicity in N2a/WT and N2a/APP cells after 24 h exposure to PcActx peptide (Figure 1(a)) were firstly detected by MTT and LDH assay. The MTT results (Figures 1(b) and 1(d)) indicated that treatment with PcActx peptide at dosages of 0.3-10 μM did not impact the viability of N2a/WT or N2a/APP cells. Similar results were obtained via LDH assay (Figures 1(c) and 1(e)): PcActx peptide did not cause obvious cytotoxicity in N2a/WT or N2a/APP cells. Moreover, precipitation was observed in the cultured medium when the concentrations were >10 μM. Therefore, N2a/WT and N2a/APP cells were treated with PcActx peptide for 24 h (maximum concentration of 10 μM) in further investigations.

3.2. PcActx Peptide Attenuated APP Processing and Amyloid Protein Accumulation in N2a/APP Cells.
Aβ protein (including Aβ 1-40/42) is the principal hallmark of AD and is generated by the alternative splicing of APP designated β-secretase (BACE1) and the γ-secretase complex [8, 9]. Presenilins (PSEN1 and PSEN2) are the major constituents of γ-secretase and mediate APP processing [37]. In addition, amyloid precursor-like proteins (APLP1 and APLP2) are highly homologous to APP, which belong to the APP family. Accumulating evidence indicates that APLPs accumulate in SP in the AD brain, suggesting that APLPs might contribute to AD-associated neurodegeneration [38–40]. In comparison with N2a/WT cells (Figures 2(a) and 2(b)), the protein levels of APP, Aβ 1-40, and Aβ 1-42 were higher in N2a/APP cells, where N2a cells were engineered to express the recombinant human APP695 gene. However, treatment with PcActx peptide for 24 h significantly decreased the levels of APP, Aβ 1-40, and Aβ 1-42 in N2a/APP cells. Consistent with the Western blot results, PcActx peptide obviously reduced the mRNA levels of APP, APLP1, and APLP2 in N2a/APP cells (Figures 2(c)–2(e)).

To further confirm the protective effect of PcActx peptides in the N2a/APP cell model of AD, the protein and mRNA levels of proteolytic enzymes implicated in APP processes were determined by Western blot and RT-PCR, respectively. As depicted in Figures 3(a) and 3(b), the protein levels of BACE1, PSEN1, and PSEN2 were markedly downregulated following 24 h PcActx peptide treatment. Moreover, both 5 and 10 μM PcActx peptide markedly inhibited the gene expression of BACE1, PSEN1, and PSEN2, in parallel to the N2a/APP group without PcActx peptide treatment (Figures 3(c)–3(e)). These results indicated that PcActx peptide could interfere with APP processing and inhibited abnormal amyloid protein production.

3.3. PcActx Peptide Inhibited TRPV1-Dependent Calcium Accumulation and TRPV1/CaMK Activation in N2a/APP Cell.
The TRPV1 channel plays a pivotal role in maintaining calcium homeostasis, which subsequently mediates mitochondrial and synaptic function, as well as neuronal survival and development [41]. In our previous study, PcActx peptide efficaciously suppressed the calcium influx evoked by capsaicin, which is a TRPV1 agonist, in HEK293/hTRPV1 cells, indicating that PcActx peptide is a potential TRPV1 channel blocker [29]. In the present study, we also investigated the effect of PcActx peptide on the TRPV1-mediated calcium response in N2a/APP cells. As can be seen in Figures 4(a) and 4(b), 250 μM capsaicin induced dramatic calcium influx into the N2a/APP cell. However, the capsaicin-stimulated calcium influx was notably counteracted by pretreatment with PcActx peptide and capsazepine (a TRPV1 channel antagonist) for 4 h. Furthermore, PcActx peptide obviously decreased the phosphorylation of CaMKII (Thr 286) and CaMKIV (Thr 196) (Figures 4(c) and 4(d)) in a dose-dependent manner, which can be aberrantly activated by intracellular calcium accumulation [42]. In contrast, PcActx peptide did not affect TRPV1 protein expression (Figures 4(c) and 4(d)). The above results demonstrated that PcActx peptide can modulate the calcium response through TRPV1 channel inactivation, without affecting the TRPV1 protein expression level.

3.4. PcActx Peptide Activated the Nrf2/Keap1 Pathway and Promoted Nrf2 Nuclear Translocation in N2a/APP Cells.
Since neurons are highly susceptible to oxidative damage, oxidative stress is commonly implicated in AD etiology. Activation of the Nrf2 pathway and its subsequent upregulation of the antioxidative system trigger protective mechanisms against oxidative insult [15]. Flow cytometry analysis (Figures 5(a) and 5(b)) showed that PcActx peptide at concentrations of 5 and 10 μM markedly attenuated the excessive production of ROS in N2a/APP cells. As shown by the Western blot results, PcActx peptide, especially at 10 μM, remarkably enhanced cytoplasmic and nuclear Nrf2 expression (Figures 5(d), 5(f), and 5(g)). The elevation of nuclear Nrf2 FITC fluorescence induced by PcActx peptide revealed by immunofluorescence assay (Figure 5(c)) also supported these observations, where PcActx peptide obviously facilitated the nuclear localization of Nrf2 in N2a/APP cells. In addition, PcActx peptide effectively decreased Keap1
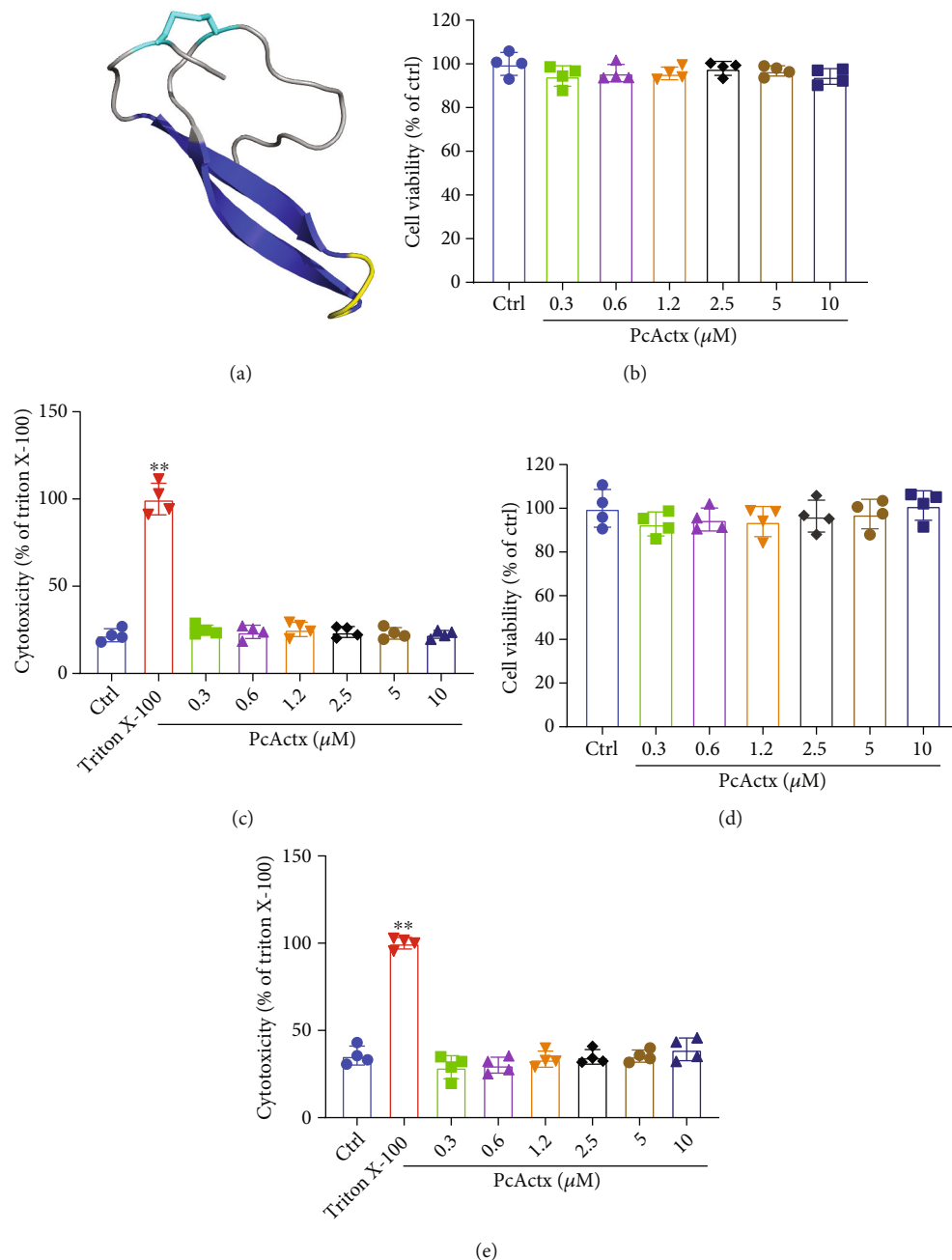
(a)

(b)

(c)

(d)

(e)

FIGURE 1: Cell viability of and cytotoxicity in N2a/WT and N2a/APP cells after 24 h of PcActx peptide treatment. MTT and LDH assay were employed to detect cell viability and cytotoxicity, respectively. (a) Homology model structure of PcActx peptide. Molecules are displayed as cartoons and colored according to the secondary structure: α-helix in purple, β-sheet in blue, β-turn in yellow, coil in gray, and disulfide bond in cyan. (b, c) Viability of N2a/WT cells and cytotoxic effects of PcActx peptide. (d, e) Viability of N2a/APP cells and cytotoxic effects of PcActx peptide. Data are expressed as the mean ± SD of four independent experiments, in duplicate or triplicate. $^{**}p < 0.01$ vs. Ctrl group.

expression and increased the levels of NQO1 and HO-1 in N2a/APP cells (Figures 5(e) and 5(h)). These data provided evidence that the activation of Nrf2/Keap1 pathways might be involved in the antioxidative effects of PcActx peptide in N2a/APP cells.

3.5. PcActx-Induced Nrf2/Keap1 Activation and Aβ Downregulation Might Be Mediated by the Akt/Gsk3β Pathway. Akt activation inhibits Gsk3β activity, thereby promoting Nrf2 activation and nuclear translocation [15]. Western blot results showed that treatment with PcActx peptide obviously increased the phosphorylation of Akt at Ser 473 (active) and Gsk3β at Ser 9 (inactive) within 60 min, without affecting the total levels of Akt or GSK3β (Figures 6(a)–6(c)). To determine whether this signaling is involved in the neuroprotective effect of PcActx peptide, N2a/APP cells were preincubated with an Akt inhibitor (LY294002) for 2 h prior to 24 h PcActx treatment. We
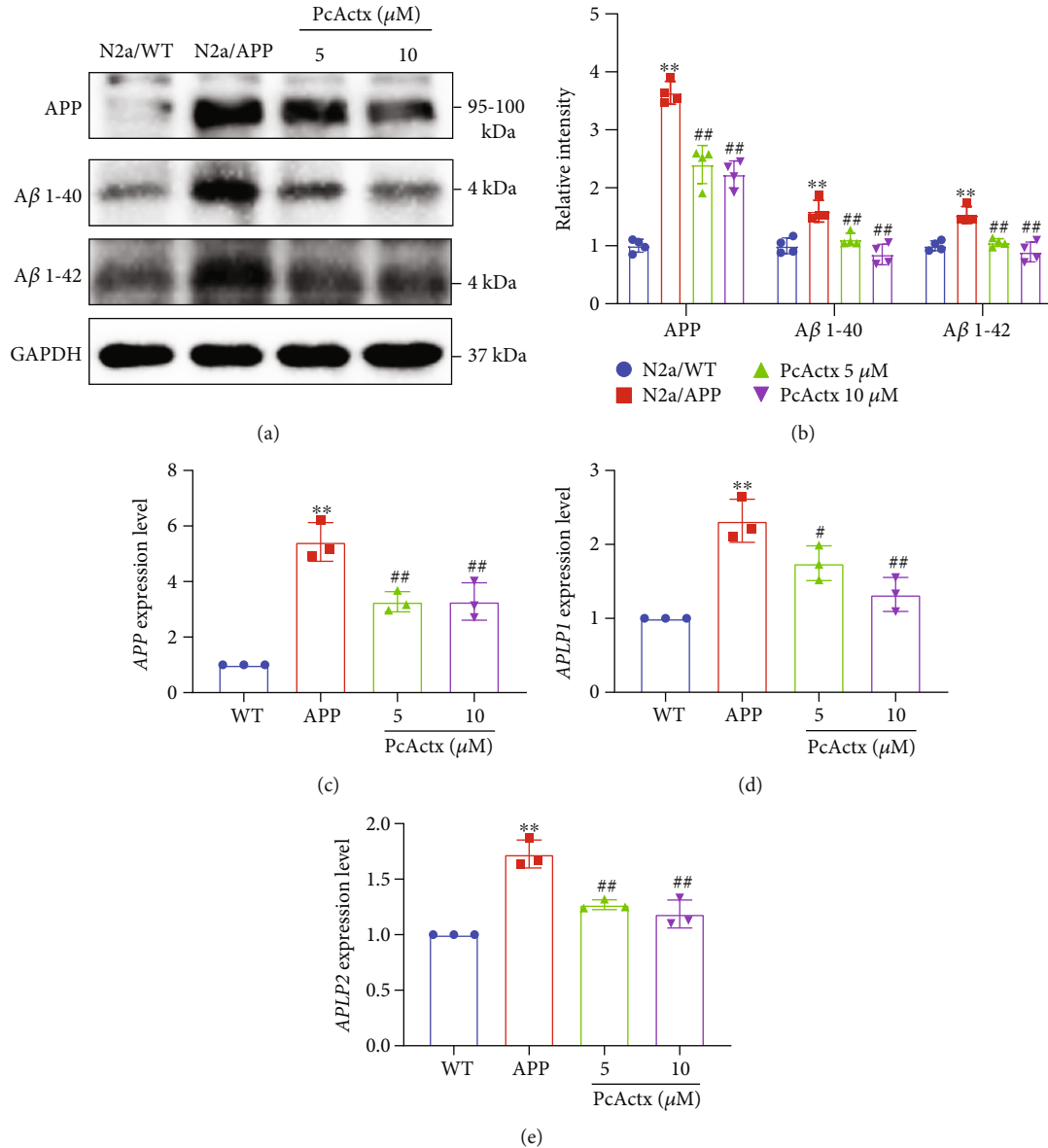
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FIGURE 2: PcActx peptide inhibited the accumulation of APP, Aβ, and APLP proteins. (a, b) The levels of APP, Aβ 1-40, and Aβ 1-42 were determined by Western blot analysis. (c–e) The expression of *APP*, *APLP-1*, and *APLP-2* was detected by RT-PCR. Data are expressed as the mean ± SD of three or four independent experiments, in duplicate or triplicate. $^{*}p < 0.05$ vs. N2a/WT group, $^{**}p < 0.01$ vs. N2a/WT group, $^{#}p < 0.05$ vs. N2a/APP group, and $^{##}p < 0.01$ vs. N2a/APP group.

found that the Akt inhibitor significantly counteracted the PcActx-induced upregulation of cytoplasmic and nuclear Nrf2 expression (Figures 6(d)–6(f)). Furthermore, compared to the PcActx-treated group, the enhanced expression of NQO1 and HO-1 seen in N2a/APP cells was also clearly reversed by the Akt inhibitor (Figures 6(g) and 6(h)). Conversely, the PcActx-mediated inhibitory effects on Keap1 were abolished by the Akt inhibitor (Figures 6(g) and 6(h)). Consistent results were observed in in terms of the expression of APP, Aβ 1-40, and Aβ 1-42 (Figures 6(i) and 6(j)). These findings demonstrated that PcActx-induced Nrf2/Keap1 activation and amyloid downregulation were mediated by Akt/Gsk3β signaling.

4. Discussion

Bioactive peptide toxins derived from cnidarians may confer neuroprotective effects due to their interaction with virous ion channels [43]. In our pervious study, PcShK and PcKuz peptides from *P. caribaeorum* exhibited potential as voltage-gated potassium channel (K_V) inhibitors and significantly suppressed 6-OHDA-induced neurotoxicity in a Parkinson's disease (PD) model [27, 44]. PpVα peptide from *P. variabilis* was shown to interact with the voltage-gated sodium (Na_V) channel and displayed neuroprotective effects against PTZ-induced epileptic seizure and 6-OHDA-induced neurotoxicity [45]. Interestingly, among all of the bioactive peptides
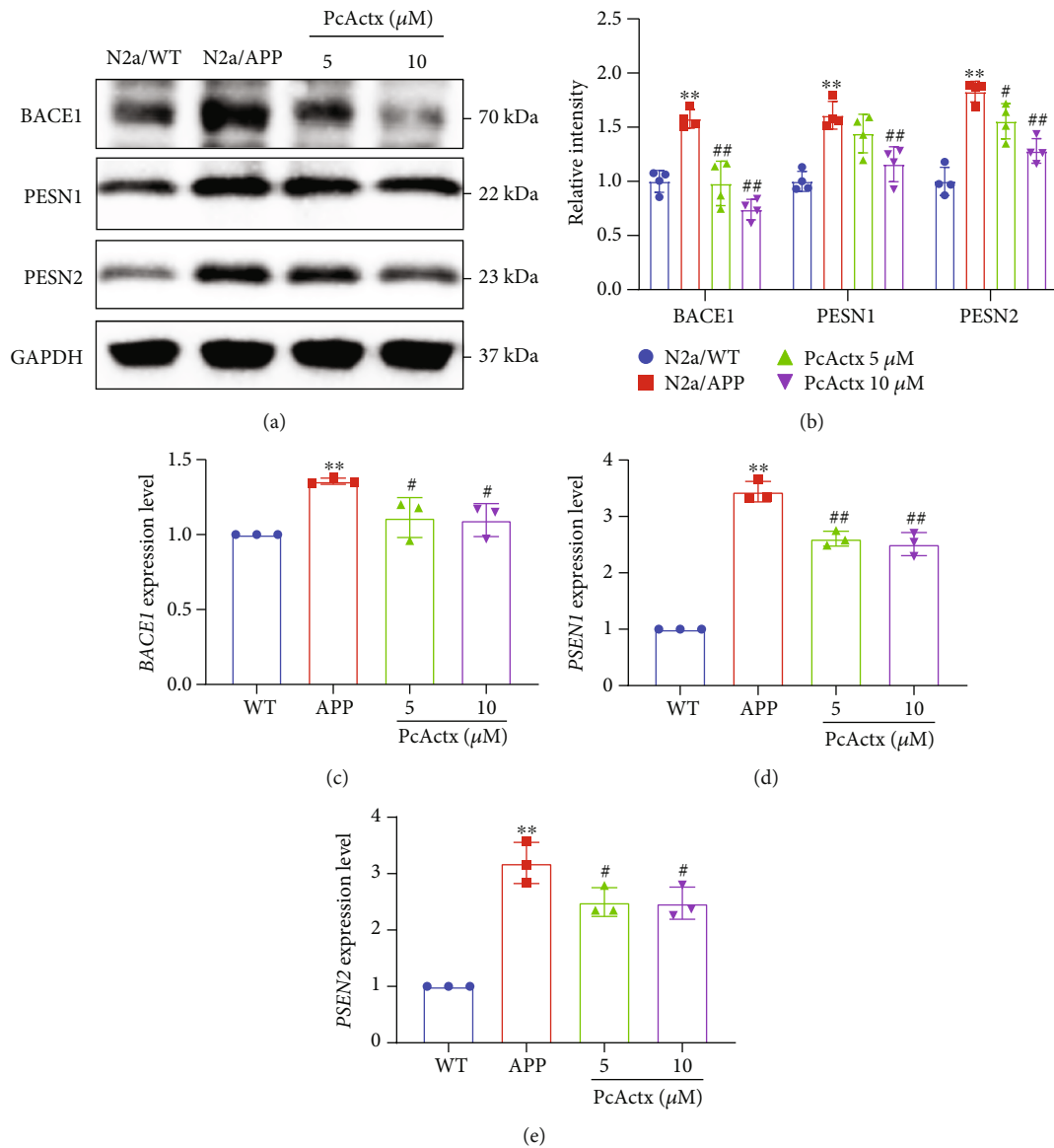
(a)

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FIGURE 3: PcActx peptide ameliorated the β- and γ-secretase-dependent cleavage of APP. (a, b) Protein levels of BACE1, PSEN1, and PSEN2 were determined by Western blot analysis. (c–e) mRNA expression of *BACE1*, *PSEN1*, and *PSEN2* was detected by RT-PCR. Data are expressed as the mean ± SD of three or four independent experiments, in duplicate or triplicate. $^*p < 0.05$ vs. N2a/WT group, $^{**}p < 0.01$ vs. N2a/WT group, $^#p < 0.05$ vs. N2a/APP group, and $^{##}p < 0.01$ vs. N2a/APP group.

from Cnidarian identified in this and previous studies, to best of our knowledge, PcActx peptide obtained from the transcriptome of *P. caribaeorum* is the first TRPV1 inhibitor identified and also exhibited antiepileptic activity by reducing ROS production and regulating calcium and GABAergic-glutamatergic signaling; thus, PcActx peptide might exert neuroprotective effects [29]. In the current study, we further demonstrated the neuroprotective potential of PcActx peptide and the underlying mechanism, from a new perspective by employing the N2a/APP cell line as an AD model.

The presence of extracellular amyloid plaques is an important histopathological characteristic in AD. Oligomeric species of Aβ (oAβ) can induce synaptic dysfunction, thereby contributing to the learning and memory impair-

ment seen during AD progression [46, 47]. Additionally, Aβ aggregation may also cause the calcium homeostasis disruption, mitochondria dysfunction, and oxidative stress, eventually leading to neuronal cell death [48]. In the present study, we employed the stably transfected N2a cell with human Swedish mutant APP, which is a well-characterized cell model of AD, to evaluate the effect of PcActx peptide on amyloid neuropathology. PcActx peptide obviously reduced the protein levels of APP, Aβ 1-40, and Aβ 1-42 (Figures 2(a) and 2(b)). Similar results were observed in terms of the mRNA expression of *APP*, *APLP1*, and *APLP2* (Figures 2(c)–2(e)). APLPs are homologues of APP, which play an important role in synaptic plasticity, neurite outgrowth, neural cell migration, and neural network function [49, 50]. APLP2 can compete with APP and be processed
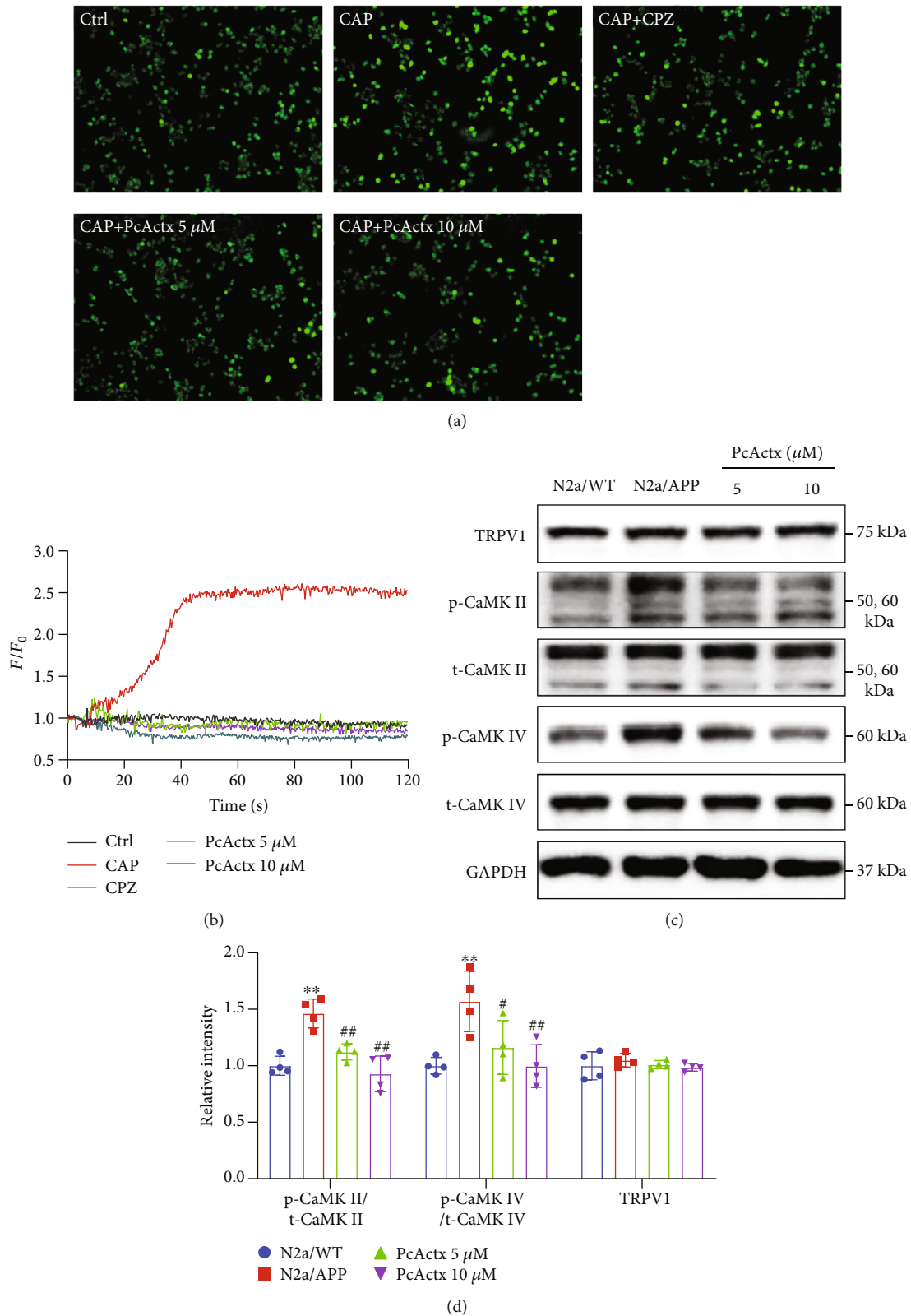
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FIGURE 4: PcActx peptide inhibited TRPV1-dependent calcium accumulation and TRPV1/CaMK activation. (a) Representative fluorescence images of the intracellular calcium response of N2a/APP cells (CAP: capsaicin and CPZ: capsazepine). (b) Time-dependent calcium response revealed by the green fluorescence intensity in representative plots ($n = 5$) before (F_0) and after capsaicin addition (F). (c, d) Protein expression levels of TRPV1 and total and phosphorylated CaMKII and CaMKIV were determined by Western blot analysis. Data are expressed as the mean ± SD of three or four independent experiments, in duplicate or triplicate. $^*p < 0.05$ vs. N2a/WT group, $^{**}p < 0.01$ vs. N2a/WT group, $^\#p < 0.05$ vs. N2a/APP group, and $^{\#\#}p < 0.01$ vs. N2a/APP group.

(a)
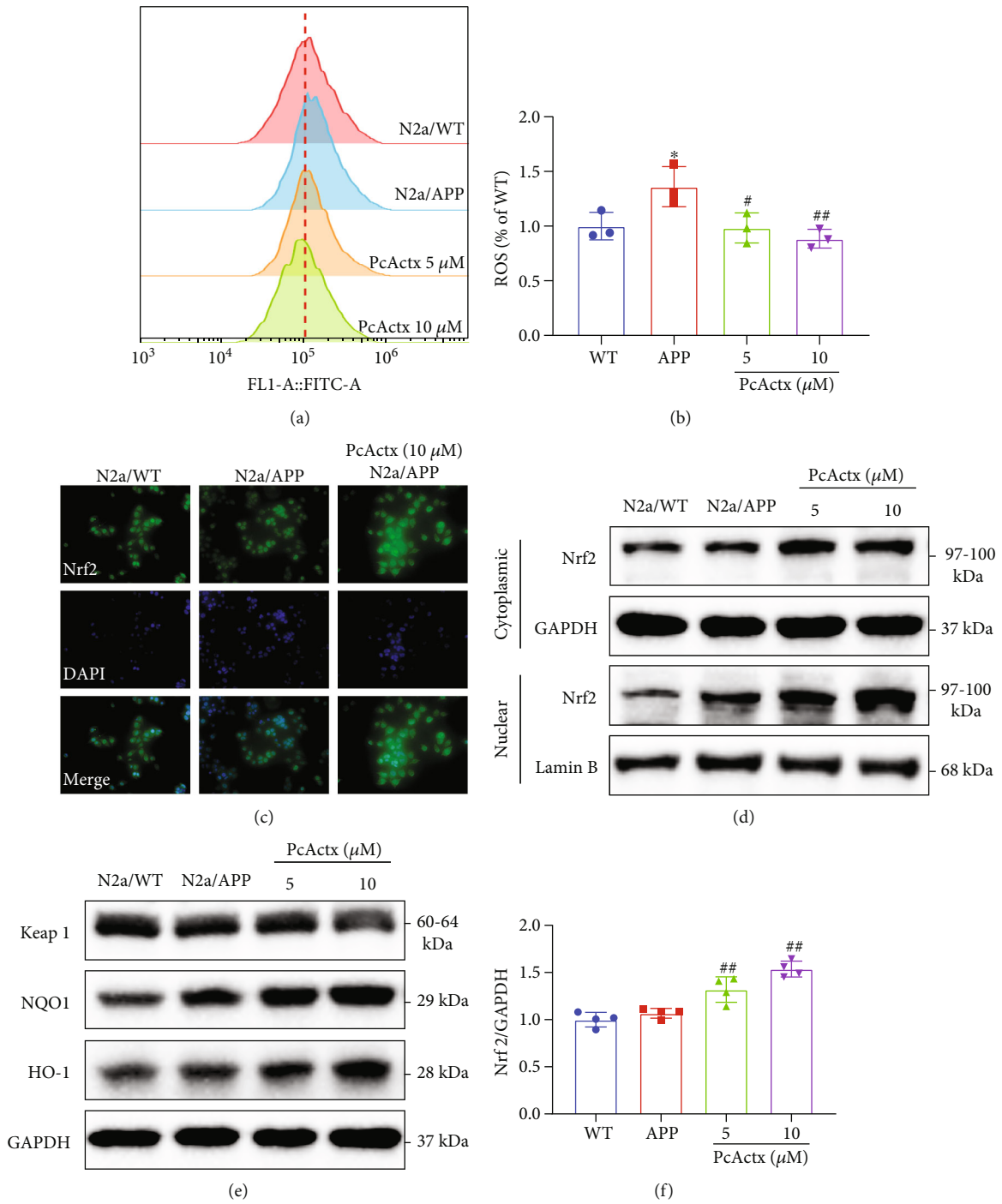

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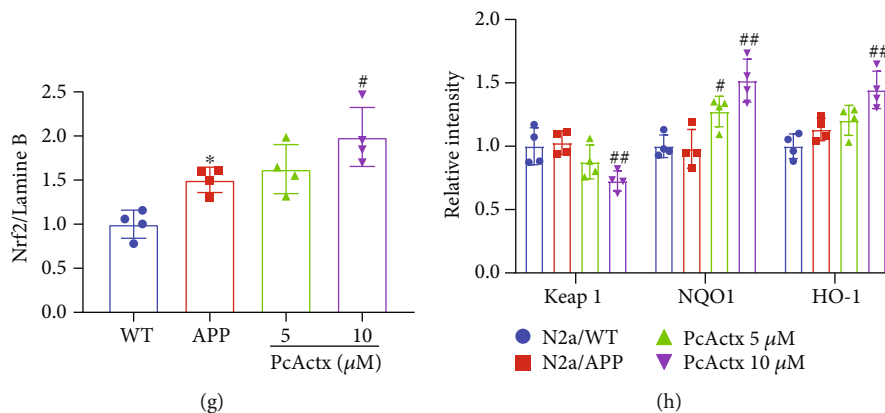

(e)

(f)

FIGURE 5: Continued.

(g)

(h)

FIGURE 5: PcActx peptide inhibited oxidative stress and induced Nrf2/Keap1 activation in N2a/APP cells. (a, b) Flow cytometry analysis of the effect of PcActx peptide on ROS production. (c) Representative immunofluorescence images of Nrf2 nuclear translocation in each group. (d–h) Levels of Nrf2, Keap1, NQO1, and HO-1 were determined by Western blot analysis. Data are expressed as the mean ± SD of three or four independent experiments, in duplicate or triplicate. $^*p < 0.05$ vs. N2a/WT group, $^{**}p < 0.01$ vs. N2a/WT group, $^#p < 0.05$ vs. N2a/APP group, and $^{##}p < 0.01$ vs. N2a/APP group.

by β- and γ-secretases, which may suppress Aβ production [50]. However, equivocal results were also found, where APLPs are highly expressed along with SP in the AD brain, suggesting that APLPs might play a role in AD pathogenesis [38–40]. In addition, the protein and mRNA levels of BACE1, PSEN1, and PSEN2 were significantly decreased by PcActx treatment (Figure 3). BACE1 and PSENs are responsible for the N-terminal and C-terminal cleavage of APP, respectively, and promote Aβ aggregation [37]. Furthermore, there is strong evidence that mutations in *PSENs* related to APP processing participate in early onset familial AD. These mutations could increase toxic Aβ production and aggregation, especially of Aβ 42, thus accelerating disease progression [51, 52]. These results suggested that one possible approach for preventing Aβ protein generation using PcActx peptide might be inhibition of β- and γ-secretase-dependent cleavage of APP.

TRPV1 is a calcium permeable channel expressed not only in the plasma membrane, but also in the endoplasmic reticulum [53]. It acts as a store-operated calcium channel and receptor-operated calcium channel and is responsible for maintaining intercellular calcium homeostasis [30]. Abnormal activation or dysregulation of TRPV1 could induce intracellular calcium overload and thus disrupt calcium homeostasis. Activation of the TRPV1 channel by capsaicinoids has been reported to increase Aβ production through effects on APP processing [34]. In turn, Aβ aggregation further disrupts the calcium-mediated channel, including the TRPV1 channel, and triggers calcium overload, subsequently exacerbating oxidative stress and mitochondrial dysfunction and finally inducing synaptic dysfunction and apoptosis [54–56]. PcActx peptide was shown to be a TRPV1 antagonist in our recent study, as evidenced by the fact that PcActx peptide markedly inhibited the capsaicin-stimulated calcium response in HEK293/hTRPV1 cells [29]. In the present study, a similar mode of action was observed in N2a/APP cells, demonstrating that PcActx peptide can mediate the calcium response through the TRPV1channel (Figures 4(a) and 4(b). Meanwhile, PcActx

peptide prominently attenuated the phosphorylation of CaMKII and CaMKIV, which are calcium-mediated proteins, in N2a/APP cells (Figures 4(c) and 4(d)). CaMKII and CaMKIV are activated by the integration of calcium/calmodulin and undergo autophosphorylation [42]. CaMKII is mainly distributed postsynaptically; aberrant activity thereof can cause synaptic dysfunction, leading to the cognitive deficits seen during AD progression [57]. Moreover, the expression and phosphorylation of CaMKIV are elevated by Aβ peptide treatment of PC12 cells [58]. CaMKIV also functions to regulate calcium-dependent gene transcription, such as that involving cAMP response element binding (CREB) protein [42]. hTau accumulation suppresses CaMKIV/CREB signaling in the nucleus and subsequently impairs synapse and memory; however, phosphorylation of CaMKIV was significantly increased in total lysates [59]. In addition, both CaMKII and CaMKIV are tau kinases, the hyperphosphorylation of which can trigger tau phosphorylation and promote NFT aggregation [57, 60]. Accumulating evidence indicates that abnormal activation of CaMKs is involved in ROS generation and oxidative damage. Inhibition of CaMKs could prevent these effects by improving antioxidant defense (e.g., via Nrf2 activation) [61–64].

Since oxidative stress can be considered as both a cause and consequence of calcium dysregulation, its role in AD pathogenesis should not be overlooked [11]. Maintaining calcium homeostasis in turn stabilizes cellular redox homeostasis in the context of oxidative damage. Nrf2 is an antioxidant transcription factor modulating endogenous antioxidant defense systems. In response to oxidative stress, Nrf2 dissociates Keap1 in the cytosol followed by nuclear translocation and binds with ARE to trigger the transcription of a series of antioxidative genes [14]. The calcium stabilizer, ITH14001, exerted a neuroprotective effect through activation of the Nrf2 pathway [65]. In the current study, PcActx peptide notably suppressed ROS overproduction (Figures 5(a) and 5(b)), which could damage various cellular biomolecules such as lipids, proteins, and nucleic acids (RNA and DNA) [66]. Next, we observed that PcActx
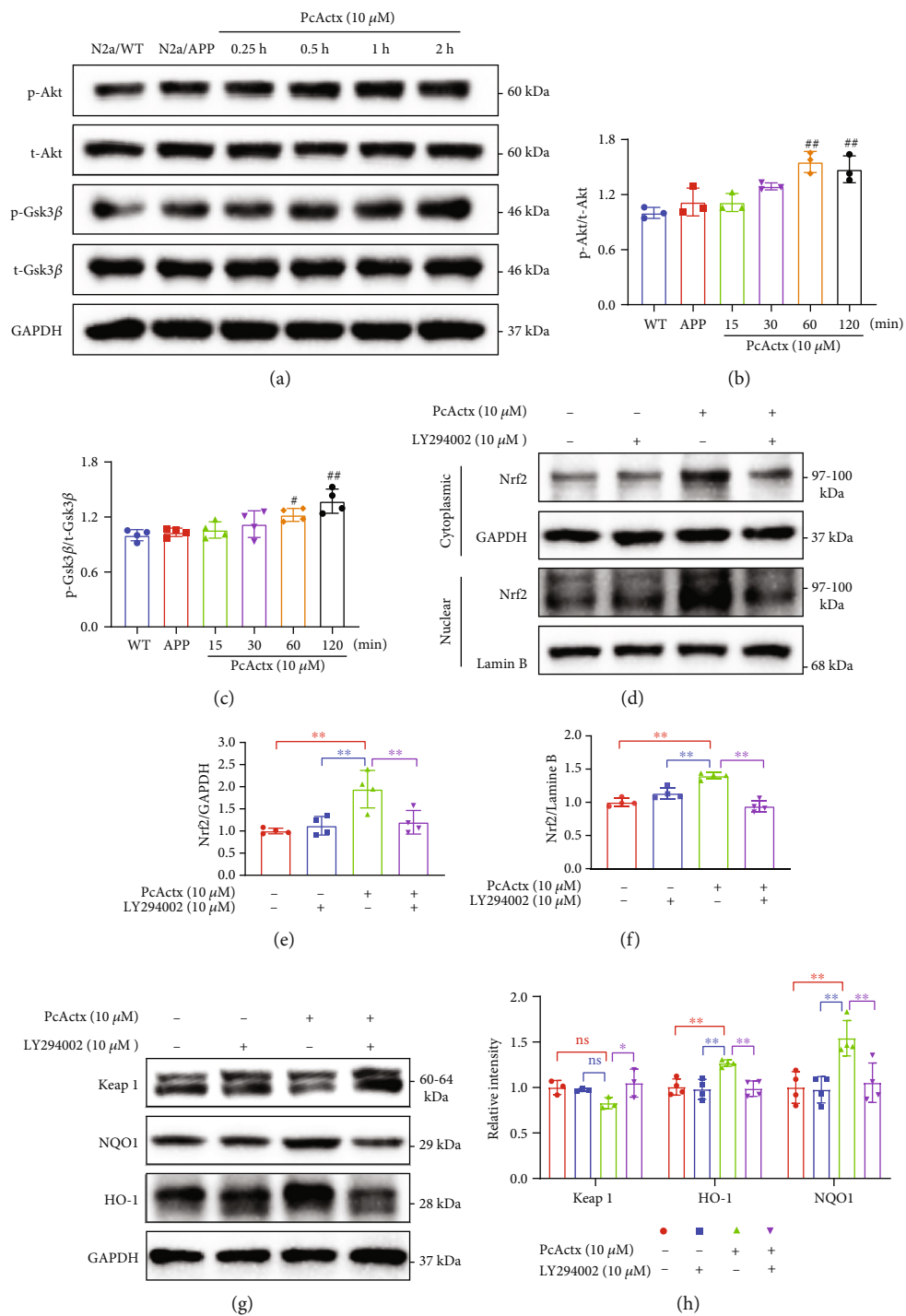
(a)

(b)

(c)

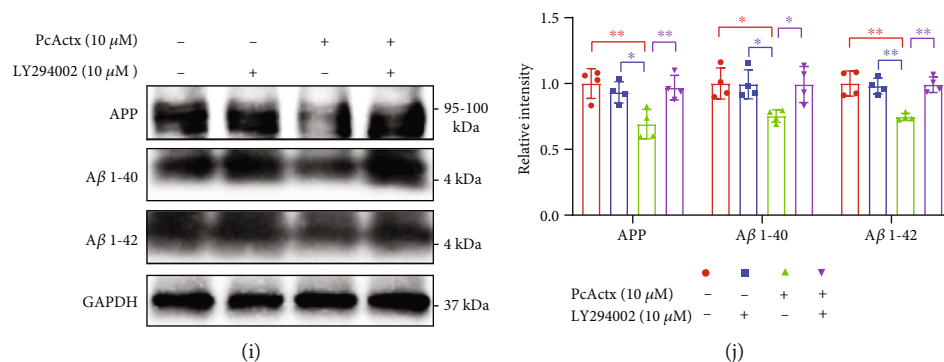(d)

(e)

(f)

(g)

(h)

FIGURE 6: Continued.

(i)

(j)

FIGURE 6: PcActx-induced Nrf2 activation and Aβ downregulation were mediated by the Akt/Gsk3β pathway. (a–c) PcActx peptide promoted the phosphorylation of Akt (Ser 473) and Gsk3β (Ser 9). (d–h) An Akt inhibitor suppressed the PcActx-induced upregulation of Nrf2, NQO1, and HO-1, as well as the downregulation of Keap1. (i, j) The reductions of APP, Aβ 1-40, and Aβ1-42 were counteracted by pharmacological inhibition of Akt/Gsk3β signaling. Data are expressed as the mean ± SD of three or four independent experiments, in triplicate. $^{\#}p < 0.05$ vs. N2a/APP group and $^{\#\#}p < 0.01$ vs. N2a/APP group. $^{*}p < 0.05$ vs. PcActx-treated group and $^{**}p < 0.01$ vs. PcActx-treated group.

peptide obviously increased the cytoplasmic and nuclear Nrf2 accumulation and favorably activated downstream antioxidative enzymes including HO-1 and NQO1 (Figures 5(c)–5(h)). Conversely, the Keap1 level was markedly reduced by treatment with PcActx peptide (Figures 5(e) and 5(h)). These data indicated that the downstream mechanism underlying the antioxidative effect of PcActx peptide might involve activation of the Nrf2/Keap1 pathway.

Activation of the Nrf2 pathway is not only directly modulated by the Nrf2-Keap1 complex but also indirectly regulated by kinases-mediated phosphorylation. Several proteins and pathways, such as Akt/Gsk3β, mitogen-activated protein kinase (MAPK), and protein kinase C (PKC), are involved in the induction of the Nrf2 pathway [15]. Gsk3β can phosphorylate Nrf2 in the Neh6 region, forming a recognition motif for β-transducin repeat containing E3 ubiquitin protein ligase (β-TrCP) and favoring Nrf2 ubiquitination and subsequent degradation [67]. Gsk3β is a downstream protein of Akt, and its inactivation via phosphorylation at Ser 9 is dependent on the activation of Akt [68]. Accordingly, Nrf2 accumulation in the nucleus may partially depend on the activation of Akt and subsequent inactivation of Gsk3β. Recent studies found that inhibition of Gsk3β activity alleviated cognitive deficits and activated the Nrf2 pathway in SAMP8 mice [69]. Huang et al. reported that calcium influx via the TRPV1 channel was responsible for UVB-induced Gsk3β activation, which stimulated the phosphorylation and ubiquitination of Nrf2 [35]. Furthermore, active Gsk3β can induce tau phosphorylation and aggregation to form NFTs [70]. In the present research, PcActx peptide obviously increased the phosphorylation of Akt at Ser 473 (active) and Gsk-3β at Ser 9 (inactive) within 60 min (Figures 6(a)–6(c)). An Akt inhibitor (LY294002) was used to further determine the specific role of the Akt/ Gsk3β pathway in PcActx-mediated Nrf2 activation and neuroprotection in N2a/APP cells. Inhibition of Akt/Gsk3β signaling efficaciously suppressed the PcActx-induced upregulation of Nrf2, HO-1, and NQO1, as well as the downregulation of Keap1 (Figures 6(d)–6(h)). Moreover, the Akt inhibitor clearly counteracted PcActx-mediated

inhibitory effects on the expression of APP, Aβ 1-40, and Aβ 1-42 (Figures 6(i) and 6(j)). On the basis of these results, we inferred that the PcActx peptide alleviated Aβ neuropathology via Akt/GSK3β-mediated Nrf2 activation in N2a/ APP cells.

In the last two decades, peptide and protein drugs have shown great promise for the treatment of a wide variety of neurodegenerative diseases [19–22]. However, a formidable hurdle to drug development for neurological disorders is the BBB, which controls the entry of agents into the brain. To overcome this barrier, multiple approaches have been developed to deliver neurotherapeutic agents to the brain. Receptor-, adsorptive-, and carrier-mediated transport systems have been introduced in recent years for delivering neuroprotective proteins and peptides to the brain [71, 72]. Studies have shown that these noninvasive approaches could increase the permeability of the peptide through the BBB, which can resolve the drug delivery problem [71, 72]. For instance, receptor-mediated transcytosis (RMT) can transport certain proteins (e.g., insulin, transferrin, and leptin) through the BBB via corresponding endogenous receptors [22], which indicates that our peptide, which was predicted to bind with ion channel, could be delivered to the brain through RMT. In our previous studies, two Kunitz-like peptides, PcKuz3 from zoantharian P. caribaeorum and Zoa-Kuz1 mat anemone Zoanthus natalensis, interacted with K_V channel and suppressed 6-OHDA-induced neuronal loss and behavioral deficits in zebrafish larvae [26, 27]. In another of our previous studies, PcActx peptide containing a Kunitz domain acted on the TRPV1 channel and prevented PTZ-induced epilepsy in zebrafish larvae [29]. Similarly, another Kunitz-type peptide called HCRG21, from sea anemone Heteractis crispa, reversed 6-OHDA-induced neurotoxicity in vitro [28]. Moreover, Angiopeps, a family of Kunitz domain-derived peptides (especially Angiopep-2), could trigger transcytosis and was transported across the BBB via interaction with low-density lipoprotein-related protein 1 (LRP-1) and serves as a new brain delivery system for neuropharmacological drugs [23–25]. All of the above studies provide evidence that Kunitz-type peptides

can potentially cross the BBB. Moreover, at a larger scale, various strategies (such as structural modification and the use of liposomes, nanoparticles, and peptide carrier systems) have been developed to enhance BBB penetration in preclinical and clinical research [22]. Our peptide could cross the BBB via any of methods. Due to the complexity of investigations of the permeability of the BBB to peptides, more experiments are needed to determine precisely how the PcActx peptide can cross the BBB.

5. Conclusion

In summary, the PcActx peptide, which is a TRPV1 blocker that could inhibit the capsaicin-induced calcium response in N2a/APP cells, prevented the abnormal accumulation of amyloid proteins by suppressing β- and γ-secretase-dependent cleavage of APP. Further analysis revealed that PcActx peptide activated the Nrf2 pathway to protect against oxidative insult via Akt/Gsk3β signaling. Hence, as a TRPV1 modulator, PcActx peptide exhibited prominent neuroprotective activity in an Alzheimer-like Aβ cell model and is therefore a promising candidate for AD therapy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Xiufen Wang and Yaqi Bian contributed equally to this work.

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References

[1] C. A. Lane, J. Hardy, and J. M. Schott, "Alzheimer's disease," *European Journal of Neurology*, vol. 25, no. 1, pp. 59–70, 2018.

[2] Alzheimer's Disease Fact Sheet, 2021, https://www.nia.nih.gov/health/alzheimers-disease-fact-sheet.

[3] A s Assoc, "Alzheimer's disease facts and figures," *Alzheimers Dement*, vol. 14, no. 3, pp. 367–425, 2018.

[4] Dementia Fact sheet, 2020, https://www.who.int/news-room/fact-sheets/detail/dementia.

[5] M. P. Mattson, "Pathways towards and away from Alzheimer's disease," *Nature*, vol. 430, no. 7000, pp. 631–639, 2004.

[6] K. L. Luan, J. L. Rosales, and K. Y. Lee, "Viewpoint: crosstalks between neurofibrillary tangles and amyloid plaque formation," *Ageing Research Reviews*, vol. 12, no. 1, pp. 174–181, 2013.

[7] W. V. Graham, A. Bonito-Oliva, and T. P. Sakmar, "Update on Alzheimer's disease therapy and prevention strategies," *Annual Review of Medicine*, vol. 68, no. 1, pp. 413–430, 2017.

[8] R. J. O'Brien and P. C. Wong, "Amyloid precursor protein processing and Alzheimer's disease," *Annual Review of Neuroscience*, vol. 34, no. 1, pp. 185–204, 2011.

[9] C. Haass, C. Kaether, G. Thinakaran, and S. Sisodia, "Trafficking and proteolytic processing of APP," *Cold Spring Harbor Perspectives in Medicine*, vol. 2, no. 5, 2012.

[10] E. Tonnies and E. Trushina, "Oxidative stress, synaptic dysfunction, and Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 57, no. 4, pp. 1105–1121, 2017.

[11] J. T. Yu, R. C. C. Chang, and L. Tan, "Calcium dysregulation in Alzheimer's disease: from mechanisms to therapeutic opportunities," *Progress in Neurobiology*, vol. 89, no. 3, pp. 240–255, 2009.

[12] M. P. Mattson and S. L. Chan, "Neuronal and glial calcium signaling in Alzheimer's disease," *Cell Calcium*, vol. 34, no. 4-5, pp. 385–397, 2003.

[13] K. Itoh, T. Chiba, S. Takahashi et al., "An Nrf2 small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements," *Biochemical and Biophysical Research Communications*, vol. 236, no. 2, pp. 313–322, 1997.

[14] Y. S. Keum and B. Y. Choi, "Molecular and chemical regulation of the Keap1-Nrf2 signaling pathway," *Molecules*, vol. 19, no. 7, pp. 10074–10089, 2014.

[15] L. Fao, S. I. Mota, and A. C. Rego, "Shaping the Nrf2-ARE-related pathways in Alzheimer's and Parkinson's diseases," *Ageing Research Reviews*, vol. 54, article 100942, 2019.

[16] C. Li, B. Tang, Y. Feng et al., "Pinostrobin exerts neuroprotective actions in neurotoxin-induced Parkinson's disease models through Nrf2 induction," *Journal of Agricultural and Food Chemistry*, vol. 66, no. 31, pp. 8307–8318, 2018.

[17] Z. C. Zou, J. J. Fu, Y. Y. Dang et al., "Pinocembrin-7-methylether protects SH-SY5Y cells against 6-hydroxydopamine-induced neurotoxicity via modulating Nrf2 induction through AKT and ERK pathways," *Neurotoxicity Research*, vol. 39, no. 4, pp. 1323–1337, 2021.

[18] Y. Bian, Y. Chen, X. Wang et al., "Oxyphylla A ameliorates cognitive deficits and alleviates neuropathology via the Akt-GSK3beta and Nrf2-Keap1-HO-1 pathways in vitro and in vivo murine models of Alzheimer's disease," *Journal of Advanced Research*, vol. 34, pp. 1–12, 2021.

[19] A. Balasubramaniam, "Clinical potentials of neuropeptide Y family of hormones," *American Journal of Surgery*, vol. 183, no. 4, pp. 430–434, 2002.

[20] I. Gozes and A. D. Spier, "Peptides as drug candidates against Alzheimer's disease," *Drug Development Research*, vol. 56, no. 3, pp. 475–481, 2002.

[21] S. A. Funke and D. Willbold, "Peptides for therapy and diagnosis of Alzheimer's disease," *Current Pharmaceutical Design*, vol. 18, no. 6, pp. 755–767, 2012.

[22] I. Brasnjevic, H. W. M. Steinbusch, C. Schmitz, P. Martinez-Martinez, and E. N. Res, "Delivery of peptide and protein drugs over the blood-brain barrier," *Progress in Neurobiology*, vol. 87, no. 4, pp. 212–251, 2009.

[23] M. Demeule, J. C. Currie, Y. Bertrand et al., "Involvement of the low-density lipoprotein receptor-related protein in the transcytosis of the brain delivery vector angiopep-2," *Journal of Neurochemistry*, vol. 106, no. 4, pp. 1534–1544, 2008.

[24] R. Gabathuler, "An engineered peptide compound platform technology incorporating Angiopep for crossing the BBB," *NeuroMethods*, vol. 45, pp. 249–260, 2010.

[25] M. Demeule, A. Regina, C. Che et al., "Identification and design of peptides as a new drug delivery system for the brain," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 324, no. 3, pp. 1064–1072, 2008.

[26] Q. Liao, G. Gong, T. C. W. Poon et al., "Combined transcriptomic and proteomic analysis reveals a diversity of venom-related and toxin-like peptides expressed in the mat anemone Zoanthus natalensis (Cnidaria, Hexacorallia)," *Archives of Toxicology*, vol. 93, no. 6, pp. 1745–1767, 2019.

[27] Q. Liao, S. Li, S. W. I. Siu et al., "Novel Kunitz-like peptides discovered in the Zoanthid Palythoa caribaeorum through transcriptome sequencing," *Journal of Proteome Research*, vol. 17, no. 2, pp. 891–902, 2018.

[28] O. Sintsova, I. Gladkikh, M. Monastyrnaya et al., "Sea anemone Kunitz-type peptides demonstrate neuroprotective activity in the 6-hydroxydopamine induced neurotoxicity model," *Biomedicine*, vol. 9, no. 3, p. 283, 2021.

[29] X. F. Wang, Q. W. Liao, H. B. Chen et al., "Toxic peptide from Palythoa caribaeorum acting on the TRPV1 channel prevents pentylenetetrazol-induced epilepsy in zebrafish larvae," *Frontiers in Pharmacology*, vol. 12, 2021.

[30] B. Minke and B. Cook, "TRP channel proteins and signal transduction," *Physiological Reviews*, vol. 82, no. 2, pp. 429–472, 2002.

[31] S. R. Kim, D. Y. Lee, E. S. Chung, U. T. Oh, S. U. Kim, and B. K. Jin, "Transient receptor potential vanilloid subtype 1 mediates cell death of mesencephalic dopaminergic neurons in vivo and in vitro," *The Journal of Neuroscience*, vol. 25, no. 3, pp. 662–671, 2005.

[32] S. R. Kim, S. U. Kim, U. Oh, and B. K. Jin, "Transient receptor potential vanilloid subtype 1 mediates microglial cell death in vivo and in vitro via Ca2+-mediated mitochondrial damage and cytochrome c release," *Journal of Immunology*, vol. 177, no. 7, pp. 4322–4329, 2006.

[33] T. Schilling and C. Eder, "Amyloid-β-induced reactive oxygen species production and priming are differentially regulated by ion channels in microglia," *Journal of Cellular Physiology*, vol. 226, no. 12, pp. 3295–3302, 2011.

[34] M. O. Grimm, T. Blümel, A. A. Lauer et al., "The impact of capsaicinoids on APP processing in Alzheimer's disease in SH-SY5Y cells," *Scientific Reports*, vol. 10, no. 1, pp. 1–16, 2020.

[35] K. F. Huang, K. H. Ma, T. Y. Jhap, P. S. Liu, and S. H. Chueh, "Ultraviolet B irradiation induced Nrf2 degradation occurs via activation of TRPV1 channels in human dermal fibroblasts," *Free Radical Biology and Medicine*, vol. 141, pp. 220–232, 2019.

[36] C. B. Yang, C. Z. Cai, J. X. Song et al., "NRBF2 is involved in the autophagic degradation process of APP-CTFs in Alzhei-

mer disease models," *Autophagy*, vol. 13, no. 12, pp. 2028–2040, 2017.

[37] Y. W. Zhang, R. Thompson, H. Zhang, and H. X. Xu, "APP Processing in Alzheimer's Disease," *Molecular Brain*, vol. 4, no. 1, pp. 1–13, 2011.

[38] B. J. Crain, W. D. Hu, C. I. Sze et al., "Expression and distribution of amyloid precursor protein-like protein-2 in Alzheimer's disease and in normal brain," *The American Journal of Pathology*, vol. 149, no. 4, pp. 1087–1095, 1996.

[39] T. A. Bayer, K. Paliga, S. Weggen, O. D. Wiestler, K. Beyreuther, and G. Multhaup, "Amyloid precursor-like protein 1 accumulates in neuritic plaques in Alzheimer's disease," *Acta Neuropathologica*, vol. 94, no. 6, pp. 519–524, 1997.

[40] M. J. McNamara, C. T. Ruff, W. Wasco, R. E. Tanzi, G. Thinakaran, and B. T. Hyman, "Immunohistochemical and in situ analysis of amyloid precursor-like protein-1 and amyloid precursor-like protein-2 expression in Alzheimer disease and aged control brains," *Brain Research*, vol. 804, no. 1, pp. 45–51, 1998.

[41] C. Hong, B. Jeong, H. J. Park et al., "TRP channels as emerging therapeutic targets for neurodegenerative diseases," *Frontiers in Physiology*, vol. 11, article 238, 2020.

[42] M. T. Swulius and M. N. Waxham, "Ca2+/calmodulin-dependent protein kinases," *Cellular and Molecular Life Sciences*, vol. 65, no. 17, pp. 2637–2657, 2008.

[43] S. Mouhat, B. Jouirou, A. Mosbah, M. De Waard, and J. M. Sabatier, "Diversity of folds in animal toxins acting on ion channels," *The Biochemical Journal*, vol. 378, no. 3, pp. 717–726, 2004.

[44] Q. Liao, G. Gong, S. W. I. Siu et al., "A novel ShK-like toxic peptide from the transcriptome of the Cnidarian Palythoa caribaeorum displays neuroprotection and cardioprotection in zebrafish," *Toxins*, vol. 10, no. 6, article 238, 2018.

[45] Q. Liao, S. Li, S. W. I. Siu et al., "Novel neurotoxic peptides from Protopalythoa variabilis virtually interact with voltage-gated sodium channel and display anti-epilepsy and neuroprotective activities in zebrafish," *Archives of Toxicology*, vol. 93, no. 1, pp. 189–206, 2019.

[46] G. M. Shankar, S. M. Li, T. H. Mehta et al., "Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory," *Nature Medicine*, vol. 14, no. 8, pp. 837–842, 2008.

[47] S. M. Li, S. Y. Hong, N. E. Shepardson, D. M. Walsh, G. M. Shankar, and D. Selkoe, "Soluble oligomers of amyloid beta protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake," *Neuron*, vol. 62, no. 6, pp. 788–801, 2009.

[48] T. T. Guo, D. H. Zhang, Y. Z. Zeng, T. Y. Huang, H. X. Xu, and Y. J. Zhao, "Molecular and cellular mechanisms underlying the pathogenesis of Alzheimer's disease," *Molecular Neurodegeneration*, vol. 15, no. 1, pp. 1–37, 2020.

[49] M. Korte, U. Herrmann, X. M. Zhang, and A. Draguhn, "The role of APP and APLP for synaptic transmission, plasticity, and network function: lessons from genetic mouse models," *Experimental Brain Research*, vol. 217, no. 3-4, pp. 435–440, 2012.

[50] D. M. Walsh, A. M. Minogue, C. S. Frigerio, J. V. Fadeeva, W. Wasco, and D. J. Selkoe, "The APP family of proteins: similarities and differences," *Biochemical Society Transactions*, vol. 35, no. 2, pp. 416–420, 2007.

[51] D. R. Dries and G. Yu, "Assembly, maturation, and trafficking of the gamma-secretase complex in Alzheimer's disease," *Current Alzheimer Research*, vol. 5, no. 2, pp. 132–146, 2008.

[52] J. Shen and R. J. Kelleher, "The presenilin hypothesis of Alzheimer's disease: evidence for a loss-of-function pathogenic mechanism," *Proceedings of the National Academy of Sciences*, vol. 104, no. 2, pp. 403–409, 2007.

[53] I. C. B. Marshall, D. E. Owen, T. V. Cripps, J. B. Davies, S. McNulty, and D. Smart, "Activation of vanilloid receptor 1 by resiniferatoxin mobilizes calcium from inositol 1,4,5-tris-phosphate-sensitive stores," *British Journal of Pharmacology*, vol. 138, no. 1, pp. 172–176, 2003.

[54] E. Ferreiro, C. R. Oliveira, and C. Pereira, "Involvement of endoplasmic reticulum Ca2+ release through ryanodine and inositol 1,4,5-triphosphate receptors in the neurotoxic effects induced by the amyloid-beta peptide," *Journal of Neuroscience Research*, vol. 76, no. 6, pp. 872–880, 2004.

[55] E. Ferreiro, C. R. Oliveira, and C. M. F. Pereira, "The release of calcium from the endoplasmic reticulum induced by amyloid-beta and prion peptides activates the mitochondrial apoptotic pathway," *Neurobiology of Disease*, vol. 30, no. 3, pp. 331–342, 2008.

[56] S. Sanz-Blasco, R. A. Valero, I. Rodriguez-Crespo, C. Villalobos, and L. Nunez, "Mitochondrial Ca2+ overload underlies Abeta oligomers neurotoxicity providing an unexpected mechanism of neuroprotection by NSAIDs," *PLoS One*, vol. 3, no. 7, article e2718, 2008.

[57] A. Ghosh and K. P. Giese, "Calcium/calmodulin-dependent kinase II and Alzheimer's disease," *Molecular Brain*, vol. 8, no. 1, 2015.

[58] T. Ye, H. W. Gao, W. T. Xuan et al., "The regulating mechanism of chrysophanol on protein level of CaM-CaMKIV to protect PC12 cells against A beta(25-35)-induced damage," *Drug Design, Development and Therapy*, vol. 14, pp. 2715–2723, 2020.

[59] Y. L. Yin, D. Gao, Y. L. Wang et al., "Tau accumulation induces synaptic impairment and memory deficit by calcineurin-mediated inactivation of nuclear CaMKIV/CREB signaling," *Proceedings of the National Academy of Sciences*, vol. 113, no. 26, pp. E3773–E3781, 2016.

[60] Y. P. Wei, J. W. Ye, X. Wang et al., "Tau-induced Ca2+/calmodulin-dependent protein kinase-IV activation aggravates nuclear tau hyperphosphorylation," *Neuroscience Bulletin*, vol. 34, no. 2, pp. 261–269, 2018.

[61] P. Zhong, D. J. Quan, J. Y. Peng et al., "Role of CaMKII in free fatty acid/hyperlipidemia-induced cardiac remodeling both in vitro and in vivo," *Journal of Molecular and Cellular Cardiology*, vol. 109, pp. 1–16, 2017.

[62] F. D. Toledo, L. M. Pérez, C. L. Basiglio, J. E. Ochoa, E. J. Sanchez Pozzi, and M. G. Roma, "The Ca2+-calmodulin-Ca2+/calmodulin-dependent protein kinase II signaling pathway is involved in oxidative stress-induced mitochondrial permeability transition and apoptosis in isolated rat hepatocytes," *Archives of Toxicology*, vol. 88, no. 9, pp. 1695–1709, 2014.

[63] Q. Lu, V. A. Harris, X. Sun, Y. Hou, and S. M. Black, "Black, Ca2+/calmodulin-dependent protein kinase II contributes to hypoxic ischemic cell death in neonatal hippocampal slice cultures," *PloS One*, vol. 8, no. 8, article e70750, 2013.

[64] L. Chen, Y. L. Tang, Z. H. Liu, Y. Pan, R. Q. Jiao, and L. D. Kong, "Atractylodin inhibits fructose-induced human podocyte hypermotility via anti-oxidant to down-regulate TRPC6/

p-CaMK4 signaling," *European Journal of Pharmacology*, vol. 913, article 174616, 2021.

[65] I. Buendia, G. Tenti, P. Michalska et al., "ITH14001, a CGP37157-nimodipine hybrid designed to regulate calcium homeostasis and oxidative stress, exerts neuroprotection in cerebral ischemia," *ACS Chemical Neuroscience*, vol. 8, no. 1, pp. 67–81, 2017.

[66] A. Misrani, S. Tabassum, and L. Yang, "Mitochondrial dysfunction and oxidative stress in Alzheimer's disease," *Frontiers in Aging Neuroscience*, vol. 13, article 617588, 2021.

[67] P. Rada, A. I. Rojo, S. Chowdhry, M. McMahon, J. D. Hayes, and A. Cuadrado, "SCF/beta-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner," *Molecular and Cellular Biology*, vol. 31, no. 6, pp. 1121–1133, 2011.

[68] M. Salazar, A. I. Rojo, D. Velasco, R. M. de Sagarra, and A. Cuadrado, "Glycogen synthase kinase-3 beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2," *The Journal of Biological Chemistry*, vol. 281, no. 21, pp. 14841–14851, 2006.

[69] S. A. Farr, J. L. Ripley, R. Sultana et al., "Antisense oligonucleotide against GSK-3 beta in brain of SAMP8 mice improves learning and memory and decreases oxidative stress: involvement of transcription factor Nrf2 and implications for Alzheimer disease," *Free Radical Biology and Medicine*, vol. 67, pp. 387–395, 2014.

[70] D. P. Hanger, B. H. Anderton, and W. Noble, "Tau phosphorylation: the therapeutic challenge for neurodegenerative disease," *Trends in Molecular Medicine*, vol. 15, no. 3, pp. 112–119, 2009.

[71] U. Bickel, T. Yoshikawa, and W. M. Pardridge, "Delivery of peptides and proteins through the blood-brain barrier," *Advanced Drug Delivery Reviews*, vol. 46, no. 1-3, pp. 247–279, 2001.

[72] F. Herve, N. Ghinea, and J. M. Scherrmann, "CNS delivery via adsorptive transcytosis," *The AAPS Journal*, vol. 10, no. 3, pp. 455–472, 2008.