Supporting Information

Hedskog et al. 10.1073/pnas.1300677110

SI Materials and Methods

Antibodies. Antibodies used were rabbit antiamyloid β-peptide (Aβ) AW7 (gift from Dominic Walsh, Harvard Institutes of Medicine, Boston), rabbit anticalnexin (Ab13504; Abcam), mouse anti-Na/K ATPase (ab7671; Abcam), mouse anti-COX1 (58347; Santa Cruz), mouse anti-GM130 (610822; BD Biosciences), rabbit antimitochondrial dynamin-related fusion protein (M6319; Sigma-Aldrich), goat antivoltage-dependent anion channel (VDAC1; sc8828; Santa Cruz), rabbit anti-σ1 receptor (σ1R; Ab53852; Abcam), mouse anti-σ1R (sc166392; Santa Cruz), rabbit anti–inositol-1,4,5-triphosphate receptor type 3 (IP3R3; Ab9076; Chemicon), mouse anti-IP3R3 (610312; BD Biosciences), rabbit anti-IP3R1 (407143; Calbiochem), goat antiphosphatidylserine synthase-1 (PSS1; sc51410; Santa Cruz), rabbit antiphosphofurin acidic cluster sorting protein-2 (PACS-2; HPA001423; Sigma Aldrich), rabbit anti–β-actin (Ab8227; Abcam), rat antitubulin (Ab6160; Abcam), rabbit anti-MAP2 (AB5622; Chemicon), mouse anti–β-tubulin (80016; Santa Cruz), mouse anti-BAF170 (17838; Santa Cruz), mouse anti-GAPDH (MAB374; Chemicon), and mouse anti-GFAP (MAB360; Chemicon).

Brain Tissue. Amyloid precursor protein $(APP)_{Swe/Lon}$ generated on a C57BL/6 F3 background (1) and WT mouse littermate brains from mice of different ages (2, 6, and 10 mo) were provided by Manfred Windisch (JSW Research, Graz, Austria). APP_{Arc} [B6CBA-Tg (Thy1.2-hAPParc)] 12-mo-old transgenic and WT mice were obtained from our in-house mouse model (2). Ethical permission was obtained from Animal Ethics Committee of South Stockholm Dnr S151-10 and S160-07. Human brain cortical tissue from Alzheimer's disease (AD) and control brains were provided by the Brain Bank at Karolinska Institutet. Ethical permissions were obtained from Regional Ethical Committee in Stockholm Dnr 024-01 and Dnr 2011/962-31/1. The mean age of the donors was 80 y, and the tissues were taken 10–30 h postmortem.

Cell Culture. Primary hippocampal neurons were derived from mouse embryos of embryonic days 16–17 (inbred strain C57B6/J) according to ref. 3. Ethical permission was obtained from the Animal Ethics Committee of South Stockholm Dnr S165-11 and S149-08. The hippocampal neurons were dissociated and plated on 35-mm glass-bottomed culture dishes (MatTek Corporation) coated with poly-D-lysine (Sigma Aldrich). They were plated at a density of 20,000 for immunocytochemistry (ICC) and 100,000 for siRNA experiments surrounded by a feeder layer of 200,000 cortical neurons and grown in 2 mL Neurobasal media [supplemented with 2% (vol/vol) B27 and 1% glutamine] at 37 °C. Astrocytes and other glial cells are present in these cultures to a limited extent. Astrocytes were identified by GFAP staining. Blastocyst-derived ES cells from mice lacking both presenilin genes (BD8 cells) were a gift from Dorit B. Donoviel (National Space Biomedical Research Institute, Houston, TX) and cultured according to the work in ref. 4. Chinese hamster ovary (CHO) cells, WT or stably transfected with the APPV717F AD mutation (CHO7PA2), were a gift from D. J. Selkoe (Harvard Medical School, Boston, MA). CHO cells and SH-SY5Y neuroblastoma cells were cultured in DMEM supplemented with 10% (vol/vol) FBS.

Subcellular Fractionation and Mitochondria-Associated ER Membrane Purification. Using the protocol from the work by Wieckowski et al. (5) to collect the fractions 20,000 × *g* (P3), 100,000 × *g* (P4), mito (percoll of P2), and mitochondria-associated endoplasmic reticulum (ER) membranes (MAMs; percoll of P2), mouse brains were cut into small pieces and homogenized using a Teflon pestle. Then, the homogenate was centrifuged two times at 800 × *g* to remove nuclei and debris. After 9,000 × *g* centrifugation, crude mitochondria pellet (P2) and S3 were collected. S3 was further centrifuged at 20,000 × *g* to generate the fraction 20,000 × *g* (P3) containing, for example, plasma membrane and thereafter, 100,000 × *g* to generate 100,000 × *g* (P4) containing, for example, ER. The crude mitochondrial fraction (P2) was put on a percoll gradient and centrifuged at 100,000 × *g* to separate mitochondria from MAM into two bands. The lower band containing mitochondria fraction mito (percoll of P2) and the upper band containing MAM fraction MAM (percoll of P2) were collected and further washed to remove percoll contaminants and mitochondria contamination from the MAM fraction. The fractions were lyzed in 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, and protease inhibitor mixture, pH 7.5 (RIPA buffer), and after protein determination, 25 μg protein from each fraction were loaded onto 4–12% (wt/vol) Bis·Tris gel (Invitrogen).

Synaptosome Preparation. To prepare synaptosomes, mouse cortexes from adult C57B6/J mice were rapidly dissected out and prepared according to ref. 6.

EM. The synaptosomes, hippocampus, and hippocampal neurons were fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer at room temperature, then dehydrated in ethanol followed by acetone, and embedded in LX-112 (Ladd). Ultrathin sections (~40–50 nm) were cut by a Leica ultracut UCT (Leica). Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10 transmission electron microscope (FEI Company) at 80 kV. Digital images were taken using a Veleta camera (Olympus Soft Imaging Solutions). Digital images were taken at a final magnification of 8,200×.

ICC. Hippocampal neurons were washed two times in PBS and subsequently fixed in 4% (vol/vol) formaldehyde solution in PBS for 10 min at room temperature. The neurons were permabilized in 0.1% Triton X-100 in PBS for 5 min, and epitopes were blocked for 30 min with 1% BSA in PBS. Primary antibodies were incubated overnight in a 1:100 dilution in 0.1% Triton X-100 at +4 °C. After washing in PBS (three times), the neurons were incubated with secondary antibodies (1:500) and/or phalloidin (1:40) for 30 min at darkness at room temperature. After washing in PBS (three times), a coverslip was mounted on top of the 35-mm glass-bottomed culture dish using mounting media containing DAPI (Vector Laboratories). The secondary antibodies Alexa-456, Alexa-555, and phalloidin-456 were purchased from Invitrogen.

Proximity Ligation Assay. Hippocampal neurons were fixed in 4% (vol/vol) formaldehyde solution as described above and incubated with anti-IP3R3 (rabbit) and anti-VDAC1 (goat) primary antibodies. Thereafter, the proximity ligation assay (PLA) probes anti-rabbit PLUS100 or MINUS100 and anti-goat PLUS100 or MINUS100 were added. Connector oligonucleotides were hybridized with the PLA probes, and after ligation, the signal was amplified by rolling circle amplification. The Duolink detection fluorophore Far-red ex644/em669 was used. For visualization of the neurons, phalloidin (Invitrogen Corporation) staining was also included. The fluorescent spots (PLA signals) were analyzed

using the software Duolink Imagetool (Olink Bioscience) by quantifying five pictures from each sample. As a negative control, the primary antibodies were omitted, and the PLA probe background staining was analyzed. The background staining was very limited for both probes.

Confocal Microscopy. The inverted microscope Axiovert 200M (Carl Zeiss MicroImaging) connected to an LSM510 META confocal unit and the LSM510 software system was used to analyze ICC and PLA staining as well as live cell imaging of GFP staining.

Knockdown Using siRNA. Hippocampal neurons seeded at a density of 100,000 cells per 35-mm poly-D-lysine–coated confocal glass-bottomed culture dish (MatTek) were grown for 6 d. BD8 cells were seeded in 60-mm culture dishes in a density to achieve 90% confluence at the day of transfection. For magnetic-assisted transfection, GFP (pEGFP-N1 vector U55762; BD Biosciences) and siRNA to PACS-2 or σ1R (Flexitube PACS-2 siRNA, gene ID: 217893, S10086564 and Flexitube σ1R siRNA, gene ID: 18391, S100197162; Qiagen) were mixed in Opti-MEM and NeuroMag according to the manufacturer's instructions (OZ Biosciences). As a control, AllStar negative control siRNA (1027280; Qiagen) was used. The final transfection solution containing 0.4 ng/μL GFP and 30 nM siRNA was added drop by drop to cells grown in 1 mL media and incubated for 25 min on a prewarmed magnetic plate at 37 °C. Thereafter, one-half of the hippocampal culture medium was changed to fresh medium, and cells were incubated at 37 °C for 16 h. For the BD8 cells, 80% of the media was changed, and cells were incubated for 24 h at 37 °C. The hippocampal neurons were either directly analyzed by live cell imaging or fixed in 4% (vol/vol) formaldehyde solution for 10 min, and GFP-positive neurons were examined by confocal microscope. BD8 cells were transfected in same way to study efficiency of knockdown. Cells were harvested 16 h posttransfection by scraping in PBS and pelleted by centrifugation at 1,500 × *g* for 5 min. Subsequently, they were lysed in RIPA buffer, and 30 μg protein were loaded onto a 4–12% (wt/vol) Bis·Tris gel.

Western Blot Analysis. Both human and mouse brain tissues were manually homogenized with a Teflon pestle in Hepes lysis buffer of 20 mM Hepes, 50 mM KCl, 2 mM EGTA, and protease inhibitor mixture, pH 7.4. Unbroken cells were spun down (800 × *g* for 5 min at 4 °C), and supernatants were further centrifuged at 12,000 × *g* for 15 min at 4 °C. Pellets were subsequently solubilized in RIPA buffer and incubated on ice for 30 min. Unsolubilized material was spun down at 4,000 × *g* for 5 min at 4 °C; 30 μg protein were loaded onto a 4–12% (wt/vol) Bis·Tris gel as described previously (4). The intensity of the bands was analyzed using the ImageJ software program.

Immunohistochemistry. Brain slices on microscopy slides stored at −20 °C or −80 °C were dried for 30–60 min at room temperature before washing two times for 1 min in tris buffer saline (TBS). Thereafter, brain slices were fixed for 5 min in 4% (vol/vol) formaldehyde solution followed by a short incubation (~1–3 min) in hydrogen peroxide containing solution [TBS, 30% (vol/vol) MeOH, 1% hydrogen peroxide] to quench endogenous peroxidase activity. After washing the slices two times for 5 min in tap water and two times for 5 min in TBS, grease pen was put around the brain slices, which were subsequently blocked with Background Punisher for 10 min at room temperature. The background punisher was removed, and the brain slices were washed in TBS (2 × 5 min) and incubated with the primary antibody (1:100 dilution in TBS) overnight at 4 °C. Slices were washed again, and biotinylated secondary antibody was put on for 30 min at room temperature followed by repeated washing in TBS and incubation with avidin-biotin complex solution for 30 min at room temperature. The staining of the brain slices was performed by incubation with 3,3′ diaminobenzidine solution for 3 min at room temperature. Slices were thoroughly rinsed in tap water, counterstained with hematoxylin for 10 s, and washed in tap water. Dehydration of the brain slices was done by successive incubation in 70%, 95%, and 99.5% EtOH [two times in 99.5% (vol/vol) EtOH] for 5 min each and terminatory two times in xylene for 10 min. Cover glasses were mounted using PERTEXsolution. Image acquisition of the immunohistochemically stained brain sections was performed with the Nikon Eclipse E800M microscope (linked to the NIS Elements F-software 3.0). Pictures were taken on sections including cortical parts, hippocampus, and the subiculum using 10–40× magnifications.

In Situ Hybridization in Tissue from Transgenic $APP_{Swe/Lon}$ Mice. For the preparation of coronal brain sections, $APP_{Swe/Lon}$ (2, 6, and 10 mo) transgenic and WT mouse brains stored at −80 °C were put into the cryostat for 30 min to reach the working temperature of −20 °C ± 1 °C. Brains were then mounted on the microtomes specimen holder and fixed with embedding freezing glue consisting of water-soluble glycols. The specimen holder was inserted into the specimen head, and 14-μm-thick sections were manually cut from $APP_{Swe/Lon}$ at prefrontal cortex (Bregma ~ 2.8–2.2) and anterior hippocampus region (Bregma ~ −1.4 to −2). Brain sections were thawed onto adhesive microscopy slides and stored at −20 °C until use. In brief, ^{33}P end-labeled oligonucleotides with a length of 50 nt were hybridized to sections from frontal cortex and anterior hippocampus. For each gene, two to four oligonucleotides complementary to different exons were tested, and the probes with the best signal-to-noise ratio were used for additional analysis. Hybridized sections were exposed for 3 wk to films, and signal densities were measured in the indicated brain areas using appropriate software. Bar graphs represent mean signals from four animals per group at 2, 6, and 10 mo of age.

Lipid Ratio Analysis of Transgenic $APP_{Swe/Lon}$ Mice and Human Brain. Lipids were extracted from 50 μg crude mitochondria from mice or human brain by adding 500 μL chloroform:methanol (2:1, vol/vol). The resulting organic phase was dried completely in a speedvac, and the samples were reconstituted in 50 μL chloroform:methanol (2:1, vol/vol). The lipids were separated for 1D thin liquid chromatography (TLC) carried out on a silica gel on 60 plates (20 × 20 cm, 0.5-mm-thick layer; Merk) using the solvent system chloroform:methanol:ammonia (60:30:5, vol/vol). The TLC plates were developed in an iodine vapor atmosphere, and the lipids of interest were identified by comparison with the spots with the commercial standards used.

Preparation of Aβ-Containing Cell Medium. CHO7PA2 cells (carrying the familial AD mutation, V717F, Indiana mutation) were cultured to obtain conditioned media (CM) containing oligomeric Aβ species as previously described (7, 8). The CHO7PA2 cells were grown in DMEM containing 10% (vol/vol) FBS in T75 culture bottles. At a cell confluence of 90%, one-half of the media was replaced by serum-free Neurobasal media. After acclimatization for 4 h, the whole media was discharged, cells were washed with PBS, and they were replaced with either 4 (CM*) or 8 mL (CM) serum-free Neurobasal medium per cell culture bottle. Cells were cultured for 16 h at 37 °C to obtain an Aβ concentration (total Aβ40 and Aβ42 concentrations) of ~2.5 (CM) and 4.5 nM (CM*), respectively. The CM was centrifuged at 1,500 × *g* for 5 min. The top media was collected and supplemented with 2% (vol/vol) B27 and 1% glutamine and used for treatment of hippocampal cells. For preparation of CHO7PA2-CM for treatment of SHSY5Y cells, CHO7PA2 cells were grown in DMEM for 16 h and used plain without supplementation.

ELISA. To measure the amount of Aβ40 and Aβ42 secreted into the conditioned media from the CHO7PA2 cells, sandwich ELISA was performed. Human/rat β-amyloid 40 and 42 ELISA kits from Wako (Wako Chemicals) were used, and the ELISA measurements were performed according to the manufacturer's instructions.

Aβ Treatment of Hippocampal Neurons and SH-SY5Y Cells. One-half of the hippocampal cell media was replaced by an equal amount of CHO7PA2-CM. Hippocampal neurons were incubated for 8 or 48 h at 37 °C. To analyze expression of MAM–mitochondria-associated proteins after Aβ treatment, the treated hippocampal neurons were fixed for immunocytochemistry according to above described protocol using VDAC1 and IP3R3. Confocal microscopy was used to measure the fluorescent intensity from the red and green channels. When acquiring the pictures from the different treatments, the same instrumental settings were used. Statistics are based on four independent experiments, in which five pictures from each sample were taken. SH-SY5Y cells were grown in serum-free DMEM 16 h before starting the exposure to CM*. One-half of the media was discharged and replaced by an equal amount of CHO7PA2-CM*.

Aequorin Ca^{2+} Measurements. SH-SY5Y cells were seeded on 13-mm coverslips and transfected with cytosolic or mitochondria-targeted aequorin constructs. After 24 h, cells were exposed to CHO7PA2-CM* (containing ∼4.5 nM Aβ) or tunicamycin (1 μg/mL) ± σ1 agonist (1 μM; SA4503 Cutamesine; M's Science Corporation) for 4 h. Thereafter, cells were incubated with coelenterazine (5 μM) for 1–2 h (to reconstitute aequorin) in 140 NaCl mM, 2.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, and 11 mM glucose, pH 7.4 (modified Krebs–Ringer buffer) and subsequently transferred to the perfusion chamber. Bradykinin (100 μM) was used to stimulate Ca^{2+} release from ER stores. All of the luminescence measurements were carried out in modified Krebs–Ringer buffer at 37 °C supplemented with $CaCl_2$ (1 mM) or EGTA (600 μM). The experiments were terminated by cell permeabilization with digitonin (100 μM) in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O) to discharge the remaining unused aequorin pool. The light signal was collected by a low-noise photomultiplier with a built-in amplifier discriminator (Thorn-EMI photon counting board) stored on an IBM-compatible computer and offline-calibrated into [Ca^{2+}] values as previously described (9).

To study if the treatments influence the uniporters in the inner mitochondrial membrane (IMM), we performed an experiment using the same procedure as described above. For permeabilization, cells were exposed for 1 min to digitonin (100 μM) in an intracellular medium containing 130 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 2 mM succinic acid, 1 mM $MgSO_4$, 20 mM Hepes, and 0.05 mM EGTA, pH 7.0 at 37 °C. After digitonin removal by 2 min of washing, mitochondrial Ca^{2+} uptake was estimated by mitochondria-targeted aequorin after cell perfusion with the same intracellular solution without EGTA and containing Ca^{2+} at different concentrations (2, 5, and 10 μM).

Statistical Analysis. Data were first analyzed for normal distribution; thereafter, statistical analysis was performed using Mann–Whitney *u* test or ANOVA followed by Games/Howell or Bonferroni posthoc test. Values are expressed as mean ± SD if not otherwise stated. *P* values <0.05 were considered to be significant.

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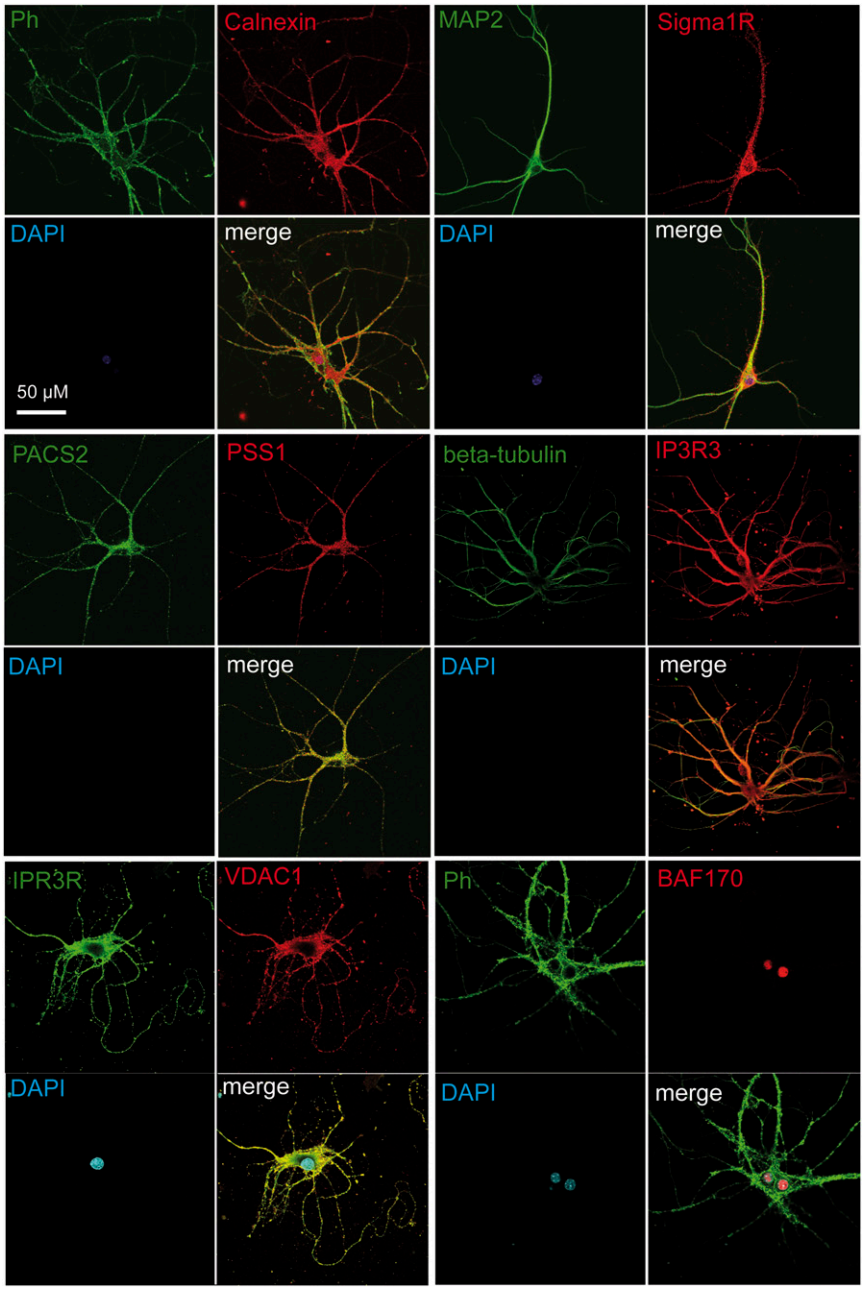

Fig. S1. Confocal images of the distribution of MAM-associated proteins (σ1R, PACS-2, PSS1, IP3R3, and VDAC1) in hippocampal neurons; 40× objective was used, and split images are shown. β-Tubulin, neuronal specific protein; calnexin, ER protein; MAP2, dendritic protein; Ph, phalloidin. As a negative control, BAF170 (nuclear protein) was used.

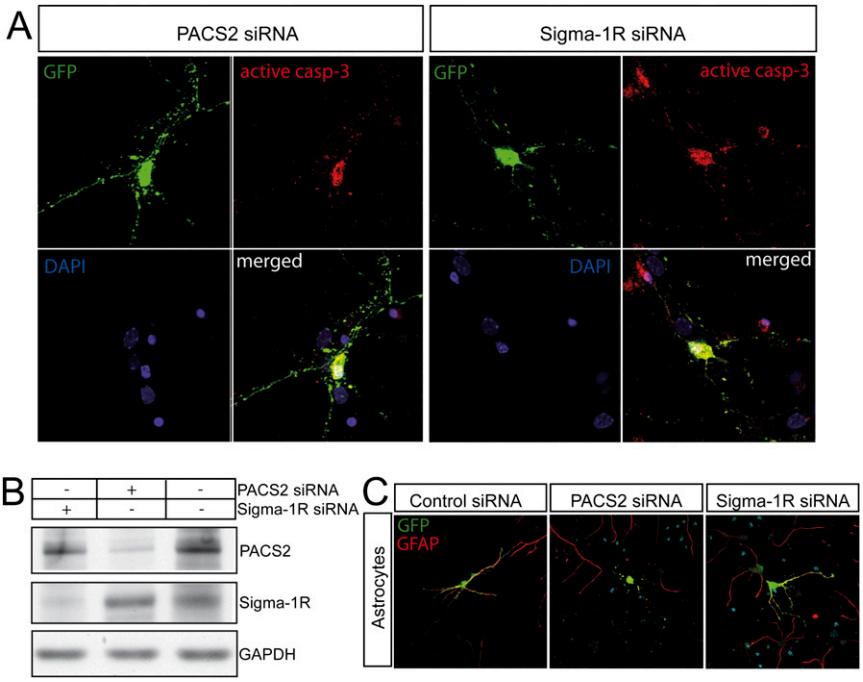

Fig. S2. MAM-associated protein depletion causes degeneration of hippocampal cultures. Hippocampal neurons and BD8 cells were transfected with AllStar negative control siRNA or siRNA to PACS-2 or σ1R (30 nM) together with pEGFP-N1 vector (0.4 ng/μL). (*A*) Fixed hippocampal neurons 16 h posttransfection stained for active caspase-3. (*B*) Western blot analysis of silencing efficiency of PACS-2 and σ1R siRNA in BD8 cells. GAPDH was used as loading control. (*C*) Hippocampal primary culture fixed 16 h after transfection with siRNA to PACS-2 or σ1R together with GFP. Astrocytes were visualized with the astrocytic marker GFAP (red).

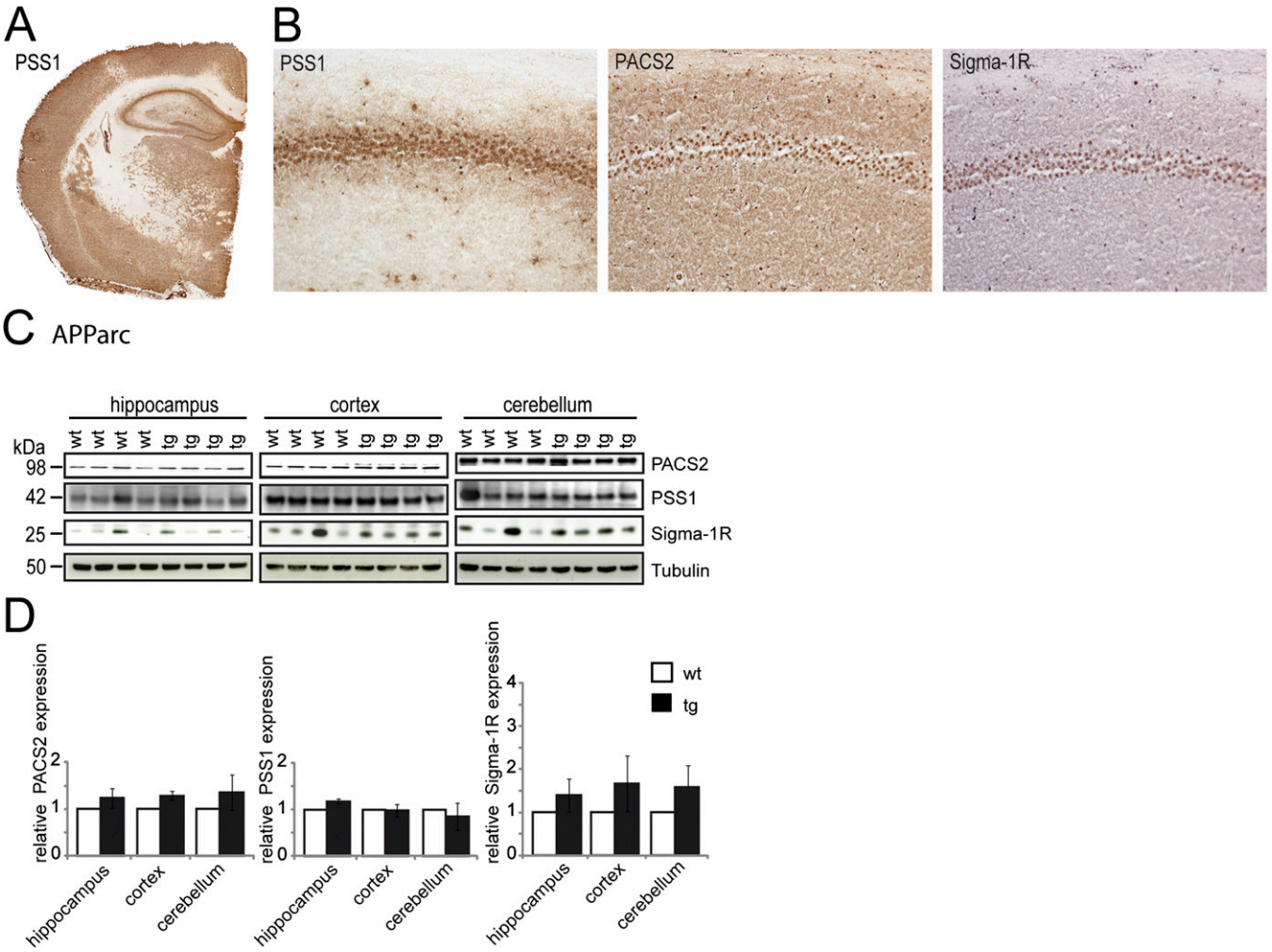

Fig. S3. Immunohistochemistry analysis of MAM-associated protein distribution in a 12-mo-old WT mouse at Bregma −1.46 revealed (*A*) widespread distribution of PSS1 in cortex and hippocampus and (*B*) neuronal staining for PSS1, PACS-2, and σ1R in the CA1 region of hippocampus. (*C* and *D*) Western blot quantification of expression of MAM-associated proteins normalized to tubulin in APP_{Arc} mouse brain (12 mo old). Data were compared using Mann–Whitney *u* test (mean ± SD, $n = 4$ for APP_{Arc}; $P < 0.05$).

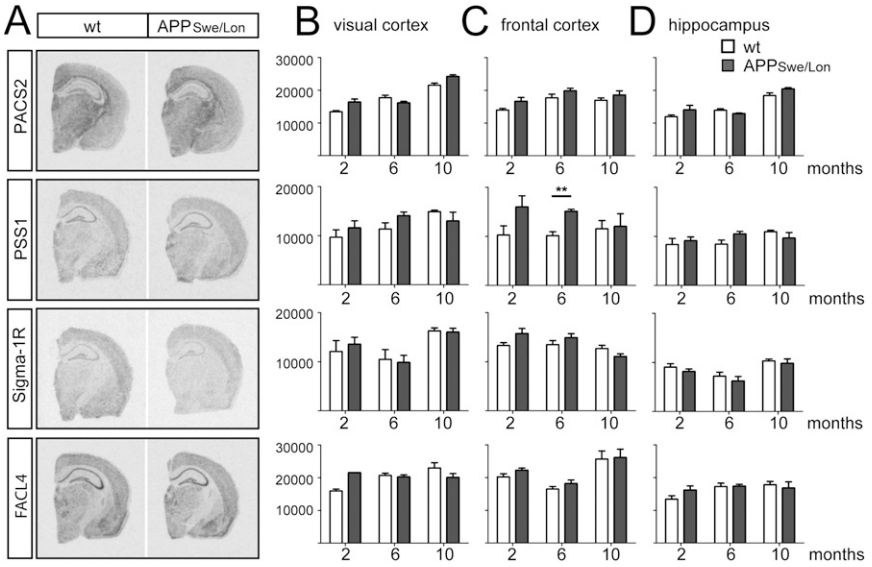

Fig. S4. In situ hybridization. (*A*) Representative staining in 6-mo-old WT and $APP_{Swe/Lon}$ mice. (*B*) Quantification of mRNA amount in visual cortex, (*C*) frontal cortex, and (*D*) hippocampus. Data were compared using ANOVA followed by Bonferroni posthoc test (mean ± SEM; $^{**}P < 0.01$).

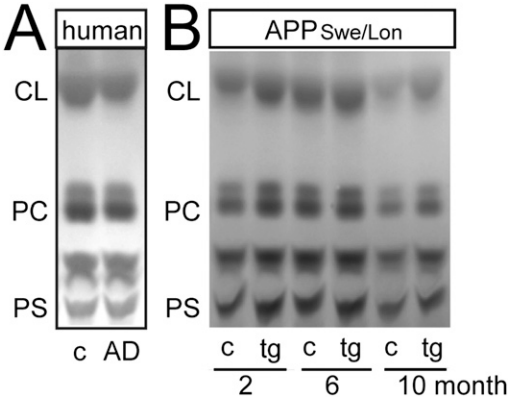

Fig. S5. TLC of expression levels of cardiolipin (CL), phosphatidylcholin (PC), and phosphatidylserine (PS) in (*A*) human postmortem cortical tissue (control and AD) and (*B*) mouse brain tissue from 2-, 6-, and 10-mo-old transgenic $APP_{Swe/Lon}$ and WT animals.