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Research Paper

Autophagy protects neuron from Aβ-induced cytotoxicity

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Abbreviations: α7nAChR or α7R, α7 nicotinic acetylcholine receptor; Aβ, beta amyloid; $Aβ_{1-42}$, beta $amyloid_{1-42}$ peptide; $Aβ_{25-35}$, beta $amyloid_{25-35}$ peptide; α-BTX, α-bungarotoxin; AD, Alzheimer disease; eAβ, extracellular beta amyloid peptides; EGFP, enhanced green fluorescent protein; LC3, microtubule-associated protein light chain 3; nAChR, nicotinic acetylcholine receptor; NeuN, neuron-specific nuclear protein; PAO, phenylarsine oxide; siRNA, small RNA interference

Key words: autophagy, Alzheimer disease, Aβ, apoptosis, α7nAChR

Autophagy is a degradation pathway for the turnover of dysfunctional organelles or aggregated proteins in cells. Extracellular accumulation of β-amyloid peptide has been reported to be a major cause of Alzheimer disease (AD) and large numbers of autophagic vacuoles accumulate in the brain of AD patient. However, how autophagic process is involved in Aβ-induced neurotoxicity and how Aβ peptide is transported into the neuron and metabolized is still unknown. In order to study the role of autophagic process in Aβ-induced neurotoxicity, EGFP-LC3 was overexpressed in SH-SY5Y cells (SH-SY5Y/pEGFP-LC3). It was found that treatment with $Aβ_{25-35}$, $Aβ_{1-42}$ or serum-starvation induced strong autophagy response in SH-SY5Y/pEGFP-LC3. Confocal double-staining image showed that exogenous application of $Aβ_{1-42}$ in medium caused the colocalization of $Aβ_{1-42}$ with LC3 in neuronal cells. Concomitant treatment of Aβ with a selective α7nAChR antagonist, α-bungarotoxin (α-BTX), enhanced Aβ-induced neurotoxicity in SH-SY5Y cells. On the other hand, nicotine (nAChR agonist) enhanced the autophagic process and also inhibited cell death following Aβ application. In addition, nicotine but not α-BTX increased primary hippocampal neuronal survival following Aβ treatment. Furthermore, using Atg7 siRNA to inhibit autophagosome formation in an early step or α7nAChR siRNA to knock down α7nAChR significantly enhanced Aβ-induced neurotoxicity. Confocal double-staining imaging shows that nicotine treatment in the presence of Aβ enhanced the colocalization of α7nAChR with autophagosomes. These results suggest that α7nAChR may act as a carrier to bind with eAβ and internalize into cytoplasm and further inhibit Aβ-induced neurotoxicity via autophagic degradation pathway. Our results suggest that autophagy process plays a neuroprotective role against Aβ-induced neurotoxicity. Defect in autophagic regulation or Aβ-α7nAChR transport system may impair the clearance of Aβ and enhance neuronal death.

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Introduction

Macroautophagy, hereafter referred to as autophagy, is an evolutionarily conserved process which is a lysosomal degradation pathway for long-lived protein and cytoplasmic organelles.[1,2] Autophagy is a complete and irreversible degradation of the substrate by lysosomal enzymes.[3] Genetic studies using various model organisms have highlighted the importance of autophagy in physiological and pathological events. The principal role of autophagy is the supply of nutrients for survival, as shown in yeast.[4] In addition to this fundamental role, autophagy also contributes to development, longevity, growth regulation, tumor suppression, immunity (intracellular clearance, degradation of invading bacteria or virus) and cell death.[5-7] Autophagy also functions in a number of neurodegenerative diseases such as Parkinson, Huntington and Alzheimer diseases, possibly by removing damaged organelles to reduce the production of reactive oxygen species and aggregate-prone proteins.[6]

LC3 (microtubule-associated protein light chain 3) is one of three mammalian homologues of yeast Atg8, which is localized in autophagosome membrane after processing and is a widely applied marker for autophagy. LC3 is not only necessary for the formation of autophagosome, but also participates in the formation of the autophagosomal membrane.[8] Following synthesis, the C-terminus of LC3 is cleaved by Atg4 to produce LC3-I (resides in the cytosol; free form), LC3-I is then further converted to LC3-II (membrane-bound form) by Atg7 and Atg3.[8] The amount of LC3-II is correlated with the extent of autophagosome formation. LC3-II is also the first mammalian protein identified to specifically associate with autophagosome membrane.[9]

Alzheimer disease (AD) is a neurodegenerative disorder characterized by progressive dementia and brain morphological change such as atrophy, senile plaques with fibrillogenic beta amyloid (Aβ), and intraneuronal neurofibrillary tangles (NFT) with hyperphosphorylated tau.[10] It has been demonstrated that a large number of autophagic vacuoles are observed in dystrophic neurites before extracellular Aβ deposition in the neurons of AD patients and presenilin-1 (PS1)/APP mice, suggesting that the autophagy system is involved in AD pathogenesis.[11,12] The mechanism related to intracellular Aβ accumulation, the association of extracellular Aβ with

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autophagosome, and the pathogenic role of autophagy process in AD are still not clear. In order to examine the role of autophagic process in Aβ-induced neurotoxicity, we here overexpressed pEGFP-LC3 in SH-SY5Y neuroblastoma cells (SH-SY5Y/pEGFP-LC3) to study the effect of autophagic process on the Aβ-induced neurotoxicity.

Results

Aβ activates the autophagic process in SH-SY5Y. We examined autophagosome formation by using two stable clones of SH-SY5Y/pEGFP-LC3 cells. Cells were treated with $A\beta_{25\text{-}35}$ or $A\beta_{1\text{-}42}$ (10 or 30 μM) for 4 h, Figure 1A shows that $A\beta_{25\text{-}35}$ or $A\beta_{1\text{-}42}$ increased the autophagosome in SH-SY5Y/pEGFP-LC3 cells as demonstrated by fluorescent punctate dots formation. Serum starvation was used as a positive control. However, there was no fluorescent punctate dot formation in two clones of SH-SY5Y/pEGFP cells (data not shown), indicating that the punctate dots formation was related to LC3. The quantitative data of EGPF-LC3-II punctate dots from two different clones in response to Aβ treatment were shown in Figure 1B. The results suggest that $A\beta_{25\text{-}35}$, $A\beta_{1\text{-}42}$ or serum starvation increased EGPF-LC3-II punctate dots formation in these two clones at 4 h. The average data of two clones in Figure 1C indicated that Aβ-induced EGPF-LC3-II punctate was dose- and time-dependent. The results revealed that autophagosome formation reached maximal effect at 4 h and 30 μM $A\beta_{25\text{-}35}$ had more autophagosomes than 10 μM at 2 h. $A\beta_{25\text{-}35}$-induced autophagosome formation declined after 24 h incubation. On the other hand, the autophagosome formation in response to serum starvation still maintained at a high level after 24 h treatment.

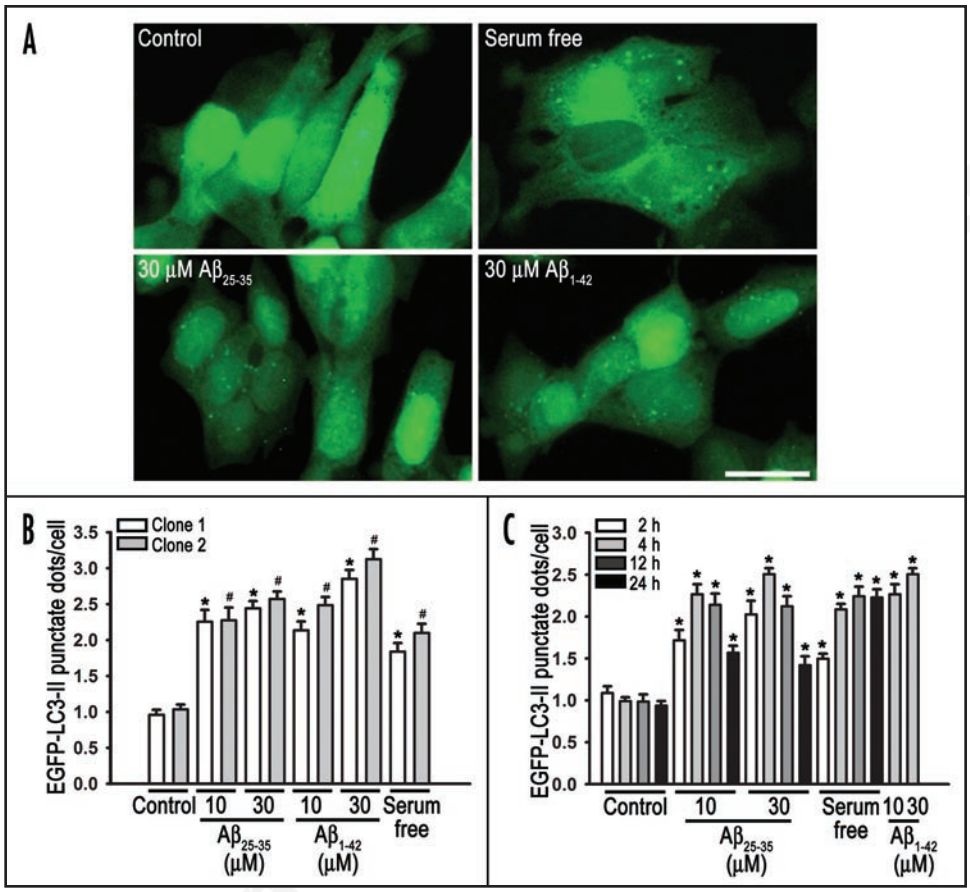

Figure 1. Aβ increases autophagosome formation in SH-SY5Y/pEGFP-LC3 cells. (A) SH-SY5Y/pEGFP-LC3 cells were treated with $A\beta_{25\text{-}35}$ or $A\beta_{1\text{-}42}$ (30 μM) or serum starvation for 4 h, autophagosome formation increased as demonstrated by the fluorescent punctate dots. Scale bar: 25 μm. (B) Summarized result of fluorescent punctate dots formation at 4 h in two different clones. $^{*,\#}p < 0.05$ as compared with respective control of clone 1 and clone 2. (C) Time-dependent effect of autophagosome formation. Data were presented as Mean ± SEM. $^{*}p < 0.05$ as compared with control.

We further used drugs which affect autophagosome formation or degradation to examine the effect of $A\beta_{25\text{-}35}$ or $A\beta_{1\text{-}42}$ on the autophagic process. Cotreatment of $A\beta_{25\text{-}35}$ or $A\beta_{1\text{-}42}$ (30 μM) with 40 nM bafilomycin A1, a H^+-ATPase inhibitor which blocks the fusion of autophagosome with lysosome, significantly increased EGPF-LC3-II fluorescent punctate dots formation (Fig. 2A). In contrast, wortmannin (20 nM), which inhibits autophagosome formation, antagonized the fluorescent punctate dots formation following $A\beta_{25\text{-}35}$ treatment or serum withdrawal (Fig. 2A). While raising bafilomycin A1 concentration from 40 nM to 200 nM, Figure 2B shows no significant difference in dot formation under serum-free condition for 4 h, suggesting that 40 nM bafilomycin A1 was high enough to exert effects in SH-SY5Y/pEGFP-LC3 cells (Fig. 2B); Zhu et al.[15] also found that treatment of SH-SY5Y cells with 10 nM bafilomycin A1 can effectively block the fusion of autophagosome with lysosome. The quantitative results of Aβ-induced autophagosome at 2~4 h in the presence of bafilomycin A1 or wortmannin were shown in Figure 2C. Aβ also concentration-dependently enhanced the number of autophagosomes following bafilomycin A1 treatment. Western blotting revealed that $A\beta_{1\text{-}42}$ enhanced the conversion of EGFP-LC3-I to EGFP-LC3-II and bafilomycin A1 (40 nM) further increased the cellular content of EGFP-LC3-II via the inhibition of autophagosome degradation (Fig. 2D; the quantitative results were shown in the right). These results demonstrate that Aβ increased autophagosome formation and bafilomycin A1 enhanced, whereas wortmannin inhibited this potentiating action.

Colocalization of $A\beta_{1\text{-}42}$ with autophagosomes in SH-SY5Y/pEGFP-LC3 cells. We further examined whether Aβ can be internalized and colocalized with autophagosome. In order to increase the autophagosome dot formation, the cells were treated with $A\beta_{1\text{-}42}$ (1 μM) for 4 h in the presence of bafilomycin A1. The immunostaining showed that $A\beta_{1\text{-}42}$ peptide was internalized and colocalized with EGFP-LC3-II punctate dots as demonstrated by confocal microscope (Fig. 3; the representative colocalization is indicated by arrow), suggesting that Aβ can be internalized and colocalized with autophagosomes.

Autophagic process protects neuron from Aβ-induced neuronal death. LC3-I is a cytosolic soluble protein and becomes membrane-associated when it is converted to LC3-II, which is dependent upon the Atg5-Atg12-Atg16 complex formation. LC3 and Atg12 are ubiquitin-

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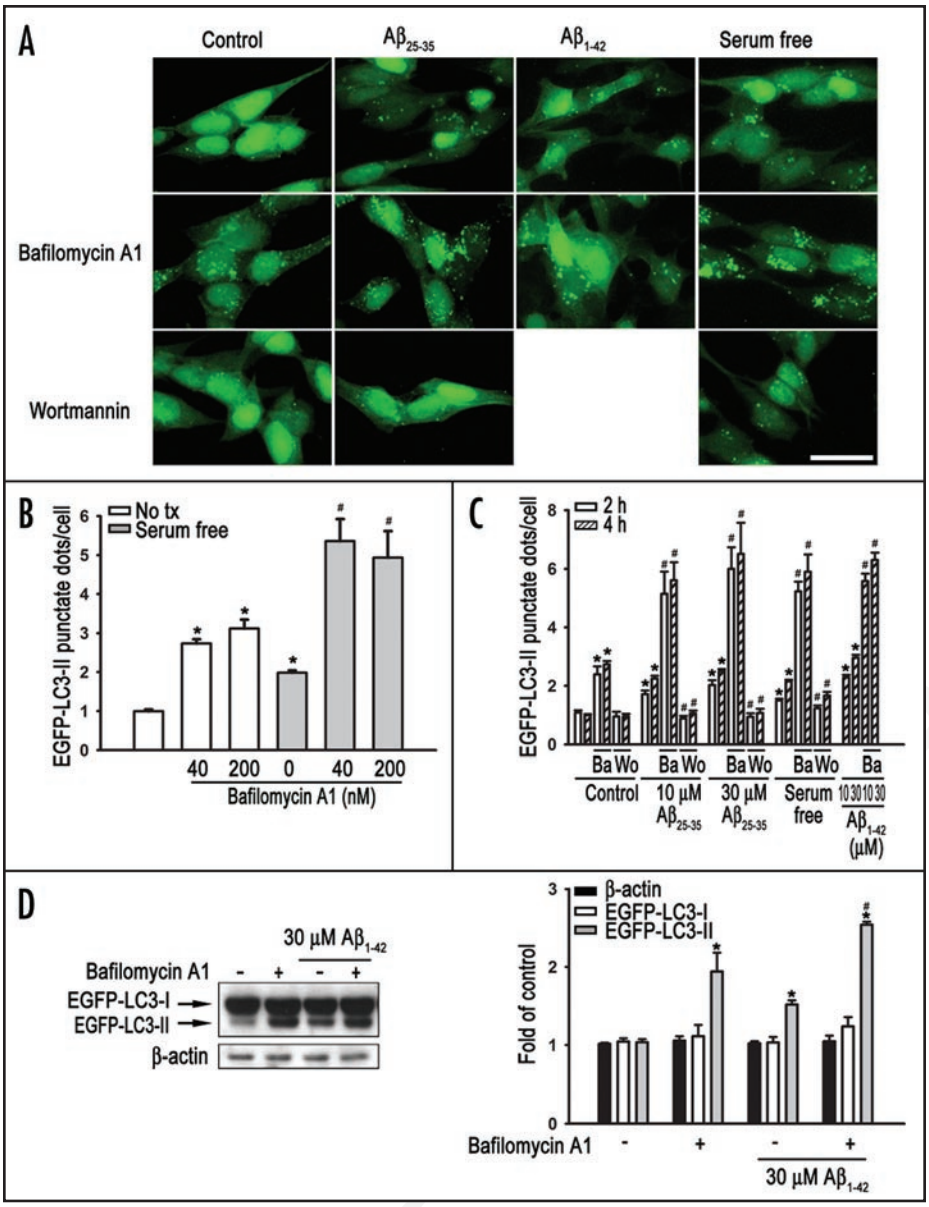

Figure 2. Aβ-induced autophagosome accumulation is enhanced by bafilomycin A1 and inhibited by wortmannin in SH-SY5Y/pEGFP-LC3 cells. (A) The photographs showed that bafilomycin A1 (40 nM) enhanced and wortmannin (20 nM) inhibited $A\beta_{25\text{-}35}$-, $A\beta_{1\text{-}42}$-(30 μM) or serum starvation-induced increase of autophagosomes after 4 h incubation. Scale bar: 25 μm (B) Bafilomycin A1 increased EGFP-LC3-II punctate formation in control cells or 4 h after serum starvation. (C) Bafilomycin A1 enhanced and wortmannin inhibited EGFP-LC3-II dots formation following Aβ treatment. *$p < 0.05$ compared with control; #$p < 0.05$ compared with $A\beta_{25\text{-}35}$-, $A\beta_{1\text{-}42}$-treatment or serum starvation alone. (D) $A\beta_{1\text{-}42}$ treatment increased the conversion of EGFP-LC3-I to EGFP-LC3-II after 4 h incubation. Cotreatment of $A\beta_{1\text{-}42}$ with bafilomycin A1 (40 nM) further increased the level of EGFP-LC3-II using western blotting analysis. The summarized data are shown in the right panel. *$p < 0.05$ compared with respective control; #$p < 0.05$ compared with bafilomycin A1 alone.

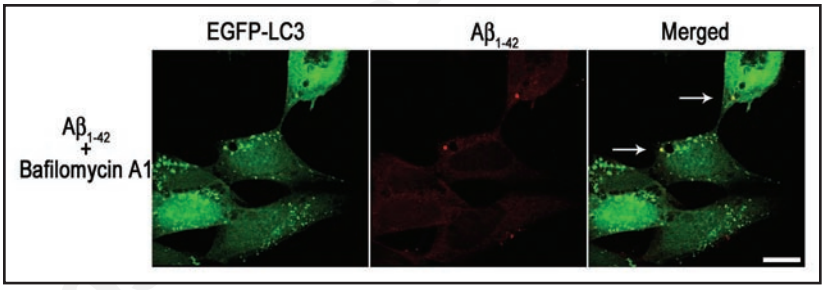

Figure 3. Colocalization of $A\beta_{1\text{-}42}$ with EGFP-LC3-II punctate dots in the cytoplasm of SH-SY5Y/pEGFP-LC3 cells. Cells were treated with $A\beta_{1\text{-}42}$ (1 μM) plus bafilomycin A1 (10 nM) for 4 h, confocal immunostaining images showed the internalization of $A\beta_{1\text{-}42}$ which were colocalized with EGFP-LC3-II autophagosome as indicated by arrows. Scale bar: 10 μm.

like proteins that are activated by the E1-like enzyme Atg7 during these processes.[8,16] SH-SY5Y cells were then transfected with 20 nM Atg7 siRNA to inhibit autophagosome formation at an earlier step. Figure 4A showed that knockdown of endogenous Atg7 expression significantly increased Aβ-induced Hoechst (+) cells as compared with control siRNA (siCon) group. The quantitative data showed that Atg7 knockdown enhanced $A\beta_{25\text{-}35}$/$A\beta_{1\text{-}42}$-induced neuronal death by 1.3/1.9 folds, respectively (Fig. 4B). Treatment of siAtg7 for 48 h inhibited endogenous Atg7 protein expression (Fig. 4C) and further antagonized $A\beta_{1\text{-}42}$-induced conversion of LC3-I to LC3-II (Fig. 4D). These results suggest that autophagic process plays an important role in protecting neurons against Aβ-induced apoptosis. Flow cytometry analysis of PI staining also revealed similar results that knockdown of Atg7 using siAtg7 significantly increased the $subG_1$ population following $A\beta_{25\text{-}35}$ treatment (Fig. 4E).

α7nAChR receptors are involved in neuroprotection following Aβ treatment. Nagele et al.[17] reported that α7nAChR in neurons facilitates intracellular accumulation of Aβ. In order to study the relationship of α7nAChR and extracellular Aβ in autophagic process, nicotinic agonist and antagonist were thus used. SH-SY5Y/pEGFP-LC3 cells were treated with 30 μM $A\beta_{25\text{-}35}$ or $A\beta_{1\text{-}42}$ in the presence of nicotine (nicotinic receptor agonist) or α-BTX (α7nAChR antagonist). Figure 5A showed that treatment with nicotine alone but not α-BTX for 4 h increased the activation of autophagic process. Concomitant treatment with bafilomycin A1 (40 nM) enhanced nicotine-induced autophagosome formation (Fig. 5B). In addition, nicotine also enhanced $A\beta_{25\text{-}35}$-induced autophagosome formation (Fig. 5A and B). In contrast, α-BTX inhibited autophagosome formation induced by $A\beta_{25\text{-}35}$ (Fig. 5A and B). On the other hand, both nicotine and α-BTX did not influence serum starvation-induced autophagosome formation in SH-SY5Y/pEGFP-LC3 cells (Fig. 5B), indicating the selectivity of nicotinic receptors in regulating autophagic process induced by different conditions. Furthermore, it was found that $A\beta_{25\text{-}35}$- or $A\beta_{1\text{-}42}$-induced neuronal apoptosis was inhibited by

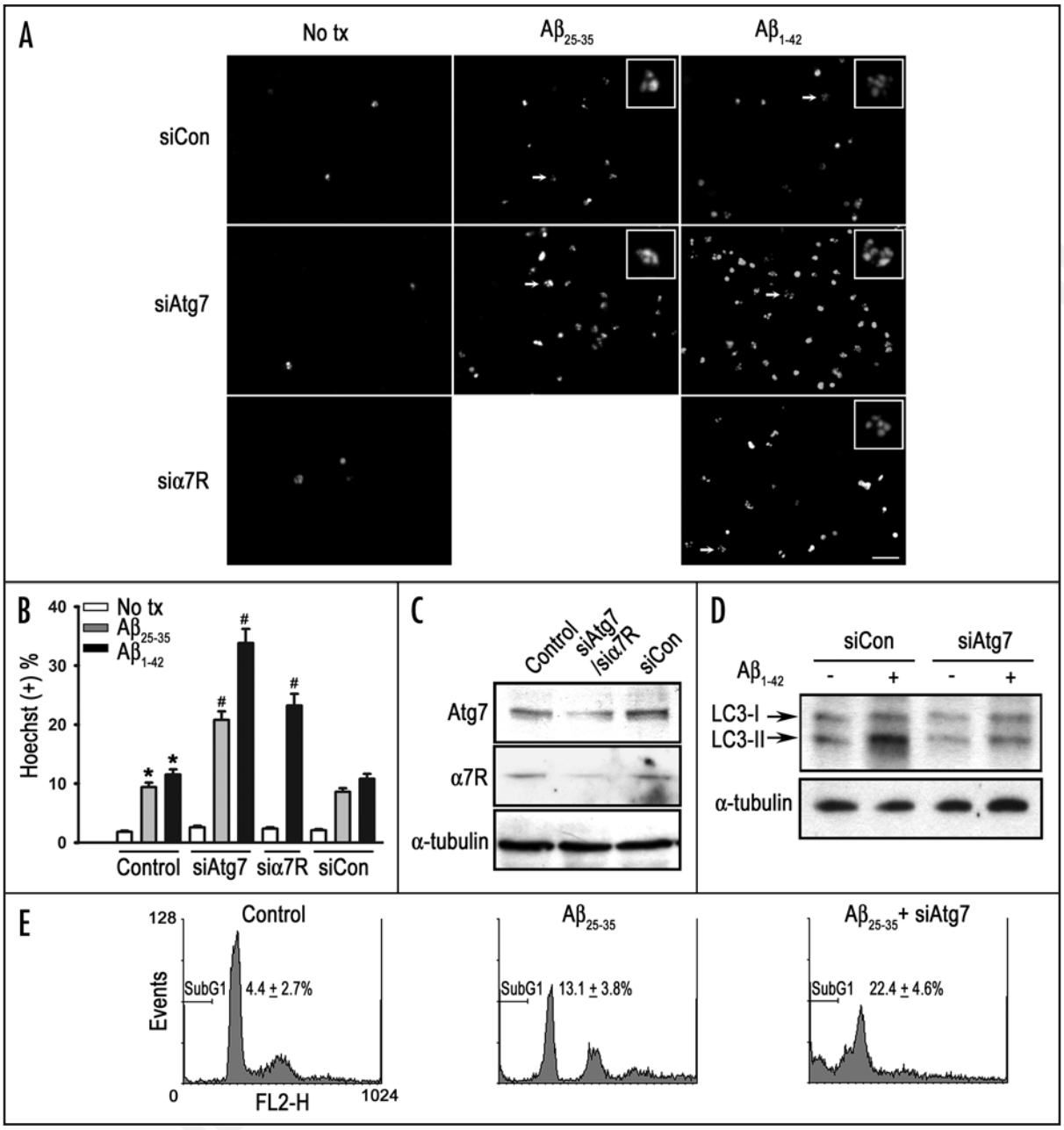

Figure 4. Knockdown of Atg7 or α7nAChR enhances Aβ-induced neuronal death. SH-SY5Y cells were pretransfected with 20 nM Atg7 siRNA for 48 h or α7nAChR (α7R) siRNA for 24 h before treatment with 30 μM $A\beta_{25-35}$ or $A\beta_{1-42}$ for another 48 h. (A) Images of Hoechst (+) stained cells showed that knockdown of Atg7 or α7R enhanced cell apoptosis induced by $A\beta_{25-35}$ or $A\beta_{1-42}$. Arrows indicated the nuclear condensation of cells, which were enlarged in the upper corner. (B) Summarized data of Hoechst (+) staining. (C) Atg7/α7R siRNA treatment reduced the protein expression of Atg7/α7R at 48/24 h. (D) Knockdown of Atg7 antagonized $A\beta_{1-42}$ (30 μM, 4 h treatment)-induced the conversion of LC3-I to LC3-II. (E) Knockdown of Atg7 enhanced $A\beta_{25-35}$ (30 μM)-induced increase of $subG_1$ cell population examined by flow cytometry analysis of PI stained cells. Scale bar: 25 μm. *$p < 0.05$ compared with control without $A\beta_{25-35}$ or $A\beta_{1-42}$; #$p < 0.05$ compared with respective $A\beta_{25-35}$- or $A\beta_{1-42}$-treatment alone. siCon: control siRNA.

nicotine (0.3 and 1 mM) and enhanced by α-BTX in SH-SY5Y (Fig. 5C). However, nicotine and α-BTX had no significant effect on serum starvation-induced apoptosis in SH-SY5Y (Fig. 5D). It has been reported that Aβ causes membrane oxidation and ROS production, which leads to neuronal apoptosis.[10] We further investigated intracellular free radical generation following $A\beta_{25-35}$ treatment. Exposure to $A\beta_{25-35}$ for 48 h resulted in a dose-dependent increase of intracellular free radicals in SH-SY5Y, as demonstrated by DCF fluorescence assay (106.1 ± 6.4%, 117.2 ± 5.9% and 147.1 ± 4.7% of control, respectively for 3, 10, 30 μM $A\beta_{25-35}$, respectively). Nicotine or rapamycin (autophagic process stimulator) inhibited $A\beta_{25-35}$-induced free radical formation, whereas α-BTX or wortmannin (autophagic process inhibitor) enhanced it (Fig. 5E). We further studied the influence of nicotine and α-BTX on the clearance of extracellular $A\beta_{1-42}$ in wild-type SH-SY5Y cells. As shown in Figure 5F, pretreatment with nicotine (1 mM) decreased and α-BTX (5 μM) increased the extracellular $A\beta_{1-42}$ content in the culture medium 2 h after incubation, suggesting that nicotinic receptor activation may

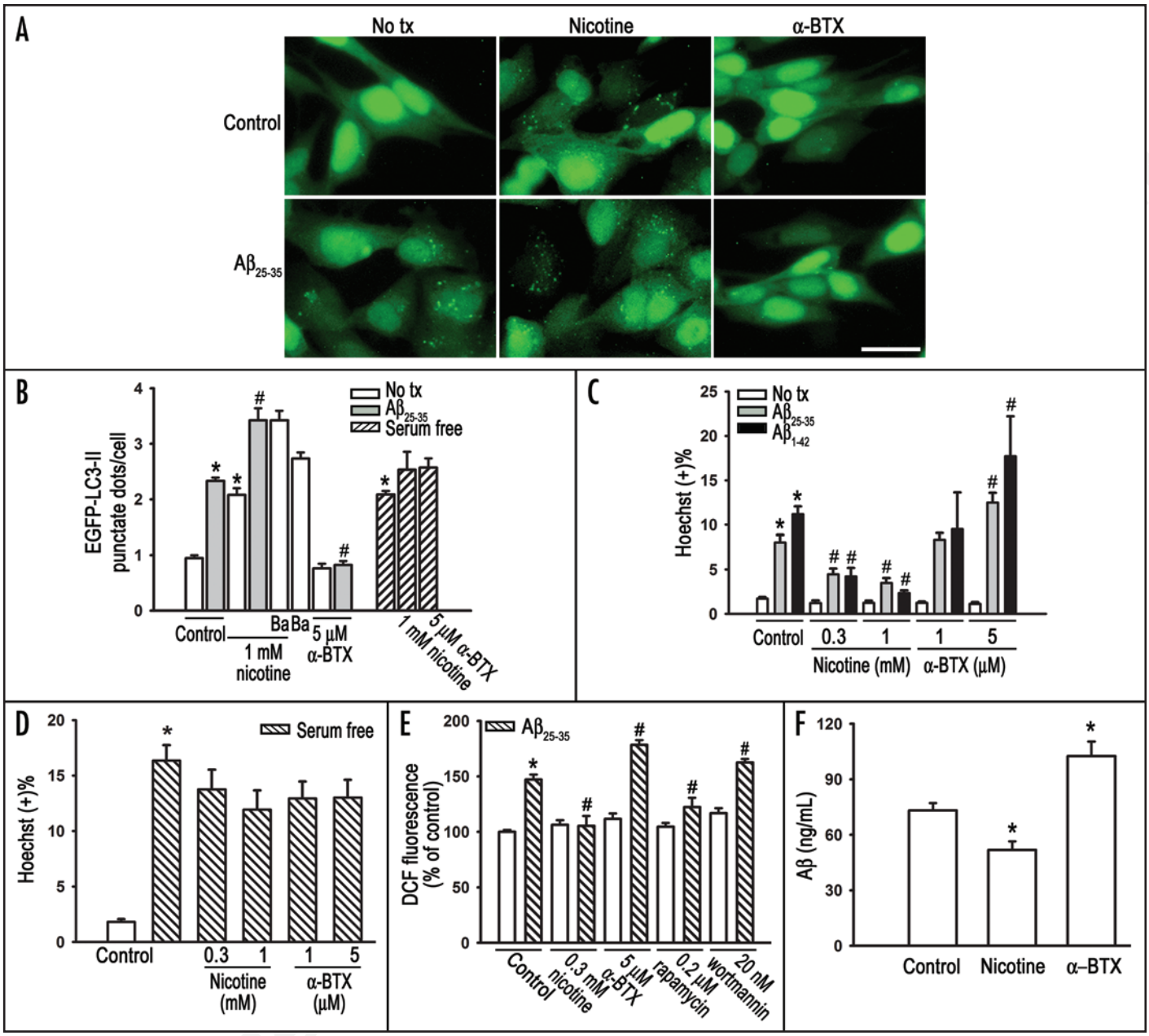

Figure 5. Nicotine inhibits and α-BTX enhances Aβ-induced neuronal death. (A) Photographs showed that treatment with nicotine (1 mM) alone but not α-BTX (5 μM) for 4 h induced the activation of autophagic process. Cotreatment with nicotine (1 mM) enhanced and α-BTX (5 μM) inhibited $A\beta_{25-35}$ (30 μM)-induced autophagosome formation in SH-SY5Y/pEGFP-LC3 cells. Scale bar: 25 μm. Quantitative analysis was shown in (B). Note that cotreatment of 1 mM nicotine with 40 nM bafilomycin A1 (Ba) enhanced autophagosome formation. (C) Nicotine inhibited and α-BTX enhanced $A\beta_{25-35}$- or $A\beta_{1-42}$-induced neuronal apoptosis, which was evaluated by Hoechst staining. (D) Nicotine and α-BTX treatment did not influence serum starvation-induced neuronal apoptosis at 24 h in SH-SY5Y cells. (E) Nicotine or rapamycin inhibited and α-BTX enhanced $A\beta_{25-35}$-induced increase of intracellular free radicals formation in SH-SY5Y using DCF assay. *$p < 0.05$ compared with control without $A\beta_{25-35}$ or $A\beta_{1-42}$; #$p < 0.05$ compared with $A\beta_{25-35}$- or $A\beta_{1-42}$-treatment alone. (F) Quantitative analysis of extracellular $A\beta_{1-42}$ of the conditioned medium in SH-SY5Y cells 2 h after $A\beta_{1-42}$ (1 μM) application. *$p < 0.05$ compared with control.

enhance the internalization of $A\beta_{1-42}$. $A\beta_{1-42}$ in the cell lysate can not be detected by ELISA, probably due to the intracellular degradation. Using siRNA to knock down α7nAChR (siα7R) in SH-SY5Y cells, the western blotting result showed that treatment of siα7R for 24 h inhibited endogenous α7nAChR protein expression (Fig. 4C). Figure 4A showed that knockdown of endogenous α7nAChR expression significantly increased $A\beta_{1-42}$-induced Hoechst (+) cells as compared with control siRNA (siCon) group. The quantitative data showed that α7nAChR knockdown enhanced $A\beta_{1-42}$-induced neuronal death by one fold (Fig. 4B). These results demonstrate the role of α7nAChR in regulating eAβ-induced neurotoxicity and ROS formation but not serum starvation-induced cell apoptosis.

Aβ-treatment induces α7nAChR internalization and colocalization with autophagosome in SH-SY5Y/pEGFP-LC3 cells. As mentioned above, α7nAChR regulates autophagosome formation induced by Aβ. We further investigated α7nAChR internalization upon Aβ treatment. Confocal image showed that α7nAChR was diffusely stained by α7nAChR antibody in control SH-SY5Y/

pEGFP-LC3 cells (Fig. 6). However, $A\beta_{25-35}$ (30 μM)-treatment for 4 h induced formation of intracellular cluster of α7nAChR. $A\beta_{25-35}$ plus nicotine greatly increased the number of α7nAChR clusters, which were colocalized with EGFP-LC3-II (Fig. 6, the co-localization is indicated by arrow). These results indicate that the surface α7nAChR may act as a carrier for Aβ and is internalized for degradation by the autophagosome-lysosome system in cytoplasm.

Autophagic process regulates Aβ-induced neurotoxicity in primary cultured hippocampal neurons. We further examined whether autophagic process affects the Aβ-induced neuronal toxicity in primary cultured neurons by using drugs related to autophagosome regulation. NeuN antibody can recognize neuron-specific nuclear protein. Figure 7 showed that $A\beta_{25-35}$ or $A\beta_{1-42}$ (20 μM) treatment for 48 h decreased the number of NeuN (+) cells of primary hippocampal neuron cultures. However, cotreatment of Aβ with nicotine (0.3 or 1 mM) or rapamycin (0.3 or 1 μM) rescued Aβ-induced neuronal death (Fig. 7A and B). In contrast, α-BTX (0.3 or 1 μM) or wortmannin (20 nM) enhanced the neuronal death caused by Aβ (Fig. 7).

Discussion

The interplay between autophagy and apoptosis is complex, with accumulating data revealing cases in which autophagy is either an antagonist, agonist or independent of canonical programmed cell death via the apoptosis pathway.[18] Several groups suggest that aggregated proteins in neurons such as htt (n-terminus of mutant huntingtin) or α-synuclein can activate autophagic process and further be degraded by autophagic pathway in the culture system.[19,20] Inhibition of autophagic process with autophagic blocker increases cell death.[19,20] On the other hand, treatment of dopamine also induces autophagic cell death and α-synuclein accumulation.[21] These studies suggest that autophagy may be involved in normal processing of these aggregated proteins in cells.

In order to evaluate the neuroprotective effect of autophagic process against Aβ-induced neurotoxicity, we established a cell clone with LC3 overexpression (SH-SY5Y/pEGFP-LC3). Here we found that extracellular Aβ treatment induced a strong autophagic process similar to serum starvation. Previous studies have shown that $A\beta_{25-35}$ or $A\beta_{1-42}$ peptide induces apoptotic cell death through the similar pathway, including ROS accumulation, mitochondrial toxicity (increase of cytochrome *C* release) and p53-Bcl2-Bax signaling pathway.[22-24] We have also demonstrated that Aβ treatment induced ROS accumulation and cotreatment of Aβ with the autophagic stimulator rapamycin decreased ROS accumulation in SH-SY5Y and enhanced hippocampal neuronal survival. In contrast, the autophagic blocker wortmannin antagonized these protective actions. Phosphatidylinositol 3-kinase (PI3K) is essential in the regulation of many cellular processes, such as signal transduction, endocytosis and endosome fusion.[25-27] Here we used wortmannin as the inhibitor of autophagy since wortmannin is reported to be a potent inhibitor of autophagic pathway at nanomolar concentration in hepatocytes and CHO cells.[28,29]

Yu et al.[11,12] found that autophagic vacuoles in the brain are the major reservoir of intracellular Aβ. They also found that autophagy in SH-SY5Y is via the mTOR kinase pathway accompanied with the increase of LC3-II level. In addition, autophagic process is impaired in AD brain and in PS1-APP mice, which leads to the accumulation

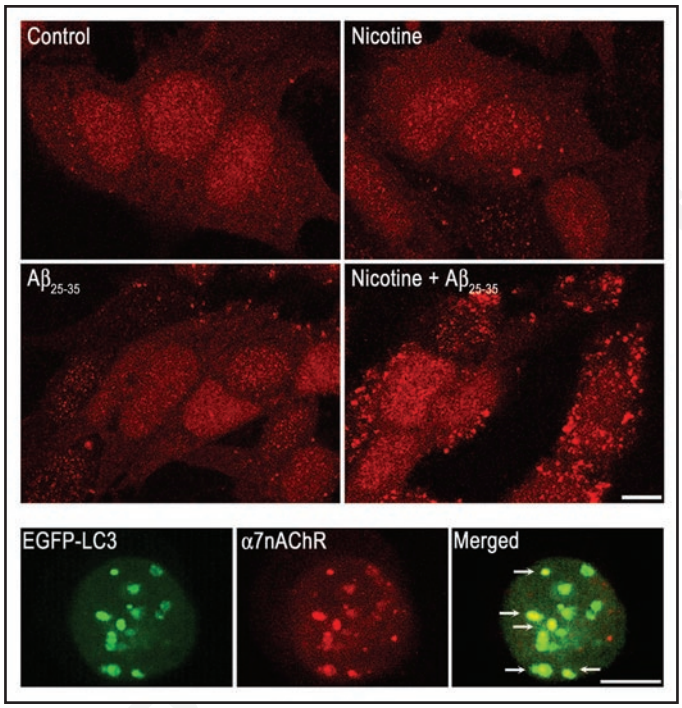

Figure 6. $A\beta_{25-35}$ increases clusters formation and colocalization of α7nAChR with autophagosome in SH-SY5Y/pEGFP-LC3. Confocal images showed that α7nAChR was diffusely stained in control cells by using α7nAChR antibody immunostaining. $A\beta_{25-35}$ (30 μM) plus nicotine (1 mM) treatment for 4 h induced a significant increase of α7nAChR internalization and clusters formation. Nicotine (1 mM) treatment enhanced $A\beta_{25-35}$-induced autophagosome accumulation and the co-localization with α7nAChR. The lower panels showed the merged image of cell treated with $A\beta_{25-35}$ + nicotine (the co-localization is indicated by arrow). Scale bar: 10 μm.

of Aβ-containing autophagic vacuoles within affected neurons.[30] We have also found that Aβ-treatment activated autophagic process because of (1) increase in the number of autophagosomes (2) increase in LC3-II protein level in SH-SY5Y/pEGFP-LC3 cell line. Impairment of autophagic process leads to the increase of neuronal death, indicating that the clearance of Aβ by autophagosome provides neuroprotective mechanism.

α7nAChR is a neuronal homopentameric cation channel, which is highly permeable to Ca^{2+}. α7nAChR modulates Ca^{2+} homeostatsis and the release of acetylcholine and binds with high affinity to α-BTX.[31] α7nAChR is found to be expressed in human neuroblastoma cell line SH-SY5Y and the neurons of human hippocampus and cortex.[17,32] Nagele et al.[17] demonstrate that binding of extracellular Aβ peptide with high affinity to α7nAChR facilitates the entry and intraneuronal accumulation of $A\beta_{1-42}$ via endocytosis, suggesting that Aβ is actively uptaken by surface receptors such as the α7nAChR. Furthermore, α7nAChR antagonist α-BTX or endocytosis inhibitor PAO significantly inhibits $A\beta_{1-42}$ internalization. Peng et al.[32] found that nicotine induces endocytosis, internalization and further lysosomal destruction of nAChRs in SH-SY5Y. We also demonstrated that nicotine treatment (1 mM) alone increased the activation of autophagic process in SH-SY5Y/pEGFP-LC3 cells. Nordberg et al.[33] and Hellstrom-Lindahl et al.[34] also demonstrate that chronic (4.5 months) or short-term (10 days) nicotine treatment effectively reduces Aβ plaque formation

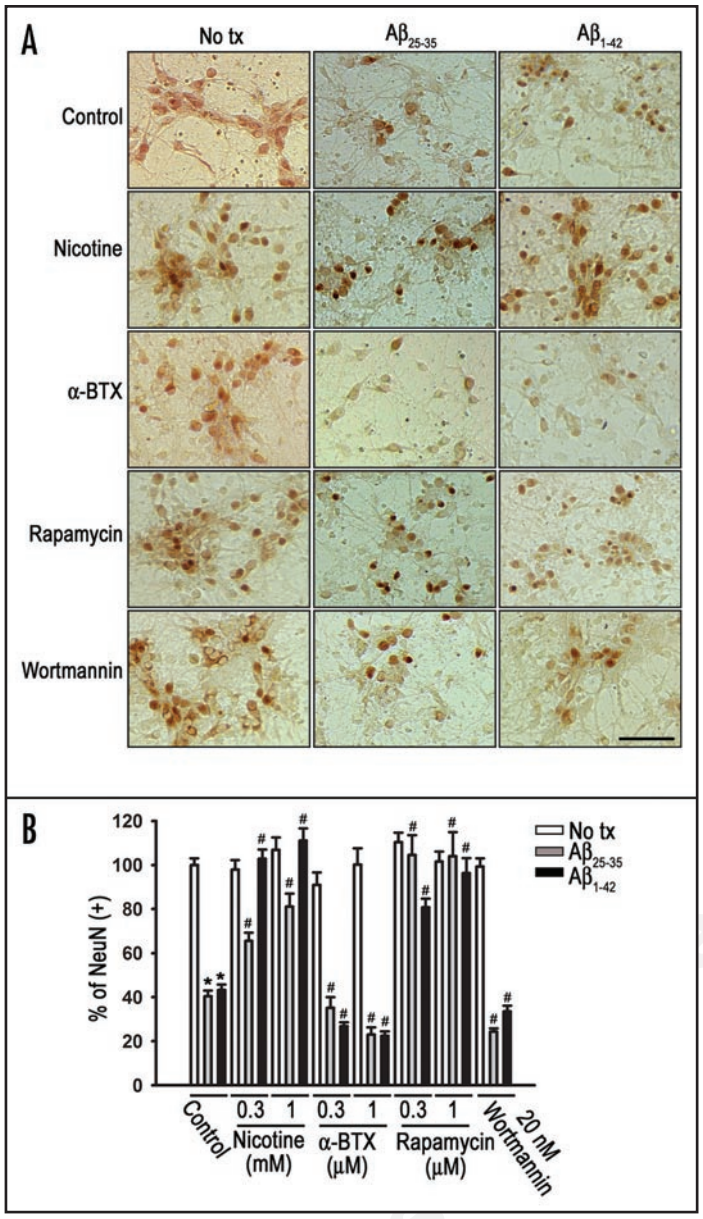

Figure 7. Effects of autophagic process-related drugs on Aβ-induced neurotoxicity in primary hippocampal neuron. (A) Photographs showed that $A\beta_{25-35}$ or $A\beta_{1-42}$ (20 μM) treatment for 48 h decreased the number of surviving NeuN (+) cells in primary hippocampal neuron-enriched cultures. Cotreatment of $A\beta_{25-35}$ or $A\beta_{1-42}$ with nicotine (0.3 mM) or rapamycin (0.3 μM) inhibited and α-BTX (0.3 μM) or wortmannin (20 nM) enhanced Aβ-induced neuronal death. Summarized results were shown in (B). Scale bar: 25 μm. *$p < 0.05$ compared with control without $A\beta_{25-35}$ or $A\beta_{1-42}$; #$p < 0.05$ compared with $A\beta_{25-35}$- or $A\beta_{1-42}$-treatment alone.

and insoluble Aβ in the brain of APPsw mice but does not influence the content of soluble Aβ, α/β/γ-secretase activity and APP synthesis, indicating that nicotine-enhanced autophagy may play a role in extracellular Aβ clearance. In addition, PI3K activity is important in the activation of autophagic process and regulation of early endosome fusion.[35] Nicotinic receptor stimulation, especially α7nAChR activation by treatment with nicotine as an example, protects neuron against Aβ-induced cytotoxicity both in cortical and hippocampal neurons.[36,37] Furthermore, we here demonstrated that knockdown of α7nAChR by siRNA significantly enhanced Aβ-induced neurotoxicity in SH-SY5Y. In contrast, nicotine, α7nAChR agonist, exerted neuroprotection against Aβ-induced neurotoxicity in SH-SY5Y cell and hippocampal neuron through the facilitation of internalization and colocalization of α7nAChR and Aβ with autophagosomes.

Autophagic process is a mechanism for cytoplasm degradation. The surface α7nAChR receptor internalization to cytoplasm upon the binding of Aβ may enhance the extracellular clearance of Aβ. Knockdown of Atg7 or α7nAChR increased Aβ-induced neurotoxicity in SH-SY5Y. Our results suggest that extracellular Aβ exerted more neurotoxicity. Once Aβ was internalized and degraded by autophagic process, extracellular Aβ-induced ROS accumulation and neuronal death decreased as a consequence.

In summary, our results indicate that autophagy process plays a neuroprotective role against Aβ-induced neurotoxicity. A defect in autophagic regulation or Aβ-α7nAChR transport system may impair the clearance of Aβ and enhance neuronal death.

Materials and Methods

Reagents. Wortmannin, bafilomycin A1 and nicotine were purchased from Sigma (Sigma, St. Louis, Missouri). $A\beta_{25-35}$ and $A\beta_{1-42}$ were purchased from AnaSpec (AnaSpec, Inc., San Jose, California). Aβ peptide at 1 mg was dissolved in 40 μL of 1% NH_4OH to get a clear solution and then diluted with ddH_2O to prepare 1 mM stock solution, which was stored at -20°C until use. α-bungarotoxin (α-BTX) was a gift from Long-Sen Chang (National Sun Yat-Sen University, Taiwan).

Cell culture of SH-SY5Y and the selection of pEGFP-LC3 stable clone. SH-SY5Y cells were maintained in a humidified incubator with 5% CO_2 at 37°C in F12/MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries, Grand Island, New York), 4,500 mg/L glucose, 100 U/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen, Carlsbad, California). SH-SY5Y cells were transfected with pEGFP-LC3 or pEGFP (pEGFP-C1; Clontech, Palo Alto, California) with 0.1% lipofectamine 2000 reagent (Invitrogen, Carlsbad, California). The culture medium was then changed by F12/MEM (Invitrogen, Carlsbad, California) containing 10% FBS and 600 μg/mL G418 (Invitrogen, Carlsbad, California) 48 h after transfection. The culture medium was changed once per week. Four to six weeks later, stable clones were isolated. Two stable clones of SH-SY5Y/pEGFP-LC3 or SH-SY5Y/pEGFP were selected for the following studies.

Quantification of EGFP-LC3-II-positive punctate dots. The SH-SY5Y/pEGFP-LC3 cells were treated with $A\beta_{25-35}$ or $A\beta_{1-42}$ peptides for 2 to 24 h. EGFP-LC3-II-positive punctate dots were taken in five random fields per slide using fluorescence microscope at 200x magnification (Leica, Heidelberg, Germany). The punctate dots were then counted in a blind manner. The data were presented as EGFP-LC3-II-positive punctate dots/cell. EGFP-LC3-II-positive punctate dot formation in serum-free medium was used as a positive control. The values are the average obtained from 3~5 experiments.

Evaluation of apoptotic neurons. To investigate the effect of autophagic process on the Aβ-induced cell apoptosis, cells were seeded on a 24-well plate and treated with 30 μM $A\beta_{25-35}$ or $A\beta_{1-42}$ in the presence or absence of autophagy-related drugs for 48 h. Cultures were then stained with Hoechst 33342 (10 μM; Sigma,

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St. Louis, Missouri) for 10 min at room temperature. Hoechst (+) cells were taken in three random fields per well by using fluorescence microscope at 200x magnification and then counted in a blind manner. The result was expressed as percentage of Hoechst (+) cells/bright field cells and the total count of the cells for each experiment was more than 5,000 cells. The values are the average of the percentage of Hoechst (+) cells obtained in 3~8 experiments.

Immunofluorescent staining. For double-immunolabeling studies, the SH-SY5Y/pEGFP-LC3 cells were stained with primary rabbit antibody against $A\beta_{1-42}$ (1:500; Sigma, St. Louis, Missouri) or α7nAChR (1:500; Abcam Inc., Cambridge, Massachusetts) and then with Alexa-543-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, California). The confocal images were obtained using excitation wavelength of 488 nm (for EGFP) and 543 nm (for Alexa-543), respectively (model SP2 TCS; Leica, Heidelberg, Germany).

RNA interference. Inhibition of the autophagy machinery via RNAi was performed as previously described.[13] Briefly, small interfering RNA (siRNA) targeting human Atg7 (sense-GGA GUC ACA GCU CUU CCU U; 20 nM; Dharmacon, Lafayette, Colorado) or α7nAChR (sense-CCA CUC ACC GUC UAC UUC UTT; 20 nM; MDBio, Taipei, Taiwan) was introduced for 24~48 h using 0.1% lipofectamine 2000. The fluorescent siRNA oligonucleotide was used to evaluate the delivery efficiency. Nontargeted RNA oligonucleotide (control siRNA; sense-UUC UCC GAA CGU GUC ACG UTT; 20 nM; MDBio, Taipei, Taiwan) was used as negative control. Efficacy and specificity of protein knockdown was assessed by western blotting.

Western blotting. The protein levels of EGFP-LC3-I, EGFP-LC3-II, EGFP, LC3-I, LC3-II, Atg7 and α7nAChR were determined by western blotting. Cells were homogenized in RIPA buffer containing 1 mM PMSF, 25 μM leupeptin and 1 μg/mL aprotinin. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, Illinois). Protein at 20~40 μg was separated by SDS-PAGE using 8~15% resolving gel under reducing conditions and electrotransferred onto immobilon-P membrane (Millipore, Bedford, Massachusetts). After being blocked with 5% non-fat milk in TBS-T (0.5% Tween 20 in 20 mM Tris and 137 mM NaCl) for 1 h at room temperature, the membranes were incubated overnight at 4°C with rabbit anti-GFP (1:5,000; Santa Cruz Biotechnology, California), anti-LC3 (1:1,000; Abgent Inc., San Diego, California), anti-Atg7 (1:1,000; Abgent Inc., San Diego, California) or anti-α7nAChR (1:5,000; Abcam Inc., Cambridge, Massachusetts) primary antibody diluted in TBS-T. The membranes were probed with a mouse anti-α-tubulin antibody as a standard (1:10,000; Santa Cruz Biotechnology, California). The blots were then incubated for 1 h at room temperature with an HRP-conjugated secondary antibody (1:20,000; Invitrogen, Carlsbad, California). Protein bands were detected using the ECL western blotting Substrate (Pierce, Rockford, Illinois).

$A\beta_{1-42}$ measurement using ELISA method. SH-SY5Y cells were pre-treated with 1 mM nicotine or 5 μM α-BTX for 30 min before incubation with $A\beta_{1-42}$ (1 μM) for another 2 h. The conditioned medium was collected for $A\beta_{1-42}$ measurement using ELISA method according to the manufacturer's protocol (Covance, Emeryville, California).

Hippocampal cultures. Neuron-enriched cultures from hippocampus of E17 Wistar rats were prepared as previously described with some modifications.[14] Briefly, the brain tissues were cleaned free of meningeal tissue, minced and mechanically dissociated by passage through a flame-polished Pasteur pipette. Dissociated cells were seeded in DMEM (Invitrogen, Carlsbad, California) containing 10% FBS, 4,500 mg/L glucose, 100 U/mL penicillin and 0.1 mg/mL streptomycin. The hippocampus neuron-enriched cultures were seeded at 3 x 10^5 cells/well on poly-D-lysine-coated 48-well plate. Twenty-four hours after plating, the cells were changed to DMEM (Invitrogen, Carlsbad, California) supplemented with 2% B27 (Invitrogen, Carlsbad, California), 4,500 mg/L glucose, 100 U/mL penicillin and 0.1 mg/mL streptomycin. The hippocampal neuron-enriched cultures were maintained in a humidified chamber at 37°C in a 5% CO_2 atmosphere for 7~10 days, the medium was changed every 3 days.

Immunocytochemistry. After treatment with 20 μM $A\beta_{25-35}$ or $A\beta_{1-42}$ for 48 h, the hippocampal neuron-enriched cultures were fixed with 4% paraformaldehyde and treated with 3% hydrogen peroxide to eliminate endogenous peroxidase activity prior to incubation of primary antibody. The cultures were then incubated with 10% BSA and 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 30 min, and then treated overnight at 4°C with the NeuN antibody (1:750; Chemicon, Temecula, California) in TBS. The cultures were subsequently incubated for 1 h with the biotinylated secondary antibody, and then for 30 min with avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories. Burlingame, California). Finally, the labeling was revealed by treatment with 0.01% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, Missouri).

Statistics. Results are expressed as mean ± SEM. Results were analyzed with one-way analysis of variance (ANOVA) and Neuman-Keuls post-hoc test to determine statistical significance between specific groups. Difference is considered significant when $p < 0.05$.

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References

1. Seglen PO, Bohley P. Autophagy and other vacuolar protein degradation mechanisms. Experientia 1992; 48:158-72.
2. Kim J, Klionsky DJ. Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. Annu Rev Biochem 2000; 69:303-42.
3. Cuervo AM. Autophagy: in sickness and in health. Trends Cell Biol 2004; 14:70-7.
4. Yorimitsu T, Nair U, Yang Z, Klionsky DJ. Endoplasmic reticulum stress triggers autophagy. J Biol Chem 2006; 281:30299-304.
5. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. Science 2004; 306:990-5.
6. Chu CT. Autophagic stress in neuronal injury and disease. J Neuropathol Exp Neurol 2006; 65:423-32.
7. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. Cell Death Differ 2005; 12:1542-52.
8. Ohsumi Y. Molecular dissection of autophagy: two ubiquitin-like systems. Nat Rev Mol Cell Biol 2001; 2:211-6.
9. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000; 19:5720-8.
10. Mattson MP. Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives. Physiol Rev 1997; 77:1081-132.
11. Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, Lee JH, et al. Macroautophagy—a novel Beta-amyloid peptide-generating pathway activated in Alzheimer disease. J Cell Biol 2005; 171:87-98.
12. Yu WH, Kumar A, Peterhoff C, Shapiro Kulnane L, Uchiyama Y, Lamb BT, et al. Autophagic vacuoles are enriched in amyloid precursor protein-secretase activities: implications for beta-amyloid peptide over-production and localization in Alzheimer disease. Int J Biochem Cell Biol 2004; 36:2531-40.

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13. Chu CT, Zhu J, Dagda R. Beclin 1-independent pathway of damage-induced mitophagy and autophagic stress: implications for neurodegeneration and cell death. Autophagy 2007; 3:663-6.
14. Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 1993; 35:567-76.
15. Zhu JH, Horbinski C, Guo F, Watkins S, Uchiyama Y, Chu CT. Regulation of autophagy by extracellular signal-regulated protein kinases during 1-methyl-4-phenylpyridinium-induced cell death. Am J Pathol 2007; 170:75-86.
16. Klionsky DJ. The molecular machinery of autophagy: unanswered questions. J Cell Sci 2005; 118:7-18.
17. Nagele RG, D'Andrea MR, Anderson WJ, Wang HY. Intracellular accumulation of beta-amyloid(1-42) in neurons is facilitated by the alpha 7 nicotinic acetylcholine receptor in Alzheimer disease. Neuroscience 2002; 110:199-211.
18. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. Nat Rev Cancer 2005; 5:726-34.
19. Qin ZH, Wang Y, Kegel KB, Kazantsev A, Apostol BL, Thompson LM, et al. Autophagy regulates the processing of amino terminal huntingtin fragments. Hum Mol Genet 2003; 12:3231-44.
20. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. Alpha-Synuclein is degraded by both autophagy and the proteasome. J Biol Chem 2003; 278:25009-13.
21. Gomez-Santos C, Ferrer I, Santidrian AF, Barrachina M, Gil J, Ambrosio S. Dopamine induces autophagic cell death and alpha-synuclein increase in human neuroblastoma SH-SY5Y cells. J Neurosci Res 2003; 73:341-50.
22. Fang F, Liu GT. Novel squamosamide derivative (compound FLZ) attenuates Abeta25-35-induced toxicity in SH-SY5Y cells. Acta Pharmacol Sin 2008; 29:152-60.
23. Lustbader JW, Cirilli M, Lin C, Xu HW, Takuma K, Wang N, et al. ABAD directly links Abeta to mitochondrial toxicity in Alzheimer disease. Science 2004; 304:448-52.
24. Zhang Y, McLaughlin R, Goodyer C, LeBlanc A. Selective cytotoxicity of intracellular amyloid beta peptide1-42 through p53 and Bax in cultured primary human neurons. J Cell Biol 2002; 156:519-29.
25. Knowles SE, Ballard FJ. Selective control of the degradation of normal and aberrant proteins in Reuber H35 hepatoma cells. Biochem J 1976; 156:609-17.
26. Schworer CM, Mortimore GE. Glucagon-induced autophagy and proteolysis in rat liver: mediation by selective deprivation of intracellular amino acids. Proc Natl Acad Sci USA 1979; 76:3169-73.
27. Kovacs AL, Seglen PO. Inhibition of hepatocytic protein degradation by methylaminopurines and inhibitors of protein synthesis. Biochim Biophys Acta 1981; 676:213-20.
28. Blommaart EF, Krause U, Schellens JP, Vreeling-Sindelarova H, Meijer AJ. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur J Biochem 1997; 243:240-6.
29. Munafo DB, Colombo MI. A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci 2001; 114:3619-29.
30. Nixon RA, Wegiel J, Kumar A, Yu WH, Peterhoff C, Cataldo A, Cuervo AM. Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. J Neuropathol Exp Neurol 2005; 64:113-22.
31. Schoepfer R, Conroy WG, Whiting P, Gore M, Lindstrom J. Brain alpha-bungarotoxin binding protein cDNAs and MAbs reveal subtypes of this branch of the ligand-gated ion channel gene superfamily. Neuron 1990; 5:35-48.
32. Peng X, Gerzanich V, Anand R, Wang F, Lindstrom J. Chronic nicotine treatment upregulates alpha3 and alpha7 acetylcholine receptor subtypes expressed by the human neuroblastoma cell line SH-SY5Y. Mol Pharmacol 1997; 51:776-84.
33. Nordberg A, Hellstrom-Lindahl E, Lee M, Johnson M, Mousavi M, Hall R, et al. Chronic nicotine treatment reduces beta-amyloidosis in the brain of a mouse model of Alzheimer disease (APPsw). J Neurochem 2002; 81:655-8.
34. Hellstrom-Lindahl E, Court J, Keverne J, Svedberg M, Lee M, Marutle A, et al. Nicotine reduces A beta in the brain and cerebral vessels of APPsw mice. Eur J Neurosci 2004; 19:2703-10.
35. Li G, D'Souza-Schorey C, Barbieri MA, Roberts RL, Klippel A, Williams LT, Stahl PD. Evidence for phosphatidylinositol 3-kinase as a regulator of endocytosis via activation of Rab5. Proc Natl Acad Sci USA 1995; 92:10207-11.
36. Kihara T, Shimohama S, Sawada H, Kimura J, Kume T, Kochiyama H, et al. Nicotinic receptor stimulation protects neurons against beta-amyloid toxicity. Ann Neurol 1997; 42:159-63.
37. Zamani MR, Allen YS, Owen GP, Gray JA. Nicotine modulates the neurotoxic effect of beta-amyloid protein(25-35)) in hippocampal cultures. Neuroreport 1997; 8:513-7.

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