Supporting Information

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SI Materials and Methods

Antibodies and Chemical Reagents. Antibodies against α-tubulin (no. 2125), APC (no. 2504), AXIN1 (no. 2087), α-tubulin (no. 2144), β-catenin (no. 9562), phospho-β-catenin (Ser33/37/Thr41; no. 9561), GSK3β (no. 9832), phospho-GSK3β (Ser-9) (no. 5558), and hemagglutinin (HA; no. 3724, no. 2367) were from Cell Signaling Technology. Anti–actin (sc-1616), GFP (sc-8334), phospho-GSK3β (Tyr-216) (sc-135653), CDK5 (C8), GAPDH, and p35/p25 (sc-820) were from Santa Cruz Biotechnology. Anti–Synapsin I (AB1543) was from Millipore (anti–FLAG (F1804) was from Sigma-Aldrich (and anti–phospho-Tau (AT8) (MN1020) was from Thermo Scientific. Anti–phospho-Tau (PHF-1) was a generous gift from Peter Davies, Albert Einstein College of Medicine, New York. Anti–phospho-Tau(T181) and anti–phospho-Tau(S396) were purchased from Abcam. Unless specified, all chemicals were obtained from Sigma-Aldrich.

Cortical Brain Tissue Isolation and Primary Neuronal Culture. Cortical brain tissue isolation was performed in adult mice of various ages. Mice were first anesthetized by i.p. administration of 1.25% (vol/vol) Avertin at a dosage of 30 mL/kg body weight. The heart of each mousse was then exposed, the left chamber was catheterized, and the right atrium was opened. Physiological saline was perfused transcardially for 5 min to remove blood from the body. After perfusion, the cranial bones were opened; the cortex of whole brain tissue was harvested, snap-frozen in liquid nitrogen, and stored in −80 °C before use.

Embryonic cortical neurons were isolated by standard procedures as reported previously (1). Briefly, embryos were collected in ice-cold PBS glucose, and the cortical lobes were dissected out. Meninges were removed, and the cortices were placed in 1× trypsin-EDTA for 10 min at 37 °C. The tissue was removed from the trypsin solution and placed in DMEM with 10% (vol/vol) FBS to inactivate the trypsin, followed by transfer to neurobasal media supplemented with B-27, penicillin/streptomycin (1×), and L-glutamine (2 mM; Invitrogen). Tissue was triturated 10× through a 5-mL pipette and allowed to settle to the bottom of a 15-mL conical tube. Cells in the solution above the 1 × G pellet were removed. Surviving cells were counted with trypan blue to identify dead cells and were plated on poly-L-lysine–coated (0.05 mg/mL) glass coverslips. Cells were plated in 24-well plates at 50,000 cells per well and allowed to mature for over 10 d in vitro (DIV10) before performing any experiments.

Stereotactic Injection. Male, adult wild-type mice were first anesthetized and placed on a stereotaxic apparatus (RWD Life Science), then 1 μL of kainic acid (1 μg/μL) or saline controls were injected into the right lateral ventricle (anteroposterior, −1.0 mm; mediolateral, −0.5 mm, dorsoventral, −2.0 mm) in a duration of 5 min (2). The needle was left in place for 10 min after the injection before being withdrawn to reduce backflow. Incision was stitched and mice were allowed to recover. After resting for 24 h, mice were killed and the hippocampus brain sections were harvested and fixed with 4% (wt/vol) PFA.

In Utero Electroporation. For in utero electroporation (IUE), expression plasmids under the control of the CAG promoter were used. IUE was conducted as previously described (3). In brief, 2 μL of plasmid mix containing Fast Green Dye (Sigma Aldrich) was injected and electroporated into the lateral ventricle of E14.5 embryos using the ElectroSquarePorator ECM 830 (BTX; Genetronics), preset at five 50-ms pulses of 40 V with 950-ms intervals. Forty-eight hours after electroporation, electroporated embryonic brains were dissected and fixed overnight in 4% (wt/vol) PFA at 4 °C, and then placed in 30% (wt/vol) sucrose in PBS for cryoprotection. Brains were frozen and sectioned coronally at 12–20 μm.

Plasmid Construction. HA-tagged CDK1-, CDK2-, CDK3-, CDK4-, CDK5-, CDK6-, and HA-GSK3β plasmids were purchased from Addgene. GFP-CDK5, GFP-GSK3β, GFP-ERK2, GFP-p35, GFP-p25, and GFP-p10 were generated by PCR in EGFP-N1 or pLVX-TRE3G-ZsGreen1 vector (Clontech). mCherry-p25 and mCherry-p35 were generated by PCR in pLV-mCherry vector (Addgene) or pLVX-TRE3G-mCherry vector (Clontech Laboratories, Inc.). HA-GSK3β truncations were fused with HA tag of pCMV-HA vector (Clontech); cyclin A and cyclin D1 were fused with EGFP in pEGFP-C1 (Clontech). Expression of gene produces on pLVX-TRE3G-ZsGreen1 and pLVX-TRE3G-mCherry vector were controlled by pLVX-Tet-OFF advanced vector purchased from Clontech. The generosity of many laboratories allowed us to assemble a majority of the vectors used in this study. pSC2-GFP-GSK3-MAPK and pSC-GFP-GSK3mut-MAPK were from Edward De Robertis (University of California, Los Angeles) (4).

Cell Culture and Transfection. Neuro2A (N2a, ATCC CCL-131) cells were cultured in DMEM/10% (vol/vol) FBS/penicillin/streptomycin medium (Life Technologies). Primary neurons were isolated from E16 embryos of B6 mice and cultured as described above. DNA constructs were transfected with Lipofectamine 2000 or Lipofectamine LTX with Plus Reagent into N2a cells and primary neuronal culture, respectively. Following the manufacturer's protocol, 6 h after transfection, cells were refreshed with culture medium and further incubated overnight for 18 h to allow recovery and ectopic expression. For siRNA pretreatments, Cy-5 labeled Accell siRNA (GE Dharmacon) were added and incubated overnight for 24 h according to manufacturer's manual, followed by transfection as mentioned above. Specific sequences of siRNA ordered were listed as below:

siRNA name	Sequence
GSK3β-1	Sense: 5′-UUGUCAGGCCUAAAGGUUAUU-3′
	Antisense: 5′-pUAACCUUUAGGCCUGACAAUU-Dy647-3′
GSK3β-2	Sense: 5′-GUGCUUGCCUGUAAAAUUUUU-3′
	Antisense: 5′-pAAAUUUUACAGGCAAGCACUU-Dy647-3′
CDK5	Sense: 5′-UUGUCAGGCUUCAUGAUGUUU-3′
	Antisense: 5′-pACAUCAUGAAGCCUGACAAUU-Dy647-3′

FRET–Acceptor Photobleaching. Primary neuronal culture at DIV12–13 cultured in a 35-mm glass-bottom culture dishes (MatTek Cooperation) were cotransfected with two constructs (GFP-GSK3β/GFP-CDK5/GFP-ERK2 or mCherry-p25/mCherry-p35) using Lipofectamine LTX with Plus Reagent (Invitrogen) as described above. After incubation for 24 h, cell images in the green and red channels were acquired using a TCS Sp8 confocal microscope (Leica Microsystems Inc.) (5).

In FRET–acceptor photobleaching, the energy transfer efficiency (E) is measured. This value is directly related to the distance (r) separating a given donor and acceptor pair by Förster equation

$$E = 1 \Big/ \left[1 + (r/R_0)^6\right], \quad \textbf{[S1]}$$

where R_0 is the Förster distance at which the transfer efficiency is 50%. The efficiency of transfer (E) can be calculated from the equation

$$E = 1 - F_{DA}/F_D, \quad \textbf{[S2]}$$

where F_{DA} and F_D are the donor fluorescence intensities in the presence and absence, respectively, of energy transfer.

FRET–acceptor photobleaching (FRET-AP) involves measuring the donor "dequenching" in the presence of an acceptor; this is done by comparing the donor fluorescence intensity in the same sample (either a whole cell or a region of interest [ROI]) before and after destroying the acceptor by photobleaching. If FRET was initially present, a resultant increase in donor fluorescence occurs upon photobleaching of the acceptor. The energy transfer efficiency is quantified by rewriting Eq. **S2** as

$$E = 1 - F_{pre}/F_{post}, \quad \textbf{[S3]}$$

where F_{pre} is the fluorescence intensity of the donor before the acceptor photobleaching, and F_{post} is the fluorescence intensity of the donor after the acceptor photobleaching.

In the photobleaching experiments, a repetitive bleaching (at excitation wavelength of 543 nm) was applied to bleach the red fluorescent signal in an ROI or a whole cell. A series of prebleaching and postbleaching donor GFP fluorescence intensities were collected. The maximum and minimum values (GFP_{max} and GFP_{min}) were used for calculation of FRET efficiency by rewriting Eq. **S3** as

$$E = 1 - FGFP_{min}/FGFP_{max}. \quad \textbf{[S4]}$$

Coimmunoprecipitation and Western Blotting. Isolated brain tissues or cell platelets were homogenized in RIPA buffer (Millipore) with 1× complete protease inhibitor mixture (Roche Applied Science) and 1× PhosSTOP phosphatase inhibitor mixture (Roche Applied Science) on ice and centrifuged for 10 min at 18,400 × g to remove large debris. The protein concentration of the supernatant was determined by Bradford Assay (Bio-Rad). One milligram of the total cell lysates were first incubated with control IgG (Santa Cruz Biotechnology) for 30 min, precleared with 50 μL of Dynabeads Protein G (Invitrogen), and then incubated with various antibodies according to the suggested dilution on the product datasheets overnight at 4 °C. Fifty microliters of Dynabeads Protein G was added and incubated for 2 h at 4 °C. Beads bound with immune complexes were collected by DynaMag-2 (Life Technologies) and washed thrice before elution in 90 μL of buffer containing 0.2 M Glycine·HCl, pH 2.5, which was neutralized with 10 μL of neutralization buffer [1 M Tris·HCl (pH 9.0)]. The eluates were subjected to 9–12% SDS/PAGE and Western blotting analysis, or enzyme activity measurement as described below.

For Western blotting, 100 μg of proteins derived from cell or tissue lysates were separated by SDS/PAGE and transferred to polyvinylidene difluoride membranes. Following blocking, membranes were probed with various primary antibodies to determine different levels of protein expressions. Immunoreactive antibody–antigen complexes were visualized with the SuperSignal West Femto Chemiluminescent Substrate (Pierce Biotechnology).

In Silico Automated Protein–Protein Docking and Contact Area Analysis. Docking of ligand protein to receptor protein, energy filtering, clustering, and ranking were done using the ClusPro 2.0 web server (cluspro.bu.edu/login.php) (6). Structures of GSK3β (PDB ID code 1Q4L, chain A), p25 (PDB ID code 1UNL, chain D), and CDK5 (PDB ID code 1UNL, chain A) were obtained from Protein Data Bank to perform docking. As a positive control, we simulated CDK5 and p25 interaction with ClusPro 2.0 using hydrophobic-favored mode, and the same set of parameters were used to simulate the interaction between GSK3β and p25. Representative structures for the cluster were obtained from each simulation and docking energy center scores of the first ranked cluster conformation were compared.

For contact area analysis, ICM-Browser Pro (Molsoft LLC) was used to analyze the key interacting residues and potential presence of interacting bonds of the most favorable confirmation obtained from ClusPro 2.0 docking.

Measurement of GSK3β Kinase Activities and in Vitro Tau Phosphorylation Levels. For visualizing the GSK3β activity in live cells, we adopted the approach developed by Taelman et al. (4) with a reporter construct. Signals from DIV14 primary neurons or N2a cells were observed and compared under the confocal microscope after cotransfection with mCherry-p25 or mCherry-p35 and GFP-GSK3, GFP-MAPKwt, or GFP-GSK3β-MAPKmut constructs for 24 h.

For in vitro tau phosphorylation assay, GSK3β was first immunoprecipitated from N2a cell lysates co-overexpressing HA-GSK3β with GFP-vector, GFP-p35, or GFP-p25 using anti–HA antibody. The active enzyme complexes were then incubated with equal amount of GST-tau40 and the degree of tau phosphorylation at sites Thr181 and Ser396 was visualized by immunoblotting.

Quantitative RT-PCR. Total RNA was isolated from N2a cells after transfection and used for the synthesis of cDNA. Quantitative RT-PCR was performed using EXPRESS SYBR Green qPCR Supermix (Invitrogen). The reactions were carried out on a 7000 Sequence Detection System (Applied Biosystems). Quantification was achieved using Ct (cycle threshold) values that were normalized with GAPDH RNA as internal controls.

Primers for *Ctnnb1*:

Forward: 5′- CTTGGACTGAGACTGCTGATCTTG-3′

Reverse: 5′-CACCAGAGTGAAAAGAACGATAGCTA-3′.

Primers for *Gapdh*:

Forward: 5′-AGG TGG TCT CCT CTG ACT TCA AC-3′

Reverse: 5′-GGT CTC TCT CTT CCT CTT GTG CT-3′.

Statistical Analysis. All data were obtained from at least three different preparations. Values are mean ± SEM. Data analysis was performed with Prism 6 (GraphPad Software Inc.) by one-way ANOVA (post hoc Bonferroni test) or t test. $P < 0.05$ was considered significant.

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2. Paxinos G, Watson C, Pennisi M, Topple A (1985) Bregma, lambda and the interaural midpoint in stereotaxic surgery with rats of different sex, strain and weight. *J Neurosci Methods* 13(2):139–143.
3. Saito T, Nakatsuji N (2001) Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. *Dev Biol* 240(1):237–246.
4. Taelman VF, et al. (2010) Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. *Cell* 143(7):1136–1148.
5. Ponsioen B, et al. (2004) Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. *EMBO Rep* 5(12):1176–1180.
6. Comeau SR, Gatchell DW, Vajda S, Camacho CJ (2004) ClusPro: A fully automated algorithm for protein–protein docking. *Nucleic Acids Res* 32(web server issue):W96–W99.

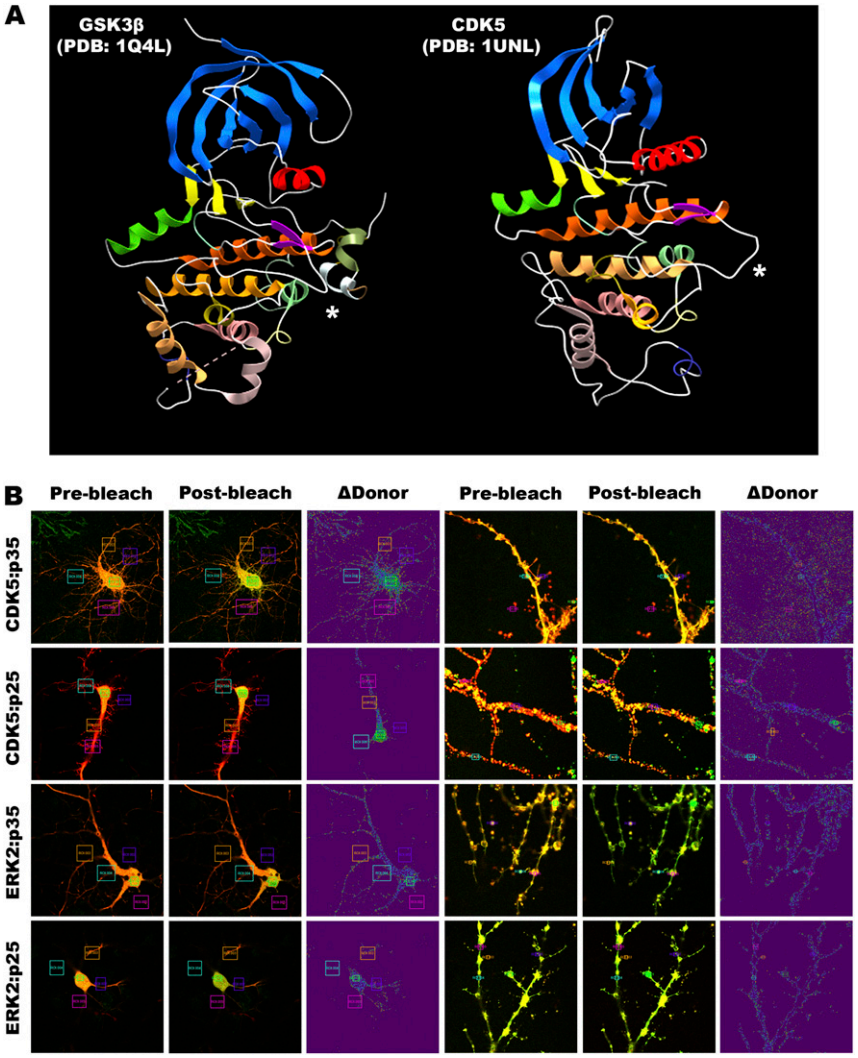

Fig. S1. Structural homology between GSK3β and controls for FRET–acceptor photobleaching. (*A*) 3D structures of GSK3β (PDB ID code 1Q4L, chain A) and CDK5 (PDB ID code 1UNL, chain A) demonstrate high degree of structural homology between the kinases. Asterisk indicates the T-loop. (*B*) In cell bodies (*Left*) or neurites (*Right*), both GFP-CDK5/mCherry-p35 and GFP-CDK5/mCherry-p25 (positive controls) show significant FRET signal, whereas cells overexpressing GFP-ERK2 plus mCherry-p35 or mCherry-p25 (negative controls) do not (total $n = 30$ from three experiments).

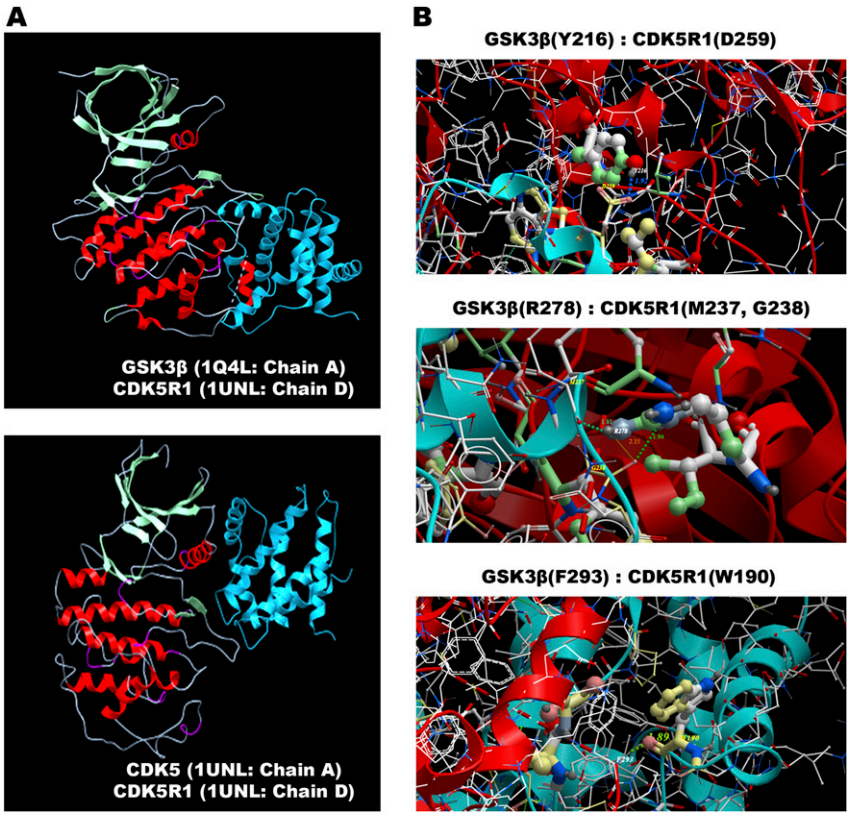

Fig. S2. The binding mode of GSK3β:p25. (*A*) The mode of interaction between GSK3β:p25 pair differs from that of the CDK5:p25 pair. p25 interacts strongly with residues at the C-terminal α-helixes of GSK3β. On CDK5, by contrast, it tends to interact with residues of N-terminal PSTAIRE helix. (*B*) Five intermolecular hydrogen bonds (indicated by dotted lines) are predicted to be formed between p25 and GSK3β.

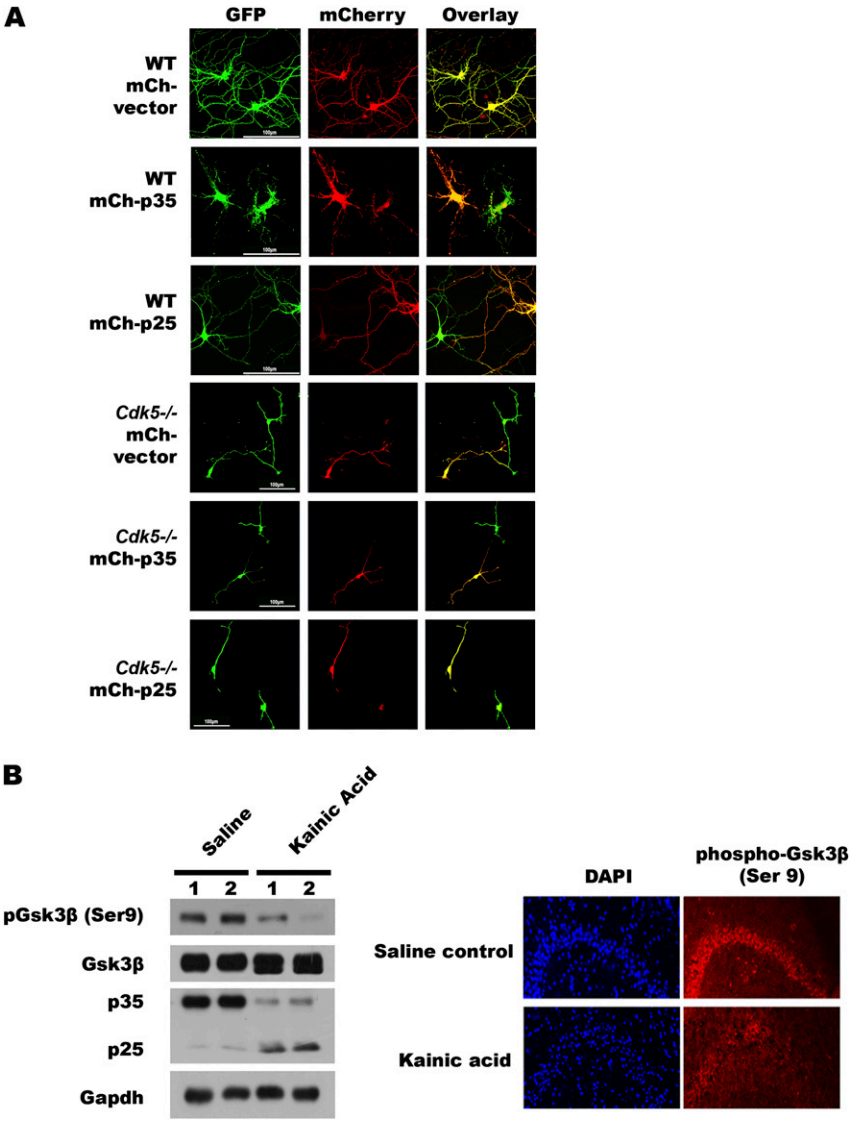

Fig. S3. Transfection controls for GSK3β reporters and phosphorylation of GSK3β at serine 9 is reduced after kainic acid treatment in hippocampus. (*A*) Representative images showing GSK3β kinase activity in both wild-type and *cdk5*$^{-/-}$ primary cortical neurons when mutant constitutively active GFP-tagged reporters are transfected with mCherry-tagged p35 or p25 (total $n = 15$ from three experiments). (*B*) Levels of phosphorylated Gsk3β at residue serine 9 in response to kainic acid treatment. Phosphorylated serine 9 level was reduced upon induction of cleavage of p35 to p25 by intracranial kainic acid injection, as reflected by Western blots and immunohistochemistry ($n = 3$ experiments).

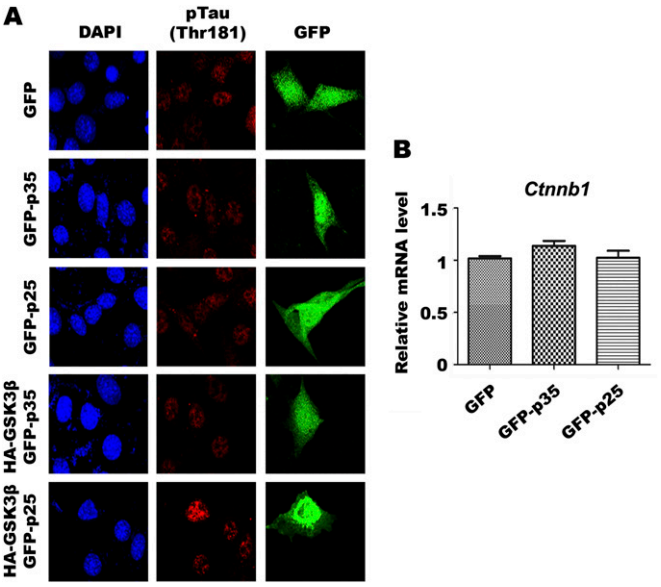

Fig. S4. Tau is aberrantly phosphorylated by GSK3β under the influence of p25 and quantification analysis for Western blotting. (*A*) Tau phosphorylation at known GSK3β sites was analyzed in N2a cells transiently overexpressing GSK3β with p25 or p35 by immunocytochemistry ($n = 3$ experiments). (*B*) Quantification of β-catenin (*CTNNB1*) gene expression by RT-PCR. p25 did not result in any significant alterations in *CTNNB1* gene transcription level. Statistical analysis among three groups were compared by one-way ANOVA with Bonferroni post hoc test ($n = 3$ experiments).

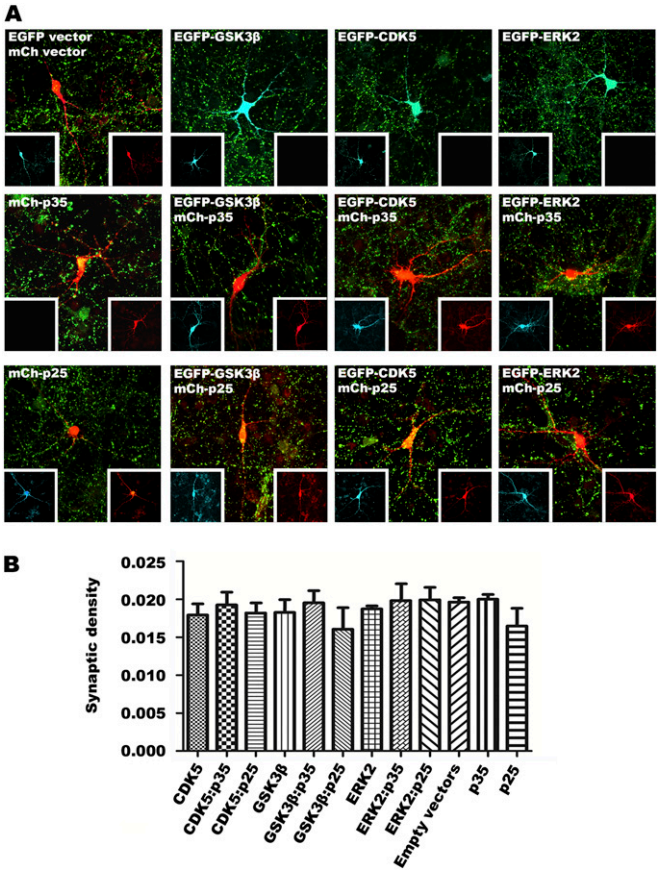

Fig. S5. Analysis of the influence of p25 on postsynaptic puncta and quantification of postsynaptic density. (*A*) Representative images of primary neurons showing different changes in postsynaptic vesicles upon ectopic expression of different constructs for 24 h. Cyan, GFP; red, mCherry; and green, PSD95 (total $n = 30$ in three experiments). (*B*) Quantitative analysis of the density of postsynaptic puncta (overlapping of green fluorescent puncta with red of transfected cell dendrite, divided by the total number of pixels of each cell) among different treatment groups. Samples were compared by one-way ANOVA with Bonferroni post hoc test.