

Supporting information

Hydrophobic tagging-mediated degradation of Alzheimer's disease related Tau

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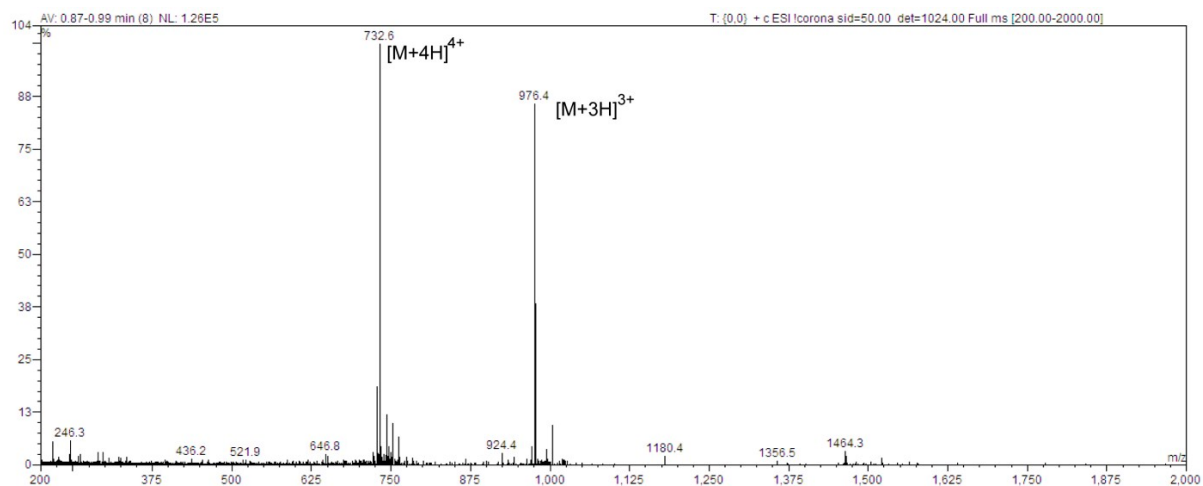
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-Materials and Methods

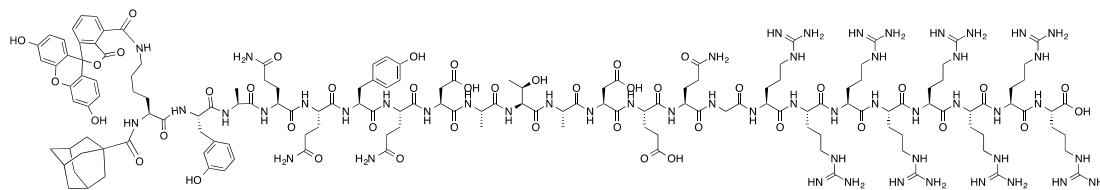
-Supplementary results

ESI-MS of HyT-Tau-CPP

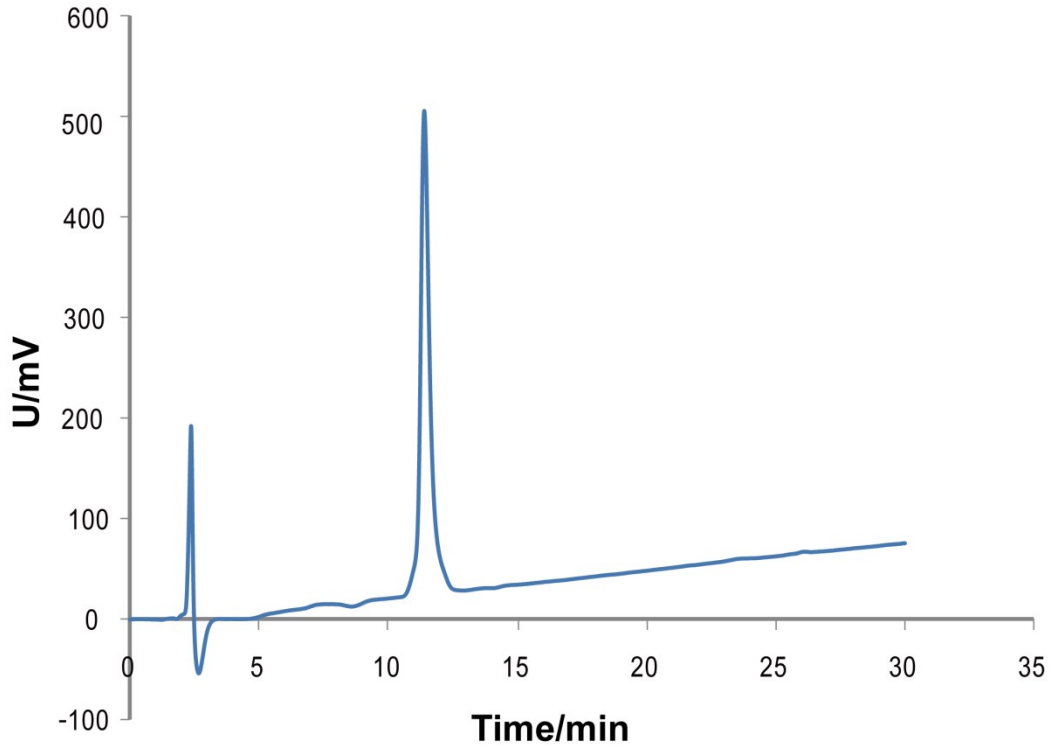


2: **CF-HyT-Tau-CPP**, MS (MALDI/TOF): Calcd. for $C_{149}H_{221}N_{51}O_{43}$, 3411 $[M+H]^+$; found 1138.7 $[M+2H]^{2+}$, 854.0 $[M+3H]^{3+}$, 683.6 $[M+4H]^{4+}$;

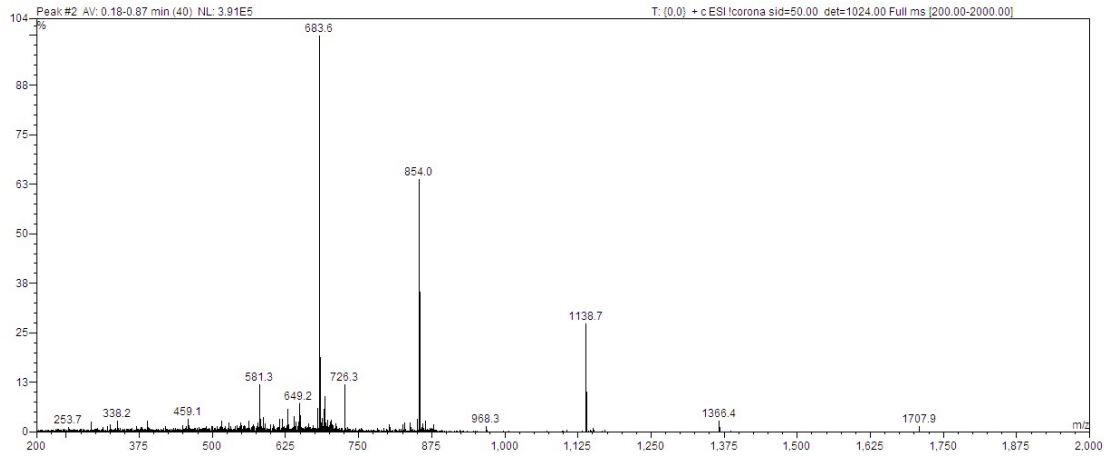
The structure of CF-HyT-Tau-CPP



Analytic HPLC trace of CF-HyT-Tau-CPP. Analytic gradient is 20% to 70% of solution B in 30 min. Retention time is 11.5 min.

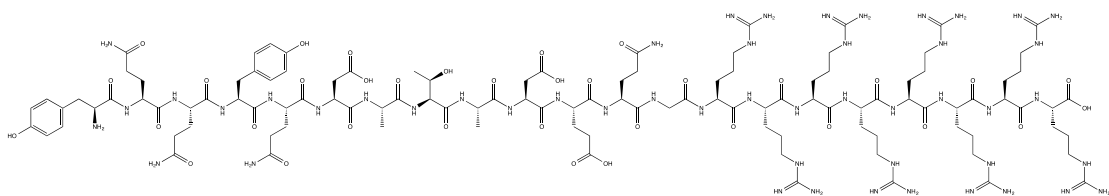


ESI-MS of CF-HyT-Tau-CPP

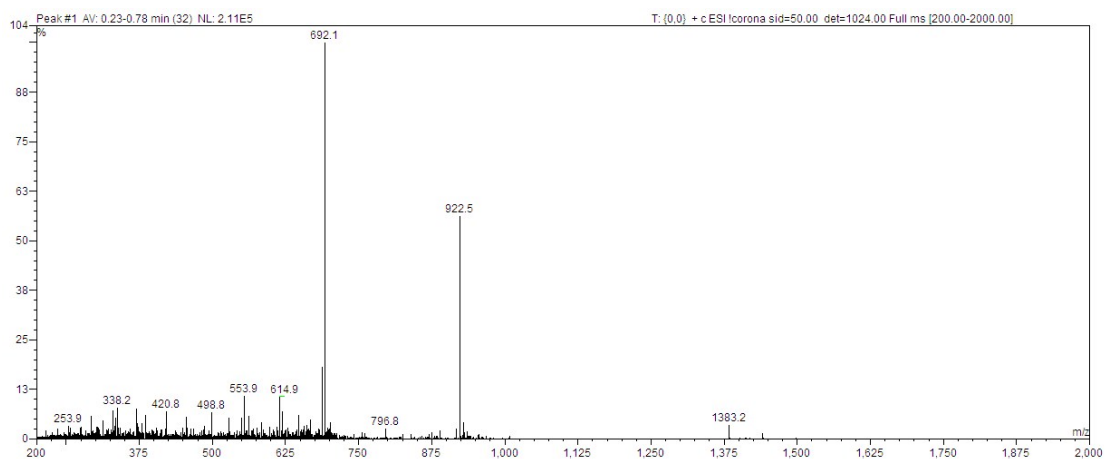


3: Tau-CPP, MS(ESI-MS): Calcd. for $C_{111}H_{185}N_{49}O_{35}$ 2764.42[M+H]⁺; found 922.5[M+3H]³⁺, 692.1[M+4H]⁴⁺ ;

The structure of Tau-CPP

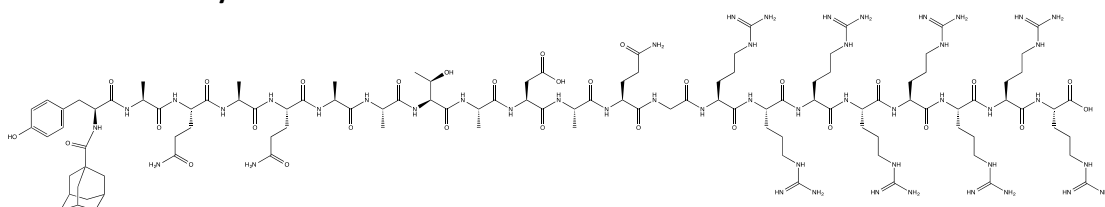


ESI-MS of Tau-CPP

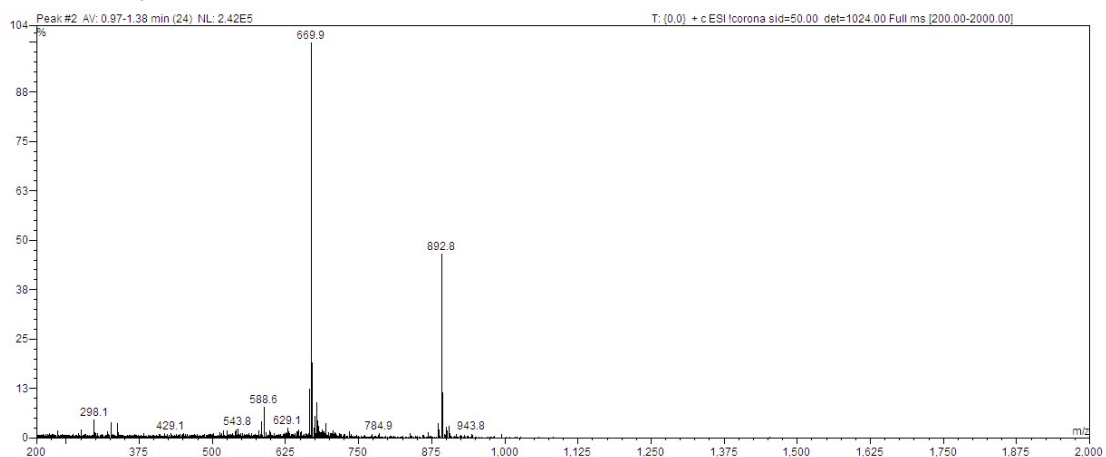


4: HyT-MTau-CPP, MS(ESI-MS): Calcd. for $C_{111}H_{190}N_{48}O_{30}$ 2675.48[M+H]⁺; found 892.8 [M+3H]³⁺, 669.9[M+4H]⁴⁺ ;

The structure of HyT-MTau-CPP

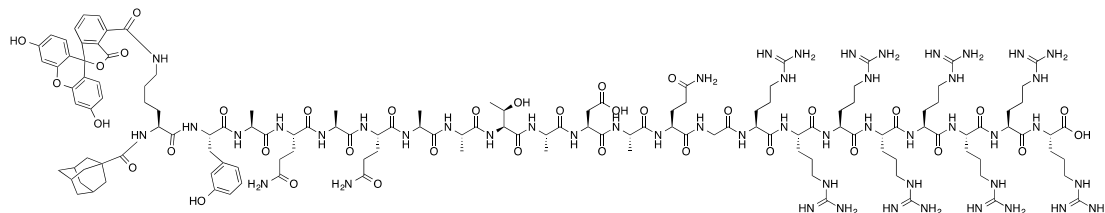


ESI-MS of HyT-MTau-CPP

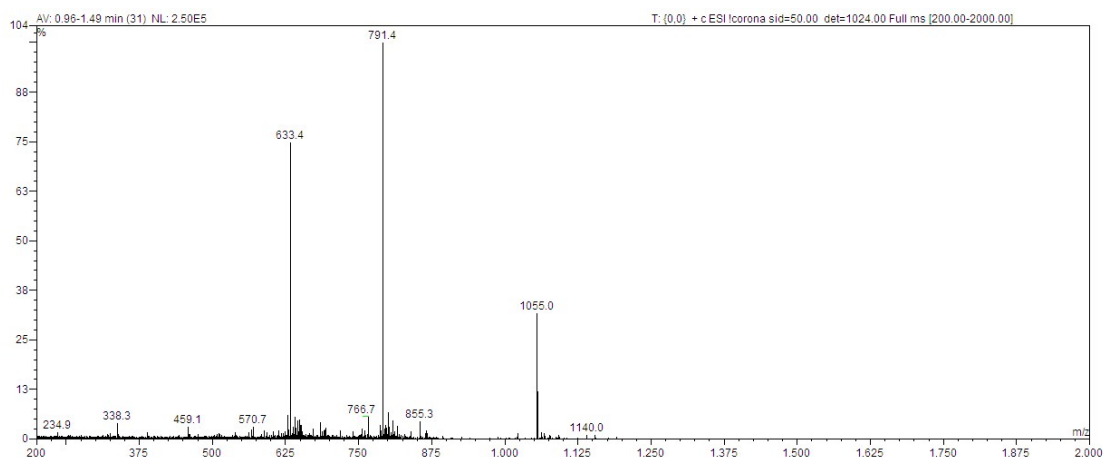


5: CF-HyT-MTau-CPP, MS(ESI-MS): Calcd. for $C_{138}H_{212}N_{50}O_{37}$ 3161.62[M+H]⁺; found 1055.0 [M+3H]³⁺, 791.4[M+4H]⁴⁺, 633.4[M+5H]⁵⁺;

The structure of CF-HyT-MTau-CPP



ESI-MS of CF-HyT-MTau-CPP



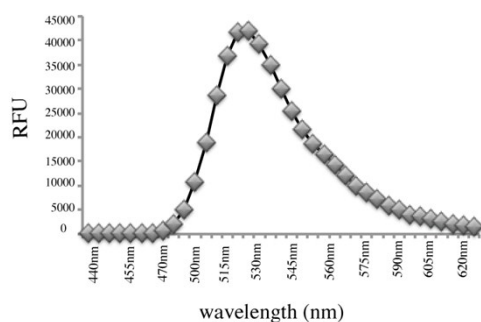
General protocol of peptide synthesis

In this work, the standard solid phase protocol of peptide synthesis was applied to synthesize all the peptides. The $N\alpha$ -Fmoc-protected amino acids and Rink Amide-AM resin was used for coupling. 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 3.6 equiv) and 1-Hydroxy-7-azabenzotriazole (HOAT, 4.0 equiv) were used as activator. Meanwhile, N, N-Diisopropylethylamine (DIEA, 8.0 equiv) was used as activator base in the coupling steps. Finally, the cleavage reagent (TFA/water/phenol/thioanisole/1, 2-Ethanethiol = 82.5/5/5/5/2.5) was used to cleave the peptides from Rink Amide-AM resin for 2.5 h. After removing TFA, peptides were precipitated by diethyl ether and centrifuged with 8000 rpm for 10min at least three times. Then the HPLC was employed to purify the peptides and ESI/MS was applied to identify the peptides. 1-adamantane carboxylic acid was coupled to the N terminus of 'No adamantane' following the same steps applied for the peptide coupling. In the same way as mentioned above, we coupled 5(6)-carboxyfluorescein (CF) to the HyT-Tau-CPP to get CF-HyT-Tau-CPP as a probe.

General protocol of RP-HPLC purification¹

A preparative C18 column of reverse phase (5 μ M, 20 \times 250 nm; flow 6.0 ml/min, YMC, Japan; solution A: 100%water + 0.06% TFA, solution B: 80% acetonitrile + 20% water + 0.06% TFA) was used to purify the peptides on a Waters-600-2487. The pure peptides we obtained were lyophilized and placed at -20 $^{\circ}$ C to store. At last, they were analyzed with an analytical C18 column of reverse phase (10 μ M, 4.6 \times 150 nm; flow 0.8 ml/min, YMC, Japan). All peptides we synthesized were >95% pure through an analytical column purification.

Fluorescence emission spectrum of CF



Plasmids construct²

To construct the stable Tau-EGFP overexpressing cell line of N2a, pEGFP-N3 plasmid was used, and then tau DNA coding sequence was inserted into restriction sites of BamH 1 and Xho 1 of the plasmid. To express the recombinant hTau40 protein, pet28a plasmid was applied, and then tau DNA coding sequence was inserted into restriction sites of Nde 1 and Xho 1 of the plasmid.

Protein expression and purification²

Recombinant hTau40 proteins with His-tag were expressed in BL21 strains. The strains were cultured in the medium of LB contained Kanamycin (100 µg/ml) at 37°C, when the value of OD600 was up to 0.6, adding 1 mM IPTG at 16°C to induce for 20 h. Profinity TM IMAC Resins (BIO-RAD) was applied to purify recombinant hTau40 proteins. The buffer that contained 500 mM NaCl and 20 mM Tris-HCl (pH=8.0) and different concentration of imidazole was used to elute proteins.

Cell culture

The medium of mouse N2a neuroblastoma cells and the Tau-EGFP overexpressing cells is 45% Dulbec-co's Modified Eagle's Medium (DMEM), 45% Minimum Essential Medium (α -MEM), 10% Fetal Bovine Serum (FBS), Streptomycin (100 µg/ml) and Penicillin (100 µg/ml). The cells were cultured in 5% CO₂ at 37°C on a high-temperature sterilization CO₂ incubator (Thermo 371).

Gene transduction²

To construct the Tau-EGFP overexpressing cell line, the reagent of Lipofectamine™2000 (Invitrogen) was used to transfect the plasmid of pEGFP-N3 tau to the mouse N2a neuroblastoma cells. After transfection, the treated cells were grown and screened in the medium that contained 800 µg/ml antibiotic G418 to get the stable cell line.

Flow Cytometry Assay

The Tau-EGFP overexpressing cells were incubated with the concentration of 0 µM, 50 µM, 100 µM, 150 µM, 200 µM HyT-Tau-CPP for 24 h or with 150µM HyT-Tau-CPP for 0 h, 4 h, 8 h, 12 h. Then the cells were washed with PBS buffer (pH=7.4) before harvest. finally, the fluorescence intensity of 10,000 cells in each experiment was observed by the FACS Calibur flow cytometer (BD Biosciences). The experiment was repeated for three times at least.

Western Blotting¹

After being incubated with cycloheximide (CHX) (10 µg/ml) for 2 h, the Tau-EGFP overexpressing cells were treated with HyT-Tau-CPP of 0 µM, 50 µM, 100 µM, 150 µM for 24 h or with 150 µM HyT-Tau-CPP for 0 h, 6 h, 12 h, 24 h. The cells were incubated with proteasome inhibitor MG132 for 6h before harvest. Then the cells were lysed with P0013 lysis buffer (P0013; Beyotime) that contained cocktail protease inhibitors (539131; Calbiochem). The primary antibodies anti-tau (sc-21796; Santa Cruz) and anti-actin (A1978; Sigma-Aldrich) as well as the secondary antibody peroxidase-conjugated anti-mouse IgG (A6154; Sigma-Aldrich) were used for western blots. Each experiment was repeated at least three times.

MTT assay

The Tau-EGFP overexpressing cells or wild N2a cells were placed in a 96 –well plate with a density of 2×10^5 /ml. The wild N2a cells were treated with an increasing concentration (0 μ M, 20 μ M, 40 μ M, 80 μ M, 100 μ M, 120 μ M, 150 μ M) of HyT-Tau-CPP for 24 h. The Tau-EGFP overexpressing cells were treated with 0 μ M, 25 μ M, 50 μ M, 75 μ M, 100 μ M, 125 μ M, 150 μ M, 200 μ M of HyT-Tau-CPP. Each concentration was repeated for five times. Then the cells were treated with MTT reagent (5 mg/ml, 20 μ l for each well) for 4 h. 150 μ l dimethyl sulfoxide (DMSO) was added to each well after removing the MTT reagent. After that, a Biotek Synergy 4 microplate reader was used to monitor the absorbance value of 490 nm wavelengths. Each experiment was repeated at least three times.

Fluorescence Polarization

The Fluorescence polarization calculation formula is:

$$F = \frac{I_{//} - I_{\perp}}{I_{//} + I_{\perp}}$$

The changing of fluorescence polarization change was accorded with Equation 1 that calculated the value of the affinity of HyT-Tau-CPP and Tau 441.

$$F = F_{\min} + \left(K_D + A_0 + E_0 - \sqrt{(K_D + A_0 + E_0)^2 - 4A_0E_0} \right) * \frac{F_{\max} - F_{\min}}{2A_0} \quad (\text{Equation 1})$$

The reaction system respectively contained 0 μ M, 0.5 μ M, 1 μ M, 2 μ M, 5 μ M, 10 μ M, 15 μ M, 20 μ M, 25 μ M Tau 441 protein with 0.1 μ M CF-HyT-Tau-CPP or 0.1 μ M CF or 0.1 μ M CF-MTau-CPP, 20 mM Tris-HCl, 150 mM NaCl, which was mixed and incubated 30min at 37 °C. Then a Biotek Synergy 4 microplate reader was applied to assay the value of fluorescence polarization. The detection excitation wavelength is at 440 nm/40 and emission wavelength is at 528 nm/20.

Animal procedures²

The mice (B6, 3xTg-AD mice, 5 to 6 months old, 129-Psen1tm1Mpm Tg [APP^{Swe}, Tau P301L]1Lfa/Mmjax) were intravenously injected with HyT-Tau-CPP (18mg/kg body weight, n=4 for each group) that was dissolved into saline solution to the concentration of 1mg/ml or saline solution for 8 days. The B6129SF2/J mice were used as control group of the experiments. The treated and untreated mice were all euthanized. Then the mice were perfused and removed the whole brain. One half brain was for western blot analysis and another half brain was for immunohistochemistry analysis. Finally, the cerebral cortex and hippocampus tissues were separated from the half brain of AD mice.

Detection of Tau levels *in vivo* by western blotting assay

The cerebral cortex and hippocampus tissues were immersed in the lysis buffer (PN78510; Thermo Scientific Pierce) that contained 1% protease inhibitor cocktails (PN87786; Thermo Scientific Pierce) and further broken by ultrasound on ice respectively. Then the mixtures were centrifuged at 15,000 rpm at 4°C for 20 min. At last, the tau level in supernatant was tested by western blotting assay. The primary antibodies anti-tau (sc-21796; Santa Cruz) and anti-actin

(A1978; Sigma-Aldrich) as well as the secondary antibody peroxidase-conjugated anti-mouse IgG (A6154; Sigma-Aldrich) were used for testing Tau level. Each experiment was repeated three times.

Immunohistochemistry²

The half brains of AD mice were immersed in 4% paraformaldehyde overnight at 4°C. The microtome was applied to get tissue sections. The tissue sections were needed to remove paraffin and then hydrated. After that, the tissue sections were blocked with 10% normal goat serum for 1 h, and then incubated with anti-tau (sc-21796; Santa Cruz) for 2 h at room temperature. After washing with PBS and PBST buffer three times, the sections were incubated with goat anti-mouse IgG (H+G) antibody that was combined with Alexa Fluor 488 (A21206, Invitrogen) at room temperature for 2 h. Then the sections were also washed with PBS and PBST buffer three times. Finally, Prolong Gold Antifade Reagent with DAPI (P36935; Invitrogen) was used to seal the sections. The inverted laser scanning confocal microscope (Zeiss LSM 780) with excitation at 415 and 488 nm and a 10 X and 20 X objective was used to observe samples.

Confocal Microscopy

The coverslips were put on the 6-well plate. Then the coverslips were covered with 0.2mg/ml poly-L-Lysine (P6282, Sigma-Aldrich) at 37°C for 3 h. After removing poly-L-Lysine, the Tau-EGFP overexpressing cells or wild N2a cells were put on coverslips. The Tau-EGFP overexpressing cells were treated with 100 μM, 150 μM HyT-Tau-CPP or vehicle for 24 h or wild N2a cells were incubated with 150 μM CF-HyT-Tau-CPP for 6 h. After washing the cells PBS buffer (pH=7.4), the cells were fixed by 4% paraformaldehyde at room temperature for 30 minutes. After that, the cells were treated with PBS buffer (pH=7.4) containing 0.2% Triton X 100 at room temperature for 10 min. Then the nucleuses of cells were stained by 1 μg/ml DAPI. At last, anti-bleaching reagent was used to seal the cells after washing with PBS buffer containing 0.02 % Triton X 100 for 3 times. The inverted laser scanning confocal microscope (Zeiss LSM 780) was used to observe samples. Wayne Rasband ImageJ (NIH) was employed to take the images. Each experiment was repeated at least three times.

1 HyT-Tau-CPP. The title peptide was synthesized by using the standard solid phase protocol. MS(ESI-MS): Calcd. for C₁₂₂H₁₉₉N₄₉O₃₆ 2926.4[M+H]⁺; found 976.4 [M+3H]³⁺, 732.6[M+4H]⁴⁺ .

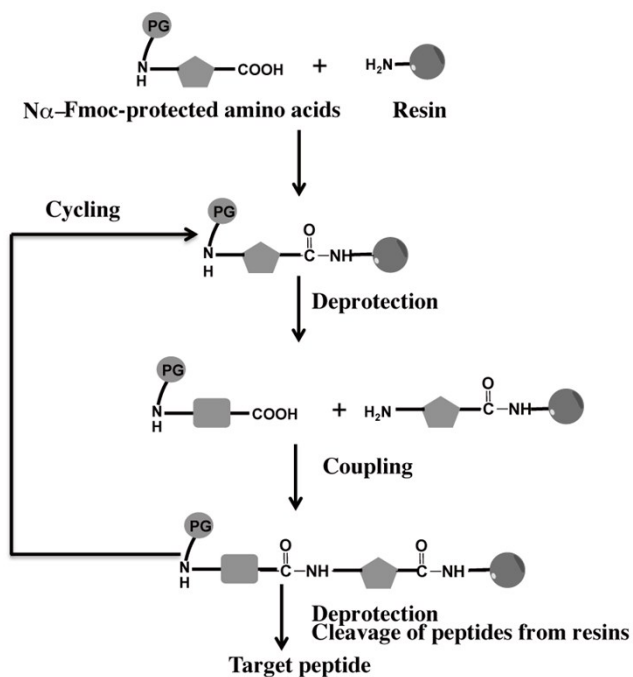
2 CF-HyT-Tau-CPP. The title peptide was synthesized by using the standard solid phase protocol. MS (ESI/TOF): Calcd. for C₁₄₉H₂₂₁N₅₁O₄₃, 3411[M+H]⁺; found 1138.7[M+2H]²⁺, 854.0 [M+3H]³⁺, 683.6[M+4H]⁴⁺ .

3 Tau-CPP. The title peptide was synthesized by using the standard solid phase protocol. MS(ESI-MS): Calcd. for C₁₁₁H₁₈₅N₄₉O₃₅ 2764.42[M+H]⁺; found 922.5[M+3H]³⁺, 692.1[M+4H]⁴⁺ .

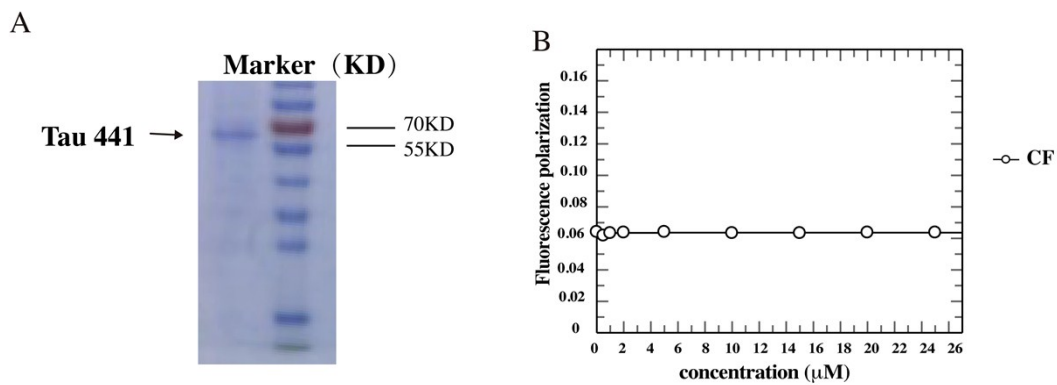
4 HyT-MTau-CPP. The title peptide was synthesized by using the standard solid phase protocol. MS(ESI-MS): Calcd. for C₁₁₁H₁₉₀N₄₈O₃₀ 2675.48[M+H]⁺; found 892.8 [M+3H]³⁺, 669.9[M+4H]⁴⁺ .

5 CF-MHyT-Tau-CPP. The title peptide was synthesized by using the standard solid phase protocol. MS(ESI-MS): Calcd. for C₁₃₈H₂₁₂N₅₀O₃₇ 3161.62[M+H]⁺; found 1055.0 [M+3H]³⁺, 791.4[M+4H]⁴⁺ , 633.4[M+5H]⁵⁺;

Supplementary results

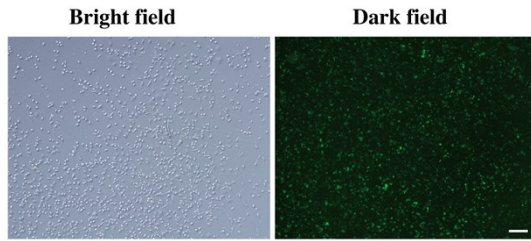


Supplementary Figure S1. Scheme of the peptide synthesis flow.

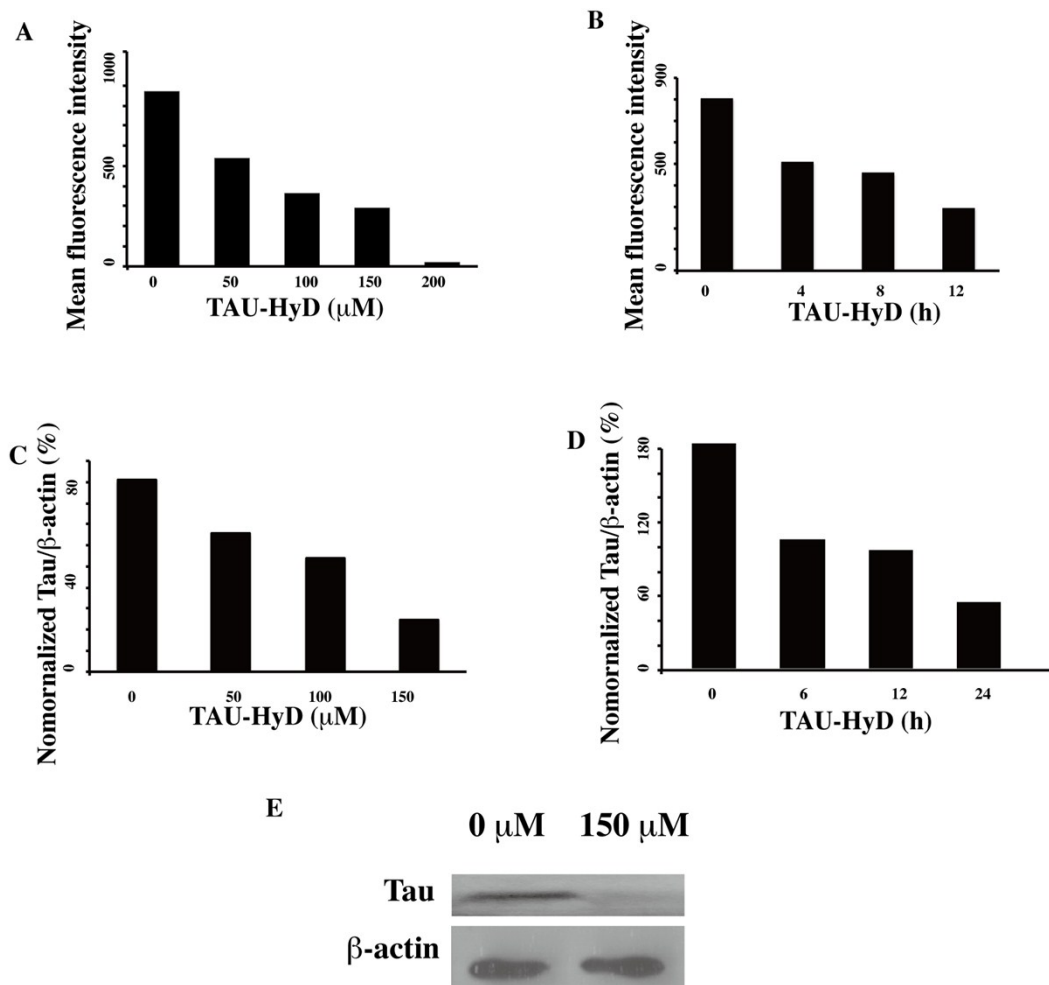


Supplementary Figure S2. (A) 15% SDS-PAGE results of expressed recombinant hTau40 protein.

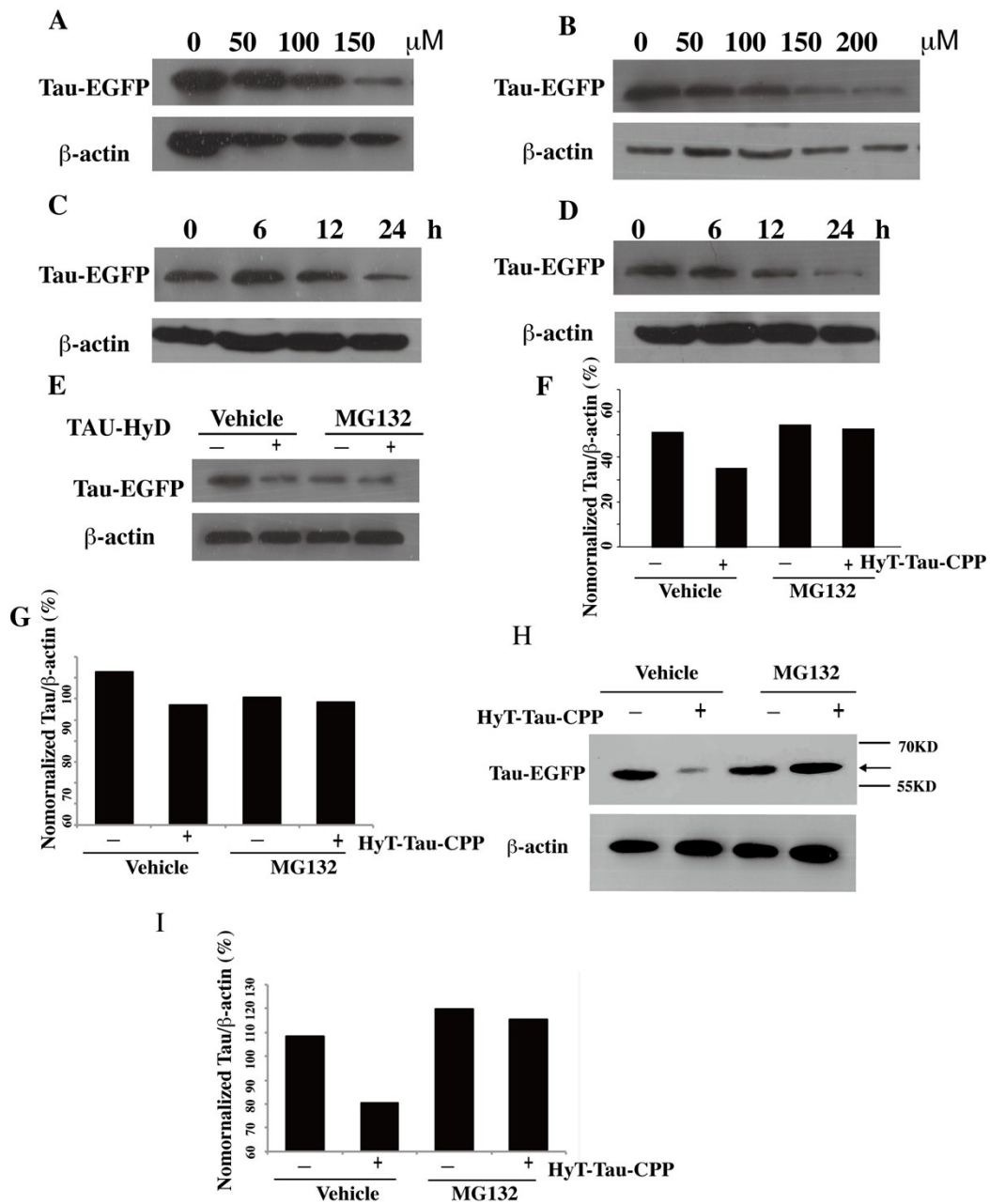
(B) The fluorescence polarization results of 0 μM , 0.5 μM , 1 μM , 2 μM , 5 μM , 10 μM , 15 μM , 20 μM , 25 μM Tau 441 protein were incubated with 0.1 μM CF respectively.



Supplementary Figure S3. The microscopic results of the wild type N2a cells with the treatment of 150 μM CF-HyT-Tau-CPP for 6 h. The results prove that HyT-Tau-CPP can penetrate into cells in 6 h. Scale bar, 10 μm .

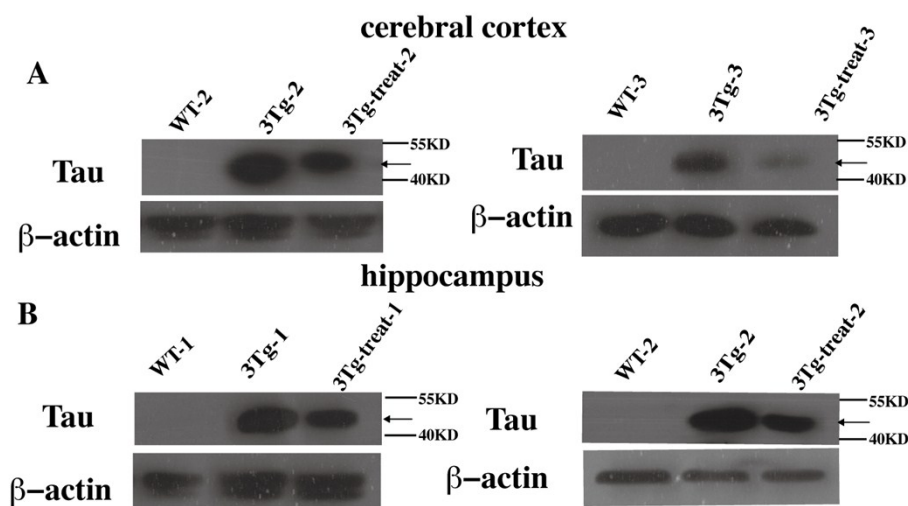


Supplementary Figure S4. (A and B) Mean fluorescence intensity of the flow plots of Figure 3A and 3B, respectively. The results reveal a concentration- and time-dependent decrease of Tau induced by HyT-Tau-CPP. (C-D) Quantification of the level of Tau based on the western blot results of Figure 3C and 3D respectively. (E) The western blotting results of wild type N2a cells with 150 μM HyT-Tau-CPP or vector for 24 h. The western blotting results showed that HyT-Tau-CPP could reduce endogenous Tau levels. Tau was probed with anti-tau antibody (sc-21796; Santa Cruz) and β -actin (A1978; Sigma-Aldrich) was applied as the control.



Supplementary Figure S5. related to Figure 3 and 4 . Whole blot images. (A-B) Whole blot images of Figure 3B. The western blot results for tau and β -actin show dose-dependent degradation induced by HyT-Tau-CPP. (C-D) Whole blot images of Figure 3C. The western blot results for tau and β -actin show time-dependent degradation induced by HyT-Tau-CPP (E,H) Whole blot images of Figure 4A. The western blot results for tau and β -actin demonstrate that HyT-Tau-CPP mediated- degradation is proteasome-dependent. Tau was probed with anti-tau antibody (Santa Cruz) and β -actin (A1978) was applied as the control. (F) Quantification of the level of Tau based on the western blot results of Figure 4A. (G) Quantification of the level of Tau based on the western blot results of Figure Supplementary Figure SE. (I) Quantification of the level

of Tau based on the western blot results of Figure Supplementary Figure SH.



Supplementary Figure S6. related to Figure 5. Whole blot images. (A) whole blot images of Figure 5A. HyT-Tau-CPP can induce the degradation of Tau in the cerebral cortex of AD mouse models. (B) whole blot images of Figure 5B. HyT-Tau-CPP can induce the degradation of Tau in the hippocampus of AD mouse models. Tau was probed with anti-tau antibody (Santa Cruz) and β -actin (A1978) was applied as the control.

Reference

- 1: T.-T. Chu, Q.-Q. Li, T. Qiu, Z.-Y. Sun, Z.-W. Hu, Y.-X. Chen, Y.-F. Zhao and Y.-M. Li, *Mol BioSyst*, 2014, **10**, 3081-3085.
- 2: T.-T. Chu, N. Gao, Q.-Q. Li, P.-G. Chen, X.-F. Yang, Y.-X. Chen, Y.-F. Zhao and Y.-M. Li, *Cell Chem Biol*, **23**, 453-461.