Activity of the AMPA receptor regulates drebrin stabilization in dendritic spine morphogenesis

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Accepted 15 December 2008
Journal of Cell Science 122, 1211-1219 Published by The Company of Biologists 2009
doi:10.1242/jcs.043729

Summary

Spine morphogenesis mainly occurs during development as a morphological shift from filopodia-like thin protrusions to bulbous ones. We have previously reported that synaptic clustering of the actin-binding protein drebrin in dendritic filopodia governs spine morphogenesis and synaptic PSD-95 clustering. Here, we report the activity-dependent cellular mechanisms for spine morphogenesis, in which the activity of AMPA receptors (AMPArs) regulates drebrin clustering in spines by promoting drebrin stabilization. In cultured developing hippocampal neurons, pharmacological blockade of AMPArs, but not of other glutamate receptors, suppressed postsynaptic drebrin clustering without affecting presynaptic clustering of synapsin I (synapsin-I). Conversely, the enhancement of the action of AMPArs promoted drebrin clustering in spines. When we explored drebrin dynamics by photobleaching individual spines, we found that AMPAR activity increased the fraction of stable drebrin without affecting the time constant of drebrin turnover. An increase in the fraction of stable drebrin corresponded with increased drebrin clustering, AMPAR blockade also suppressed normal morphological maturation of spines and synaptic PSD-95 clustering in spines. Together, these data suggest that AMPAR-mediated stabilization of drebrin in spines is an activity-dependent cellular mechanism for spine morphogenesis.

Key words: Dendritic spine, AMPA receptor, Drebrin, Actin cytoskeleton, Synapse development, Synaptic plasticity

Introduction

Dendritic spines are the receptive sites of most glutamatergic synapses in adult brains (Harris and Kater, 1994; Hering and Sheng, 2001). A typical case of a synaptically functional spine is represented by a mushroom-shaped protrusion possessing a bulbous head and narrow neck (Matsuzaki et al., 2001; Noguchi et al., 2005). Various morphological changes of spines have been reported, such as spine enlargement (Matsuzaki et al., 2004; Okamoto et al., 2004), shrinkage (Zhou et al., 2004), new formation (Dunaevsky et al., 1999; Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999) and retraction (Hashani et al., 2001). Spine morphogenesis during development mainly occurs as the morphological shift from filopodia, long headless protrusions, to mushroom-shaped spines (Fiala et al., 1998). Previous studies have shown that chronic perturbation of neuronal activity with tetrodotoxin (TTX) or picrotoxin during development changes spine number (Riccio and Matthews, 1985; Papa and Segal, 1996; Collin et al., 1997), suggesting that synaptic activity regulates spine morphogenesis during development. Given the importance of morphological changes of spines in synaptic plasticity of mature neurons (Bourne and Harris, 2007; Segal, 2005; Yuste and Bonhoeffer, 2001), it is proposed that activity-dependent mechanisms for spine morphogenesis during development are crucial for the development and plasticity of neuronal circuits. However, the cellular mechanisms of the activity-dependent regulation of spine morphology during development remain unclear.

Spine morphology depends on two major structural elements: the actin cytoskeleton and the postsynaptic density (PSD) (Kennedy, 1997; Matus et al., 1982; Sekino et al., 2007). We previously demonstrated that the postsynaptic actin-binding protein drebrin is involved in spine morphogenesis (Takahashi et al., 2003). Drebrin clustering occurs at nascent synapses on dendritic filopodia, and clustering of actin filaments (F-actin) and PSD-95, a PSD scaffold protein, at postsynaptic sites depends on pre-existing drebrin clusters (Takahashi et al., 2003). However, the mechanism that regulates how drebrin is clustered at postsynaptic sites remains unclear.

One possible mechanism is the conversion of drebrin isoform expression from a drebrin embryonic isoform (drebrin E) to an adult isoform (drebrin A), because the inhibition of the expression of drebrin A during neuronal development suppresses spine morphogenesis (Takahashi et al., 2003). However, the premature expression of drebrin A does not promote either drebrin clustering at spines or spine morphogenesis, but induces abnormally large headless protrusions with the unrestricted accumulation of F-actin, PSD-95 and drebrin itself (Mizui et al., 2005). By contrast, the synchronous development of drebrin clustering and functional turnover of synaptic vesicles (Renger et al., 2001) suggests that synaptic activity regulates drebrin clustering. Therefore, we hypothesize that an activity-dependent mechanism regulates drebrin clustering during development.

To examine this hypothesis, we pharmacologically manipulated glutamate-receptor activity and then analyzed the distribution of drebrin in developing hippocampal neurons in cultures by immunocytochemistry. Furthermore, we performed fluorescence recovery after photobleaching (FRAP) analysis to explore a cellular
basis for activity-dependent drebrin clustering. In this study, we show that AMPA-receptor (AMPAR)-activity-mediated stabilization of drebrin plays a pivotal role in the drebrin clustering at postsynaptic sites that underlies spine morphogenesis during development.

Results

Synaptic activity regulates drebrin clustering during development

We first investigated whether synaptic activity is involved in drebrin clustering during development. After 8 days in vitro (DIV), we treated hippocampal neurons in low-density cultures with TTX, which blocks action potentials, or with the GABA<sub>A</sub>-receptor blocker picrotoxin, which enhances the excitatory component of synaptic transmission. At 15 DIV, we performed drebrin immunostaining and then analyzed the cluster densities (number of clusters in a given area) of drebrin along the dendrites of the treated neurons. The cluster density of drebrin was decreased by chronic treatment with TTX, as compared with control neurons (Fig. 1). By contrast, the cluster density of drebrin was increased by treatment with picrotoxin (Fig. 1). These data indicate that spontaneous synaptic activity enhances drebrin clustering during development.

AMPAR activity regulates synaptic clustering of drebrin in dendritic spines

To investigate which subtype of glutamate receptors is involved in drebrin clustering, cultured neurons were chronically treated with subtype-specific blockers of glutamate receptors (Fig. 2A,B). CNQX (blocker for AMPARs and kainite receptors) and GYKI (blocker for AMPARs) reduced the cluster density of drebrin at 15 DIV. By contrast, drebrin clustering was unaffected by phlanthotoxin (PhTX), a drug that blocks only a subset of AMPARs—those that are Ca<sup>2+</sup>-permeable because they lack the GluR2 subunit. Neither AP5 [blocker for NMDA receptors (NMDARs)] nor MCPG (blocker for metabotropic glutamate receptors) affected the cluster density of drebrin. According to western blot data (Fig. 2C), GYKI and CNQX treatments had no significant effects on the total expression level of drebrin protein (n=3 separate cultures; GYKI, 109.8±7.7% of control, P=0.33; CNQX, 89.1±8.3% of control, P=0.32; Student’s t-test), suggesting that the reduction of drebrin clustering by these treatments is not due to the change of protein expression level of drebrin.

To exclude the possibility that chronic AMPAR blockade secondarily suppresses drebrin clustering by impaired formation of presynaptic contact sites, we investigated the effects of AMPAR blockade on cluster densities of drebrin and synapsin I (synapsin I), a synaptic vesicle marker, along the same dendrites (Fig. 3). At 15 and 22 DIV, we performed double immunostaining with drebrin and synapsin I of the neurons that were chronically treated with GYKI from 8 DIV onwards. In control neurons, the densities of both drebrin clusters and synapsin-I clusters were increased during development (Fig. 3). By contrast, AMPAR blockade significantly reduced the developmental increase of the cluster density of drebrin, as compared with control neurons (Fig. 3A-D). Unlike its effect on drebrin, AMPAR blockade did not affect the developmental increase of cluster density of synapsin I (Fig. 3A-C,E).

According to our previous study (Takahashi et al., 2003), drebrin clustering occurs at postsynaptic sites in dendritic filopodia. Furthermore, drebrin clustering in dendritic filopodia is important for spine morphogenesis. Thus, we compared the effects of AMPAR.

![Fig. 1. Synaptic activity regulates drebrin clustering.](Image)

(A) Drebrin immunolabeling of cultured hippocampal neurons treated with vehicle (control), TTX or picrotoxin from 8 DIV for 7 days. Upper panels show the representative low-magnification images of each treated neuron. Lower panels shows the boxed region in the respective upper panel. Scale bars: 20 μm (upper), 10 μm (lower). (B) Quantitative analysis of the densities of drebrin clusters. TTX treatment suppressed drebrin clustering whereas picrotoxin treatment enhanced it (>14 neurons for each condition). **P<0.01, *P<0.05 versus control.

![Fig. 2. AMPAR activity is involved in drebrin clustering.](Image)

(A) Drebrin immunolabeling of neurons treated with vehicle (control) or the following agents from 8 DIV for 7 days: the AMPAR and kainite-receptor blocker CNQX (40 μM), the specific AMPAR blocker GYKI (10 μM), the Ca<sup>2+</sup>-permeable (GluR2-subunit-lacking) AMPAR blocker PhTX (10 μM), the NMDAR blocker AP5 (50 μM), GYKI (10 μM) plus AP5 (50 μM), or the metabotropic glutamate receptor blocker MCPG (100 μM). Scale bar: 5 μm. (B) Quantitative analysis of the densities of drebrin clusters after these treatments. CNQX, GYKI and GYKI plus AP5 treatments suppressed drebrin clustering (>14 neurons for each treatment). *P<0.01 versus control. (C) Western blots showing the representative effect of GYKI and CNQX treatment on the expression of total drebrin (drebrin E and A) and β-actin. According to the densitometric analysis, GYKI and CNQX treatments do not change the expression level of drebrin protein (n=3 separate cultures; GYKI, 109.8±7.7% of control; P=0.33; CNQX, 89.1±8.3% of control, P=0.32).
activity on the cluster density of drebrin between dendritic protrusions and dendritic shafts by triple-staining for drebrin, synapsin I and F-actin. On the basis of F-actin images, we distinguished drebrin clustering in dendritic protrusions from that in dendritic shafts. AMPAR blockade reduced drebrin cluster density on dendritic protrusions from that in dendritic shafts. AMPAR blockade reduced drebrin cluster density on dendritic protrusions, especially at synaptic sites, without affecting it on dendritic shafts (Fig. 4A,B). By contrast, enhancement of AMPAR activity with the AMPAR desensitization blocker, cyclothiazide (CTZ), increased total drebrin cluster density on dendritic protrusions in the presence of NMDAR blocker (Fig. 4A,B). Enhancement of AMPAR activity did not affect drebrin cluster density on dendritic shafts (Fig. 4A,B). Although CTZ treatment was performed in the presence of AP5 to block the secondary activation of NMDARs, AP5 treatment itself had no significant effect on total drebrin cluster density on dendritic protrusions (Fig. 4A,B). In addition, cluster density of synapsin I was not changed by these treatments (Fig. 4A,C).

Taken together, these data indicate that the spontaneous activation of Ca²⁺-impermeable (GluR2-containing) AMPARs specifically promotes drebrin clustering on dendritic protrusions without affecting the formation of presynaptic contact sites.

FRAP analysis reveals that AMPAR activity increases the fraction of stable drebrin in dendritic spines

We next explored the dynamic properties of these clusters within the spine in order to demonstrate the cellular basis of activity-dependent drebrin clustering. Neurons were transfected with vectors that encoded drebrin A fused to enhanced green fluorescent protein (eGFP). Individual eGFP molecules can be rendered nonfluorescent, or ‘bleached’, with high-intensity laser pulses. Such pulses darken the target area until new, unbleached eGFP-drebrin replaces the bleached molecules during normal protein turnover. This technique, called fluorescence recovery after photobleaching (FRAP), enabled us to study drebrin dynamics within individual spines in real time (Fig. 5).

We first investigated drebrin dynamics in spines under normal physiological conditions. In drug-free controls, the stable fraction of drebrin at 14-16 DIV was 22.7±3.0% (Fig. 5A-F), and the drebrin exchange rate followed a time constant of 5.8±0.4 minutes (Fig. 5D,E,G). These data indicate that about a quarter of total drebrin within a single spine is stabilized under normal physiological conditions that allow spontaneous neuronal activity.

We next investigated whether and how AMPAR activity affects drebrin dynamics in spines. Application of CNQX decreased the stable drebrin fraction to 8.9±3.6% without affecting the exchange rate (Fig. 5C,D,F,G). The fraction of stable drebrin increased when we applied AMPA or CTZ in the presence of AP5 to enhance only AMPAR activity, but the exchange rate remained the same (Fig. 5D,F,G). AP5 treatment itself did not alter the size of the stable drebrin fraction, but the time constant for drebrin turnover fell to 4.0±0.3 minutes (Fig. 5E-G). These data show that the activity of AMPARs, but not that of NMDARs, regulates the level of stable drebrin in spines.

An increase in size of the stable drebrin fraction corresponds with increased drebrin clustering

We next sought to determine whether the change in size of the stable drebrin fraction corresponds with altered drebrin clustering (Fig. 6). The previous FRAP study of GFP-tagged actin showed that short-term (15- to 30-minute) treatment with the F-actin capping molecule cytochalasin D (cytoD) increases the size of the stable fraction of actin in dendritic spines (Star et al., 2002). Furthermore, in non-neuronal cells, short-term (1-hour) treatment with cytoD causes drebrin-associated F-actin to aggregate in the cytoplasm (Asada et al., 1994), presumably by changing the dynamics of drebrin-actin complexes. As expected, we found that a 1-hour cytoD treatment...
increased the fraction of stable drebrin in cultured neurons (Fig. 6A-C). Interestingly, the short-term cytoD treatment also promoted the formation of drebrin clusters along dendrites, and these clusters colocalized with F-actin (Fig. 6D-F). These results suggest that the increased fraction of stable drebrin led to the formation of drebrin clusters. Thus, we conclude that AMPAR activity promotes drebrin clustering by enhancing drebrin stability in spines.

Chronic inhibition of AMPARs causes filopodia-like thin spines during development

Given the importance of AMPAR activity in drebrin clustering, which governs dendritic spine morphogenesis, we next investigated whether AMPAR activity is involved in spine morphogenesis of developing hippocampal neurons in culture. At 7 DIV, the cells were transfected with an expression vector for membrane-targeted GFP (mGFP) (Matus et al., 2000), which allowed us to visualize spine morphology. At 8-9 DIV, we added to the culture medium the drug that blocked a specific glutamate-receptor subtype: GYKI for AMPARs, AP5 for NMDARs and MCPG for mGluRs. At the time of transfection and drug application, the dendritic spines were long, thin and headless. These 'filopodia-like' characteristics are typical of immature spines (Papa et al., 1995; Takahashi et al., 2003). At 15-16 DIV, the cultures that did not receive drugs showed mature spines, which have shorter necks and defined heads (Fig. 7A). The 7 days of treatment with blockers for NMDARs or mGluRs did not obviously affect spine development and morphology (Fig. 7A). However, 7 days of treatment with the AMPAR blocker resulted in dendrites that still displayed thin, headless protrusions, some of which were malformed with Y-shaped or multi-branched structures (Fig. 7A).

To quantify the effects of each blocker on the morphological shift from filopodia-like thin protrusions to bulbous ones during development, we measured the width of dendritic protrusions of each treated neuron. Compared with drug-free controls, AMPAR blockade caused a decrease of more than 25% in the width of dendritic protrusions (including immature filopodia and mature spines) (Fig. 7B,C). Neither NMDAR blockade nor mGluR blockade altered the width of dendritic protrusions (Fig. 7B,C). These data indicate that AMPAR activity is required for normal spine maturation.

Chronic AMPAR inhibition suppresses synaptic clustering of PSD-95 in dendritic spines

Our previous study demonstrated that drebrin clustering is required for synaptic targeting of PSD-95 during spine morphogenesis (Takahashi et al., 2003). We finally investigated whether AMPAR activity is also required for PSD-95 clustering in spines during development. When we examined the localization of PSD-95 during chronic AMPAR blockade, we saw a lower density of PSD-95 clusters at synaptic sites on dendritic protrusions but not on dendritic shafts (Fig. 8). These data support a model in which spontaneous AMPAR activity stabilizes drebrin, which in turn promotes the formation of PSD-95 clusters within spines, thereby enabling the normal maturation of these structures.

Discussion

In this study, we found that inhibition of AMPARs, but not other glutamate receptors, prevented drebrin clustering in spines. By contrast, enhancement of AMPAR activity promoted drebrin clustering. Furthermore, we demonstrated that AMPAR activity
regulated drebrin stabilization, which caused drebrin clustering, in dendritic spines. We propose the AMPAR-dependent stabilization of drebrin in spines as a novel activity-dependent mechanism for spine morphogenesis.

**AMPAR-mediated drebrin stabilization and clustering**

A significant finding of the present study is that AMPAR activity positively regulates the size of the stable fraction of drebrin within spines. Imaging studies have shown that filopodia rapidly turn over, undergoing de novo formation and elimination, whereas spines persist for a long time (Dailey and Smith, 1996; Grutzendler et al., 2002; Marrs et al., 2001; Portera-Cailliau et al., 2003). Spines have a large amount of F-actin that is clustered with drebrin (Hayashi et al., 1996; Takahashi et al., 2003), and these clusters are resistant to cytoD (Allison et al., 1998). Additionally, our previous studies using cultured cell lines have shown that the binding of drebrin to F-actin is resistant to cytoD (Asada et al., 1994; Ikeda et al., 1996), suggesting that drebrin at the spines is responsible for the cytoD-resistance of actin filaments at spines and eventually for spine persistence. The study using glutamate uncaging has shown that functional AMPARs are sparsely distributed in dendritic filopodia and thin spines but abundant in mushroom-shaped large spines,
indicating the correlation between spine volume and its expression of functional AMPAR (Kasai et al., 2003; Matsuzaki et al., 2001). Given that drebrin content is correlated with spine volume (Kobayashi et al., 2007), our present study suggests that the rapid turnover of filopodia is due to an insufficient amount of stable drebrin, whereas spine persistence is due to an AMPAR-regulated abundance of stable drebrin. This is consistent with evidence that shows that AMPAR activity contributes to spine maintenance (McKinney et al., 1999). Thus, we propose that AMPAR-mediated drebrin stabilization underlies the difference of persistence between dendritic filopodia and spines.

Our previous biochemical studies showed that drebrin is a side-binding protein to F-actin (Ishikawa et al., 1994), and can change F-actin into thick and curving bundles (Shirao et al., 1994), suggesting that the F-actin-remodeling activity of drebrin is one of the factors that induce clustering of drebrin with F-actin in spines (for a review, see Sekino et al., 2007). We have previously reported that drebrin clustering governs synaptic clustering of F-actin and PSD-95 in the process of spine morphogenesis (Takahashi et al., 2003). In the present study, we show that the stable fraction of drebrin in spines is positively correlated with drebrin cluster density. In addition, our cytoD experiments indicate that increased drebrin stability in spines leads to drebrin clustering. These data suggest that an increase in stabilized drebrin in spines is a key cytoskeletal reorganization that can lead to synaptic clustering of postsynaptic proteins, including of drebrin itself, via the F-actin-remodeling activity of drebrin. Because the induction of long-term potentiation (LTP) is known to be accompanied by drebrin accumulation (Fukazawa et al., 2003), AMPAR-mediated drebrin stabilization might be involved in the enlargement of stimulated spines (Honkura et al., 2008; Matsuzaki et al., 2004; Okamoto et al., 2004).

In addition to the importance of the stable drebrin fraction, our FRAP data show that, under normal physiological conditions, which allow spontaneous neuronal activity, about 70% of drebrin in spines is in a dynamic fraction. Interestingly, NMDAR blockade decreases the time constant of dynamic drebrin without affecting the stable drebrin fraction, suggesting that spontaneous NMDAR activity regulates the transport efficiency of drebrin into spines. Our previous study demonstrated that drebrin is involved in the synaptic targeting of NMDARs that is induced by chronic blockade of NMDARs (Takahashi et al., 2006), conditions known as homeostatic scaling of NMDARs (Perez-Otano and Ehlers, 2005). Because NMDAR blockade has no effect on the stable fraction of drebrin, our data suggest that dynamic drebrin contributes to the transport of NMDARs into spines.
Involvement of AMPAR activity in spine morphogenesis

A previous report implicated AMPAR-mediated synaptic transmission in the maintenance of dendritic spines (Mateos et al., 2007; McKinney et al., 1999), and our present study extends this hypothesis by showing that AMPAR activity is also required for the morphological maturation of dendritic spines.

In contrast to these observations, some studies have shown that the mere presence of the extracellular domain of the AMPAR subunit GluR2 has a trophic effect on spine formation through interaction with N-cadherin (Passafaro et al., 2003; Saglietti et al., 2007). Those reports suggest that AMPAR-channel activity is not important for spine maturation and/or maintenance. However, the overexpression of GluR2 in young (11 DIV) neurons promotes the formation of headless, filopodia-like protrusions rather than headed spines (Passafaro et al., 2003; Saglietti et al., 2007). Because presynaptic maturation at this developmental stage is far from complete (Reger et al., 2001), it is suggested that synaptic transmission in these neurons is not enough for the activation of postsynaptic AMPARs. Furthermore, a mutation in GluR2 that renders it Ca\(^{2+}\)-permeable decreases the trophic effects on spine width and length (Passafaro et al., 2003). In view of these data, our results suggest that the transformation from filopodia into headed spines containing dendritic clusters and PSD-95 clusters requires AMPAR-channel activity, although the sprouting of new filopodia and the stabilization of pre-existing filopodia require the trophic effects of the GluR2 extracellular domain.

Activation of the AMPAR channel not only causes ion influx but also activates intracellular signaling pathways (Wang and Durkin, 1995; Wang et al., 1997; Hayashi et al., 1999; Kim et al., 2004). Furthermore, a recent study demonstrated the functional interaction between drebrin and Ras in spine plasticity (Biou et al., 2008). We expect that future studies will determine what signaling pathway from AMPAR to the drebrin-based actin cytoskeleton underlies spine maturation, maintenance and/or plasticity.

Impairment of AMPAR-mediated drebrin clustering and dendritic-spine pathology

A specific decrease in drebrin has been observed not only in neurodevelopmental disorders such as Down syndrome but also in neurodegenerative diseases such as Alzheimer disease and mild cognitive impairment (Harigaya et al., 1996; Shim and Lubec, 2002; Calon et al., 2004; Counts et al., 2006) – all of which are associated with abnormal spine morphology (Fiala et al., 2002; Hering and Sheng, 2001). Our recent studies involving a mouse model of Alzheimer disease showed that a decrease in AMPARs at the synapse was followed by a decrease in the proportion of drebrin-positive spines (Chang et al., 2006; Mahadomrongkul et al., 2005). Normal spine morphogenesis is impaired by declustering of drebrin using antisense oligonucleotides against drebrin A (Takahashi et al., 2003) and also by knockdown of drebrin using RNA interference (Biou et al., 2008). Furthermore, our present study shows that AMPAR blockade causes malformed dendritic spines, such as filopodia-like thin spines with Y-shape and multi-branched structures. Taken together, these data suggest that the declustering of drebrin in spines, as caused by impaired AMPAR-mediated synaptic transmission, represents part of the underlying etiology of spine malformation that is associated with these neurological conditions.

In this study, we found that AMPAR activity regulates drebrin clustering in spine morphogenesis during development via the stabilization of drebrin in spines. Our findings suggest that AMPAR-mediated stabilization of drebrin is an activity-dependent cellular mechanism for spine morphogenesis. In addition, because it has been well established that decreased drebrin in spines is a pathognomonic feature of Down syndrome (a developmental disorder) and Alzheimer disease (a degenerative disease), we propose the impairment of AMPAR-mediated clustering of drebrin in spines as one of the etiologies of spine abnormality in many neurological conditions.

Materials and Methods

cDNA constructs

The cDNA construct for rat drebrin A with an N-terminal eGFP tag was described previously (Takahashi et al., 2003). To form a membrane-targeted marker (mGFP), the cDNA for eGFP was spliced to DNA encoding the membrane-anchoring domain (last 20 amino acids) of a farnesylation signal of K-ras 4B oncogene (Matus et al., 2000).

Hippocampal cell culture and transfection

Primary hippocampal cultures were prepared with previously described methods (Takahashi et al., 2003); cDNA transfection was carried out at 7 DIV using the calcium phosphate method. All animal experiments were carried out according to the Animal Care and Experimentation Committee, Gunma University, Showa Campus (Maebashi, Japan) and conformed to NIH guidelines for the use of animals in research. Every effort was made to minimize animal suffering and reduce the number of animals used.

Pharmacological experiments, immunocytochemistry, F-actin staining and fluorescence microscopy

Neurons were treated with the following drugs at 7-8 DIV: tetrodotoxin (TTX; 1 μM; Sigma, St Louis, MO), picrotoxin (50 μM; Sigma), 6-cyano-7-nitroquinoxaline-2,3-
ion (CNQX; 40 μM, Tocris, Ellisville, MO), GYK123466 (GYK; 10 μM, Tocris), philanthotoxin (PTX; 10 μM; Tocris), cyclothiazide (CTZ; 100 μM; Tocris), D-(+)-2-amino-5-phosphonopentanoic acid (AP5; 50 μM; Tocris) or α-methyl-4-carboxyphenylglycine (MCPG; 100 μM; Tocris). When we analyzed neurons at 22 DIV, we treated the neurons with drugs from 7-8 DIV every seventh day. In cytoD experiments, neurons at 14 DIV were incubated for 1 hour with 10 μM cytoD (Sigma) and then fixed. Immunocytochemistry, F-actin staining and fluorescence microscopy were performed as previously described (Takahashi et al., 2003) using the following primary antibodies: mouse mononuclear anti-drebrin (clone M2F6, MBL, Aichi, Japan), mouse mononuclear anti-PSD-95 (clone 7E3-1B8, Affinity Bioreagents, Golden, CO) and rabbit polyclonal anti-synapsin-I (Chemicon, Temecula, CA). F-actin staining utilizing rhodamine-phalloidin (Molecular Probes, Eugene, OR). Fluorescent images were acquired with a Zeiss Axioplan 2 microscope equipped with a Cool Snap f cooled CCD camera (Photometrics, Tucson, AZ), and analyzed using MetaMorph software (Universal Imaging, West Chester, PA). The images used for comparison were collected under identical conditions.

Western blot analysis
Cell lysates from ten coverslips of each treated culture were solubilized in 200 μl of sample buffer composed of 2% SDS, 5% mercaptoethanol, 10% glycerol, 1 mM EDTA, 40 mM Tris and 240 mM glycine at pH 8.5, and one-twentieth of the extract was loaded in each lane. The samples were subjected to SDS-PAGE (8% acrylamide) and transferred to an Immobilon transfer membrane (Millipore, Bedford, MA) by electroblotting. The blots were blocked in 10% non-fat milk in PBS, immunostained with the mouse mononuclear anti-drebrin antibody (clone M2F6, MBL, Aichi, Japan) or mouse mononuclear anti-β-actin antibody (clone AC-15, Sigma, St Louis, MO) and visualized with enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). The chemiluminescent signals were captured by LAS-3000 mini (Fujifilm, Tokyo, Japan), and the signal on the digital image was quantitated using NIH Image, a public-domain program developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/.

FRAP analysis
Neurons were imaged at 14-16 DIV with a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany) and a 40X water-immersion objective lens (NA, 1.2) in Tyrode’s solution [in mM: 119 NaCl, 2.5 KCl, 2 CaCl2, 2 MgCl2, 25 HEPES-NaOH (pH 7.4), 30 glucose]. Coverslips with cultured neurons were transferred to a bath chamber (RC-21BR/18; Warner Instruments, Hamden, CT) where the cultures were imaged for <2 consecutive hours. For FRAP analysis using a bath chamber, both image acquisition and morphometric quantification at least three neurons were analyzed per culture. In all experiments other than those involving FRAP analysis, all image acquisition and morphometric quantification were performed by investigators who were blind to the experimental conditions. For FRAP analysis, we selected neurons and dendrites, and defined protein clusters and dendritic filopodia in developing hippocampal area CA1. Dendritic spine pathology: cause or effect of the degenerative process? J. Neurosci. 2008, 28(51), 13438-13443.

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We thank Tomoko Takahashi for assistance with hippocampal culture preparation; and Ann Marie Craig, George J. Augustine, Kunihiko Obata, Alaa El-Husseini and Joshua N. Levinson for critical reading of the manuscript. This work was supported by Grants-in-Aid for Scientific Research (19200029) and those on Priority Areas (Elucidation of neural network function in the brain) (18021004, 20021002) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. H.T. was supported by the postdoctoral fellowship for research abroad from Japan Society for the Promotion of Science (JSPS) and the research fellowship from Uehara Memorial Foundation. Y.S. was supported by CREST, JST.

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