Activation of Rheb, but not of mTORC1, impairs spine synapse morphogenesis in tuberous sclerosis complex

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Mutations in the Tsc1 or Tsc2 genes cause tuberous sclerosis complex (TSC). Tsc1 and Tsc2 proteins form a complex that inhibits mammalian target of rapamycin complex 1 (mTORC1) signalling through Rheb-GTPase. We found that Tsc2¹²/² neurons showed impaired spine synapse formation, which was resistant to an mTORC1 inhibitor. Knockdown of mTOR also failed to restore these abnormalities, suggesting mTORC may not participate in impaired spine morphogenesis. To address whether Rheb activation impairs spine synapse formation, we expressed active and inactive forms of Rheb in WT and Tsc2¹²/² neurons, respectively. Expression of active Rheb abolished dendritic spine formation in WT neurons, whereas inactive Rheb restored spine synapse formation in Tsc2¹²/² neurons. Moreover, inactivation of Rheb with farnesyl transferase inhibitors recovered spine synapse morphogenesis in Tsc2¹²/² neurons. In conclusion, dendritic spine abnormalities in TSC neurons may be caused through activation of Rheb, but not through of mTORC1.

In the mammalian brain, dendritic spines make most excitatory synapses. During development, synapse formation and elimination are accompanied by changes in dendritic spine number and morphology, allowing the establishment and remodelling of neuronal circuits. Structural plasticity of dendritic spines is tightly correlated with synaptic function and plasticity; even subtle changes in dendritic spines may have marked effects on synaptic plasticity. In particular, a large number of brain disorders, including developmental disorders are accompanied by disease-related changes in dendritic spine density and/or morphology.

Tuberous sclerosis complex (TSC) is a systemic genetic disease that causes the growth of hamartomas in the brain and other organs¹. The neurological symptoms of TSC include mental retardation, intractable epilepsy, and autism¹. TSC results from inactivating mutations in either Tsc1 or Tsc2, which encode hamartin and tuberin, respectively. Hamartin and tuberin form a complex that exhibits guanosine triphosphatase (GTPase)-activating protein (GAP) activity toward Rheb, a Ras-like small G-protein⁴. Inactivating mutations in Tsc1 or Tsc2 result in the accumulation of the GTP-bound form of Rheb, which activates mammalian target of rapamycin complex 1 (mTORC1)⁷. The mTOR is a serine/threonine protein kinase that stimulates translation initiation processes involving both eIF4E/4E-binding protein (4EBP) and p70S6 kinase/ribosomal S6 protein.

Mouse models that harbor mutations in the Tsc1 or Tsc2 genes have been extensively investigated to clarify the mechanisms of the pathogenesis of TSC. Several lines of evidence suggest that these TSC model mice exhibit impairments in dendritic spine formation⁴, long-term depression and cognitive behaviors⁸, suggesting that activation of mTORC1 in TSC neurons may cause memory disturbance probably through dendritic spine abnormalities. However, the mTOR signaling has been demonstrated to be activated during learning and is required for spatial memory formation⁴. This controversy may suggest that another mechanism other than the mTOR signaling could be involved in memory impairment in TSC.

In the present study, we first confirmed that dendritic spine formation was abrogated and also excitatory spine synapses were drastically reduced in cultured rat Tsc2¹²/² neurons. This observation was consistent with the finding that filopodia-like protrusions were increased in Tsc2¹²/² rat brain. We treated cultured Tsc2¹²/² neurons with rapamycin, a specific mTORC1 inhibitor; however, it did not restore spine morphology but rather elongated dendritic protrusions. Knockdown of mTOR did not restore spine synapse formation either, suggesting that the mTORC pathway may not participate in spine synapse morphogenesis. We therefore focused on Rheb upstream...
of mTORC1, and expressed the GDP-bound form of Rheb in \( \text{Tsc}2^{-/-} \) neurons, resulting in restoration of spine synapse formation. Conversely, expression of the GTP-bound Rheb abolished spine formation in WT neurons. Furthermore, treatment with farnesyl transferase inhibitors (FTIs) lonafarnib and tipifarnib also rescued spine synapse formation in \( \text{Tsc}2^{-/-} \) neurons. Taken together, these results suggest that spine synapse deformity in TSC neurons may be dependent on the state of Rheb, but not on mTORC activation.

**Results**

**\( \text{Tsc}2^{-/-} \) neurons show impaired spine synapse formation.** In this study, we used spontaneous \( \text{Tsc}2^{-/-} \)-mutated Eker rats as animal models of TSC\(^{11} \). We first compared dendritic spine morphology of cultured hippocampal neurons of wild type (WT) and heterozygous Eker (\( \text{Tsc}2^{+/+} \)) rats. At 21 days in vitro (DIV), the dendritic spines of WT neurons displayed mushroom-type spines (Fig. 1a)(top), whereas the dendrites of \( \text{Tsc}2^{-/-} \) neurons possessed long, thin filopodia (Fig. 1a)(bottom). Quantification showed the Tsc2 mutation reduced the width of dendritic protrusions (Fig. 1a, b) and increased their length (Fig. 1a, c). To assess the influence of Tsc2 haploinsufficiency on spine synapse formation, we immunolabelled neurons with vesicular glutamate transporter 1 (vGlu1), a presynaptic excitatory marker. In \( \text{Tsc}2^{-/-} \) neurons, dendritic vGlu1 puncta were rarely located on protrusions but preferentially localized to dendritic shafts (Fig. 1a, d, e), indicating that \( \text{Tsc}2^{-/-} \) growth cones form synapses with dendritic shafts.

Next, to confirm whether dendritic protrusions of in vivo \( \text{Tsc}2^{-/-} \) neurons exhibit similar morphological changes to those in cultured \( \text{Tsc}2^{-/-} \) neurons, we labeled hippocampal CA1 neurons in WT and \( \text{Tsc}2^{-/-} \) fixed brain tissues with biolistic-DiI (Fig. 1f). We observed similar morphological changes in spine width and length in \( \text{Tsc}2^{-/-} \) neurons in vivo (Fig. 1g, h) to those observed in cultured \( \text{Tsc}2^{-/-} \) neurons.

**The mTORC1 inhibitor rapamycin does not restore spine synapse morphogenesis in \( \text{Tsc}2^{-/-} \) neurons.** To address whether the effects of Tsc2 haploinsufficiency on neuronal morphology are mediated by elevated mTORC1 activity, we examined the ability of rapamycin to reverse defects in neuronal morphology. WT and \( \text{Tsc}2^{-/-} \) hippocampal neurons were treated with rapamycin and maintained for 6 days. Application of rapamycin to \( \text{Tsc}2^{-/-} \) neurons completely abolished the phosphorylation of S6 ribosomal protein, which is a substrate of the mTORC1 downstream target S6 kinase\(^{12} \) (Fig. 2a, Supplementary Fig. S4a, b). Although rapamycin did not affect spine morphology in WT neurons (Fig. 2b, c, d), it rather induced the growth of long, thin protrusions in \( \text{Tsc}2^{-/-} \) neurons (Fig. 2b, c, d) without restoration of spine synapse formation (Fig. 2b, e, f). To test whether mTORC2 participates in spine synapse formation, we next knocked down mTOR in WT and \( \text{Tsc}2^{-/-} \) neurons by genetic silencing with siRNA (Supplementary Fig. S1). However, mTOR siRNA did not reverse spine synapse formation but rather elongated dendritic protrusions in \( \text{Tsc}2^{-/-} \) neurons, like rapamycin treatment (Fig. 2g–k). The mTOR inhibition by either rapamycin or siRNA did not significantly change the number of spine synapses (Fig. 2e, f, j, k). Thus, neither mTORC1 nor mTORC2 may participate in the impaired spine synapse morphogenesis in TSC neurons.

**Inactivation of Rheb recovers spine synapse formation in \( \text{Tsc}2^{-/-} \) neurons.** We therefore focused on Rheb, a GTPase located downstream of the Tsc1/2 complex and upstream of mTORC1, and examined whether Rheb activation is directly involved in spine synapse abnormalities in \( \text{Tsc}2^{-/-} \) neurons. We first asked whether activation of Rheb in WT neurons causes morphological changes similar to those in \( \text{Tsc}2^{-/-} \) neurons. To address this question, we expressed two kinds of Rheb mutants Glu64Val (Rheb\(^{64V} \)) and Asp60Ile (Rheb\(^{60I} \)) in WT or \( \text{Tsc}2^{-/-} \) neurons (Supplementary Fig. S2a, b). Glu64 mutant of Rheb displays a higher basal GTP level, whereas Rheb\(^{60I} \) mutation interferes selectively with GTP binding\(^{13} \). Expression of the Rheb\(^{60I} \) completely abolished spine synapse formation in WT neurons, whose dendritic morphology was similar to that of \( \text{Tsc}2^{-/-} \) neurons (Fig. 3a–c). In contrast, Rheb\(^{60I} \) expression did not affect filopodia-like morphology of dendritic protrusions in \( \text{Tsc}2^{-/-} \) neurons (Fig. 3a–c). Conversely, the expression of Rheb\(^{60I} \) restored spine synapse formation in \( \text{Tsc}2^{-/-} \) neurons by increasing the ratio of spine synapses (Fig. 3f, i, j). Rheb\(^{60I} \) expression also rescued spine width in \( \text{Tsc}2^{-/-} \) neurons to a similar extent to those in WT neurons (Fig. 3f, g). However, overexpression of Rheb\(^{60I} \) in WT neurons differentially modified their spine structure from those of \( \text{Tsc}2^{-/-} \) neurons. Rheb\(^{60I} \) expression unexpectedly caused narrower and longer dendritic protrusions (Fig. 3f, g, h), and reduced the ratio of spine synapses in WT neurons (Fig. 3f, i, j). These results suggest that appropriate temporal activation of Rheb may be required for normal spine development in WT neurons. Thus, impaired spine synapse morphogenesis in \( \text{Tsc}2^{-/-} \) neurons may be mediated by constitutively active state of Rheb and could be rescued by inactivation of Rheb.

**FTI treatment restores spine synapse morphogenesis in \( \text{Tsc}2^{-/-} \) neurons.** To address whether pharmacological inactivation of Rheb recovers spine synapse formation in \( \text{Tsc}2^{-/-} \) neurons, we utilized two kinds of FTIs, lonafarnib and tipifarnib. FTIs have been shown to reverse growth and actin filament distributions in \( \text{Tsc}1^{-/-} \) and \( \text{Tsc}2^{-/-} \) cells in a rapamycin-sensitive and -insensitive manners\(^{14} \). Therefore, we treated \( \text{Tsc}2^{-/-} \) neurons with lonafarnib or tipifarnib\(^{15} \) for 3 days. These FTI treatments resulted in recuperation of F-actin accumulation in dendritic spines (Fig. 4a, b) and increases in spine synapse ratio (Fig. 4c–g) in \( \text{Tsc}2^{-/-} \) neurons. On the other hand, either lonafarnib or tipifarnib treatment did not affect spine synapse formation in WT neurons (Fig. 4c, f, g). In conclusion, persistent activation of Rheb may abolish dendritic spine morphogenesis and that inactivation of Rheb with FTI may restore spine synapse formation in TSC neurons. Finally, to reveal the functional differences in excitatory synaptic transmission between WT and \( \text{Tsc}2^{-/-} \) neurons, we measured evoked EPSC amplitude, mEPSC amplitude/frequency, readily releasable pool size and vesicular release probability from both cultured neurons. However, unexpectedly, synaptic transmission was sufficiently observed in cultured \( \text{Tsc}2^{-/-} \) neurons without any significant difference in presynaptic or postsynaptic features between WT and \( \text{Tsc}2^{-/-} \) neurons (Supplementary Fig. S3a–f). These results suggest that basal synaptic transmission may be preserved in \( \text{Tsc}2^{-/-} \) neurons as WT neurons, irrespective of spine synapse abnormalities. Other electrophysiological properties, such as long-term depression, might be affected by impaired spine morphogenesis in \( \text{Tsc}2^{-/-} \) neurons. Further studies are necessary for understanding the effects of morphological abnormalities of dendritic spines on the pathogenesis of TSC.

**Discussion**

In this paper, we have demonstrated activation of Rheb, not of mTORC1, is a main cause of dendritic spine synapse abnormality in \( \text{Tsc}2^{-/-} \) neurons. We showed that active Rheb mutant abolished spine formation in WT neurons, whereas the inactive mutant of Rheb restored spine synapse morphogenesis in \( \text{Tsc}2^{-/-} \) neurons. Furthermore, we demonstrated that FTI treatment, but not rapamycin treatment, rescued spine synapse morphology in \( \text{Tsc}2^{-/-} \) neurons. These results, taken together, suggest that Rheb inhibition by FTI may be necessary for recovery of spine synapse formation in the TSC brain.
Mouse models of TSC have demonstrated dendritic spine abnormalities that are resistant to rapamycin treatment. For example, Tsc1-deficient mice develop several pathological features observed in human TSC patients. Rapamycin treatment improved neurofilament abnormalities, myelination and cell enlargement in Tsc1-ablated neurons, but it did not reverse dendritic spine morphology. Additional studies have revealed that the application of rapamycin to Tsc1- or Tsc2-reduced neurons did not restore spine formation. F-actin is the major cytoskeletal component in dendritic spines and has been thought to provide the structural basis for dendritic spines. However, rapamycin has been shown to rather suppress F-actin reorganization in an mTOR-dependent manner. Therefore, inhibition of F-actin reorganization by rapamycin might cause an elongation of existing filopodial protrusions, consistent with our results. Taken together, the impaired spine formation in TSC neurons may not be mediated by mTORC1.

Our results indicate that FTI treatment could restore morphological abnormalities in Tsc2+/− neurons. Farnesyl transferase (FT) recognizes the carboxyl-terminal CAAX box of small GTP-binding proteins and catalyses the transfer of lipid onto the cysteine residue. Following carboxymethylation of the farnesyl cysteine renders the carboxyl terminus more hydrophobic. Membrane localization is essential for Rheb activity as for other small GTP-binding proteins. To prevent Rheb farnesylation and membrane localization, FTIs have been used. Unlike Ras, Rheb is not a substrate for geranylgeranyltransferases and can only undergo farnesylation. Therefore, FTIs may suppress the Rheb activity mainly. On the other hand, another small GTP-binding protein RhoB has been shown to be an additional target of FTIs. Because FTI treatment induces apoptosis in the endothelial cells of sprouting and developing blood vessels via inhibition of RhoB, pharmaceutical inactivation of RhoB could cause possible side effects as cardiovascular disorders where the angiogenesis would be desirable.

The finding that FTI was capable of restoring dendritic spine formation suggests that there are two different pathways downstream of Rheb in neurons, one signalling to neuronal growth and the other regulating neuronal morphology. The pathway affecting growth is likely to be mTORC1-dependent, whereas the pathway regulating neuronal morphology is rapamycin-insensitive. This mTORC1-dependent signalling is activated during learning and plays a crucial role in synaptic plasticity.
Figure 2 | Rapamycin fails to reverse spine synapse formation in Tsc2+/− neurons. (a) Rapamycin treatment completely abolished the phosphorylation of S6 in both WT and Tsc2+/− neurons. These blots are cropped, and full-length blots are presented in Supplementary Figure 1. (b) High-power images of the dendrites of DMSO- or 100 nM rapamycin-treated WT and Tsc2+/− neurons. Scale bar, 10 μm. (c, d) Cumulative probability plots of dendritic protrusion width (c) and length (d) shown in (b). Note that rapamycin treatment increases dendritic protrusion lengths compared with DMSO treatment in Tsc2+/− neurons (D = 0.1717, P < 0.001, Kolmogorov-Smirnov test). (e) The densities of the vGlut1-positive spine synapses shown in (b). (f) The percentage of spine synapses in (b) (n = 400/7/3 (DMSO, WT), 400/7/3 (Rapa, WT), 1091/12/6 (DMSO, Tsc2+/−) and 1020/12/6 (Rapa, Tsc2+/−) protrusions/neurons/experiments). Note that rapamycin treatment did not significantly change the number of spine synapses. (g) Dendrites from control-, or mTOR siRNA-transfected WT and Tsc2+/− neurons. Scale bar, 10 μm. (h, i) Cumulative probability plots of protrusion width (h) and length (i) shown in (g). Note that mTOR siRNA expression causes increases in dendritic protrusion lengths in both WT and Tsc2+/− neurons, compared with scrambled siRNA (D = 0.1684, P < 0.01 (WT), D = 0.3676, P < 0.001 (Tsc2+/−), Kolmogorov-Smirnov test). (j) The densities of the vGlut1-positive spine synapses shown in (g). (k) The percentage of spine synapses in (g) (n = 400/7/3 protrusions/neurons/experiments). Note that treatment with mTOR siRNA did not significantly change the number of spine synapses.
Figure 3 | Rheb regulates spine synapse formation. (a) Dendrites of EGFP- and mock- or FLAG-RhebQ64V-expressing hippocampal neurons prepared from WT and Tsc2−/− rats at DIV 19. Neurons were co-immunostained with anti-GFP and vGlut1 antibodies. Scale bar, 10 μm. (b, c) Cumulative probability distributions of protrusion width (b) and length (c) in (a). RhebQ64V-expressing WT neurons showed narrower (Δ = 0.2690, P < 0.001) and longer (Δ = 0.2391, P < 0.001) dendritic protrusions than mock transfectants (Kolmogorov-Smirnov test). (d) Quantification of the spine synapse densities shown in (a). (e) Percentage of spine synapses shown in (a).FLAG-RhebQ64V-expressing WT neurons showed few vGlut1-positive spines compared with mock-transfected neurons. The histograms show the mean values ± SEM (***P < 0.001, Two-Way ANOVA, post-hoc Tukey test). A total of 803/10/5 (WT, mock), 1,029/12/6 (WT, RhebQ64V), 501/6/3 (Tsc2−/−, mock), 320/6/3 (Tsc2−/−, RhebQ64V) protrusions/neurons/experiments, respectively, were analysed. (f) Dendrites of EGFP- and mock- or FLAG-RhebD60I-expressing hippocampal neurons prepared from WT and Tsc2−/− rats at DIV 19. Neurons were co-immunostained with anti-GFP and vGlut1 antibodies. Scale bar, 10 μm. (g, h) Cumulative probability plots of dendritic protrusion width (g) and length (h) in mock- or RhebD60I-expressing WT and Tsc2−/− neurons in (f). Note that RhebD60I increased dendritic protrusion width (Δ = 0.2903, P < 0.001) and decreased length (Δ = 0.4306, P < 0.001) in Tsc2−/− neurons, whereas RhebD60I decreased protrusion width (Δ = 0.638, P < 0.001) and increased length (Δ = 0.509, P < 0.001) in WT neurons, compared with mock transfectants (Kolmogorov-Smirnov test). (i) Quantitative analysis of the spine synapse density shown in (f). (j) The percentage of spine synapses in (f) (n = 300/6/3 (WT, mock), 300/6/3 (WT, RhebQ64V), 988/12/6 (Tsc2−/−, mock) and 1,044/12/6 (Tsc2−/−, RhebD60I) protrusions/neurons/experiments, *P < 0.05, ***P < 0.001, Two-Way ANOVA, post-hoc Tukey test).
role in the spatial learning\textsuperscript{10}. By contrast, the TSC mice develop significant cognitive deficits\textsuperscript{9}. This discrepancy might be explained by the notion that activation of the mTORC1-independent signalling may impair spine synapse formation in TSC and dominantly disturb memory acquisition or retention despite the mTORC1 activation.

What could be involved in the mTORC1-independent pathway to control neuronal morphology? We initially considered the possibility of the mTORC2 pathway; however, this seems unlikely, because mTOR knockdown fails to reverse spine synapse formation in \textit{Tsc2}\textsuperscript{1/2} neurons (Fig. 2g–k). Further experiments may provide important insights into how Rheb regulates neuronal morphology. As Rheb-mediated regulation of cytoskeleton has not been reported so far, we are currently searching Rheb-binding protein(s) in neurons. Clarification of this mechanism could extend our understanding of the prevailing role of Rheb in memory impairments in TSC patients.

**Methods**

**Reagents.** Rapamycin and FTIs (lonafarnib and tipifarnib) were purchased from LKT Laboratories and Toronto Research Chemicals, respectively. The used siRNAs are as follows: mTOR siRNA (CAGCAGCUGGUACUGACAAGUCU and...
AGUACUUGUCAUGUCCACUGUCGCU and Stealth RNAi siRNA negative control kit. Oligonucleotides were synthesized and duplexed by Invitrogen.

**Animals.** The Eker (Tsc2+/−) and wild-type (WT) genotypes were determined using PCR. All animal experiments were approved by the Committee on the Animal Care and Use Committees of the Tokyo Metropolitan Institute of Medical Science and Fukuoka University, and were performed according to their recommendation.

**Primary culture and transfection.** Primary culture and transfection of hippocampal neurons from Tsc2−/− and WT littermates (E20) have been previously described25–27. Cultured neurons were maintained in Neurobasal Medium supplemented with B27, gentamicin and GLUTAMAX. The neurons were transfected at DIV11–12 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cultured hippocampal neurons were treated with 100 nM rapamycin for 6 days, 2 μM lonafarnib or 2 μM tipifarnib for 3 days. All experiments comparing WT and Tsc2−/− neurons were performed in parallel.

Spine and/or shaft synapse density analysis were performed as described previously9. Spines were defined as any protrusions from the dendritic shaft (rabbit, CST) or anti-vGlut1 (guinea pig, Millipore). The immunoreactivity was overnight at 4°C or 3% bovine serum albumin with 0.1% Triton X-100 in PBS), and incubated paraformaldehyde, blocked, permeabilized with BL solution (3% normal goat serum, 3% normal rabbit serum, 1% bovine serum albumin, 0.1% Triton X-100 in PBS) and incubated with primary antibody (1:100) at 4°C overnight. The bleached slices were re-fixed with 4% PFA at 4°C for 1 h. The immunoreactivity was visualized using secondary antibody (1:100) and Alexa Fluor 568 phallidin (Invitrogen).

**Immunostaining.** Rat cultured hippocampal neurons were fixed with 4% paraformaldehyde, blocked, permeabilized with BL solution (3% normal goat serum or 3% bovine serum albumin with 0.1% Triton X-100 in PBS), and incubated with primary antibody (1:100) at 4°C overnight. The bleached slices were re-fixed with 4% PFA at 4°C for 1 h. The immunoreactivity was visualized using species-specific, fluorochrome-conjugated secondary antibodies.

Neuronal F-actin was visualized by staining with Alexa Fluor 647 phallolidin (Invitrogen).

**Image acquisition and quantification.** Confocal images of neurons were obtained using a Zeiss 63× lens (NA 1.4) with sequential acquisition settings at the resolution of the confocal microscope (512 × 512 pixels). Each image was a composite constructed from a series of images captured throughout the 2 aspect of each cell. The parameters of each composite image were optimized for the particular lens and pinhole setting. Identical confocal microscope settings were maintained for all scans in each experiment. All morphometric analyses and quantifications were performed using MetaMorph image analysis software (Universal Imaging). For the spine morphology studies, individual dendritic protrusions were manually traced, and the maximum length and head width of each dendritic protrusion were measured and recorded in Microsoft Excel.

**Diolistic labelling.** Eight-week-old rats were deeply anesthetized with 0.1 mg/kg phenobarbital perfused transcardially with 0.1 M PB, followed by 50 ml of 2% PFA. The brains were removed and post-fixed in the same fixative for 4 hr. Subsequently, sagittal brain sections were obtained using a vibratome and collected in PBS. Twenty-five milligrams of gold particles (Bio-Rad) were coated with 7.0 mg of the lipophilic dye DiI (Invitrogen) and injected into Tefzel tubing, which was subsequently cut into pellets. These pellets were delivered diolistically at 40 psi using a Helios gene-gun system (Bio-Rad). The slices were fixed with 4% PFA at 4°C for 1 hr and mounted on slides in VectaShield Mounting Medium (Vector Laboratories).

**Western blot analysis.** Western blot analysis of rat hippocampal neurons with anti-phospho S6 (Ser235/236) (rabbit, CST) antibody59, anti-S6 (rabbit, CST) antibody59 or HEK 293T cells with anti-miTOR antibody59 were performed as described previously59.

**Autaptic culture.** Astrocytes were obtained from cerebral cortices of newborn (P0) ICR mice (Kyudo). The brains were removed and post-fixed in the same fixative for 4 hr. Subsequently, sagittal brain sections were obtained using a vibratome and collected in PBS. Twenty-five milligrams of gold particles (Bio-Rad) were coated with 7.0 mg of the lipophilic dye DiI (Invitrogen) and injected into Tefzel tubing, which was subsequently cut into pellets. These pellets were delivered diolistically at 40 psi using a Helios gene-gun system (Bio-Rad). The slices were fixed with 4% PFA at 4°C for 1 hr and mounted on slides in VectaShield Mounting Medium (Vector Laboratories).

**Electrophysiology.** Synaptic responses from WT and Tsc2−/− autaptic neurons were recorded in the whole-cell configuration under the voltage-clamp mode at a holding potential (Vh) of −70 mV (MultiClamp 700B, Molecular Devices) and room temperature. The patch pipette resistance was 3–5 MΩ, and a series resistance was compensated by 70–90%. The AMPA receptor-mediated evoked glutamatergic synaptic transmission was recorded in response to an action potential elicited through a brief (2 ms) somatic depolarization pulse (to +20 mV) from the patch pipette. Spontaneous glutamatergic mEPSCs were recorded in the presence of 0.5 μM tetrodotoxin (TTX). The size of the readily releasable pool (RRP) of synaptic vesicles was estimated using a synaptic transient charge elicited through a 10 sec application of 0.5 M sucrose. The number of synaptic vesicles in the RRP was subsequently calculated by dividing the RRP size by an individual average mEPSC charge. The vesicular release probability (Pv) was calculated as the ratio of the charge of the evoked EPSC to the RPP charge successfully obtained from the same neuron. The synaptic responses were recorded at a sampling rate of 20 kHz and filtered at 10 kHz. The template for the mEPSC data analysis was set as a rise time constant of 0.5 ms, a decay time constant of 4 ms, a baseline of 5 ms, and a template length of 10 ms. The data were analysed off-line using Axograph X 1.2 software (AxoGraph Scientific). Recordings of synaptic responses were performed at DIV 7–9 for immature synapses and at DIV 19–22 for mature synapses.

The standard extracellular solution contained (in mM) NaCl 140, KCl 2.4, HEPES 10, glucose 10, CaCl2 1, and MgCl2 1, with pH 7.4 and an adjusted osmotic pressure of 295–320 mOsm. Patch pipettes were filled with an intracellular solution containing (in mM) K-glucate 146.3, MgCl2 0.6, ATP-Na 4, GTP-Na3 0.3, phosphocreatine 12.1, EGTA 1, and HEPES 17.8, as well as creatine phosphokinase 50 μM (pH 7.4). The extracellular solutions were applied using a fast-flow application system (Warner Instruments). All chemicals were purchased from Sigma-Aldrich unless otherwise specified.

**Statistical analysis.** The data were statistically analysed using Student’s t-test, two-way ANOVA or the Kolmogorov-Smirnov test. Differences were considered significant when p < 0.05.
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**Author contributions**

K.Y., S.Y. and H.S. designed research; S.Y., H.S., S.K., T.S., K.T., K.I., T.K., O.H. and K.Y. performed experiments; H.T. analysed data; and K.Y., S.Y. and H.S. wrote the manuscript. All authors reviewed the manuscript.

**Additional information**

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