



Amyloid β 1-42 ($A\beta$ 1-42) Induces the CDK2-Mediated Phosphorylation of Tau through the Activation of the mTORC1 Signaling Pathway While Promoting Neuronal Cell Death

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Alzheimer's disease (AD) is a neurodegenerative disorder, characterized by cognitive impairment and memory loss. Amyloid β 1-42 ($A\beta$) and hyper-phosphorylation of microtubule-associated protein tau have been considered as major histological features in AD. However, the mechanism of how $A\beta$ induces the hyper-phosphorylation of tau remains to be clarified. In the present study, we investigated the underlying cellular mechanisms of $A\beta$ with regard to the cell cycle regulatory protein-mediated phosphorylation of tau in promoting neuronal cell death. The oligomer $A\beta$ (5 μ M) significantly increased the level of caspase 3 cleavage and has the ability to induce cytotoxicity in human neuroblastoma SK-N-MC cells. $A\beta$ induced the degree of extracellular calcium influx via the L-type channel to facilitate the production of reactive oxygen species (ROS). $A\beta$ signaling through ROS production is uniquely mediated by the activation of PI3K/Akt, which is in turn required for mammalian target of rapamycin complex 1 (mTORC1) phosphorylation. mTORC1 activated by $A\beta$ further increased the phosphorylation of eukaryotic translation initiation factor 4E (eIF4E), a binding protein (4E-BP1) and p70S6K1 to stimulate the HIF1 α synthesis responsible for the induction of cyclinD₁/cyclin-dependent kinase 4 (CDK4) and cyclinE/CDK2, whereas it significantly attenuated the activation of autophagy. $A\beta$ distinctively induced the CDK2-mediated phosphorylation of tau, which is responsible for microtubule destabilization in promoting neuronal apoptosis. In mouse hippocampal primary neurons, the apoptotic cell death induced by $A\beta$ is highly susceptible to the mTORC1 signaling pathway. These results demonstrate that $A\beta$ efficiently stimulates the mTORC1 signaling pathway to facilitate HIF1 α synthesis and autophagy inhibition to promote the expression of cell cycle regulatory proteins, during which CDK2 uniquely stimulates tau phosphorylation for microtubule destabilization-mediated neuronal apoptosis.

Keywords: Alzheimer's disease, mammalian target of rapamycin, cyclin dependent kinase 2, tauopathy, neuronal cell death

INTRODUCTION

Alzheimer's disease (AD) is a common aging-associated disease causing cognitive impairment and memory loss. Two histopathological hallmarks are generally found in most AD patients: Amyloid β 1-42 (A β) deposition and neurofibrillary tangles (NFTs; Spires-Jones and Hyman, 2014; Kim et al., 2017). Senile plaque is an extracellular accumulation of A β which is composed of soluble oligomer A β , while NFTs are intraneuronal aggregates of the highly phosphorylated microtubule-associated protein tau, also referred to as tauopathy (Spires-Jones and Hyman, 2014). In AD, however, tau is abnormally hyper-phosphorylated and detached from the microtubule, which leads to microtubule destabilization (Johnson and Stoothoff, 2004). The increased and detached soluble tau undergoes conformational changes and forms paired helical filaments which are believed to be toxic to cells (Wischik et al., 1995; Hyman et al., 2005). Previous research has suggested that tauopathy is induced by specific phosphorylation sites in tau, such as Thr 212 and Ser 396 (Alonso et al., 2010). Thus, many researchers have recently focused on elucidating how A β induces the hyper-phosphorylation of tau, which is a critical therapeutic target of tauopathy in AD patients.

A nutrient-sensing serine/threonine protein kinase, the mammalian target of rapamycin (mTOR), was shown to be hyper-phosphorylated in AD patients. In addition, previous studies have discovered that mTOR is a critical candidate molecule; it phosphorylates tau protein (Sun et al., 2014; Tramutola et al., 2015), and the inhibition of mTOR can ameliorate AD symptoms (Wang et al., 2014). On the other hand, healthy neurons are not believed to proceed to the G1/S phase and do not replicate DNA (Copani et al., 2007). However, cell cycle regulatory proteins are increased in AD patients which is associated with cell cycle re-entry process (Moh et al., 2011). And, the dysregulation of cell cycle regulator protein could be involved in the microtubule-mediated mechanisms associated with neuroplasticity (Arendt and Brückner, 2007). Although A β is known to increase cell cycle regulatory proteins through reactive oxygen species (ROS)-mediated signaling, the mechanism acting between A β and the accumulation of cell cycle regulatory proteins is poorly understood (Folch et al., 2012). Hence, discovering the functional mechanism by which A β regulates cell cycle re-entry by exploiting the mTOR signaling pathway serves as a novel target for the treatment and/or prevention of AD pathogenesis. Autophagy regulation is also another major role of mTOR. Autophagy is a conserved physiological pathway consisting of several steps, including initiation, autophagosome formation, fusion with lysosomes, and degradation (Klionsky and Emr, 2000; He and Klionsky, 2009). Recent reports have shown that hyper-activated mTOR in AD patients accelerates apoptosis by inhibiting autophagy (Son et al., 2012; Liang and Jia, 2014). Given that the increased expression of cell cycle regulatory proteins is toxic to neuronal cells and that toxic molecules are degraded by autophagy for cell survival, we investigated the roles and interplay of mTOR, cell cycle re-entry, and autophagy in the tauopathy of AD.

In the present study, we used the neuroblastoma SK-N-MC cell line and mouse hippocampal primary neurons to study the role of A β in promoting neuronal cell death. SK-N-MC neuronal cell line has been widely used for studying the pathogenesis and molecular mechanism of neurodegenerative diseases including AD due to its advantages, such as high stability and homogeneity (Chan et al., 2007; Shaykhalishahi et al., 2010; Kuo and Chou, 2014; Lee et al., 2016). In addition, the hippocampus, a major component of the brains of vertebrates, is known as the first area of the brain affected by the pathogenic mechanisms occurring in AD. In the present study, therefore, we investigated the underlying cellular mechanisms of A β with regard to the cell cycle regulator-mediated AD pathogenesis with SK-N-MC cells and mouse hippocampal primary neurons.

MATERIALS AND METHODS

Materials

The neuroblastoma SK-N-MC cell lines were obtained by Korean Cell Line Bank (Seoul, South Korea). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, MD, USA). The antibodies of p-Akt (Thr 308, sc-16646-R), p-Akt (Ser 473, sc-7985-R), Akt (sc-8312), eukaryotic translation initiation factor 4E (eIF4E; sc-9976), cyclin D₁ (sc-753), cyclin E (sc-481), cyclin-dependent kinase 4 (CDK4; sc-749), CDK2 (sc-748), tau (sc-390476), p-tau (Thr 212, sc-135643), p-tau (Ser 396, sc-101815), rapamycin-insensitive companion of mTOR (RICTOR; sc-271081), α -tubulin (sc-32293) and β -actin (sc-47778) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated IgG was obtained from Jackson ImmunoResearch (West Grove, PA, USA). The antibodies of cleaved caspase 3 (#9661), mammalian target of rapamycin (mTOR; #2983), p-mTOR (Ser 2448, #5536), p-mTOR (Ser 2481, #2974), p-regulatory-associated protein of mTOR (RAPTOR; Ser 792, #2083), p70 S6 kinase 1 (S6K1, #2708), p-p70S6K1 (Thr 389, #9234), eIF4E binding protein (4EBP1; #9644), p-4EBP1 (Thr 37/46, #9459) and p35/25 (#2680) were acquired from Cell Signaling Technology (Beverly, MA, USA). The antibodies of hypoxia inducible factor (HIF1 α ; NB100-105), p70S6K1 (NB600-1049), LC3 (NB100-2220) and p62 (NBP1-48320) were obtained from Novus Biologicals (Littleton, CO, USA) and the HRP-conjugated goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology. NeuN (ab77315), RAPTOR (ab26264) antibodies were purchased from Abcam (Cambridge, England). Small interfering RNA (siRNA) for *HIF1A* was purchased from GenePharma (Sanghai, China). *CDK2*, *CDK4* and non-targeting (NT) siRNAs were purchased from Dharmacon (Lafayette, CO, USA). EGTA, BAPTA-AM, ionomycin, nifedipine, *N*-acetyl-L-cysteine (NAC), PF4708671, cycloheximide, LY294002, rapamycin, trehalose, flavopiridol, paclitaxel were purchased from Sigma Aldrich (St. Louis, MO, USA). Akt inhibitor was purchased from Calbiochem (La Jolla, CA, USA). All chemicals used in this study were of the highest quality commercially available forms.

Cell Culture

The SK-N-MC cells were cultured with Dulbecco Modified Eagle Medium (DMEM, Thermo Fisher, Waltham, MA, USA). Cells were grown in 10% FBS with a 1% antibiotics mixture. Cells were plated on 35, 60, or 100 mm diameter culture dishes, or in 6- or 12-well plates in an incubator kept at 37°C with 5% CO₂. The medium was changed to serum-free medium. After 24 h of incubation in serum-free medium, cells were washed twice with phosphate buffered solution (PBS), and placed in medium that included supplements.

Primary Culture of Hippocampal Neurons from Mice

Hippocampal primary neurons were isolated from prenatal ICR mice (18–19 days) to confirm the effect of A β in neurons. Isolation was performed as described (Seibenhener and Wooten, 2012). Hippocampus was isolated from the prenatal mice (18–19 days), and gently minced by using sterile scalpel. Minced hippocampus was treated with trypsin (0.025%) and dissociated by trituration. 2.5×10^6 cells were plated at poly-D-lysine-coated 35 mm dish in neurobasal plating media (neurobasal Media containing B27 Supplement [1 ml/50 ml], 0.5 mM glutamine, 25 μ M glutamate, 1% antibiotics, 1 mM HEPES, 10% heat inactivated donor horse serum) and placed in an incubator kept at 37°C with 5% CO₂. After 24 h, growth media is changed to neurobasal feeding media (Neurobasal media containing B27 supplement [1 ml/50 ml], 0.5 mM glutamine, 1% antibiotics, 1 mM HEPES. To avoid glial cell contamination, cytosine arabinoside (AraC) was treated early time points in the culture. NeuN antibody was used for neuronal nucleus staining. The protocol for mouse hippocampal neuron primary culture was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-151116-1). All procedures involving mice were performed following the National Institutes of Health Guidelines for Humane Treatment of Animals.

Preparation of A β

The β -Amyloid [1-42] (Human) peptide was purchased from Invitrogen Corporation (Camarillo, CA, USA). Peptide was dissolved at a concentration of 1 mg/ml in 100% HFIP (Sigma Aldrich), and incubated at room temperature for 1 h with occasional vortexing. The sonicated for 10 min in a water bath sonicator. After sonication, it was freeze-dried for 3 h. When the film is formed, it dissolved in DMSO. Scrambled β -Amyloid [1-42] (scrambled A β) was purchased from ANASPEC (San Jose, CA, USA). Protein sequence of scrambled A β is as follows. AIAEGDSHVLKEGAYMEIFDVQGHVFGGKIFRVV DLGSHNVA.

Real Time Polymerase Chain Reaction (Real Time-PCR)

The total RNA of cells was extracted using MiniBEST Universal RNA Extraction Kit (TaKaRa, Otsu, Shinga, Japan). Reverse transcription (RT) was carried out using a MaximeTM RT-PCR PreMix kit (Intron Biotechnology, Seongnam, Korea) with the oligo(dT18) primers. RT was performed at 45°C for 60 min to

cDNA synthesis and 95°C for 5 min RTase inactivation step. RT products were amplified using QuantiSpeed SYBR Kits (Life technologies, Gaithersburg, MD, USA). Real-time quantification of RNA targets was performed in a Rotor-Gene 6000 real-time thermal cycling system (Corbett Research, NSW, Australia). The primers used are described in the Supplementary Table S1. The reaction mixture (10 μ l) contained 50 ng of total RNA, 0.5 mM of each primer, and appropriate amounts of enzymes and fluorescent dyes as recommended by the supplier. The Real-time PCR were performed as follows: 15 min at 95°C for DNA polymerase activation; 15 s at 95°C for denaturing; and 40 cycles of 15 s at 94°C, 30 s at 54°C and 30 s at 72°C. Data was collected during the extension step (30 s at 72°C) and analysis was performed using the software provided. Following real-time PCR, melting curve analysis was performed to verify the specificity and identity of the PCR products.

Western Blot Analysis

Harvested cells were washed once with cold PBS prior to incubation in lysis buffer (1 mM EDTA, 1 mM EGTA, 20 mM Tris (pH 7.5), 1% Triton X-100, 1 mg/ml aprotinin, and 1 mM phenylmethylsulfonylfluoride (PMSF)) for 30 min on ice. The lysates were then cleared by centrifugation (16,000 g at 4°C for 30 min). The BCA assay with lysates was performed to determine protein concentration. Samples containing 10 μ g of protein were prepared for 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane. Protein-containing membranes were washed Tris-buffered saline containing 0.1% Tween-20 (TBST) solution (10 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.1% Tween-20) blocked with 5% bovine serum albumin (BSA) for 30 min. Blocked membranes were washed with TBST for three times every 10 min, and incubated with primary antibody overnight at 4°C. The membranes were then washed and incubated with HRP-conjugated secondary antibody at room temperature for 2 h. The western blotting bands were visualized by using chemiluminescence (Bio-Rad, Hercules, CA, USA). Densitometric analysis was carried out by using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA¹).

Transfection of Small Interfering RNA

Prior to A β treatment, siRNAs specific for *HIF1A*, *CDK2*, *CDK4*, or NT were transfected to cell for 24 h with TurbofectTM (Thermo Fisher) according to the manufacturer's instructions. The concentration of each transfected siRNA was 25 nM. The NT siRNA was used as the negative control. The siRNA sequences are described in Supplementary Table S2.

Trypan Blue Exclusion Test for Measuring Cell Viability

Cells were incubated with a 0.05% Trypsin and 0.5 mM EDTA solution. Detached cells were treated with soybean trypsin inhibitor (0.05 mg/ml) to quench trypsin. To exclude dead cells, 0.4% trypan blue was added to the cell suspension. Subsequently,

¹<https://imagej.nih.gov/ij/download.html>

unstained and stained cells were counted by using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA, USA). The equation used to determine cell viability was Cell viability = $[(1 - (\text{number of trypan blue-stained cells}/\text{number of total cells}))] \times 100$.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

The cells were cultured in a 12-well plate. Prior to harvesting, each well was treated with 100 pg/ml of MTT reagent (Sigma Aldrich) for 4 h. Harvested cells were washed with PBS and incubated with 150 μ l of DMSO (Sigma Aldrich) for 20 min. 1×10^5 cells were collected, and resuspended in 150 μ l of PBS. Absorbance at 540 nm was measured by using a micro plate reader (Bio-Rad).

Immunofluorescence Staining

Cells were fixed with 4% paraformaldehyde (Sigma Aldrich) for 10 min, followed by permeabilization with 0.1% Triton X-100 (Sigma Aldrich) in PBS followed by washing with PBS. Cells were incubated with 1% normal goat serum to block non-specific binding of antibody, and then incubated with 1:100 dilution of primary antibody for overnight in 4°C. Next, the cells were incubated for 2 h at room temperature with Alexa Fluor[®]-conjugated secondary antibodies (1:100 dilution) and propidium iodide (PI, Invitrogen, Carlsbad, CA, USA) in PBS. Images were obtained by using a FluoViewTM 300 fluorescence microscope (Olympus, Tokyo, Japan). Three independent experiments were performed and 60 cells (20 cells per experiment) were used for statistical analysis.

Measurement of $[Ca^{2+}]_i$

The changes in $[Ca^{2+}]_i$ were observed using Fluo 3-AM dissolved in DMSO. The cells were washed once with a PBS, incubated in PBS containing 3 μ M of Fluo 3-AM with 5% CO₂ at 37°C for 40 min, and washed once with the PBS and scanned every second using confocal microscopy (FluoViewTM 300, Olympus, Tokyo, Japan). The fluorescence was excited at 488 nm and the emitted light was read at 515 nm. In order to verify the assay, ionomycin was applied to the cells as a positive control. All of the analysis of $[Ca^{2+}]_i$ were processed at a single cell level are expressed as the relative fluorescence intensity (RFI).

Measurements of Intracellular ROS Levels

The cells were plated on 12-well dish and confocal dishes. The cells were then incubated in the dark with 10 μ M CM-H₂DCFDA for 1 h at 37°C. The cells were visualized by using laser confocal microscopy (FluoViewTM 300, Olympus, Tokyo, Japan) with an excitation wavelength of 448 nm and an emission wavelength of 515 nm. To quantify the ROS production, cells were incubated with 10 μ M of CM-H₂DCFDA, and washed twice with PBS. The 1×10^5 cells were loaded into a 96-well black plate and assessed by using a luminometer (Victor3, Perkin-Elmer, Waltham, MA, USA).

Co-Immunoprecipitation

Cells were lysed with the co-immunoprecipitation buffer (1% Triton X-100 in 50 mM Tris-HCl [pH 7.4] containing 150 mM NaCl, 5 mM EDTA, 2 mM Na₃VO₄, 2.5 mM Na₄PO₇, 100 mM NaF, 200 nM microcystin lysin-arginine, and protease inhibitor). Cell lysates (300 μ g) were incubated with 10 μ g of anti-tau antibody. The samples were incubated for 4 h, and Protein A/G PLUS-agarose immunoprecipitation reagent was added. The samples were incubated for an additional 12 h. The beads were washed three times with the co-immunoprecipitation buffer. Beads were added to sample buffer, and eluted by boiling. Samples were analyzed by western blotting.

AnnexinV/PI Double Staining

Apoptosis and necrosis of cells were measured with an Annexin V and PI staining kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, the cells were detached with 0.05% trypsin/EDTA and 1×10^5 cells were suspended with Annexin V binding buffer. The cells were stained with Annexin V and PI and incubated for 20 min at room temperature in the dark. The sample was read by flow cytometry and analyzed using CXP software (Beckman Coulter, Brea, CA, USA). Samples were gated to exclude debris (forward light scatter [FSC] area vs. side scatter area), and then any cell doublets were excluded using FSC area vs. FSC width analysis.

Statistical Analysis

Results are expressed as mean value \pm standard error of mean (SE). All experiments were analyzed by analysis of variance (ANOVA), and some experiments which needed to compare with two groups were examined by comparing the treatment means to the control using a Student's test. A result with a *p* value of < 0.05 was considered statistically significant.

RESULTS

Effect of A β on Neuronal Cell Death

A β significantly induced the cytotoxicity of SK-N-MC cells from 1 μ M to 20 μ M for 48 h (**Figure 1A**). For instance, cell viability decreased by 36% at 5 μ M of A β compared to a control sample. The results of a trypan blue exclusion assay also revealed that cell viability decreased by 34% when the cells were treated with 5 μ M of A β (**Figure 1B**). In addition, we found that A β has the ability to induce caspase 3 cleavage, suggesting that neuronal cell death as induced by A β is related to apoptotic cell death (**Figure 1C**). It was also found that A β increased ROS production by as much as 87% in 3 h, with the rate strongly maintained for up to 48 h (**Figure 1D**). The stimulatory effect of A β on ROS production was further visualized with a fluorescent dye, CM-H₂DCFDA (Supplementary Figure S1A). Importantly, the cell viability decreased by the A β treatment was significantly recovered by a treatment with the ROS scavenger NAC (**Figure 1E**). Consistent with this, we found through flow cytometric analyses that A β significantly

induced the necrotic cell death (a 1.3 ± 0.8 -fold increase compared to a control) as well as apoptosis (a 3.0 ± 0.2 -fold increase compared to a control; **Figure 1F**). In addition, we performed CM-H₂DCFDA staining to confirm the effect of NAC in ROS production. We observed that ROS level of A β -treated SK-N-MC was increased to 143%. And, those of NAC-pretreated SK-N-MC with either vehicle or A β were decreased to 85% and 72%, respectively. (Supplementary Figure S1B). A β has greater stimulatory potency on apoptotic cell death than necrotic cell death, confirming that A β is essential for triggering apoptotic cell death. However, this increase in apoptotic cell death was significantly blocked by a treatment with NAC. Next, we determine whether ROS production induced by A β causes alterations in the Ca²⁺ influx [Ca²⁺]_i level. As shown in **Figure 1G**, A β caused an increased level of [Ca²⁺]_i, which was enhanced by the Ca²⁺ ionophore ionomycin as a positive control. However, ROS production as induced by A β was significantly blocked by a pretreatment with the extracellular calcium chelator EGTA but not with the intracellular calcium chelator BAPTA-AM in SK-N-MC cells, indicating that A β -induced ROS is dependent on extracellular [Ca²⁺]_i (**Figure 1H**). Interestingly, the [Ca²⁺]_i induced by A β was blocked by a treatment with an L-type calcium channel blocker, Nifedipine (**Figure 1I** and Supplementary Figure S2). These results suggest that [Ca²⁺]_i is highly dependent on extracellular calcium influx.

The Role of A β in the Activation of mTORC1

As shown in the **Figure 2A** and Supplementary Figure S3, A β significantly induced the phosphorylation of Akt at Thr 308 and Ser 473, which was blocked by NAC pretreatment in SK-N-MC and mouse hippocampal neuron. A β also significantly induced the phosphorylation of mTOR at Ser 2448, but not at Ser 2481 (**Figure 2B**). mTOR was more likely to bind RAPTOR, while decreasing the binding of RICTOR when the cells were treated with A β for 24 h, suggesting that A β uniquely regulates the phosphorylation of mTOR Complex 1 (mTORC1) rather than mTORC2 (**Figure 2C**). Importantly, the inhibition of PI3K with LY294002 and Akt with an Akt inhibitor significantly blocked the phosphorylation of mTORC1 and RAPTOR, suggesting that A β triggers mTORC1 activation through the PI3K/Akt pathway (**Figure 2D**). The mTOR phosphorylation at Ser 2448 residue in A β -treated SK-N-MC cells and hippocampal primary neurons were increased to 238% and 208%, respectively (**Figures 2E,F**). Given that A β specifically induces mTOR phosphorylation, we further assessed whether mTOR activation is involved in the regulation of neuronal apoptosis. The cytotoxicity (**Figure 3A**) and reduced number of cells (**Figure 3B**) induced by A β were significantly inhibited by a treatment with the mTOR inhibitor, rapamycin. The result in flow cytometric analyses also revealed that the rapamycin significantly blocked the apoptosis induced by A β (**Figure 3C**). Moreover, the levels of caspase-3 cleavage (**Figure 3D**) and cytotoxicity (**Figure 3E**) induced by A β in primary neurons were significantly blocked by rapamycin. These results indicate that A β stimulates the

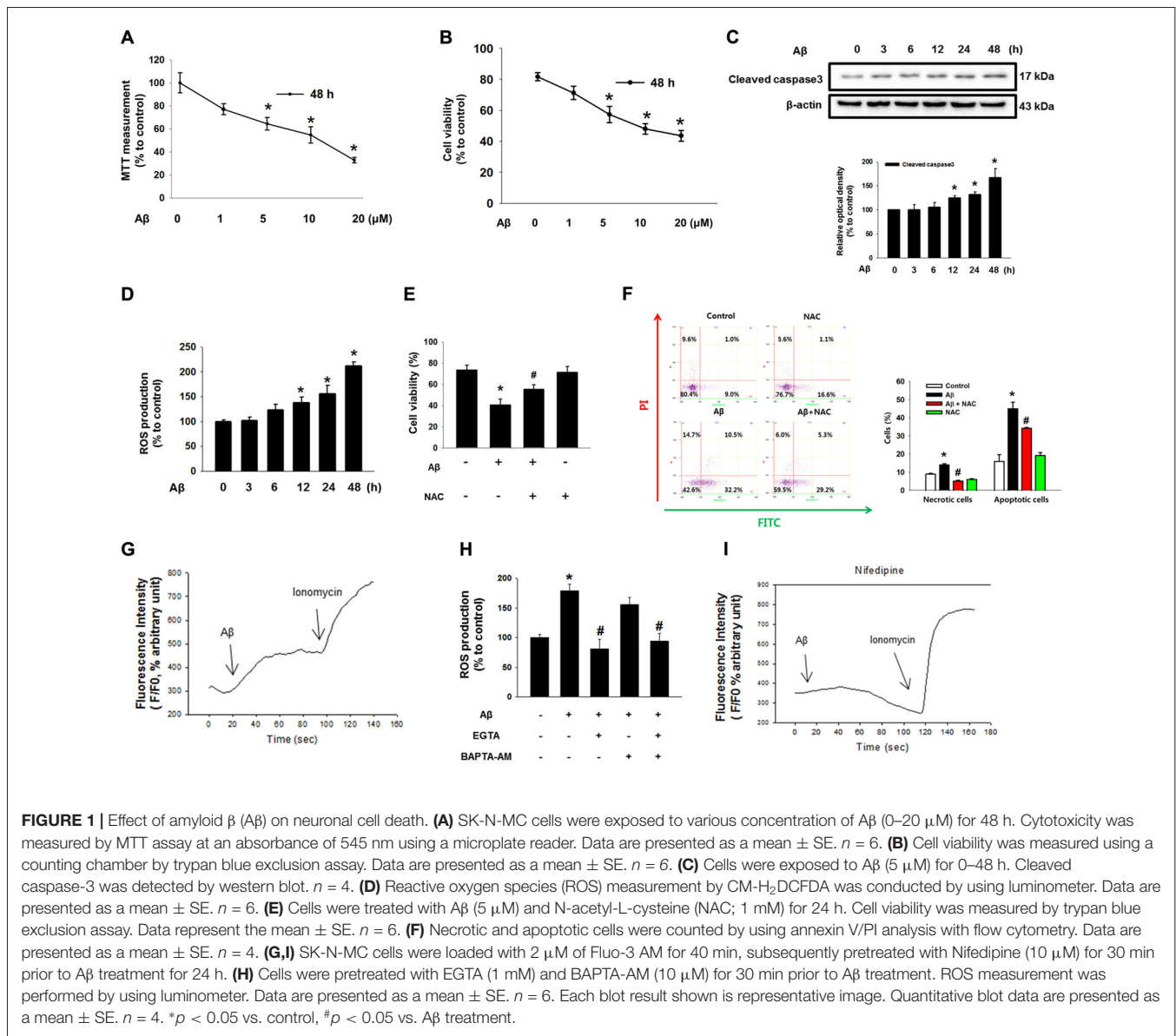
PI3K/Akt pathway required for mTORC1 activation to stimulate neuronal apoptosis.

A β Facilitates HIF1 α Synthesis and Autophagy Inhibition via mTOR Activation

Despite the fact that A β is a strong regulator of ROS production, it was previously reported that A β reduces HIF1 α expression via a ROS-independent mechanism in astrocytes (Schubert et al., 2009). However, our results showed that the protein level of HIF1 α is increased by A β for 48 h in a time-dependent manner (**Figure 4A**). The increased HIF1 α level was significantly inhibited by the ROS scavenger NAC (**Figure 4B**). To determine how ROS induced by A β regulates the level of the HIF1 α protein, we focused on 4EBP1 and S6K1, which are involved in the mRNA translation of HIF1 α . As shown in **Figure 4C**, A β induced the phosphorylation of 4EBP1 and p70S6K1, which was blocked by rapamycin, suggesting that mTOR activation is mediated by the activation of 4EBP1 and p70S6K1 in A β -treated SK-N-MC cells. In addition, A β -mediated 4EBP1 phosphorylation was closely related to a decreased level of 4EBP1 interaction with its binding protein eIF4E, in which a rapamycin treatment significantly recovered this interaction to the control level (**Figure 4D**). Importantly, the increased protein level of HIF1 α induced by A β was markedly inhibited by a treatment with rapamycin (**Figure 4E**), the p70S6K1 inhibitor PF4708671 (**Figure 4F**), or the translation inhibitor cycloheximide (**Figure 4G**). These results indicate the novel role of A β in the promotion of HIF1 α synthesis through the mTORC1 pathway, which is required for the phosphorylation of 4EBP1 and p70S6K1. Another major role of mTOR is known to be the inhibition of autophagy. As shown in **Figure 4H**, we found that A β reduces the expression of LC3-II and the degradation of p62, and that the inhibition of autophagy induced by A β significantly blocked by a treatment with rapamycin. We confirmed using confocal microscopy that A β inhibits the formation of autophagic vesicles via mTOR (**Figure 4I**). Interestingly, the cytotoxicity induced by A β was recovered by a treatment with the autophagy inducer trehalose (**Figures 4J,K**). Together, these results suggest that A β has the ability to induce two pathogenic pathways related to HIF1 α synthesis and autophagy inhibition through the regulation of mTORC1 activation to promote neuronal apoptosis. Moreover, we pretreated inhibitors used in this study to demonstrate the sequential activation of A β -induced ROS/Akt/mTOR/HIF1 α pathway. As shown in the Supplementary Figure S4, mTOR phosphorylation (Ser 2448) and HIF1 α expression were suppressed by NAC or Akt inhibitor or rapamycin. However, Akt phosphorylations at Thr 308 and Ser 473 residues were inhibited by NAC or Akt inhibitor, but not rapamycin. These findings indicate the ROS-induced Akt is an upstream regulator of Akt/mTOR/HIF1 α pathway.

A β Induces the Expression of Cell Cycle Regulatory Proteins

To determine how two pathogenic pathways triggered by A β influence the neuronal cell death process, we subsequently investigated the role of A β at the level of cell cycle regulatory



proteins including cyclin 2, cyclin 4, CDK2, CDK4 and CDK5, which are known as critical regulators of neuronal cell death in AD patients (Frade and Ovejero-Benito, 2015; Liu et al., 2016). Interestingly, A β did not have an effect on the mRNA expression of CDK5 or its regulators, p35 and p39, which are well-known candidate molecules responsible for the hyper-phosphorylation of tau protein in AD patients (Figure 5A). Moreover, our result showed that A β did not affect p35/p25 expression in SK-N-MCs (Supplementary Figure S5). Instead, we found that A β significantly induced the expression of cyclin D₁/CDK4 and cyclin E/CDK2 (Figures 5B,C). However, the level of cell cycle regulatory proteins was inhibited by the knockdown of *HIF1A* (Figure 5B) and by a treatment with the trehalose (Figure 5C), respectively. In addition, the expression of cell cycle regulatory proteins induced by A β is markedly inhibited by a treatment with

the rapamycin (Figure 5D), the p70S6K1 inhibitor PF4708671 (Figure 5E), and the translation inhibitor cycloheximide (Figure 5F). Importantly, our results with A β -treated primary hippocampal neurons revealed that the increase in the level of cell cycle regulatory proteins was blocked by a treatment with *HIF1A* siRNA (Figure 5G) and trehalose (Figure 5H). These results indicate that A β -induced mTOR signaling pathway stimulates the expression of cell cycle regulatory proteins.

CDK2 Plays a Critical Role in Tau Phosphorylation and Microtubule Destabilization

A β significantly induced the phosphorylation of tau at Thr 212 and Ser 396 (Figure 6A). Interestingly, the A β -induced phosphorylations of tau (Thr 212, Ser 396 and Ser 262)

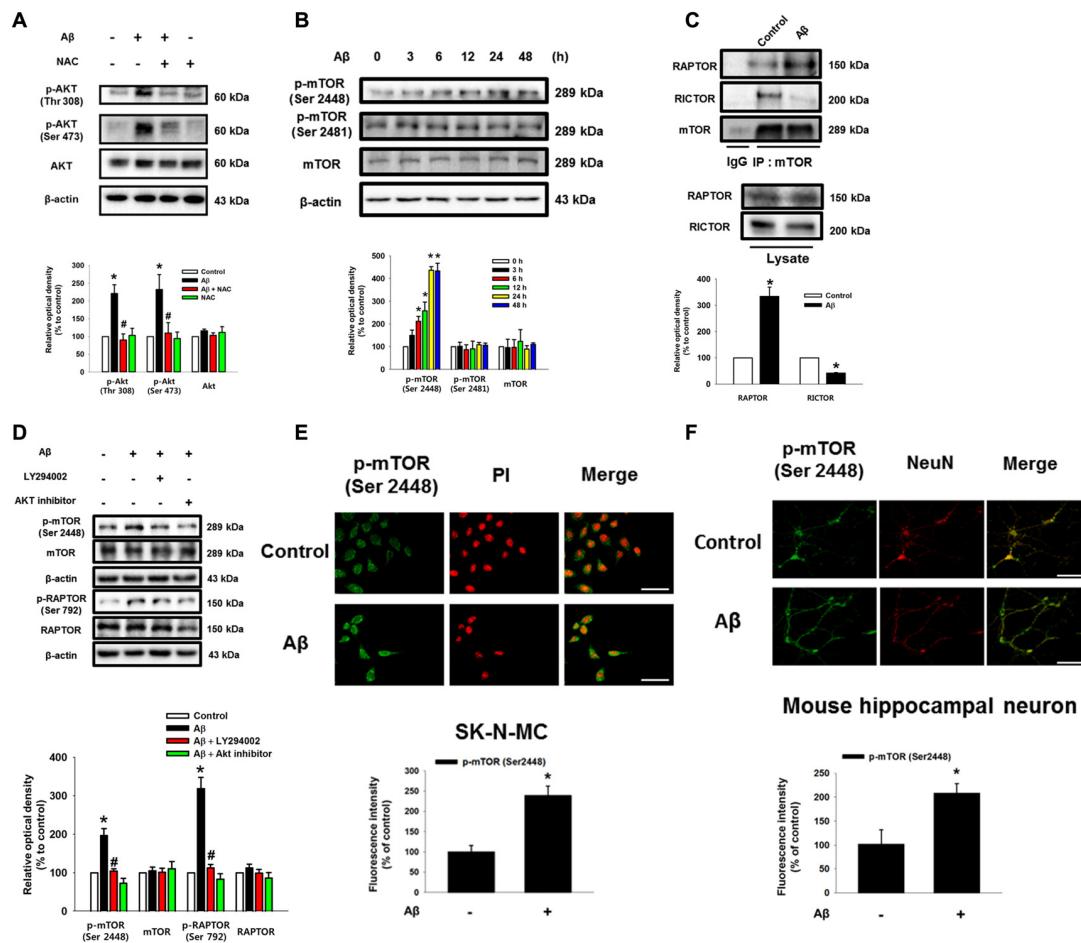


FIGURE 2 | Role of A β in the activation of Akt and mammalian target of rapamycin complex 1 (mTORC1). **(A)** SK-N-MC were incubated with A β (5 μ M) and NAC (1 mM) for 24 h. Phosphorylated Akt (Thr 308 or Ser 473), Akt and β -actin were detected by western blot. $n = 6$. **(B)** Cells were incubated with A β for 0–48 h. Phosphorylated mTOR (Ser 2448 and Ser 2481), mTOR and β -actin were detected by western blot. $n = 3$. **(C)** Cells were treated with A β for 24 h. Protein samples were immunoprecipitated by using mTOR antibody-conjugated protein A/G agarose beads. Immunoprecipitation assay was described in “Materials and Methods” section. Raptor, Rictor and mTOR were detected by western blot. $n = 3$. **(D)** Cells were pretreated with LY294002 (20 μ M) or Akt inhibitor (20 μ M) for 30 min prior to A β treatment for 24 h. Cells were blotted with p-mTOR (Ser 2448), mTOR, p-Raptor (Ser 792), Raptor and β -actin specific antibodies. $n = 4$. Each western blot image was presented as representative image. Quantitative blot data are presented as a mean \pm SE. $n = 3$. * $p < 0.05$ vs. control, # $p < 0.05$ vs. A β treatment. **(E,F)** SK-N-MC cells and mouse hippocampal neurons were immunostained with p-mTOR (Ser 2448) specific antibodies, and visualized by confocal microscopy. Scale bars, 100 μ m (magnification, $\times 400$). Data are presented as a mean \pm SE. $n = 3$.

were significantly blocked by the knockdown of CDK2 in SK-N-MC and mouse hippocampal neuron (Figure 6B and Supplementary Figures S6A,B), suggesting that tau phosphorylation induced by A β is CDK2-dependent. Furthermore, pretreatment with the CDK inhibitor flavopiridol also prevented the tau phosphorylation induced by A β in SK-N-MC cells (Supplementary Figure S7A) and primary neurons (Supplementary Figure S7B). It was noted that tau is co-immunoprecipitated with CDK2, and importantly, that these interactions were enhanced by the A β treatment (Figure 6C). A β also decreased tubulin interaction with tau, which was blocked by the knockdown of CDK2 (Figure 6C). Consistent with this, pretreatment with the CDK inhibitor flavopiridol also prevented microtubule destabilization as

induced by A β (Supplementary Figure S7C). Finally, the silencing of CDK2 attenuated the level of cleavage of caspase-3 (Figure 6D), cytotoxicity (Figure 6E) and apoptotic cell death (Figure 6F) as stimulated by an A β treatment. In addition, we screened the effect of scrambled A β (1–42) peptide to check the nonspecific effect of A β . Our results showed that scrambled A β treatment did not affect the phosphorylations of Akt (Thr 308 and Ser 473), mTOR (Ser 2448), and tau (Thr 212 and Ser 396) and expressions of HIF1 α and CDK2 (Supplementary Figure S8). These findings suggest that CDK2 plays a critical role in tau activation and in the detachment of tau from tubulins in promoting neuronal apoptosis. Interestingly, treatment with a microtubule stabilizer, paclitaxel, significantly blocked A β -induced neuronal cell death, although paclitaxel

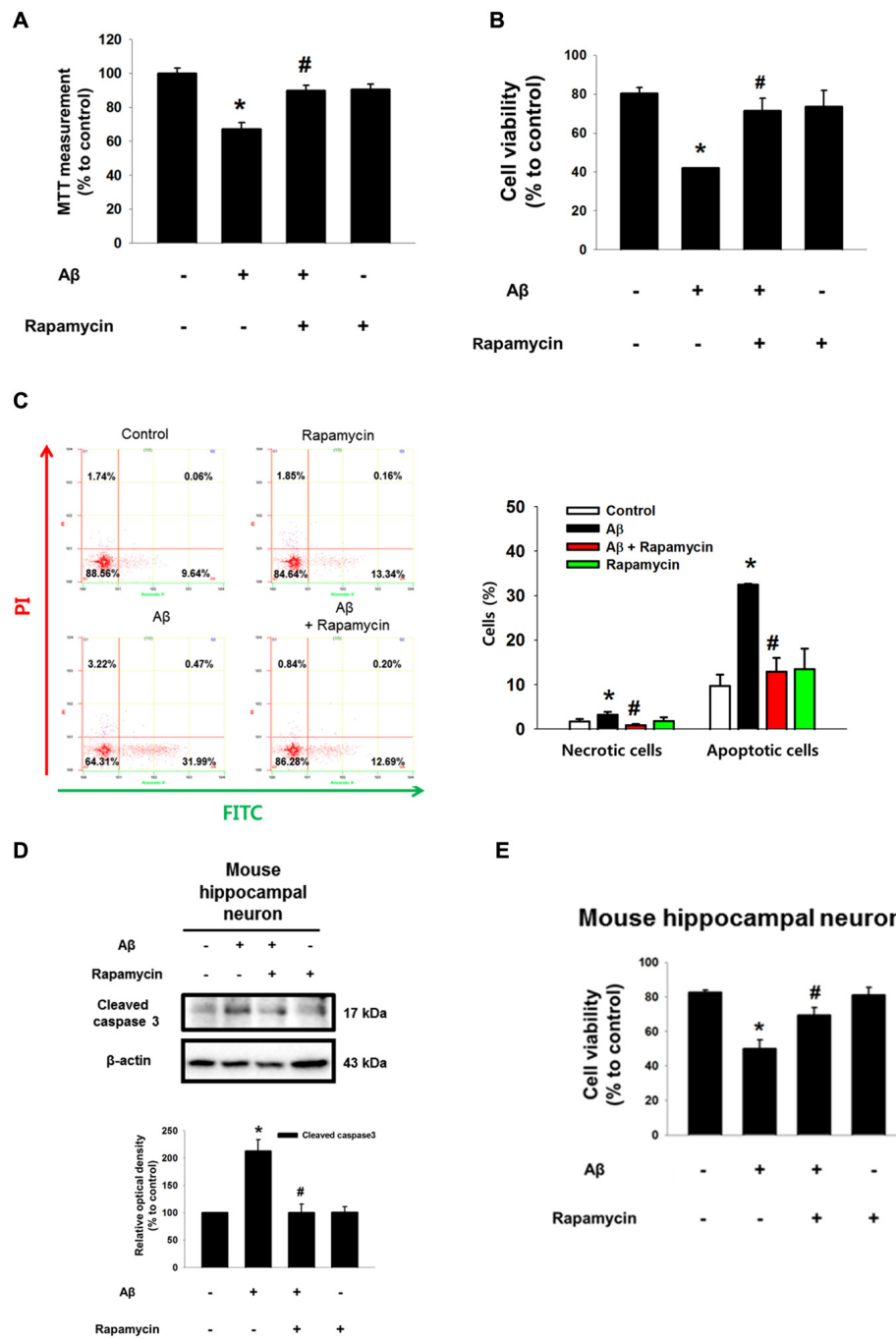


FIGURE 3 | Role of mTORC1 in the neuronal cell death induced by A β . **(A)** SK-N-MC cells were pretreated to rapamycin 10 nM for 30 min prior to A β (5 μ M) treatment for 24 h. Cytotoxicity was measured by MTT assay at an absorbance of 545 nm using a microplate reader. $n = 6$. **(B)** Cell viability was measured by trypan blue exclusion assay. $n = 6$. **(C)** Necrotic and apoptotic cells were counted by using annexin V/PI analysis with flow cytometry. $n = 4$. **(D)** Mouse hippocampal neurons were pretreated with rapamycin (10 nM) for 30 min prior to A β (5 μ M) for 24 h. Samples were blotted with cleaved caspase-3 and β -actin specific antibodies. $n = 6$. **(E)** Cell viability of mouse hippocampal neuron was counted by trypan exclusion assay. Data are presented as a mean \pm SE. $n = 6$. Each western blot image was presented as representative image. Quantitative blot data are presented as a mean \pm SE. * $p < 0.05$ vs. control, # $p < 0.05$ vs. A β treatment.

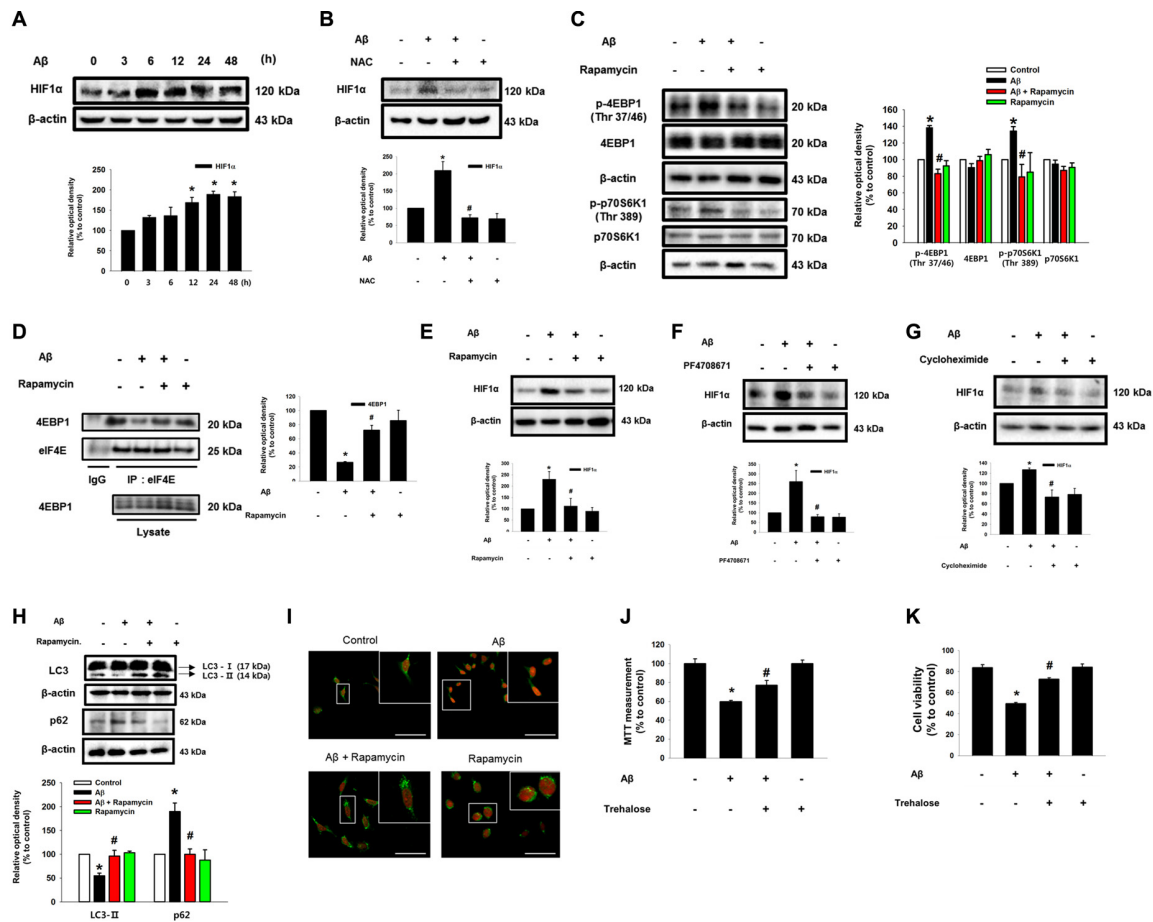


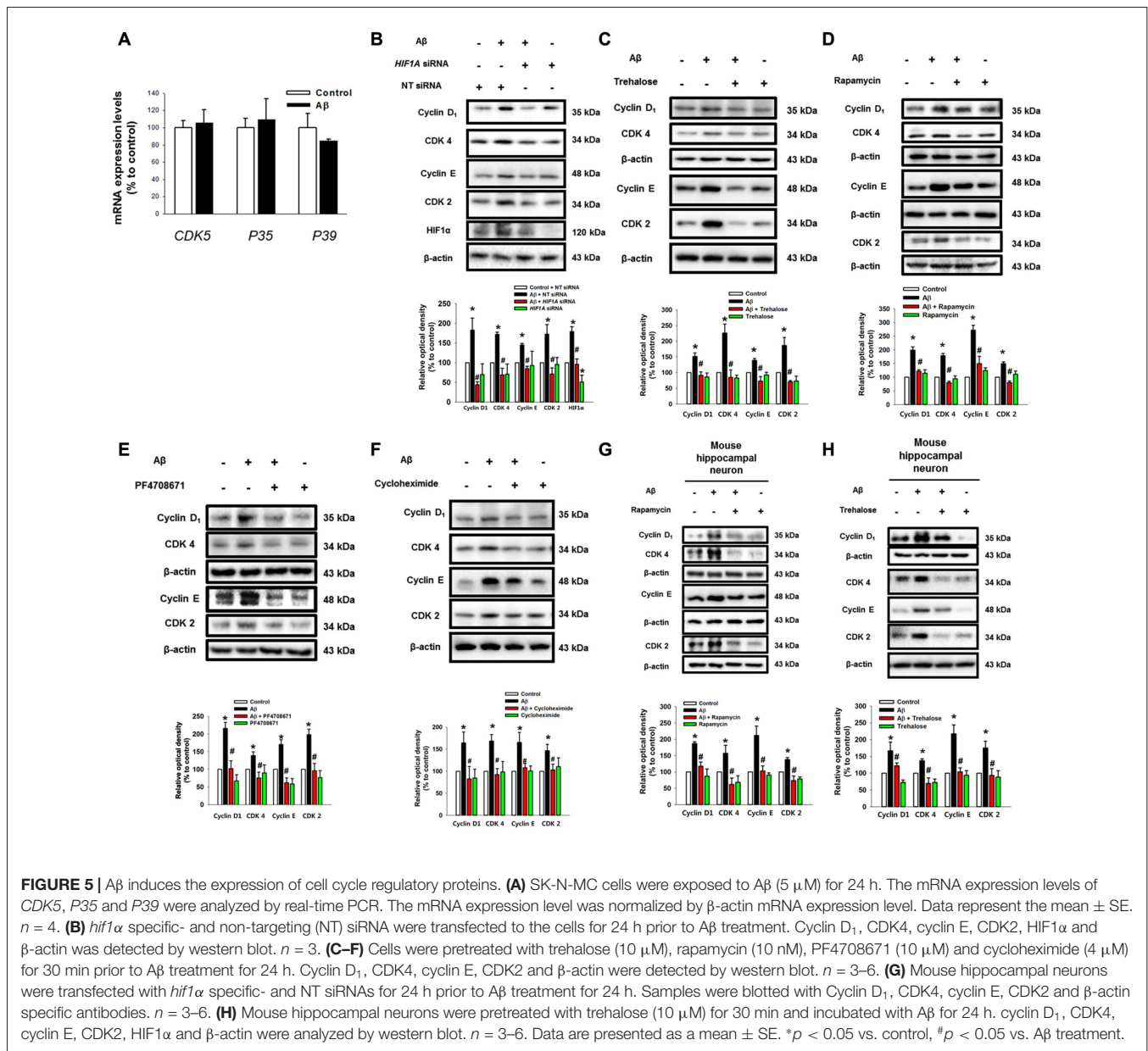
FIGURE 4 | A β facilitates HIF1 α synthesis and autophagy inhibition via mTOR activation. **(A)** SK-N-MC cells were exposed to A β (5 μ M) for 0–48 h. HIF1 α and β -actin expression was analyzed by western blot. $n = 3$. **(B)** Cells were pretreated with NAC (1 mM) for 30 min prior to A β treatment for 24 h. HIF1 α and β -actin expression were analyzed by western blot. $n = 3$. **(C,E)** Cells were incubated with rapamycin (10 nM) for 30 min prior to A β treatment for 24 h. Phosphorylation of 4EBP1 (Thr 37/46) and 4EBP1, phosphorylation of p70S6K1 (Thr 389), HIF1 α and β -actin were analyzed by western blot. $n = 6$. **(D)** Protein samples were immunoprecipitated by eukaryotic translation initiation factor 4E (eIF4E) antibody-conjugated protein A/G agarose beads. Samples were blotted with 4EBP1 and eIF4E-specific antibodies. $n = 3$. **(F)** Cells were exposed to PF4708671 (10 μ M) for 30 min prior to A β treatment for 24 h. HIF1 α and β -actin expression was detected by western blot. $n = 6$. **(G)** Cells were exposed to cycloheximide (4 μ M) for 30 min prior to A β treatment for 24 h. HIF1 α and β -actin expressions were detected by western blot. $n = 6$. **(H)** Cells were pretreated with rapamycin (10 nM) for 30 min, incubated with A β for 24 h and analyzed by western blotting with LC3, p62 and β -actin specific antibodies. $n = 3$ –6. **(I)** LC3 puncta was visualized by confocal microscopy. Presented results are merged images. Green and red fluorescents indicate LC3 and PI respectively. Scale bars, 50 μ m (magnification \times 600). **(J)** Cells were pretreated with trehalose (10 μ M) for 30 min prior to A β treatment for 24 h. Cytotoxicity was measured by MTT assay at an absorbance of 545 nm using a microplate reader. Data present the mean \pm SE. $n = 6$. **(K)** Cell viability was measured by trypan blue exclusion assay. Data are presented as a mean \pm SE. $n = 6$. Each blot image was presented as representative image. * $p < 0.05$ vs. control, # $p < 0.05$ vs. A β treatment.

reduced cell viability by 20% (Figure 6G). As tau proteins aggregate after detachment from microtubules, we investigated whether autophagy is associated in any way with aggregated tau. Interestingly, the induction of autophagy with trehalose significantly inhibited the tau phosphorylation induced by A β but not the total level of the tau protein, suggesting that autophagy negatively regulates the accumulation of cell cycle regulatory proteins, but not tau itself (Supplementary Figure S9A). Moreover, tau proteins aggregated by A β did not co-localize with LC3 puncta despite the fact that autophagy was induced by trehalose (Supplementary Figure S9B). These results suggest that the accumulation of cell cycle regulatory

proteins induced by A β is necessary for tauopathy, which leads to neuronal apoptosis.

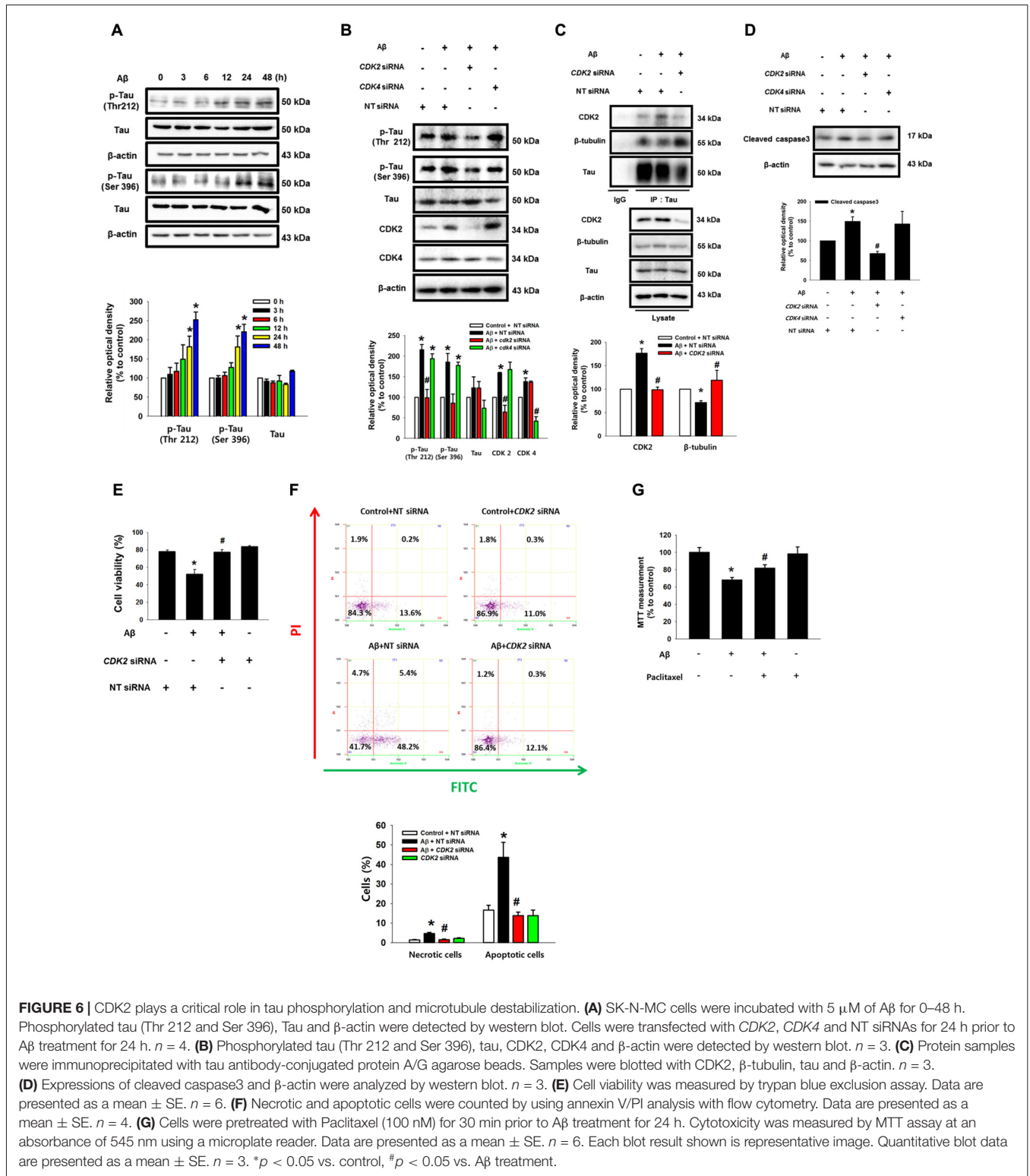
DISCUSSION

Our results here clearly show that A β has the ability to facilitate HIF1 α synthesis and autophagy inhibition via the PI3K/Akt-dependent distinctive mTORC1 pathway, which is critical for the expression of cyclins and CDKs, where CDK2 plays a unique role in the neuronal cell death mediated by tau phosphorylation (Figure 7). Concerning the cellular mechanisms of A β with regard to neuronal apoptosis, our data revealed



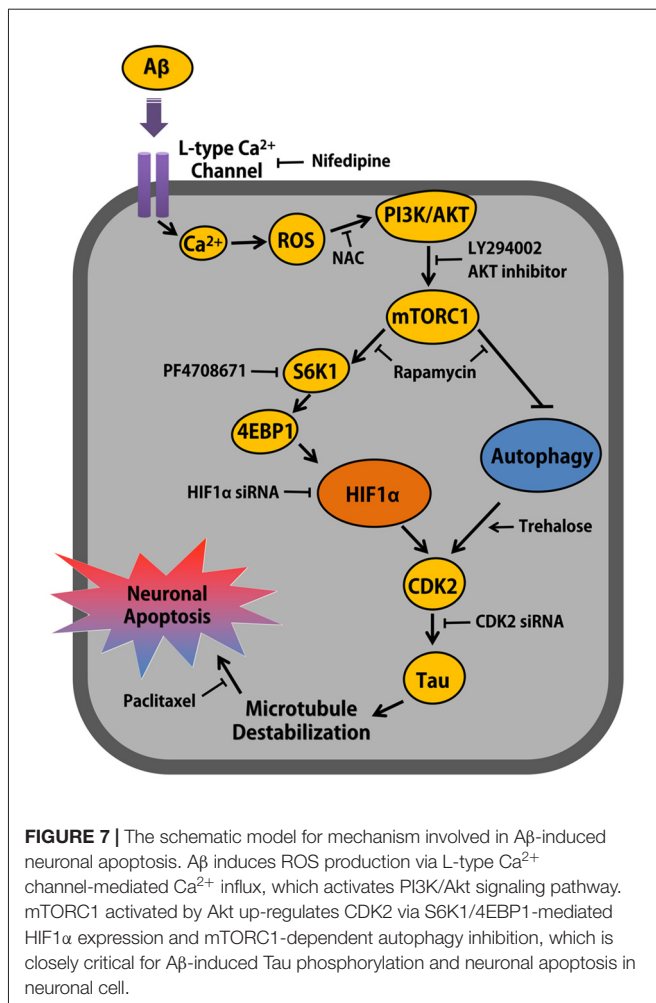
that a unique relationship between the A β signaling pathway and ROS production via the L-type calcium channel in the regulation of neuronal cell death. Evidence has demonstrated that oxidative stress is closely connected with several major pathological processes in AD, including tauopathy, mitochondria dysfunction and A β -induced neurotoxicity (Zhao and Zhao, 2013). Moreover, an increased cytosolic concentration of Ca²⁺ was shown to trigger mitochondrial damage, possibly increasing mitochondrial ROS production in various ways, such as by stimulating the TCA cycle and oxidative-phosphorylation or by blocking the respiratory chain at complex III (Brookes et al., 2004; Irigoien et al., 2009). Indeed, the L-type calcium channel was shown to possess a clear link to Ca²⁺ dysregulation induced by A β (Smith et al., 2005; Small et al., 2009). Thus, these results suggest that A β -induced ROS involves

an alteration in extracellular Ca²⁺ influx, where the L-type calcium channel is an essential mediator of the A β signaling pathway. One of the responsive molecules associated with ROS is the mTOR, which is hyper-activated in both mild and severe AD patients (Hudson et al., 2002). While the mTOR inhibitor has been well studied for its action to ameliorate symptoms in AD models (Hudson et al., 2002), the detailed mechanisms of mTOR in promoting neuronal apoptosis and its role in AD pathogenesis remain a topic of much debate. In addition, it has been reported that PI3K/Akt/mTOR signaling pathway has differential role in mental illnesses including depression. Previous researchers reported that the PI3K/Akt/mTOR signaling regulates neuronal inflammation and apoptosis via GSK3 inhibition, which is closely associated with neuroprotection (Kitagishi et al., 2012). In the present study,



we showed that ROS production generated by A β distinctively triggers mTORC1 activation through the PI3K/Akt signaling pathway. Given that PI3K/Akt signaling is regulated by growth factors and related receptors, we suggest that the PI3K/Akt pair has ability to activate downstream nutrient-sensing protein

kinases, including mTORC1 in A β -treated SK-N-MC cells. In addition, it was clearly shown that ROS production induced by an A β treatment stimulates the PI3K/Akt-mediated mTORC1 signaling pathway in neuronal cells (Maiese et al., 2012), suggesting that the ROS-dependent activation of the



PI3K/Akt/ mTORC1 pathway has a key function in A β -induced neuronal cell death.

A key finding of our study is that mTORC1 activated by A β stimulates HIF1 α synthesis in promoting neuronal cell death. This result differs from that in a previous report which revealed that A β actually reduces the level of HIF1 α via a ROS-independent mechanism in astrocytes (Schubert et al., 2009). Thus, some scientists have proposed that hypoxic preconditioning could prevent the deterioration of neuronal cells in AD as an alternative therapy (Crapper McLachlan et al., 1991; Ogunshola and Antoniou, 2009). However, many contradictory results have also been reported, showing that the manipulation of hypoxic pathways has many different outcomes (Ogunshola and Antoniou, 2009). For instance, we have shown that HIF1 α binding to the *BACE1* promoter induces *BACE1* expression and results in increased A β production in neuroblastoma cells (Lee et al., 2016). Moreover, hypoxia-mediated HIF1 α signaling is involved in the amyloidogenic processing of the amyloid precursor protein, and subsequent downstream events influence the activation of the pro-death gene BNIP3, thus leading to an increased incidence of AD and neurodegeneration after cerebral ischemia and stroke (Zhang

et al., 2007; Ogunshola and Antoniou, 2009). Although the reasons for the discrepancy with regard to the functional role of A β in the regulation of HIF1 α remain unknown, one possibility in the present study is that A β induces the co-operative regulation of mTORC1 downstream substrates, eIF4E, 4E-BP1, and p70S6K1, to stimulate mRNA translation during the synthesis of relevant proteins, including HIF1 α . This indicates that p70S6K1 activated by A β phosphorylates the ribosomal S6 protein and 4E-BP1, which in turn prevents the interaction between 4E-BP1 and eIF-4E ultimately to enhance the cap-dependent mRNA translation of HIF1 α and HIF1 α synthesis. In agreement with our current findings, mTORC1 was shown to act as a central mediator of the HIF1 α translation process in promoting the expression of VEGF-A (Dodd et al., 2015). A question remains as to how A β induces neuronal cell death via HIF1 α synthesis. We have shown that the HIF1 α synthesis induced by A β promotes the expression of cell cycle regulatory proteins. Indeed, mTORC1 signaling acting on HIF1 α was shown to promote the entering of the cell cycle, which can be harmful to neurons and which can result in neuronal apoptosis rather than replication (Bowser and Smith, 2002; Folch et al., 2012). This result is consistent with the notion that neurons are constantly struggling to keep their cell cycle in check, and negligence in this surveillance often leads to cell cycle re-entry and then to neuronal apoptosis (Herrup and Yang, 2007). Thus, our results in the present study suggest that the increased level of HIF1 α synthesis is able to enhance AD pathogenesis by inducing the expression of cell cycle regulatory proteins and allow us to speculate that HIF1 α inhibition provides an alternative approach for therapeutic targeting. On the other hand, we found that mTORC1 activated by A β facilitates autophagy inhibition to stimulate the expression of cell cycle regulatory proteins during neuronal cell death. This result is consistent with the notion that a treatment with pharmacological inhibitors of lysosomal degradation, such as bafilomycin and chloroquine, increases the expression levels of many proteins, including cell cycle regulatory proteins and even HIF1 α (Hubbi et al., 2013). Indeed, it was proven that mTORC1 plays an inhibitory role in autophagy by the blocking of ULK-1 activation, which is required for autophagosome formation (Kim et al., 2011; Russell et al., 2013). Thus, these data indicate that A β may cause autophagy inhibition and in turn attenuate the lysosomal degradation of downstream signaling molecules in the A β signaling pathway. Collectively, these results provide important evidence of a pathogenic mechanism of the A β -mediated mTORC1 signaling pathway by which the level of cell cycle regulatory proteins is enhanced in a multifaceted manner through not only the stimulation of HIF1 α synthesis but also the inhibition of autophagy.

Among cell cycle protein kinases, we identified here that A β facilitates CDK2 interaction with a tau protein and that CDK2 is highly involved in the processes of tau phosphorylation and microtubule destabilization during neuronal cell death. In support of our data, the tau protein was shown to have several motifs which can interact with cell cycle protein kinases, including CDK2 (Schmetsdorf et al., 2009). NMR spectroscopy

analysis results also revealed that the CDK2 protein complex has the ability to phosphorylate the tau protein (Welburn and Endicott, 2005). Thus, these results present the possibility that CDK2 interacting with tau protein may be an important signaling event for tau phosphorylation induced by A β . Previous studies have discovered many candidate molecules that phosphorylate tau, including the mTOR, glycogen synthase kinase 3 β (GSK3 β) and CDK5 (Rankin et al., 2007; Caccamo et al., 2013; Kimura et al., 2014). Although we have not studied the role of A β on GSK3 β , we found that A β did not have an effect on the expression of CDK5. Indeed, CDK5 was reported to play a positive role in neuronal survival under hypoxic conditions (O'Hare et al., 2005). In contrast, previous reports provided compelling evidence that CDK2 plays a critical role in the neuronal apoptosis induced by A β , while a specific inhibitor of CDK1 and CDK2, flavopiridol, shows neuroprotective effects (Copani et al., 1999, 2001; Folch et al., 2012). Thus, these results indicate that A β facilitates the interaction between CDK2 and tau protein and uniquely regulates tau phosphorylation during neuronal apoptosis as induced by A β . On the other hand, it is well known that tau is a neuronal-specific protein associated with microtubules and that it plays a pivotal role in the maintenance of the neuronal cytoskeleton and neurofilaments. However, given that microtubule destabilization in neuronal axons attenuates synaptic plasticity and may also lead to neuronal apoptosis and thus causing clinical symptoms of AD (Bendiske et al., 2002; Conde and Cáceres, 2009; Tu et al., 2014), our findings here suggest that A β induces the CDK2-mediated phosphorylation of tau and is then responsible for microtubule destabilization ultimately to promote neuronal apoptosis. Several reports have suggested that tau aggregates induced by A β can be removed from cells by autophagy to then be rescued from apoptosis (Bandyopadhyay et al., 2007; Flach et al., 2012; Krüger et al., 2012). However, our results in the present study revealed that autophagy induction does not affect the degradation of tau aggregates but blocks the phosphorylation of tau. These findings suggest that A β contributes to tauopathy via the CDK2-mediated phosphorylation of tau by preventing autophagy induction. Our results and schematic model were based on the data analyzed

by quantitative western blot. Western blot-based analysis is highly depending on the specificity of antibodies. Therefore, further analysis of protein concentration and phosphorylation (such as proteomics and transcriptomics) will support our observation.

Overall, the results of this study suggest that A β is responsible for the AD pathogenesis by stimulating neuronal apoptosis, through which A β stimulates the ROS-mediated PI3K/Akt pathway to activate mTORC1, which is essential for the processes of autophagy inhibition and HIF1 α synthesis. The A β signaling pathway through mTORC1 activation ultimately triggers the CDK2-mediated phosphorylation of tau and the destabilization of microtubules for neuronal cell death. Thus, highlighting the signaling pathways involved in the A β -stimulated cell death pathway may provide potential therapeutic targets for strategic modulations of AD.

AUTHOR CONTRIBUTIONS

KHL: conception and design, collection of data, data analysis and interpretation, manuscript writing; S-JL and HJH: conception and design, data analysis and interpretation, manuscript writing; HJL: conception and design, collection of data, data analysis and interpretation; GEC, YHJ and DIK: conception and design, data analysis and interpretation; AAG: data analysis and interpretation; JMR: conception and design.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fnmol.2017.00229/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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