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Supplementary Materials for

Disrupted hippocampal growth hormone secretagogue receptor 1α interaction with dopamine receptor D1 plays a role in Alzheimer's disease

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Other Supplementary Material for this manuscript includes the following:

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Method and Materials

Human samples

Frozen postmortem hippocampal tissues and paraffin-embedded hippocampal slices were requested from the University of Texas (UT) Southwestern Medical Center ADC Neuropathology Core supported by ADC grant (AG12300) under a protocol approved by The UT Southwestern Medical Center as well as the University of Kansas (KU) Alzheimer's disease center Neuropathology Core under a protocol approved by KU Medical Center supported by NIH GRANT (P30 AG035982). Informed consent was collected from all subjects and the study adhered to the Declaration of Helsinki principles.

Mice

Animal studies were approved and performed under the guidelines of the University of Texas at Dallas (UTD) Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH). 5×FAD mice (B6SJL-Tg (APPSwFlLon, PSEN1*M146L*L286V) 6799Vas/Mmjax) (*28*) were originally obtained from Jackson Laboratory. *Ghsr* null mice on a pure C57BL/6N genetic background (*20*) from a colony maintained at UT Southwestern Medical Center were crossed with 5×FAD mice to generate litters including non-transgenic (nonTg), *Ghsr* null, 5×FAD, and *Ghsr* null/5×FAD mice. Genotypes of animals were confirmed using PCR and/or amyloid plaques staining. The number of mice was determined by our previous data and power calculation to ensure that the minimal number of mice as required were used in the experiments.

Antibody validation

For accurate and reproducible results, we validated all antibodies used in the current study as previously described (*65*). Most antibodies passed the validation; however, all commercially

available antibodies against GHSR1α or DRD1 performed poorly in immunoblotting. Further antibody validation using non-Ghsr1α expressing mouse tissues and cells as well as non-Drd1 expressing cells as critical negative controls and GHSR1α- and DRD1-expressing tissues and cells as critical positive controls showed that anti-GHSR1α from Santa Cruz Biotechnology (#sc-10359) and anti-DRD1 from Abcam (#ab81296) exhibited specific and reproducible results in immunostaining and membrane blotting. The results suggest that the above mentioned anti-GHSR1α and anti-DRD1 antibodies only recognize antigens preserved in their natural structures in brain slices and isolated membranes. Therefore, in the current study we conducted immunostaining and/or membrane blotting to reflect the expression of GHSR1α and DRD1, as well as membrane-incorporated GHSR1α and DRD1, respectively.

Oligomeric Aβ preparation

Aβ42 peptide (GenicBio, A-43-T-1000) was diluted in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich) to 1 mM. After centrifugation, the clear solution was then aliquoted in microcentrifuge tubes, and it was dried overnight in the fume hood. Peptide film was diluted in DMSO to 5 mM and sonicated for 10 minutes in bath sonicator. The peptide solution was resuspended in cold HAM'S F-12 (Sigma-Aldrich) to 100 μM and immediately vortexed for 30 seconds. The solution was then incubated at 4 °C for 24 hours to prepare oligomeric Aβ42.

Duolink in Situ Proximity Ligation Assay (PLA)

Protein interactions between GHSR1α/DRD1 and Aβ/GHSR1α, respectively, in human/mouse brain slices, cultured hippocampal neurons, and transfected HEK 293T cells were detected using Duolink In Situ PLA detection kits (Sigma-Aldrich, #DUO92008, #DUO92012), following the manufacturer's instruction. The following primary antibodies were used: goat polyclonal anti-GHSR1α (Santa Cruz Biotechnology, #sc-10359, 1:100), rabbit polyclonal anti-DRD1 (Abcam,

#ab81296, 1:200), rabbit polyclonal anti-β-amyloid (CST, #8243, 1:1,000), mouse monoclonal anti-β-amyloid (CST, #15126, 1:1,000). Mouse anti-FLAG-tag (Thermo Fisher Scientific, #MA1-91878, 1:400) was used to recognize Ghsr1α-3×FLAG and its mutants in transiently transfected HEK 293T cells. The following Duolink in Situ PLA Probes were used: anti-Rabbit PLUS (Sigma-Aldrich, #DUO92002), anti-Goat MINUS (Sigma-Aldrich, #DUO92006), and anti-Mouse MINUS (Sigma-Aldrich, #DUO92004). Images were collected on a Nikon confocal microscope or Olympus upright microscope. PLA analysis was referred to previous report (*66, 67*). NIH Image J was used for PLA signal quantification. The threshold was adjusted to attain the best visualization of the PLA-positive signal. The numbers and intensity of PLA-positive dots were counted and analyzed using NIH Image J "analyze particles" plug-in. The number of PLA-positive dots were counted and divided by the area to represent specific protein interaction. For mouse brain slice, HEK 293T cells and cultured hippocampal neurons (Aβ/GHSR1α only for cultured hippocampal neurons), due to the dense signal, the meanintensity (defined as arbitrary unit) of positive fluorescence signals was quantified instead of number of dots.

Immunocytochemistry

Mouse brains were dissected and immediately fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 24-26 hours at 4 °C. The frozen tissue sections were prepared as previously described (*68*). Primary cultured neurons on Lab-Tek chamber slides were fixed in 4% PFA for 30 minutes at 37 °C. The slices or neurons were blocked with blocking buffer (5% goat or donkey serum, 0.3% Triton X-100 in PBS, pH 7.4) for 1 hour, then incubated with primary antibodies at room temperature overnight. Dilutions of antibodies were as follows: goat-anti-GHSR1α (Santa Cruz Biotechnology, #sc-10359, 1:100), rabbit-anti-DRD1 (Abcam, #ab81296, 1:200), rabbit-anti-β-amyloid (CST, #8243,1:1,000) for Aβ deposition detection, mouse-anti-β-

amyloid (CST, #15126,1:1,000), rabbit-anti-PSD 95 (CST, #3450, 1:400), guinea pig-anti-vGLUT1 (Synaptic system, #135304, 1:400), mouse-anti-MAP2 (Sigma-Aldrich, #M4403, 1:300), rabbit-anti-MAP2 (CST, #4542, 1:600), mouse-anti-NeuN (Millipore, #MAB377, 1:600), mouse-anti-FLAG-tag (Thermo Fisher Scientific, #MA1-91878, 1:400), rabbit-anti-HA-tag (CST, #3724S, 1:400), mouse-anti-HA-tag (CST, #2367, 1:400), mouse-anti-Doublecortin (Santa Cruz Biotechnology, #sc271390, 1:100). After washing with PBS, the slices or neurons were probed with appropriate cross-adsorbed secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 594 or Alexa Fluor 647 (Thermo Fisher Scientific, 1:500). Images were collected on a Nikon confocal microscope or inverted fluorescence microscope. NIS element software was used for image analysis. "Objective Count" dialog was used for intensity measurement. Positive staining was identified in the image using an intensity threshold set at the mean pixel intensity for the entire image plus the standard deviation of the pixel intensity. Total fluorescence intensity was taken as the sum of all intensity in these objects, which then was divided by the area of objects to calculate the average fluorescence intensity per area. For synapse density counting, vGLUT1 and PSD95 stained channels were saved as two binary layers which were overlapped by using "AND" operation in "binary operation" dialog of NIS element software. The overlapped dots represent synapses. For images from brain slice, average synapse number per volume was calculated. For images from primary neurons, the synapse number per length of dendritic segment was calculated. All confocal images were converted to 3 dimensional images by using NIS element software "3D reconstruction" module for analysis.

Plasmid construction

Mouse full length *Ghsr1α* cDNA (NCBI Gene ID: 208188) was purchased from OriGene (MR226073). *Drd1* cDNA (NCBI Gene ID: 13488) was isolated by PCR from mouse brain

cDNA. *Ghsr1α* cDNA and C terminal 3×FLAG tag, or *Drd1* cDNA and C terminal HA tag, were subcloned into pcDNA3 to generate pcDNA3-*Ghsr1α*-3×*FLAG* and pcDNA3-*Drd1-HA*. To screen the Aβ binding site on Ghsr1α, seven deleted forms of Ghsr1α were generated (fig. S3). The integrity of all constructs generated by PCR and subcloning was confirmed by nucleotide sequencing.

HEK 293T cell culture and transfection

HEK 293T cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) with 10% FBS (Sigma-Aldrich) and penicillin–streptomycin (Thermo Fisher Scientific) at 37 °C and 5% CO_2.Trypsinized cells were seeded on poly-D-lysine-coated culture dishes for Co-IP, Lab-Tek chamber slides (Nunc, #177445) for immunostaining or black culture plates (Corning, 3603 or 4580) for FlAsH-Fret assay. Calcium-phosphate method was used for plasmid transfection on HEK 293T cells.

Co-immunoprecipitation assay (Co-IP)

pcDNA3-*Ghsr1α*-3×*FLAG* or its mutants transfected HEK 293T cells were collected in IP buffer containing 50 mM Tris-HCl (Fisher Scientific), pH 7.4, 150 mM NaCl (Fisher Scientific), 1 mM EDTA (Fisher Scientific), 0.1% NP-40 (Fisher Scientific) and protease inhibitor cocktail (Calbiochem, set V, EDTA free). After three freeze-thaw cycles, the cell lysates were centrifuged at 12,000×*g* for 5 minutes at 4 °C. The supernatants were incubated with mouse anti-FLAG (Thermo Fisher Scientific, # MA1-91878, 0.5 μg IgG per 100 μg protein ratio) at 4 °C overnight, followed by an incubation with pre-washed protein A/G agarose beads (Pierce) for 2 hours at room temperature. Non-immune mouse IgG was used as negative control. After 6 times of washing with IP buffer, the protein complexes were eluted by boiling in 1×NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) and subjected to immunoblotting with antibody

to Aβ (CST, #8243, 1: 1,000). Anti-FLAG (Thermo Fisher Scientific, # MA1-91878, 1:2,000) was used for input detection of Ghsr1α-3×FLAG and its mutants.

Drd1 and Aβ interaction was tested from pcDNA3-*Drd*1-*HA* transfected HEK 293T cell lysates. Mouse anti-HA (CST, #2367, 1:400) was used for Drd1-HA immunoprecipitation. Rabbit anti-HA (CST, #3724S, 1:400) was used for Drd1-HA immunoblotting.

FlAsH-based FRET

The cDNA encoding the enhanced CFP (ECFP) was fused to C terminus of mouse *Ghsr1α* cDNA (Gene ID: 208188) or mouse *Drd1* cDNA (Gene ID: 13488). The CCPGCC motif which has higher binding affinity for FlAsH was inserted in the third intracellular loop between Val247 and Gly248 of *Ghsr1α* cDNA or between Thr245 and Gly246 of *Drd1* cDNA. The cDNAs were cloned into pcDNA3 to generate construct pcDNA3-$Ghsr1\alpha^{FIAsH/ECFP}$ and pcDNA3-$Drd1^{FIAsH/ECFP}$ using in-fusion system from Takara and verified by sequencing. HEK 293T cells were cultured on black 96 well plate with clear bottom (Corning) and transfected using calcium phosphate method. FlAsH labeling was performed as previously described(*18*). 50 μM MK0677 or 100 μM SKF81297 and / or 2 μM Aβ were used as the treatments. JMV2959 at a concentration of 50 μM was used to offset the effect of MK0677. Fluorescence signals were monitored on microplate reader (BioTek Cytation 5) before and after the treatments. The following excitation/emission wavelengths were used: 425±20 nm/475±20 nm for ECFP; 500±20 nm/535±20 nm for FlAsH; 425±20 nm/535±20 nm for FRET between ECFP and FlAsH. Area scanning read mode was used to correct artificial inaccuracies. The FRET ratio ($F_{\mathrm{FIAsH}}/F_{\mathrm{ECFP}}$) was calculated according to equation as previously reported (*69*):

$$\text{Ratio } (F_{\mathrm{FIAsH}}/F_{\mathrm{ECFP}}) = (F_{\mathrm{FIAsH}}^{\mathrm{ex425/em535}} - a \times F_{\mathrm{ECFP}}^{\mathrm{ex425/em475}} - b \times F_{\mathrm{FIAsH}}^{\mathrm{ex500/em535}}) / F_{\mathrm{ECFP}}^{\mathrm{ex425/em475}}$$

In this equation, the leakage from ECFP into 535 nm channel ($F_{ECFP}^{ex425/em475}$) and from the direct FIAsH excitation by light at 425 ($F_{FIAsH}^{ex500/em535}$) were subtracted from the FIAsH signal after ECFP excitation ($F_{FIAsH}^{ex425/em535}$). The terms *a* and *b* are correction factors for the two leakages mentioned above. *a* is the ratio of the direct emission of donor, ECFP at 535 nm, to its emission at 475 nm when excited at 425 nm which was calculated with cells transfected with pcDNA3-$Ghsr1\alpha^{FIAsH/ECFP}$ or pcDNA3-$Drd1^{FIAsH/ECFP}$ only. *b* is the ratio of the direct emission of FlAsH at 535 nm when excited at 425 nm to its emission at 535 nm when excited at 500 nm which was calculated with FlAsH labeled untransfected cells.

For FRET assay with Ghsr1α/Drd1 co-activation, we generated pcDNA3-$Ghsr1\alpha^{FIAsH/ECFP}$-T2A–*Drd1*-HA construct. $Ghsr1\alpha^{FIAsH/ECFP}$ and mouse *Drd1-HA* cDNA were cloned into pcDNA3 with T2A sequence (GSGEGRGSLLTCGDVEENPGP) inserted between those two cDNAs to express both proteins simultaneously. 50 μM MK0677 and 100 μM SKF81297 were added on HEK 293T cells at the same time to co-activate both Ghsr1α and Drd1 proteins in the presence or absence of Aβ (2 μM).

For cell imaging, HEK 293T cells were cultured on 96 well plate with cover glass bottom (Corning). The images were taken using an Olympus FV3000RS confocal microscope equipped with oil immersion 40× objective. ECFP was excited at 445 nm and images were taken with the factory setting for ECFP fluorescence (460 nm-500 nm). FlAsH was excited at 488 nm and images were taken with the factory setting for EYFP fluorescence (530 nm-580 nm). FRET between ECFP and FlAsH was excited at 445 nm and images were taken with the factory setting for EYFP fluorescence (530 nm-580 nm). The images on the same cell areas were taken before and after treatment. Image J software (NIH) was used to calculate FRET ratio ($FlAsH^{exECFP/emFIAsH}$/$ECFP^{exECFP/emECFP}$) and generate pseudocolor images.

Mouse behavioral test

Morris water maze test was performed as previously described in order to test changes in mice spatial learning and memory (*70*). Mice were allowed to acclimate to the testing environment at least 0.5 hours before tests. Mice were randomized by gender and genotypes to which the experimenter was blinded during the tests. In brief, mice were trained to find a hidden platform (20 cm diameter) in an open swimming pool (200 cm diameter) filled with 21°C water. Four trials were performed each day for 12 days. Each trial started at a different position (NW, N, E, SE) while the platform was kept in a single location (SW). Each trial lasted 60 seconds, followed by 30 seconds during which mice were allowed to remain on the platform to give them an opportunity to memorize the location of the platform. After 12 days of training, mice were subjected to a probe test in which the platform was removed. The latency they needed to reach the platform or the number of times they passed the previous platform location were analyzed using HVS Image 2015 software (HVS Image) to present mice learning curves and probe results.

Primary hippocampal neuron culture

Hippocampal neuron cultures were prepared as previously described (*15*). In brief, mouse hippocampi were dissected from postnatal day 0–1 pups in cold HBSS. Cells were dissociated by using 0.025% trypsin at 37 °C for 15 minutes, followed by 10 times homogenization in ice cold DMEM. Dissociated cells were then passed through a 100 μm cell strainer (Corning) and centrifuged for 5 minutes at 210×*g*. The pellet was gently resuspended in neuron culture medium (Neurobasal A with 2% B27 supplement, 0.5 mM L-glutamine, Invitrogen) and plated on poly-D-lysine (Sigma-Aldrich) coated Lab-Tek chamber slides (Nunc, 177445) with appropriate densities.

Agonist treatment on primary cultured neurons

At 14 days in vitro (DIV14) hippocampal neurons were exposed to the synthetic growth hormone secretagogue receptor agonist MK0677 (Tocris, #5272, 1.5 μM), the dopamine D1-like receptor agonist SKF81297 (Sigma-Aldrich, #S143, 2 μM), or a mixture of MK0677 and SKF81297 for 5 minutes. The exposure was followed by immunostaining to examine the effects of Ghsr1α/Drd1 co-activation on Aβ induced synaptic loss or Ghsr1α/Drd1, Aβ/Ghsr1α interaction as described in previous sections.

Electrophysiology

For electrophysiological experiments, mice were anesthetized with isoflurane and decapitated. Brains were extracted and transverse sections (350 μm) of the hippocampus were cut on a vibratome (VT1200S, Leica) in ice-cold oxygenated (95% O_2, 5% CO_2) ACSF containing the following: 110 mM choline (Sigma-Aldrich), 25 mM $NaHCO_3$ (Fisher Scientific), 1.25 mM NaH_2PO_4 (Fisher Scientific), 2.5 mM KCl (Sigma-Aldrich), 7 mM $MgCl_2$ (Sigma-Aldrich), 0.5 mM $CaCl_2$ (Sigma-Aldrich), 10 mM dextrose (Fisher Scientific), 1.3 mM L-ascorbic acid (Fisher Scientific), and 2.4 mM sodium pyruvate (Sigma-Aldrich). Slices were incubated for at least 1 hour in normal recording ACSF consisting of: 126 mM NaCl (Fisher Scientific), 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM dextrose, 2.4 mM sodium pyruvate, and 1.3 mM L-ascorbic acid, bubbled with 95% O_2 / 5% CO_2. Slices were allowed to rest for 30 minutes after being transferred to the recording chamber before recordings began. Recordings of local field potentials (LFPs) were performed on an Axon Multiclamp 700B amplifier (Molecular Devices), and data were acquired and analyzed using AxoGraph X (AxoGraph Scientific). A tungsten concentric bipolar microelectrode (World Precision Instruments), and a recording glass electrode (1.5 MΩ) filled with recording ACSF, were placed approximately 200 μm apart in the Schaffer collateral-commissural pathway in the CA1 region

of the hippocampus. Pulses were delivered in 30 seconds intervals. Before baseline recordings commenced, input-output curves were taken, using 25-150 µA stimulation currents in 25 µA steps. Three sweeps were sampled at each stimulation intensity and averaged to represent the voltage response at each step. Recordings of baseline responses lasted for at least 40 minutes. Stimulation intensity was set to approximately 40% of the minimum intensity required to evoke the maximum response (based on the input-output curve). The stimulation intensity was kept consistent throughout the duration of the experiment. All drugs (1.5 µM MK0677 and 2 µM SKF81297) were bath applied for 20 minutes prior to long-term potentiation (LTP) induction. LTP was induced using a theta burst stimulation (TBS) protocol consisting of 10 bursts (5 pulses at 100Hz) repeated at 5 Hz delivered two times 30 seconds apart. After LTP induction, LFPs were recorded for an additional 60 minutes. The slope of the field excitatory postsynaptic potential (fEPSP) was measured in Axograph scientific software and sweeps were averaged in bins of 5 for both the baseline and post LTP induction periods. Changes in LFPs are expressed as percentage change from the averaged baseline values.

Whole-cell voltage-clamp recordings were obtained at room temperature using oxygenated (95% O_2, 5% CO_2, pH 7.3) recording ACSF containing the following: 120 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM Na_2HCO_3, 10 mM dextrose, 2 mM $CaCl_2$, and 2 mM $MgCl_2$, 2.4 mM sodium pyruvate, and 1.3 mM L-ascorbic acid. Slices treated with MK0677/SKF81297 were incubated for at least 1 hour in ACSF containing 1.5 µM MK0677 and 2 µM SKF81297 prior to the start of recordings. For voltage-clamp recordings, electrodes (WPI; 3–5 MΩ open tip resistance) were filled with the following: 130 mM CsCl (Sigma-Aldrich), 20 mM tetraethylammonium chloride (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 2 mM $MgCl_2$, 0.5 mM EGTA (Sigma-Aldrich), 4 mM Na_2-ATP (Sigma-Aldrich), 0.3 mM Lithium-GTP

(Sigma-Aldrich), 14 mM phosphocreatine (Sigma-Aldrich), and 2 mM QX-314 bromide (Tocris Bioscience) and brought to a pH of 7.2 with CsOH (Fischer Scientific). AMPA-mediated miniature excitatory postsynaptic currents (AMPA-mEPSCs) were pharmacologically isolated by adding 75 μM picrotoxin (Sigma-Aldrich), 1 μM tetrodotoxin (Alomone Labs) and 10 μM CPP ((±)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid, Sigma-Aldrich) to the recording ACSF. Access resistance was monitored throughout the recording, and a 20% change was deemed acceptable. The frequency and amplitude of AMPA-mEPSCs were measured from 200 seconds of continuous recording using MiniAnalysis (Synaptosoft) with a threshold set at two times the RMS baseline noise.

Agonist treatment on mice

3 months old nonTg and 5×FAD mice received daily intraperitoneal (i.p.) injections of saline (sterilized 0.9% NaCl), or a combination of MK0677 (Tocris, #5272, a dose of 1 or 3 mg/kg) and SKF81297 (Sigma-Aldrich, #S143, a dose of 1.5 or 4.5 mg/kg) diluted in saline for one month. After the treatment the mice were subjected to behavioral test, then euthanized for tissue collection.

Cell membrane isolation and membrane blotting

Human or mouse hippocampal cell membranes were extracted using a previously published protocol (*71*). In brief, hippocampi were homogenized and incubated in ice-cold isolation buffer containing 40 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$ (Fisher Scientific), 0.15 U/μl benzonase (EMD Millipore) for 10 minutes. Three times 12,000g × 10 minutes centrifugation were performed to isolate and wash cell membrane. Purified hippocampal cell membrane were then fixed in 4% PFA for 0.5 hours followed by 1 hour blocking (5% donkey/goat serum, 0.3% Trition-X-100, PBS, pH 7.4). Membranes were incubated overnight in primary anti-GHSR1α or

anti-DRD1 antibody at 4 °C, followed by 1 hour incubation with anti-goat or anti-rabbit HRP-conjugated secondary antibody at room temperature. In order to remove non-specific binding of primary and secondary antibodies membranes were washed in PBST (PBS containing 0.05% Tween-20) for three times followed by centrifugation at 16,500×*g* for 20 minutes. Cell membrane protein were then extracted by using urea buffer containing 50 mM Tris-HCl, pH 6.8, 8 M urea (Fisher Scientific), 2% SDS (Fisher Scientific), 10% glycerol (Fisher Scientific). Cell membrane extracts were loaded onto nitrocellulose membrane (Bio-Rad). The nitrocellulose membrane was dried, then subjected to imaging immediately by using Bio-Rad Chemidoc Imaging System. The membrane was reprobed with mouse anti-β-III-tubulin (Proteintech, #66240, 1:1,000) to normalize protein expression.

Mouse serum total ghrelin ELISA

Mice were fasted for 8 hours before blood collection. Rat/Mouse total Ghrelin ELISA kits (Millipore, EZRGRT-91K) were used for serum total ghrelin measurement following the manufacturer's instruction. Data were collected on a microplate reader (BIOTEK) and the concentration of ghrelin was calculated (ng/ml).

Postsynaptic density isolation

Hippocampal postsynaptic densities (PSD) were prepared based on a published protocol (*72*). In brief, the hippocampi were dissected and homogenized in ice-cold homogenizing buffer (25 mM Tris-HCl, 0.32 M sucrose (Fisher Scientific), 1mM phenylmethylsulfonyl fluoride (PMSF, Fisher Scientific), 1 mM EDTA, 1mM EGTA (Fisher Scientific), 10 mM Na_3VO_4 (Fisher Scientific), 25 mM NaF (Fisher Scientific), 10 mM $Na_4P_2O_7$ (Fisher Scientific) and protease inhibitor cocktail, pH 7.5) with a Dounce homogenizer (Wheaton). The resultant homogenates were centrifuged at 1,000×*g* for 10 minutes to remove cell debris and nuclei. The supernatants were then centrifuged

at 12,000×*g* at 4 °C for 15 minutes. Then the pellets (crude synaptosome) were resuspended in homogenizing buffer containing 1% Triton X-100 and 300 mM NaCl on ice for 30 minutes. After centrifugation at 16,000 g for 30 minutes the pellets were obtained as PSD (post synaptic density) fraction. Purified hippocampal PSD samples were resuspended in urea buffer for immunoblotting.

Immunoblotting

Samples were prepared in 1×NuPAGE LDS Sample Buffer or urea buffer as needed. Proteins were separated in 10% or 12% Bis-Tris Gel (Thermo Fisher Scientific) and then transferred to PVDF membrane (Bio-Rad). After blocking in 5% non-fat milk (Labscientific Inc) in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.6) for 1 hour at room temperature, the membranes were probed with appropriate primary antibodies overnight at 4 °C followed by incubation with the corresponding secondary antibody for 1 hour at room temperature. The following antibodies were used: rabbit monoclonal anti-Phospho-CaMKII (Thr286) (CST, #12716, 1:2,000), mouse monoclonal anti-CaMKII-α (CST, #50049, 1:5,000), mouse monoclonal anti-β-III-tubulin (Proteintech, #66240, 1:5,000), mouse monoclonal anti-Phospho-Tau (Ser202, Thr205) (Thermo Fisher, #MN1020, 1:1,000), mouse monoclonal anti-Phospho-Tau (Ser396) (CST, #9632, 1: 5,000), rabbit monoclonal anti-Phospho-Tau (Ser404) (CST, #20194, 1:5,000), mouse monoclonal anti-T-Tau (Tau46) (CST, #4019, 1: 1,000), mouse anti-β actin (Sigma-Aldrich, #5441, 1:10,000), mouse anti-amyloid precursor protein (BioLegend, #803002, 1:2000), goat anti-mouse IgG HRP conjugated and goat anti-rabbit IgG HRP conjugated secondary antibodies (Thermo Fisher Scientific, #31430 and 31460, 1: 2,000–8,000). Images were collected on a Bio-Rad Chemidoc Imaging System. Image J software (NIH) was used for analysis.

Immunohistochemistry and H&E staining analysis

Paraffin-embedded human hippocampal sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Heat induced antigen retrieval was performed in boiling citrate buffer (pH 6.0) for 15 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. Slides were blocked in PBS containing 5% goat or donkey serum (Sigma-Aldrich) and 0.3% Triton X-100 (Fisher Scientific) for 1 hour, then incubated with following primary antibodies at room temperature overnight: anti-GHSR1α (Santa Cruz Biotechnology, #sc-10359, 1:100) or anti-DRD1 (Abcam, #ab81296, 1:200) followed by 1 hour room temperature incubation of Biotin-conjugated secondary antibody (anti-goat from Invitrogen, #A16003, 1:500; anti-rabbit from Sigma-Aldrich, #B8895, 1:500) and ExtrAvidin–peroxidase (Sigma-Aldrich, #E2886, 1:500). Signal was developed using DAB (Sigma-Aldrich, #D4168). Hematoxylin (Sigma-Aldrich, MHS-16) was used for nuclear counterstain. Images were collected on Olympus upright microscope, mean intensity of the DAB signal were analyzed using Image J software (NIH) to represent human hippocampal GHSR1α and DRD1 expression.

Mice brain, kidney and liver sections were freshly dissected and fixed in 4% PFA overnight at 4 °C then proceeded to frozen tissue sectioning. Hematoxylin-Eosin (H&E) staining were performed following the commercial protocol. Briefly, slices are air dried overnight then proceeded to rehydration with 100%, 95%, 80%, 75% and 50% ethanol. Slices were stained with hematoxylin (Sigma-Aldrich, MHS-16) for 10 minutes followed by 5 minutes tap water rinsing. Next, we put the slices into eosin (Sigma-Aldrich, HE110316) for 1 minute then dipped into ddH2O. Dehydration with reversed order of ethanol as rehydration were performed after the staining. Images were collected on Olympus upright microscope. Cell density were counted using Image J software (NIH).

Parenchymal amyloid plaques staining/Congo red staining

Parenchymal amyloid plaques were stained as previous described (*73*). The brain slices were dried overnight then rinsed in ddH_2O for 30 seconds. Afterwards, the brain slices were immersed in saturated NaCl solution (over saturated NaCl in 80% ethanol) with 10 mM NaOH for 20 min, followed by the immersion in 0.2% Congo red solution (0.2% Congo red in saturated NaCl solution, 10 mM NaOH) for 30 min. After the staining, the brain slices were quickly dipped in 90% ethanol for 8 times followed by 8 × quick dip in 100% ethanol and then 3 × 5min's xylene incubation. Images were collected on Olympus upright microscope. The occupied area of Congo red-labelled Parenchymal amyloid plaques was analyzed using Image J software (NIH).

Intraneuronal amyloid β staining

PFA fixed frozen tissue were sectioned and proceeded to intraneuronal amyloid β staining as previously described (*13*). Briefly, mouse brain sections were blocked with 5% goat serum and 0.3% Triton X-100 for 1 hour. Slices were then probed with rabbit monoclonal anti-Aβ (1:500, Invitrogen, #700254) to detect intraneuronal amyloid β expression. Mouse monoclonal anti-β-III-tubulin (Proteintech, #66240, 1:1,000) was used to determine neurons and DAPI was used for nuclear staining. Images were collected on a Nikon confocal microscope. Aβ positive signals which were overlapped with β-III-tubulin were considered as intraneuronal amyloid β staining and its occupied area were analyzed by using "AND" operation in "binary operation" dialog of NIS element software. All confocal images were converted to 3 dimensional images by using NIS element software "3D reconstruction" module for analysis.

ELISA assay for soluble Aβ measurement

Aβ amounts in human or mouse hippocampal samples were measured by using human Aβ40 and Aβ42 ELISA kits (Thermo Fisher Scientific, KHB3481 for Aβ40, KHB3441 for Aβ42)

following the manufacturer's instructions. Tissues were homogenized thoroughly with cold 5 M guanidine HCl/50 mM Tris HCl. The homogenates were incubated at room temperature for 4 hours. The samples were diluted with cold reaction buffer (Dulbecco's phosphate buffered saline with 5% BSA and 1× protease inhibitor cocktail) and centrifuged at 16,000 × g for 20 minutes at 4°C. The supernatants were diluted with standard diluent buffer provided in the kit and quantified by ELISA kits. Aβ amounts were normalized to total protein content in the samples.

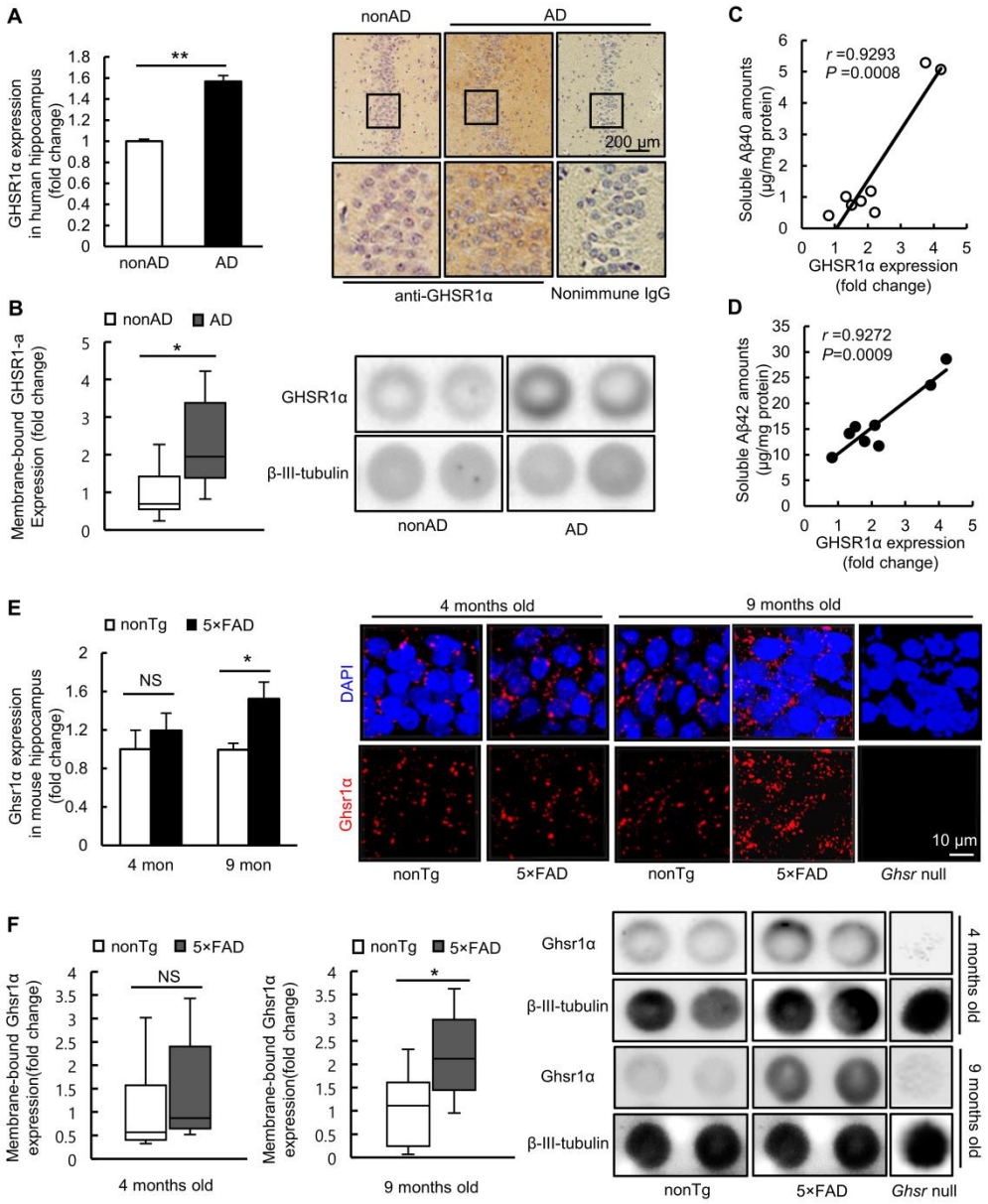

Fig. S1. Expression of GHSR1α increased in hippocampi from subjects with AD and 9-month-old 5×FAD mice. (**A**) Analysis of GHSR1α expression in the hippocampal region from subjects with AD and nonAD healthy controls. Unpaired Student's *t*-test. ** $P < 0.01$. $n = 4$ per group. The right panel is representative images. Nonimmune IgG to replace specific GHSR1α

antibody was used to reflect the specificity of the staining. Scale bar = 200 μm. (**B**) Analysis of cell membrane-incorporated GHSR1α in the hippocampal tissues from subjects with AD and nonAD healthy controls. Unpaired Student's *t*-test. * $P < 0.05$. nonAD, $n = 9$; AD, $n = 8$. The right panel is representative membrane blotting images. β-III-tubulin was used as the loading control. (**C&D**) Correlation between GHSR1α expression and soluble Aβ40 (**C**) ($r = 0.9293$, $P = 0.0008$) or Aβ42 amount (**D**) ($r = 0.9272$, $P = 0.0009$). Pearson correlation coefficient. $n = 8$. (**E**) Analysis of Ghsr1α expression in the hippocampal region from 4 and 9 month-old nonTg and 5×FAD mice. Unpaired Student's *t*-test. NS, not significant. * $P < 0.05$. 4 month-old mice, nonTg, $n = 6$; 5×FAD, $n = 5$. 9 month-old mice, nonTg, $n = 4$; 5×FAD, $n = 4$. The right panel are representative images. 9 month-old *Ghsr* null mice were used as a critical negative control. Scale bar = 10 μm. (**F**) Analysis of cell membrane-incorporated Ghsr1α in the hippocampal tissues from 4 and 9 months old nonTg and 5×FAD mice. Unpaired Student's *t*-test. NS, not significant. * $P < 0.05$. 4 month-old mice, nonTg, $n = 6$; 5×FAD, $n = 6$. 9 month-old mice, nonTg, $n = 7$; 5×FAD, $n = 7$. The right panel is representative membrane blotting. β-III-tubulin was used as the loading control.

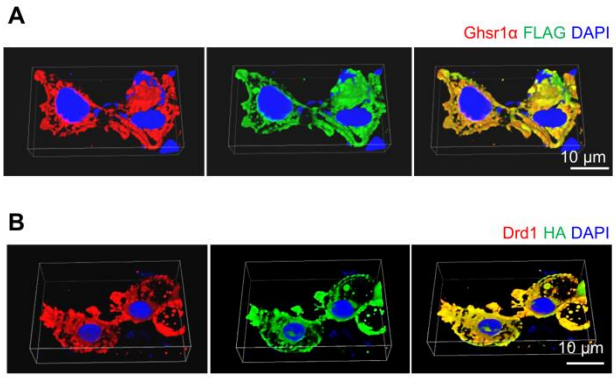

Fig. S2. Expressions of Ghsr1α and Drd1 were validated in transfected HEK 293T cells. FLAG-tagged mouse Ghsr1α and HA-tagged mouse Drd1 were transiently expressed in otherwise non-expressing HEK 293T cells. The antibody specificity and expression of Ghsr1α (**A**) and Drd1 (**B**) were determined by immunofluorescence staining. The nuclei were visualized by DAPI staining. Scale bar = 10 μm.

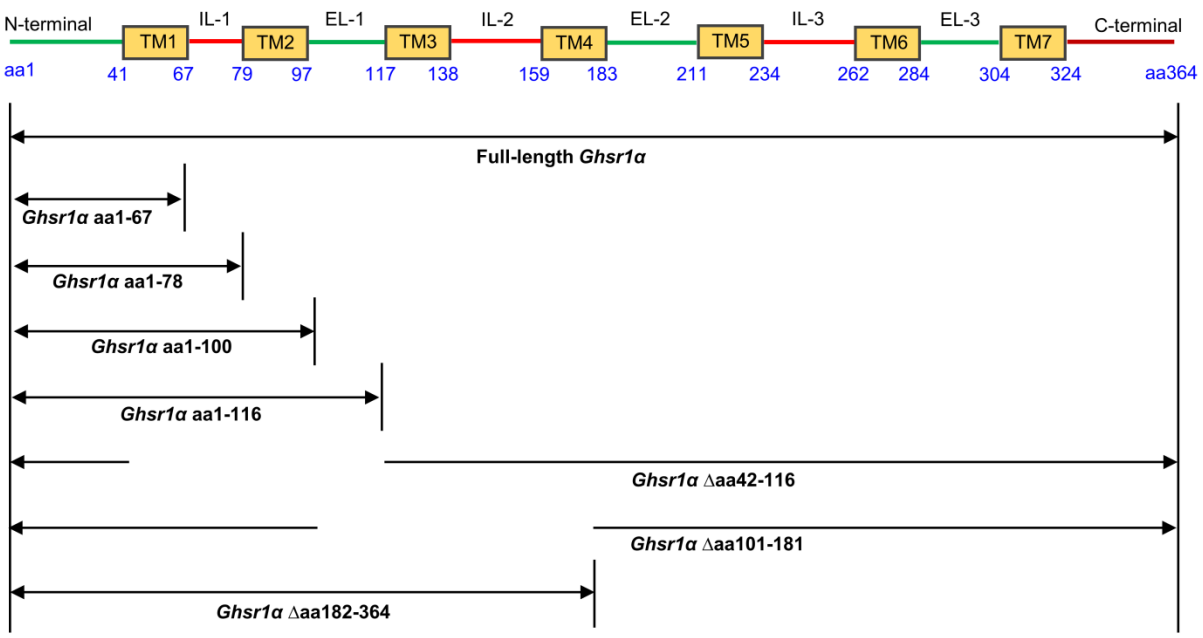

Fig. S3. Schematic diagram shows the sequence of full-length *Ghsr1α* and its truncating mutants. Schematic map of full-length *Ghsr1α*, *Ghsr1*α aa1-67, *Ghsr1α* aa1-78, *Ghsr1α* aa1-100, *Ghsr1α* aa1-116, *Ghsr1α* Δaa42-116, *Ghsr1α* Δaa101-181 and *Ghsr1α* Δaa182-364. IL, TM and EL stand for intracellular loop, transmembrane domain and extracellular loop, respectively. Δ stands for deletion of fragment.

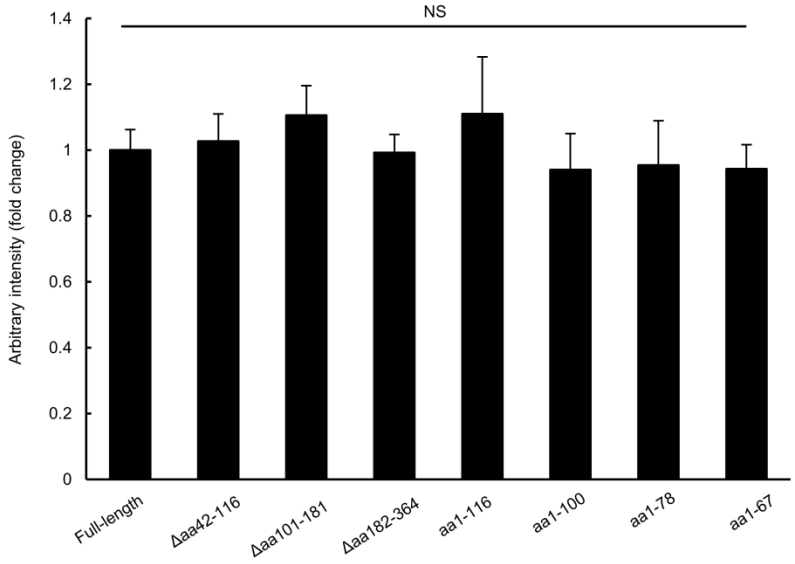

Fig. S4. FLAG-tagged Ghsr1α and its truncating mutants were similarly expressed in HEK 293T cells. Anti-FLAG-tag was used to recognize FLAG-tagged Ghsr1α and its mutants in transiently transfected HEK 293T cells, followed by incubation with Alexa Fluor 488 conjugated secondary antibody. The images were collected on a Nikon confocal microscope. The expressions of FLAG-tagged Ghsr1α and its mutants were quantified by measuring the intensity of fluorescence signals. One-way ANOVA followed by Bonferroni post hoc analysis. NS, not significant. n = 8-11 cells.

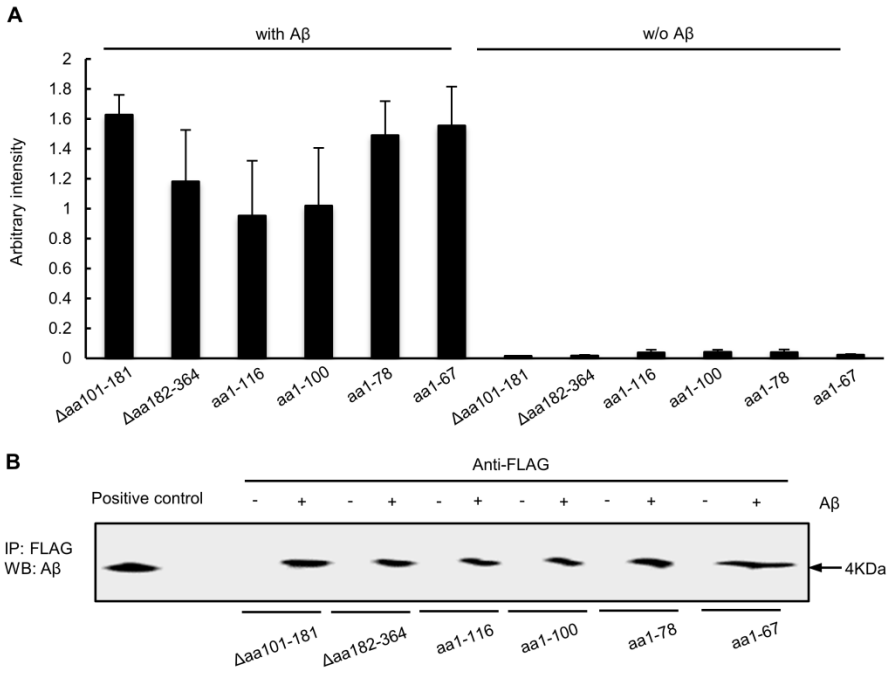

Fig. S5. The interaction between Ghsr1α mutants and Aβ42 was assessed by using Co-IP. (**A**) Densitometric analysis of protein interaction between oligomeric Aβ42 and Ghsr1α mutants including aa1-67, aa1-78, aa1-100, 1-116, Δaa182-364 and Δaa101-181. *n* = 3. (**B**) Representative immunoblots showing the interactions between oligomeric Aβ42 and Ghsr1α mutants.

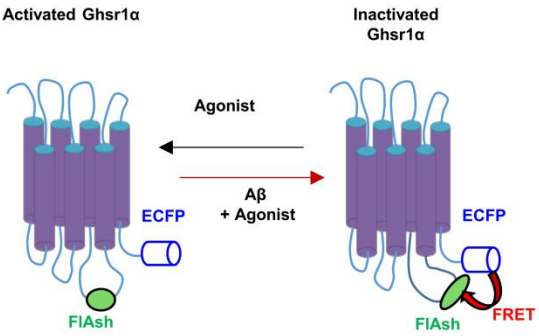

Fig. S6. Schematic diagram represents FlAsH-FRET assay for Ghsr1α activity and the impact of oligomeric Aβ42 on Ghsr1α activation. Ghsr1α activation was monitored by recording changes in FRET between ECFP (the donor) and FlAsH (the acceptor) introduced respectively into the C-terminal tail and the third intracellular loop of the *Ghsr1α*. Decreased FRET ratio in the presence of agonist reflected the activation of Ghsr1α by its agonist. Co-incubation of oligomeric Aβ42 blunted agonist-induced reduction of FRET ratio, indicating that oligomeric Aβ42 inhibits agonist-induced Ghsr1α activation.

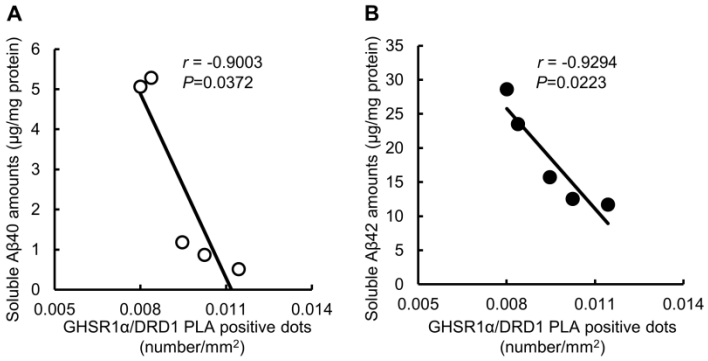

Fig. S7. GHSR1α/DRD1 complex density was negatively correlated with hippocampal soluble Aβ40 and 42 amounts in subjects with AD. GHSR1α/DRD1 complex density exhibited a strong correlation with the amounts of soluble Aβ40 (**A**) (r = -0.9003, P = 0.0372) or Aβ42 (**B**) (r = -0.9294, P = 0.0223) in hippocampi from patients with AD. Pearson correlation coefficient. n =5.

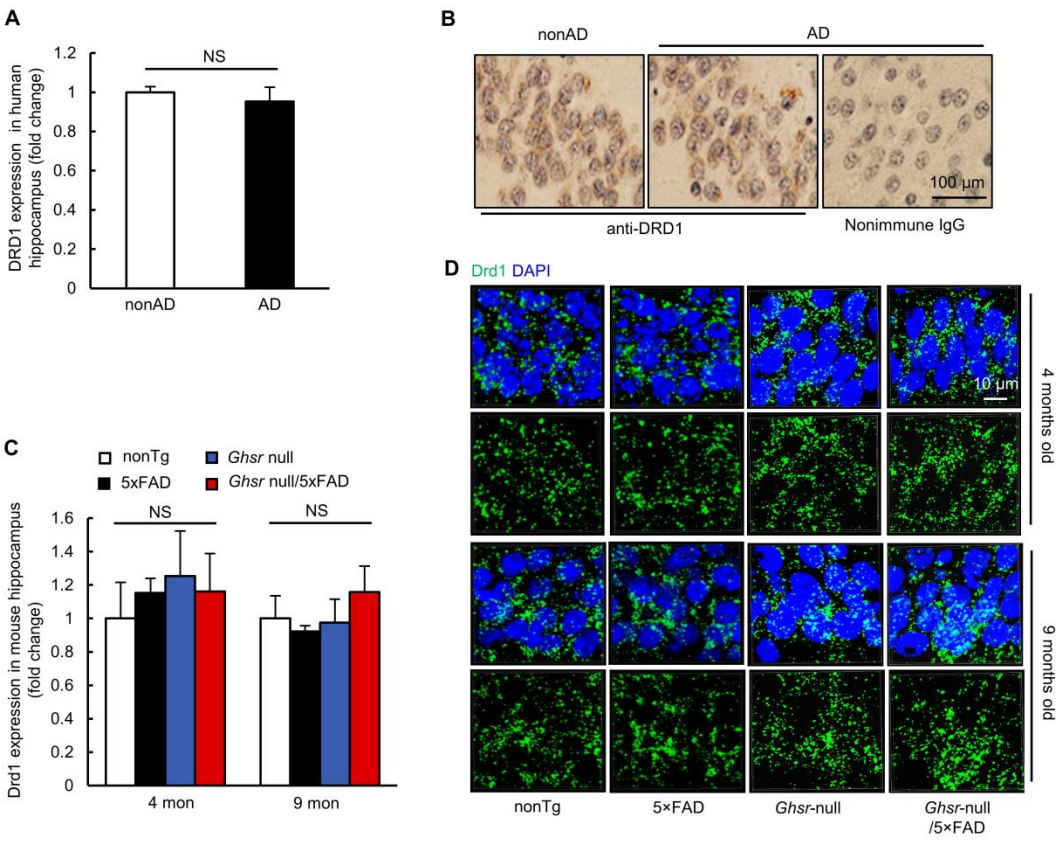

Fig. S8. Expression of hippocampal DRD1 remained unaltered in hippocampi from subjects with AD and 5×FAD mice. (**A&B**) Analysis of DRD1 expression in the hippocampal region from subjects with AD and nonAD healthy controls. Unpaired Student's *t*-test. NS, not significant. *n* = 4 cases per group. (**B**) Representative images. Nonimmune IgG to replace specific DRD1 antibody was used to determine the specificity of the staining. Scale bar = 100 μm. (**C&D**) Analysis of Drd1 expression in the hippocampal CA1 region from 4 and 9 month-old four genotypes of mice. One-way ANOVA followed by Bonferroni post hoc analysis. NS, not significant. 4 month-old mice, nonTg, *n* = 5; 5×FAD, *n* = 5; *Ghsr* null, *n* = 3; *Ghsr* null/5×FAD, *n* = 3. 9 month-old mice, nonTg, *n* = 4; 5×FAD, *n* = 4; *Ghsr* null, *n* = 4; *Ghsr* null/5×FAD, *n* = 4. (**D**) Representative images. Scale bar = 10 μm.

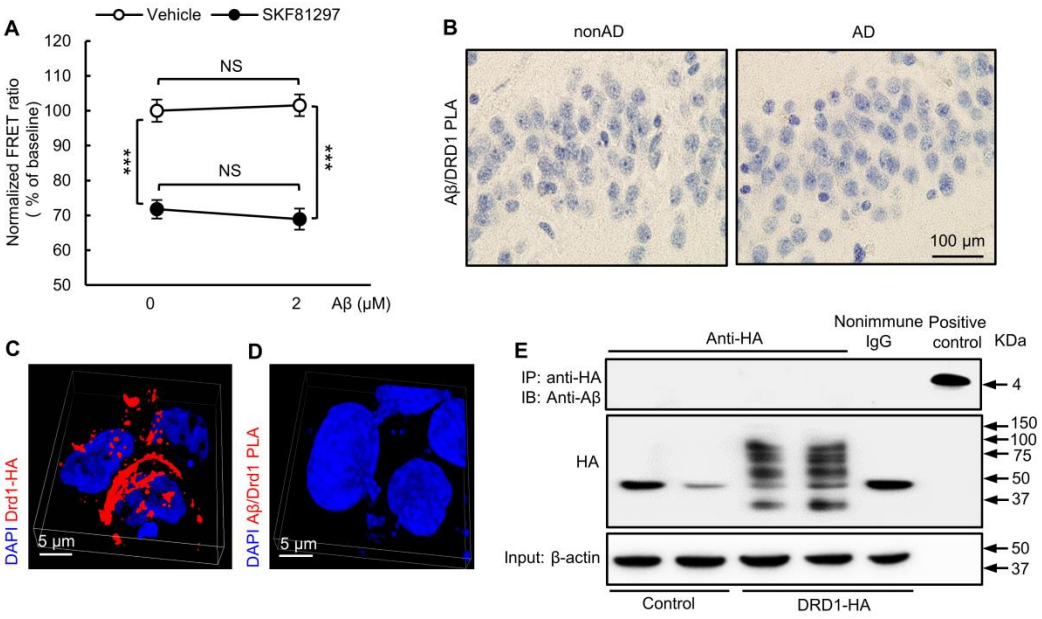

Fig. S9. Oligomeric Aβ42 did not affect agonist-induced activation of Drd1 or form complex with Drd1. (A) Oligomeric Aβ42 (2 μM) exhibited little interference with Drd1 FlAsH-FRET response in the presence or absence of Drd1 agonist SKF81297 (100 μM). Two-way ANOVA followed by Bonferroni post hoc analysis. *** $P < 0.001$. n = 18-20 per group. Moreover, our in vitro and in vivo PLA assay did not support the interaction between Aβ42 and DRD1 in hippocampal tissues from subjects with AD (**B**) or Drd1-expressing HEK 293T cells (**C, D&E**). (**B**) Aβ/DRD1 PLA detection in hippocampi from AD and nonAD cases. n = 4 per group. Scale bar = 100 μm. (**C**) Expression of transfected Drd1-HA in HEK 293T cells. Scale bar = 5 μm. (**D**) No PLA-positive dot was detected in oligomeric Aβ42-treated Drd1-HA expressing HEK 293T cells. Scale bar = 5 μm. (**E**) Interaction between Drd1 and oligomeric Aβ42 was not detected in oligomeric Aβ42-treated Drd1-HA expressing HEK 293T cells by using Co-IP. Mouse HA antibody was used for immunoprecipitation of Drd1-HA. Drd1-HA immunoblots was detected with rabbit anti-HA antibody.

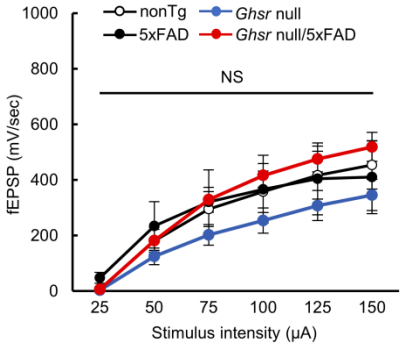

Fig. S10. Input/output curves of fEPSPs were similar in four types of transgenic mice. Input/output (I/O) curves of fEPSPs were obtained by plotting the slope of fEPSPs recorded in the CA1 area of the hippocampus as a function of the stimulation intensity (from 25 to 150 μA). A one-way ANOVA followed by Bonferroni post hoc analysis revealed no significant differences between the four groups at 9 months old. NS, not significant. nonTg, n = 8; 5×FAD, n = 4; *Ghsr* null, n = 6; *Ghsr* null/5×FAD, n = 6.

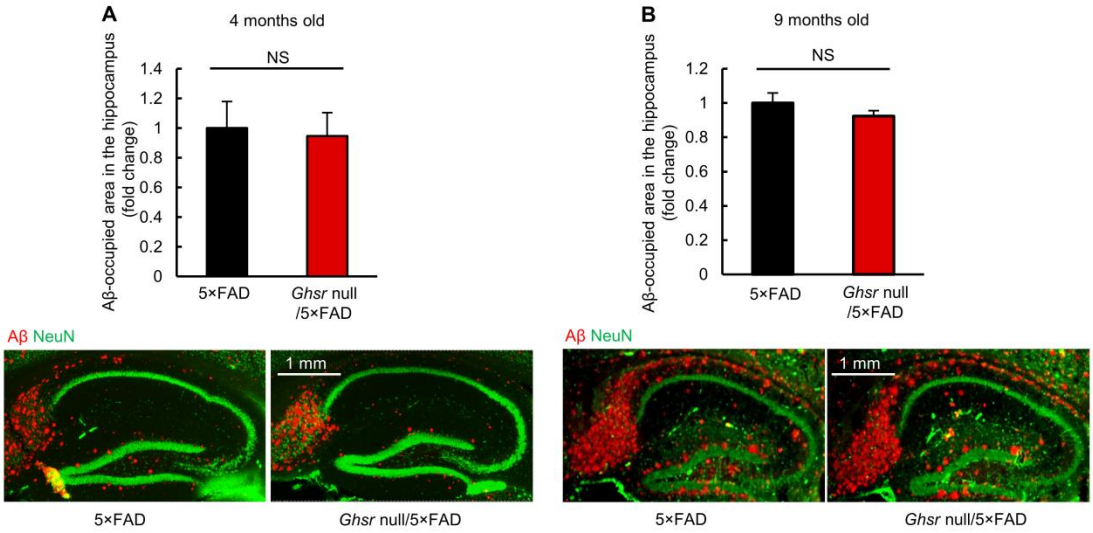

Fig. S11. Aβ deposition in the hippocampus remained unchanged in *Ghsr* null/5×FAD mice as compared with their 5×FAD littermates. Aβ deposition in the hippocampal region was measured and analyzed from 4 (**A**) and 9 (**B**) month-old mice. Unpaired Student's *t*-test. NS, not significant. 4 month-old mice, 5×FAD, $n = 7$; *Ghsr* null/5×FAD, $n = 5$. 9 month-old mice, 5×FAD, $n = 5$; *Ghsr* null/5×FAD, $n = 4$. The lower panels are representative images for Aβ staining (red color). Neurons were identified by NeuN staining (green color). Scale bar = 1 mm.

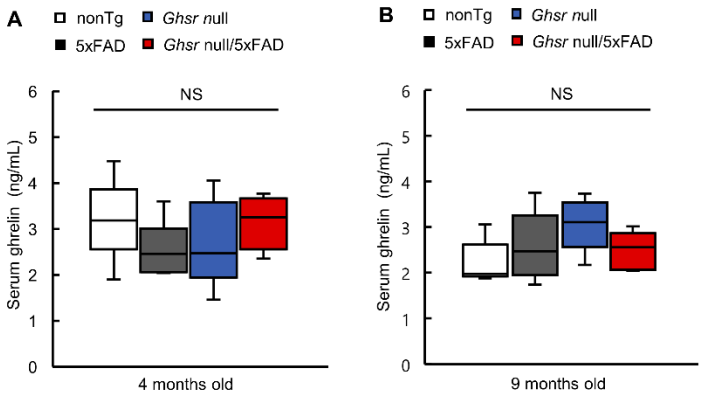

Fig. S12. Serum ghrelin amounts were similar in four types of transgenic mice. The amounts of serum total ghrelin in four groups of mice at 4 (**A**) and 9 (**B**) months old. One-way ANOVA followed by Bonferroni post hoc analysis. NS, not significant. 4 month-old mice, nonTg, n = 6; 5×FAD, n = 6; *Ghsr* null, n = 8; *Ghsr* null/5×FAD mice, n = 8. 9 month-old mice, nonTg, n = 8; 5×FAD, n = 8; *Ghsr* null, n = 6; *Ghsr* null/5×FAD mice, n = 7.

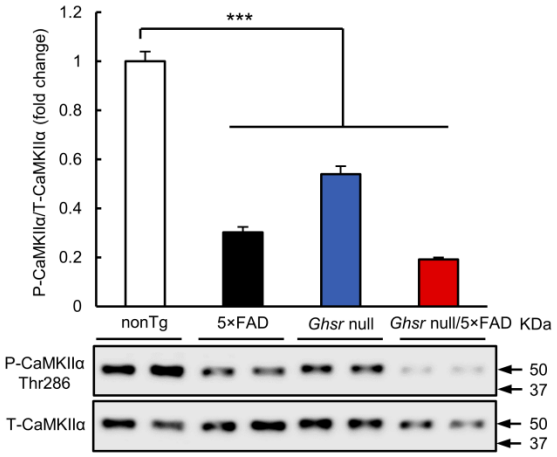

Fig. S13. Loss of Ghsr1α suppressed postsynaptic CaMKII activation in the hippocampus. Analysis of CaMKII phosphorylation (Thr286) in postsynaptic density from the hippocampus of four groups of mice at 9 months old. One-way ANOVA followed by Bonferroni post hoc analysis. *** $P < 0.001$. nonTg, $n = 3$; 5×FAD, $n = 4$; *Ghsr* null, $n = 3$; *Ghsr* null/5×FAD, $n = 4$. The lower panels are representative immunoreactive bands of phosphorylated CaMKII (P-CaMKII Thr286) and total CaMKII (T-CaMKII).

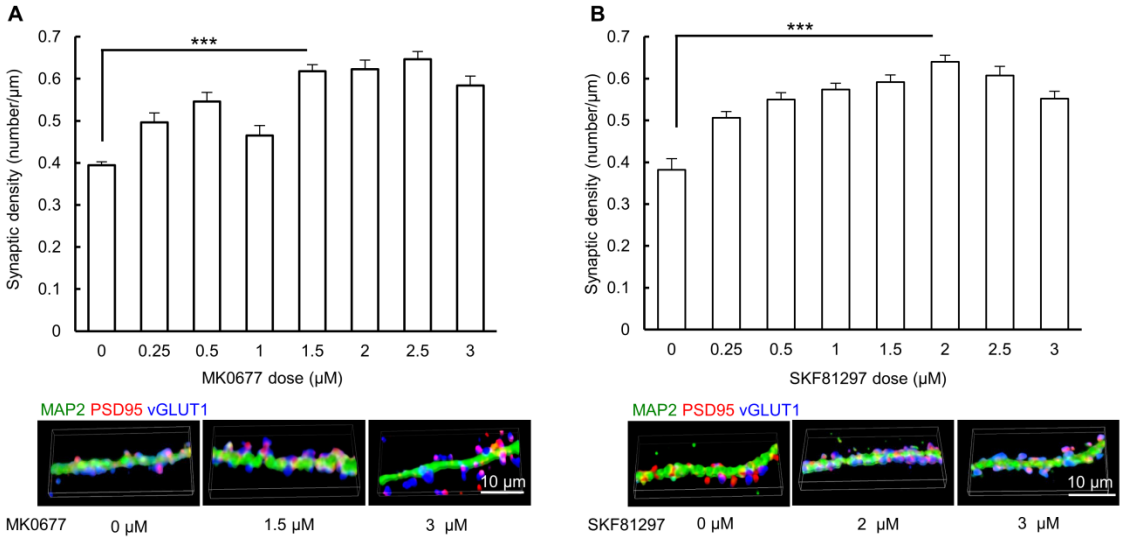

Fig. S14. The optimal doses of MK0677 and SKF81297 were determined by their augmenting effect on synaptogenesis in cultured hippocampal neurons. To determine the appropriate doses of MK0677 and SKF81297 for in vitro studies on primary hippocampal neuron culture, we exposed the neurons to different doses of the indicated drugs and examined the change of synapse density. Application of MK0677 at 1.5 μM (**A**) and SKF81297 at 2 μM (**B**) greatly promoted synaptogenesis. Unpaired Student's *t*-test. *** $P < 0.001$. n = 33-45 neurons from 3 independent experiments. The lower panels are 3D-reconstructed representative images of synaptic staining in the presence of different doses of drugs. The pre- and post-synaptic content was determined by the staining of vGLUT1 (blue color, presynaptic marker) and PSD95 (red color, postsynaptic marker), respectively. The dendrites were identified through staining for MAP2 (green color). Scale bar = 10 μm.

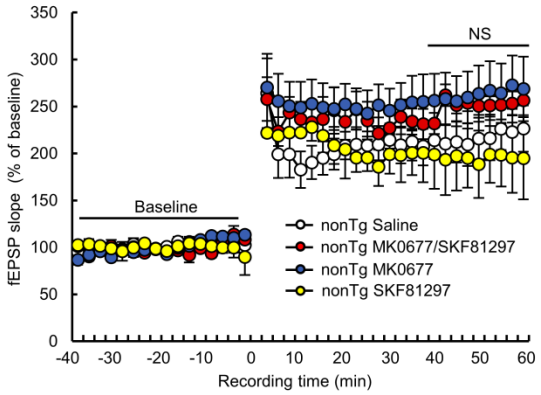

Fig. S15. Time course of LTP and fEPSP amplitudes was not changed by different treatments on hippocampal slices from nonTg mice. Hippocampal slices from 4 months-old nonTg mice were used for experiments. One-way ANOVA followed by Bonferroni post hoc analysis. NS, not significant. nonTg saline, n = 9; nonTg MK0677/SKF81297, n = 7; nonTg MK0677, n = 7; nonTg SKF81297, n = 2.

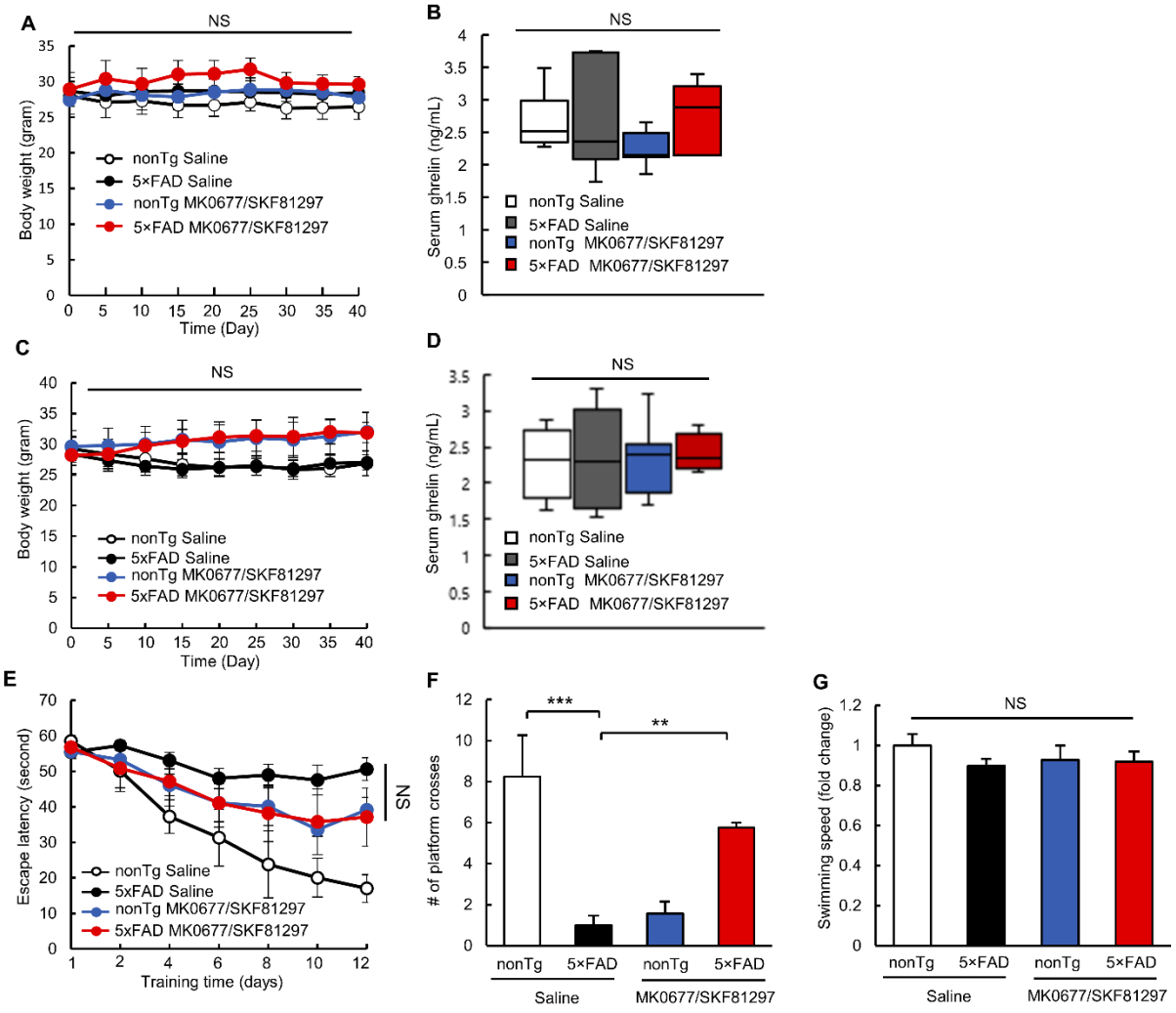

Fig. S16. The doses of MK0677/SKF81297 treatment were optimized on the basis of the influence on body weight, serum ghrelin, and behavioral performance. NonTg and 5×FAD mice at 3 months old received daily intraperitoneal (i.p.) injections of saline or MK0677/SKF81297 combination therapy for 30 days, then the mice were subjected to behavioral experiments at 4-5 months of age. The doses of the treatment were MK0677 1 mg/kg and SKF81297 1.5 mg/kg (A-B), or MK0677 3 mg/kg and SKF81297 4.5 mg/kg (C-G). **(A)** The mice body weights were measured every 5 days. Two-way ANOVA followed by Bonferroni post hoc analysis. NS, not significant. nonTg saline, *n* = 8; 5×FAD saline, *n* = 8; nonTg

MK0677/SKF81297, n = 7; 5×FAD MK0677/SKF81297, n = 5. **(B)** Total ghrelin amounts in mice serum were measured after treatment. Two-way ANOVA followed by Bonferroni post hoc analysis. NS, not significant. nonTg saline, n = 6; 5×FAD saline, n = 6; nonTg MK0677/SKF81297, n = 7; 5×FAD MK0677/SKF81297, n = 5. **(C)** Body weight was measured every 5 days. Two-way ANOVA followed by Bonferroni post hoc analysis. NS, not significant. **(D)** Ghrelin amount in mice serum was measured after treatments. Two-way ANOVA followed by Bonferroni post hoc analysis. NS, not significant. **(E-G)** Spatial navigation analysis in four groups of mice treated with vehicle (saline) or MK0677/SKF81297 performing the Morris water maze test. **(E)** Spatial learning. Two-way ANOVA followed by Bonferroni post hoc analysis. There is no significant difference (NS) between 5×FAD saline and 5×FAD MK0677/SKF81297. **(F)** Spatial reference memory. Two-way ANOVA followed by Bonferroni post hoc analysis. ** $P < 0.01$, *** $P < 0.001$. **(G)** Swimming speed. Two-way ANOVA followed by Bonferroni post hoc analysis. NS, not significant. nonTg saline, n = 4; 5×FAD saline, n = 8; nonTg MK0677/SKF81297, n = 7; 5×FAD MK0677/SKF81297, n = 4.

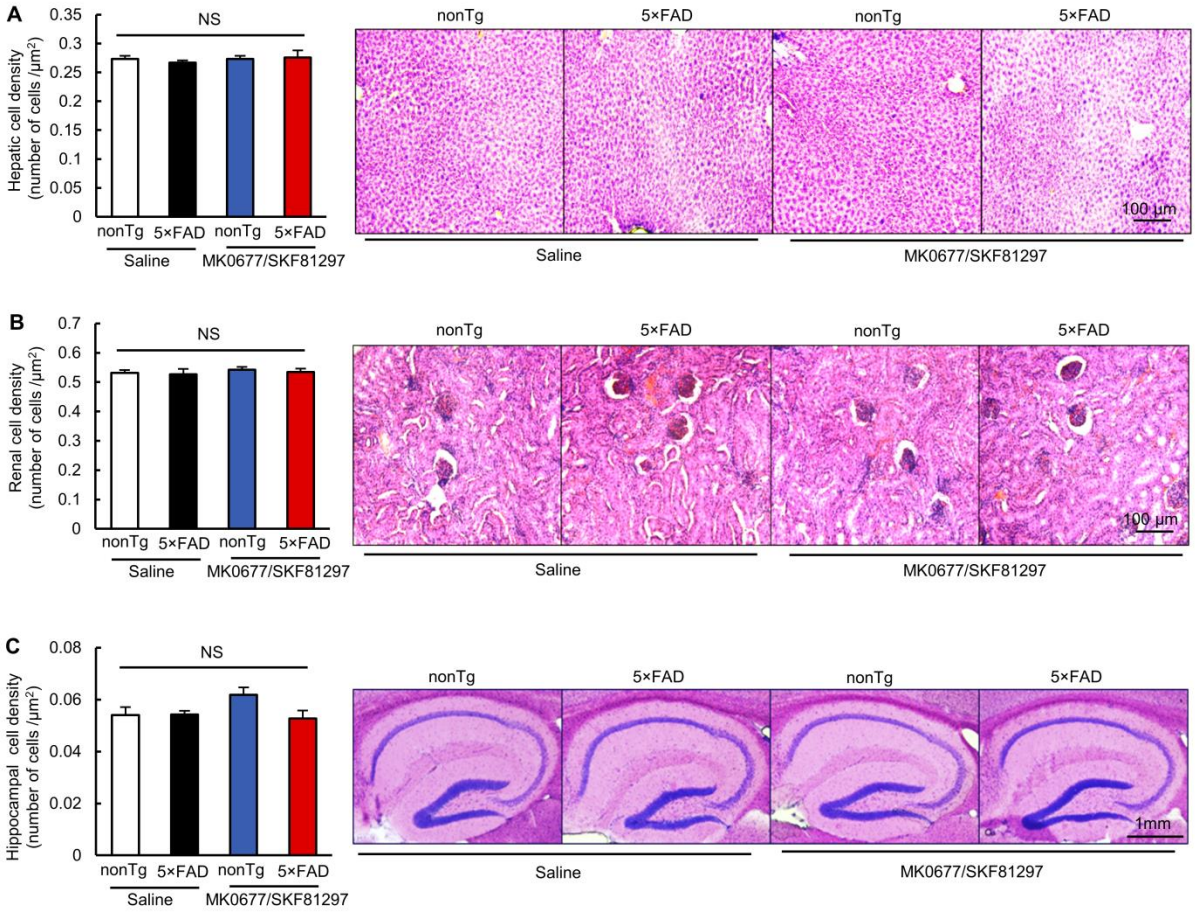

Fig. S17. MK0677/SKF81297 [MK0677 (1 mg/kg) and SKF81297 (1.5 mg/kg)] treatment on mice had no effect on hepatic, renal, and hippocampal cell density. The cell density in liver (**A**), kidney (**B**) and hippocampus (**C**) in different groups of mice with saline or MK0677/SKF81297 treatment. Two-way ANOVA followed by Bonferroni post hoc analysis. NS, not significant. *n* = 5 mice per group. The right panels are the representative images of HE staining, scale bar = 100 μm (**A&B**), = 1 mm (**C**).

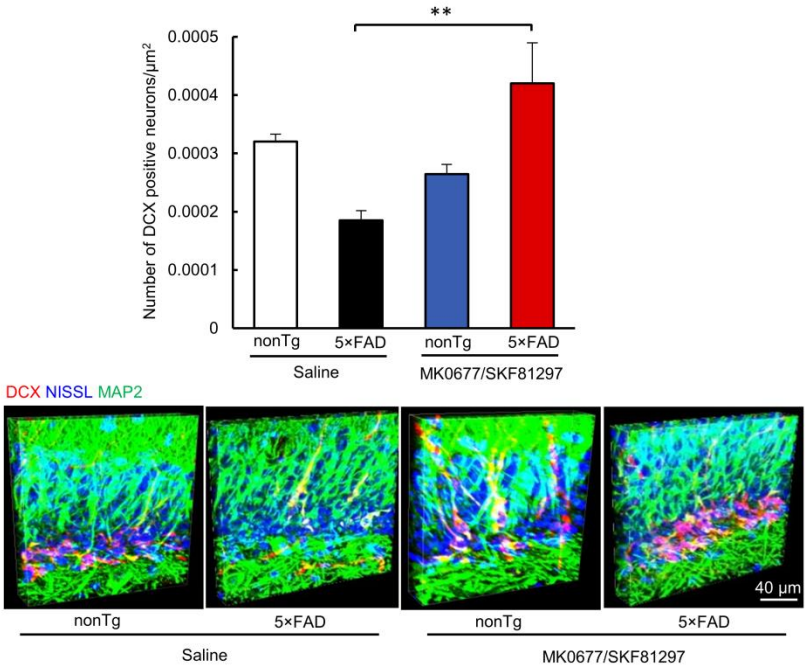

Fig. S18. MK0677/SKF81297 [MK0677 (1 mg/kg) and SKF81297 (1.5 mg/kg)] treatment improved neurogenesis in the dentate gyrus of 5×FAD mice. The number of doublecortin (DCX)-positive neurons were counted in the dentate gyrus from four groups of mice. Two-way ANOVA followed by Bonferroni post hoc analysis. ** $P < 0.01$. $n = 3$ mice per group. The lower panel are 3D-reconstructed representative images. Neurons were identified by the staining of NISSL blue (blue color) and MAP2 (green color). Adult neurogenesis was determined by DCX positive staining (red color). Scale bar = 40 μm.

Table S1. Human brain tissue information.

Clinical Dx	Case number	Gender	Age	PMI(Hr)	Braak	CERAD score
nonAD	36359	M	84	24	IV	Normal
nonAD	42133	F	100	12	IV	Normal
nonAD	42990	F	84	14	I	Normal
nonAD	45329	M	78	21	I	Normal
nonAD	39146	F	67	12	II	Normal
nonAD	46202	M	77	20	II	Normal
nonAD	K45	M	66	18	N/A	Normal
nonAD	K46	M	79	17	I	Normal
nonAD	K101	M	76	21.25	N/A	Normal
nonAD	K108	F	75	20	0	Normal
Mean ± SE		4F/6M	78.4 ± 2.8	18.61 ± 1.6		
AD	46090	F	75	27	VI	Definite
AD	46121	F	74	16	VI	Definite
AD	46991	M	62	23	V	Definite
AD	47586	F	78	18	V	Definite
AD	K01	M	84	15.5	V	Definite
AD	K26	F	79	10	V/VI	Definite
AD	K44	M	87	17.75	V/VI	Probable
AD	K55	F	87	28.5	V	Definite
Mean ± SE		5F/3M	78.25 ± 2.93	19.49 ± 2.21		