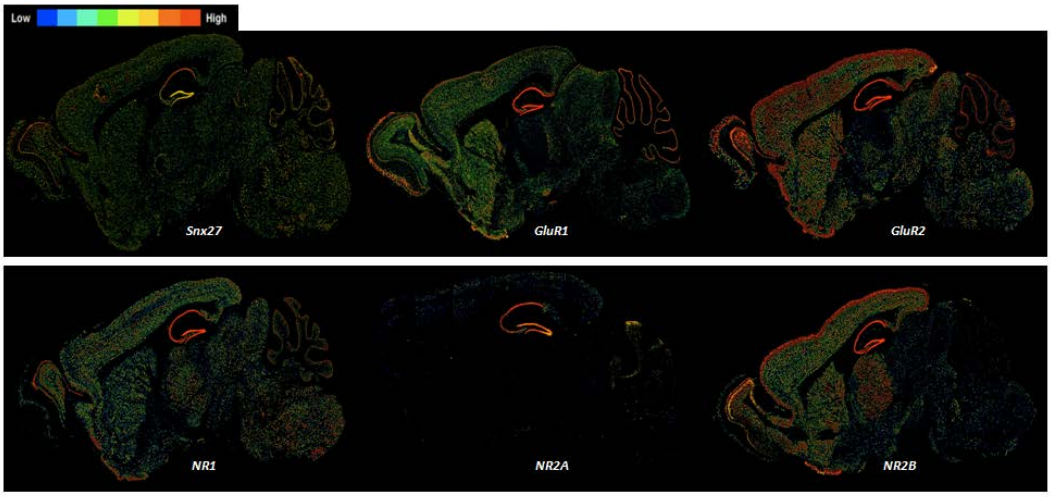
Supplementary materials for

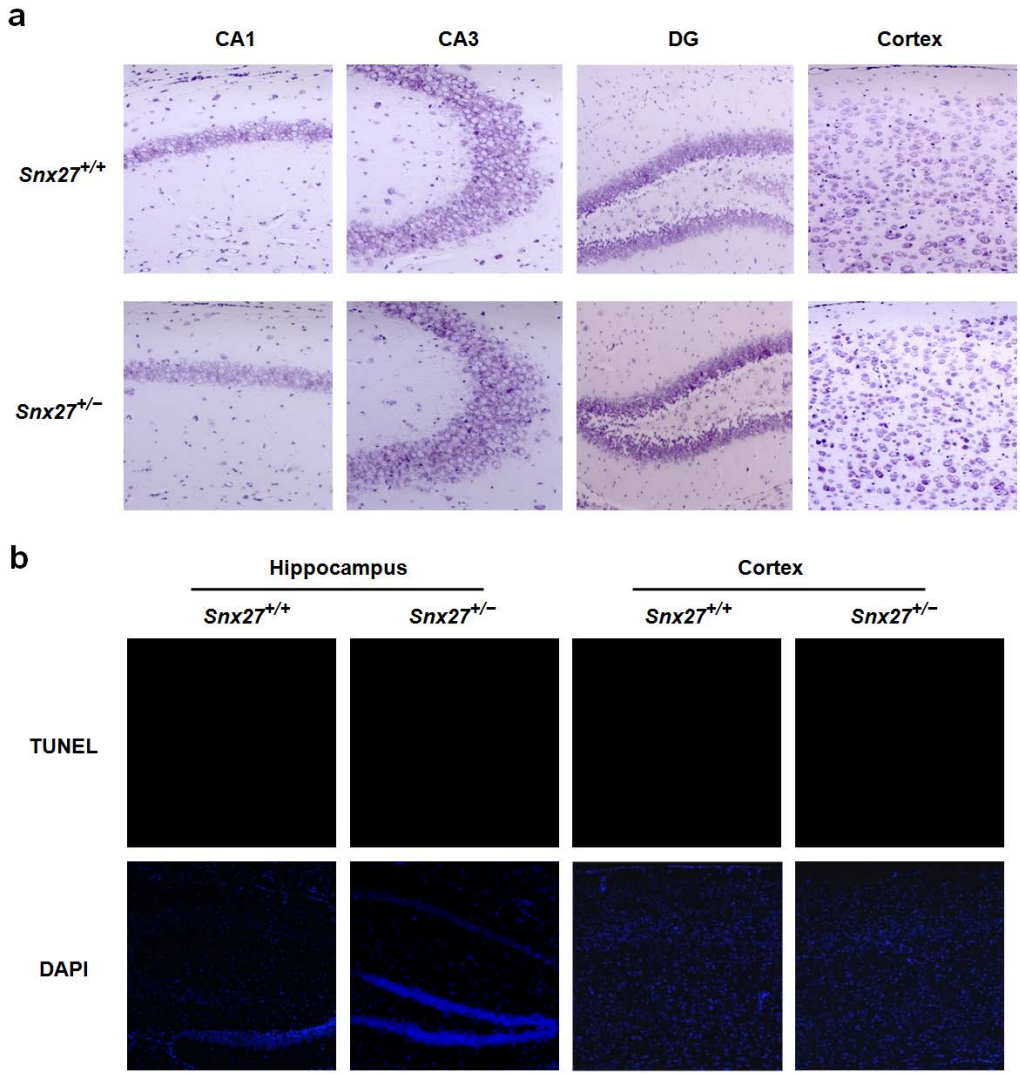
Wang, et al. Loss of sorting nexin 27 contributes to excitatory synaptic dysfunction via modulation of glutamate receptor recycling in Down's syndrome



from: Allen Brain Atlas

Supplementary Figure 1. *Snx27* mRNA is highly expressed in the hippocampus and frontal cortex of mouse brains

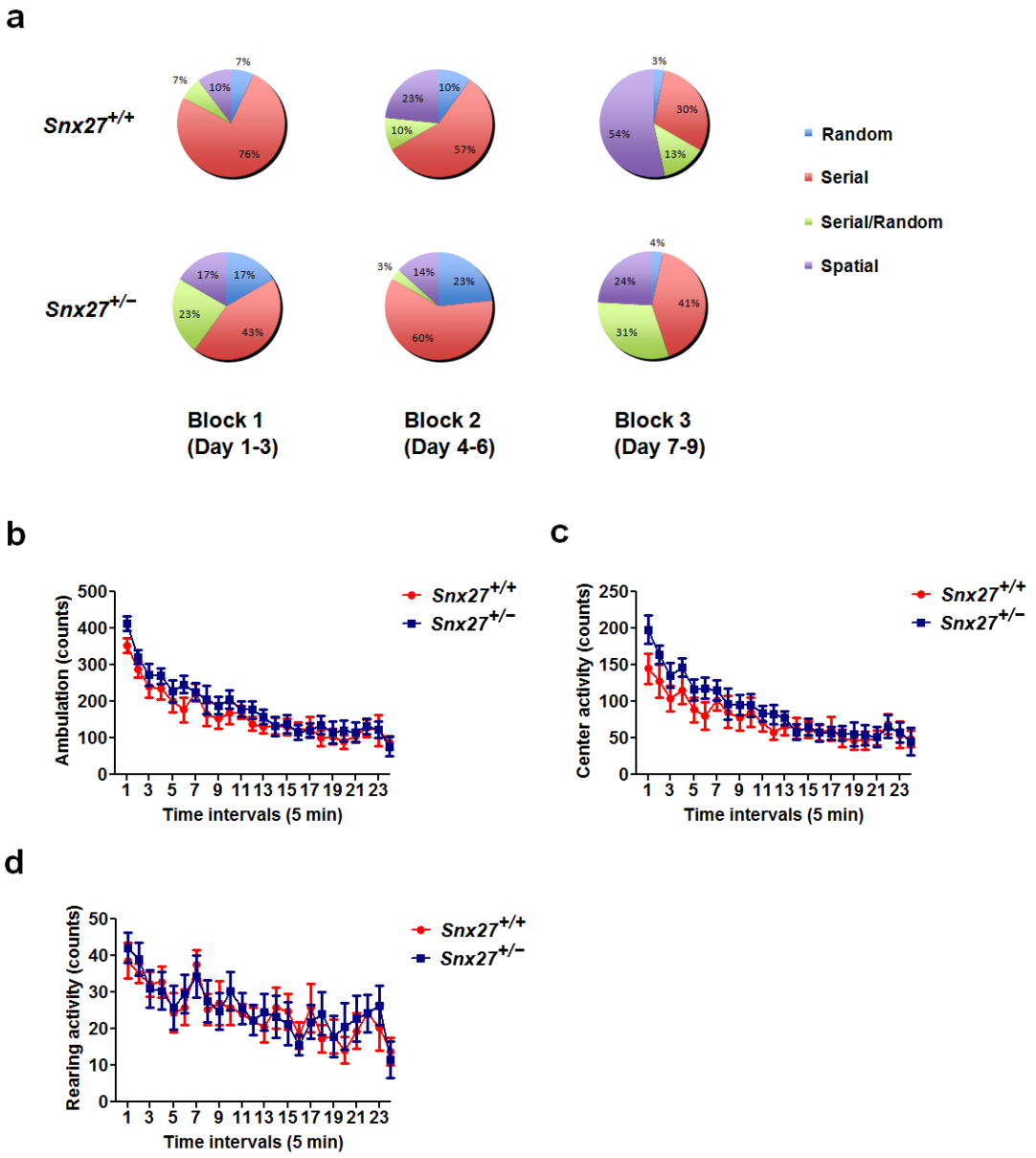
Snx27 mRNA expression in the hippocampus and frontal cortex. Images are reproduced from the Allen Brain Atlas (www.brainatlas.org).



Supplementary Figure 2. Normal brain morphology of *Snx27*$^{+/-}$ mice

(a) Nissl staining of brain morphology of *Snx27*$^{+/+}$ and *Snx27*$^{+/-}$ mice.

(b) No apoptotic cells were detected in the *Snx27*$^{+/+}$ and *Snx27*$^{+/-}$ mouse brains using TUNEL staining.



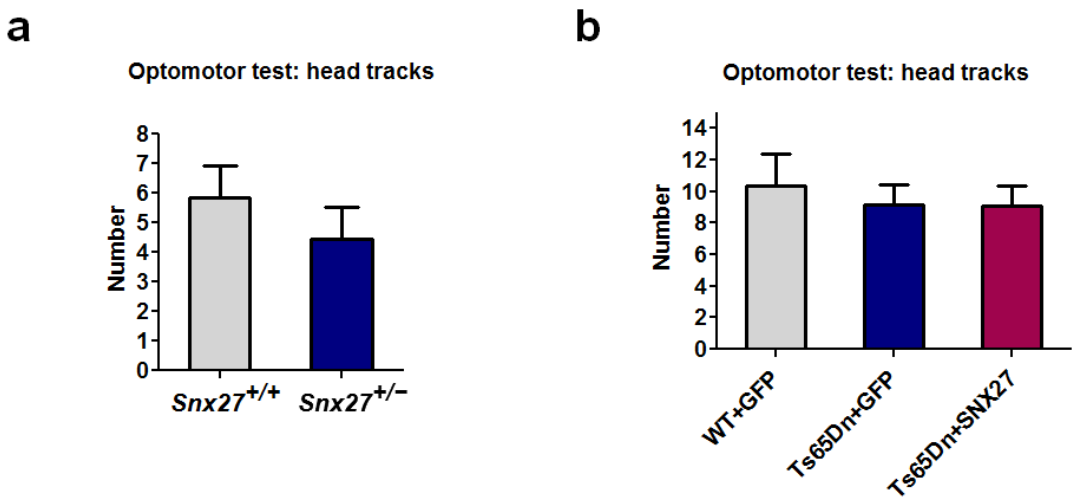
Supplementary Figure 3. *Snx27*$^{+/-}$ mice exhibit delay in using spatial strategy

(a) Percentage of *Snx27*$^{+/+}$ and *Snx27*$^{+/-}$ mice using each search strategy across blocks in the Barnes maze test (values represent group means, n = 10). Search strategies are defined as spatial and nonspatial (serial, random, random/serial).

(b) Locomotor activity test (ambulation activity) for *Snx27*$^{+/+}$ and *Snx27*$^{+/-}$ mice. Data represent mean ± s.e.m., n = 10 mice per genotype. P values were calculated using repeated-measures ANOVA with Bonferroni's *post hoc* analysis, $P > 0.05$.

(c) Locomotor activity test (center activity) for *Snx27*$^{+/+}$ and *Snx27*$^{+/-}$ mice. Data represent mean ± s.e.m., n = 10 mice per genotype. P values were calculated using repeated-measures ANOVA with Bonferroni's *post hoc* analysis, $P > 0.05$.

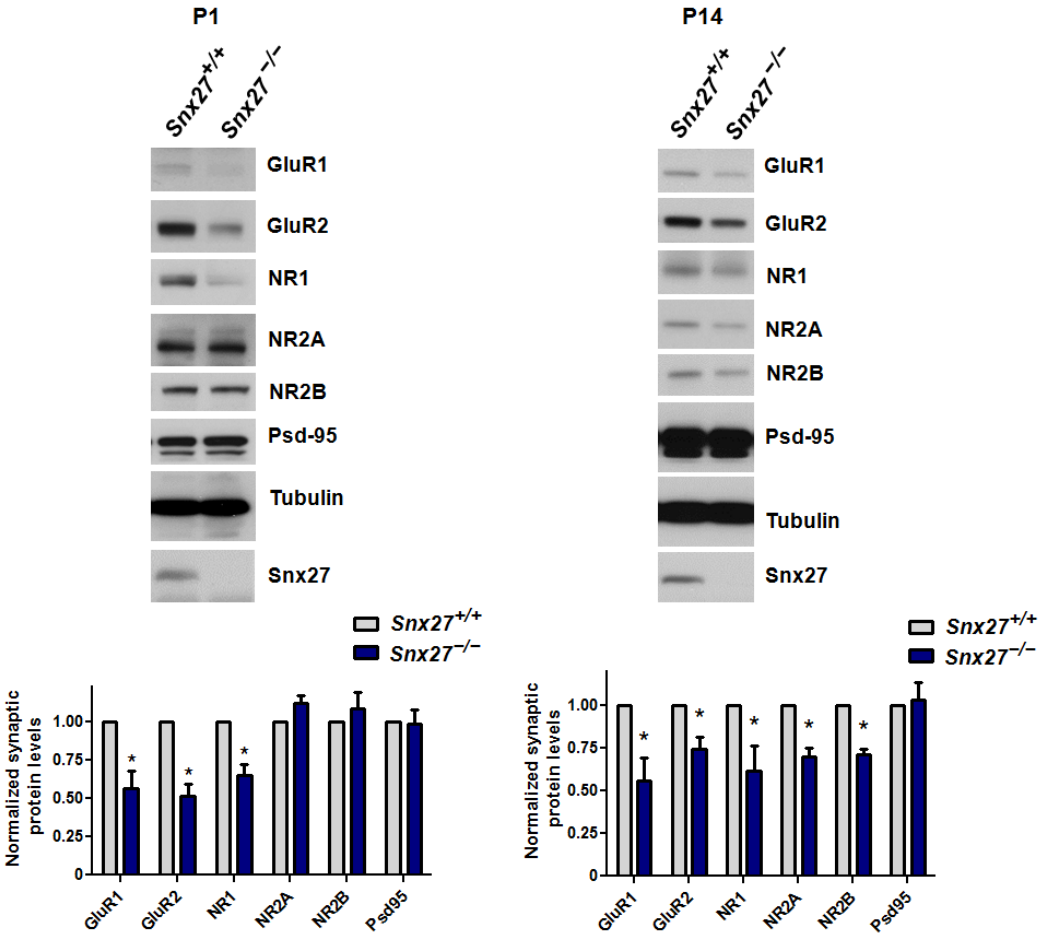
(d) Locomotor activity test (rearing activity) for *Snx27*$^{+/+}$ and *Snx27*$^{+/-}$ mice. Data represent mean ± s.e.m., n = 10 mice per genotype. P values were calculated using repeated-measures ANOVA with Bonferroni's *post hoc* analysis, $P > 0.05$.



Supplementary Figure 4. Normal optomotor activity in *Snx27*$^{+/-}$ and Ts65Dn mice

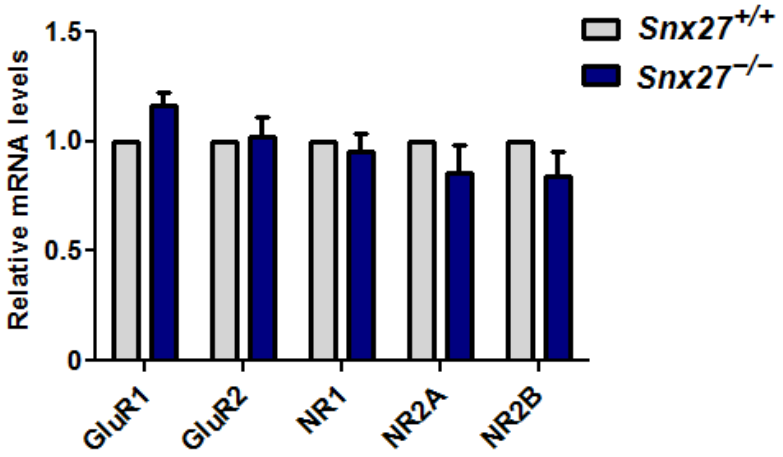
(a) *Snx27*$^{+/+}$ and *Snx27*$^{+/-}$ mice behavior in the optomotor test. Data represent mean ± s.e.m., n = 10 mice per genotype. P values were calculated using nonparametric t test, $P > 0.05$.

(b) Ts65Dn mice behavior in the optomotor test. Data represent mean ± s.e.m., WT+GFP (n = 8 mice), Ts65Dn+GFP (n = 5 mice), Ts65Dn+SNX27 (n = 7 mice). P values were calculated using one-way ANOVA with Dunnett's *post hoc* analysis, $P > 0.05$.



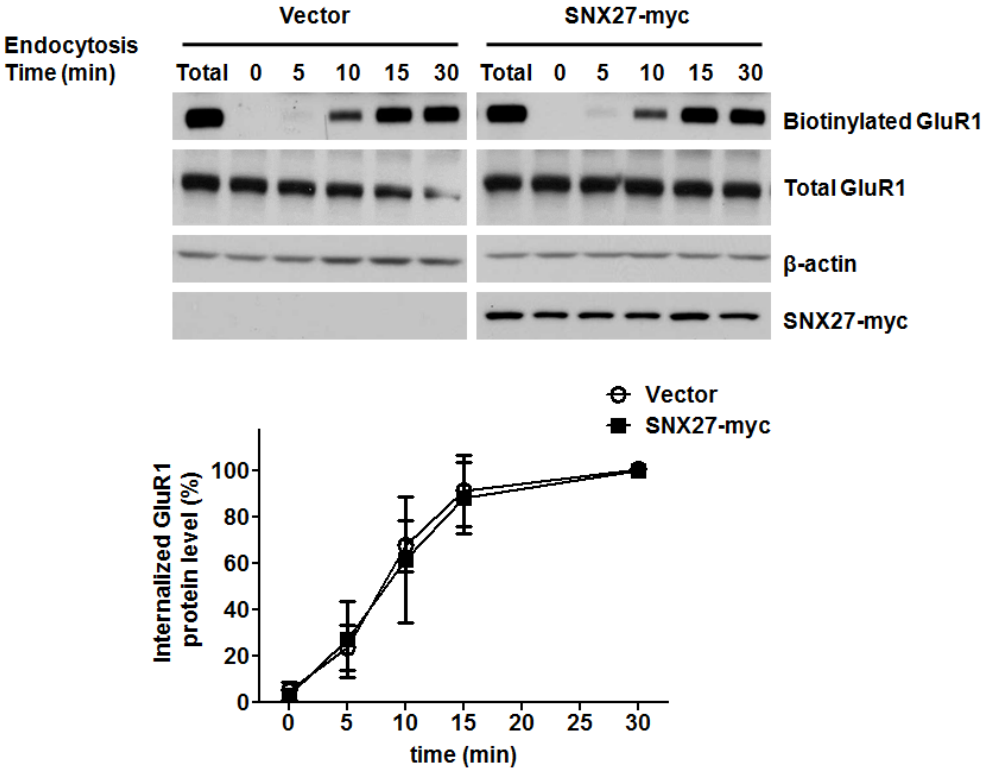
Supplementary Figure 5. Reduced amounts of glutamate receptors in the brain of *Snx27*$^{-/-}$ mice

Expression of AMPARs, NMDARs, and other synaptic proteins in hippocampal extracts from *Snx27*$^{+/+}$ and *Snx27*$^{-/-}$ mice at P1 and P14. Data represent mean ± s.e.m., n = 4 mice per genotype. P values were calculated using two-tailed Student's t test, *$P < 0.05$.



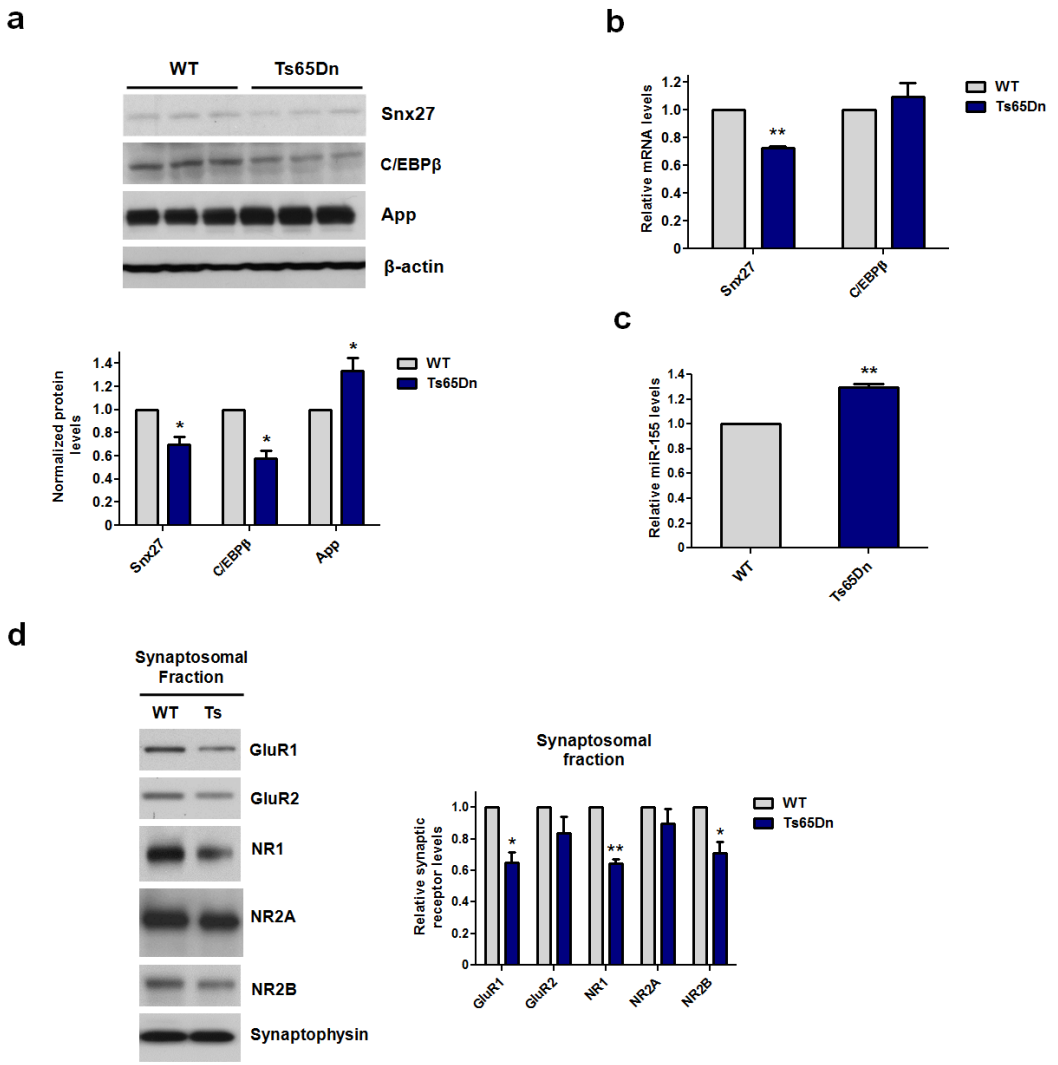
Supplementary Figure 6. mRNA amounts of glutamate receptors were unchanged in *Snx27*$^{-/-}$ mouse brains

The mRNA amounts of GluR1, GluR2, NR1, NR2A and NR2B relative to that of β-actin from *Snx27*$^{-/-}$ mouse brains were analyzed and normalized to that of *Snx27*$^{+/+}$ controls (set as one arbitrary unit). Data represent mean ± s.e.m., n = 3 mice per genotype. P values were calculated using two-tailed Student's t test, $P > 0.05$.



Supplementary Figure 7. SNX27 does not influence endocytosis of GluR1

The amount of internalized GluR1 at different time points was compared to total cell surface GluR1 to quantify the percent of internalization. Data represent mean ± s.e.m., $n = 3$. P values were calculated using repeated-measures ANOVA with Bonferroni's *post hoc* analysis, $P > 0.05$.



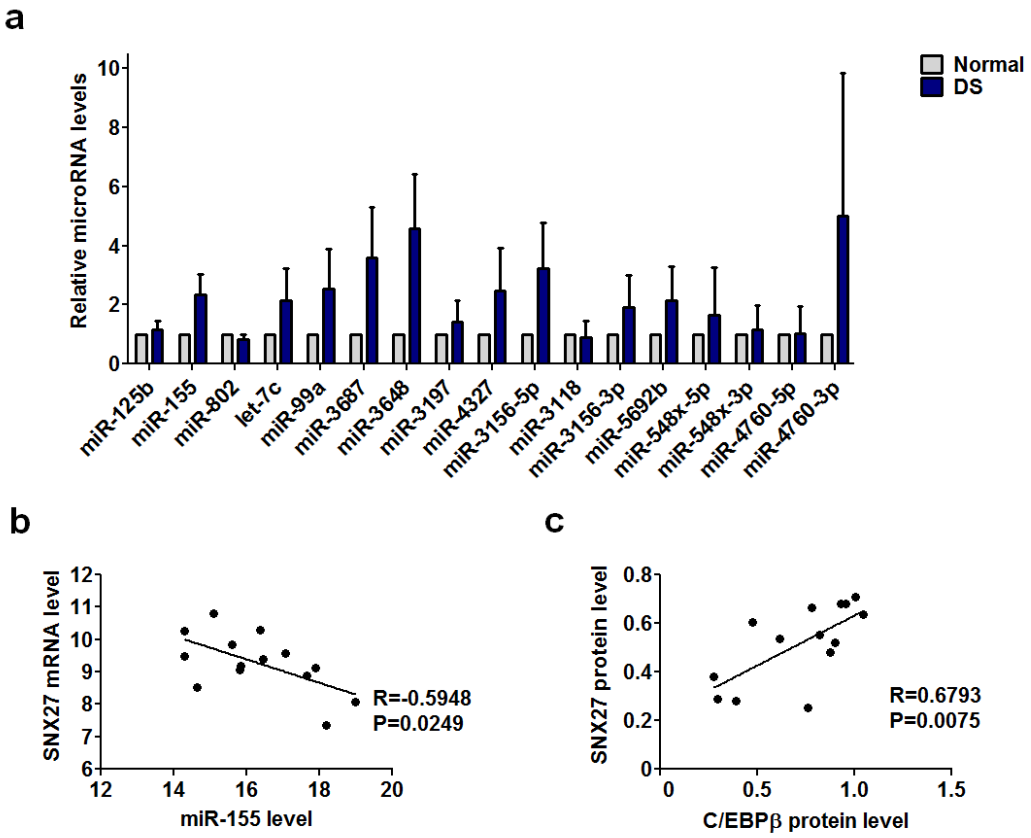
Supplementary Figure 8. Increased amounts of miR-155 and decreased amounts of C/EBPβ and Snx27 in Ts65Dn mouse brains

(a) Western blot analyses of Snx27, C/EBPβ, App, and β-actin in the hippocampi of Ts65Dn mice and WT controls. Signal intensity of the immunoblots was calculated and normalized to β-actin; $n = 3$ mice per genotype. P values were calculated using two-tailed Student's t test, $^*P < 0.05$.

(b) SNX27 mRNA amounts in the hippocampi of Ts65Dn mice and WT controls were determined by qRT-PCR. Data represent mean ± s.e.m., $n = 3$. P values were calculated using two-tailed Student's t test, $^{**}P < 0.01$.

(c) Relative expression amounts of miR-155 (normalized to U6 snRNA) were examined by qPCR in the hippocampi of Ts65Dn mice and WT controls, $n = 3$. P values were calculated using two-tailed Student's t test, $^{**}P < 0.01$.

(d) Protein amounts of glutamate receptor subunits GluR1, NR1 and NR2B in synaptosomal fractions from Ts65Dn mice and WT controls. Data represent mean ± s.e.m., $n = 3$. P values were calculated using two-tailed Student's t test, $^{*}P < 0.05$, $^{**}P < 0.01$.

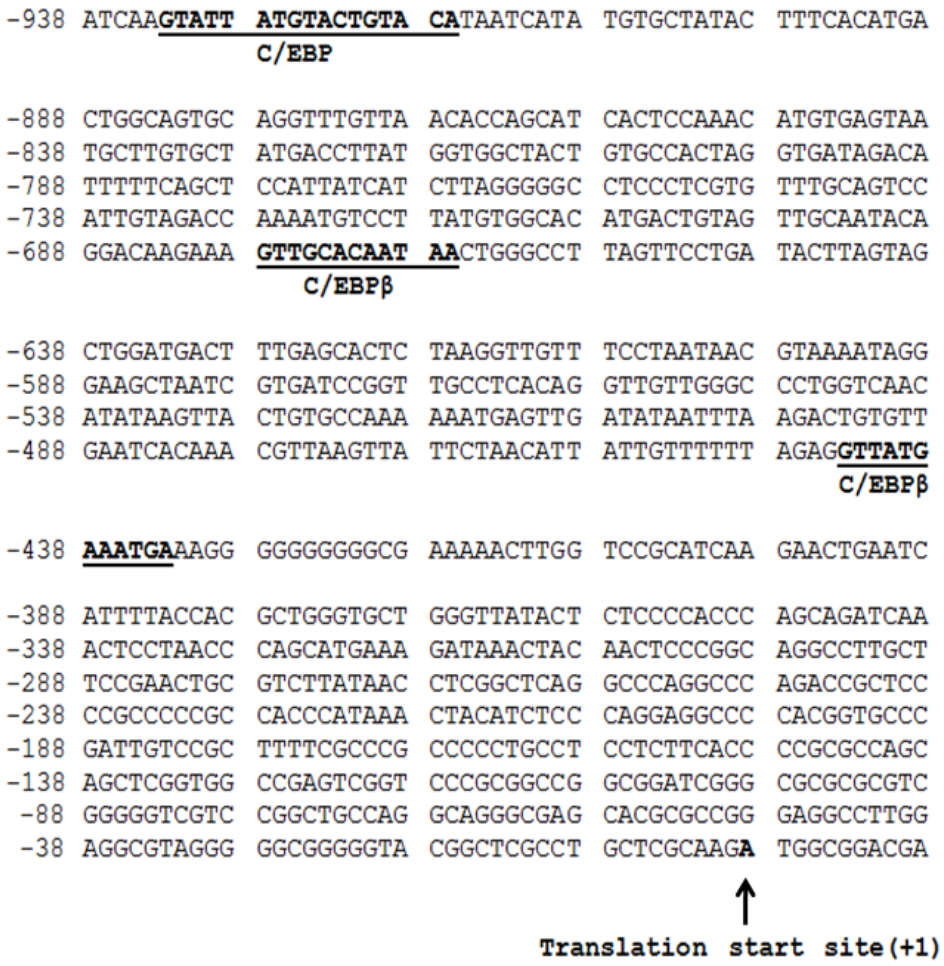


Supplementary Figure 9. Expression of chromosome 21-encoded microRNAs in Down's syndrome brains vs. normal controls

(a) Relative amounts of chromosome 21-encoded microRNAs (normalized to U6 snRNA) are examined by qPCR in the cortices of Down's syndrome brains and compared to age-matched controls, $n = 7$.

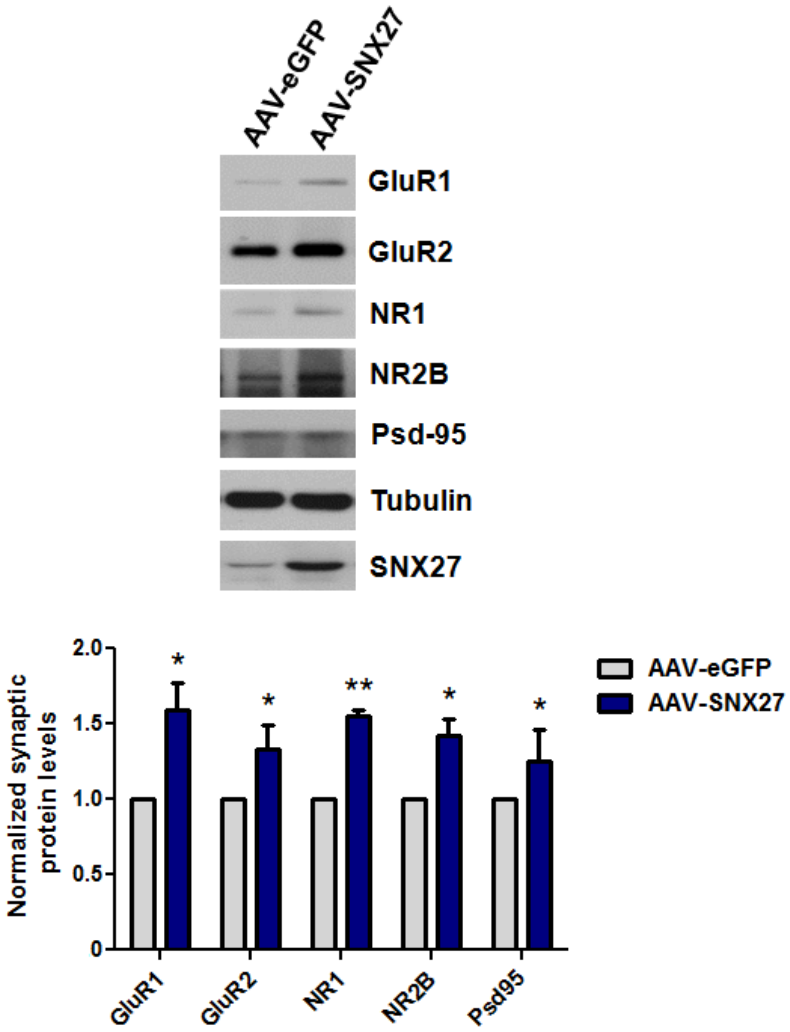
(b) Correlation analysis of miR-155 amounts and SNX27 mRNA amounts in human brains. Regression analysis, $n = 14$.

(c) Positive correlation between C/EBPβ and SNX27 protein amounts in Down's syndrome brains. Regression analysis, $n = 14$.



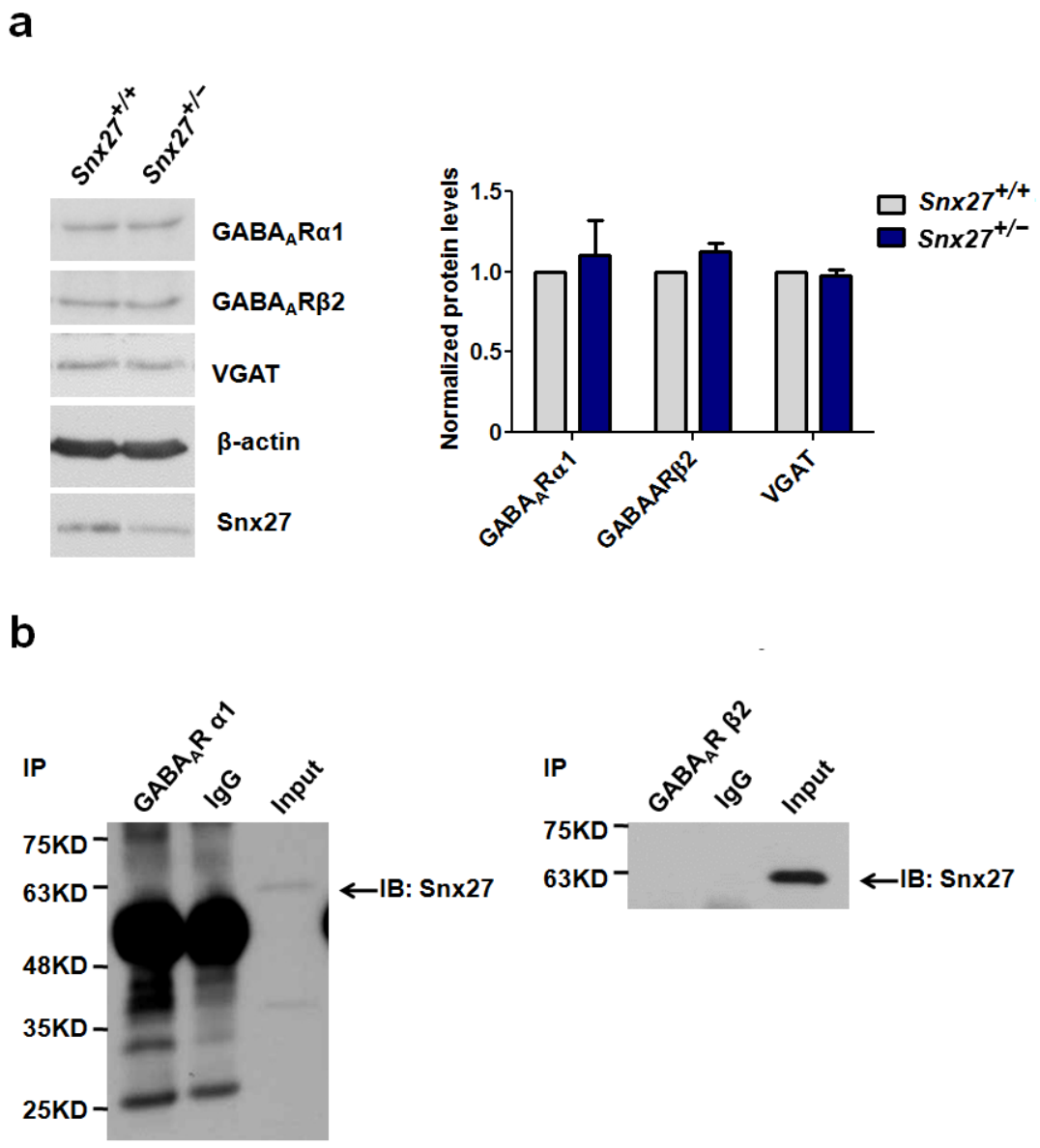
Supplementary Figure 10. Human *SNX27* promoter region analysis and prediction of C/EBPβ binding sites

Potential C/EBPβ binding sites identified on the minus strand of the human *SNX27* promoter. Numbering is relative to the translation start site (defined as +1).



Supplementary Figure 11. Over-expression of AAV-SNX27 increases expression of glutamate receptors *in vitro*

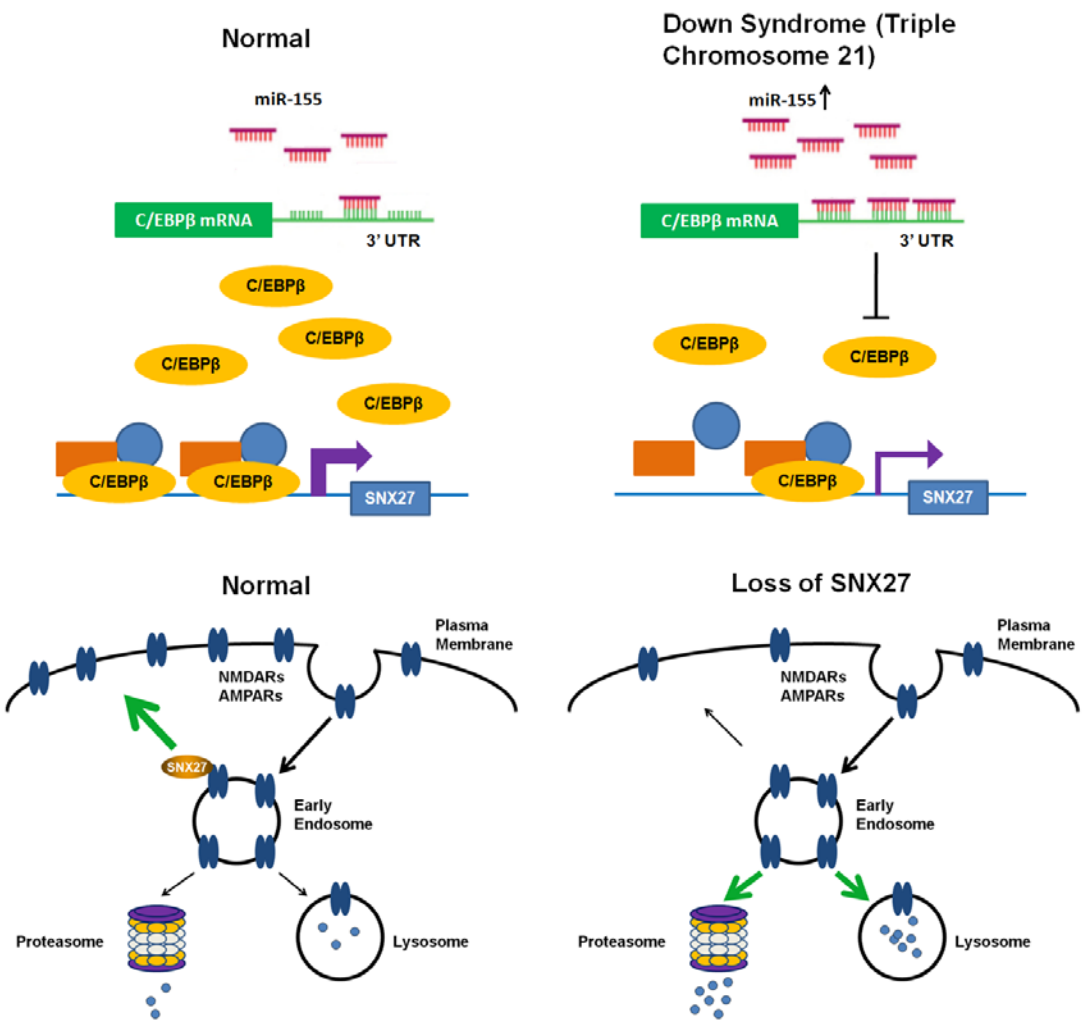
The amounts of different synaptic proteins in rat primary neurons infected with AAV containing SNX27-IRES-GFP or GFP (control) as measured by Western blot. Data represent mean ± s.e.m., $n = 4$. P values were calculated using two-tailed Student's t test, $^{*}P < 0.05$, $^{**}P < 0.01$.



Supplementary Figure 12. Normal $GABA_A$ receptor expression in *Snx27*$^{+/–}$ mouse brain

(a) Expression of $GABA_AR\alpha1$, $GABA_AR\beta2$ and GABAergic presynaptic marker VGAT in hippocampal extracts from *Snx27*$^{+/+}$ and *Snx27*$^{+/–}$ mice at P30. Data represent mean ± s.e.m., $n = 3$ mice per genotype. P values were calculated using two-tailed Student's t test, $P > 0.05$.

(b) Co-immunoprecipitation assays for endogenous Snx27 and endogenous $GABA_AR\alpha1$ or $GABA_AR\beta2$ in C57Bl/6 mouse brains. Brain lysates were immunoprecipitated with IgG control or $GABA_AR\alpha1$ or $GABA_AR\beta2$ antibodies and immunoblotted with SNX27 antibody.



Supplementary Figure 13. A schematic model of miR-155-C/EBPβ-SNX27 pathway in Down's syndrome pathogenesis

SNX27 is required for maintaining synaptic function through modulation of NMDAR and AMPAR recycling. In Down's syndrome, higher than normal amounts of the Chromosome 21-encoded miR-155 suppresses the expression of C/EBPβ, which results in reduced amounts of SNX27 and eventually contributes to synaptic deficits in Down's syndrome brains.

Supplementary Tables

Supplementary Table 1. Primer sequences

Target gene	Primer Sequences
human SNX27 F	5′-GGAGCAGAATGGTGAGAAGTTT-3'
human SNX27 R	5'-TCCCGGTACCGCTTAGAAC-3'
human CEBPB F	5'-CGCTTACCTCGGCTACCA-3'
human CEBPB R	5'-ACGAGGAGGACGTGGAGAG-3'
mouse Snx27 F	5'-GCAGAATGGTGAGAAGTTTGTG-3'
mouse Snx27 R	5'-TCCCGGTACCGCTTAGAAC-3'
mouse CEBPB F	5'-TGATGCAATCCGGATCAA-3'
mouse CEBPB R	5'-CACGTGTGTTGCGTCAGTC-3'
pGEX-4T2-SNX27FL F	5'-AGTCGGATCCATGGCGGACGAGGACGG-3'
pGEX-4T2-SNX27FL R	5'-AGTCGTCGACCTAATATTCCTCTTTTCTCC-3'
pGEX-4T2-SNX27PDZ F	5'-AGTCGGATCCATGGCGGACGAGGACGG-3'
pGEX-4T2-SNX27PDZ R	5'-AGTCGTCGACCTAAGGTACAGATAACAC-3'
mouse GluR1 F	5'-AGGGATCGACATCCAGAGAG-3'
mouse GluR1 R	5'-TGCACATTTCCTGTCAAACC-3'
mouse GluR2 F	5'-CAGTTTCGCAGTCACCAATG-3'
mouse GluR2 R	5'-ACCCAAAAATCGCATAGACG-3'
mouse NR1 F	5'-GGATACCAGATGTCCACCAGACTAAAG-3'
mouse NR1 R	5'-AACGCAGAAGCCATAACAGCAC-3'
mouse NR2A F	5'-CGGGTCTCATTTCAGTCTCTTACG-3'
mouse NR2A R	5'-GGTTGTCATCTGGCTCACAGTCAG-3'
mouse NR2B F	5'-GCGATTTGGTTACTCTGGGGTC-3'
mouse NR2B R	5'-GTCTCTGGAACTTCTTGTCACTCAGG-3'
SNX27 Chip F	5'-TAATCGTGATCCGGTTGCCTCAC-3'
SNX27 Chip R	5'-AGTATAACCCAGCACCCAGCGTG-3'

Supplementary Table 2. Information of the tissue samples from Down's syndrome and normal individuals

Related to Figure 5a, 5c and Supplementary Figure 9.

Case number	Disorder	Age	Gender	Tissue
952	Control	Fetal/16 wks	M	Cortex
1152	DS	Fetal/16 wks	F	Cortex
4509	Control	Fetal/18 wks	F	Cortex
1478	DS	Fetal/18 wks	F	Cortex
205	Control	Fetal/19 wks	M	Cortex
956	DS	Fetal/19 wks	M	Cortex
1537	Control	31 days	F	Cortex
1273	DS	30 days old	M	Cortex
615	Control	163 days	M	Cortex
M1974M	DS	148 days old	F	Cortex
M1931M	Control	332 days old	M	Cortex
832	DS	339 days old	M	Cortex
931	Control	13 yrs 119 days old	M	Cortex
1276	DS	13 yrs 286 days old	M	Cortex

Down's syndrome cortices used for immunoblot analysis in Figure 5a. Abbreviations:

DS, Down's syndrome.

Supplementary Table 3. Information of the tissue samples from Alzheimer's disease and normal individuals

Related to Figure 5b.

	Case	Gender	Age	Tissue
Normal control	99-25	F	76	Cortex
	98-19	F	87	Cortex
	98-32	M	83	Cortex
	98-34	F	73	Cortex
	98-23	M	68	Cortex
	00-49	F	86	Cortex
	97-17	M	78	Cortex
	96-35	M	84	Cortex
	99-44	M	69	Cortex
SAD	99-39	M	72	Cortex
	99-15	M	83	Cortex
	01-05	M	88	Cortex
	01-13	M	87	Cortex
	01-09	F	83	Cortex
	03-02	M	76	Cortex
	01-17	F	78	Cortex
	01-34	F	89	Cortex
	03-07	M	67	Cortex

SAD cortices used for immunoblot analysis in Figure 5b. Abbreviations: SAD, Sporadic Alzheimer’s disease.

SUPPLEMENTAL METHODS

Antibodies

The SNX27-specific antibodies used in these experiments include the previously described polyclonal (dilution 1:2,000) and monoclonal (dilution 1:1,000) antibodies to SNX27[1,2]. Other antibodies acquired from commercial sources were: GluR1 (Chemicon, 04-855, 1:500), GluR2 (Millipore, AB1768, 1:1,000), PSD-95 (Millipore, MAB1598, 1:500), synaptophysin (Sigma, SVP-38, 1:200), NR1 (BD Biosciences, 556308, 1:1,000), NR2A (Millipore, 07-632, 1:500), NR2B (BD Biosciences, 610416, 1:500), VGAT (Millopore, AB5062P, 1:500), $GABA_AR\alpha1$ (Millipore, 06-868, 1:500), $GABA_AR\beta2$ (Millipore, MAB341, 1:500), C/EBPβ (Santa Cruz, sc-7962, 1:100), Transferrin receptor (Invitrogen, 13-6800, 1:500), c-Myc (Invitrogen, 9E10, 1:500), α-tubulin (Sigma, T5168, 1:10,000) and β-actin (Sigma, A5316, 1:5,000).

Constructs

The following vectors were used in this study: pCI-neo (Promega), pDMyc-SNX27, GluR1 and GluR2, NR1 and NR2A, and pDMyc-Neo and pDHA-Neo (Modified from pCI-neo). Both Full-length and PDZ domain sequences of SNX27 were amplified by PCR and then inserted into pGEX-4T2 (GE Healthcare) for GST pull down assays. GluR1 and GluR2 constructs are from Paul Greengard's lab. Constructs expressing LAP2 and LIP (Addgene plasmid 15738 and 12561) were ordered from Addgene and used in previous studies[3].

Mouse strains

Snx27$^{+/+}$, *Snx27*$^{+/-}$ and *Snx27*$^{-/-}$ mice[1] were generated by crossing heterozygotes on C57Bl/6 and 129SV mixed backgrounds to produce F1 hybrid background animals.

Cebpb$^{+/-}$ and *Cebpb*$^{-/-}$ mice[4] were generated by crossing heterozygotes on C57Bl/6 and 129SV backgrounds to produce F1 hybrid background animals.

Segmental trisomy 16 (Ts65Dn) mice were obtained by mating female carriers of the 17^{16} chromosome (B6EiC3H-a/A-Ts65Dn) with (C57BL/6JEi X C3H/HeJ) F1 (JAX #JR1875) males[5]. Ts65Dn mice were thus maintained on the B6/C3H background. Diploid (2N) littermate mice served as WT control. All mice were also screened for retinal degeneration due to Pde6brd1 homozygosity and only animals free of retinal degeneration were used for behavioral tests.

Both male and female were used for biochemical experiments, only male mice were used for electrophysiological and behavioral experiments.

All procedures involving animals were performed under the guidelines of Sanford-Burnham Medical Research Institute (SBMRI) Institutional Animal Care and Use Committee.

Preparation of synaptosomal and PSD fractions from mouse hippocampus

Mouse hippocampi were dissected and homogenized on ice in 10 volumes of cold sucrose buffer (0.32M Sucrose, 25mM HEPES pH 7.4). The homogenates were centrifuged at 300g for 5 min to separate supernatant (S1) from the nuclei and large debris fraction. The S1 fraction was centrifuged at 10,000g for 12 min to separate the

supernatant (S2: light membrane and cytosolic fraction) and the pellet (P2: crude synaptosomal fraction). The P2 fraction was washed twice by sucrose buffer and resuspended in cold HBS buffer (25mM HEPES pH 7.4, 150mM NaCl) to get the synaptosomal fraction. The PSD fraction was prepared by solubilizing the synaptosomal fraction in 1% Triton HBS buffer at 4 °C for 30 min, then centrifuging at 10,000g for 20 min.

siRNA, miRNA mimic and quantitative Real-Time PCR

The human SNX27 siRNA used was: 5'-CCAGAUGGAACAACGGUUATT-3'. The control siRNA was from QIAGEN. The miRIDIAN miR-155 mimic was from Dharmacon. siRNA or miRNA was transfected into HEK293T or MC-IXC cells using Lipofectamine RNAiMAX reagent (Invitrogen), following the manufacturer's protocol. After siRNA/miRNA transfection, total RNA was extracted from cells with Trizol reagent (Invitrogen). After reverse transcription to first strand cDNA using standard conditions, samples were analyzed independently by real-time PCR using an iCycler iQ with SYBR green supermix (Bio-Rad). The primer sequence is in Supplementary Table 1. β-actin served as internal control. Relative microRNA expression was determined using real-time quantitative PCR and normalized to U6 expression as an internal control. Total RNA was isolated using Trizol Reagent (Invitrogen) and 500ng of total RNA was reverse transcribed using MicroRNA First-Strand Synthesis and Quantitation Kits (Clontech).

GST pull-down assays

Full-length (FL) SNX27 and SNX27 PDZ domain cDNAs were cloned into the pGEX4T1 vector. Glutathione S-transferase (GST)-fused recombinant proteins were purified as described previously[1]. For *in vitro* pull-down assays, 0.2 mg of GST or GST-SNX27 PDZ or GST-SNX27 FL was incubated with 20 ml of glutathione sepharose beads for 1 hour at 4 °C. After washing, GST or GST-SNX27 PDZ or GST-SNX27 FL conjugated beads were re-suspended in 100 ml binding buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% NP-40, supplemented with protease inhibitors), and incubated overnight at 4 °C with the mouse brain lysates (lysis buffer is the same as the binding buffer). After washing, retained proteins were eluted by boiling in SDS protein loading buffer and analyzed by Western blot using the indicated antibodies.

Co-immunoprecipitation

293T cells transfected with different expression constructs were lysed in NP-40 buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% NP-40, supplemented with protease inhibitors). Lysates were immunoprecipitated using mouse IgG, rabbit IgG, and antibodies against Myc or HA and Trueblot IP beads (eBioscience), followed by Western blot.

Promoter luciferase assay

Briefly, the human *SNX27* promoter was amplified using genomic DNA from 293T cells as templates. After amplification, PCR products were inserted into the pGL3-Basic vector containing the firefly luciferase gene (Promega). Firefly luciferase vectors were cotransfected with phRL-SV40 containing the *Renilla* luciferase gene (Promega) into Hela cells with altered C/EBPβ (over-expressed or down-regulated) for 48 h. Firefly luciferase activities were assayed and normalized to those of *Renilla* luciferase.

Chromatin IP

ChIP assays were performed with a commercial kit (Upstate, Chicago, IL), following the manufacturer's instructions with minor modifications. Briefly, 293T cells were incubated with 1% formaldehyde in tissue culture media to cross-link proteins to DNA. The cell pellet was lysed and sonicated. After centrifugation, the supernatant was incubated overnight at 4 °C with antibodies against C/EBPβ (H-7, Santa Cruz) and normal mouse IgG (Sigma). After immunoprecipitation, the antibody/protein/DNA complex was incubated at 65 °C for 4 h to reverse the protein/DNA cross-links. The DNA was purified and used as a template for PCR amplification. *SNX27* promoter primers are shown in Supplementary Table 1. PCR products were resolved on 2% agarose gels and visualized after ethidium bromide staining.

Pharmacological treatments of cycloheximide, and proteasomal and lysosomal inhibitors

Cells were incubated for 8 hours with cycloheximide (CHX, 500 μM), in the presence or absence of lysosomal inhibitor (100 μg ml^{-1} leupeptin and 50mM NH_4Cl, Sigma) or proteasomal inhibitor (10 μM MG132 and 10 μM lactacystin from Calbiochem). After treatment, cells were lysed and subjected to western blot analysis.

Immunohistochemistry and data analyses

Snx27$^{-/-}$ or *Snx27*$^{+/-}$ mice and *Snx27*$^{+/+}$ littermate controls were anesthetized and fixed by cardiac perfusion with 4% PFA. Whole brains were excised and post-fixed in 4% PFA overnight. Tissue blocks were embedded in paraffin and 5μm sections were cut and stained with Nissl (cresyl violet). Immunostained sections were examined and fluorescence images collected using a Zeiss fluorescence microscope with AxioVision software.

Golgi staining

Golgi staining was performed by using the FD Rapid GolgiStain Kit (FD NeuroTechonologies, Baltimore, MD, USA). Images were acquired by using a Zeiss fluorescence microscope using a 10× or 20× objective under differential interference contrast (DIC). Dendritic branching and length were measured using NIH Image J-software with a previously described sholl analysis plug-in[6]. Student's t test was used to determine the significance between *Snx27*$^{+/+}$ and *Snx27*$^{-/-}$ mouse neurons.

Cell surface biotinylation assay

Biotinylation was carried out following a previously described protocol[7]. Briefly, cells were washed with ice-cold phosphate-buffered saline containing 1 mM each of $CaCl_2$ and $MgCl_2$ and incubated at 4 °C with 0.5 mg ml^{-1} Sulfo-NHS-LC-biotin (Pierce) for 20 min and this process was repeated once. Cell lysates were prepared in 1% Nonidet P-40 lysis buffer. After affinity precipitation with streptavidin beads (Pierce), biotinylated proteins were eluted with SDS-PAGE sample buffer (Invitrogen) and loaded directly on SDS-PAGE gels for electrophoresis, followed by Western blot analysis.

Receptor endocytosis assay

The receptor endocytosis assays were performed as described previously[8]. After labeling with 0.5 mg ml^{-1} Sulfo-NHS-SS-biotin (Pierce) at 4 °C, the cells were placed back at 37 °C for the indicated time. Endocytosis was then stopped by transferring cells back to 4 °C. After treatment with reducing solution (15.5 mg ml^{-1} glutathione, 75 mm NaCl, 75 mm NaOH, and 10% fetal bovine serum) and 5 mg ml^{-1} iodoacetamide (Sigma) in phosphate-buffered saline containing 0.8 mm $MgCl_2$, 1.0 mm $CaCl_2$ plus 1% bovine serum albumin, the cells were lysed in TNE (10 mm Tris, pH 7.5, 150 mm NaCl, 0.5% NP-40, and 1 mm EDTA). Equal amounts of cell lysates were used for precipitation of biotinylated proteins with streptavidin beads (Pierce).

Precipitated proteins were eluted and analyzed by Western blot analysis with GluR1 and β-actin-specific antibodies and then quantified by densitometry.

Receptor recycling experiment

Receptor recycling experiments were performed as described[9]. Briefly, cells were specifically labeled with Sulfo-NHS-SS-biotin at 4 °C. After washing, cells were incubated at 37 °C for 30 min to allow endocytosis to occur. Cells were then cooled to 4 °C to stop membrane trafficking and biotin was cleaved from biotinylated proteins remaining at the cell surface with glutathione. Cells were then incubated with serum-free growth media containing 50 mM glutathione at 37 °C for various times to allow internalized receptors to recycle before the cells were cooled to 4 °C again. Cells were then incubated with glutathione cleavage buffer (twice, 15 min each at 4 °C) to ensure complete cleavage of any newly appearing surface biotin. Residual biotinylated (internalized) receptors were pulled down from cell lysates by streptavidin precipitation, and detected by Western blot analysis with GluR1 and β-actin-specific antibodies and then quantified by densitometry. The rate of reduction of biotinylated AMPARs provides a measure of the receptor recycling rate.

Cell culture and adeno-associated virus infection

For primary neuronal cultures, cortices from rat pups of different genotypes were isolated on postnatal day 0. Dissociated cells were plated at 800,000 cells ml^{-1} in

Neurobasal A medium supplemented with B27, 100 U ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin.

To over-express hSNX27, an adeno-associated viral vector (AAV1 serotype: titer 3×10^{12}) encoding human SNX27 cDNA, under control of the cytomegalovirus promoter, was used to infect rat primary neurons after 7 days in culture. Cells were harvested for Western blotting analysis at 6 days post-infection.

Stereotactic injection of adeno-associated virus

Recombinant human SNX27 and GFP adeno-associated virus (2μl, titer 3×10^{12}) were stereotactically injected into the hippocampus of Ts65Dn or WT mice (7~8 months old) at the following coordinates: anterior posterior, 1.8; medial lateral, ±1.8; dorsal ventral, 1.8. To confirm region-specific over-expression of SNX27 in mouse brains, 4 weeks after injection mice were anesthetized and sacrificed, whereupon brain tissues were rapidly removed. Hippocampal lysates were prepared by homogenizing tissue in RIPA buffer in the absence of protease inhibitors, for Western blotting analysis.

Miniature EPSC recording

For hippocampal slice recordings, horizontal slices were prepared from *Snx27*$^{+/-}$ and *Snx27*$^{+/+}$ mice at 4 to 5 weeks of age. Brains were removed from isoflurane-anesthetized mice and placed in an ice-cold buffer equilibrated with 95% O_2 and 5% CO_2 (consisting of 246 mM sucrose; 2 mM KCl; 1.25 mM NaH_2PO_4; 26 mM $NaHCO_3$; 10 mM D-glucose; 3 mM $MgSO_4$; 0.5 mM $CaCl_2$; 1 mM

Na-L-ascorbate; pH 7.4). Slices of 350 μm-thickness were cut on a vibratome (VT1000S, Leica) and recovered at room temperature for at least 1 hr prior to recording in equilibrated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) solution (containing 124 mM sucrose; 3 mM KCl; 1.2 5mM NaH_2PO_4; 26 mM $NaHCO_3$; 10 mM D-glucose; 1 mM $MgSO_4$; 2 mM $CaCl_2$; pH 7.4). All recordings were performed at room temperature, 1 to 6 hr after slices were cut. To monitor mEPSCs, slices were perfused at 2 ml min^{-1} with equilibrated ACSF supplemented with 50 μM picrotoxin and 1 μM TTX for at least 20 min prior to initiating the recording. CA1 neurons were visually identified using DIC optics. Whole-cell patch clamp recordings were performed using pipettes of 3-5 MΩ resistance when filled with internal solution (composed of 120 mM K-gluconate; 15 mM KCl; 1 mM $MgCl_2$; 5 mM HEPES; 5 mM EGTA; 2 mM Mg-ATP; pH 7.4; 290 mOsm). For mEPSC analysis, Mini Analysis software (version 6.0.3, Synaptosoft, Inc.) was used. The same parameters were used to detect mEPSC for both *Snx27*$^{+/-}$ and *Snx27*$^{+/+}$ mice; 200 consecutive events were analyzed from cells of either *Snx27*$^{+/-}$ or *Snx27*$^{+/+}$ mice.

Extracellular electrophysiology

The acute hippocampal slices were obtained and maintained as described above. For recording purposes, one slice was transferred to the recording chamber where it was continuously superfused (4-5 ml min^{-1}) with oxygenated ACSF. The Schaffer collateral inputs to the CA1 region were stimulated with a bipolar tungsten electrical stimulating electrode at three different intensities (min, half-max. and max.). Using a

low resistance recording electrode, the field Excitatory Postsynaptic potential (fEPSP) responses from the stratum radiatum region of CA1 were recorded using a MultiClamp (Axon Instrument). The initial slope of the fEPSP response was measured using Clampex software. Synaptic transmission of CA1 neurons was determined as input-ouput curves for fEPSP slope response to Schaffer collateral stimulation.

Long-term synaptic plasticity

A paired-pulse facilitation (PPF) response was induced by stimulating the Schaffer collateral pathway twice at various intervals (15, 30, 50, 100, 200 msec) at half-max intensity. To obtain the PPF ratio, the initial slope of the second fEPSP response was divided by the first. Long-term potentiation (LTP) was induced following PPF and establishing baseline fEPSP recordings for 10-15 minutes. LTP induction consisted of five high frequency trains (HFTs) of stimuli (100 Hz – 20 sec intervals) at half-maximum stimulus intensity. Thirty seconds following LTP induction, fEPSP recordings of the CA1 region were resumed for a total duration of 60 minutes. All data for LTP experiments was normalized to baseline recordings.

Neurobehavioral tests

Locomotor activity test - Locomotor activity was measured in a polycarbonate cage (42 x 22 x 20 cm) placed into frames (25.5 x 47 cm) mounted with two levels of photocell beams at 2 and 7 cm above the bottom of the cage (San Diego Instruments,

San Diego, CA). Both horizontal (locomotion) and vertical (rearing) behavior were recorded. A thin layer of bedding material was placed on the bottom of the cage. Mice were tested over 120 minutes.

Novel object exploration tests – In general, these tests allowed for the assessment of learning and memory by looking at whether mice can distinguish a newly experienced object from familiar ones (Novel Object test) or a moved object relative to its original position (Novel Location test). This was accomplished by comparing exploration of the objects (number of contacts with objects or time spent contacting objects) before and after changes were made.

Combined novel object/novel location test. The location novelty recognition test (NL) assesses the ability of mice to recognize a novel spatial arrangement of familiar objects and is sensitive to hippocampal damage. The object novelty recognition test assesses the ability to recognize a novel object in the environment. Mice were individually habituated to a 51 × 51 × 39 cm open field for 5 min. For the familiarization trials, three plastic toy objects were placed in the open field (one in each of three corners), and an individual animal was allowed to explore for 5 min. After three familiarization trials (separated by 1 minute in a holding cage), the mouse was tested by a location novelty recognition test in which one of the familiar objects was moved to a novel location in the arena. The same object was moved to the same new location for every mouse tested. The mouse was then tested in an object novelty recognition test in which a novel object replaced one of the familiar objects. All

objects and the arena were thoroughly cleaned with 70% ethanol between trials to remove odors. The four different objects required for this study were chosen based on previously published methods[10] and a lack of statistically significant preference for any object in pilot studies using C57BL/6J mice. These included a toy farmer boy, horse and cow, and queen (Playmobil, Geobra Brandstatter GmbH & Co. KG, Zirndorf, Germany). The objects chosen were made of durable non-toxic plastic and were fixed to 10 × 7 × 0.5 cm square clear Plexiglas bases to prevent mice from moving the objects during testing. 'Exploration' is defined as approaching the object nose-first within 2–4 cm. The numbers of approaches toward each object were calculated for each trial. Habituation to the objects across the three familiarization trials (decreased contacts) is an initial measure of learning and then renewed interest (increased contacts) in the moved object and new object indicate evidence of spatial memory and object memory, respectively.

Barnes maze test - The Barnes maze test is a spatial learning and memory test originally developed in rats[11], but also adapted for mice[12]. The Barnes maze task has the benefit of minimizing pain and distress to the animal. Traditional spatial tests involve swimming in opaque water to locate a hidden platform (the Morris water maze). The Barnes maze is less physically taxing than the Morris water maze, making it more suitable for mice, which are not as strong as rats, and also suitable for studies of mice of all ages and abilities[12]. In addition, the strategies used by the animals to perform the task are readily revealed. The Barnes maze used was an opaque Plexiglas

disc 75 cm in diameter elevated 58 cm above the floor by a tripod. Twenty holes, 5 cm in diameter, are located 5 cm from the perimeter and a black Plexiglas escape box (19 × 8 × 7 cm) was placed under one of the holes. Distinct spatial cues were located all around the maze and were kept constant throughout the study.

ACQUISITION: On the first day of testing a training session was performed, consisting of placing the mouse in the escape box and leaving it there for one minute. One minute later, the first session was started. At the beginning of each session, the mouse was placed in the middle of the maze in a 10 cm high cylindrical black start chamber. After 10 seconds the start chamber was removed, a buzzer (80 dB) and a light (400 lux) were turned on, and the mouse was set free to explore the maze. The session ended when the mouse entered the escape tunnel or after 3 min elapsed. When the mouse entered the escape tunnel, the buzzer was turned off and the mouse was allowed to remain in the dark for one minute. If the mouse did not enter the tunnel by itself it was gently put in the escape box for one minute. The tunnel was always located underneath the same hole (stable within the spatial environment) and was randomly determined for each mouse. Mice were tested once a day for 9 days for the acquisition portion of the study.

PROBE TEST: For the 10^{th} test (probe test), the escape tunnel was removed and the mouse was allowed to freely explore the maze for 3 min. The time spent in each quadrant was determined and the percent time spent in the target quadrant (the one

originally containing the escape box) was compared with the average percent time in the other three quadrants. This is a direct test of spatial memory as there is no potential for local cues to be used in the mouse's behavioral decision.

Each session was videotaped and scored by an experimenter blind to the genotype/treatment of the mouse. Measures recorded include the number of errors made per session and the strategy employed by the mouse to locate the escape tunnel. Errors are defined as nose pokes and head deflections over any hole that did not have the tunnel beneath it. Search strategies are determined by examining each mouse's daily session and classifying it into one of three operationally defined categories: 1) Random search strategy - localized hole searches separated by crossings through the center of the maze, 2) Serial search strategy - systematic hole searches (every hole or every other hole) in a clockwise or counterclockwise direction, or 3) Spatial search strategy - reaching the escape tunnel with both error and distance (number of holes between the first hole visited and the escape tunnel) scores of less than or equal to 3.

Optomotor test - The optomoter is a stationary elevated platform surrounded by a drum with black and white striped walls. The mouse was placed on the platform to habituate for 1 minute and then the drum rotated at 2rpm in one direction for 1 minute, stopped for 30 sec, and then rotated in the other direction for 1 minute. The number of head tracks (15 degree movements at speed of drum) were recorded.

Human brain specimens

Human Down's syndrome brain cortex samples were obtained from the Brain and Tissue Bank for Developmental Disorders, University of Maryland at Baltimore, in contract with the National Institutes of Health, NICHD. Additional human Alzheimer's disease brain samples used in this project were provided by UCSD, San Diego, CA and were analyzed with institutional permission under California and National Institutes of Health guidelines. For specimen information see supplementary Table 2, 3.

Statistical analyses

Statistical analyses were performed with GraphPad Prism. Data distribution was assessed by a Kolmogorov-Smirnoff nonparametric test of equality. Differences between two means were assessed by paired or unpaired *t* test. Differences among multiple means were assessed, as indicated, by one-way, two-way or repeated-measures ANOVA, followed by Bonferroni's, Dunnett's or Tukey's *post-hoc* analysis. Error bars represent s.e.m. Null hypotheses were rejected at the 0.05 level.

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