APP-C31 is an intracellular promoter of amyloid- β aggregation and toxicity

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Abstract

Intracellular C-terminal cleavage of the amyloid precursor protein (APP) is elevated in the brain of Alzheimer's disease (AD). Emerging evidence proposes a pathological relationship between the production of a C-terminal APP fragment, called APP-C31, and the toxicity induced by amyloid- β (A β) that is a major contributor towards AD; however, the interaction between the two peptides and the consequent impact of APP-C31 on A β -related toxicity were unknown thus far. Here we report the discovery that APP-C31 facilitates the aggregation of A β and aggravates its toxicity at the intracellular level, with escalating neurodegeneration. APP-C31 forms a heterodimer with A β through the contacts onto the *N*-terminal and self-recognition regions of A β and induces its conformational transition accelerating amyloid fibrillization. APP-C31 promotes the perinuclear and intranuclear deposition of enlarged A β aggregates and, consequently, damages the nucleus leading to apoptosis. A β -induced degeneration of neurites in human neurons is also intensified by APP-C31. Our studies demonstrate a new function of APP-C31 as an intracellular factor of the proteopathy found in AD.

Proteolysis of the amyloid precursor protein (APP) is associated with the pathology of Alzheimer's disease (AD). As a type-I transmembrane protein, APP is localized in cell surfaces or membranes of organelles^{1,2}. Cleavage of APP by proteolytic enzymes such as α -, β -, and γ -secretases releases various fragments that participate in neurotrophic activities, synaptic plasticity, and intracellular signaling¹. Amyloid- β (A β) peptides with 38-43 amino acid residues are generated *via* the sequential cleavage of APP by β - and γ -secretases,^{1,3} as illustrated in Fig. 1a. Intrinsically disordered A β peptides self-assemble into oligomers and fibrils that trigger neurodegeneration observed in AD¹⁻⁴. As the primary constituent of senile plaques, A β peptides were first found outside the neuron^{2,3}. Extracellular A β aggregates are thought to promote synaptic loss and memory impairment^{2,4}. Curiously, recent studies supported the production of intracellular A β by cellular secretory pathways with its pathological relevance towards AD². *In vivo* studies have identified that the intraneuronal accumulation of A β aggregates could cause cognitive deficits prior to forming senile plaques^{2,5}. Thus, A β inside the cell may be a source of neurotoxicity.

As depicted in Fig. 1a, a cytoplasmic peptide carrying 31 amino acid residues denoted APP-C31 (APP₆₆₅₋₆₉₅) is produced upon cleavage of the C-terminal region of APP by caspases^{1,6-8}. In AD-affected brains, the levels of activated caspases are elevated and give rise to caspase-mediated APP fragments⁶⁻⁸. Like A β , APP-C31 contributes to neuronal toxicity leading to apoptosis and synaptic depression^{7,9-13}. Mutation of APP from Asp664 to Ala that prevents the production of APP-C31 could diminish synaptic loss and neurodegeneration in AD transgenic mice^{11,12}. Thus, these findings imply that the amount of intracellular APP-C31 is augmented and, consequently, neurotoxicity is enhanced when AD develops. Recently, the co-localization of the C-terminal region of APP with A β was observed in intraneuronal perinuclear compartments and multivesicular bodies in the brains of AD transgenic mice^{14,15}. Moreover, studies with the C-terminal truncated APP, APP₁₋₆₆₄, and mutant APP(Asp664Ala) suggested that A β could activate caspases that induce the C-terminal cleavage of APP generating APP-C31¹¹⁻¹³. Despite these indications, the mechanism of how APP-C31 is involved in the pathology driven by A β is not known to date.

We questioned whether APP-C31 can directly bind to A β and alter the aggregation and toxicity profiles of A β . Thus, binding of APP-C31 to A β at the molecular level of details and its influence on the aggregation of A β were evaluated. In addition, we determined the effects of APP-C31 on modifying the aggregation and toxicity of intracellular A β through the live-cell investigations employing the microinjection methods. Moreover, the impact of A β incubated with APP-C31 on human neuronal growth was probed. Overall, our work substantiates that an

intracellular C-terminal fragment, APP-C31, can provoke the aggregation behaviors and toxicity of A β via direct interactions between the two peptides, which illuminates a new role of APP-C31 as an intracellular factor in the pathology of AD.

Fig. 1 | APP-C31, A β , and their direct interactions. **a**, Amino acid sequences of APP-C31 (APP₆₆₅₋₆₉₅), A β_{40} , and A β_{42} that are generated from the proteolytic cleavage of APP. The self-recognition site of A β is highlighted in bold. **b**, ESI-MS spectra of APP-C31, A β_{40} , and APP-C31 incubated with A β_{40} . Charge states are marked in the MS spectra. Conditions: [APP-C31] = 100 μ M; [A β_{40}] = 100 μ M; 20 mM ammonium acetate, pH 7.3; 37 $^{\circ}$ C; 2 h incubation; no agitation; 10-fold diluted samples were injected to the mass spectrometer. **c**, ITC thermogram (upper panel) and binding isotherm (lower panel) obtained upon titration of APP-C31 to A β_{40} . Conditions: [A β_{40}] = 30 μ M; [APP-C31] = 600 μ M; 20 mM HEPES, pH 7.4; 10 $^{\circ}$ C. **d,e**, 2D ^1H - ^{15}N HSQC spectra (900 MHz) and CSPs of ^{15}N -labeled A β_{40} monomer by treatment of APP-C31. The average of CSPs and the average + one standard deviation are indicated with solid and dashed lines, respectively. Conditions: [^{15}N -labeled A β_{40}] = 40 μ M; [APP-C31] = 200 μ M; 20 mM HEPES, pH 7.4; 5 $^{\circ}$ C.

Results

Direct binding of APP-C31 to A β . The molecular-level interaction between APP-C31 and A β was first monitored by electrospray ionization–mass spectrometry (ESI–MS), a soft ionization method used in the characterization of protein complexes¹⁶. A β_{40} was used for binding studies because it is a major isoform of A β found in AD-affected brains and is less aggregation-prone than A β_{42} ³. As shown in Fig. 1b, new peaks were observed upon incubation of APP-C31 with A β_{40} at 1342, 1610, and 2012 m/z that correspond to the complex between APP-C31 and A β_{40} with +6, +5, and +4 charge states, respectively. Furthermore, the molecular binding of APP-C31 with A β_{40} was thermodynamically analyzed by isothermal titration calorimetry (ITC). When APP-C31 was titrated into the solution of A β_{40} , the reaction was spontaneous and exergonic with $\Delta G = -5.8 (\pm 0.3)$ kcal/mol and slightly exothermic at $\Delta H = -0.4 (\pm 0.2)$ kcal/mol, as shown in Fig. 1c. The $T\Delta S$ term was found to be $5.4 (\pm 0.4)$ kcal/mol. The binding affinity of APP-C31 for A β_{40} was measured to be $35.2 (\pm 17.6)$ μ M, indicating the relatively weak binding between the two peptides according to previously reported K_d values for protein–protein interactions¹⁷. These thermodynamic properties are curious on first sight, as a reaction in which two particles associate to form a single species is expected to be entropically disfavored. Specifically, it is the translational entropy associated with each particle amounting to roughly 10 kcal/mol at room temperature that disfavors associative processes reducing the number of free particles¹⁸. A minor but relevant feature is the loss of structural flexibility upon aggregation, which also reduces the conformational entropy. The increase in entropy observed here is likely due to the release of water molecules that were tightly bound to the protein surfaces and become free water molecules as the two peptides form an association complex.

Direct binding of APP-C31 to A β in solution was supported by two dimensional (2D) ^1H – ^{15}N heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy at the amino acid residue level. As presented in Fig. 1d and 1e, upon treatment of APP-C31 with a solution of uniformly ^{15}N -labeled A β_{40} , we detected notable chemical shift perturbations (CSPs) of the amino acid residues Arg5, Glu11, Gln15, Leu17, Phe20, and Ala21 in A β . This observation indicates that APP-C31 could interact with the *N*-terminal region and the self-recognition site of A β (*i.e.*, Leu17 to Ala21; Fig. 1a). Given that the self-recognition site of A β is a central hydrophobic region responsible for the oligomerization and fibrilization^{3,4}, APP-C31 may alter the conformation and aggregation propensity of A β (*vide infra*). Together, the abovementioned studies by MS, ITC, and 2D NMR verify that APP-C31 can interact with the *N*-terminal and self-recognition regions of A β in a relatively weak binding manner.

APP-C31–A β binding modes. To obtain atomistic insight into the binding of APP-C31 to A β ₄₀, a series of molecular dynamics (MD) simulations was carried out¹⁹⁻²⁷. In these computer simulations, A β ₄₀ (PDB 2LFM²⁸; Fig. 2a) obtained by solution NMR was used as the starting conformation of monomeric A β . Unfortunately, the exact structure of APP-C31 is not known; thus, the X-ray crystal structure of APP₆₆₆₋₆₉₃ excised from the intracellular domain of APP complexed with Fe65 protein (PDB 3DXC²⁹; Fig. 2a) was selected to provide the starting guess structure of APP-C31. Faithfully, modelling slow processes like the folding and association of peptides taking place in minutes to hours time-scale with MD simulations is challenging. One solution to accelerating the simulation and obtaining meaningful data within a tractable computing time is to first conduct metadynamics MD simulations that allow for a more rapid and efficient sampling of the unique configurational space to identify the most relevant adduct structures. We sampled dimeric configurations with the gross metadynamics simulation time being 8.4 μ s. These simulations facilitate the conformational sampling along the center-of-mass (COM) coordinate between the pair of proteins, and provide the Gibbs free energy profile along the reaction coordinate¹⁹. The Gibbs free energy profile of the APP₆₆₆₋₆₉₃–A β ₄₀ adduct suggests that the association is fully reversible. As shown in Supplementary Fig. 1, we found an ensemble of hetero-dimer conformers at the COM distance of 13 Å.

To verify and rank the relative stabilities of the dimeric structures, additional 500 ns of unbiased MD simulations without the metadynamics acceleration were performed, where the initial structures were taken from the metadynamics MD simulations. The adduct structures from the MD simulations were evaluated based on two criteria: (i) the monomers of A β ₄₀ and APP₆₆₆₋₆₉₃ maintain contacts throughout the course of unbiased MD simulations (Supplementary Fig. 2); (ii) the six amino acid residues displaying the pronounced CSPs are at the dimeric interface (Supplementary Fig. 3). As illustrated in Fig. 2b, we identified two classes of stable APP₆₆₆₋₆₉₃–A β ₄₀ dimeric adduct structures that are most consistent with our experimental results described in Fig. 1b-e. In Model I and II, the dimeric interfaces were formed by favorable intermolecular contacts and hydrogen bonding. The amino acid residues in the *N*-terminal region and the self-recognition site of A β ₄₀ exhibited intermolecular ionic interactions with APP₆₆₆₋₆₉₃. Both models presented a salt bridge between Arg5 of A β ₄₀ and Glu683 of APP₆₆₆₋₆₉₃. In Model I, another salt bridge was detected between Asp7 of A β ₄₀ and Lys688 of APP₆₆₆₋₆₉₃. Additionally, the intermolecular hydrophobic contacts between the aliphatic side chain of Val18 in A β ₄₀ and the aromatic rings of Tyr682, Phe689, and Phe690 in APP₆₆₆₋₆₉₃ were revealed in both models. Moreover, multiple hydrogen bonds were observed in the APP₆₆₆₋₆₉₃–A β ₄₀ dimeric interfaces. The

Fig. 2 | Interactions between APP-C31 and A β ₄₀ visualized by metadynamics MD simulations. **a**, Conformations of A β ₄₀ (PDB 2LFM²⁸) and APP₆₆₆₋₆₉₃ (PDB 3DXC²⁹) as a part of APP-C31 (APP₆₆₅₋₆₉₅) used for metadynamics MD simulations. The self-recognition site of A β is highlighted in purple. **b**, Representative models (I and II) of the APP₆₆₆₋₆₉₃–A β ₄₀ interfaces from the trajectories of MD simulations. Possible hydrogen bonds within 3.0 Å and hydrophobic interactions observed within 4.0 Å are indicated with dashed black lines. The amino acid residues involved in hydrophobic interactions are presented in the space-filling representation.

Asp7 residue of A β ₄₀ constructed hydrogen bonds with Tyr687 and Asn684 of APP₆₆₆₋₆₉₃ in Model I and II, respectively. Furthermore, the backbone carbonyl group of Val12 and the imidazole nitrogen atom of His13 in A β ₄₀, as shown in Model I and II, respectively, produced the backbone–side chain and side chain–side chain hydrogen bonds with Asn684 of APP₆₆₆₋₆₉₃, respectively. Hydrogen bonding between the backbone carbonyl and hydroxyl groups of Gly681 and Thr686 in APP₆₆₆₋₆₉₃, respectively, and Lys16 that is adjacent to the self-recognition site of A β ₄₀ was also monitored in Model I. As indicated in Model II, Thr686 of APP₆₆₆₋₆₉₃ additionally formed hydrogen bonding with the backbone amide moiety of Val18 in A β ₄₀.

Interestingly, our MD simulations indicated a conformational change in A β upon binding to APP-C31. As displayed in Fig. 2b, the secondary structure of A β ₄₀ spanning from His13 to Asp23 became disordered, which is distinct from its monomeric form exhibiting an α -helical structure in

the same region (Fig. 2a). In particular, the self-recognition site of A β ₄₀ highlighted in purple adopted an extended conformation. The Ramachandran plots for the amino acid residues in the self-recognition site (Leu17 to Ala21) presented their dihedral angles mostly in the region of a β -sheet and an extended helix³⁰, as summarized in Supplementary Fig. 4. This observation suggests that the conformation of A β ₄₀ can be easily converted to a β -strand in the presence of APP-C31. Although structural changes are expected during protein association, the extent of change we observed is notable and offers additional explanations for the dominance of the entropy term in the binding free energy. The α -helical form of A β ₄₀ is the most stable form allowing for favorable interactions with the surrounding water molecules. As the peptide undergoes a transition to the extended form and is incorporated into the heterodimer with APP-C31, water molecules from the α -helix are liberated³¹. Furthermore, the transition to the extended motif increases the intrastrand conformational entropy that will help to compensate for the translational entropy penalty that must be paid during the hetero-dimer formation. Ultimately, in addition to intermolecular ionic and hydrophobic interactions and hydrogen bonds, such structural alteration in A β can modify its oligomerization and fibrillization, as the self-recognition site is critically involved in peptide aggregation^{3,4}. Overall, the results and observations by MD simulations support that APP-C31 can directly interact with the *N*-terminal region and the self-recognition site of A β and induce its conformational transition.

Effect of APP-C31 on A β aggregation. To assess whether APP-C31 can influence the aggregation of A β , the samples prepared with the two peptides, as described in Fig. 3a and Supplementary Fig. 5a, were first analyzed by the thioflavin-T (ThT) assay that quantifies the degree of β -sheet formation^{3,32}. We tested the two major isoforms of A β , A β ₄₀ and A β ₄₂^{3,4}. As summarized in Fig. 3b, β -sheet-rich species were generated in the sample of A β ₄₀ after a lag time for 1.7 h and the ThT fluorescence intensity plateaued at 6 h. In the presence of APP-C31, the lag time of A β ₄₀ was notably shorter than that of A β ₄₀ only. When APP-C31 was treated with A β ₄₀, the ThT fluorescence intensity increased after 1 h and reached a plateau at 3.5 h. These results suggest that APP-C31 facilitates the aggregation of A β ₄₀. Different from A β ₄₀, the aggregation kinetics of A β ₄₂ was not significantly affected by APP-C31 (Supplementary Fig. 5b). It should be noted that APP-C31 by itself did not change the ThT fluorescence even with longer incubation time; thus, it could not generate β -sheet-rich assemblies under our experimental conditions if no A β was present. Notable aggregates of APP-C31 were not observed by transmission electron microscopy (TEM) (data not shown). Analysis of APP-C31 by circular dichroism (CD)

Fig. 3 | Impact of APP-C31 on $A\beta_{40}$ aggregation. **a**, Scheme of the aggregation experiments. **b,c**, Analysis of the degree on peptide aggregation upon incubation of either $A\beta_{40}$, APP-C31, or both by the ThT and dot blot assays, respectively. **d**, Morphologies of the peptide aggregates produced upon 2 h and 8 h treatment of $A\beta_{40}$ with or without APP-C31 detected by TEM. Scale bars = 200 nm. Conditions: $[A\beta_{40}] = 25 \mu\text{M}$ and $[\text{APP-C31}] = 25 \mu\text{M}$ (for ThT assay and TEM); $[A\beta_{40}] = 100 \mu\text{M}$ and $[\text{APP-C31}] = 100 \mu\text{M}$ (for dot blot assay); 20 mM HEPES, pH 7.4, 150 mM NaCl; 37°C ; constant agitation.

spectroscopy further delineated that its secondary structure was not changed after 24 h incubation, as presented in Supplementary Fig. 6.

Next, the impact of APP-C31 on the oligomerization of $A\beta$ was probed by the dot blot assay employing three different antibodies including an anti-oligomer antibody (A11)³³, an anti- $A\beta$

antibody (6E10)³⁴, and an anti-APP C-terminus antibody (Y188)³⁵. As illustrated in Fig. 3c, the A11 signal appeared from the sample of A β ₄₀ incubated with APP-C31 after 2 h while A β ₄₀ in the absence of APP-C31 showed the A11 signal after 4 h. These results are consistent with the accelerated oligomerization of A β ₄₀ by interacting with APP-C31, as proposed above. Similar to the ThT assay, the dot blot indicated no noticeable effect of APP-C31 on the oligomerization of A β ₄₂ (Supplementary Fig. 5c). Lastly, morphologies of the A β aggregates produced upon addition of APP-C31 were visualized by TEM. Long linear A β ₄₀ fibrils were detected from the sample containing A β ₄₀ and APP-C31 even at the early aggregation stage, compared to short fibrils formed by A β ₄₀ only, as depicted in Fig. 3d. The change in the morphology of A β ₄₂ aggregates was also observed by treatment of APP-C31, as shown in Supplementary Fig. 5d, which is distinct from the results by the ThT and dot blot assays. When A β ₄₂ was incubated with APP-C31 for 2 h and 8 h, long straight A β ₄₂ fibrils were monitored in contrast to smaller aggregates or bundled fibrils generated by incubation of A β ₄₂ only. Collectively, our aggregation studies corroborate that APP-C31 is capable of promoting the oligomerization and fibrilization of A β .

Interactions of APP-C31 with metals and metal–A β and its impact on metal–A β aggregation.

Under normal brain conditions, metal ions are tightly regulated and play crucial roles in neurotransmission, protein folding, and enzymatic reactions³. In AD-afflicted brains, however, metal ion dyshomeostasis and highly concentrated metals trapped in senile plaques such as *ca.* 400 μ M of copper and *ca.* 1 mM of zinc are found^{3,36}. Additionally, the accumulation of A β aggregates with metal ions is detected in intracellular systems^{37,38}. Metal ions including Cu(I/II) and Zn(II) can coordinate to A β forming metal-bound A β (metal–A β) and subsequently affect A β aggregation pathways to different degrees depending on the type of metal ions and metal-to-A β stoichiometry^{3,36}. Under these notions, the interactions of APP-C31 with metal ions and metal–A β were investigated.

Binding of APP-C31 to Cu(II) and Zn(II) was determined by ESI–MS and ITC. As presented in Supplementary Fig. 7, when APP-C31 was added with Cu(II), ESI–MS studies exhibited the Cu(II)–APP-C31 complexation with indication of the peak corresponding to [Cu(II) + APP-C31]⁴⁺. Zn(II)-bound APP-C31 was not observed under our experimental conditions for our MS studies. Binding events between APP-C31 and Cu(II) or Zn(II) were further analyzed by ITC, as shown in Supplementary Fig. 8. When Cu(II) was titrated to APP-C31, an exergonic reaction was observed ($\Delta G = -6.5 (\pm 0.1)$ kcal/mol; $\Delta H = -0.5 (\pm 0.1)$ kcal/mol; $T\Delta S = 6.0 (\pm 0.1)$ kcal/mol) with the dissociation constant (K_d) value of $8.7 (\pm 1.5)$ μ M. Similarly, binding of APP-C31 to Zn(II) was also

exergonic ($\Delta G = -6.2 (\pm 0.1)$ kcal/mol; $\Delta H = 0.3 (\pm 0.1)$ kcal/mol; $T\Delta S = 6.5 (\pm 0.1)$ kcal/mol). The K_d value of Zn(II)–APP-C31 was determined to be $18.0 (\pm 4.6)$ μ M. Curiously, the metal-binding enthalpies are essentially thermoneutral at -0.5 and 0.3 kcal/mol for Cu(II) and Zn(II), respectively. Thus, metal binding is dominated by favorable entropy components of the free energy. As explained when discussing the heterodimer formation, this finding is counter intuitive, but it is likely caused by the release of water molecules that were tightly bound to the metal ion in the first solvent shell and become free water molecules as the metal ion binds to the peptide. The signs of enthalpy of binding may imply that APP-C31 presents either different binding sites or the structural arrangements around Cu(II) and Zn(II) distinctively different^{39,40}. Given that the K_d values of Cu(II)–A β_{40} and Zn(II)–A β_{40} are 10^{-10} M and 10^{-6} M, respectively^{3,36}, APP-C31 may not be able to extract the metal ion from metal-bound A β . As illustrated in Fig. 4a and Supplementary Fig. 9, the interaction of APP-C31 with Cu(II)-treated A β was verified by ESI–MS. The sample containing APP-C31, A β_{40} , and Cu(II) in a molar ratio of 1:1:1 presented the peaks assigned to $[\text{Cu(II)} + \text{A}\beta_{40} + \text{APP-C31}]^{5+}$ and $[2\text{Cu(II)} + \text{A}\beta_{40} + \text{APP-C31}]^{5+}$. Furthermore, the peak corresponding to $[\text{Zn(II)} + \text{A}\beta_{40} + \text{APP-C31}]^{5+}$ with a low intensity was monitored when APP-C31 was incubated with Zn(II)-added A β , as presented in Fig. 4a and Supplementary Fig. 10. Together, our binding studies confirm the direct interactions of APP-C31 with metal ions and metal–A β .

Moving forward, the effects of APP-C31 on the aggregation of metal–A β were examined by the ThT assay, the dot blot assay, and TEM. As depicted in Fig. 4b and 4c, APP-C31 expedited the aggregation of both Cu(II)–A β_{40} and Zn(II)–A β_{40} to different extents. A β_{40} treated with Cu(II) and APP-C31 rapidly aggregated displaying a lag phase of approximately 4 h and produced more ThT-reactive β -sheet-rich aggregates, relative to Cu(II)–A β_{40} only. The oligomerization of Cu(II)–A β_{40} was also promoted by APP-C31, as analyzed by the dot blot assay (Supplementary Fig. 11). In the case of Zn(II)-treated A β_{40} , incubation with APP-C31 led to the facilitated aggregation kinetics with the saturated ThT fluorescence at 8 h and the reduction of forming ThT-reactive β -sheet-rich A β_{40} aggregates by *ca.* 25%. It should be noted that APP-C31 with Cu(II) and Zn(II) in the absence of A β_{40} did not change the ThT fluorescence, suggesting no conformational transition to a β -sheet-rich form under our experimental conditions.

As illustrated in Fig. 4d, when Cu(II)-added A β_{40} was incubated with APP-C31 for 8 h, 24 h, and 72 h, shorter and thicker fibrils were detected by TEM, different from fibrils produced from APP-C31-untreated Cu(II)–A β_{40} . Additionally, short fibrils over amorphous aggregates were present from the sample of Zn(II)–A β_{40} treated with APP-C31 for 8 h and 24 h. After longer incubation (*i.e.*, 72 h), no significant morphological difference between the samples of Zn(II)-

Fig. 4 | Interactions of APP-C31 with metal-treated A β and its influence on metal-induced A β aggregation. **a**, ESI-MS spectra of Cu(II)- or Zn(II)-added A β_{40} with and without APP-C31 and Cu(II)- or Zn(II)-treated APP-C31. The full MS spectra are depicted in Supplementary Fig. 9 and 10. Conditions: [APP-C31] = 100 μ M; [A β_{40}] = 100 μ M; [CuCl₂ or ZnCl₂] = 100 μ M; 20 mM ammonium acetate, pH 7.3; 37 $^\circ$ C; 2 h incubation; no agitation; 10-fold diluted samples were injected to the mass spectrometer. **b**, Scheme of the aggregation experiments. **c**, Analysis of the degree on peptide aggregation upon incubation of either metal-added A β_{40} , metal-treated APP-C31, or both by the ThT assay. **d**, Morphologies of the peptide aggregates generated by 8 h, 24 h, and 72 h incubation of metal-treated A β_{40} with and without APP-C31 monitored by TEM. Scale bars = 200 nm. Conditions for the ThT assay and TEM: [APP-C31] = 25 μ M; [A β_{40}] = 25 μ M; [CuCl₂ or ZnCl₂] = 25 μ M; 20 mM HEPES, pH 7.4, 150 mM NaCl; 37 $^\circ$ C; constant agitation.

added A β ₄₀ with and without APP-C31 was monitored. Similar to metal-free A β ₄₂, the aggregation of metal-A β ₄₂ was much less modified by APP-C31. As depicted in Supplementary Fig. 12 and 13, APP-C31 did not alter the aggregation kinetics of metal-added A β ₄₂. The amount of ThT-reactive β -sheet-rich aggregates produced by interacting Zn(II)-A β ₄₂ with APP-C31 was lowered by approximately 35%, compared to that from Zn(II)-A β ₄₂ only, but morphologies of the resultant A β ₄₂ aggregates from both samples were similar. Overall, our investigations confirm that APP-C31 accelerates the aggregation of metal-A β ₄₀.

Cytotoxicity of A β associated with APP-C31. We performed microinjection experiments in living cells to identify the impact of APP-C31 towards the toxicity triggered by intracellular A β . As described in Fig. 5a, A β ₄₀ with or without APP-C31 in the absence and presence of metal ions was introduced inside the cells with Texas Red-labeled dextran that is an indicator for the cells injected with the samples⁴¹. After 24 h incubation, cell viability was determined by counting the Texas Red-positive cells following previously reported methods⁴². The cells injected with either APP-C31 or A β ₄₀ under our experimental conditions exhibited ca. 95% of cell survival, as presented in Fig. 5b. When both APP-C31 and A β ₄₀ were administered inside the cells, cell death was enhanced by ca. 40%. In the case of Cu(II)- and Zn(II)-added A β ₄₀, the viability was approximately 80% and 90%, respectively. Upon addition of metal-treated A β ₄₀ with APP-C31 inside the cells, cell survival was further decreased by ca. 10%. Thus, our microinjection studies demonstrate that APP-C31 aggravates the cytotoxicity of intracellular A β with and without metal ions to different degrees.

To further identify whether the cell death induced by treating both APP-C31 and A β is associated with apoptosis that is a type of programmed cell death⁴³, live-cell imaging experiments using annexin V staining were carried out. Annexin V conjugated with Alexa Fluor 488 can stain externalized phosphatidylserine in apoptotic cells, thereby emitting green fluorescence⁴³. The overlap between green and red fluorescence from annexin V and Texas Red-labeled dextran, respectively, indicates the cells that are injected with the samples and undergo apoptosis. The degree of the colocalization between green and red fluorescence was analyzed by the Pearson's coefficient (r), a type of the correlation coefficient that represents the strength of the association between the two variables⁴⁴. When APP-C31 or A β ₄₀ was added inside the cells followed by 24 h incubation, the cells did not show significant green fluorescence of annexin V, as displayed in Fig. 5c. The r value was approximately 0.2, indicative of the poor association between apoptosis and the internalization of APP-C31 or A β ₄₀ under our experimental conditions. Notably, the injection

Fig. 5 | Influence of APP-C31 on the toxicity and aggregation of intracellular Aβ. **a**, Scheme of the microinjection experiments. **b**, Cell viability of SH-SY5Y cells microinjected with either APP-C31, Aβ₄₀, or both in the absence and presence of metal ions. Measurements of cell survival were described in detail in the experimental section. Conditions: [Aβ₄₀] = 10 μM; [APP-C31] = 10 μM; [CuCl₂ or ZnCl₂] = 10 μM; [Texas Red-labeled dextran] = 0.4 mg/mL; 24 h incubation. All values denote mean ± s.e.m. **P* < 0.05; ****P* < 0.001; *n* = 3–5; Student's *t*-test. **c**, Apoptosis monitored using Alexa Fluor 488-tagged annexin V. Pearson's correlation coefficients were calculated to

present the colocalization of the fluorophore-tagged dextran and Annexin V (mean $\pm$ s.e.m, $**P < 0.01$; $n = 5$; Student's t -test). White arrows indicate apoptotic cells microinjected with peptide samples. Conditions: $[A\beta_{40}] = 10 \mu\text{M}$; $[APP\text{-}C31] = 10 \mu\text{M}$; [Texas Red-labeled dextran] = 0.4 mg/mL; 24 h incubation. Scale bars = 100 μm . **d**, Mean size of intracellular $A\beta_{40}$ aggregates in the absence and presence of APP-C31 observed in the airyscan confocal images. Aggregates (red) with dimensions exceeding the resolution limit were analyzed. Conditions: $[^{HF647}A\beta_{40}] = 10 \mu\text{M}$; $[APP\text{-}C31] = 10 \mu\text{M}$; 24 h incubation. Scale bars = 5 and 10 μm . Data are represented as mean $\pm$ s.e.m. $n = 52$ for the group of $^{HF647}A\beta_{40}$; $n = 63$ for the group of $^{HF647}A\beta_{40}$ with APP-C31; $*P < 0.05$; Student's t -test. The size distributions of $A\beta_{40}$ aggregates in both groups were summarized in Supplementary Fig. 14. **e**, Intranuclear transmission of $A\beta_{40}$ aggregates (red) with and without APP-C31. Nuclei were stained with SYTO16 (green). Conditions: $[^{HF647}A\beta_{40}] = 10 \mu\text{M}$; $[APP\text{-}C31] = 10 \mu\text{M}$; $[SYTO16] = 2.5 \mu\text{M}$; 24 h incubation. Scale bars = 10 μm .

of both APP-C31 and $A\beta_{40}$ into the cells resulted in the intense green fluorescence of annexin V with the r value of *ca.* 0.64. Clearly, these results illuminate that the intracellular presence of the two peptides can cause apoptosis.

Given that intracellular $A\beta$ species can induce apoptosis *via* damaging organelles and membranes^{2,4,43}, we examined the influence of APP-C31 on accumulating $A\beta$ aggregates at the intracellular level employing a fluorophore-labeled $A\beta_{40}$ and high-resolution confocal microscopy. The size of individual $A\beta_{40}$ aggregates was determined by counting the pixels of the fluorescent area based on previously reported procedures^{45,46}. The HiLyte Fluor 647-conjugated $A\beta_{40}$ ($^{HF647}A\beta_{40}$) with and without APP-C31 was injected into the cytoplasm of the cells. After 24 h incubation, $^{HF647}A\beta_{40}$ in the absence of APP-C31 exhibited a cluster of spherical aggregates with an average size of *ca.* 0.19 μm^2 , as illustrated in Fig. 5d and Supplementary Fig. 14. When $^{HF647}A\beta_{40}$ with APP-C31 was introduced inside the cells, we identified enlarged $A\beta_{40}$ aggregates with a cluster size of *ca.* 0.42 μm^2 . The intracellular distribution of $^{HF647}A\beta_{40}$ aggregates was further monitored in the cells stained with the fluorescent SYTO16 dye that can visualize nuclei by binding to nucleic acids⁴⁷, as depicted in Fig. 5e. $A\beta_{40}$ aggregates without APP-C31 were mostly located in the cytoplasm and a few clusters of them were internalized at the nucleus, as highlighted with white arrows shown in Fig. 5e. When $^{HF647}A\beta_{40}$ was injected with APP-C31 into the cells, we observed intranuclear $A\beta_{40}$ aggregates. Furthermore, the bulky $A\beta_{40}$ aggregates were invaded

into the nucleus. Therefore, our findings support that APP-C31 can promote the deposition of A β aggregates at the nuclear and perinuclear regions and, consequently, damage the nucleus leading to apoptosis.

Moreover, the effect of A β species treated with APP-C31 towards human neuronal growth was investigated with human neurons that are differentiated from H9 human embryonic stem cells. A β_{40} was introduced with and without APP-C31 into the media of human neurons. After 24 h and 72 h incubation, the length of neurites was measured to monitor the neuronal growth, as shown in Fig. 6a. Neurons incubated with A β in the absence and presence of APP-C31 for 24 h exhibited slightly shortened neurites by ca. 10%, compared to those of vehicle-added neurons. Upon treating neurons with A β_{40} only for 72 h, as indicated in Fig. 6b and 6c, the length of neurites was reduced by ca. 35%. More notably, administration of both APP-C31 and A β to neurons led to an approximately 70% decrease in the length of neurites exhibiting the fragmentation of neurites. These observations suggest that A β -induced degeneration of neurites can be intensified by APP-C31. Taken together, our studies employing cells and neurons manifest that APP-C31 can detrimentally impact the toxicity of A β .

Fig. 6 | Impact of APP-C31 on the neurodegeneration induced by A β . **a**, Scheme of the experiments. **b**, Representative images of neurons after 72 h incubation with either APP-C31, A β_{40} , or both. **c**, Average length of neurites quantified by NeuronJ in Fiji software. At least 25 neurons were analyzed per each condition. Conditions: [A β_{40}] = 20 μ M; [APP-C31] = 20 μ M; incubation for 24 and 72 h. Scale bars = 100 μ m. Data are represented as mean $\pm$ s.e.m. *** $P < 0.001$; Student's t -test.

Discussion

APP-C31 is a peptide composed of 31 amino acid residues that is generated by cleaving the

intracellular C-terminal region of APP by caspases¹. This peptide has been suggested to be a toxic APP fragment, along with A β , that could cause synaptic damage and neurodegeneration^{1,7,9,10}. An inter-relationship among the activation of caspases, the intracellular C-terminal cleavage of APP, and A β -induced toxicity towards amyloid pathology has been recently proposed¹¹. But to date, it was not known that APP-C31 can directly interact with A β and alter the folding behavior that ultimately leads to the A β -related toxicity. To the best of our knowledge, this work is the first report to illuminate the effects of APP-C31 on the aggregation and toxicity of A β through their direct contacts at both the intracellular and extracellular levels. In addition, such influence can be varied in the presence of metal ions such as Cu(II) and Zn(II) that bind to A β species in the AD-affiliated brain.

The interaction between APP-C31 and A β_{40} is relatively weak exhibiting a micromolar binding affinity that is still in the range of triggering conformational variations in intrinsically disordered proteins¹⁷. Through the contacts onto the *N*-terminal region and the self-recognition site of A β_{40} , APP-C31 forms a hetero-dimer with A β . Our computer simulations suggest that upon complexation with APP-C31, A β_{40} unfolds the α -helical segment at the self-recognition site that is critical for the aggregation of A β ^{3,4}. This structural rearrangement facilitates the β -sheet formation. The interactions between the two peptides change the aggregation of A β_{40} and promote the generation of elongated amyloid fibrils. In the case of A β_{42} , the aggregation is much less affected by APP-C31, which may be due to the fast kinetics and polymorphism of A β_{42} fibrillization leading to fewer contacts between APP-C31 and A β_{42} ^{4,48}. APP-C31 can interact with Cu(II)–A β_{40} and Zn(II)–A β_{40} and accelerate their aggregation, similar to metal-free A β_{40} . In living cells, larger A β_{40} aggregates are intracellularly produced in the presence of APP-C31. APP-C31 can noticeably induce the perinuclear and intranuclear deposition of A β_{40} aggregates. APP-C31 may assist in translocating A β into the perinuclear region or nuclei because the C-terminal fragments of APP are reported to transfer into nuclei after being complexed with other proteins^{49,50}. This is further supported by the recent findings that perinuclear A β aggregates are colocalized with the C-terminal region of APP^{14,15}. Moreover, A β_{40} incubated with APP-C31 significantly suppresses the outgrowth of neurites in human neurons. Clearly, APP-C31 can exacerbate the toxicity mediated by A β . Our combined experimental and theoretical studies demonstrate that direct interactions of APP-C31 with metal-free A β and metal–A β transform their aggregation and toxicity profiles under intracellular and extracellular environments.

Methods

Materials and methods. All reagents were purchased from commercial suppliers and used as received unless otherwise stated. APP-C31 (AAVTPEERHLSKMQQNGYENPTYKFFEQMQN) was purchased from AnaSpec (Fremont, CA, USA). A β ₄₀ (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) and A β ₄₂ (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) were obtained from Peptide Institute, Inc. (Osaka, Japan). ^{HF647}A β ₄₀ was purchased from AnaSpec. Double-distilled water (ddH₂O) used for all experiments was obtained from a Milli-Q Direct 16 system (Merck KGaA, Darmstadt, Germany). Trace metal contamination was removed from the solutions used for A β experiments by treating Chelex (Sigma-Aldrich, St. Louis, MO, USA) overnight. The concentrations of peptides were determined by an Agilent 8453 UV-visible spectrophotometer (Agilent, Santa Clara, CA, USA). Experiments by ESI-MS were performed by an Agilent 6530 Accurate Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer with an ESI source (Agilent). NMR spectroscopy was conducted by an AVANCE II-900 spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a cryogenic probe. ITC was performed by a VP-ITC instrument equipped with a motor driven syringe (Malvern Panalytical, Malvern, UK). The secondary structure of APP-C31 was analyzed by a JASCO-815 150-L CD spectropolarimeter [KAIST Analysis Center for Research Advancement (KARA), Daejeon, Republic of Korea]. The membranes obtained by the dot blot assay were visualized on a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). Morphological changes of peptide aggregates were monitored by a Tecnai F20 transmission electron microscope (KARA, Daejeon, Republic of Korea). A SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) was employed to measure fluorescence values for the ThT fluorescence assay. Live-cell microinjection experiments were carried out by the combined system composed of a micromanipulator (InjectMan 4, Eppendorf, Hamburg, Germany) and a microinjector (Femtojet 4i, Eppendorf, Hamburg, Germany). Fluorescence analysis of living cells was conducted by an EVOS FL fluorescence microscope (Advanced Microscopy Group, Bothell, WA, USA). Confocal microscopic images were taken by Zeiss LSM 880 (KARA, Daejeon, Republic of Korea).

Preparation of APP-C31 and A β . APP-C31 and A β were dissolved in ddH₂O and ammonium hydroxide (NH₄OH; 1% w/w, aq), respectively. After lyophilizing the resulting solutions, the peptides were stored at -80 °C. A stock solution of A β was prepared by dissolving the lyophilized peptide with NH₄OH (1% w/w, aq; 10 μ L) and diluting with ddH₂O, as previously reported^{32,34}. A stock solution of APP-C31 was prepared by dissolving the lyophilized peptide in ddH₂O. The concentration of A β ₄₀ or A β ₄₂ was determined by measuring the absorbance of the solution at 280

nm ($\epsilon_{280} = 1,450 \text{ M}^{-1}\text{cm}^{-1}$ and $1,490 \text{ M}^{-1}\text{cm}^{-1}$, respectively)^{32,34}. The concentration of APP-C31 was determined based on the extinction coefficient ($\epsilon_{280} = 2,980 \text{ M}^{-1}\text{cm}^{-1}$) obtained from the ExPASy ProtParam online tool.

ESI-MS. APP-C31 (100 μM) was incubated with A β (100 μM) in the absence and presence of CuCl₂ or ZnCl₂ (100 μM) in 20 mM ammonium acetate, pH 7.3 at 37 °C for 2 h without agitation. Before injection into the mass spectrometer, samples were diluted by 10 fold with ddH₂O. The capillary voltage, the drying gas flow, and the gas temperature were set to 58 kV, 12 L/min, and 300 °C, respectively. The fragment voltage was set to 170 V.

2D ¹H–¹⁵N HSQC NMR spectroscopy. NMR samples were prepared as described in the previous study⁵¹. Uniformly ¹⁵N-labeled A β_{40} (rPeptide Inc., Bogart, GA, USA) was initially dissolved in NH₄OH (1% w/w, aq; 10 μL). After lyophilizing the resulting solution, the peptide was stored at –80 °C. A solution of the ¹⁵N-labeled A β_{40} monomer was prepared by dissolving the lyophilized peptide in the chilled NaOH solution (10 mM) to make a stock concentration of ca. 200 μM . For 2D ¹H–¹⁵N HSQC measurements, the stock solution was further diluted to be 40 μM of ¹⁵N-labeled A β_{40} in the buffered solution [20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4] containing 10% v/v D₂O, and then treated with APP-C31 (200 μM). All HSQC spectra were obtained at 5 °C. Each spectrum was obtained from 256 t_1 experiments using 64 transients and 1 s recycle delay. Data were processed by NMRPipe⁵² and analyzed by Sparky⁵³. The assignment of backbone resonance was carried out based on the previously assigned information⁵¹. CSP ($\Delta\delta_{\text{NH}}$) was calculated by the equation below:

$$\Delta\delta_{\text{NH}} = \sqrt{\Delta\delta_{\text{H}}^2 + \left(\frac{\Delta\delta_{\text{N}}}{6.5}\right)^2}$$

where $\Delta\delta_{\text{H}}$ and $\Delta\delta_{\text{N}}$ represent the change of chemical shift in the proton and nitrogen dimensions, respectively.

ITC. All samples were dissolved in the buffered solution (20 mM HEPES, pH 7.4), and the resultant solutions were degassed for 3 min prior to the loading into the ITC instrument. To investigate the binding affinity of APP-C31 to A β_{40} , the final concentrations of APP-C31 (in the syringe) and A β_{40} (in the cell) were adjusted to 600 μM and 30 μM , respectively. To measure the

binding affinity of metal ions to APP-C31, 2.1 mM of CuCl₂ or ZnCl₂ (in the syringe) and 100 μM of APP-C31 (in the cell) were used for experiments. Titration experiments composed of 28 injections were conducted. The injection volume was 2 μL for the first injection to minimize effects of bubbles and 10 μL for the remaining injections. To prevent Aβ₄₀ aggregation, the temperature and stirring speed were set to 10 °C and 260 rpm, respectively. The initial delay was 1,800 s and the reference power was 10 μcal/s. The heats of dilution of APP-C31, CuCl₂, and ZnCl₂ titrated into the same buffer were also examined. The ITC thermogram and binding isotherm were displayed after subtracting the dilution heat. The binding isotherms after baseline correction were fitted to the one set of sites binding model incorporated in the MicroCal Origin 7.0 software⁵⁴.

Preparation of MD simulations. The atomistic NMR structure of Aβ₄₀ deposited in the protein data bank (PDB 2LFM²⁸) was used as the initial conformation of Aβ monomer. For APP-C31, the X-ray crystal structure of APP₆₆₆₋₆₉₃ excised from the intracellular domain of APP complexed with Fe65 protein (PDB 3DXC²⁹) was selected as the starting conformation of the protein. MD simulations of APP₆₆₆₋₆₉₃ monomer, Aβ₄₀ monomer, and the APP₆₆₆₋₆₉₃–Aβ₄₀ dimer were conducted by a GPU enabled Amber16 software suite²⁰. Each monomer was immersed in an octahedral solvation box filled with TIP3P water molecules²¹. For the APP₆₆₆₋₆₉₃–Aβ₄₀ dimer, 34 distinct dimeric conformations that were sampled from metadynamics MD simulations were used as the initial conformations (*vide infra*). The minimal distance from the protein surface to the solvation boundary was at least 12 Å. Monovalent counterions (Na⁺ or Cl[−]) were added to ensure the charge neutrality of the simulated systems. To model the peptides, the ff14SB all atom amino acid force fields parameter set was adopted²². MD simulations were applied to 0.2 fs time integration steps in conjunction with the Settle algorithm that fixes the bond distance between a hydrogen atom and its heavy atom partner²³. Particle Mesh Ewald (PME) method was employed to accelerate the calculations of long-range electrostatic interactions²⁴. The simulations began by 4,000 steps of energy minimizations. The temperature of each solvated model was increased from 0 K to 100 K in 2 ns while restraining each carbon atom with a harmonic potential of spring constant as 2 kcal/mol/Å². The temperature was then increased from 100 K to 300 K in 10 ns while restraining each Cα atom with a harmonic potential of spring constant as 10 kcal/mol/Å². Prior to the production periods, additional 10 ns of equilibrations was performed while keeping the temperature at 300 K and the pressure at 1 atm. The production runs lasted for 500 ns for the monomer and dimer models without any restraints to the solutes.

Metadynamics MD simulations. Metadynamics MD simulations were employed to investigate the interfaces of the APP₆₆₆₋₆₉₃–A β ₄₀ dimer. The COM distance of the two monomeric peptides, APP₆₆₆₋₆₉₃ and A β ₄₀, was used as the reaction coordinate of the dimerization. Gibbs free energy profile was computed along the chosen reaction coordinates without additional processing of the simulation data¹⁹. Using the metadynamics MD simulations, reversible association of the two monomers was monitored followed by achieving an enriched set of the associated dimer conformations. Metadynamics MD simulations were initiated from the last step of the constant temperature-pressure MD simulations of the APP₆₆₆₋₆₉₃–A β ₄₀ dimer. To enhance the convergence of the simulations, the well-tempered metadynamics method was applied with the bias temperature of 500 K and the energy bin height of 0.005 kcal/mol^{25,26}. The multi-walker method was used to diversify the dimer conformations²⁷. Total 12 replicas of the dimers were simulated. Each replica was simulated for 700 ns resulting in 8.4 μ s of gross simulation time. The COM distance was sampled from 5 Å to 40 Å. The simulation temperature was held constant at 300 K.

Structural clustering of the dimer interfaces sampled from metadynamics MD simulations.

Upon completion of metadynamics MD simulations, structural cluster analysis was carried out to identify the distinct APP₆₆₆₋₆₉₃–A β ₄₀ dimer interfaces. First, the dimer interfaces that show the COM distances between 10 Å to 15 Å were collected, where the computed Gibbs free energy profile was minimized. Those collected interfaces were subject to the structural clustering analysis using VMD software. Two conformers having the backbone RMSD less than 3.0 Å were considered to be similar structures, which resulted in 34 different structural clusters. A representative conformation of each structural cluster was subject to additional 500 ns MD simulations without structural constraints.

ThT fluorescence assay. The kinetics of A β aggregation were detected by the ThT fluorescence assay. Samples containing A β (25 μ M) with or without APP-C31 (25 μ M) in 20 mM HEPES, pH 7.4, 150 mM NaCl (40 μ L) were incubated for 0 h, 0.3 h, 0.5 h, 0.7 h, 1 h, 1.3 h, 1.7 h, 2 h, 3 h, 3.5 h, 4 h, 6 h, and 8 h (under metal-free A β ₄₀ conditions), for 0 h, 0.25 h, 0.5 h, 0.75 h, 1 h, 1.25 h, 1.5 h, 2 h, 2.5 h, 4 h, and 8 h (under metal-free A β ₄₂ conditions), and for 0 h, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, and 72 h (under Cu(II)- or Zn(II)-present conditions) at 37 °C with constant agitation. Upon 20 min treatment of ThT into each sample, the fluorescence intensity was measured with excitation at 440 nm and emission at 490 nm.

Dot blot assay. Samples containing A β (100 μ M) with and without APP-C31 (100 μ M) in the absence and presence of CuCl₂ or ZnCl₂ (100 μ M) were incubated in 20 mM HEPES, pH 7.4, 150 mM NaCl at 37 °C with constant agitation. After 0 h, 1 h, 2 h, 4 h, and 8 h incubation (under metal-free conditions) or 0 h, 1 h, 2 h, 4 h, 8 h, and 24 h incubation (under Cu(II)- or Zn(II)-added conditions), the samples (4 μ L) were spotted on a nitrocellulose membrane. The membrane was blocked with bovine serum albumin (BSA; 3% w/v; Biosesang, Seongnam, Republic of Korea) in Tris-buffered saline (TBS) containing 0.01% v/v Tween-20 (TBS-T) for 1.5 h at room temperature. Then, the membrane was incubated with a primary antibody [an anti-oligomer antibody (A11; 1:2,500, Invitrogen, Carlsbad, CA, USA), an anti-A β antibody (6E10; 1:2,000, Covance, Princeton, NJ, USA), or an anti-APP antibody (Y188; 1:2,000, Abcam, Cambridge, MA, USA)] for 1.5 h at room temperature. After washing with TBS-T three times (7 min each), the horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2,500 for 6E10; Cayman, Ann Arbor, MI, USA) or the goat anti-rabbit secondary antibody (1:2,000 for A11 and an Y188 antibody; Promega, Madison, WI, USA) in the solution of BSA (2% w/v in TBS-T) was added to the membrane and incubated for 1 h at room temperature. The membrane was visualized by an imaging system with a homemade ECL kit⁵⁵. To monitor both peptides in the samples, the same membrane was stripped after the visualization by treating it with hydrogen peroxide (H₂O₂) for 30 min at room temperature followed by washing four times with TBS-T for 10 min each, blocking with the solution of BSA [3% w/v in TBS-T (0.01% v/v)], and incubating with the other antibody.

CD spectroscopy. APP-C31 (100 μ M) was incubated in 20 mM HEPES, pH 7.4, 150 mM NaF for 0 and 24 h. The CD spectra of APP-C31 samples were collected in the range from 190 to 250 nm with a cell path length of 0.5 mm. The digital integration time, the bandwidth, and the scanning speed were 4 s, 2 nm, and 20 nm/min, respectively. Each spectrum was smoothed by Fourier transforms. The secondary structure of APP-C31 was analyzed following the previously reported method⁵⁶.

TEM. Samples for TEM measurements were prepared based on previously published methods³⁴. Glow-discharged grids (Formvar/Carbon 300-mesh, Electron Microscopy Sciences, Hatfield, PA, USA) were treated with the resultant samples for 2 min at room temperature. Excess sample was removed using a filter paper. Each grid was washed three times with ddH₂O and incubated with uranyl acetate (1% w/v ddH₂O; 5 μ L) for 1 min. After removing excess uranyl acetate, the grids were dried for at least 20 min at room temperature. Images of each grid were taken at 200 kV with a magnification of 9,900x or 29,000x.

Cell culture and microinjection. The human neuroblastoma SH-SY5Y cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cell line was maintained in media containing 50% v/v Dulbecco's Modified Eagle Medium and 50% v/v Nutrient Mixture F12 (phenol red-free DMEM/F12; GIBCO, Grand Island, NY, USA) supplemented with 10% v/v fetal bovine serum (GIBCO) and 100 U/mL penicillin-streptomycin (GIBCO). Cells were grown and maintained at 37 °C in a humidified atmosphere with 5% CO₂. The cells used for our studies did not indicate mycoplasma contamination. Cells were plated on an imaging dish (35 mm; 1.0 x 10⁵ cells per 1 mL) and incubated for 48 h before microinjection. The solutions of peptides were centrifuged at 5,000 rpm for 1 min to remove large aggregates that can clog a microinjection glass capillary. The solutions of either A β ₄₀ (10 μ M), APP-C31 (10 μ M), or both peptides in the absence and presence of CuCl₂ or ZnCl₂ (10 μ M) were mixed with dextran labeled with Texas Red (0.4 mg/mL, 3,000 MW; Invitrogen, Carlsbad, CA, USA) followed by injection into the cells using an InjectMan 4 micromanipulator and a Femtojet 4i microinjector. The injection pressure (P_i), the compensation pressure (P_c), and the injection time (t_i) were set to 50 hPa, 20 hPa, and 0.2 s, respectively. Cell viability was determined based on the previously reported procedures⁴². After microinjection, the number of Texas Red-positive cells was first counted after 3 h and it was assigned as 100% of cell viability to exclude the cells damaged by microinjection. Cells were counted after 24 h incubation and cell viability (%) was calculated. At least 100 cells from each sample were injected and analyzed. Experiments were performed at least triplicate. Data were represented as mean $\pm$ standard error of the mean (s.e.m). Fluorescence analysis of the cells was conducted by a fluorescence microscope with a RFP light cube [excitation at 531 ($\pm$ 40) nm and emission at 593 ($\pm$ 40) nm].

Annexin V staining. SH-SY5Y cells were plated on an imaging dish (35 mm; 0.3 x 10⁵ cells per 1 mL) and incubated for 48 h. The solutions of either A β ₄₀ (10 μ M), APP-C31 (10 μ M), or both peptides were mixed with dextran labeled with Texas Red, and the resultant solutions were microinjected into the cells, as described above. After 24 h incubation, the cells were washed with annexin-binding buffer and stained with annexin V-Alexa Fluor 488 for detecting apoptosis following the manufacturer's instruction (FITC Annexin V/Dead Cell Apoptosis Kit; V13241, Invitrogen, Carlsbad, CA, USA). Fluorescent responses of annexin V and Texas Red-labeled dextran were identified by a fluorescence microscope with an GFP light cube [excitation at 470 ($\pm$ 22) nm and emission at 525 ($\pm$ 50) nm] and a Cy5 light cube [excitation at 628 ($\pm$ 40) nm and emission at 685 ($\pm$ 40) nm], respectively. Pearson's correlation coefficient for the indication of co-

localization was calculated with Coloc2 plugin in Fiji software⁵⁷.

Confocal microscopy. All experiments were conducted with ^{HF647}A β ₄₀ (Anaspec). The concentration of ^{HF647}A β ₄₀ was determined by measuring the absorbance at 649 nm (ϵ_{649} = 250,000 M⁻¹cm⁻¹)⁴⁵. ^{HF647}A β ₄₀ (10 μ M) with or without APP-C31 (10 μ M) was microinjected into SH-SY5Y cells followed by incubation for 24 h. Images were obtained by adopting a Zeiss LSM 880 confocal microscope with a lateral resolution of 120 nm (excitation at 633 nm). ^{HF647}A β ₄₀ aggregates were monitored from three or more cells. The size of ^{HF647}A β ₄₀ aggregates was determined by the “analyze particles” program of Fiji software based on the previously reported reports⁴⁶. All pixels above the threshold value were selected to calculate the area of ^{HF647}A β ₄₀ aggregates. Our analysis included ^{HF647}A β ₄₀ aggregates with the size larger than 0.05 μ m².

Neuronal differentiation from embryonic stem cells. Human neurons were differentiated from neural progenitor cells (NPCs), which are derived from human embryonic stem cells (hESCs), WA09 (also known as H9). hESCs were maintained on inactivated mouse embryonic fibroblasts (MEFs) in hESC-media comprising DMEM/F12 (GIBCO) with MEM non-essential amino acids (MEM NEAA; GIBCO), sodium bicarbonate (14 mM; Sigma-Aldrich), L-glutamine (1 mM; Sigma-Aldrich), β -mercaptoethanol (100 μ M; Merck Millipore, Billerica, MA, USA), knockout serum replacement (20% v/v; GIBCO), and bFGF (10 ng/mL; R&D systems, McKinley Place, MN, USA). MEFs were collected from E13.5 CrljOri:CD1 (ICR) mice (Orientbio, Seongnam, Republic of Korea) and inactivated with 10 μ g/mL of mitomycin C (AG Scientific, San Diego, CA, USA) in 2 h. hESC colonies were dissociated with Collagenase IV and plated onto Petri dishes in hESC-media to form embryoid bodies (EBs). At the following day, floating EBs were treated with LDN (Selleckchem, Huston, TX, USA) and SB-431542 (Cayman) in DMEM/F12+glutamax (GIBCO) with N2 and B27 supplements (GIBCO). The treatment was continued for a week, followed by plating onto growth factor reduced matrigel (BD biosciences)-coated dishes in DMEM/F12+glutamax (GIBCO) with N2 and B27 supplements (N2/B27 media) and laminin (1 mg/mL; GIBCO). Within a few days, neural rosettes were manually picked and dissociated with Accutase (Innovative Cell Technologies, San Diego, CA, USA) and plated onto poly-L-ornithine/laminin-coated dishes with NPC media (N2/B27 media with 20 ng/mL of bFGF). To differentiate NPCs into neurons, NPCs were plated onto poly-L-ornithine/laminin-coated dishes in N2/B27 media in the presence of ascorbic acid (200 nM; Sigma-Aldrich), dcAMP (500 mg/mL; Selleckchem), BDNF (20 ng/mL; Peprotech, London, UK), GDNF (20 ng/mL; Peprotech), and

laminin (1 mg/mL), as previously described⁵⁸. Protocols describing the use of MEFs and hESCs were approved by the ethical requirements and regulations of the Institutional Review Board of KAIST (IRB #KA2020-37 for MEFs and IRB #KA2018-61 for hESCs).

Measurement of neurite outgrowth. Human NPCs were differentiated into neurons for 2 d and the media was replaced by fresh media. Neurons were treated with either A β (20 μ M), APP-C31 (20 μ M), or both peptides followed by incubation for 24 h and 72 h. Under each condition, at least 5 randomly selected fields were captured with an Olympus IX71 microscope. The neurite of a neuron measured from the soma to the tip was analyzed using NeuronJ in Fiji software⁵⁹.

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