Supplementary Information for

Filamin A-interacting protein (FILIP) is a region-specific modulator of myosin 2b and controls spine morphology and NMDA receptor accumulation

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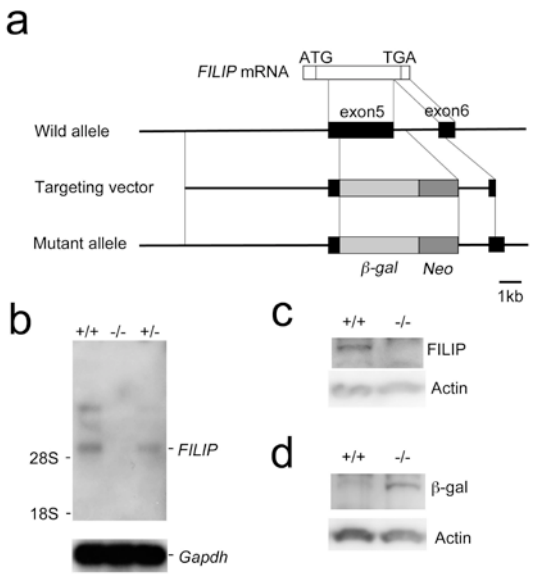
Supplementary Figures S1-S8
Supplementary Methods

SUPPLEMENTARY FIGURES



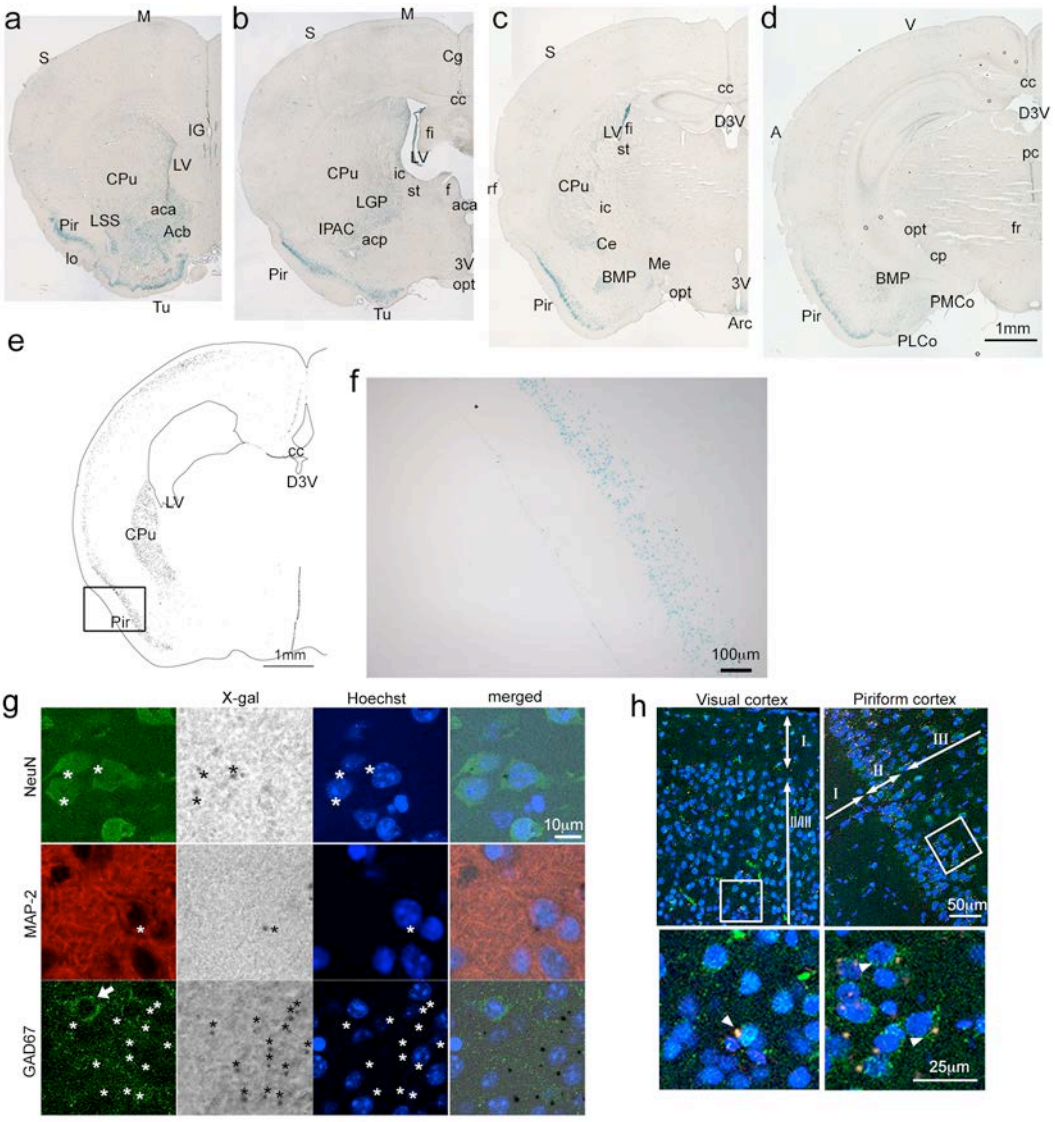
Supplementary Figure S1 Generation of *FILIP*-knockout mice

(a) The targeting vector and mutant allele used to create the *FILIP*-knockout mice are shown. The *β-galactosidase gene* (*β-gal*) was fused to the 5' fragment of exon 5 in frame in the targeting vector.

(b) Deletion of full-length *FILIP* mRNA in *FILIP*-knockout mice was confirmed through northern blot analysis. PolyA RNAs were obtained from the heads at E16.5. +/+ wild-type littermates, +/- heterozygous-knockout mice, -/- homozygous-knockout mice.

(c) FILIP was not expressed in the *FILIP*$^{-/-}$ mice. FILIP was detected using anti-FILIP antibody. The samples were obtained from whole embryos at E14.5; actin was used as the loading control.

(d) Expression of the β-galactosidase fusion protein was confirmed in *FILIP*$^{-/-}$ mice. Actin was used as the loading control.



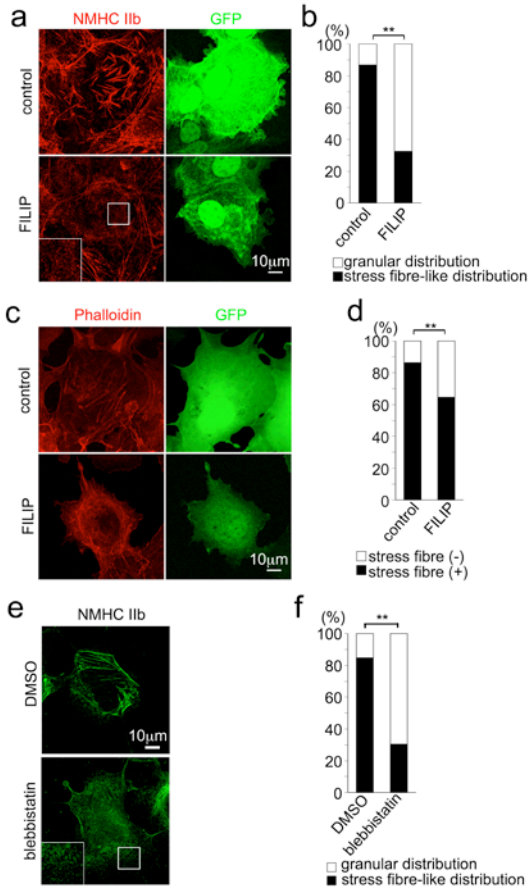
Supplementary Figure S2 FILIP-containing cells are visualised using β-galactosidase expression

(a)-(d) β-galactosidase expression in the coronal sections of an adult (12 week-old) *FILIP*$^{-/-}$ mouse brain is shown; (a)-(d) are equivalent to Figure 2, panels (d)-(g).

(e) The signals from β-galactosidase expression in the coronal sections of a *FILIP*$^{+/-}$ mouse brain at P15 are represented by black dots. The box indicates the area shown in (f).

(f) β-galactosidase expression in the piriform cortex of a *FILIP*$^{+/-}$ mouse brain at P15.
(g) High-magnification images of upper layer III of the piriform cortex (upper and middle rows) and layer II of the piriform cortex (bottom row). β-galactosidase-positive cells were NeuN and MAP-2 positive but GAD 67 negative. The nuclei were visualised using Hoechst 33258. The asterisks (*) indicate β-galactosidase-positive dots. The white arrow indicates a GAD 67 positive cell.
(h) The colocalisation of the β-galactosidase-positive cells in the *FILIP*$^{-/-}$ mouse adult brain with Brn1 was examined. The X-gal staining is indicated in orange, and the expression of Brn1 is indicated in green. The cells expressing β-galactosidase were Brn1-positive in the occipital cortex (left column) and piriform cortex (right column) of *FILIP*$^{-/-}$ mice. The rectangles in the upper panels indicate the higher magnification of the images shown in the bottom panels. The arrowheads indicate the Brn1- and β-galactosidase-expressing cells. The following abbreviations are used in the figures: A, auditory cortex; aca, anterior commissure, anterior part; Acb, nucleus accumbens; acp, anterior commissure, posterior; Arc, arcuate nucleus; BMP, basomedial amygdaloid nucleus, posterior part; cc, corpus callosum; Ce, central amygdaloid nucleus; Cg, cingulate cortex; cp, cerebral peduncle, basal part; CPu, caudate putamen; D3V, dorsal third ventricle; f, fornix; fi, fimbria of the hippocampus; fr, fasciculus retroflexus; ic, internal capsule; IG, indusium griseum; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; LGP, lateral globus pallidus; lo, lateral olfactory tract; LSS, lateral stripe of the striatum; LV, lateral ventricle; M, motor cortex; Me, medial amygdaloid nucleus; opt, optic tract; pc, posterior commissure; Pir, piriform cortex; PLCo, posterolateral cortical amygdaloid nucleus; PMCo, posteromedial cortical

amygdaloid nucleus; rf, rhinal fissure; S, somatosensory cortex; st, stria terminalis; Tu, olfactory tubercle; V, visual cortex and 3V, third ventricle.



Supplementary Figure S3 FILIP regulates the subcellular distribution of NMHC IIb and modulates NMHC IIb binding to F-actins

(a) In most FILIP-expressing cells, NMHC IIb localised to particle-like (granular-like) structures, whereas NMHC IIb exhibited a stress fibre-like appearance in most cells without FILIP. The transfected cells expressed GFP.

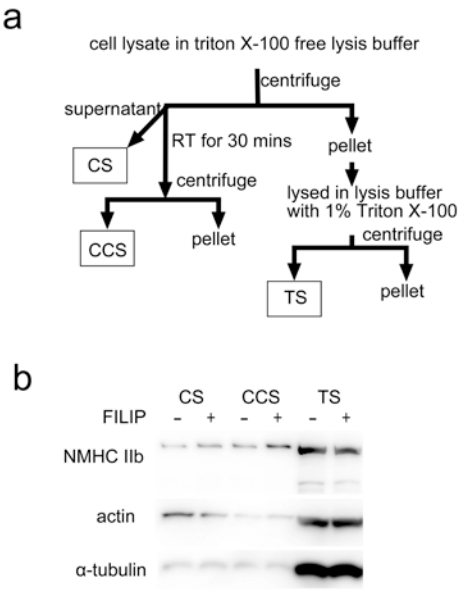
(b) The data from 3 independent culture dishes, with 319 control cells and 311 FILIP-expressing cells, are shown; ** $p<0.00001$ (Fisher's exact test, two-tailed).

(c) FILIP is involved in actin localisation. Actin fibres were visualised using Alexa 568-conjugated phalloidin. The transfected cells expressed GFP.

(d) The data from 3 independent culture dishes, with 308 control cells and 311 FILIP-expressing cells, are shown; ** $p<0.00001$ (Fisher's exact test, two-tailed).

(e) Treatment with vehicle (DMSO) did not influence the stress fibre-like subcellular distribution of NMHC IIb. In contrast, treatment with 100 μM blebbistatin altered the subcellular distribution of NMHC IIb from fibre-like to granular. An inset shows the granular distribution of NMHC IIb.

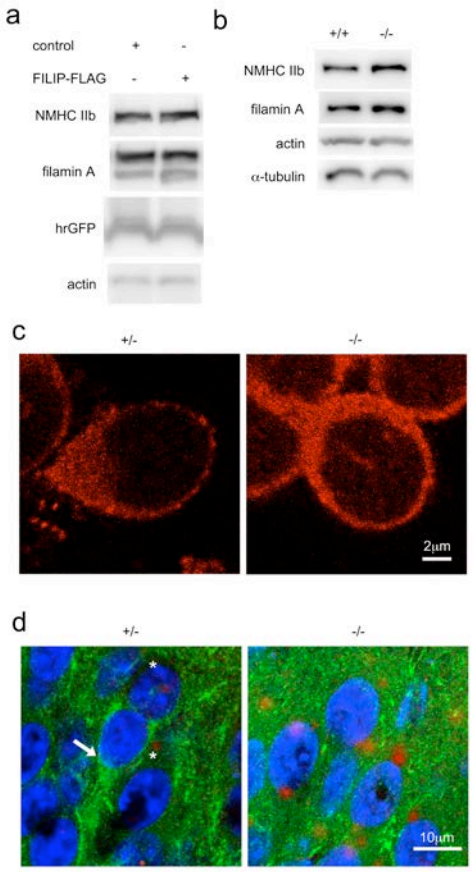
(f) The distribution of NMHC IIb was analysed in 3 independent culture dishes in which cells were treated with vehicle only (DMSO, n = 309) and 100 μM blebbistatin (n = 309); ** $p<0.00001$ (Fisher's exact test, two-tailed).



Supplementary Figure S4 FILIP modulates NMHC IIb binding to F-actins

(a) The cytosolic fraction (CS), Triton-soluble fraction (TS) and cleared cytosolic fraction (CCS) were obtained as shown in this flow chart. RT, room temperature.

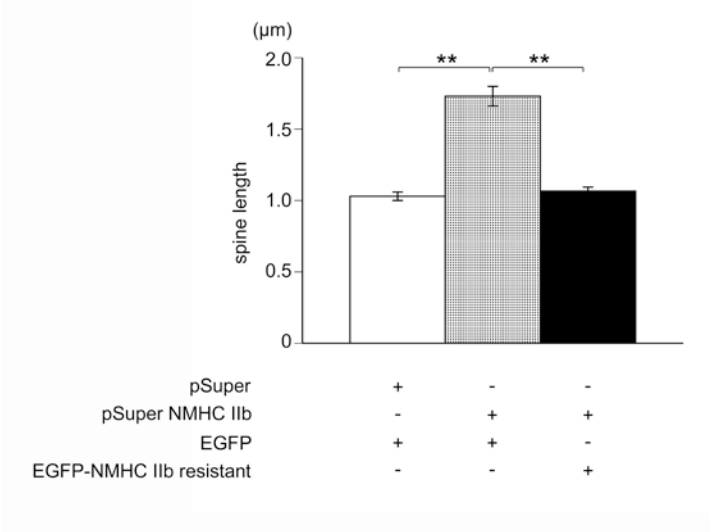
(b) NMHC IIb shifted from the F-actin-containing fraction (TS) to the G-actin-rich fraction (CS) in COS-7 cells in the presence of FILIP. Five microliters of sample was loaded in each lane. +, sample derived from FILIP-expressing cells; -, sample derived from control cells.



Supplementary Figure S5 Overexpression of FILIP influences the amount of NMHC IIb *in vivo*

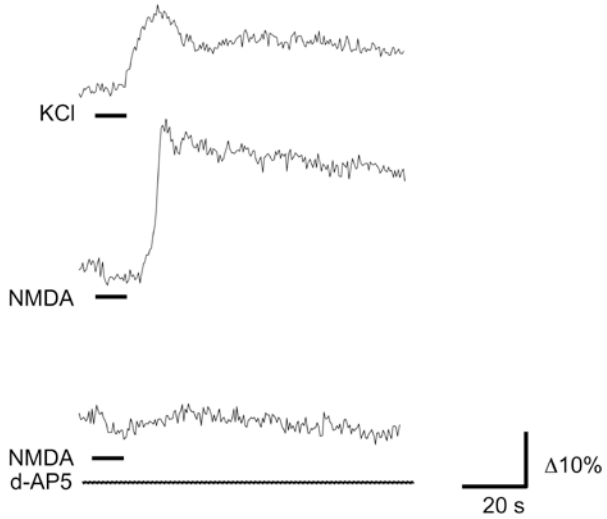
(a) COS-7 cells were transfected with the vectors indicated in the figure. At 48 hr after transfection, the cells were lysed, and the protein amounts were estimated. Actin was used as a loading control. hrGFP represents the amount of GFP for evaluating transfection efficiency. (b) Deletion of FILIP resulted in an increase in filamin A and NMHC IIb levels in the heart. (c) The primary cultured piriform cortical neurons at DIV2 are shown. NMHC IIb expression is shown. (d) Expression of NMHC IIb and β-galactosidase in the piriform cortex are shown. An arrow indicates a β-galactosidase

negative (non-FILIP expressing), NMHC IIb-containing cell in the piriform cortex of *FILIP*$^{+/-}$ mice.



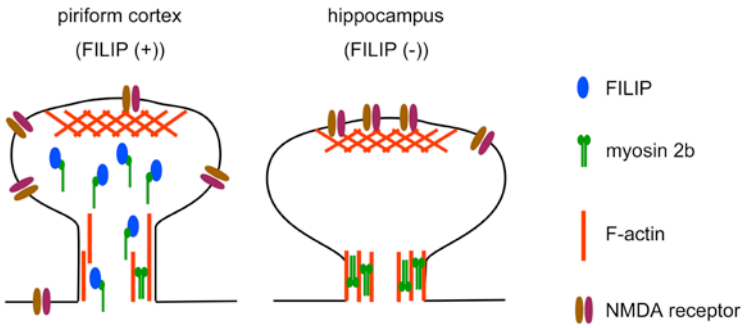
Supplementary Figure S6 Knockdown of *NMHC IIb* results in the elongation of spine length

The primary cultured hippocampal neurons were transfected with the *NMHC IIb* knockdown vector. The exogenous expression of resistant NMHC IIb rescued the effect of *NMHC IIb* knockdown. The data were obtained from 558 spines (pSuper/ EGFP, 1.03 ± 0.03, mean ± s.e.m.), 392 spines (pSuper NMHC IIb/ EGFP, 1.73 ± 0.07) and 545 spines (pSuper NMHC IIb/ EGFP-NMHC IIb resistant, 1.07 ± 0.03). Error bars, s.e.m.; **p<0.00001 (Welch's *t*-test, two-tailed).



Supplementary Figure S7 Bath application of NMDA results in increased calcium concentration in neurons located in the piriform cortex

The fluorescence intensity was altered by an application of NMDA (50 μM). The antagonist of NMDA receptor, d-AP5, inhibited the changes of fluorescence intensity by NMDA application. KCl was applied to stimulate responsive neurons.



Supplementary Figure S8 FILIP activities in the spine

FILIP expression resulted in the disturbance of localisation of myosin 2b and inhibited its function at the spine neck. The accumulation of NMDA receptors within the synapses was also modified in the presence/absence of FILIP.

SUPPLEMENTARY METHODS

Animals

The mice were maintained in the animal room at the Division of Laboratory Animal Resources, University of Fukui and Hyogo College of Medicine. The day of birth was designated P0. All experiments were conducted in accordance with the Regulations for Animal Research at University of Fukui and the Regulations for Animal Experimentation in Hyogo College of Medicine. The Animal Research Committee, University of Fukui and the President of Hyogo College of Medicine under the review of the Hyogo College of Medicine Animal Experiment Committee approved the experiments.

Construction of targeting vectors

To generate the *FILIP*-knockout mice, a targeting vector was constructed in which *β-galactosidase* followed the first 162 bp of exon 5 of FILIP in frame, and the rest of exon 5 and a portion of intron 5 were replaced with the *PGK-neo* cassette[45]. To construct the targeting vector, a *Bam*HI-*Spe*I fragment that contained 162 bp of exon 5 was ligated to the *β-galactosidase* gene followed by the *PGK-neo* cassette. We inserted this 5' region of exon 5-, *β-galactosidase-*, *PGK-neo*-containing fragment and a 1.8 kb *FILIP* genomic fragment, which consisted of intron 5 and a portion of exon 6, into a *Cla*I site and a *Sal*I-*Kpn*I site of the pBluescript SK (Agilent Technologies, Inc., Santa Clara, CA), respectively, in which the *Ssp*I fragment was replaced with the *PGK-tk* cassette[44].

Generation of *FILIP*-knockout mice

The targeting vectors were linearised by *Not*I digestion, electroporated into 1 x 10^7 embryonic stem (ES) cells and selected with G418 (Life Technologies Corporation, Grand Island, NY) and ganciclovir (F. Hoffmann-La Roche, Basel, Switzerland). Resistant colonies were selected, expanded and screened for homologous recombination by conventional Southern blot analyses[40]. ES cells that were heterozygous for the *FILIP* mutation were injected into E3 blastocysts taken from C57BL/6J mice. The ES-cell-injected blastocysts were transferred into the uteri of pseudopregnant ICR females (Japan SLC, Shizuoka, Japan). Chimeric mice were mated with C57BL/6J mice (Japan SLC), and germline transmission was confirmed through Southern blot analyses. Brother-sister mating was then performed to generate homozygous mutants.

Vector construction

The full-length rat *L-FILIP* cDNA was amplified using PCR and inserted into the pCAGGS vector[38], which contains 3 x FLAG sequences and IRES GFP (pCAGGS FILIP IRES GFP). The empty-vector control was the pCAGGS vector expressing IRES GFP (pCAGGS IRES GFP). Vectors that express a truncated form of FILIP were constructed using the KOD-plus-mutagenesis kit (TOYOBO CO., LTD, Tokyo, Japan). The primers for the truncated forms of FILIP were as follows: FILIP 687-1212, gccaagcacaaagccatagag and cattcccaccagatctcgag; FILIP 960-1212, tcgcaaaagcccaaaagtgca and cattcccaccagatctcgag; FILIP 1-652, caagtccccctccaccacctc and ggaggggggaagggatatc; and FILIP 687-960, catgacattgggtgatggaat and gccaagcacaaagccatagag. The full-length and various fragments of *NMHC IIb* were amplified using PCR and inserted into the pCMV VSV vector to produce fragments

tagged with VSV-G under the *CMV* promoter. The PCR primers for the construction of the constructs were as follows: full length NMHC IIb, atagatctatggcgcagagaactggactcgaggat and tacgcggccgccttactctgactggggtggctgcgtct; NMHC IIb 1-782 atagatctatggcgcagagaactggactcgaggat and tcccgcggcagccactcatctgcctt; NMHC IIb 1-331 atagatctatggcgcagagaactggactcgaggat and tcccgcggcatggtctcctggaa; NMHC IIb 90-331 tcagatctcatggcagaattgcatgc and tcccgcggcatggtctcctggaa. The shRNA vector for *NMHC IIb* and *NMHC IIb*-resistant vector were constructed as previously reported[7]. The conditional knockdown vector for *FILIP* was constructed using the Tol2 transposon-mediated technique[39]. The target nucleotide was bp 1433-1453 of mouse FILIP cDNA (GenBank accession number: BC131965.1).

Northern blot analysis

Two or 5 μg of poly(A)+ RNA from embryonic brains was blotted onto a Hybond N nylon membrane (GE Healthcare Life Sciences, Piscataway, NJ) after agarose-formaldehyde gel electrophoresis. Details of the mRNA extraction and hybridisation procedures are described elsewhere[40,45]. A probe matching approximately 1 kb of the mouse *FILIP* cDNA was used for northern blot analyses. Filters were re-hybridised with a [α-^{32}P]-labelled, 0.85-kb fragment of mouse *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*).

Histochemical detection of β-galactosidase and immunostaining

After fixation with 4% paraformaldehyde (PFA), the brains were cut into 30-μm sections with a cryostat. The sections were stained with X-gal staining solution (1

mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.01% NP-40) at 37°C for 40-48 hr. For the presentation of the β-galactosidase distribution, the images were combined using Adobe Photoshop CS5 (Adobe systems Inc., San Jose, CA). For immunohistochemical analyses, the sections were incubated with antibody dilution solution (0.01 M phosphate buffered saline (PBS) containing 1% bovine serum albumin, 1% normal goat serum and 0.25% Triton X-100) for 30 min and incubated overnight at 4°C with the antibody dilution buffer containing anti-NeuN antibody (1:500, EMD Millipore Corporation, Billerica, MA), anti-MAP-2 antibody (1:200, EMD Millipore Corporation), anti-GAD67 antibody (1:500, EMD Millipore Corporation) or anti-Brn-1 (1:50, Santa Cruz, Santa Cruz, CA). The signals were visualised with Alexa Fluor 568-conjugated anti mouse IgG or Alexa Fluor 488-conjugated anti mouse IgG (Life Technologies Corporation, Grand Island, NY).

Golgi staining method

The FD Rapid GolgiStain kit (FD Neurotechnologies, Inc., Ellicott City, MD) was used for Golgi-Cox staining according to the manufacturer's protocol. The spine morphology in piriform layer II neurons was classified according to Harris's report[41].

Cell culture, DNA transfection and immunocytochemistry

COS-7 cells cultured on fibronectin-coated cover glasses in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal bovine serum (FBS) were transfected with the pCAGGS FILIP IRES GFP vector or the pCAGGS IRES GFP vector using FuGENE 6 transfection reagent (Roche-Diagnostics, Tokyo, Japan). Twenty-four hours after

transfection, the cells were fixed with 4% PFA. NMHC IIb or actin fibres were visualised using polyclonal anti-NMHC IIb antibody (1:1000, Covance, Emeryville, CA) followed by Alexa Fluor 568-conjugated anti-rabbit IgG or Alexa Fluor 568-conjugated phalloidin (Life Technologies Corporation). NMHC IIb distribution in the cells was categorised into stress fibre-like distribution or granular distribution. "Stress fibre-like distribution" indicates that the cells had thick fibres that are longer than the diameter of the nucleus. "Granular distribution" indicates that the cells lacked thick, long fibres but had intracellular particle-like spots.

Primary culture of neurons from the hippocampus and the piriform cortex

The hippocampi and piriform cortices were dissected at E17.5. The samples were incubated in an enzyme solution that contained 90 U of papain in 1 ml PBS for 20 min at 37°C. Then, the dissociated cells were collected and plated on polyethyleneimine-coated coverslips in DMEM containing 10% FBS. After the cells attached to the coverslips, the medium was changed to growth medium, which was a neurobasal medium (Life Technologies Corporation) containing MACS Supplement B27 PLUS (Miltenyi Biotec, Bergisch Gladbach, Germany) and l-glutamine. For the analysis of the spine morphology, the pCAGGS FILIP IRES GFP vector and pCAGGS tdTomato vector were transfected using Lipofectamine 2000 (Life Technologies Corporation) at DIV17 or 18. To calculate the spine head volume and length, the neurons were fixed, and Z-stacked images were captured using a confocal microscope (LSM 5 Pascal, Carl Zeiss MicroImaging, GmbH, Jena, Germany) at DIV21. The spine head width and spine head length of the mushroom- or thin-type spines were measured using the ImageJ image analysis program (NIH, Bethesda, MD)[42]. The spine head

volume was calculated according to the method of Knafo[13]. To treat cells with glycine, at DIV12, pCAGGS FILIP IRES GFP vector and pCAGGS tdTomato vector were transfected as described above. At DIV15, the cells were incubated with 200 μM glycine-containing HEPES-buffered balanced salt solution (HEPES-BSS; 25 mM HEPES, pH 7.4, 119 mM NaCl, 2.5 mM KCl, 10 mM NaH_2PO_4, 2 mM $CaCl_2$ and 30 mM glucose) supplemented with 0.5 μM tetrodotoxin, 1 μM strychnine and 20 μM bicuculline for 10 min following incubation with normal HEPES-BSS for 20 min. D-AP5 (100 μM) was added to the HBSS-BSS for the pretreatment and the glycine treatment. The cells were fixed with 4% PFA in 0.1 M PB (pH 7.2) and were observed as described above. To be treated with 10 μM blebbistatin during the glycine treatment, the cells were preincubated with the growth medium containing10 μM blebbistatin for 30 min; then, the cells were treated with glycine as described above, with the exception of using HEBES-BSS supplemented with 10 μM blebbistatin. NMDA receptors were visualised using polyclonal anti-NR1 antibody (Sigma-Aldrich Co. LLC, St. Louis, MO) or polyclonal anti-NMDAR2A antibody (EMD Millipore Corporation) followed by Alexa Fluor 633-conjugated anti-rabbit IgG (Life Technologies Corporation). The knockdown of *FILIP* in the cultured piriform neurons was performed as previously reported[32]. pCAGGS-tdTomato, pT2K-CAGGS-rtTAM2, pT2K-TBI-shRNAmir and pCAGGS-T2TP (Tol2 transposase) were transfected at a ratio of 1:4:2:4 using Lipofectamine 2000 (Life Technologies Corporation) at DIV16. The medium was changed to doxycycline-containing medium (1 μg/ml) at DIV17. The cells were fixed and observed at DIV21. We selected pyramidal-shaped neurons for the *in vitro* observations of spine morphology. We visualised the distribution of NMHC IIB in the

neurons in the piriform cortex with a polyclonal anti-NMHC IIB antibody (1:1,000, Covance, Inc.) followed by an Alexa Fluor 568-conjugated anti-rabbit IgG or an Alexa Fluor 633-conjugated anti-rabbit IgG (Life Technologies Corporation). The intensity of NMHC IIB in the spine was measured with the ImageJ image analysis program. Spines in which the fluorescent intensity of NMHC IIB was altered more than twice were designated as NMHC IIB-intensified spines.

Immunoprecipitation

COS-7 cells that had been cultured in a 6-cm dish were lysed in 400 μl of ice-cold lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 5 mM sodium pyrophosphate, 50 mM NaF, 10 mM β-glycerophosphate, proteinase inhibitor cocktail; Nacalai tesque, Kyoto, Japan) 24 hr after transfection with the plasmid vectors. Pre-cleared lysates were incubated with antibody-bound Protein G Dynabeads (Life Technologies Corporation) at 4°C for 3 hr. The antibodies used for immunoprecipitation were as follows: 0.5 μl of polyclonal anti-NMHC IIb antibody for endogenous NMHC IIb; 0.2 μl of monoclonal anti-FLAG M2 antibody (Sigma-Aldrich Co. LLC) for FLAG-tagged FILIP and its truncated form; and 0.1 μl of monoclonal anti-VSV Glycoprotein antibody (Sigma-Aldrich Co. LLC) for truncated NMHC IIb. After the Dynabeads were rinsed three times in lysis buffer, the immunoprecipitated protein was eluted in SDS sample buffer and boiled for 3 min.

Preparation of cytosolic and Triton-soluble fractions

This method was modified from the method reported by Taguchi et al[46]. COS-7 cells that had been cultured in a 10-cm dish were collected 24 hr after transfection with plasmid vectors. The cells were resuspended in 200 μl of ice-cold lysis buffer (40 mM HEPES-KOH (pH 7.5), 5 mM ATP, 2 mM EDTA, proteinase inhibitor cocktail). The lysate was centrifuged at 17,000 x g for 60 min, and the supernatant was recovered as the cytosolic fraction (CS). The pellet was resuspended in 200 μl of ice-cold lysis buffer with 1% Triton X-100 and centrifuged at 17,000 ×*g* for 60 min. The supernatant was recovered as the Triton-soluble fraction (TS). Half of the CS was incubated at RT for 30 min to allow the actin to polymerise in the solution. The solution was centrifuged at 17,000 ×*g* for 60 min, and the supernatant was recovered as the cleared cytosolic fraction (CCS).

Western blot analyses and anti-FILIP antisera

The cortices of the ICR mouse brains were dissected out and homogenised in lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 5 mM sodium pyrophosphate, 50 mM NaF, 10 mM β-glycerophosphate and proteinase inhibitor cocktail. The insoluble materials were removed by centrifugation at 14,500 ×*g* for 60 min at 4°C. The hearts were extracted from the mice and homogenised in lysis buffer containing 40 mM HEPES-KOH (pH 7.5), 5 mM ATP, 2 mM EDTA and proteinase inhibitor cocktail. The insoluble materials were removed by centrifugation at 14,500 ×*g* for 60 min at 4°C. The protein concentration was measured using protein assay CBB solution (Nacalai tesque). Protein lysates (5 μg of cortices and heart protein) or immunoprecipitation products were separated by SDS-PAGE and transferred onto

PVDF membranes (EMD Millipore Corporation). After the membranes were blocked with 5% fat-free milk in PBS containing 0.1% Tween 20, they were incubated with primary antibodies as follows: polyclonal anti-FILIP antibody (1:500); monoclonal anti-filamin A antibody (1:1,000; Abnova Corporation, Taipei City, Taiwan); monoclonal anti-actin antibody (1:500; Sigma-Aldrich Co. LLC); polyclonal anti-β-galactosidase antibody (1:100; Life Technologies Corporation); polyclonal anti-NMHC IIb antibody (1:500); monoclonal anti-FLAG M2 antibody (1:2,000; Sigma-Aldrich Co. LLC); and monoclonal anti-VSV-Glycoprotein antibody (1:5,000; Sigma-Aldrich Co. LLC), followed by secondary antibodies coupled to HRP (1:2,000; BD Biosciences, Franklin Lakes, NJ). The peroxidase activity was detected using enhanced chemiluminescence and visualised using LAS-3000mini (Fujifilm Corporation, Tokyo, Japan). The blot densities were quantified using the ImageJ program. Anti-FILIP antisera were raised against a recombinant peptide of rat FILIP (ESQEMPMGRTILK) in rabbit.

Statistical analyses

To analyse the statistical significance of averages, we used an unpaired two-tailed Student *t*-test when two samples had equal variances and Welch's *t*-test when two samples had unequal variances. To analyse statistical significance of the spine head volume, we used the Wilcoxon rank sum test or Welch's *t*-test. To analyse statistical significance of ratio, we used Fisher's exact test. The criterion for statistical significance was $p < 0.05$.

SUPPLEMENTARY REFERENCE

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46. Taguchi, K., Yoshinaka, K., Yoshino, K., Yonezawa, K. & Maekawa, S. Biochemical and morphologic evidence of the interaction of oligodendrocyte membrane rafts with actin filaments. *J. Neurosci. Res.* **81,** 218-225 (2005).