EXTENDED EXPERIMENTAL PROCEDURES

Primary Neuronal Cultures

Cortices and hippocampi were dissected, trypsinized, and plated in cell culture plates coated with poly-D-lysine and laminin (BD Biosciences) at a density of 5.0 × 10^5 cells/ml in the plating media (Neurobasal media (GIBCO/Invitrogen) containing 10% horse serum (Invitrogen), penicillin/streptomycin (Invitrogen) and Glutamax (Invitrogen)). And the plating media was replaced with the serum-free neuron culture media (Neurobasal media containing B-27 (Invitrogen), penicillin/streptomycin and Glutamax) 2 to 4 hr after plating. For NMDA or glycine experiments, to ensure that p25 generation was unlikely to be caused by any cellular stress resulting from the handling of cells, vehicle (PBS) was added to all control groups, which went through all the media changing and washing steps together with the treated groups. For Aβ experiments, oligomeric Aβ was prepared as described previously (Gräff et al., 2012). Primary cultured neurons were incubated in Aβ on DIV14 for 48 hr, and then fixed for immunocytochemistry.

Immunoblot Analysis

Primary neurons were lysed or hippocampi were homogenized in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors. Lysates were spun at 13,000 rpm for 15 min, after which supernatants were collected and analyzed for protein concentration (Bio-Rad Protein Assay). SDS buffer was added to equal amounts of protein.

Preparation of PSD Fractions

Hippocampi were homogenized in Solution A containing (mM) 320 sucrose, 6 Tris (pH 8.0), 1 $MgCl_2$ and 0.5 $CaCl_2$ with protease/phosphatase inhibitors using Teflon/glass homogenizer (H). The homogenate was then centrifuged at 1,400 × *g* for 10 min. Supernatants (S1) were saved and pellets were resuspended in Solution A (P1), and centrifuged at 710 × *g* for 10 min. Supernatants from the above two steps were combined and centrifuged at 710 × *g* for 10 min, and supernatants were further spun at 31,800 × *g* for 10 min. Supernatants were saved (S2), and pellets were resuspended in Solution B containing (mM) 320 sucrose, 6 Tris (pH 8.0) with protease/phosphatase inhibitors (P2). The homogenate was layered on a 0.85/1/1.5 M discontinuous sucrose gradient, and centrifuged at 82,500 × *g* for 2 hr at 4°C. The synaptosomal fraction was collected from between the 1 M and 1.15 M layers (LP1). Synaptosomes were further fractionated by adding Solution C containing 12 mM Tris (pH 8.0) and 1% Triton X-100, and centrifugated at 32,000 × *g* for 20 min. The PSD fractions were resuspended in 40 mM Tris (pH 8.0).

DNA Constructs

C-terminally triple-HA (3xHA) tagged wild-type p35, was created by first amplifying the p35 (*Cdk5r1*) sequence from a mouse BAC clone (RP23-252E4 clone obtained from the Children's Hospital of Oakland Research Institute; CHORI), using the primers MmutP35-NheI and MmutP35-4xh. This PCR product was subcloned into the pC2HA vector, a modified pDsRed1-N1 (Clontech) vector in which the RFP fragment is replaced by a two tandem HA epitopes. Addition of a third HA tag was achieved by first annealing the SalIHA-F and SalHA-R primers, followed by subcloning the third HA epitope before the stop codon of the p35-C2HA plasmid. C-terminally triple-HA tagged Δ93-98, A99L p35 (Δp35) was made by generating two PCR products using the MmutP35-NheI, MmutP35-2, MmutP35-3 and MmutP35-4xh primer pairs. These two PCR products were subcloned into the C2HA vector, to which the third HA tag was also added as described above.

HSV Production

The WT-p35-3xHA or Δp35-3xHA inserts were subcloned into the p1005+ vector using BamHI/XhoI. Herpes simplex viruses were packaged at the Viral Vector Core Facility at MIT, and neurons were transduced with HSV-WT-p35-3xHA or HSV-Δp35-3xHA for 24 hr before performing treatments.

Protein Stability

Protein stability was determined by treating the transfected SH-SY5Y cells with 5 μM cycloheximide for the indicated time-points.

Generation of Δp35-3xHA Knockin Mouse

In order to generate a targeting construct, the Δp35-3xHA construct was subcloned into the pL452 vector, containing loxP sites surrounding a neomycin cassette, using SalI/EcoRI. A 200 bp homology arm, encoding the 200 bp immediately following the p35 stop codon, was amplified from the RP23-252E4 BAC clone and subcloned into the NotI/SacII sites of pL452. Generation of the knockin cassette was carried out through two sequential recombination reactions. We employed this strategy after unsuccessfully trying to directly recombine our pL452-3xHA with our BAC clone. The first recombination reaction consisted of a plasmid intermediate using the TK-Amp vector (provided by Dr. Jianping Jin), in which we subcloned 55 nucleotide upstream and downstream homology arms. The homology arms were generated through sequential PCR reactions using Taq polymerase, first using the P35L_in/P35S_in and EGFPc1 as a template. This fragment was then purified and amplified using the P35L_out/P35S_out. This PCR product was then transferred onto the TOPO TA vector (Invitrogen), and then subcloned into the TK-Amp vector using NotI/KpnI. This plasmid intermediate was linearized using XhoI and the purified DNA was used to recombine with the RP23-252E4 BAC using lambda

phage-based recombineering (Red/ET; Gene Bridges GmbH), effectively subcloning the genomic region comprising the long and short homology arms flanking the p35 gene into the TK-Amp vector. We then performed a second recombineering reaction using a positive TK-Amp-BAC clone with the AflI/SalI/SacII linearized Δp35-3xHA- loxP-neo-loxP-200 bpHomo. Positive clones were confirmed using HindIII and KpnI, and by sequencing using multiple primers encompassing the Δp35, neo cassette, and 3′UTR region. The correctly identified clones were linearized using EcoRV, and this targeting construct was recombined onto mouse ES cells using standard procedures. Successful targeting in ES cells was confirmed by Southern blot. Probes were generated outside the 13.5 kb homologous region. The 5′ probe (P1; P35_P1_For2, P35_P1_Rev2), upstream of the long homology arm, was ~500 bp, while the 3′ probe (P2; P35_P2_For2, P35_P2_Rev1), just downstream of the short homology arm, was ~600 bp. Wild-type DNA digested with HindIII and EcoRV and probed with P1 yielded a 10.6 kb band, while the Δp35KI band was 12.6 kb. Probing against P2, after digestion of genomic DNA with EcoRI and SpeI yielded a 4.4 kb or 3.9 kb band in DNA from wild-type and Δp35KI, respectively. Positive ES cell clones were injected onto blastocysts from pseudopregnant females. Chimeric mice were crossed to wild-type C57BL/6J mice, and germline transmission was assessed using Southern blot and genotyping PCRs. Positive Δp35-3xHA-loxP-neo-loxP heterozygote males were crossed to Meox2-Cre females. Adequate removal of the neomycin cassette was confirmed by PCR. Mice were backcrossed with C57BL/6J mice for at least five generations.

Microscopy

All images were captured using a Zeiss LSM 510 confocal microscope and LSM 510 software, and analyzed using ImageJ software.

Calcium Imaging

Primary cultured neurons were prepared from WT or Δp35KI embryos, and experiments were performed on DIV 14 to 16. Neurons on coverslips coated with poly-D-lysine and laminin were incubated in Tyrode's solution (in mM: 129 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 HEPES, 30 glucose, pH 7.4) containing 5 μM Fluo4-AM (Invitrogen, diluted in Tyrode's from 4.55 mM fresh stock in DMSO/20% F-127) for 45 min in the dark on the stage of a Nikon TE-2000S inverted microscope inside an environmental chamber (In Vivo Scientific) at room temperature and 5% CO_2. The solution was replaced with Tyrode's solution not containing Fluo4-AM and the cells were incubated for an additional 45 min at room temperature for dye de-esterification. Imaging was conducted inside the environmental chamber at room temperature and a 5% CO_2 atmosphere, and the cells were perfused with Tyrode's solution at a rate of 2 ml/min. After a baseline of 60 s, NMDA (50 μM) was added. With a standard FITC excitation filter, time-series images were captured using a Photometrics CoolSnap EZ camera controlled by the NIS-Elements AR2.3 software package. Time series experiments consisted of 600 512 × 512 frames with an interval of 1 s. Image stacks were exported to ImageJ (NIH), and fluorescence intensities were measured. Only cells that responded to KCl (30 mM) at the end of the imaging session were quantified. Measurements shown represent $\Delta F = F/F_o$ (increase in fluorescence over baseline following background subtraction).

Real-Time PCR

Mouse cortex was homogenized and total RNA was extracted using an RNeasy plus mini kit (QIAGEN) following the manufacturer's instructions. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen), and Quantitative real time PCR was performed on a thermal cycler (Bio-Rad) using SYBR green (Bio-Rad) and gene-specific primers (Table S2). The Ct values were determined to address the difference in gene expression and all values were normalized to *Gapdh*.

Behavioral Experiments

Open Field Test

Activity in an open field (41 cm × 40 cm × 30 cm) was measured over a 30 min period with an AccuScan Instruments VersaMax Animal Activity Monitoring System. Time spent in margin and center, total distance, and horizontal activity were measured in 1 min bins using automated activity monitors.

Light-Dark Exploration Test

A testing box (41 cm × 41 cm × 30 cm) was divided into two sections of equal size with a partition that allowed for free movement from one side to the other side. One chamber was brightly illuminated, while the other chamber was dark. Mice were placed into the dark side, and measurements were taken for latency to enter the light side, total number of transitions, and total amount of time in the light chamber.

Elevated Plus-Maze Test

Activity in an elevated plus maze apparatus (each arm being 45 cm × 9.5 cm, and the height of the closed arms being 29.5 cm) was measured over a 5 min period. The time spent in the open arms and the closed arms were measured using the Noldus tracking system.

Morris Water Maze Test

Spatial reference memory testing was performed in a circular tank (diameter 1.2 m) filled with opaque water, 22°C. The walls surrounding the tank contained bright and contrasting shapes that served as reference cues. The tank contained a fixed platform (10 cm diameter) in a target quadrant. On the test days, the platform was submerged and the mice were place into the maze at one of four points randomly facing the wall of the tank. Mice were allowed to search for a platform for 60 s and, if they did not find a platform, they were gently guided to it. Animals were kept on the platform for 10 s. Two trials a day were conducted with at

least 1 hr intertrial interval. Between the trials, mice were warmed on a heating pad. Mouse behavior was video-recorded and analyzed using Videomot 2 software (TSE systems). The escape latency, an indication of learning progress, was scored for each trial. On day 9 of the experiment, the platform was removed and a memory test (probe test) was conducted. The time spent in each of the 4 quadrants and the number of crossing the area, where the platform used to be, was recorded automatically. Repeated probe tests were performed on day 10 and 11 for examination of memory extinction.

Fear Conditioning Test

For analysis of Δp35KI mice and their WT littermates, mice were put in the conditioning chamber (TSE systems) for 3 min, after which they received a one-time 2 s foot-shock (0.8 mA). Animals were then left in the box for another 30 s. One day later, mice were re-exposured to the training context for 3 min and their freezing behavior was scored for memory acquisition test. The following 5 days, mice were also put into the same context for 3 min and their freezing behavior was scored for examination of memory extinction. For analysis of WT, 5XFAD, 5XFAD;Δp35KI and Δp35KI mice, they were put in the conditioning chamber for 3 min, and then cue (sound) was applied for 30 s right before a one-time 2 s foot-shock. One day later, mice were re-exposured to the training context for 3 min without cue to address contextual fear memory. The following day, mice were exposure to novel chamber, and then cue was applied for 3 min and their freezing behavior was scored for examination of cued memory.

Novel Object Recognition

The novel object recognition task consisted of habituation, training, and testing phases performed on separate days. Before training, mice were habituated to the open testing arena (40 cm × 40 cm × 35 cm) for 10 min per day for three days. No data was recorded during habituation. During training, mice were placed into the same box for 10 min with two identical objects placed in the left and right corners. Object memory was tested 24 hr later by using the same procedure as on the training day but replacing one of the objects with a novel one and measuring exploration over a 5 min session. Object exploration was recorded when the front paws or nose contacted either object and was expressed by a discrimination index, $DI = T_{novel} / (T_{novel} + T_{familiar})$, where T_{novel} and $T_{familiar}$ indicate the time spent with the novel and familiar object, respectively.

Electrophysiology

The brain was rapidly removed and transferred to ice-cold, oxygenated (95% O_2 and 5% CO_2) dissection buffer containing (mM) 211 sucrose, 3.3 KCl, 1.3 NaH_2PO_4, 0.5 $CaCl_2$, 10 $MgCl_2$, 26 $NaHCO_3$ and 11 glucose. Transverse hippocampal slices were prepared by cutting with a Leica VT1000S vibratome (Leica) and transferred for recovery to a holding chamber containing oxygenated ACSF consisted of (mM) 124 NaCl, 3.3 KCl, 1.3 NaH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgCl_2$, 26 $NaHCO_3$ and 11 glucose at 28-30°C for at least 1 hr before recording. For extracellular recording, CA1 field potentials evoked by Schaffer collateral stimulation with bipolar electrode were measured. After recording of stable baseline for at least 20 min, LTP was induced by repeated theta-burst stimulation (TBS, containing 10 brief bursts which consisted of four pulses at 100 Hz), or LTD was induced by single-pulse low-frequency stimulation (LFS, 1 Hz for 900 s). For intracellular recording, CA1 pyramidal neurons were held at −70 mV with recording pipettes, which containing (mM) 145 CsCl, 5 NaCl, 10 HEPES-CsOH, 10 EGTA, 4 MgATP and 0.3 Na_2GTP. TTX (1 μM) and picrotoxin (50 μM) were added to ACSF for mEPSC measurements, and picrotoxin was replaced with CNQX (10 μM) and D-APV (50 μM) for mIPSC measurements. For Aβ experiment, oligomeric Aβ was prepared as described previously (Gräff et al., 2012). Recordings were performed using an AM-1800 Microelectrode amplifier (A-M systems) for extracellular recording, or a MultiClamp 700B amplifier (Axon Instruments) with a Digidata 1440A A-D converter (Axon Instruments). All data were digitized and analyzed by the use of pClamp 10 software (Axon Instruments).

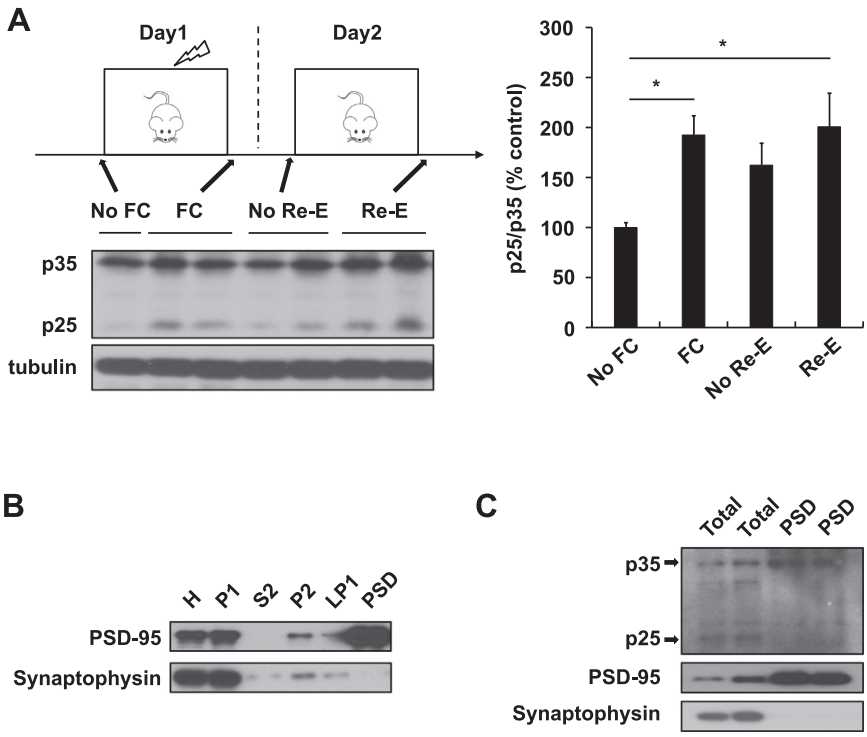

Figure S1. Fear-Conditioning-Induced p25 Generation and the Liberation of p25 from Hippocampal PSD, Related to Figure 1

(A) WT mice were sacrificed either before or after FC, and with or without re-exposure to the conditioning chamber one day after FC. (Right) The bar graph represents relative immunoreactivity of p25/p35 compared to control, n = 8 per group.

(B) PSD-95 and synaptophysin distribution patterns in hippocampal PSD fractions.

(C) The levels of p35 and p25 in total hippocampal lysates or PSD fractions from WT mice.

(*$p < 0.05$ by Student's t test; error bars ± SEM).

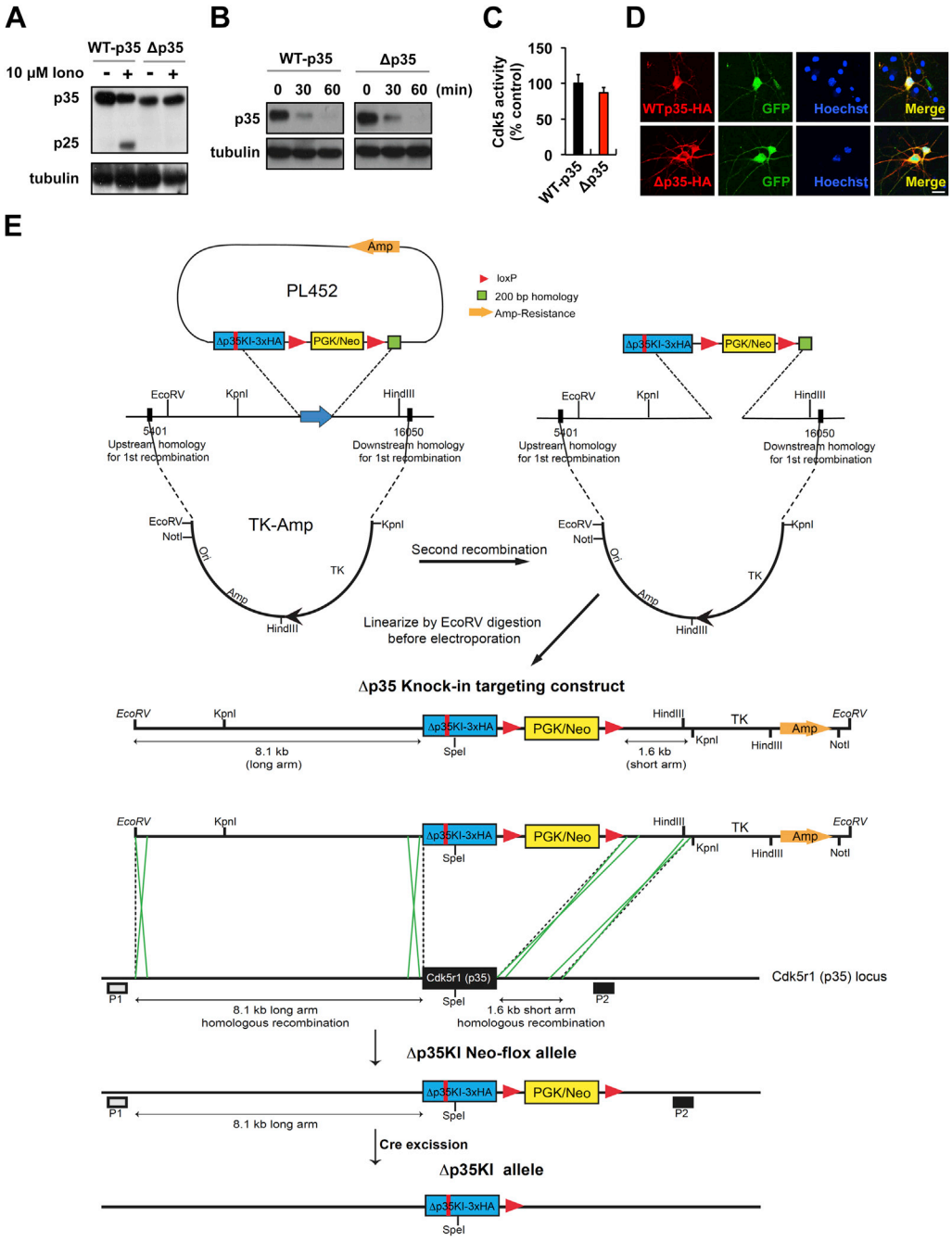

Figure S2. Generation of Cleavage-Resistant p35 Mutant (Δp35) and Δp35KI Mice, Related to Figure 2

(A) SH-SY5Y cells were transfected with either WT-p35 or Δp35, and treated with ionomycin for 1 hr.

(B) Half-life experiments were performed with the protein synthesis inhibitor cycloheximide (5 μM) in SH-SY5Y cells following transfection with WT-p35 or Δp35.

(C) Cdk5 kinase assay was performed on cell lysates from SH-SY5Y cells transfected with WT-p35 or Δp35.

(D) Subcellular localization of WT-p35 or Δp35 protein in cultured hippocampal neurons after infection with HSV virus expressing WT-p35-3xHA or Δp35-3xHA. Scale bar = 10 μm.

(E) Strategy of generating the Δp35KI mouse line (See Extended Experimental Procedures).

Error bars, ± SEM.

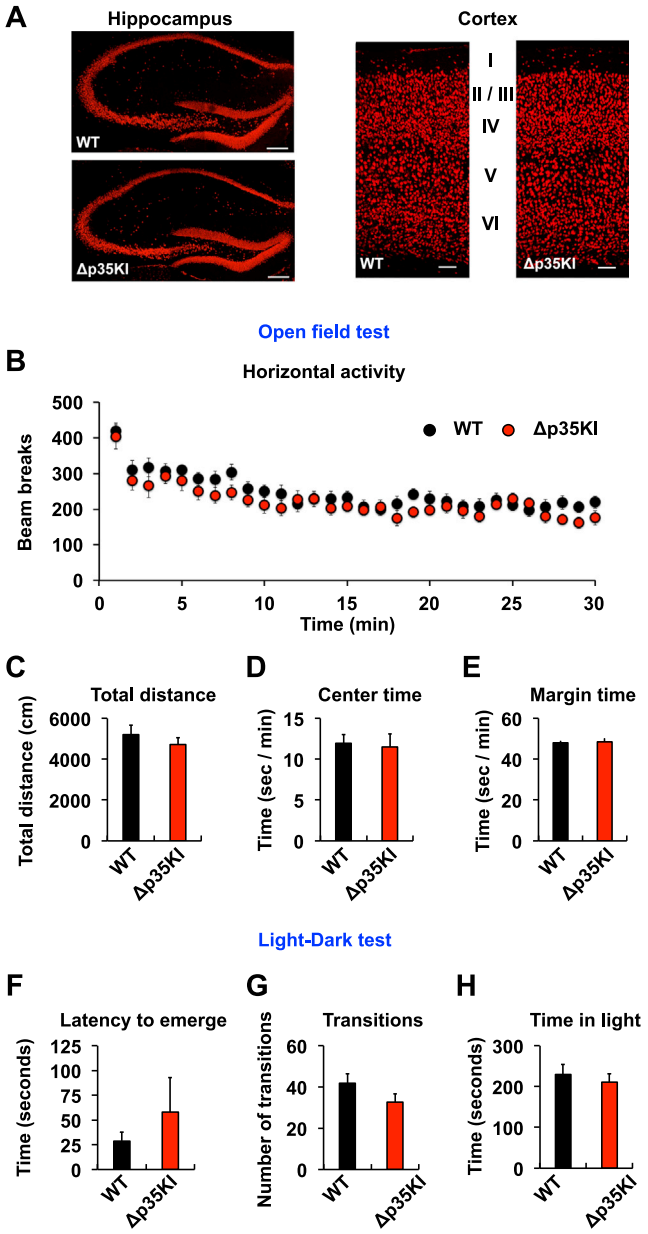

Figure S3. Δp35KI Mice Exhibit Normal Neurodevelopment and Exploratory Behavior, Related to Figure 2

(A) Brain slices from WT or Δp35KI mice were stained with an anti-NeuN antibody. Scale bar = 100 μm.

(B–E) Open field test was performed with WT and Δp35KI mice (11 mice per group).

(B) Horizontal activity of WT and Δp35KI mice.

(C) Total distance traveled in the chamber during the test.

(D) Time spent in the center of the chamber.

(E) Time spent in the margin of the chamber.

(F–H) Light-dark test was performed with WT and Δp35KI mice (6 mice per group).

(F) The latency to emerge from the dark during the test.

(G) The number of transitions from the light to the dark side.

(H) Time spent in the light.

Error bars, ± SEM.

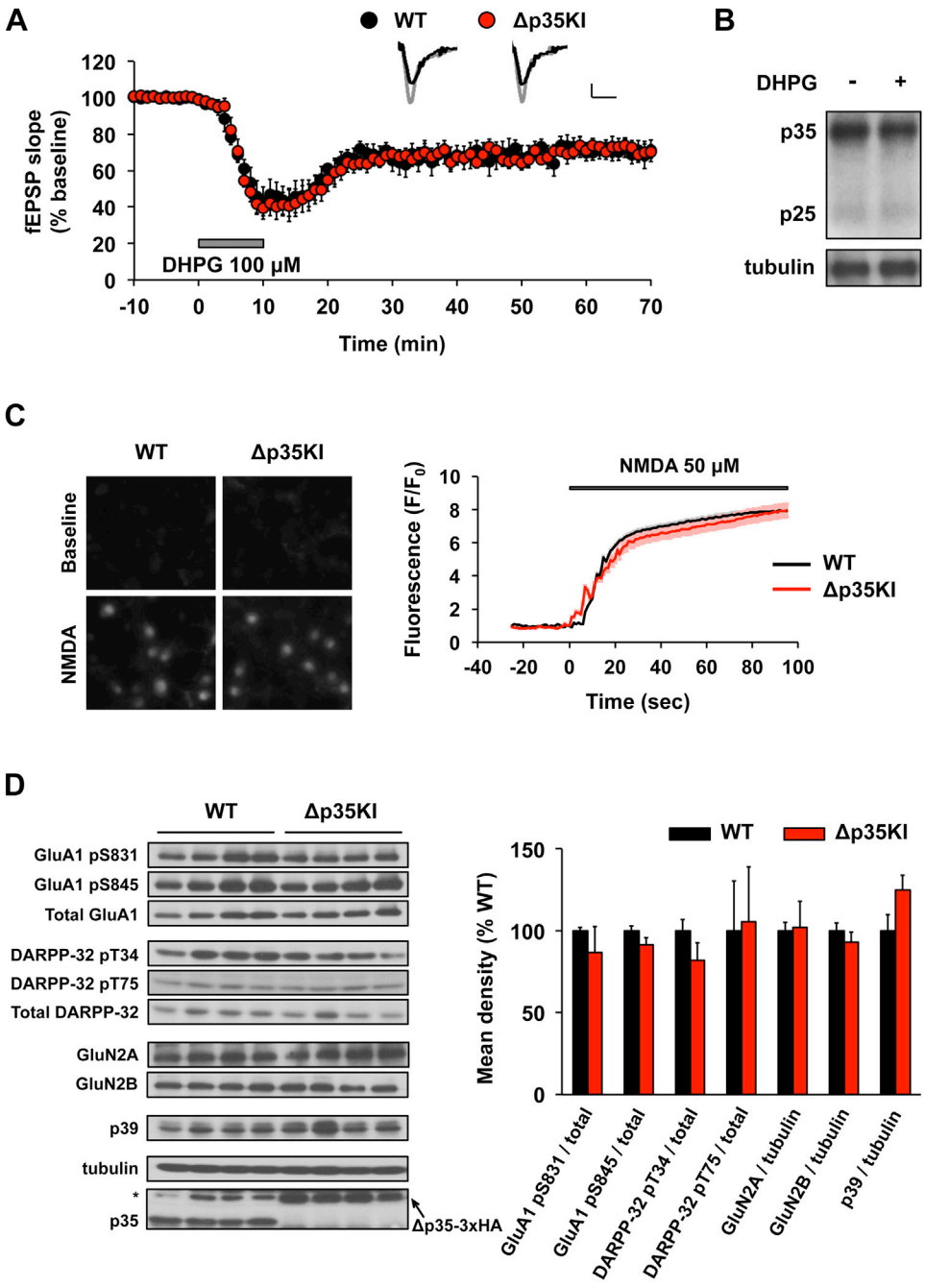

Figure S4. Normal DHPG-Induced LTD, NMDA-Induced Intracellular Calcium Flux, and Expression of Synaptic Proteins in the Δp35KI Mouse Hippocampus, Related to Figures 3 and 4

(A) LTD was induced by DHPG (100 μM for 10 min) at Shaffer collateral-CA1 synapses (WT: 70.6% ± 5.0%, 5 slices from 3 mice; Δp35KI: 71.8% ± 3.7%, 5 slices from 3 mice). Scale bar = 0.5 mV and 10 ms. Sample traces represent fEPSPs at 1 min before (gray) and 1 hr after (black) DHPG treatment.

(B) DHPG was applied to acute hippocampal slices for 10 min and slices were allowed to recover for an additional 15 min before lysed for immunoblotting.

(C) Intracellular calcium flux in WT and Δp35KI neurons on DIV 14-16 following NMDA (50 μM) treatment. (Right) The bar graph represents $\Delta F = F/F_o$ (increase in fluorescence over baseline following background subtraction), n = 135-150 neurons per group.

(D) Basal levels of various synaptic proteins in WT and Δp35KI hippocampus. The asterisk represent a nonspecific background band. (Right) Relative immunoreactivity of indicated proteins was normalized to WT.

Error bars, ± SEM.

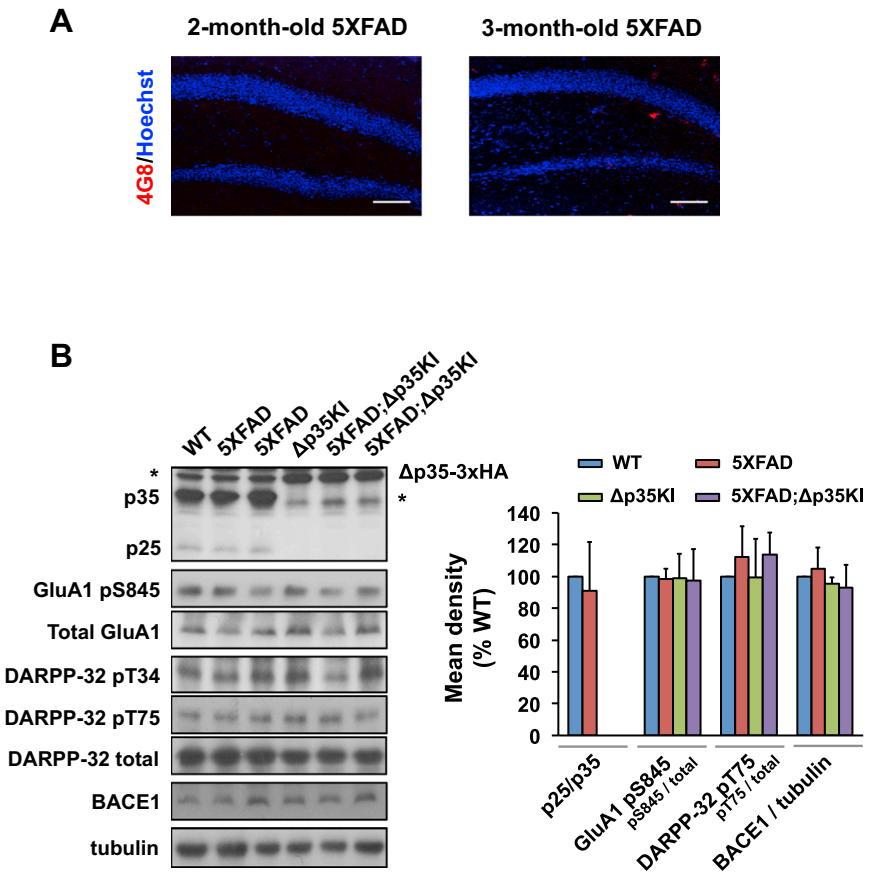

Figure S5. Normal p25, GluA1 pS845, DARPP-32 pT75, and BACE1 Levels in the 5XFAD Mouse Hippocampus Prior to Aβ Plaque Deposition, Related to Figure 5

(A) Hippocampal slices from 2-month-old or 3-month-old 5XFAD mice were stained with an anti-4G8 antibody. Aβ deposition in hippocampus of 5XFAD mice becomes apparent at 3 months of age. Scale bar = 50 μm.

(B) The levels of p25/p35, GluA1, DARPP-32 and BACE1 in the hippocampus of 2-month-old WT and 5XFAD mice. (Right) Relative immunoreactivity of indicated proteins was normalized to WT, n = 3-6 per group. The asterisks represent nonspecific background bands.

Error bars, ± SEM.

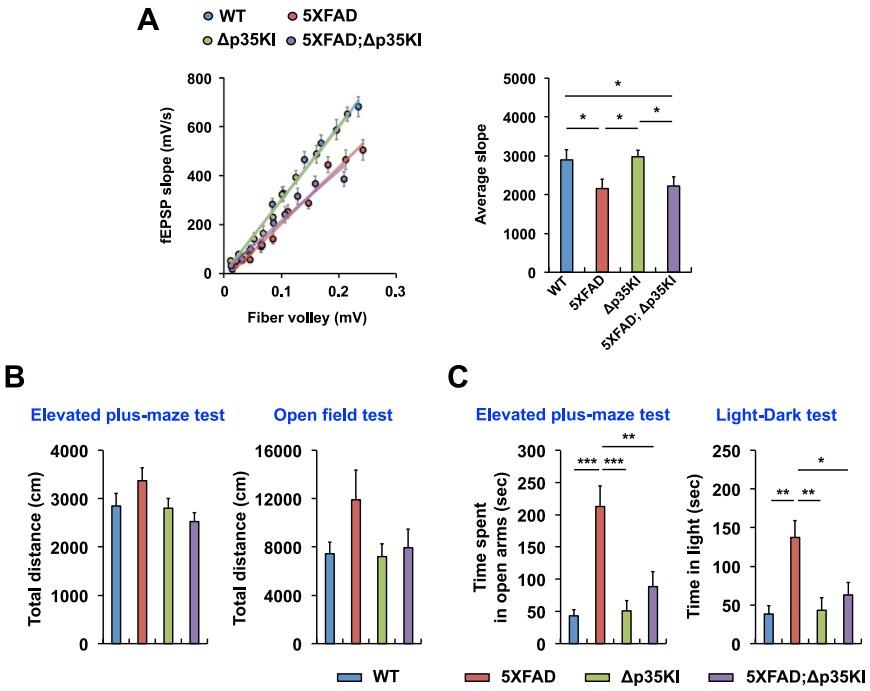

Figure S6. Inhibition of p25 Generation Is Ineffective in Impaired Basal Synaptic Transmission, but Rescues Reduced Anxiety in 5XFAD Mice, Related to Figure 6

(A) Input-output ratio was examined at Shaffer collateral-CA1 synapses, and fPESPs were plotted against presynaptic fiber volley amplitude. ($p < 0.05$ by ANOVA).

(B) Total distance traveled in the elevated plus-maze or in the open field.

(C) Time spent in open arms during the elevated plus-maze test or in the light side during light-dark test. (Elevated puls-maze test, WT: 43.2 ± 9.4 s, n = 6; 5XFAD: 212.8 ± 31.6 s, n = 6; Δp35KI: 50.9 ± 15.5 s, n = 7; 5XFAD;Δp35KI: 88.5 ± 23.6 s, n = 5, $p < 0.001$ by ANOVA; Light-dark test, WT: 38.3 ± 11.0 s, n = 6; 5XFAD: 137.3 ± 21.5 s, n = 6; Δp35KI: 43.1 ± 15.9 s, n = 7; 5XFAD;Δp35KI: 63.0 ± 16.5 s, n = 5, $p < 0.01$ by ANOVA).

(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Tukey's post hoc analysis; error bars ± SEM).

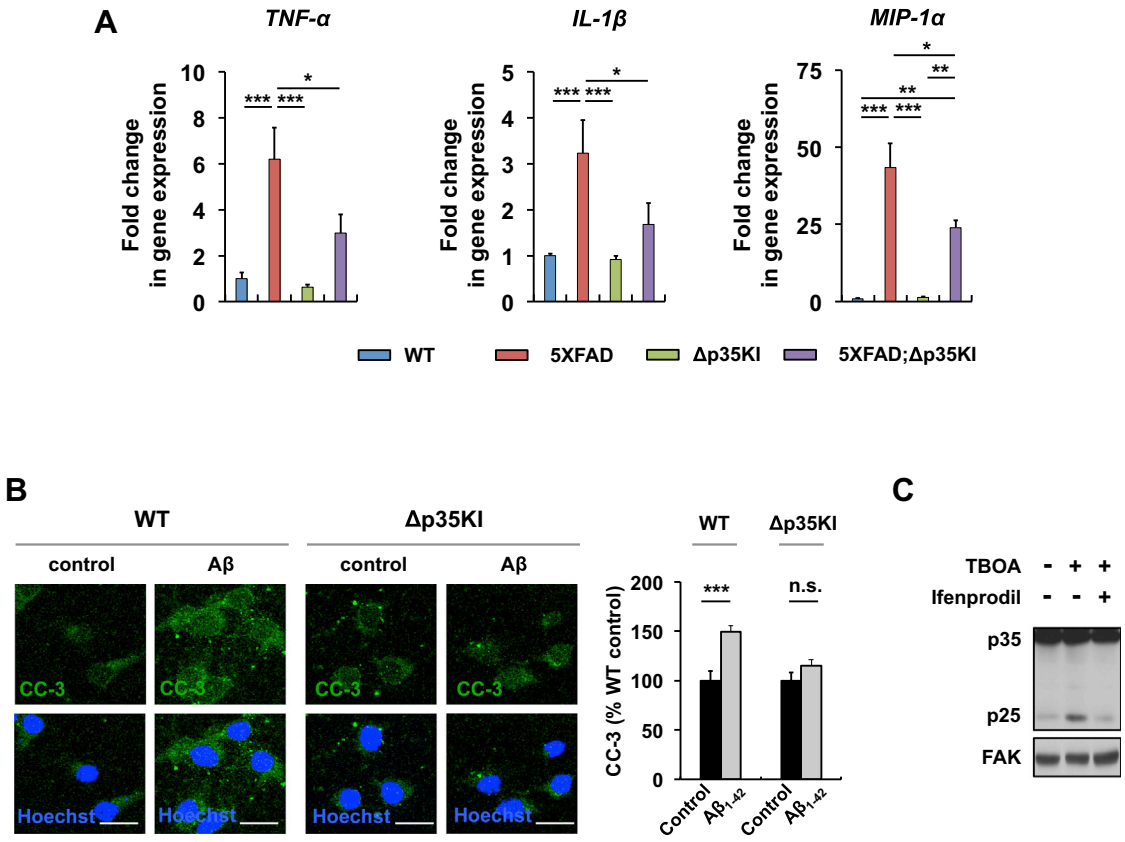

Figure S7. Suppression of Aβ-Induced Elevation of Cytokine/Chemokine mRNA Levels and Caspase-3 Activation by Blockade of p25, and p25 Generation Following Inhibition of Glutamate Uptake, Related to Figure 7

(A) Cytokines/chemokines levels in WT, 5XFAD, Δp35KI and 5XFAD;Δp35KI mice. ($p < 0.001$ by ANOVA).

(B) WT or Δp35KI neurons were stained with an anti-cleaved caspase-3 (CC-3) antibody following $A\beta_{1-42}$ (2.5 μM) treatment. Scale bar = 10 μm. (Right) The bar graph represents relative immunoreactivity of CC-3 compared to each untreated control, n = 10 per group. (Student's t test).

(C) p25 generation in acute hippocampal slices following TBOA (100 μM) treatment with or without preincubation with ifenprodil (10 μM) for 1 hr.

(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student's t test or Tukey's post hoc analysis; error bars ± SEM).