Activity-dependent localization in spines of the F-actin capping protein CapZ screened in a rat model of dementia

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Actin reorganization in dendritic spines is hypothesized to underlie neuronal plasticity. Actin-related proteins, therefore, might serve as useful markers of plastic changes in dendritic spines. Here, we utilized memory deficits induced by fimbria-fornix transection (FFT) in rats as a dementia model to screen candidate memory-associated molecules by using a two-dimensional gel method. Comparison of protein profiles between the transected and control sides of hippocampi after unilateral FFT revealed a reduction in the F-actin capping protein (CapZ) signal on the FFT side. Subsequent immunostaining of brain sections and cultured hippocampal neurons revealed that CapZ localized in dendritic spines and the signal intensity in each spine varied widely. The CapZ content decreased after suppression of neuronal firing by tetrodotoxin treatment in cultured neurons, indicating rapid and activity-dependent regulation of CapZ accumulation in spines. To test input specificity of CapZ accumulation in vivo, we delivered high-frequency stimuli to the medial perforant path unilaterally in awake rats. This path selectively inputs to the middle molecular layer of the dentate gyrus, where CapZ immunoreactivity increased. We conclude that activity-dependent, synapse-specific regulation of CapZ redistribution might be important in both maintenance and remodeling of synaptic connections in neurons receiving specific spatial and temporal patterns of inputs.

Introduction

The vast majority of excitatory synapses in the central nervous system occur on dendritic spines (Harris & Stevens 1989). Recent findings have revealed that the shape of spines (e.g., head size, length and neck width) is closely associated with their functional properties such as size of excitatory currents (Matsuzaki et al. 2001) and biochemical and electrical properties (Bloodgood & Sabatini 2005; Noguchi et al. 2005; Araya et al. 2006). Furthermore, spines undergo activity-dependent morphological plasticity. The induction of long-term potentiation (LTP) is associated with long-lasting enlargement of existing spines and de novo emergence of spines (Engert & Bonhoeffer 1999; Matsuzaki et al. 2004), while long-term depression leads to shrinkage and retraction of them (Nagerl et al. 2004; Zhou et al. 2004). Filamentous actin (F-actin) is a crucial component of the spine cytoskeleton, and its dynamic behavior regulates the shape of spines (Matus et al. 2000). Thus, quantitative monitoring of actin-related proteins in spines may provide a useful readout of the ongoing plastic changes in neural networks that are responsible for memory formation and maintenance.

Fimbria-fornix transection (FFT) in animals has long been used as a model of memory deficit (Gaffan
It involves the axotomy of septohippocampal projection neurons, leading to a loss of theta-wave activity (Oddie et al. 2002) and inhibition of c-fos expression in the hippocampus ipsilateral to unilateral FFT (Vann et al. 2000), both of which are implicated in memory formation. Within 7–10 days after FFT surgery, deficits in hippocampus-dependent spatial learning and memory function become apparent without detectable histological damage to the hippocampal trisynaptic glutamatergic circuits (Cassel et al. 1997). Bilateral FFT has been shown to induce memory deficit similar to that observed in Alzheimer’s disease within 10 days (Gaffan 1972). In addition, our previous study of the bilateral FFT protocol and its subsequent effects on animal behavior confirmed the initiation of learning deficits by 10 days after the surgery (Nakao et al. 2001).

The animals with bilateral FFT at 10 days show marked learning deficits, which would accompany changes in protein markers of plasticity independent of compensatory mechanisms. In this study, we designed an experiment with animals that had undergone unilateral FFT instead of bilateral transection to obtain control protein samples within the same animals. We believe that comparison of protein profiles between the ipsilateral and contralateral hippocampi after unilateral FFT in the same rats can reduce the variability associated with inter-individual genetic, physiological and environmental differences. Using a proteomic approach (fluorescent two-dimensional differential gel electrophoresis, 2D-DIGE), we identified the F-actin capping protein CapZ (Schafer & Cooper 1995) among the candidate proteins showing changes after FFT. CapZ is known to regulate actin dynamics in non-neuronal cells (Mejillano et al. 2004) and regulate growth cone morphology and neurite outgrowth in cultured hippocampal neurons (Davis et al. 2009). However, its localization and activity-dependent regulation in mature neurons are not yet clear. To evaluate its ability to mark a specific subset of synapses undergoing activity-dependent changes, we further examined the localization and dynamics of CapZ in hippocampal neurons, both in vitro and in vivo.

Results

Changes in 2D-DIGE profile of CapZ after unilateral FFT

By using 2D-DIGE, we analyzed the difference in protein profiles in an intact hippocampus and a transected side at 10 days after unilateral FFT in rats. Of more than 400 spots detected by the 2D-DIGE system (Fig. 1A), 27 spots exhibited statistically significant differences in signal intensity between the transected and intact hemispheres in the same animal (P < 0.01 by Student’s t-test, N = 5 rats). While the intensity in the lesioned side had increased in most of proteins (e.g., GFAP, vimentin, HSP90 and 14-3-3), it decreased in seven proteins: drebrin-like SH3P7r3, CRMP4, profilin Iia, the guanine nucleotide-binding protein transducin, chaperonin-containing Tcp1, the

Figure 1 2D-DIGE analysis showing a decrease in the amount of CapZ beta 2 in the fimbria-fornix transection (FFT) hippocampus. (A) A representative image of the 2D-DIGE analysis showing protein spots at 10 days after unilateral FFT. The CapZ beta spot is indicated by a red box. M.W.: molecular weight. (B) Three-dimensional views of the signal intensity around the CapZ beta spot indicated in A show that it was well isolated from neighboring spots. The spot from the transected hippocampus had lower intensity than that from the control side. (C) The signal intensity of the spots in B was significantly decreased in the transected sides of all rats (N = 5 rats, ** P < 0.01 by Student’s t-test). The mean signal intensities for each condition are connected by a line. Cont: control side; Trans: transected side.
transcriptional activator protein PUR-alpha and F-actin capping protein (CapZ). Among these candidate proteins, CapZ is directly involved in the reorganization of the F-actin network and is likely to play an important role in stabilizing changes in neuronal morphology, especially at synaptic connections. Therefore, we subsequently examined the localization and dynamic redistribution of CapZ during activity-dependent alterations in the neural network.

CapZ is a heterodimer consisting of alpha and beta subunits. Here, we analyzed the CapZ splicing variant beta 2 subunit, which is more abundantly expressed in the brain than the other variant, beta 1 (Hart & Cooper 1999; Huang et al. 1999). The intensity of the spot corresponding to the beta subunit was significantly decreased in the transected side of all five rats (Fig. 1B,C). However, 1D SDS-PAGE immunoblot analysis of the same samples as those used in the 2D-DIGE analysis of CapZ beta 2 showed no statistically significant differences in the signal intensity of this protein (104 ± 1% of that in the control, N = 5 rats, P = 0.12 in Student’s t-test, Supplementary Fig. S1B). The specificity of the monoclonal antibody for CapZ beta 2, 3F2.3, was confirmed (see Supplementary Fig. S1A), wherein a single band of approximately 30 kDa (deduced molecular weight is 31.3 kDa) was detected. Three smaller spots with identical molecular weight but with different isoelectric values in 2D immunoblot of CapZ suggest protein modifications (Supplementary Fig. S1C). Thus, the decrease in the signal observed in the 2D-DIGE analysis was not because of the change in the net amount of CapZ protein but because of a change in the protein state (e.g., tight association with other molecules) that hampers the isoelectric focusing of the protein spot in one-dimensional separation.

Enrichment of CapZ in a subset of dendritic spines and its dependency on neuronal activity

To examine the expression of CapZ in neurons and its subcellular localization, we immunocytochemically analyzed dissociated and cultured hippocampal neurons with anti-CapZ beta 2 antibody. A punctate pattern of CapZ immunoreactivity (i.r.) was observed along the dendrites and in dendritic spines, where all protrusions whose length exceeded 0.5 μm were defined as spines (Fig. 2A. rhodamine-phalloidin was used to detect F-actin, which is abundant in spines.). CapZ i.r. was also observed in cell body with moderate reactivity and in thin processes corresponding to isolated and bundled axons with low reactivity. Glial cells showed less i.r. than neurons (data not shown). To exclude the possibility that the immunoreactive puncta corresponded to focal adhesion contacts, we examined the distance in the z-direction between CapZ-immunopositive puncta and the culture surface. The presence of a space between them indicated that CapZ was accumulated in structures distinct from focal adhesion contacts.

Interestingly, the intensity of the i.r. signal in each spine (average pixel intensity in a spine) varied

Figure 2 Heterogeneous localization of CapZ among spines and its regulation by neuronal activity. (A) Dissociated hippocampal neuronal cultures immunostained with anti-CapZ beta 2 antibody and counterstained with rhodamine-phalloidin (purple). Punctate CapZ signal (green) was observed in dendrites and heterogeneously in dendritic spines. Some spines were labeled strongly (arrowheads), while others were moderately labeled (yellow arrows). The right panels represent the CapZ immunoreactivity (i.r.) in each spine against its head size and length. N = 265 spines from 16 cells in three independent experiments. Scale bar, 2 μm. (B) CapZ i.r. in the spines of hippocampal neurons decreased after treatment with the sodium channel blocker tetrodotoxin (TTX; 1 μM) for 60 min. Each symbol represents an i.r. value from a spine (N = 265 spines from 16 cells for the control group and 219 spines from 11 cells for the TTX-treated group in 3 independent experiments) and **P < 0.01 by Mann–Whitney’s U-test. The line in the middle of each box-and-whisker plot indicates the median, that at the top of the box indicates the 75th quartile, that at the bottom of the box indicates the 25th quartile, and the whiskers indicate the extent of the 10th and 90th percentiles, respectively. The right panel shows the frequency distribution (P) of the i.r. values in percentage from the control and TTX-treated spines.
widely, ranging from a value below the level of detection to a value 10-fold greater than the median intensity level (Fig. 2A, right graphs). When the intensity was plotted against the spine head size and spine length, a positive (N = 265 spines from 16 cells, \( r^2 = 0.14, \ P < 0.01 \) by Pearson’s correlation test) and a negative (\( r^2 = 0.13, \ P < 0.01 \) correlations were found, respectively. Considering that short spines tend to have a large head (N = 265 spines, \( r^2 = 0.046, \ P < 0.01 \) by Pearson’s correlation test), these data indicate that short and large-head spines tend to have relatively high levels of CapZ. However, considerable variation in CapZ intensity was observed among spines having similar head size and length (Fig. 2A, white arrow heads and yellow arrows), suggesting that spine morphology is not the sole determinant of the CapZ content. Conversely, the observation of spines with widely varying morphology but similar CapZ reactivity indicates that the CapZ content is less likely to determine spine morphology. To rule out the possibility that the observed localization might be an aberration observed because of antibody accessibility, we co-expressed CapZ tagged with EGFP together with monomeric red fluorescent protein and found similar expression pattern (Supplementary Fig. S2).

As we found that spine morphology is not the sole determinant of CapZ accumulation, we hypothesized that previous synaptic transmission may regulate the determinant of CapZ accumulation. To test this hypothesis, cultured neurons were treated with 1 mM of the sodium channel blocker tetrodotoxin (TTX) for 60 min. The level of CapZ beta 2 i.r. in each individual spine was found to be significantly lower in the TTX-treated group than in nontreated controls (Fig. 2B), these results raised the possibility that CapZ accumulation was closely regulated by the activity-dependent signaling system. As neighboring spines along the dendritic shafts showed wide heterogeneity in the content of CapZ, synapse-specific signaling mechanisms might lead to heterogeneous nature of CapZ expression in selective spines. To determine whether selective stimulation of particular axons can induce input-specific CapZ accumulation in individual spines, we unilaterally delivered a high-frequency stimulus (HFS) to the medial perforant path (MPP) axons in awake animals (Fig. 4). The axons in the MPP selectively make synapses on dendritic segments of the middle molecular layer (MML) of ipsilateral dentate gyrus granule cells. The HFS used here has been shown to induce robust LTP and actin-related structural reorganization at MPP-MML synapses (Fukazawa et al. 2003).

At 45 min after HFS delivery, the CapZ beta 2 i.r. increased significantly at the ipsilateral MML (Fig. 4A–D). The i.r. at the ipsilateral MML increased at multiple test time points (30 and 60 min, \( N = 2 \) rats each) after HFS delivery. The CapZ signal did not increase in the neighboring inner and outer molecular layers or in the contralateral MML. The staining intensity of rhodamine-phalloidin, with which F-actin is visualized, was also increased only in the ipsilateral MML (Fig. 4E–H), and this finding is

**Input layer-selective increase in CapZ after LTP-inducing stimuli**

Detailed *in vitro* and *in vivo* analysis of CapZ localization revealed its accumulation in a specific subset of spines. Together with the experiment with TTX treatment (Fig. 2B), these results raised the possibility that CapZ accumulation was closely regulated by the activity-dependent signaling system. As neighboring spines along the dendritic shafts showed wide heterogeneity in the content of CapZ, synapse-specific signaling mechanisms might lead to heterogeneous nature of CapZ expression in selective spines. To determine whether selective stimulation of particular axons can induce input-specific CapZ accumulation in individual spines, we unilaterally delivered a high-frequency stimulus (HFS) to the medial perforant path (MPP) axons in awake animals (Fig. 4). The axons in the MPP selectively make synapses on dendritic segments of the middle molecular layer (MML) of ipsilateral dentate gyrus granule cells. The HFS used here has been shown to induce robust LTP and actin-related structural reorganization at MPP-MML synapses (Fukazawa et al. 2003).

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consistent with previous reports (Fukazawa et al. 2003). Thus, within a single dentate gyrus granule cell, CapZ selectively accumulated in the MML that received synaptic stimulation (Fig. 4D). Schematic illustration of dentate granule cells and the stimulated axons within MML are shown in Fig. 4I. The staining was seen as puncta that matched the sizes of spines, and the intensity of each puncta increased in the MML (Fig. 4J). In Fig. 4K, we have shown partial colocalization of CapZ and phosphorylated Cofilin (pCofilin), that is induced by LTP-inducing stimulation and is mostly restricted to dendritic spines (Chen et al. 2007b; Fedulov et al. 2007). According to Fedulov et al., pCofilin was sparsely found as 1.4 puncta/100 μm³ in a normal condition, while total spines are 300/100 μm³. The result shows that CapZ and pCofilin had similar density and co-localized only in a limited portion in the ipsilateral MML, suggesting that CapZ accumulation in spines is limited and heterogeneous even in the MML that received HFS. The difference in the distribution between two activity-dependent molecules may be attributable to the difference in the time course, because pCofilin is rapidly accumulated (within 2–7 min) and disappeared to half at 15–30 min after the stimulation.

**Discussion**

CapZ is a well-known F-actin capping protein named after the Z-band of muscle fibers, where it stabilizes, holds and aligns F-actin (Casella et al. 1987). Few studies have focused on the role of CapZ in the CNS. In the present study, we found a decrease in the intensity of the CapZ spot in 2D-DIGE analysis of the hippocampus immediately after the induction of dementia, and we revealed that CapZ accumulates in a subset of dendritic spines and is regulated by neuronal activity. Previous studies have revealed that CapZ is found in the postsynaptic density (Jordan et al. 2004; Li et al. 2004; Peng et al. 2004; Yoshimura et al. 2004), and its content is decreased in the brains of individuals with fetal Down’s syndrome (Guesserian et al. 2002). Interestingly, CapZ was recently reported to bind to disabled-1 (Sato et al. 2007), which regulates amyloid beta production (Hoe et al. 2006). These reports together with our present findings suggest that CapZ regulation...
might be associated with the neural network remodeling underlying higher cognitive processes such as learning and memory.

**Possible CapZ function in spine shape regulation**

CapZ binds to the fast-growing barbed end of actin filaments (Caldwell et al. 1989; Schafer et al. 1993). Gelsolin, the other well-known capping protein, exhibits severing activities (Yin et al. 1981) and has been shown to destabilize F-actin in spines (Hayashi et al. 1996; Star et al. 2002). In contrast, CapZ has been found to exhibit only stabilizing activities, e.g., on F-actin in muscle cells. Thus, CapZ might preferentially function to stabilize F-actin in the CNS as well.

Considering that the shape of dendritic spines is primarily regulated by F-actin (Ackermann & Matus 2003), it is logical to suggest that CapZ may be involved in the regulation of spine shape. In a melanocytoma cell line, depletion of CapZ by short hairpin RNA caused loss of lamellipodia and an explosive increase in the number of filopodia (Mejillano et al. 2004). We therefore suggest that CapZ suppresses the formation of thin actin-fiber bundles and negatively regulates filopodia formation in neurons. In the present study, large amounts of CapZ were rarely found in thin filopodia (Figs 2, 3). In some cases, CapZ was found at the tip of thin filopodia with small globular expansions (Fig. 2A, arrowhead at the bottom left), where it may inhibit further elongation and thereby promote spherical expansion at the tip to generate new spine heads.

The CapZ content varied greatly among large spines, even those with similar head size and length.
Localization of CapZ in dendritic spines

(Figs 2,3), suggesting that this parameter is not the sole or critical determinant of spine shape. Therefore, the role(s) played by CapZ in some large dendritic spines is an interesting subject for future investigation. Typically, the F-actin in dendritic spines is in dynamic equilibrium with globular actin (G-actin). That is, the actin components are treadmilling from one end of the fiber to another, continually turning over. Synaptic inputs shift the F-actin/G-actin equilibrium toward F-actin predominance (Okamoto et al. 2004) and slow down the turnover rate of the fiber in an NMDA receptor-dependent manner (Star et al. 2002). Thereafter, stable F-actin appears in spines in association with enlargement of the spine head (Honkura et al. 2008). CapZ may contribute to the activity-regulated stabilization of actin architecture.

The results obtained here are consistent with the above-mentioned hypothetical role of CapZ. We found that LTP-inducing electrical stimulation led to CapZ accumulation in the stimulated layer (MML) within 45 min and conversely that the CapZ content in spines decreased after a 60-min blockade of neuronal activity. This activity-dependent CapZ accumulation supports the possible role of CapZ in the regulation of actin dynamics in response to synaptic inputs.

CapZ as a possible molecular marker of spine plasticity

The characteristic distribution of CapZ shown in this study indicates the heterogeneity of CapZ content in spines even with similar morphological characteristics. Interestingly, this variability might reflect the history of input given to the individual synapses. Our in vivo experiments involving an LTP-inducing stimulus suggest that the input-specific marking of synapses by CapZ accumulation occurs in a time scale of 30 min to 1 h. In turn, the clearance of CapZ accumulation by the overall suppression of neuronal firing observed in vitro also occurs in a similar time scale. The kinetics of CapZ accumulation and clearance is comparable to the previously reported time course of spine structural remodeling (Honkura et al. 2008), suggesting the role of CapZ in marking spines with previous synaptic inputs.

Previously, we reported that, in only a limited number of spines in Arc-expressing cells (Kitanishi et al. 2009), exploring activity of an animal was accompanied by morphological alterations in its dendritic spines in the hippocampal neurons. In addition, we showed that learning enhances brain-derived neurotrophic factor expression (Chen et al. 2007a) in a limited number of neurons. Together, these data imply that natural sensory and learning stimuli cause changes only in a limited number of synapses. To capture such a limited behavior-dependent synaptic plasticity in vivo, a highly specific molecular marker is necessary. Although 2 actin-related proteins, namely, profilin (Ackermann & Matus 2003; Lamprecht et al. 2006) and coflin (Chen et al. 2007b; Fedulov et al. 2007), have been proposed to show activity-dependent accumulation or phosphorylation in spines, the input specificity of accumulation in vivo has not been proved so far.

The findings of the present study show that CapZ is heterogeneously localized and shows input layer-specific accumulation as well as a response to increased or decreased neuronal activity. We suggest that CapZ serves as a useful marker for activity-dependent spine plasticity and possibly for memory-related neuronal network reorganization.

Experimental procedures

Unilateral FFT

All experiments were performed according to the Japan Neuroscience Society guide for the care and use of laboratory animals. Wistar/ST male rats (aged 8–9 weeks; SLC, Shizuoka, Japan) were deeply anesthetized with pentobarbital (50 mg/kg i.p.; Dainippon Sumitomo Pharma, Osaka, Japan) and placed in a stereotaxic apparatus. Unilateral FFT was performed by vertical insertion of a coronally positioned razor blade (5.0–mm wide, an end at the midline) through a lined skull burr hole into the right hemisphere (6.0-mm deep at a position 1.1 mm posterior to bregma) (Nakao et al. 2002). The rats after surgery were not tested in any behavioral paradigm. We have purposely left rats in novel environment (laboratory room) at least for 1 h before sampling.

2D-DIGE and LC-MS/MS (liquid chromatography with tandem mass spectrometry) analysis

Ten days after unilateral FFT, each side of the hippocampus was rapidly dissected, separately frozen in liquid N₂, powdered and homogenized in cold lysis buffer (7 m urea, 2 m thiourea, 4% CHAPS, 5 mm magnesium acetate, 2 mm sodium vanadate, 2 mm phosphatase SC, 5 mm Tris/Cl (pH 8)). After centrifugation (20 min, 12 000 g, 4°C), each supernatant from 10 samples was labeled with the indodicarbocyanine dye Cy5. As a standard, a mixed pool containing equal amounts of some samples (6 of the 10 samples; from three rats) labeled with the indodicarbocyanine Cy3 was used to prevent labeling bias. Each Cy5-labeled sample and the Cy3-labeled standard (50 μg each) were mixed and applied to an 18-cm immobilized pH gradient strip (pH 4–7). Isoelectric focusing was carried out using Multiphor II according to the manufacturer’s protocol (GE
Healthcare Bioscience, Tokyo, Japan). Electrophoresis in the second dimension (molecular weight) was carried out with 12% SDS-PAGE gels. Gel images were collected by scanning with a 2920-2D Master Imager (GE Healthcare Bioscience). Quantitative statistical analysis was performed using DeCyder software (GE Healthcare Bioscience).

Spots of differentially expressed proteins were excised from the 2D gels by using an Etant Spot Picker (GE Healthcare Bioscience) and digested with trypsin (Promega, Mannheim, Germany). After digestion, the products were recovered from the gels by sequential extraction with 25 mM ammonium bicarbonate and 100% acetonitrile. The extracts were dried in a SpeedVac evaporator (Thermo Savant, Waltham, MA, USA) and resuspended in 10 μL of 0.1% TFA. The digested samples were injected onto a capillary high-performance liquid chromatography system equipped with a HP1100 solvent delivery pump (Agilent Technologies, Waldbronn, Germany), an Acquity UPLC HSS T3 analytical column (150 mm × 0.15 mm, 1.8 μm), a peptide trap column (0.5 mm × 5 mm; Michrom BioResources, Auburn, CA, USA) and a PepMap analytical column (0.075 mm × 150 mm; LC Packings). The extracted peptides were analyzed using an LCQ ion-trap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA) in the data-dependent MS/MS mode. The MASCOT program (Matrix Science, London, UK) was used for protein identification, and mammalian proteins in the NCBI nr database were used as references.

Primary culture and immunocytochemistry

Cultured hippocampal neurons (DIV14) were prepared as described previously (Ohba et al. 2005) with minor modifications. Briefly, whole brains were isolated from embryonic day 18 Wistar rats (SLC); the hippocampi were removed and treated with 0.25% trypsin (Difco Laboratories, Detroit, MI, USA) and 0.01% deoxyribonuclease I (Sigma, St. Louis, MO, USA) at 37°C for 30 min. The cells were suspended in Neurobasal medium (Invitrogen, Eugene, OR, USA) containing 10% fetal bovine serum (Sanko-junyaku, Tokyo, Japan) and were plated at a density of 4.5–5.0 × 10^4 cells/cm^2 on polyethyleneimine (Sigma)-coated glass coverslips (Matsunami Glass, Osaka, Japan), each equipped with flexiPERM (Sartorius, Gottingen, Germany), which created eight wells of dimensions 0.8 cm^2 on polyethyleneimine-coated glass coverslips (Matsunami Glass, Osaka, Japan), each equipped with flexiPERM (Sartorius, Gottingen, Germany), which created eight wells of dimensions 0.8 cm^2 (LC Packings, Amsterdam, The Netherlands), a peptide trap column (0.5 mm × 5 mm; Michrom BioResources, Auburn, CA, USA) and a PepMap analytical column (0.075 mm × 150 mm; LC Packings). The extracted peptides were analyzed using an LCQ ion-trap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA) in the data-dependent MS/MS mode. The MASCOT program (Matrix Science, London, UK) was used for protein identification, and mammalian proteins in the NCBI nr database were used as references.

Immunohistochemical analysis

Male Thy1-mGFP mice [5 months old, line 21 (DePaola et al. 2003)], which express membrane-targeted green fluorescent protein (mGFP) in a subset of neurons, were used to visualize fine dendritic structures including spines. Anesthetized mice or rats were transcardially perfused with 4% PFA in 0.1 M PB. Dissected brains were soaked in fixative for 2 h. For antigen retrieval, Microslicer (Dosaka, Japan) sections of thickness 100 μm (Fig. 3A) or 50 μm (Fig. 3B) were treated with LAB solution (Polysciences, Warrington, PA, USA) for 5 min, 0.1% Triton X-100 for 30 min and 1 mg/mL of pepsin (DAKO, Carpenteria, CA, USA) in 0.2 N HCl at 37°C for 3 min (Watanabe et al. 1998; Fukaya & Watanabe 2000). The sections were then sequentially incubated with 2% or 10% normal goat or donkey serum for 60 min, anti-CapZ beta 2 antibody (0.3 μg/mL; Developmental Studies Hybridoma Bank, University of Iowa, IA, USA) and rat monoclonal anti-GFP antibody [1:400; Nalai Tesque, Japan, (Fig. 3A)] or anti-pCofilin (1:100, Abcam, Cambridge, UK) overnight at 4°C, and finally with Alexa-594 anti-mouse IgG and Alexa-488 anti-rat IgG antibodies (1 : 400; Invitrogen) or Cy3-labeled donkey anti-mouse IgG (1 : 200; Jackson ImmunoResearch, West Grove, PA, USA). The sections were then mounted on Mermafluor (Thermo Shandon, Pittsburgh, PA, USA). Fluorescent images were acquired using a confocal laser scanning microscope (LSM510 Meta, Carl Zeiss) equipped with a 100× magnification, 1.3 NA, oil immersion objective and 488-nm argon and 543-nm helium/neon lasers. Images (16-bit) were acquired with a fixed exposure time that was determined to avoid saturation. At first, rhodamine-phalloidin (33 μm) images were processed with a convolution filter (Laplacian) to detect the edges of protrusions: those whose length exceeded 0.5 μm were objectively defined as dendritic spines. After the background was automatically subtracted from the CapZ beta 2 immunocytochemical images, the average intensity in each spine was measured.

For immunoelectron microscopic examination, the fixative was 4% PFA and 0.1% glutaraldehyde in 0.1 M PB. For postembedding immunogold processing, the slices were cryo-protected by immersion in 30% sucrose in 0.1 M PB and frozen rapidly in liquid propane in a Leica EM CPC unit. The frozen sections were immersed in 0.5% uranyl acetate in methanol at −90°C in a Leica AFS freeze substitution unit, embedded at −45°C in Lowicryl HM-20 resin (Lowi, Waldkraiburg, Germany) and polymerized with UV light. After etching with saturated sodium ethanolate solution for 3 s, ultra-thin sections on nickel grids were successively treated with 1% human serum albumin (Wako, Osaka, Japan) in 0.1% Tween 20 in Tris-buffered saline (pH 7.5) for 1 h, anti–CapZ antibody (15 μg/mL) overnight and colloidal gold (10 nm)-conjugated anti-mouse IgG (1 : 100; British Biocell International, Cardiff, UK) for 2 h. Finally, the grids were stained with uranyl acetate for 15 min. Electron micrographs were taken randomly with an H7100 electron microscope (Hitachi, Tokyo, Japan).
Localization of CapZ in dendritic spines

Dentate gyrus LTP and histochemical analysis

LTP experiments on freely moving rats were carried out as described previously (Fukazawa et al. 2003). Briefly, a bipolar tungsten electrode (8.7 mm posterior, 5.3 mm lateral and 5.3 mm ventral to bregma) and a recording electrode (4.0 mm posterior, 2.5 mm lateral and 3.8 mm ventral to bregma) were unilaterally implanted into the rats under pentobarbital anesthesia (i.p., 50 mg/kg body weight). The rats were individually housed in cages and allowed to recover for at least 2 weeks. After the stability of the basal fEPSP was monitored, an HFS was applied; this stimulus consists of 500 pulses (10 trains of 5 bursts of ten 400-Hz pulses with a 1-s interburst interval and a 1-min intertrain interval) and has been shown to induce LTP. The rats were sacrificed under anesthesia at 30, 45, or 60 min after stimulation. The brain was immediately removed, frozen in dry ice and cut coronally with a cryostat microtome into 10- to 30-μm sections. These were fixed in 4% PFA in 0.1M PB for 30 min. The subsequent histochemical procedures were as described for Fig. 3A. When F-actin was to be detected, the step involving LAB solution was omitted. The secondary antibody solution contained NeuroTrace 435/455 blue fluorescent Nissl stain (1:50; Invitrogen) to enable visualization of cell layers. Images acquired using an LSM 510 Meta microscope (Carl Zeiss) were analyzed using the MetaMorph software (Universal Imaging, West Chester, PA, USA).

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References


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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Immunoblot for CapZ in 1D (A, B) and 2D (C) SDS-PAGE.

Figure S2 Localization and dynamics of EGFP-tagged CapZ in cultured hippocampal neurons.

Additional Supporting Information may be found in the online version of this article.

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