Loss of sorting nexin 27 contributes to excitatory synaptic dysfunction by modulating glutamate receptor recycling in Down’s syndrome

Xin Wang1,2, Yingjun Zhao1,3, Xiaofei Zhang1, Hedieh Badie4, Ying Zhou2, Yangling Mu5, Li Shen Loo6, Lei Cai6, Robert C Thompson1, Bo Yang1, Yamin Chen1, Peter F Johnson7, Chengbiao Wu8, Guojun Bu3, William C Mobley8, Dongxian Zhang1, Fred H Gage5, Barbara Ranscht4, Yun-wu Zhang1,3, Stuart A Lipton1,8, Wanjin Hong6,9 & Huaxi Xu1,3

Sorting nexin 27 (SNX27), a brain-enriched PDZ domain protein, regulates endocytic sorting and trafficking. Here we show that Snx27−/− mice have severe neuronal deficits in the hippocampus and cortex. Although Snx27+/− mice have grossly normal neuroanatomy, we found defects in synaptic function, learning and memory and a reduction in the amounts of ionotropic glutamate receptors (NMDA and AMPA receptors) in these mice. SNX27 interacts with these receptors through its PDZ domain, regulating their recycling to the plasma membrane. We demonstrate a concomitant reduced expression of SNX27 and CCAAT/enhancer binding protein β (C/EBPβ) in Down’s syndrome brains and identify C/EBPβ as a transcription factor for SNX27. Down’s syndrome causes overexpression of miR-155, a chromosome 21–encoded microRNA that negatively regulates C/EBPβ, thereby reducing SNX27 expression and resulting in synaptic dysfunction. Upregulating SNX27 in the hippocampus of Down’s syndrome mice rescues synaptic and cognitive deficits. Our identification of the role of SNX27 in synaptic function establishes a new molecular mechanism of Down’s syndrome pathogenesis.

SNX proteins comprise a large group of proteins that contain a conserved PX (or phagocyte oxidase homology) domain targeting SNX proteins to endosomes. SNX27 was originally identified in rats as an alternative splicing product of the Mrt1 (methamphetamine responsive transcript 1) gene. SNX27 contains a PDZ (PSD-95, Disc-large and ZO-1) domain, making it unique among the PX domain proteins. PDZ domains are protein-protein interaction domains that are often found in the postsynaptic density (PSD) of neuronal excitatory synapses.

SNX27 reportedly participates in the dynamic trafficking of receptors and ion channels such as β2-adrenergic receptors (β2-ARs), G protein–activated inward rectifying potassium type 2 (GIRK2), serotonin receptor subunit 4a (5-HT4a) and NMDA-type glutamate receptor subunit 2C (NR2C). However, the physiological role of SNX27 at synapses and whether SNX27 regulates the trafficking of other major types of glutamate receptors remains unknown.

In the PSDs of excitatory synapses, different classes of glutamate receptors are responsible for transducing the presynaptic signal into both biochemical and electrical events in the postsynaptic neuron. The two major types of glutamate receptors are AMPA-type glutamate receptors (AMPA-Rs) and NMDA receptors (NMDA-Rs). Dysregulation of AMPA-Rs and NMDA-Rs is involved in several neurodegenerative diseases.

Down’s syndrome, or trisomy 21, is a congenital disorder that manifests as defects in multiple organs and causes developmental delays and learning disabilities. People with Down’s syndrome have an extra copy of chromosome 21, leading to an overdosage of the gene products and noncoding RNAs encoded by this chromosome. Down’s syndrome pathology includes neuropathology of the cortex and hippocampus in both developmental and aging processes. Substantial dendritic and synaptic abnormalities, including decreased dendritic arborization and a reduction in synaptic number, have been observed in both prenatal and postnatal Down’s syndrome brains. The balance between excitatory and inhibitory synapses is reportedly impaired in the brains of both humans with Down’s syndrome and mouse models of the disease. Impaired long-term potentiation (LTP) has also been detected in the hippocampal CA1 region of Ts65Dn mice, a widely used Down’s syndrome mouse model. Although several chromosome 21–encoded products, such as β-amyloid...
precursor protein (APP), are thought to contribute to the pathology of Down’s syndrome, the detailed molecular mechanisms remain largely unclear.

Here we demonstrate a new role for SNX27 in the dysregulation of synaptic function in Down’s syndrome. We show that chromosome 21–encoded miR-155 targets and downregulates C/EBPβ, which is a transcription factor for SNX27. Thus, the lower amounts of C/EBPβ in Down’s syndrome lead to reduced SNX27 expression. We show that SNX27 promotes recycling of AMPARs and NMDARs from early endosomes to the plasma membrane through direct PDZ binding and thus prevents their degradation. Deletion of Snx27 in mice results in synaptic dysfunction and cognitive deficits. Further, overexpressing Snx27 in the hippocampus of Ts65Dn mice reverses the impairments in receptor amounts and synaptic functions, as well as in hippocampus-dependent memory. Therefore, SNX27 is crucial for maintaining glutamate receptors through post-translational mechanisms and is required for normal synaptic activity and memory formation.

RESULTS
Cortical and hippocampal pathology of Snx27−/− mice

We first examined the developmental expression pattern of Snx27 in postnatal mouse brains and found that Snx27 expression can be detected at postnatal day 0 (P0) and reaches a plateau at P7. The developmental expression pattern of Snx27 is similar to those of glutamate receptor, ionotropic, AMPA1 (GluR1, also known as Gria1) and glutamate receptor, ionotropic, NMDA1 (NR1, also called Grin1) (Fig. 1a). In situ hybridization results, as reported by the Allen Brain Atlas, revealed that Snx27 mRNA is highly expressed in the cortex, hippocampus and cerebellum (Supplementary Fig. 1). To investigate the physiological function of Snx27, we analyzed Snx27 knockout mice and found that most Snx27−/− mice were viable from birth until P14. Their growth rate then slowed, and the mice died by week 4 (data not shown).

Microscopic histological examination of Snx27−/− brains revealed degenerating neurons in the cortex at P14, with reduced somal size and hyperchromicity (Fig. 1b).

Brain development during the early postnatal period involves increases in dendritic branching and synapse formation, both of which were greatly compromised in Snx27−/− mice at P14. Although the orientation of the apical dendrites was unaffected, the total dendritic lengths of both cortical layer 5 and hippocampal CA1 neurons were substantially reduced. There was also a marked decrease in dendritic branching in cortical neurons (Fig. 1c,d).

Impaired learning and memory in Snx27+/− mice

Complete loss of Snx27 results in severe neuronal death and eventual lethality in mice, making it impossible to determine how Snx27 influences memory formation and synaptic function. However, Snx27+/− mice are viable and have grossly normal neuroanatomy (Supplementary Fig. 2) and lifespan compared to Snx27+/+ littermates; thus, we examined the role of Snx27 in memory and synaptic function using Snx27+/− mice.

As intellectual disability is a primary aspect of Down’s syndrome, we assessed potential cognitive deficits in Snx27+/− mice using behavioral tests. We first used the Barnes maze to assess learning and memory and found that Snx27+/− mice made more errors at days 6–8 after training (Fig. 2a) and used less spatial strategies than Snx27+/+ mice (Supplementary Fig. 3a). Snx27+/− mice did not spend significantly more time in the target quadrant than in other quadrants in the probe test (Fig. 2b). Furthermore, Snx27+/− mice spent much less time exploring novel objects than familiar objects in the novel object recognition task compared to Snx27+/+ mice (Fig. 2c). We performed additional behavioral tasks to test for locomotor activity.
Figure 2 Cognitive and synaptic deficits in Snx27−/− mice. (a) Barnes maze test results for error counts in finding the escape chamber. Data represent the mean ± s.e.m. n = 10 mice per genotype. *P < 0.05 determined by repeated-measures analysis of variance (ANOVA) with Bonferroni’s post hoc analysis. (b) Barnes maze probe test results. Data represent the mean ± s.e.m. n = 10 mice per genotype. **P < 0.01, NS, not significant determined by nonparametric t test. (c) Novel object recognition test results. Data represent the mean ± s.e.m. n = 10 (Snx27+/+ mice) and n = 8 (Snx27−/− mice). *P < 0.05 determined by nonparametric t test. (d) Input-output curves for basal synaptic transmission in hippocampal slices from Snx27+/+ (n = 4 slices) and Snx27−/− (n = 8 slices) mice. Data represent the mean ± s.e.m. **P < 0.05 determined by two-tailed Student’s t test. (e) PPF, field excitatory postsynaptic potential. (f) PPF in Snx27+/+ (n = 6 slices) and Snx27−/− (n = 8 slices) hippocampal neurons. Data represent the mean ± s.e.m. Slope2/Slope1 is an indicator of paired-pulse facilitation. (g) Representative mEPSC recordings in CA1 neurons from hippocampal slices of Snx27+/+ and Snx27−/− mice at 1 month of age. (h) Cumulative probability distributions of mEPSC amplitude and frequency for Snx27+/+ (n = 8 cells) and Snx27−/− (n = 9 cells) mice. (i) Mean mEPSC amplitude and frequency in Snx27+/+ (n = 8 cells) and Snx27−/− (n = 9 cells) neurons. Data represent the mean ± s.e.m. ***P < 0.001 determined by two-tailed Student’s t test. (i) CA1 LTP in Snx27+/+ (n = 8) and Snx27−/− (n = 9) mouse hippocampal slices. Note, the arrow indicates where time 0 is. Data represent the mean ± s.e.m. **P < 0.01 determined by repeated-measures ANOVA with Bonferroni’s post hoc analysis on the last 10 min of data.

Impaired synaptic functions in Snx27−/− mice

We took extracellular recordings of hippocampal slices to examine LTP in the hippocampal CA1 region. Input-output response results showed that Snx27−/− mice had decreased excitatory synaptic transmission compared to Snx27+/+ mice (Fig. 2d). The attenuation of basal synaptic transmission in Snx27−/− may arise from a reduced number of synapses or defects in either presynaptic or postsynaptic function. To distinguish between these possibilities, we used a paired-pulse facilitation (PPF) paradigm to examine the regulation of synaptic activity through Ca2+-mediated presynaptic neurotransmitter release. We found that the PPF ratio was unchanged in Snx27−/− brain slices compared to Snx27+/+ controls (Fig. 2e). In contrast, when we recorded miniature excitatory postsynaptic currents (mEPSCs) from CA1 hippocampal neurons, the amplitude was significantly decreased in Snx27+/+ neurons compared to Snx27+/− neurons, whereas the frequency remained unaltered (Fig. 2f–h).

Given that the defect is postsynaptic, we reasoned that synaptic plasticity should also be disrupted. When we examined NMDAR-dependent synaptic plasticity in Snx27+/+ mice, we found that LTP induced by high-frequency trains (100 Hz for 1 s) was decreased compared to Snx27+/+ controls (Fig. 2i). As the mEPSCs data represent AMPAR-mediated responses, and the induction of LTP is dependent on intact NMDAR activity, our electrophysiological data suggest that Snx27+/+ mice have defects in both AMPAR- and NMDAR-dependent synaptic events.

Loss of SNX27 increases degradation of AMPARs and NMDARs

Using western blot analysis, we determined that Snx27+/− mice had lower amounts of GluR1, GluR2, NR1, NR2A and NR2B in both synapticosomal membranes and PSD fractions compared to Snx27+/+ littermates (Fig. 3a). We examined the total amounts of glutamate receptor subunits in the hippocampus and found that the amounts of GluR1, GluR2 and NR1, but not NR2A and NR2B, were decreased in Snx27−/− mice compared to Snx27+/+ mice at P1. However, by P14, the amounts of NR2A and NR2B were also decreased (Supplementary Fig. 5). The amount of PSD-95 (also called Dlg4) was unaffected at both time points.

We performed quantitative RT-PCR to measure receptor mRNA amounts and found that they were unaffected in the hippocampus of Snx27−/− mice compared to littermate controls (Supplementary Fig. 6). To determine whether SNX27 influences turnover of the receptors, we performed cycloheximide chase assays in HEK293 cells overexpressing either GluR1 (GluR1-HEK293) or both NR1 and NR2A (NR1-NR2A-HEK293). The turnover of both GluR1 and NR1 was markedly accelerated by the downregulation of SNX27 using siRNA (Fig. 3b, c). Treatment of GluR1-HEK293 or NR1-NR2A-HEK293 cells with the proteasomal inhibitor lactacystin or MG132 in the presence of cycloheximide largely rescued the degradation of GluR1 and NR1 caused by SNX27 knockdown. Treatment with the lysosomal inhibitor leupeptin or NH4Cl also rescued receptor degradation, although to a lesser extent (Fig. 3d, e).

SNX27 promotes glutamate receptor recycling to cell surface

We found that both a recombinant SNX27 PDZ domain and full-length SNX27 protein linked with GST can pull down glutamate receptors (GluR1, GluR2, NR1 and NR2A) but not the presynaptic vesicle protein synaptophysin from mouse brain lysates (Fig. 4a). We co-transfected Myc-tagged SNX27 and constructs expressing various glutamate receptors into HEK293T cells and verified their interaction by coimmunoprecipitation (Fig. 4b). Moreover, endogenous Snx27 coimmunoprecipitated with endogenous glutamate receptors from mouse brain lysates (Fig. 4c).
Cell surface biotinylation experiments showed that overexpression of SNX27 resulted in increased cell surface amounts of GluR1 and NR1 in Glur1–HEK293 and NR1-NR2A-HEK293 cells (Fig. 4d). Conversely, knockdown of SNX27 resulted in reduced cell surface expression of GluR1 and NR1 (Fig. 4e). This effect was also evident in primary neurons; we found a decrease in cell surface expression of GluR1 and NR1 in primary neurons derived from Snx27+/- mice (Fig. 4f).

Figure 3 Downregulation of SNX27 affects the stability of NMDARs and AMPARs. (a) Amounts of the glutamate receptor subunits GluR1, GluR2, NR1, NR2A and NR2B in synaptosomal and PSD fractions derived from Snx27+/+ and Snx27+/- mice. Data represent the mean ± s.e.m. n = 3 mice per genotype. *P < 0.05; **P < 0.01 determined by two-tailed Student’s t test. (b,c) Time course of Glur1 degradation after SNX27 knockdown in Glur1-HEK293 or NR1-NR2A-HEK293 cells. Shown are results of western blot analyses of GluR1 (b) or NR1 (c) and β-actin performed after treatment with cycloheximide (CHX; 500 µM) for indicated time periods. The signal intensities of Glur1 and NR1 were standardized to the signal intensity of β-actin and normalized to 100% at time 0. Data represent the mean ± s.e.m. n = 3. **P < 0.05; ***P < 0.01 determined by repeated-measures ANOVA with Bonferroni’s post hoc analysis. Ctrl, control. (d,e) Analysis of degradation of Glur1 (d) and NR1 (e) after addition of lysosomal and proteasomal inhibitors in Glur1-HEK293 or NR1-NR2A-HEK293 cells that express SNX27 siRNA or control siRNA. Cells were treated with CHX (500 µM) for 8 h in the presence of DMSO (−), lysosomal inhibitors (100 µg ml⁻¹ leupeptin (Leu) or 50 mM NH4Cl) or proteasomal inhibitors (10 µM lactacystin (Lac) or 10 µM MG132), as indicated. Data represent the mean ± s.e.m. n = 3. *P < 0.05, **P < 0.01, ***P < 0.001, NS, not significant determined by one-way ANOVA with Dunnett’s post hoc analysis.
The steady-state expression of cell surface receptors is the net result of both endocytosis and recycling of the receptors. As SNX27 did not influence the endocytosis of GluR1 (Supplementary Fig. 7), we postulated that SNX27 affects the recycling of the receptors. To test this hypothesis, we carried out receptor recycling assays and found that overexpression of SNX27 decreased the amount of internalized biotinylated GluR1 in GluR1-HEK293 cells after SNX27 overexpression (Fig. 4g). Consistent with these overexpression experiments, we found slower recycling of biotin-labeled GluR1 after treatment with SNX27 siRNA as compared to treatment with control siRNA (Fig. 4b).

Reduction of SNX27 expression in Down’s syndrome brains

We examined the amount of SNX27 in brain samples from humans with Down’s syndrome and age-matched controls and found that both protein and mRNA amounts of SNX27 were markedly decreased in the cortex of individuals with Down’s syndrome (Fig. 5). Consistent with changes in SNX27, the amount of GluR1 was also decreased in Down’s syndrome brains, whereas the amount of APP was markedly increased (Fig. 5a). However, the amount of SNX27 was not altered in the cortex of humans with sporadic Alzheimer’s disease compared to controls (Fig. 5b). We also found that the amounts of both Snx27 and C/EBPβ were lower in the hippocampus of Ts65Dn mice than in wild-type (WT) controls (Supplementary Fig. 8a). However, only Snx27, but not Cebpβ, mRNA expression was reduced in the hippocampus of Ts65Dn mice (Supplementary Fig. 8b).

Regulation of SNX27 by the miR-155–C/EBPβ pathway

We next examined the expression of chromosome 21–derived microRNAs (miRNAs) in Down’s syndrome cortices. Our findings were consistent with previous studies demonstrating increased expression of chromosome 21–encoded miRNAs in the brains of both humans with Down’s syndrome and Ts65Dn mice (Supplementary Figs. 8c and 9a). Among these miRNAs, only miR-155 expression was negatively correlated with SNX27 mRNA expression (Supplementary Fig. 9b). We transfected a miR-155 mimic into human neuroblastoma MC-IXC cells and found decreased amounts of both SNX27 protein and mRNA (Fig. 5d,e). However, we found no consensus miR-155 binding sites on the SNX27 3’ untranslated region, suggesting that SNX27 is not the direct target. We found a positive correlation between the amounts of C/EBPβ and SNX27 in the cerebral cortex of humans with Down’s syndrome (Supplementary Fig. 9c). Additionally, in silico analysis predicted several C/EBPβ binding sites in the SNX27 promoter.
region (Supplementary Fig. 10), and the binding of miR-155 to the C/EBPβ 3′ untranslated region was recently reported22.

We overexpressed an active isoform of C/EBPβ, liver-enriched activator protein (LAP2), in MC-IXC human neuroblastoma cells and found that SNX27 was significantly upregulated (Fig. 5f). Moreover, we determined that the amount of SNX27 was decreased in the cortex and hippocampus of Cebpb−/− mice compared to Cebpb+/− controls (Fig. 5g). A luciferase reporter assay using HeLa cells showed that overexpression of LAP2 enhanced the promoter activity of human SNX27, whereas overexpression of another C/EBPβ isoform, liver inhibitory protein (LIP), did not affect SNX27 promoter activity (Fig. 5h).

We carried out a chromatin immunoprecipitation (ChIP) assay and found that C/EBPβ binds to the endogenous SNX27 promoter in HEK293T cells (Fig. 5i).

Figure 6 SNX27 rescues cognitive and synaptic deficits in Ts65Dn mice. (a) Schematic illustration of the AAV construct of human SNX27 with IRES-GFP. (b) Workflow of stereotactic AAV injection rescue experiments. (c) GFP fluorescence in the hippocampus showing the infected region of a mouse brain. Scale bar, 200 μm. (d) The effect of hippocampal injection of AAV-SNX27-IRES-GFP on cognitive deficits in 7- to 8-month-old Ts65Dn mice in the novel object recognition test. Data represent the mean ± s.e.m. WT + GFP, n = 8 mice; Ts65Dn + GFP, n = 5 mice; Ts65Dn + SNX27, n = 7 mice. *P < 0.05 determined by one-way ANOVA with Tukey’s post hoc analysis. (e) The effect of hippocampal injection of AAV-SN X27-IRES-GFP on CA1 LTP deficits in 7- to 8-month-old Ts65Dn mice. Data represent the mean ± s.e.m. WT + GFP, n = 7 slices; Ts65Dn + GFP, n = 9 slices; Ts65Dn + SNX27, n = 10 slices. *P < 0.05 determined by repeated-measures ANOVA with Bonferroni’s post hoc analysis on the last 10 min of data. Note, the arrow indicates where time 0 is. (f) Western blot analysis of GluR1, NR1, NR2B and other synaptic proteins after intrahippocampal injection of AAV-SNX27-IRES-GFP or AAV-GFP into Ts65Dn mice. Data represent the mean ± s.e.m. n = 3 mice per genotype. *P < 0.05 determined by one-way ANOVA with Tukey’s post hoc analysis.
SNX27 expression rescues synaptic deficits in Ts65Dn mice

To test whether SNX27 expression could rescue the synaptic deficits and impaired learning and memory of Down’s syndrome, we generated an adeno-associated virus type 1 (AAV1) containing human SNX27 complementary DNA (Fig. 6a). Overexpression of human SNX27 increased the amounts of GluR1, GluR2, NR1, NR2A and NR2B in rat primary neurons (Supplementary Fig. 11). We next injected AAV-SNX27–internal ribosome entry site (IRES)-GFP or an AAV-GFP control bilaterally into the hippocampal CA1 area of 7- to 8-month-old Ts65Dn or WT mice and analyzed the subsequent behavioral changes (Fig. 6b,c). Because CA1-specific deletion of NMDAR1 can result in impaired performance on novel object recognition tests and Ts65Dn mice show impaired LTP and novel object recognition memory, this test may allow for the evaluation of hippocampus-dependent memory. Notably, the cognitive and LTP deficits in Ts65Dn mice were rescued by increased SNX27 expression in the hippocampus (Fig. 6b–e). All mice behaved normally on the optomotor test (Supplementary Fig. 4b), excluding the possible confounding effects of visual problems (because a small portion of Ts65Dn mice may become blind). Four weeks after injection of AAV-SNX27–IRES-GFP, the amounts of SNX27 were markedly higher in AAV-SNX27–IRES-GFP–injected Ts65Dn mice than in AAV-GFP–injected Ts65Dn mice (Fig. 6f). In synaptosomal preparations from Ts65Dn and WT mice, the amounts of glutamate receptor subunits GluR1, NR1 and NR2B were lower in Ts65Dn mice than in WT mice (Supplementary Fig. 8d). Reduced amounts of synaptosomal glutamate receptors were rescued by exogenous expression of SNX27 in the hippocampus of the Ts65Dn mice (Fig. 6f).

DISCUSSION

Substantial dendritic and synaptic abnormalities, such as decreased dendritic arborization and reduced synapse numbers, have been reported in both prenatal and postnatal Down’s syndrome brains. Similarly, dendritic growth and branching is greatly compromised in Snx27−/− mice analyzed at early postnatal stages. The decreased dendritic length and branching suggest that Snx27 is essential for postnatal brain development.

Abnormalities in excitatory neurotransmission and cognitive function have been reported in humans with Down’s syndrome, as well as in Down’s syndrome mouse models. Decreased LTP has been observed in both Ts65Dn and Tc1 Down’s syndrome mouse models. To investigate whether SNX27 deficiency results in synaptic and cognitive deficits, we used Snx27−/− mice for electrophysiological and behavioral studies, as Snx27−/− mice show severe neuropathology and early lethality. Additionally, the Snx27−/− mouse model might better mimic the pathology of human Down’s syndrome, which is caused by a partial loss of SNX27 expression only.

mEPSC recordings reveal that AMPAR-mediated postsynaptic currents are reduced in the hippocampus of Snx27−/− mice compared to controls. Furthermore, NMDAR-dependent Schaffer-collateral CA1 LTP induction is attenuated in Snx27−/− mice, indicating a possible role for Snx27 in the LTP mechanism.

The balance between the insertion and internalization of AMPARs at the postsynaptic membrane is an important mechanism for regulating synaptic strength. Our biochemical and cell biological data show that SNX27 promotes NMDAR and AMPAR recycling back to the plasma membrane and is not involved in the internalization of these receptors.

In the future, it will be interesting to study whether SNX27 regulates the recycling of NMDARs and AMPARs in cooperation with other components of the trafficking machinery. For example, SNX27, similarly to SNX17, contains a C-terminal PX–FERM domain, and the FERM domain can reportedly associate with the activated Ras small GTPase. It is still unclear, however, whether SNX27 interacts with the small GTPase to regulate the sorting and recycling of NMDARs and AMPARs. Additionally, the retromer complex has also been shown to regulate the transport of various cargos from the endosome to the trans-Golgi network and plasma membrane directly. Previous studies showed cooperation between SNX27 and the retromer complex in the regulation of β2-AR recycling. Mutation and dysfunction of the retromer complex have been linked with neurodegenerative diseases, including Alzheimer’s disease and Parkinson’s disease, and thus further investigation on whether SNX27 regulates the recycling of AMPARs and NMDARs in a retromer-dependent manner will be interesting.

miR-155, encoded on chromosome 21 and thus overexpressed in Down’s syndrome, is a negative regulator of C/EBPβ. Previous studies have suggested that C/EBP is crucial for the consolidation of long-term memory in both invertebrates and mice. C/EBPβ is an evolutionarily conserved gene with a selective role in the consolidation of new memories in the hippocampus. Our results indicate that SNX27, a downstream target of C/EBPβ, is downregulated in Down’s syndrome brains. Notably, restoration of SNX27 by site-specific delivery of AAV reverses the LTP and cognitive deficits of Ts65Dn mice. Loss of Snx27 may partially explain the mechanism whereby trisomy 21 leads to synaptic dysfunction and memory impairments.

We found nearly normal protein amounts of GABA A receptor subunits (α1 and β2) in the hippocampus of Snx27+/− mice. Using coimmunoprecipitation, we also determined that SNX27 does not interact with GABA A receptor subunits (α1 and β2) (Supplementary Fig. 12). These results suggest that SNX27 does not affect GABA A receptors. However, previous studies have reported that SNX27 negatively regulates GIRK2 (ref. 5), which is encoded by chromosome 21 and is highly expressed in Down’s syndrome brains. As GIRK2 controls the excitability of neurons through GIRK-mediated self-inhibition, its overexpression is involved in the excessive inhibition in Down’s syndrome brains. Therefore, SNX27 expression could contribute to the restoration of balance between excitation and inhibition through both upregulation of excitatory transmission and suppression of GIRK2-mediated inhibition.

In summary, we have identified a signaling pathway in which the decreased amount of C/EBPβ resulting from excessive miR-155 in Down’s syndrome represses SNX27 expression (see Supplementary Fig. 13 for a schematic model). This leads to synaptic dysfunction and neuropathological changes. Thus, the miR-155–C/EBPβ–SNX27 pathway is a newly identified contributory mechanism to the neuropathogenesis of Down’s syndrome.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
X.W. and H.X. conceptualized the study, X.W. designed and performed morphological analysis and biochemical assays, and Y. Zhao performed receptor endocytosis and recycling experiments. X.Z. performed mEPSC and LTP morphological analysis and biochemical assays, and Y. Zhao performed receptor trafficking experiments. H.B. and Y.M., respectively. Y. Zhou analyzed miRNA expression in humans recordings in the SNX27 rescue experiments, and S.A.L. designed and helped endocytosis and recycling experiments. X.Z. performed mEPSC and LTP expression in humans. Excellent Talents in Universities (NCET), the Fundamental Research Funds for the collection and A. Brzozowska-Prechtl and L. Lacarra for technical help. This work was supported in part by US National Institutes of Health grants (R01 AG038710, R01 AG021173, R01 NS046673, R01 AG030197 and R01 AG044420 to H.X.; and P01 HD29587, P01 ES016738, P30 NS076411 to S.A.L.) and grants from the Alzheimer’s Association (to H.X. and Y. Zhang), the American Health Assistance Foundation (to H.X.), National Natural Science Foundation of China (30973150 and 81161120496 to Y. Zhang), the 973 Prophecy Project (2010CB539904 to Y. Zhang) and Natural Science Funds for Distinguished Young Scholar of Fujian Province (2009J06022 to Y. Zhang). Y. Zhang is supported by the Program for New Century Excellent Talents in Universities (NCET), the Fundamental Research Funds for the (2009J06022 to Y. Zhang). Y. Zhang is supported by the Program for New Century Excellent Talents in Universities (NCET), the Fundamental Research Funds for the

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ONLINE 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 (refs. 7,40). Other antibodies acquired from commercial sources were to the following: GluR1 (Chemicon, 04-855, 1:500), GluR2 (Millipore, AB1768, 1:1,000), NR1 (BD Biosciences, 556308, 1:1000), NR2A (Millipore, 01-632, 1:500), NR2B (BD Biosciences, 610416, 1:500), VGAT (Millipore, AB5062P, 1:500), GABA_A_R1 (Millipore, 06-868, 1:500), GABA_A,β2 (Millipore, MAB341, 1:500), C/EBPβ (Santa Cruz, sc-7962, 1:100), Transferrin receptor (Invitrogen, 13-6800, 1:500), c-Myc (Invitrogen, 9E10, 1:1000), 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-Neon and pDHANeo (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 pulldown assays. GluR1 and GluR2 constructs were from Paul Greengard’s lab. Constructs expressing LAP2 and LIP (Addgene plasmid 15738 and 12561) were ordered from Addgene and described in a previous study41.

Mouse strains. Snx27+/+, Snx27+/− and Snx27−/− mice were generated by crossing heterozygotes on C57BL/6 and 129/SV mixed backgrounds to produce F1 hybrid background mice. Cebpb+/− and Cebpb−/− mice51 were generated by crossing heterozygotes on C57BL/6 and 129/SV backgrounds to produce F1 hybrid background mice. T65Dn mice were obtained by mating female carriers of the 17 (ref. 16) chromosome (B6Eic3H-A-T65Dn) with (C57BL/6/Ei × C3H/He) F1 (JAX JR1875) males25. T65Dn mice were maintained on the B6/C3H background. Diploid (2n) littermate mice were used as WT controls. All mice were also screened for retinal degeneration caused by Pde6brd1 homozygosity, and only mice that were free of retinal degeneration were chosen 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 (0.32 M sucrose and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4). The homogenates were centrifuged at 300g for 5 min to separate the 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 with sucrose buffer and resuspended in cold HBS buffer (25 mM HEPES, pH 7.4, and 150 mM 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 and then centrifuging at 10,000g for 20 min.

siRNA, miRNA mimic and quantitative real-time PCR. The human SNX27 siRNA was purchased from Qiagen. The miRIDIAN miR-155 mimic was from Dharmacon. siRNA or miRNA was transfected into HEK293T or MC-1Xc cells using Lipofectamine RNAiMAX. The real-time PCR primer sequence is given in Supplementary Table 1. β-actin served as the control. Relative miRNA 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 500 ng of total RNA was reverse transcribed using miRNA First-Strand Synthesis and Quantitation Kits (Clontech).

GST pulldown assays. Full-length SNX27 and SNX27 PDZ domain complementary DNAs were cloned into the pGEX-4T1 vector. GST-fused recombinant proteins were purified as described previously42. We performed GST pulldown assays in the binding buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and 1% NP-40 supplemented with protease inhibitors). After washing, retained proteins were eluted by boiling in SDS protein loading buffer and analyzed by western blot using the indicated antibodies.

Immunoprecipitation. HEK293T 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 and 1% NP-40 supplemented with protease inhibitors). Lysates were immunoprecipitated using mouse IgG, rabbit IgG and antibodies to Myc or hemagglutinin (HA) and Trueblot IP beads (eBioscience) followed by western blot.

Promoter luciferase assay. Briefly, the human SNX27 promoter was amplified using genomic DNA from HEK293T cells as templates. After amplification, PCR products were inserted into the pGL3-Basic vector containing the firefly luciferase gene (Promega). Firefly luciferase vectors were co-transfected with pRL-SV40 containing the Renilla luciferase gene (Promega) into HeLa cells with altered C/EBPβ (overexpressed or downregulated) for 48 h. Firefly luciferase activities were assayed and normalized to those of Renilla luciferase.

ChIP. ChIP assays were performed with a commercial kit (Upstate) following the manufacturer’s instructions with minor modifications. The 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 with cycloheximide, and proteasomal and lysosomal inhibitors. Cells were incubated for 8 h with cycloheximide (500 µM) in the presence or absence of lysosomal inhibitor (100 µg ml−1 leupeptin and 50 mM NH4Cl; Sigma) or proteasomal inhibitor (10 µM MG132 and 10 µM lactacystin; Calbiochem). After treatment, cells were lysed and subjected to western blot analysis.

Immunohistochemistry and data analyses. Snx27+/+ or Snx27+/− mice were anesthetized and fixed by cardiac perfusion with 4% paraformaldehyde (PFA). Whole brains were excised and fixed in 4% PFA overnight. Tissue blocks were embedded in paraffin, and 5-µm sections were cut and stained with Nissl (creosyl violet). Immunostained sections were examined, and fluorescence images were collected using a Zeiss fluorescence microscope with AxioVision software.

Golgi staining. Golgi staining was performed using the FD Rapid GolgiStain Kit (FD NeuroTechnologies). Images were acquired using a Zeiss fluorescence microscope with 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 in43. Student’s t-test was used to determine significance between Snx27+/+ and Snx27−/− neurons.

Cell surface biotinylation assay. Biotinylation was carried out following a previously described protocol44.

Receptor recycling experiment. Receptor recycling experiments were performed as described45. Briefly, cells were specifically labeled with Sulfo-NHS-Ssucc-biotin at 4 °C. After labeling, 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 medium 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 at 4 °C) to ensure complete cleavage of any newly present surface biotin. Residual biotinylated (internalized) receptors were pulled down from cell lysates by streptavidin precipitation, detected by western blot analysis with GluRI-specific 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.

Stereotactic injection of AAV. Recombinant human SNX27 and GFP AAV (2 µl, titer 3 × 1012) were stereotactically injected into the hippocampus of T65Dn 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 overexpression of SNX27 in mouse brains, 4 weeks after injection, mice were anesthetized and euthanized, after which 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.

**mEPSC recording.** For hippocampal slice recordings, horizontal slices were prepared from Snx27+/− and Snx27+/+ mice at 4–5 weeks of age. We performed electrophysiological recordings on transverse hippocampal slices (350-µm thickness); 200 consecutive events were analyzed from cells of either Snx27+/− or Snx27+/+ mice. For details, see the Supplementary Methods.

**Extracellular electrophysiology.** The acute hippocampal slices were obtained and maintained as described above. The Schaffer collateral inputs to the CA1 region were stimulated with a bipolar tungsten electrical-stimulating electrode at three different intensities (minimum, half maximum and maximum). Using a low-resistance recording electrode, the 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-output curves for the fEPSP slope response to Schaffer collateral stimulation.

**Neurobehavioral tests.** See the Supplementary Methods.

**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 US National Institutes of Health, National Institute of Child Health and Human Development. Additional human Alzheimer's disease brain samples used in this project were provided by UCSD and were analyzed with institutional permission under California and US National Institutes of Health guidelines. For specimen information, see Supplementary Tables 2 and 3.

**Statistical analyses.** Statistical analyses were performed with GraphPad Prism. Data distribution was assessed by a Kolmogorov-Smirnov 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, Dunnets’ or Tukey’s post-hoc analysis. Error bars represent the s.e.m. Null hypotheses were rejected at the 0.05 level.

**Additional methods.** Detailed methodology is described in the Supplementary Methods.

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