Supplementary Materials

A CBS3-targeting direct AMPK activator ameliorates Alzheimer's disease pathology and cognitive decline

Kyung-Hwa Jeon[1,2,†], Hyunji Jo[1,†], Soyeon Cho[3,†], Jae-Ho Shin[4], Seojeong Park[1], Kyu-Yeon Jun[1], Seojeong Kim[1], Seung Hee Seo[1], Soo-Yeon Hwang[1], Dabin Song[3], Yumi Son[3], Zhi Zheng[5], Hyunjeong Kim[3], Younghwa Na[4,*], Eosu Kim[3,*], and Youngjoo Kwon[1,2,6,*]

[1]*College of Pharmacy, Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul, 03760, Korea*

[2]*Graduate Program in Innovative Biomaterials Convergence, Ewha Womans University, Seoul 03760, Korea*

[3]*Department of Psychiatry, Institute of Behavioral Science in Medicine, Yonsei Metabolism-Dementia Research Institute, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, 03722, Korea.*

[4]*College of Pharmacy, CHA University, Pocheon, 11160, Korea*

[5]*Department of Toxicology, School of Public Health, Xinxiang Medical University, Henan, China*

[6]*Graduate Program in Regulatory Science Convergence, Ewha Womans University, Seoul, 03760, Republic of Korea*

[†]These authors equally contributed.

Contents

	Page
Material and methods	**4-24**
Chemistry general	4
General synthetic methods	4-6
^{1}H NMR and ^{13}C NMR Spectra of the synthesized compounds	7-8
HPLC Chromatogram of the synthesized compounds	9-10
In silico docking study	11
Cell culture	11
Plasmid transfection of AMPK wild type and mutants and drug treatment in cells	11
AMPK kinase assay	12
Conditioned Medium Preparation and Treatment	12
Cell viability assay	13
Cellular thermal shift assay (CETSA)	13
Immunofluorescence assay	13
Subcellular fractionation	14
Dot blot analysis	14
ELISA assay	15
DCFH-DA assay	15
NAD^{+} and NADH quantification	15
ADP and ATP quantification	16
$A\beta_{1-42}$ fibril synthesis and stereotaxic surgery	16
Drug administration	16
Morris water maze test	17
Passive avoidance test	17
Rotarod test	18
Vertical pole test	18
Novel object recognition test	18
Touchscreen behavioral assessment	19
Slice preparation	20
Electrophysiology recording	20
Measurement of acetylcholine (Ach) content and acetylcholinesterase (AChE) activity	21
TUNEL assay	21
Mouse primary neuron culture	22

In vivo hippocampal western blot	22-24
Supplementary Tables	**25-27**
Supplementary Table S1. The antibodies used in this study	25
Supplementary Table S2. Gene-specific primers used for RT-qPCR and RT-PCR	26
Supplementary Table S3. The antibodies used for in vivo western blot	27
Supplementary Figures	**28-39**
Supplementary Figure S1. GSEA hallmark pathway analysis	28
Supplementary Figure S2. The ability of YE-06 to activate AMPK in an AMPKα isotype-non-specific and AMP-competitive manner via AMPK-SIRT1-Nampt pathway.	29
Supplementary Figure S3. Downregulation of BACE1 expression by AMPK in an enzymatic activity–dependent manner	31
Supplementary Figure S4. Mode of action of YE-06 with multiple effects on the pathogenesis of AD	32
Supplementary Figure S5. Effects of YE-06 on short- and long-term memory in Aβ-injected mice	33
Supplementary Figure S6. Sex-specific effects of YE-06 on novel object recognition, body weight, and food intake in 3xTg-AD mice.	34
Supplementary Figure S7. Effects of YE-06 on motor function of 10-month-old 3xTg-AD mice in 5-CSRT task.	35
Supplementary Figure S8. Effects of Compound C on cognitive and motor function in the 5-CSRT task.	36
Supplementary Figure S9. Effects of YE-06 on synaptic and neuronal markers.	37
Supplementary Figure S10. Effects of YE-06 treatment on hippocampal AD-related molecular markers in two independent 3xTg-AD behavioral cohorts	38

Chemistry general

Chemicals and reagents used were obtained from Sigma-Aldrich (St. Louis, MO, USA) and TCI Chemicals (Tokyo, Japan). All HPLC-grade solvents were purchased from Burdick and Jackson, USA and were used without distillation. Melting points were measured without correction in open capillaries with Barnstead Electrothermal melting point apparatus, Manual MELTEMP (Model No: 1202D). Chromatographic separations were monitored by thin-layer chromatography using a commercially available pre-coated Merck Kieselgel 60 F254 plate (0.25 mm) and detected by visualizing under UV at 254 and 365 nm. Silica gel column chromatography was carried out with Merck Kieselgel 60 (0.040-0.063 mm). The purity of all synthesized compounds was assessed by HPLC (Shimadzu LC-20AD) analysis under the following conditions; column, SunFire C18 (4.6 mm × 150 mm, 5 μm); mobile phase, condition A: isocratic 40% acetonitrile in water with 0.5% TFA, condition B: isocratic 50% acetonitrile, condition C: isocratic 30% acetonitrile in water with 0.5%-TFA, or condition D: isocratic 70% acetonitrile in water over 15 min; flow rate, 1.0 mL/min; detector, UV detector (Shimadzu Spd-M20A diode array detector). NMR spectra were recorded on Varian AS 400 (^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz) equipped at Ewha Drug Development Research Core Center or JEOL JNM-ECZ500R/S1 (^{1}H NMR at 500 MHz and ^{13}C NMR at 125 MHz) with tetramethylsilane as an internal standard. Chemical shift (d) values are expressed in ppm and coupling constant *(J)* values in hertz (Hz). Mass spectral investigations were performed on an AB SCIX API 4000 equipped with an electrospray ionization (ESI) source.

General Synthetic Methods

Method **1**: The reaction mixture of acetophenone analogue (1 equiv.), benzaldehyde analogue (1 equiv.), and NaOH (1 equiv.) in ethanol was stirred at room temperature (1-2 d), and water

was added. The reaction mixture was extracted with ethyl acetate (x 2 times). The organic layer was collected and washed with water and then dried over anhydrous $MgSO_4$. Solvent was removed under reduced pressure and the residue was purified by silica gel chromatography to give the desired product.

(*E*)-1-(4-Aminophenyl)-3-(2,5-dimethoxyphenyl)prop-2-en-1-one (YE-02)

Following method **1**, 4-aminoacetophenone (0.50 g, 3.70 mmol), 2,5-dimethoxybenzaldehyde (0.62 g, 3.70 mmol), and NaOH (0.15 g, 3.70 mmol) were used. Purification by silica gel chromatography (eluent: ethyl acetate/*n*-hexane = 1:2 → 1:1) gave YE-02 (0.66 g, 62.5%) as a yellow solid. R_f 0.36 (ethyl acetate/*n*-hexane = 1:1); m.p. 125.8 ~ 127.3 °C; HPLC : R_T 5.27 min (purity: 99.9%); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.81 (s, 3H), 3.85 (s, 3H), 4.19 (br s, 2H), 6.69 (d, *J* = 8.8 Hz, 2H), 6.86 (d, *J* = 8.8 Hz, 1H), 6.91 (dd, *J* = 8.8, 2.8 Hz, 1H), 7.16 (d, *J* = 2.8 Hz, 1H), 7.58 (d, *J* = 15.6 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 2H), 8.04 (d, *J* = 15.6 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) 56.0, 56.3, 112.7, 113.9, 114.1, 116.8, 123.3, 125.2, 128.9, 131.3, 138.6, 151.2, 153.4, 153.7, 188.8 ppm; HRMS-ESI (m/z) $[M+H]^+$ $C_{17}H_{17}NO_3$ calcd 284.1281, found 284.1281.

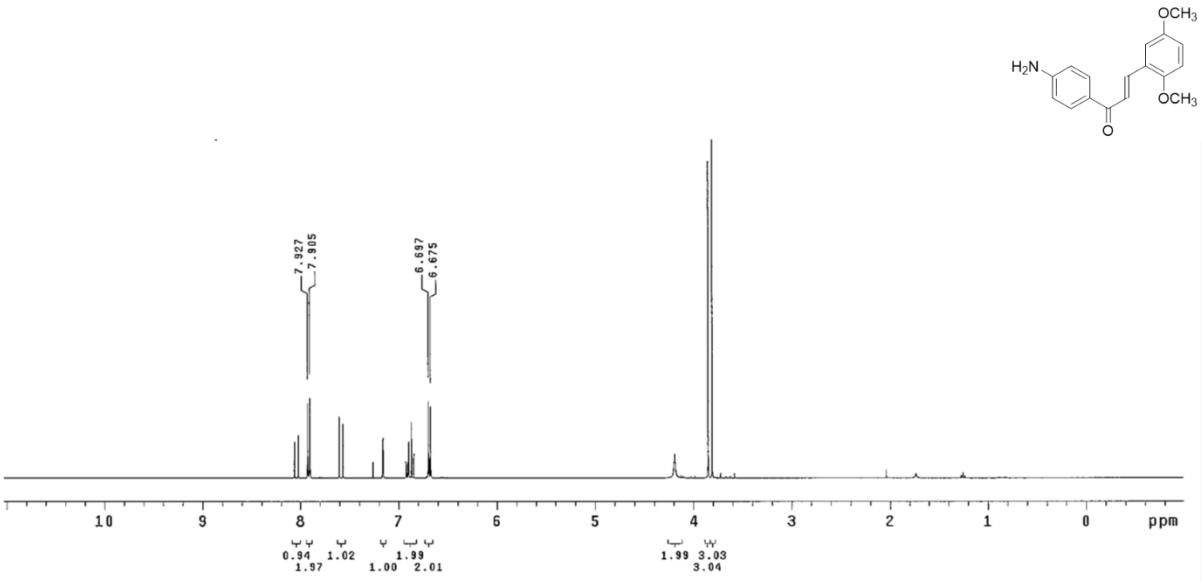
(*E*)-4-Chloro-*N*-(4-(3-(2,5-dimethoxyphenyl)acryloyl)phenyl)benzenesulfonamide (YE-06)

To a reaction mixture of YE-02 (0.67 g, 2.36 mmol) and triethylamine (TEA) (0.26 g, 2.60 mmol) in CH_2Cl_2 was added 4-chlorobenzenesulfonyl chloride (0.75 g, 3.54 mmol). The reaction mixture was stirred at room temperature (1 d) and water was added, and then the

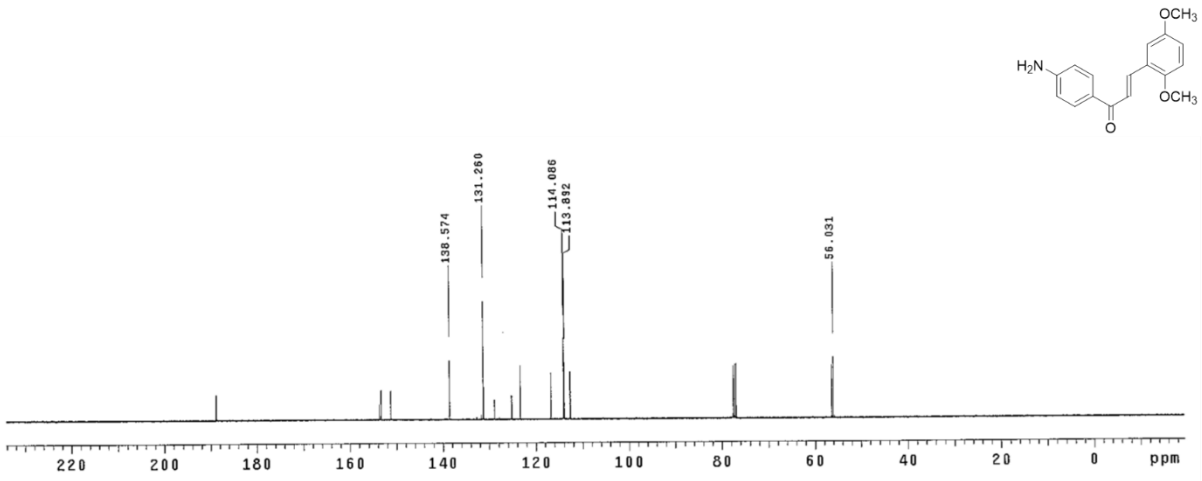
product was extracted with ethyl acetate. The organic layer was collected and washed with sat. $NaHCO_3$, and then dried over anhydrous $MgSO_4$. Solvent was removed under reduced pressure, and the residue was purified by silica gel chromatography (eluent: ethyl acetate/*n*-hexane = 1:2) to give YE-06 (0.55 g, 50.9%) as a yellow solid. R_f 0.18 (ethyl acetate/*n*-hexane = 1:1); m.p. 144.0-144.9 °C; HPLC : R_T 5.49 min (purity: 96.1%); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 3.74 (s, 3H), 3.80 (s, 3H), 6.84 (d, *J* = 8.8 Hz, 1H), 6.88 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.16 (d, *J* = 2.4 Hz, 1H), 7.20 (d, *J* = 8.4 Hz, 2H), 7.40 (d, *J* = 8.4 Hz, 2H), 7.54 (d, *J* = 15.6 Hz, 1H), 7.74 (d, *J* = 8.4 Hz, 2H), 7.85 (d, *J* = 8.4 Hz, 2H), 7.91 (d, *J* = 15.6 Hz, 1H), 10.58 (s, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6) 55.2, 55.6, 112.1, 112.7, 117.0, 118.0, 121.6, 123.5, 128.0, 128.1, 128.7, 129.4, 132.9, 137.8, 138.3, 141.5, 152.5, 152.9, 187.9 ppm; HRMS-ESI (m/z) $[M+H]^+$: $C_{23}H_{20}ClNO_5S$, calc'd: 458.0823, found: 458.0825.

^{1}H NMR and ^{13}C NMR Spectra of the synthesized compounds

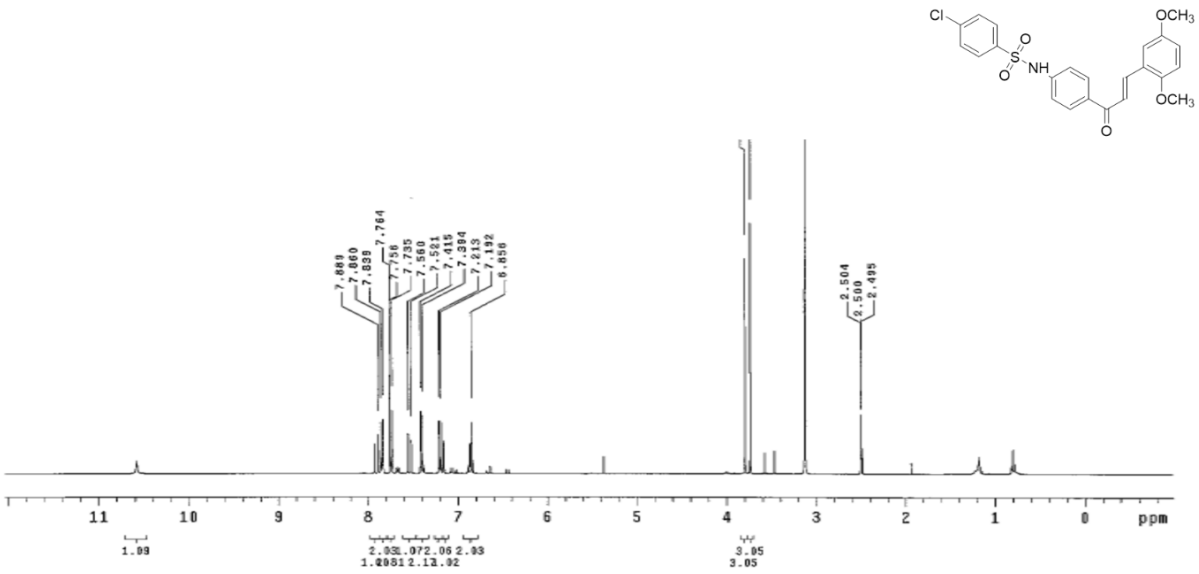
^{1}H NMR Spectrum of YE-02



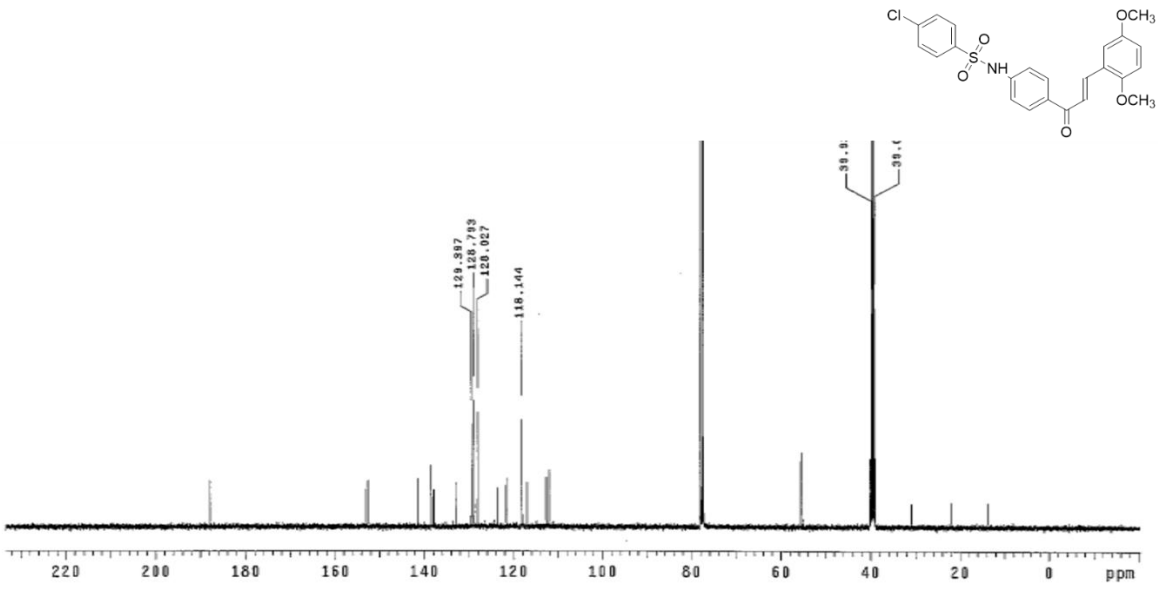
^{13}C NMR Spectrum of YE-02



^{1}H NMR Spectrum of YE-06

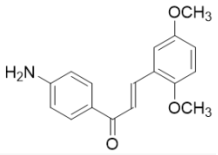


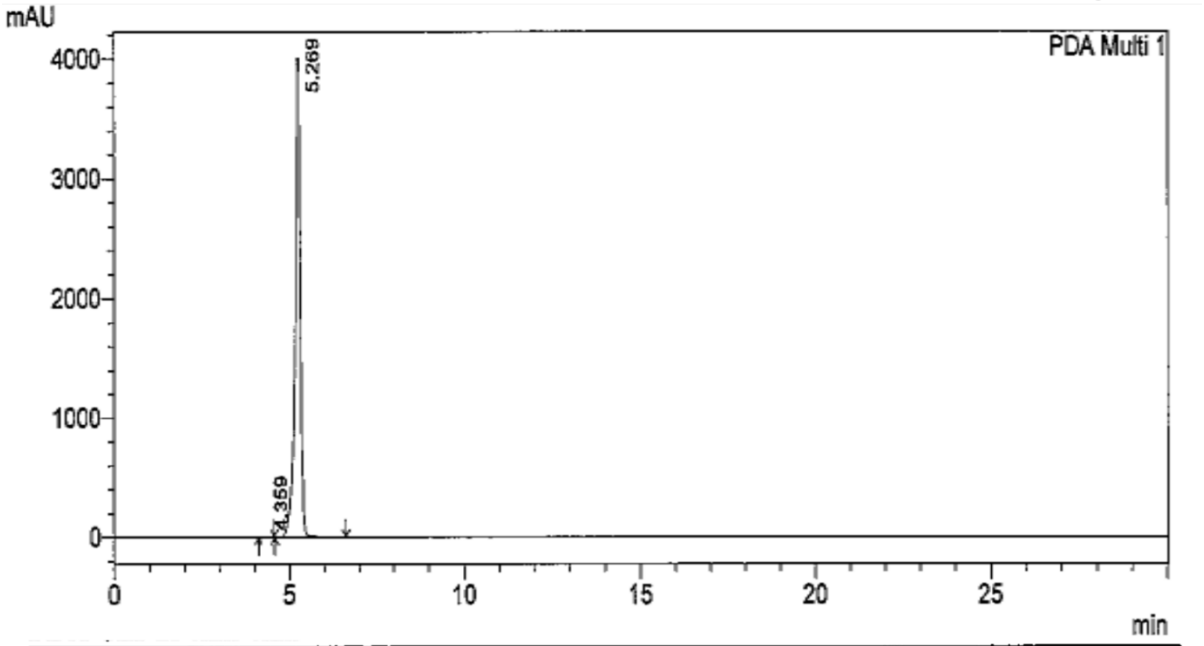
^{13}C NMR Spectrum of YE-06



HPLC Chromatogram of the synthesized compounds

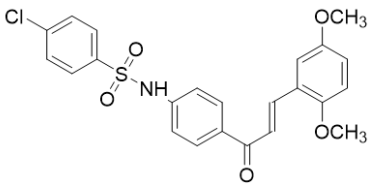
YE-02

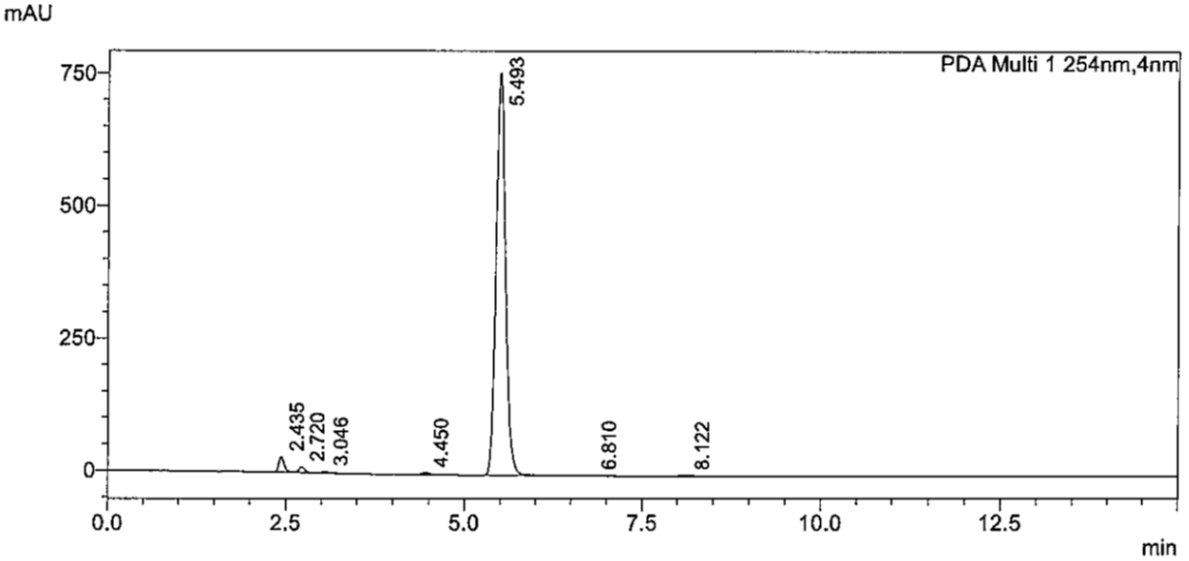




Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.359	28371	3074	0.063	0.077
2	5.269	44850018	3999685	99.937	99.923
Total		44878389	4002759	100.000	100.000

YE-06





Peak#	Ret. Time	Area	Height	Area%	Height%
1	2.435	155119	28961	2.113	3.587
2	2.720	62791	10572	0.856	1.310
3	3.046	19442	3109	0.265	0.385
4	4.450	28718	3683	0.391	0.456
5	5.493	7054493	759380	96.117	94.061
6	6.810	8467	819	0.115	0.101
7	8.122	10463	805	0.143	0.100
Total		7339494	807329	100.000	100.000

In silico docking study

Docking studies were conducted utilizing the X-ray crystal structures of AMPK γ-subunit (4CFH and 4RER, downloaded from Protein Data Bank) for all compounds, employing Cresset Flare™ software V6.1. Prior to docking, energy minimization of the compounds was performed. The docking grid for the compound was positioned at AMP-binding site 3 of AMPK γ. The LF rank score index was utilized for ranking the poses of the compounds, while the LF VS score index was employed for ranking the compounds docked during screening.

Cell culture

N2a (mouse neuroblastoma cell line) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The human Swedish mutation (K670M/N671L) APP_{695} N2a APP swe cell line was generously provided by Prof. E.S. Kim (Yonsei University, Seoul, Korea). HT-22 (a mouse hippocampal neuronal cell line) was obtained from Prof. H. J. Kim (Ewha Womans University, Seoul, Korea). BV2 (a mouse microglia cell line) was purchased from Elabscience® (Houston, USA). N2a, N2a APP swe and HT22 were maintained in high-glucose Dulbecco's modified Eagle medium (Welgene, Gyeongsan, Korea), and BV2 cells were grown in minimum essential medium (Welgene). All cells were supplemented with 10% fetal bovine serum (FBS; Corning, New York, USA) and 1% penicillin streptomycin (HyClone, Logan, USA) and were cultured in a humidified incubator at 37 ℃ with 5% CO_2.

Plasmid transfection of AMPK wild type and mutants and drug treatment in cells

pcDNA3.1-AMPKα1 and -AMPKγ1 were provided by Prof. S.B. Rho (National Cancer Center, Korea, Goyang). Sub-cloning procedures were undertaken to incorporate protein tags. Specifically, AMPKα1 and γ1 were ligated into the p3xFLAG-Myc-CMV-26 plasmid. Mutant variants including AMPKα1 T183A (dominant negative; DN), AMPKα1 T183D (constitutively active; CA), and AMPKγ1 H298G/R299F were generated using a mutagenesis kit (Takara, Kyoto, Japan). For plasmid transfection, cells were cultured until reaching approximately 70% confluency and were

then transfected with plasmids using Polyplus transfection reagent (jetPRIME®RI, Illkirch, France). Plasmids and reagents were mixed in a buffer and incubated for 10 min at room temperature (RT). Cells were treated with DMSO or compounds prior to harvesting. AICAR, donepezil and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA), and A-769662 and STO-609 were purchased from Selleck Chemicals (Houston, TX, USA). The dosage and duration of treatment varied across experiments, and specific details are provided in the respective results.

AMPK kinase assay

The AMPK kinase *in vitro* assay was performed in two steps using the AMPK kinase assay kit (Promega, Madison, USA). Initially, AMPK enzyme, SAMS peptide, and ATP were mixed in a kinase buffer and incubated for 1 h at RT to activate AMPK kinase. In the second step, following the AMPK reaction, ADP-Glo™ reagent was added to each well and incubated for 40 min to deplete any unreacted ATP using the ADP-Glo™ assay kit (Promega). This step enabled the measurement of the amount of ADP converted specifically due to AMPK activation. Subsequently, a second reagent was added to each well and incubated for 30 min to convert ADP to ATP. The newly synthesized ATP produced light via a luciferase/luciferin reaction, and the resulting light intensity was quantified using a Microplate reader M200 Pro (Tecan, Männedorf, Switzerland).

Conditioned Medium Preparation and Treatment

For conditioned medium experiments, BV2 cells were treated with YE-06 in the presence or absence of LPS as indicated. Following treatment, the culture medium was completely removed, and the cells were washed with PBS. The cells were then incubated for 24 h in fresh YE-06-free culture medium, after which the conditioned medium (CM) was collected. The CM was used for cytokine analysis or transferred to HT22 cells for subsequent assessment of apoptosis. This procedure was designed to minimize direct carryover of YE-06 from BV2 cells to HT22 cells.

Cell viability assay

Cells were seeded into 96-well plates at a density of 1.5×10^4 cells per well in 100 μL of media. The next day, cells were treated with varying concentrations of compounds at for 24 h at 37ºC. Then, 5 μl of EZ-CytoX (DoGen, Seoul, Korea) was added to each well. After a further 2h incubation, the absorbance was measured at a 450 nm using a Microplate Reader M200 Pro (Tecan). The IC_{50} values were calculated using the Table Curve 2D program (SPSS, Chicago, USA).

Cellular thermal shift assay (CETSA)

Cells were cultured in 100 mm plates and transfected with either AMPK-WT or AMPK mutant ($AMPK^{H298G/R299F}$). Following transfection, cells were treated with either DMSO or YE-06 (10 μM) for 3 h prior to harvesting. Cells were harvested and resuspended in PBS, and then divided into seven PCR tubes. Subsequently, all samples in the PCR tubes were heated at temperatures ranging from 48 ºC to 57 ºC for 3 min. Following heat treatment, cell lysates were obtained using liquid nitrogen and centrifuged at 13,000 rpm for 15 min at 4°C to separate the supernatant. Flag-tagged wild-type AMPKγ (WT-AMPKγ) and mutant AMPKγ (MU-AMPKγ) were then detected through Western blot analysis.

Immunofluorescence assay

Cells were seeded onto 8-well chamber slides (SPL, Pocheon, Korea) at a density of 20,000 cells per well in 300 μL medium. After washing once with PBS, the cells were fixed by incubating with 4% paraformaldehyde for 20 min at RT. The fixative was washed twice with PBS, and the cells were incubated in a blocking solution consisting of 5% Blocking one-P (Nacalai Tesque, Kyoto, Japan) and 0.1% Triton X-100 (Sigma-Aldrich) for 20 min at RT. After washing twice again with PBS, the cells were incubated at 4ºC overnight with the primary antibody dilution solution (1:50). After washing three times with PBS, the cells were incubated with the secondary antibody (Alexa 488 green) in dilution buffer (mixed with blocking solution and PBS) for 1 h at RT. Nuclei were stained using DAPI at a concentration of 0.1 μg/mL for 10 min. After washing again three times,

the cells were treated with mounting solution (Agilent, Santa Clara, USA), and a cover glass was placed thereon. Images were captured by an apotome laser-scanning microscope (ZEISS, Jena, Germany) and analyzed using Zen pro software (ZEISS).

For nuclear translocation analysis, nuclei were identified and segmented based on DAPI staining. The DAPI-defined nuclear regions were used to distinguish the nuclear and cytoplasmic compartments, and fluorescence intensities of the target protein were quantified separately in each compartment.

Subcellular fractionation

Cytoplasmic and nuclear fractionation was performed using NE-PER® nuclear and cytoplasmic extraction reagents (Thermo). Harvested cells were lysed using a cytoplasmic extraction reagent I and II, followed by centrifugation at 14,000 g for 5 min at 4 ºC. The supernatant was transferred to a new tube. The insoluble pellet was lysed using nuclear extraction reagent and centrifuged at 14,000 g for 10 min at 4ºC, with the resulting supernatant transferred to a new tube. The protein concentration of each fraction was determined using the BCA protein assay kit (Thermo). Equal amounts of proteins from each fraction were loaded onto the gel for subsequent analysis of protein expression. Lamin A/C was employed as the loading control for the nuclear fraction and α-tubulin for cytoplasmic fraction.

Dot blot analysis

Debris-free conditioned media were prepared for dot blots by centrifugation at 3,200 rpm for 5 min. The resulting supernatant was transferred to a new tube, and samples were dropped on a nitrocellulose membrane and air-dried. This step was repeated until 30 μg protein was loaded. After loading, the membrane was dried for 1 h at RT. Subsequent steps for Western blot analysis were carried out in the same manner.

ELISA assay

ELISA measurements were performed to determine the levels of secreted IL-1β and TNF-α using ELISA kits (R&D systems, Minneapolis, USA). Conditioned media, cleared of debris by centrifugation at 3,200 rpm for 5 min, were prepared for analysis. Samples were added to each well coated with monoclonal antibody specific for IL-1β and TNF-α, followed by 2 h incubation at RT. Standard dilutions were tested in parallel to generate standard curves for IL-1β and TNF-α in the conditioned media. After 2h incubation, each well was washed to remove unbound material, followed by incubation with each secondary antibody for 2 h at RT. Subsequently, the substrate solution was added to the wells and the absorbance of each well was measured at 450 nm using a Microplate Reader M200 Pro (Tecan). Quantitative values were obtained by applying the sample values to the standard curve.

DCFH-DA assay

Intracellular ROS levels were assessed using DCFH-DA assay. DCFH-DA penetrates cells and is converted to DCFH by esterase. In the presence of ROS, DCFH is oxidized to form the fluorescent compound DCF. Cells seeded overnight in 6-well plates were washed with PBS and then incubated with DCFH-DA (10 μM) for 30 min at 37 ºC in the dark. Subsequently, images were captured using an apotome laser-scanning microscope (ZEISS) and analyzed with Zen pro software (ZEISS).

NAD^+ and NADH quantification

The concentration of NAD^+ and NADH in N2a cell extracts was measured using a NAD^+/NADH quantification kit (Biovision, Milpitas, CA, USA) according to the manufacturer's protocol. Total intracellular nicotinamide adenine dinucleotides, NADt (comprising NAD^+ and NADH), as well as NADH specifically, were measured. The obtained values were normalized to the protein concentration of the cell extracts, determined using the DC protein assay kit (Bio-Rad).

ADP and ATP quantification

The ADP and ATP levels of N2a cells were measured using an ADP/ATP Ratio Assay kit (abcam, Cambridge, UK) according to the manufacturer's protocol. The levels of ATP from N2a cell extracts were measured by a luminometer (Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany). For detecting ADP levels, samples were measured again after applying ADP converting enzyme.

$A\beta_{1-42}$ fibril synthesis and stereotaxic surgery

$A\beta_{1-42}$ was dissolved in tertiary distilled water to achieve a concentration of 0.25 mM and then incubated for 5 days at 37ºC to form fibrils. For the development of AD mouse model, mice were anesthetized with 2.5% isoflurane (Goyang, Republic of Korea) inhalation and placed in a stereotaxic instrument (Jeung Do Bio & Plant, Seoul, Korea). The $A\beta_{1-42}$ solution was injected into the hippocampus at coordinates AP: -2.3 mm, L: ±2 mm, and H: -1.5 mm, using a 10.0-μL gas-tight glass syringe (Hamilton, Reno, NV, USA) at a rate of 0.2 μL/min. The syringe was left in place for 5 min post-injection to ensure proper diffusion of the solution, and then slowly removed. Behavioral assessments began the day following the surgery.

Drug administration

Donepezil and YE-06 were administered to 6-week-old $A\beta_{1-42}$-injected ICR mice via oral gavage for 14 days, starting 7 days before $A\beta_{1-42}$ injection and continuing until the completion of behavioral assessments. Compound C was prepared at a concentration of 2 mg/kg and intraperitoneally injected into 15-month-old C57BL/6 mice, 3 h before each probe session. For chronic YE-06 treatment, 20 mg/kg YE-06 was mixed into chow and orally administered to 6- or 9-month-old male and female 3xTg-AD mice for 3 months. Food intake was recorded every 3 days on a per-cage basis, normalized to grams per mouse per day, and statistically analyzed using the cage as the experimental unit. For acute YE-06 treatment, 10 mg/kg YE-06 was diluted in a DMSO-based solvent (DMSO:PEG-400:Saline = 1:25:25) and intraperitoneally injected into 10-

month-old male 3xTg-AD mice, 2 h before each probe session.

Morris water maze test

Morris water maze test was performed according to standard methods [18] in a circular pool (diameter: 90 cm; depth: 45 cm; Iwoo Scientific Co., Seoul, Korea) filled with water to a depth of 31 ± 1 cm. To make the water opaque, non-fat milk was added and the water temperature was maintained at 20 ± 2°C throughout the experiment. A platform (diameter: 10 cm) was submerged approximately 1 cm below the water surface. On day 1, mice were allowed to freely swim for 60 sec in the absence of the platform to acclimatize them to the water environment. On day 2, mice underwent 4 training sessions with an intertrial interval of 5 min, conducted over four consecutive days. Each training session commenced from a different starting position within the pool, while the platform remained in the same quadrant during all training trials. Mice were required to find the platform within 200 sec and stay on the platform for 20 sec. If a mouse failed to find the location of the platform within 180 sec, the researcher gently guided the mouse to the platform by hand and allowed it to stay on the platform for 20 sec. The time taken by each mouse to locate the platform was measured as escape latency. A probe session was conducted 24 h after the final training session, in which the platform was removed, and the mice were required to search for the target quadrant (where the platform had been located). Each mouse was given 90 sec to find the target quadrant, and the time spent in that quadrant was recorded as a measure of spatial memory retention. Data acquisition and analysis were performed using a smart video tracking system (Smart version 2.5.19; Panlab, Barcelona, Spain).

Passive avoidance test

Mice were tested to detect memory retention deficit using a passive avoidance apparatus (Iwoo Scientific Co.). The apparatus consisted of a two-compartment; dark/light shuttle box (20 × 20 × 15 cm), separated by a guillotine door. The dark chamber had a stainless-steel grid floor (5 × 5 cm) designed to deliver an electric shock. During the acquisition trial, each mouse was placed in the

light chamber. After a 60-sec habituation period, the guillotine door was opened, and the time taken for the mouse to enter the dark chamber was recorded as the initial latency. Mice with an initial latency greater than 60 sec were excluded from further experiments. When a mouse instinctively entered the dark chamber, the guillotine door was closed, and the mouse received an electric foot shock (75 V, 0.2 mA, 50 Hz) through the floor grid for 3 sec. Five seconds later, the mouse was removed from the dark chamber and returned to its home cage. After 4 h, the retention trial was conducted. The mouse was again placed in the light chamber, and the time it took to enter the dark chamber was recorded as the retention latency.

Rotarod test

The rotarod test was utilized to evaluate motor coordination and balance using a five-lane rotarod apparatus (Jeung Do Bio & Plant). Mice underwent two habituation sessions, each lasting 1 min, where they were placed on a horizontal rotarod set to a fixed speed (diameter: 8 cm; rotation speed, 20 rpm). After a 24-h interval, the mice were reintroduced to the rotarod for 1 min or until they fell off. The latency for each mouse to fall off the rotarod was recorded. Mice that passively rotated without walking were considered to have fallen.

Vertical pole test

The vertical pole test was employed to evaluate grip strength and sensorimotor performance in mice. Each mouse was placed on a vertical pole (diameter: 1 cm; height: 60 cm; Iwoo Scientific Co.), positioned at a 45° angle with its head facing upward. The latency for the mouse to fall off the pole was recorded as a measure of performance.

Novel object recognition test

The novel object recognition test was conducted to assess cognitive function in mice. Initially, mice were habituated to an open field (50 cm x 50 cm x 38 cm) for 10 min per day over 2 days.

During the acquisition phase, the mice were presented with two familiar objects and allowed to explore them for 10 min per session for a total of 4 days. One hour after the final acquisition session, the probe test was conducted, in which one familiar object was replaced with a novel object. Mice were allowed to explore both the novel and familiar objects for 10 min. The discrimination index was calculated using the formula: (Novel object exploration time – Familiar object exploration time) / Total exploration time. The exploration times were measured in sec. This index served as a measure of the mouse's preference for exploring the novel object compared to the familiar one.

Touchscreen behavioral assessment

The touchscreen behavioral assessment was conducted using the standard Bussey–Saksida mouse touchscreen chambers (Campden Instruments Ltd., Loughborough, UK), following the established protocol [19]. Mice first underwent habituation and shaping, then were trained on the 5-choice serial reaction time (5-CSRT) task as described elsewhere [14, 20]. Briefly, mice were considered trained when they met specific criteria: completing 60 trials within 60 min, achieving an accuracy of at least 80%, and maintaining omission rates of no more than 20% for 2 consecutive days, all under a stimulus duration (SD) of 2 s. Upon meeting these criteria, baseline sessions were set at a 2-s SD for 2 consecutive days. The YE-06 cohort subsequently underwent a 2-day probe test using a shorter SD (0.5 s), whereas the Compound C cohort underwent a 4-day within-session probe test in which four different SDs (2.0, 1.5, 1.0, and 0.5 s) were presented in a psudo-random order within each session to increase attentional demands. Subsequently, a probe test was conducted over the following 2 days using the same or shorter SDs. Accuracy and omission rates were measured to assess attention. Premature responses (indicative of impulsivity) and perseverative responses to both correct and incorrect stimuli (indicative of compulsivity) were measured to assess inhibitory control ability. For locomotor functions, beam break rates and response/reward latencies were analyzed.

Slice preparation

Mice were deeply anesthetized with avertin (250 mg/kg, intraperitoneal injection) and transcardially perfused with ice-cold, sucrose-based ACSF containing 87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1 mM Na-pyruvate, 1 mM Na-ascorbate, 25 mM D-glucose, 75 mM sucrose, 7 mM $MgCl_2$, and 0.5 mM $CaCl_2$ (pH 7.4; cutting ACSF). Following perfusion, the brain was quickly removed and submerged in the cutting ACSF. Using a vibratome (VT7000smz-2, Campden Instruments, Ltd., Loughborough, UK), coronal hippocampal slices (300 μm thick) were prepared from the brain tissue in the carbogenated (5% CO_2/95% O_2) cutting ACSF. These slices were then transferred to the carbogenated recording ACSF (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1 mM Na-pyruvate, 1 mM Na-ascorbate, 20 mM D-glucose, 1 mM $MgCl_2$, and 2 mM $CaCl_2$ at pH 7.4) and allowed to recover for 30 min at 33°C, followed by 1 h at room temperature.

Electrophysiology recording

Hippocampal slices were transferred to an individual incubation chamber and treated with either 0.1% DMSO or 10 μM YE-06 for 1 h. Subsequently, the slices were transferred to a recording chamber and perfused with carbogenated recording ACSF at a flow rate of 2 mL/min and maintained at 31°C. Hippocampal CA1 pyramidal cells were visualized using infrared optics with an upright fixed microscope equipped with 4X and 40X water-immersion lenses (FN1, Nikon), along with a Charge-Coupled Device (CCD) monochrome video camera (IR-1000, DAGE-MTI). Glass pipettes for patching the cells were made by pulling thick-walled borosilicate glass (2.0 mm diameter; Science Products, Hofheim, Germany) with a vertical glass puller (Tritech Research, Inc., CA, USA) at a resistance of 3–5 MΩ. The pipettes were filled with an internal solution containing 127 mM K-gluconate, 5 mM KCl, 10 mM HEPES (pH 7.3), 0.3 mM EGTA-K, 4 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM phospho-creatine, and 0.1% biocytin (pH 7.3). Whole-cell patch-clamp recordings were performed using a Multipatch 700B preamplifier, digitized with a Digidata 1440A, and acquired using pCLAMP 10 (Molecular Devices). Action potentials were elicited by current injection with 200 pA for 200 ms. Only recordings with series resistance (Ra)

under 30 MΩ were considered valid. Following recording, the brain slice was fixed with 4% paraformaldehyde for 48 h and stained with streptavidin conjugated to Alexa Fluor 488 (Invitrogen, MA, USA) for 24 h to trace the patched cells.

Measurement of acetylcholine (Ach) content and acetylcholinesterase (AChE) activity

Ach content was determined using a method based on the reaction of *o*-acyl derivatives with alkaline hydroxylamine [21]. Initially, 50 µL of 1% hydroxylamine was added to 50 µL of brain homogenate, and the pH of the solution was adjusted to 1.2 ± 0.2 using HCl solution. Then, 500 µL of $FeCl_3$ (10% in 0.1 N HCl) was added, and the absorbance was measured at 540 nm. AChE activity was determined using the colorimetric method established by Ellman [22]. In a 96-well plate, 300 µL of 0.1 M Tris buffer (pH 8.0), 20 µL of 0.01 M 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB; Sigma-Aldrich), and 10 µL of brain homogenate were sequentially added. The enzymatic reaction was initiated by adding 10 µL of 0.1 M acetylthiocholine chloride (Sigma-Aldrich) as the substrate. Upon hydrolysis of acetylthiocholine by AchE, the resulting formation of the yellow 2-nitro-5-sulfidobenzene-carboxylate anion from DTNB with thiocholine was monitored by measuring absorbance at 412 nm for 5 min.

TUNEL assay

Mice were perfused and fixed with PBS containing 4% formaldehyde. The brains were then dehydrated, embedded in paraffin, sectioned in 5 µm thick slices, and mounted onto gelatin-subbed slide glass. Apoptotic cells in the brain sections were analyzed using a TUNEL assay kit (in situ cell death detection kit, fluorescein; Roche) according to the manufacturer's protocol. Cell nuclei exhibiting dark green-fluorescent staining were identified as apoptotic cells. The green fluorescence-positive cells were visualized using a fluorescence microscope (Olympus, Tokyo, Japan). Cell nuclei were counterstained with 6-diamidino-2-pheylindole (DAPI; 1:1000; Sigma-Aldrich).

Mouse primary neuron culture

E17 embryos were harvested from the uterus of deeply anesthetized pregnant female C57BL/6 mice (Koatech, Pyeongtaek, Korea). After transferring the embryos to ice-cold dissection medium (HBSS without calcium and magnesium (Gibco, New York, USA), 10 mM HEPES, and 100 U/mL penicillin-streptomycin (PS, Sigma-Aldrich)), cortices and hippocampi were dissected. The minced tissues were dissociated using a chopping solution (140 U/mL DNase, 0.05% Trypsin-EDTA in dissection medium) at 37°C for 15 min, then transferred to dissection medium. The cells were then centrifuged at 1,000 rpm for 5 min at room temperature, and the resulting pellet was resuspended in culture medium (Neurobasal medium (Gibco), B-27 supplement (1:50, Gibco), 2 mM Glutamax (Gibco), 100 U/ml PS). Cells were plated in 12-well plates pre-coated with poly-D-lysine (Sigma-Aldrich). After 2 h, the medium was replaced with fresh culture medium containing PS. During incubation, half of the medium was exchanged every 3-4 days with fresh culture medium without PS. On DAY 18, mouse primary neurons grown on coverslips were washed twice with ice-cold PBS and fixed with 4% paraformaldehyde for 20 min for immunocytochemistry. After washing five times with PBS, cells were permeabilized with PBS containing 0.15% Triton X-100 for 30 min and blocked with PBS containing 5% BSA for 1 h. The cells were then incubated overnight at 4°C with primary antibodies against synaptophysin, PSD-95, NeuN, and MAP2 (1:500) diluted in PBS containing 2% BSA. After washing three times with PBS, the cells were incubated with secondary antibodies (1:500) diluted in PBS containing 2% BSA for 60 min at room temperature. All cells were washed three times with PBS, counterstained with DAPI (1:2000) in PBS, and mounted with Vectashield mounting medium. Immunoreactivity was observed under a fluorescence microscope. For western blot analysis, primary neurons were washed with ice-cold PBS and directly lysed in Laemmli sample buffer. Samples were denatured at 95°C for 5 min and subjected to SDS-PAGE and western blot analysis for p-AMPK, AMPK, p-ACC, and ACC.

In vivo hippocampal western blot

For hippocampal molecular analyses, tissues were collected from mice of 3xTg-AD behavioral

cohorts associated with the novel object recognition (NOR) and touchscreen behavioral paradigms. Following the NOR test, oral YE-06 administration was continued, and hippocampal tissues were subsequently collected from female mice at 11 or 14 months of age for molecular analyses. To minimize the potential influence of disease stage, only the 11-month-old cohort was included in the present western blot analyses (n = 3 per group). For hippocampal molecular analysis from touchscreen cohort (n = 5 per group), tissues were collected 2 h after the final intraperitoneal administration of vehicle or YE-06, following the repeated treatment schedule used during the touchscreen paradigm. Hippocampal tissues were homogenized in ice-cold lysis buffer containing 20 mM HEPES (pH 7.0), 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1.5 mM $MgCl_2$, 250 mM sucrose, phosphatase inhibitors (1 mM NaF, 1 mM Na_3VO_4, 1.15 mM Na_2MoO_4, 2 mM sodium pyrophosphate, and 4 mM sodium tartrate), and a protease inhibitor cocktail (Complete, EDTA-free; Roche, Indianapolis, IN, USA). Tissues were homogenized at a ratio of 10 μL of lysis buffer per mg of wet tissue, incubated on ice for 15 min with intermittent vortexing, and centrifuged at 8,000 ×g for 30 min at 4°C. The supernatants were collected, and protein concentrations were measured using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples (20 μg per lane) were denatured at 95°C for 5 min before electrophoresis.

Proteins were separated on 8%, 10%, or 15% SDS-polyacrylamide gels and transferred onto PVDF membranes (Merck Millipore, Burlington, MA, USA). Membranes were blocked with 5% skim milk in TBST for 1 h at room temperature and incubated overnight at 4°C with primary antibodies (1:1,000–1:5,000). After washing with TBST, membranes were incubated for 1 h at room temperature with either HRP-conjugated secondary antibodies (1:5,000–1:10,000) for chemiluminescent detection or IRDye® 680RD/800CW-conjugated secondary antibodies for fluorescent detection. Chemiluminescent signals were developed using Amersham™ ECL™ Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ, USA) and acquired with an ImageQuant LAS 4000 mini (GE Healthcare), whereas fluorescent signals were acquired using an Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Band intensities were quantified using Multi Gauge software (Fujifilm, Tokyo, Japan) for chemiluminescent images and Image Studio software (LI-COR Biosciences) for fluorescent images. Protein expression levels were normalized to GAPDH. Details of all primary and secondary antibodies, including host

species, catalog numbers, and working dilutions, are provided in Supplementary Tables S3.

Supplementary Tables

Supplementary Table S1. The antibodies used in this study

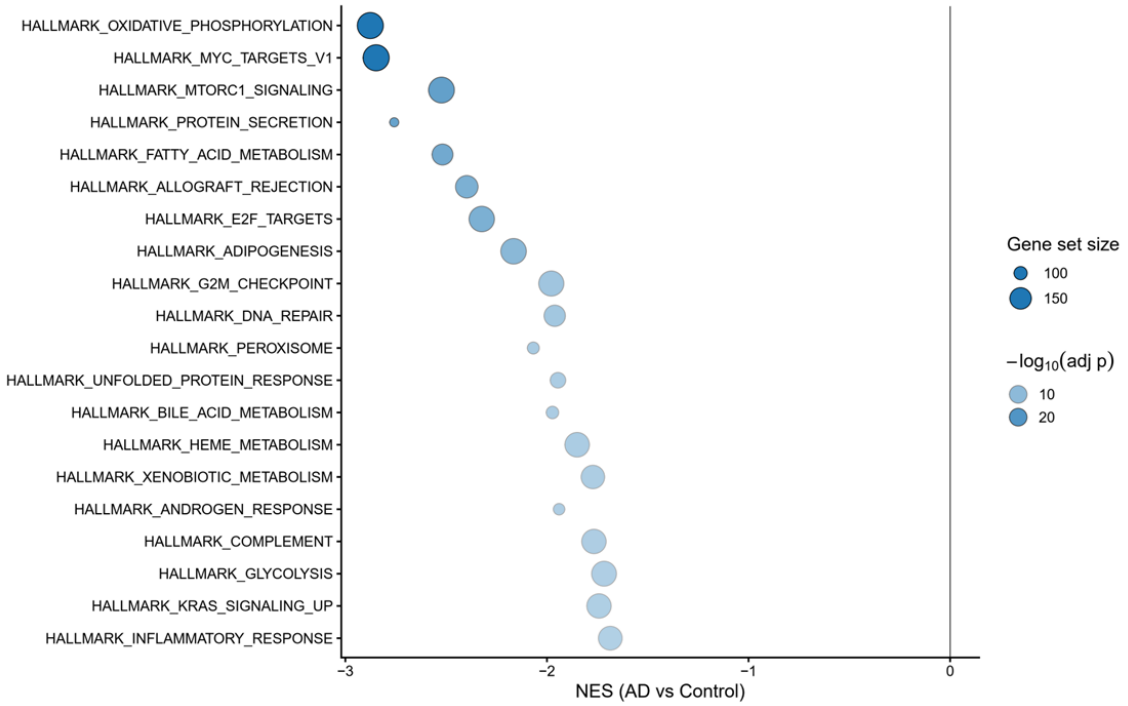
Antibody	Vendor	Catalogue number
p-AMPKα	Cell Signaling Technology	2535S
AMPKα	Thermo	#AHO1332
FLAG	MBL International Corporation	M185-3L
vinculin	MBL International Corporation	PM-088
p-ACC	Cell Signaling Technology	11818S
ACC	Cell Signaling Technology	3662S
p-raptor	Cell Signaling Technology	2083S
raptor	Cell Signaling Technology	2280S
α-tubulin	MBL International Corporation	M175-3
LKB1	Cell Signaling Technology	3050S
Aβ (application: dot blot)	Cell Signaling Technology	2454S
APPβ (application: dot blot)	Immuno-Biological Laboratories, Inc.	10321
BACE	Cell Signaling Technology	5606S
GAPDH	MBL International Corporation	M171-3
GST	MBL International Corporation	M209-3
Lamin A/C	Cell Signaling Technology	2032S
acetylated NF-κB	Cell Signaling Technology	3045S
NF-κB	Santa Cruz Biotechnology, Inc.	sc-8008
Nrf2	Cell Signaling Technology	12721S
Cleaved PARP	Cell Signaling Technology	9541S
Cleaved caspase 3	Cell Signaling Technology	9661S

Supplementary Table S2. Gene-specific primers used for RT-qPCR and RT-PCR

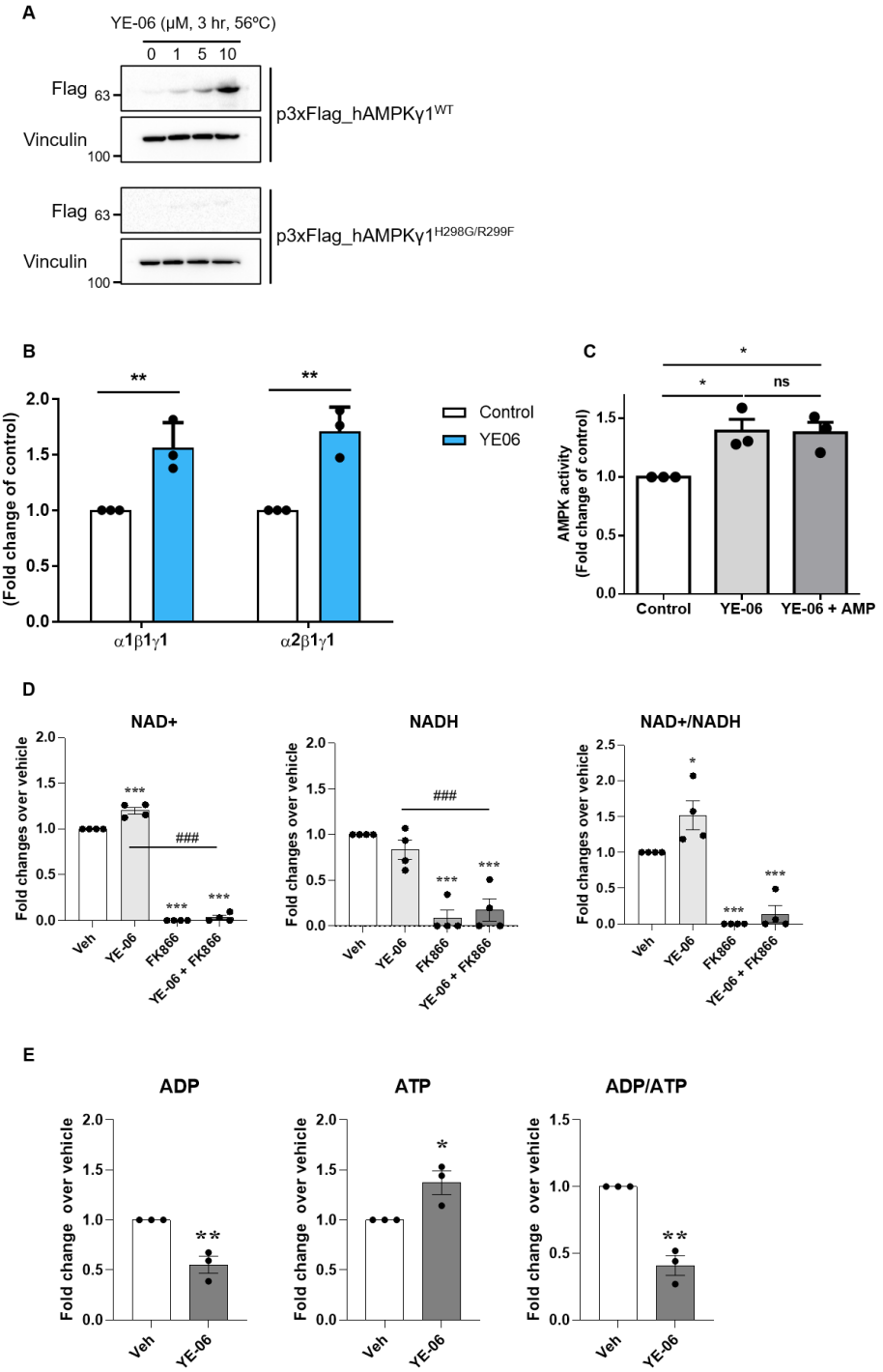
Usage	Name	Forward primer (5'→3')	Reverse primer (5'→3')
RT-qPCR	BACE	GATGGTGGSCAACCTGAG	CTGGTAGTAGCGATGCAG
	TNFα	GCCTCTTCTCATTCCTGCTT	TGGGAACTTCTCATCCCTTTG
	IL-1β	TGGCAACTGTTCCTG	GGAAGCAGCCCTTCATCTTT
	HO-1	CAAGCCGAGAATGCTGAGTTCATG	GCAAGGGATGATTTCCTGCCAG
	NQO1	TTCTCTGGCCGATTCAGAG	GGCTGCTTGGAGCAAAATAG
	GAPDH	AATGGTGAAGGTCGGTGTG	GTGGAGTCATACTGGAACATGTAG
Reverse transcript -ion PCR	BACE	CCACAGACGCTCAACATCCTG	CAATGATCATGCTCCCTCCCA
	β-actin	CCGTGAAAAGATGACCCAGAT	CCATACCCAAGAAGGAAGGCT

Supplementary Table S3. The antibodies used for in vivo western blot

Antibody	Vendor	Catalogue number
Phospho-AMPKα (Thr172)	Cell Signaling Technology	2535
AMPKα	Cell Signaling Technology	5831
Phospho-ACC (Ser79)	Cell Signaling Technology	11818
ACC	Cell Signaling Technology	3662
PSD-95	Synaptic Systems	124 011
Synaptophysin	Abcam	Ab32127
NeuN	Invitrogen	PA5-78499
MAP-2	Synaptic Systems	188-004
Phospho-Tau (Ser199/202)	Invitrogen	44-768G
Phospho-Tau (Thr231)	Invitrogen	44-746G
Phospho-Tau (Ser262)	Santa Cruz Biotechnology	sc-32828
Phospho-Tau (Ser356)	Santa Cruz Biotechnology	sc-101814
Phospho-Tau (Ser396)	Cell Signaling Technology	9632
Total Tau	Abcam	ab80579
GAPDH	Santa Cruz Biotechnology	sc-25778
Goat anti-rabbit IgG-HRP	Cell Signaling Technology	7074
Horse anti-mouse IgG-HRP	Cell Signaling Technology	7076
Goat anti-rabbit IRDye 800CW	Licorbio	926-32211
Goat anti-mouse IRDye 680RD	Licorbio	926-68070

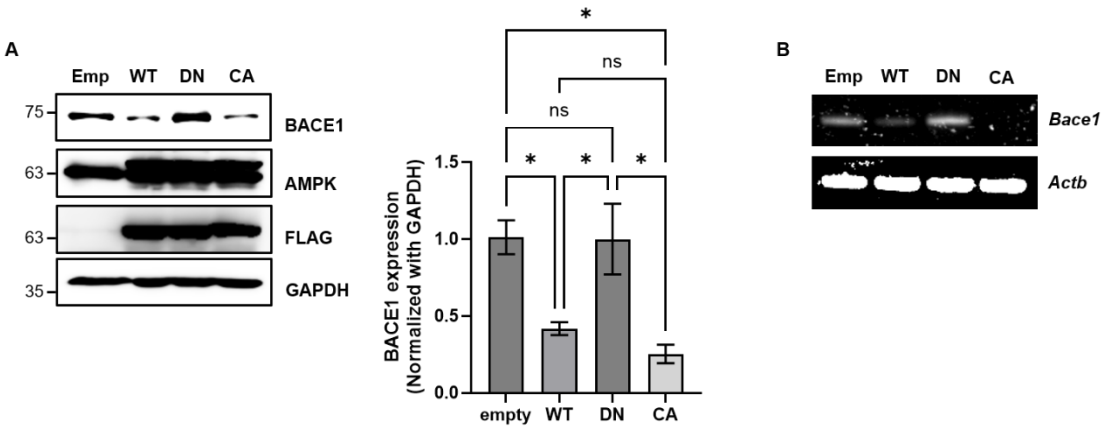


Supplementary Figure S1. GSEA hallmark pathway analysis. Gene set enrichment analysis (GSEA) was performed using the Hallmark gene sets. Pathways are ordered by $-\log_{10}$(adjusted p-value), with statistical significance increasing accordingly. The x-axis represents the normalized enrichment score (NES). Bubble size indicates the gene set size for each pathway.

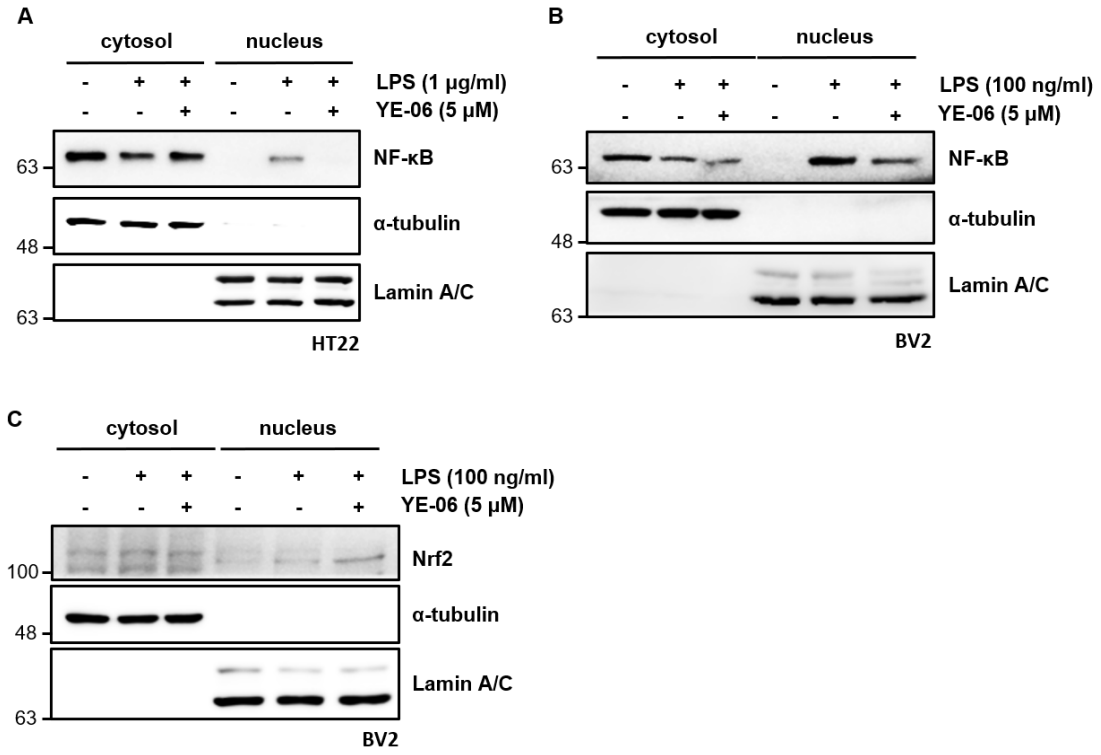


Supplementary Figure S2. The ability of YE-06 to activate AMPK in an AMPKα isotype-non-specific and AMP-competitive manner via AMPK-SIRT1-Nampt pathway. (A) CETSA at constant temperature but varied concentration of YE-06. **(B, C)** To evaluate whether YE-06 activates AMPK in an isotype-specific and AMP-competitive manner, assays were conducted. An AMPK kinase assay was performed *in vitro* using AMPK substrate-derived SAMS peptide and ATP (n=3). **(B)** Each of the AMPK isoforms, composed of α1β1γ1 and

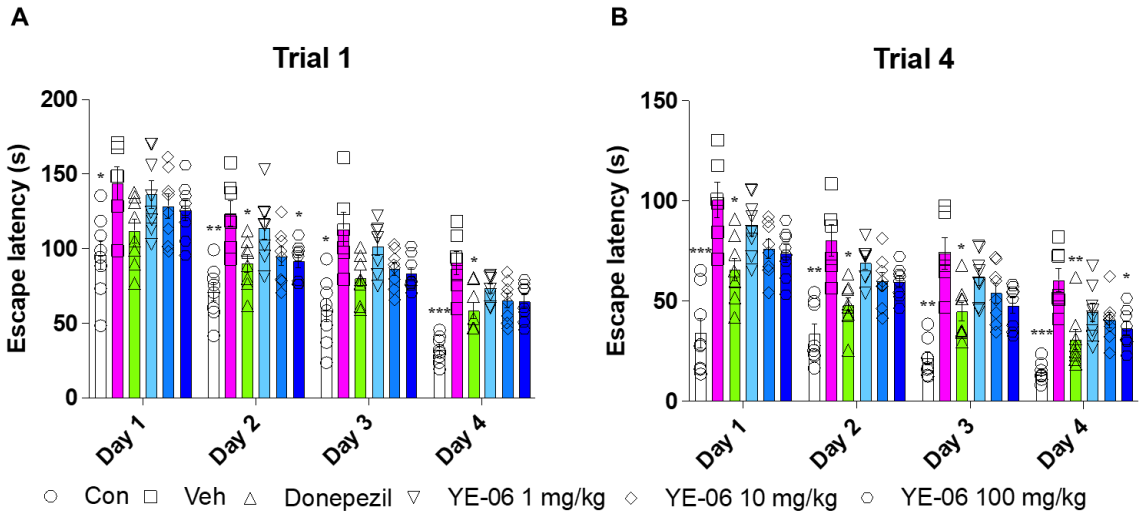
α2β1γ1, was treated with YE-06. Results are expressed as mean ± SEM. Welch's t-test, $**p < 0.01$ *vs.* control. **(C)** AMPK activity was compared by adding YE-06 (10 μM) alone and YE-06 in combination with AMP (10 μM). Data shown as mean ± SEM. One-way ANOVA, $*p < 0.05$ *vs.* control. **(D)** After exposing N2a cells to YE-06 (10 μM, 24 h) and FK866 (20 nM, 24 h), individually and in combination, intracellular nucleotide levels (NAD^+, NADH, and the NAD^+/NADH ratio) were measured using a NAD^+/NADH quantification colorimetric kit (n = 4). Results are expressed as mean ± SEM. One-way ANOVA followed by Dunnett's multiple comparisons test. Relative to Veh; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Relative to YE-06; $###p < 0.001$. Veh, vehicle. **(E)** After treatment of N2a cells with YE-06 (10 μM, 24 h), intracellular ATP, ADP, and the ADP/ATP ratio were measured using an ADP/ATP Raio Assay Kit (n = 4). Results are expressed as mean ± SEM. Unpaired Student's t-test, $p < 0.05$, $**p < 0.01$ vs. Veh. Veh, vehicle.



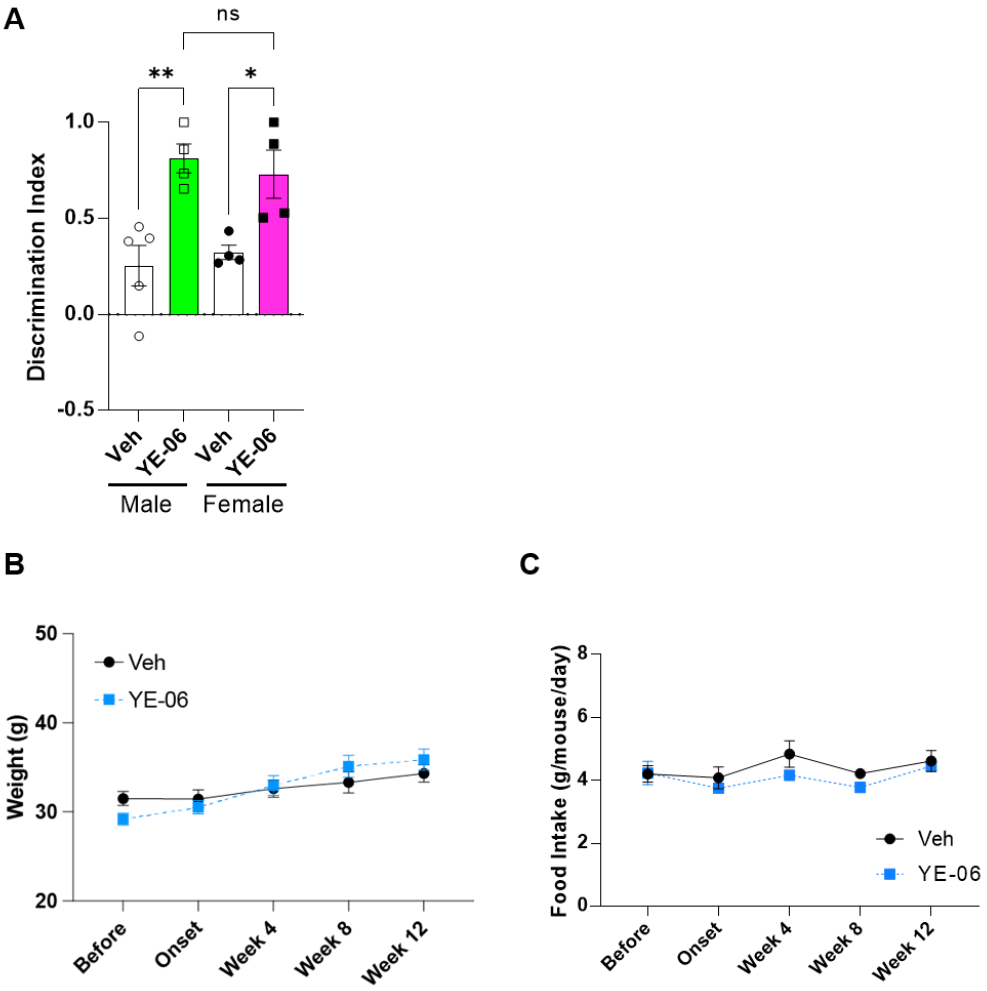
Supplementary Figure S3. Downregulation of BACE1 expression by AMPK in an enzymatic activity–dependent manner. (A, B) Western blot (A) and RT–qPCR (B) analyses of BACE1 expression in N2a cells expressing control, wild-type (WT), dominant-negative (DN), or constitutively active (CA) AMPKα1.



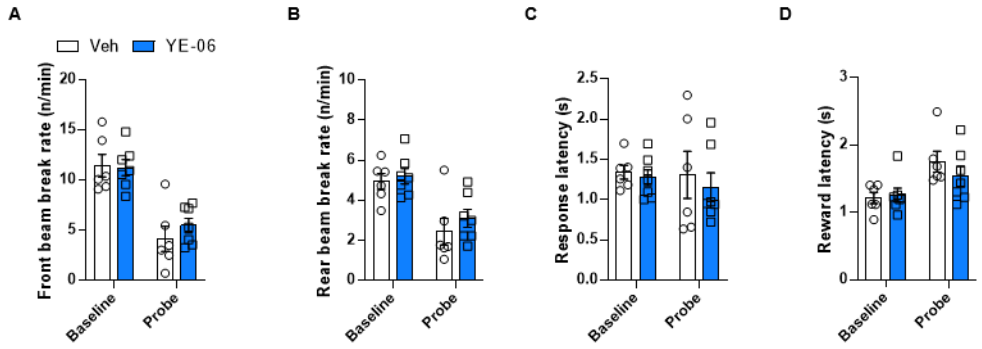
Supplementary Figure S4. Mode of action of YE-06 with multiple effects on the pathogenesis of AD. HT22 and BV2 cells were exposed to LPS for 1 h **(A, B)** or for 12 h **(C)** in the absence and presence of YE-06. **(A)** HT22 Cells were pre-treated with YE-06 for 12 h before treatment with LPS. Western blot analysis was performed to evaluate the subcellular localization of NF-κB in HT22 cells. **(B, C)** BV2 cells were treated with YE-06 for 12 h before treatment with LPS. Western blot analysis was performed to evaluate the subcellular localization of NF-κB **(B)** or Nrf2 **(C)** in BV2 cells.



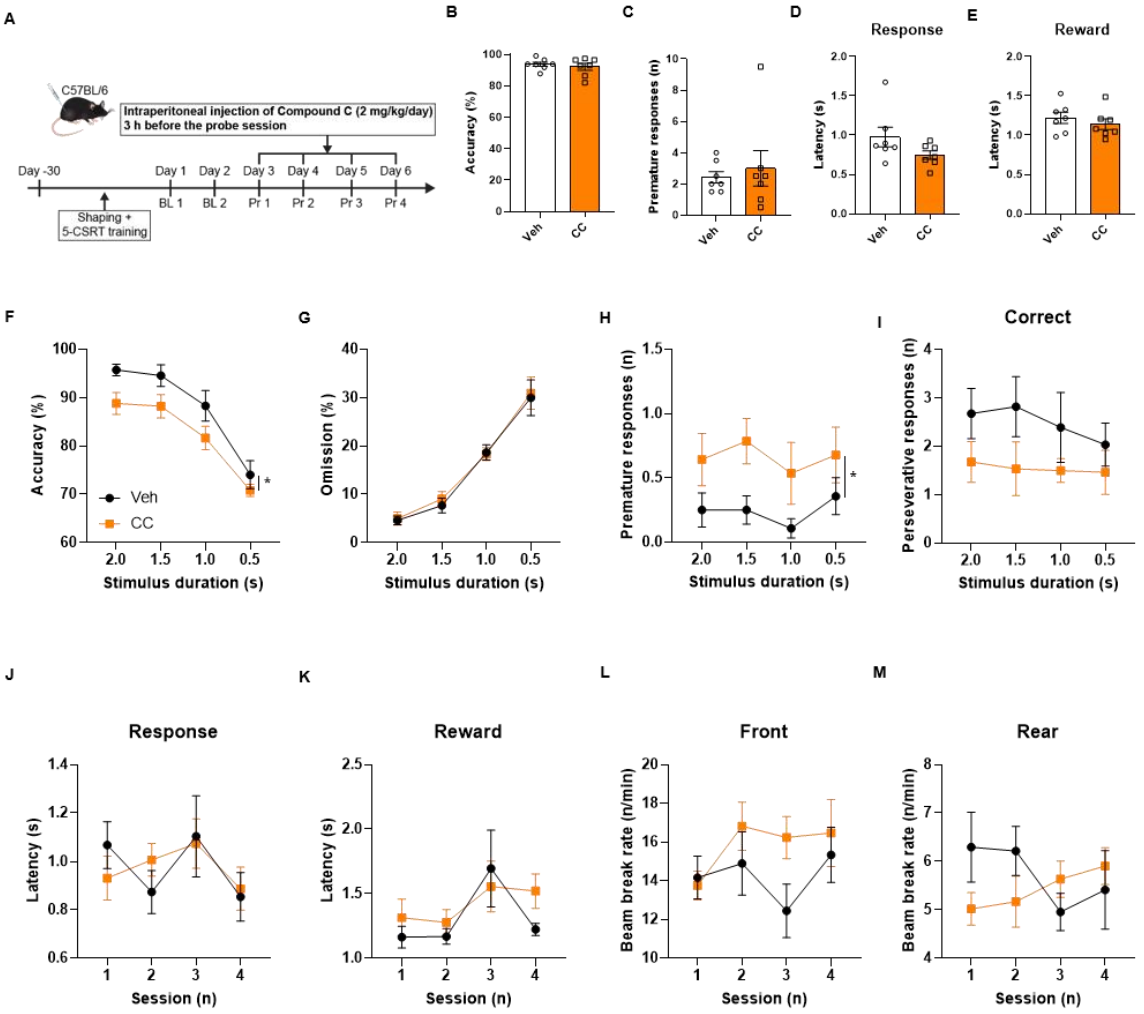
Supplementary Figure S5. **Effects of YE-06 on short- and long-term memory in Aβ-injected mice.** Escape latency was measured during trial 1 **(A)** and trial 4 **(B)** of the acquisition sessions of Morris water maze test in $A\beta_{1\text{-}42}$-injected mice (n = 8 per group). Data are presented as mean ± SEM. One-way ANOVA with Dunnett's multiple comparison post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 *vs.* Veh. Con, $A\beta_{1\text{-}42}$ non-injected and drug-nontreated mice; Veh, $\beta_{1\text{-}42}$-injected and drug-nontreated mice.



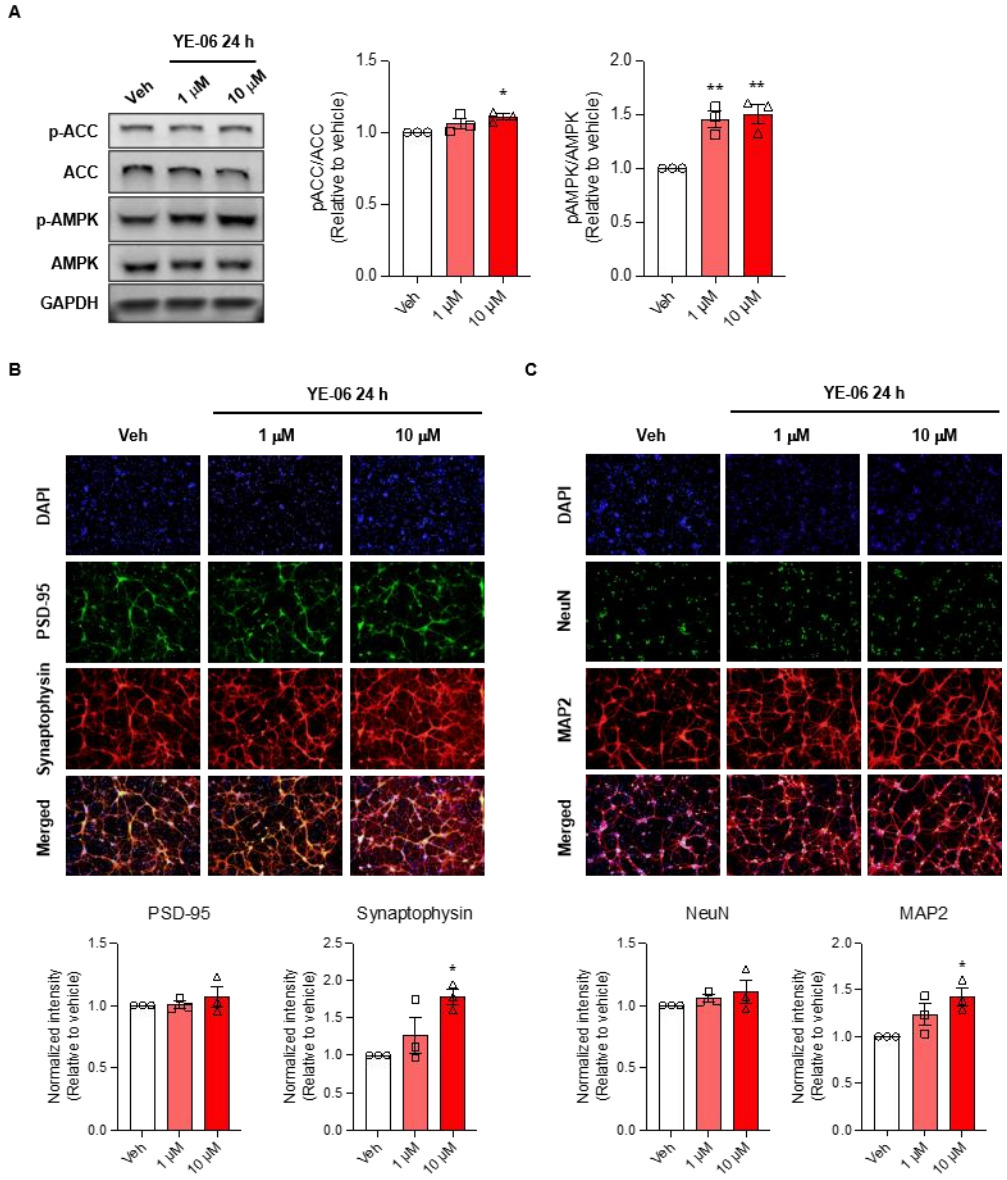
Supplementary Figure S6. Sex-specific effects of YE-06 on novel object recognition, body weight, and food intake in 3xTg-AD mice. (A) The discrimination index in the novel object recognition test was analyzed separately in male (Veh, n = 5; YE-06, n = 4) and female (Veh, n = 4; YE-06, n = 4) 3xTg-AD mice after 12 weeks of treatment with vehicle (Veh) or YE-06 (20 mg/kg). Data are presented as mean ± SEM. Unpaired Student's t-test, *p < 0.05 vs. Veh. **(B)** Body weights of 3xTg-AD mice were measured before treatment onset and throughout the 12-week treatment period with vehicle (Veh, n = 9) or YE-06 (20 mg/kg, n = 8). **(C)** Food intake was measured before treatment onset and throughout the 12-week treatment period and is presented as grams per mouse per day (g/mouse/day). Data are presented as mean ± SEM. Veh, vehicle.



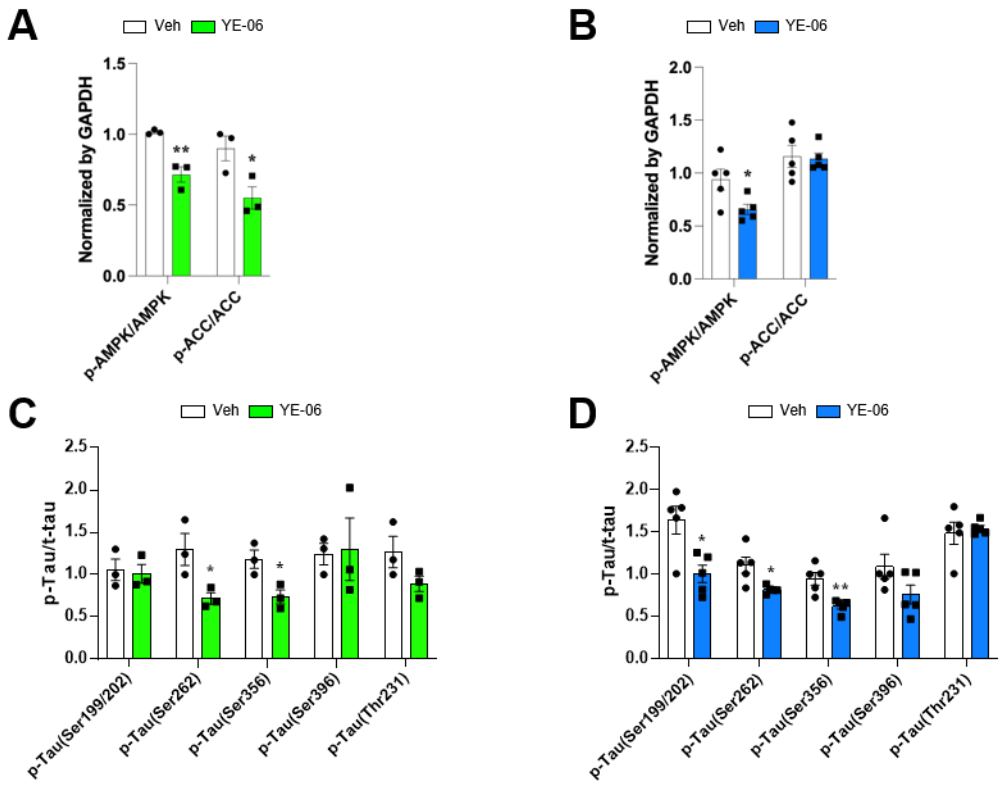
Supplementary Figure S7. Effects of YE-06 on motor function of 10-month-old 3xTg-AD mice in 5-CSRT task. (A) Front beam break rate, **(B)** rear beam break rate, **(C)** response latency, and **(D)** reward collection latency were analyzed during the baseline (2.0 s SD) and probe sessions (0.5 s SD) of the 5-CSRT task in 10-month-old 3xTg-AD mice with vehicle (n = 6) or YE-06 (20 mg/kg, n = 7) administered 2 h before the probe sessions. Data are presented as mean ± SEM. Unpaired Student's t-test. Veh, Vehicle.



Supplementary Figure S8. Effects of Compound C on cognitive and motor function in the 5-CSRT task. (A) Experimental timeline for the 5-CSRT task. C57BL/6 mice received 2 mg/kg Compound C 3 h before each probe session (n = 7 per group) at 15 month of age. **(B)** Accuracy, **(C)** premature responses, **(D)** response latency, and **(E)** reward collection latency were analyzed during the baseline sessions. **(F)** Accuracy, **(G)** omission, **(H)** premature responses, and **(I)** perseverative responses to correct stimuli were analyzed during the probe sessions, in which four stimulus durations (2.0, 1.5, 1.0, and 0.5 s) were presented in a randomized order **(J)** Response latency, **(K)** reward collection latency, **(L)** front beam break rate, and **(M)** rear beam break rate were analyzed across the four probe sessions. Data are presented as mean ± SEM. **(B-E)** Unpaired Student's t-test, **(F-M)** mixed-effects model followed by Tukey's multiple comparisons test. *$p < 0.05$ vs. Veh. Veh, vehicle; CC, Compound C.



Supplementary Figure S9. Effects of YE-06 on synaptic and neuronal markers. (A) Representative western blot images and quantification of pACC/ACC and pAMPK/AMPK ratios in mouse primary neuron culture following YE-06 treatment (3 independent experiments). **(B)** Representative immunocytochemistry images and quantification of PSD-95 (green) and synaptophysin (red) fluorescence intensity in mouse primary neuron culture (4 independent experiments). **(C)** Representative immunocytochemistry images and quantification of NeuN (green) and MAP2 (red) fluorescence intensity in mouse primary neuron culture (4 independent experiments). Scale bar, 200 μm. One-way ANOVA followed by Dunnett's multiple comparisons test. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ versus Veh. Veh, vehicle



Supplementary Figure S10. Effects of YE-06 treatment on hippocampal AD-related molecular markers in two independent 3xTg-AD behavioral cohorts. Hippocampal protein expression was analyzed in two independently conducted 3xTg-AD mouse cohorts associated with the novel object recognition (NOR) and touchscreen behavioral paradigms. (A, C) NOR-associated cohort: hippocampal tissues were obtained from 11-month-old female 3xTg-AD mice following prolonged oral administration of vehicle (Veh) or YE-06 (20 mg/kg; n = 3 per group). (B, D) Touchscreen-associated cohort: hippocampal tissues were obtained from 10-month-old male 3xTg-AD mice receiving repeated intraperitoneal administration of vehicle or YE-06 (10 mg/kg; n = 5 per group) during the touchscreen testing period, with tissue collected 2 h after the final administration. (A, B) Quantitative analyses of AMPK signaling, including p-AMPK/AMPK and p-ACC/ACC. (C, D) Quantitative analyses of tau phosphorylation at Ser199/202, Ser262, Ser356, Ser396, and Thr231 relative to total tau. GAPDH was used as a loading control where indicated. The NOR- and touchscreen-associated cohorts represent independent experiments with different treatment schedules and were analyzed separately. Data are presented as mean ± SEM. Unpaired Student's t-test. *$p < 0.05$, **$p < 0.01$ vs. Veh. Veh, vehicle.