Synaptic Scaffolding Molecule α Is a Scaffold To Mediate
N-Methyl-d-Aspartate Receptor-Dependent RhoA
Activation in Dendrites

Junko Iida,1 Hiroyoshi Ishizaki,2 Miki Okamoto-Tanaka,2 Akira Kawata,1 Kazutaka Sumita,1
Shintaro Ohgake,1 Yuji Sato,1 Hiroshi Yorifuji,3 Nobuyuki Nukina,4 Kazumasa Ohashi,5
Kensaku Mizuno,5 Tomonari Tsutsumi,6 Akira Mizoguchi,6 Jun Miyoshi,2
Yoshimi Takai,2 and Yutaka Hata1+

Department of Medical Biochemistry, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan1; Department of Molecular Biology, Osaka Medical Center for Cancer and Cardiovascular Disease, Osaka 537-8511, Japan2; Department of Neuromuscular and Developmental Anatomy, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi 371-8511, Japan3; Laboratory for Structural Neuropathology, RIKEN Brain Science Institute, Saitama 351-0198, Japan4; Department of Biomolecular Sciences, Graduate School of Life Sciences, Tohoku University, Sendai 980-8578, Japan5; Department of Anatomy, Faculty of Medicine, Meie University, Tsu 514-8507, Japan6; and Department of Molecular Biology and Biochemistry, Osaka University Graduate School of Medicine/Faculty of Medicine, Suita 565-0871, Japan7

Received 8 October 2006/Returned for modification 17 November 2006/Accepted 3 April 2007

Synaptic scaffolding molecule (S-SCAM) interacts with a wide variety of molecules at excitatory and inhibitory synapses. It comprises three alternative splicing variants, S-SCAMα, -β, and -γ. We generated mutant mice lacking specifically S-SCAMα. S-SCAMα-deficient mice breathe and feed normally but die within 24 h after birth. Primary cultured hippocampal neurons from mutant mice have abnormally elongated dendritic spines. Exogenously expressed S-SCAMα corrects this abnormal morphology, while S-SCAMβ and -γ have no effect. Active RhoA decreases in cortical neurons from mutant mice. Constitutively active RhoA and ROCKII shift the length of dendritic spines toward the normal level, whereas ROCK inhibitor (Y27632) blocks the effect by S-SCAMα. S-SCAMα fails to correct the abnormal spine morphology under the treatment of N-methyl-d-aspartate (NMDA) receptor inhibitor (AP-5), Ca2+/calmodulin kinase inhibitor (KN-62), or tyrosine kinase inhibitor (PP2). NMDA treatment increases active RhoA in dendrites in wild-type hippocampal neurons, but not in mutant neurons. The ectopic expression of S-SCAMα, but not -β, recovers the NMDA-responsive accumulation of active RhoA in dendrites. Phosphorylation of extracellular signal-regulated kinase 1/2 and Akt and calcium influx in response to NMDA are not impaired in mutant neurons. These data indicate that S-SCAMα is a scaffold required to activate RhoA protein in response to NMDA receptor signaling in dendrites.

Various scaffold proteins, such as PSD-95 and gephyrin, play an important role at excitatory and inhibitory synapses in the brain. The physiological importance of these proteins is underscored by the results of studies using mutant mice. PSD-95 mutant mice show impaired learning and lack of neuropathic sensitization (9, 28). Gephyrin-deficient mice fail to suckle and die within 1 day of birth (6). Glycine receptor clustering is sensitized (9, 28). Gephyrin-deficient mice fail to suckle and die within 1 day of birth (6). Glycine receptor clustering is disrupted in these mice. Synaptic scaffolding molecule (S-SCAM)/membrane-associated guanylate kinase inverted-2 (MAGI-2) was originally characterized as a scaffold protein interacting with N-methyl-d-aspartate (NMDA) receptors at excitatory synapses (16). The protein comprises a guanylate kinase domain, two WW domains, and PSD-95/Discs large/zebrafish homolog.

Zonula Ocludens-1 (PDZ) domains. S-SCAM interacts with various components of excitatory synapses (4). Moreover, we have recently reported that S-SCAM interacts with neuroligin 2 and β-dystroglycan at inhibitory synapses in rat hippocampal neurons (42). These interactions of S-SCAM with a wide variety of synaptic molecules lead us to speculate that S-SCAM plays an important role as a scaffold at both excitatory and inhibitory synapses. However, direct evidence that S-SCAM is a critical component of synapses is missing. To address this question, we generated mice lacking S-SCAM.

The S-SCAM gene is located on human chromosome 7 and mouse chromosome 5. It spans more than 1.4 Mb. There are three alternative splicing variants, S-SCAMα, -β, and -γ, which start with different initiation methionines. S-SCAMα is the longest and has an additional N-terminal PDZ domain (17). S-SCAMβ and -γ start in the guanylate kinase domain. The exon coding the initiation methionine of S-SCAMα is separated by more than 1,000 kb from the first noncoding exon of S-SCAMβ. We replaced the first exon of the coding region of S-SCAMα with a neomycin resistance (neo3) gene cassette.

* Corresponding author. Mailing address: Department of Medical Biochemistry, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: (81) 3 5803 5164. Fax: (81) 3 5803 0121. E-mail: yuhamench@tdm.ac.jp.

* Published ahead of print on 16 April 2007.
FIG. 1. S-SCAM domain structure and gene targeting strategy. (A) N-terminal splicing leads to different start sites and sequences in S-SCAMα, -β, and -γ. S-SCAM protein contains a guanylate kinase (GK), two WW (WW), and PSD-95/Discs-large/Zonula Ocludens-1 (PDZ) domains. (B) The 5' region of the S-SCAM gene structure is shown (not drawn to scale). Mouse S-SCAM gene is located in mouse chromosome 5. The first and second exons of S-SCAMα and the first noncoding exon of S-SCAMβ are unique for each splicing variant. The subsequent exons are shared by S-SCAMα and -β. The exon containing the initiation methionine of S-SCAMα is separated by more than 1,000 kb from the first (1st.) exon of S-SCAMβ. (C) Targeting of the S-SCAMα gene was performed by replacing the first exon with the neomycin resistance gene cassette. The targeting construct is flanked by a 5' short arm (2.3-kb MboI-HindIII fragment) and a 3' long arm (7.5-kb EcoRV-HindIII fragment). Outer and inner probes were used for Southern blotting. DT-A, diphtheria toxin A gene cassette. (D) Genotype analyses of tail DNA of second filial generation (F2) mice by Southern blotting. The wild-type allele is detected by Southern blotting after digestion with XbaI or EcoRI. (E) Western blot analysis of brain lysates. Brain lysates were immunoblotted (IB) by anti-MAGI-2 and anti-S-SCAMα-specific antibodies. The leftmost three lanes contain the lysates of COS-7 cells expressing Myc-tagged S-SCAMα, -β, and -γ for reference. Arrows point to S-SCAMα.
FIG. 2. Thinner elongated filopodia predominate in S-SCAMα-deficient neurons at 14 DIV. (A) Postsynaptic density and synaptic vesicles in wild-type (+/+ ) and S-SCAMα-deficient (−/− ) cerebral cortex. The black arrows and arrowheads indicate postsynaptic density and synaptic

B

Phalloidin/Synapsin  PSD-95/Synapsin  Merge

+/+

−/

PSD-95  NR1  Merge

+/+

−/

VGAT  Phalloidin/VGAT  VGAT/Synaptophysin  Merge

+/+

−/

C  +/+  −/

D

Cumulative (%)  Spine length (μm)

E

Cumulative (%)  Head area (μm²)

F

Cumulative (%)  Head width (μm)

G

Cumulative (%)  Filopodia length (μm)

FIG. 2. Thinner elongated filopodia predominate in S-SCAMα-deficient neurons at 14 DIV. (A) Postsynaptic density and synaptic vesicles in wild-type (+/+ ) and S-SCAMα-deficient (−/− ) cerebral cortex. The black arrows and arrowheads indicate postsynaptic density and synaptic
and obtained mice with a deletion of S-SCAMα. The mutant mice, which still express S-SCAMβ and -γ, are developed and born normally but die within 24 h after birth. Although brain anatomy does not show significant abnormalities at birth, hippocampal neurons cultured from mutant mice exhibit remarkably altered spine morphologies with elongated necks. Mounting evidence shows that dendritic spines are highly plastic and can undergo rapid structural changes in response to physiological stimuli (27, 30, 51). This morphological plasticity is intimately related to synaptic function and is thought to be part of the cellular basis for learning and memory (3, 19, 23, 49). Genetic and environmental changes that impact dendritic spine structure have an effect on cognitive function (7). It is therefore important to study the molecular basis that determines the morphology of dendritic spines. We have studied here the S-SCAMα-specific role in the regulation of spine morphology.

MATERIALS AND METHODS

Construction of targeting vector. A 1.2-kb BamHI-digested cDNA fragment containing the initiation methionine of the S-SCAMα gene was used to isolate genomic clones from a 129/sv mouse genomic DNA library (Stratagene). Overlapping genomic clones were obtained and mapped with respect to the mouse S-SCAMα cDNA sequence. The targeting construct was made to replace the DNA region of about 0.9 kb covering the first coding exon of the S-SCAMα gene with a neo5 gene cassette. A 2.3-kb MboI-HindIII fragment and 7.5-kb EcoRV-HindIII fragment were used as short and long arms, respectively. The neo5 gene cassette contained the exogenous MC1 promoter. A diphtheria toxin A cassette at the 3′ end of the targeting vector was used for negative selection.

Animals and generation of S-SCAMα−/− mice. C57BL/6 and BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). The animals were cared for as described previously (22). The procedures used on the animals in this study were in accordance with the guidelines and approval of the Osaka University Medical School and Tokyo Medical and Dental University Animal Care and Use Committee. CCE embryonic stem (ES) cells were cultured essentially as described previously (39). The linearized targeting vector was electroporated into ES cells as described previously (22). Homologous recombinants were verified by Southern blotting using the neo5 gene probe. A 2.3-kb MboI-HindIII fragment and 7.5-kb EcoRV-HindIII fragment were used as short and long arms, respectively. The neo5 gene cassette contained the exogenous MC1 promoter. A diphtheria toxin A cassette at the 3′ end of the targeting vector was used for negative selection.

Hippocampal neuron culture and cortical neuron culture. Hippocampal neuron and cortical neuron cultures were performed from embryonic day 15 (E15) embryos as described previously (10, 47). Cortical neurons were plated at 2 × 104 cells/3.5-cm dish in poly-L-lysine-coated dishes in Dulbecco’s modified Eagle medium and Ham’s F-12 medium (1:1) supplemented with 5% fetal bovine serum and 5% horse serum. Cytosine arabinoside (10 μM) (Sigma-Aldrich) was added to the culture medium 2 days after plating to inhibit proliferation of nonneuronal cells.

Sindbis virus production and infection. Sindbis virus was produced using baby hamster kidney 21 (BHK21) cells as described previously (48). Briefly, BHK21 cells were transfected with RNA derived from pSindbis vector and cultured. Viral stock was prepared from the medium and stocked at −80°C. Primary cultured hippocampal neurons were infected 13 to 16 days after plating. Hippocampal neurons were fixed 24 h after the infection and immunostained with specified primary antibodies.

Immunocytochemistry. Hippocampal neurons were fixed with phosphate-buffered saline (PBS) containing 4% (wt/vol) paraformaldehyde and 4% (wt/vol) sucrose for 15 min or with cold methanol for 10 min at −20°C and permeabilized with 0.25% (wt/vol) Triton X-100 in PBS for 5 min. For the immunocytochemistry, anti-NMDAR1 and anti-GluR1 antibodies, methanol fixation was used. After the nonspecific reaction was blocked with PBS containing 10% (wt/vol) BSA, cells were incubated with the first antibody in PBS containing 3% (wt/vol) bovine serum albumin (BSA) overnight, washed with PBS, and incubated with the secondary antibody in PBS containing 3% (wt/vol) BSA for 2 h. After the samples were washed with PBS, they were embedded in 95% (wt/vol) glycerol in PBS. The images were obtained by using an Olympus IX71 charge-coupled-device microscope (Olympus Corp.) with a 60× objective. Images of endogenous and overexpressed proteins were taken with 100- to 500-ms and 100-ms exposures, respectively.
The brains of S-SCAM-deficient (−/−) neurons were infected after 13 DIV with Sindbis virus engineered to express Myc-tagged S-SCAM or − (Myc-S-SCAM) or −γ, rescues abnormal spine morphology in hippocampal neurons at 14 DIV. Cortical neurons at 8 days in vitro (DIV) were used. To assess GTP-bound RhoA, cortical neurons (2 × 10^6 cells) were washed with ice-cold PBS, lysed with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 30 mM MgCl2, 0.1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM Na3VO4), and incubated for 5 min at 4°C. Cell lysates were centrifuged for 5 min at 10,000 × g at 4°C, and the supernatant was incubated with 20 μg of GST-robotkin-RBD for 10 min at 4°C and incubated with 25 μl of glutathione-Sepharose 4B beads for 1 h at 4°C. The beads were washed twice by lysis buffer. To assess GTP-bound Rac1 and Cdc42, cortical neurons (2 × 10^6 cells) were washed with ice-cold PBS, lysed with lysis buffer containing 10 μg GST-PAK-CRIB (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 μM 4-aminophenylmethanesulfonyl fluoride, and 1 mM Na3VO4), and incubated for 30 min at 4°C. Cell lysates were centrifuged for 15 min at 10,000 × g at 4°C, and the supernatants were incubated with glutathione-Sepharose 4B beads for 1 h at 4°C. The beads were washed twice by lysis buffer. RhoA, Rac1, and Cdc42 trapped by the beads were detected by immunoblotting. Densitometric analyses of detected signals were performed using NIH Image software. The amounts of GTP-bound RhoA, Rac1, and Cdc42 were normalized to the total amounts of RhoA, Rac1, and Cdc42 in cell lysates, respectively.

### Image quantification

Neurons from three independent cultures were quantified for each experimental condition. Neurons were stained with rhodamine-phalloidin to demonstrate the spine morphology. Dendrites from cell body, 50 to 200 μm, were analyzed for quantification. The length of the spines is defined as the length starting from the base of the neck to the furthest point on the spine head. The width of a spine is the maximum width of the spine head perpendicular to the long axis of the spine head. Protrusions with a length of >0.5 μm and with a width in the range of 0.3 to 1.0 μm were defined as spines, while headless protrusions with a length of >2.5 μm were defined as filopodia. Data from different cultures were pooled. Spine area, length, and width were measured using the measurement tool in Metamorph (Roper Industries, Inc.). Two hundred spines in 20 dendrites of 10 neurons were analyzed for each experimental condition. For statistical analyses of spine width, the lamellipodium-like structures (defined by a protrusion width of >2.5 μm) were excluded.

### Ultrastructural analysis

The brains of S-SCAM-deficient mice were perfused with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and fixed further in the same fixative for 12 h. The samples were washed with 10% sucrose in 0.1 M sodium cacodylate buffer (pH 7.4) for 15 min and postfixed with 1% osmium tetroxide in the same buffer for 90 min. They were then washed with cold water for 10 min and stained with 1% uranyl acetate for 1 h. After dehydration with a graded series of ethanol, the samples were embedded in Spurr’s resin. Ultrathin sections about 70 nm thick were cut using an ultramicrotome (Richter Ultracut FCR; Leica). They were then double stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (JEM-1010; JEOL).

### Pull-down assay for RhoA, Rac1, and Cdc42

The pull-down assay was performed as described previously with some modifications (8). Cortical neurons at 8 days in vitro (DIV) were used. To assess GTP-bound RhoA, cortical neurons (2 × 10^6 cells) were washed with ice-cold PBS, lysed with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 30 mM MgCl2, 0.1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM Na3VO4), and incubated for 5 min at 4°C. Cell lysates were centrifuged for 5 min at 10,000 × g at 4°C, and the supernatant was incubated with 20 μg of GST-robotkin-RBD for 10 min at 4°C and incubated with 25 μl of glutathione-Sepharose 4B beads for 1 h at 4°C. The beads were washed twice by lysis buffer. To assess GTP-bound Rac1 and Cdc42, cortical neurons (2 × 10^6 cells) were washed with ice-cold PBS, lysed with lysis buffer containing 10 μg GST-PAK-CRIB (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 μM 4-aminophenylmethanesulfonyl fluoride, and 1 mM Na3VO4), and incubated for 30 min at 4°C. Cell lysates were centrifuged for 15 min at 10,000 × g at 4°C, and the supernatants were incubated with glutathione-Sepharose 4B beads for 1 h at 4°C. The beads were washed twice by lysis buffer. RhoA, Rac1, and Cdc42 trapped by the beads were detected by immunoblotting. Densitometric analyses of detected signals were performed using NIH Image software. The amounts of GTP-bound RhoA, Rac1, and Cdc42 were normalized to the total amounts of RhoA, Rac1, and Cdc42 in cell lysates, respectively.

### In situ RhoA, Rac1, and Cdc42 GTPase activity assays

In situ RhoA, Rac1, and Cdc42 GTPase activity assays were performed using recombinant GST-robotkin-RBD and GST-PAK-CRIB were performed as described previously with some modifications (25). When indicated, neurons were equilibrated with Tyrode’s solution (25 mM HEPES-NaOH [pH 7.4], 129 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 30 mM d-glucose) for 60 min at 37°C, incubated in Tyrode’s solution without MgCl2 but with 100 mM NMDA for the indicated periods at room temperature, and then fixed. For the high-potassium treatment, Tyrode’s solution containing 90 mM KCl was used. Neurons were fixed with PBS containing 4% (vol/vol) paraformaldehyde and 4% (wt/vol) sucrose for 15 min and permeabilized with 0.25% (wt/vol) Triton X-100 in PBS for 5 min. After the non-specific

### Table 1: Colocalization of various synaptic marker proteins

<table>
<thead>
<tr>
<th>Synaptic marker protein</th>
<th>% Colocalization (mean ± SEM) of synaptic marker protein in mice:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
</tr>
<tr>
<td>PSD-95/synapsin</td>
<td>84.6 ± 4.0</td>
</tr>
<tr>
<td>VGAT/synaptophysin</td>
<td>88.0 ± 5.0</td>
</tr>
<tr>
<td>GluR1/synaptophysin</td>
<td>66.8 ± 6.9</td>
</tr>
<tr>
<td>GluR1/PSD-95</td>
<td>93.0 ± 3.2</td>
</tr>
<tr>
<td>NMDAR1/PSD-95</td>
<td>90.9 ± 2.36</td>
</tr>
<tr>
<td>PSD-95/NMDAR1</td>
<td>82.5 ± 6.6</td>
</tr>
<tr>
<td>Neurogli/synaptophysin</td>
<td>80.9 ± 4.9</td>
</tr>
</tbody>
</table>

* Percentages of colocalization of various synaptic marker-positive puncta in primary cultured hippocampal neurons from wild-type and S-SCAM-deficient mice at 15 DIV.

### Figure 3

**A** −/− Myc-SCα Myc-SCβ Myc-SCγ

**B** −/− Myc-SCα Myc-SCβ Myc-SCγ

**C** Cumulative frequency plots of spine length. Cumulative frequency plots were obtained as described in the legend to Fig. 2. Closed circles, Myc-S-SCAMα; open circles, Myc-S-SCAMβ; closed triangles, Myc-S-SCAMγ.
reaction was blocked with PBS containing 10% (wt/vol) BSA, cells were incubated with GST-rhotekin-RBD and GST-PAK-CRIB domain as described in Materials and Methods. GTP-bound RhoA, Rac1, and Cdc42 were detected by immunoblotting. The amount of GTP-bound form was normalized against the total amount, and then, the ratio of wild-type mice was set at 100%. GTP-bound RhoA decreases in mutant mice, while no significant difference is detected for GTP-bound Rac1 or Cdc42. Error bars indicate standard errors of the means. Values that are significantly different (P < 0.01 by t test) from the value for the wild-type mice are indicated by an asterisk. (C) Ectopic expression of constitutively active RhoA (Myc-RhoAV14) induces the loss of spines in wild-type neurons (+/+) (white arrows), while it shifts the appearance of spines back toward that of wild-type neurons in mutant neurons (+/−) (white arrows). Control GFP expression has no effect. Ectopic expression of constitutively active Rac1 (Myc-Rac1V12) and Cdc42 (Myc-Cdc42V12) induced lamellipodium-like veils (white arrowheads) and stubby type protrusions (white arrows), respectively, in both wild-type and mutant neurons. Bar, 10 μm. (D) Close-up views of dendritic spines expressing control GFP or Myc-tagged RhoAV14 in mutant neurons. Bar, 1 μm. (E) Cumulative frequency plots of spine length. Closed circles, Myc-RhoAV14; open circles, control GFP. Cumulative frequency plots were obtained as described in the legend to Fig. 2. Ectopic expression of Myc-RhoAV14 in mutant neurons shifts the length of spines back toward the normal level.

Assays of NMDA receptor-dependent ERK activation, PI3K activation, and calcium influx. Primary cultured cortical neurons at 8 DIV were used for these experiments. The activation of ERK1/2 in cortical neurons was evaluated by immunoblotting the cell lysates with anti-ERK1/2 and anti-phospho-ERK1/2 antibodies at 5 min and 10 min after the application of NMDA (100 μM). To evaluate the activation of phosphatidylinositol 3-kinase (PI3K), the cell lysates were immunoblotted by the phosphorylation site (Thr at position 308)-specific anti-Akt antibody. We confirmed that wortmannin (200 nM) blocks this NMDA-induced phosphorylation of Akt. This is evidence that we can evaluate PI3K activation by monitoring this phosphorylation. The assay of calcium influx was performed as described previously with some modifications (5, 45). Cortical neurons were plated at a density of 5 × 10^4 cells/cm² and were loaded with 2 μM fluo-4 AM (Invitrogen) for 40 min at 37°C in the CO2 incubator in Tyrode’s solution. Cells were then washed three times with Tyrode’s solution and left for an additional 30 min at 37°C in Tyrode’s solution in the CO2 incubator to permit
FIG. 5. ROCK is involved in the rescue of abnormal spine morphology by S-SCAMα. (A) ROCK inhibitor Y27632 causes the elongation of spines in wild-type (+/+) neurons. Neurons from wild-type mice were treated with Y27632 (100 μM) at 14 DIV and fixed. Close-up views (bottom) show the representative spines of the mock-treated and Y27632-treated neurons. The treatment with Y27632 induced spine elongation. In the
complete hydrolysis of the probe. NMDA (100 μM) was added to the wells directly above the microscope objective. Fluo-4 was excited with 488-nm argon laser. Images were acquired from five fields for each plate at 5- to 30-s intervals using Metamorph (Roper Industries, Inc.). Fluorescence signals were adjusted by autothreshold using Image J. Particles of 7 to 10 μm in diameter were defined as cells. Fluorescence intensities were averaged over 200 cells for each plate. Experiments were carried out using three different cultures, and data were combined to allow quantitative estimates of changes in fluo-4 fluorescence signal intensity.

**RESULTS**

**S-SCAMx mutant mice die shortly after birth.** To explore the physiological importance of S-SCAM, we generated mutant mice lacking the protein. There exist three alternative splicing isoforms of S-SCAM, S-SCAMα, -β, and -γ (Fig. 1A). All of them have two WW domains and five PDZ domains in common. S-SCAMα has one additional PDZ domain in the N terminus. S-SCAMβ and -γ start in the middle of the guanylate kinase domain. The genomic DNA containing the 5′ end of the mouse S-SCAM gene was isolated. The exons containing the initiation methionines of S-SCAMα and -β were separated by more than 1,000 kb (Fig. 1B). The exon containing the first methionine of S-SCAMα was selected for deletion and replaced with a neoR gene cassette (Fig. 1C). The first exon of S-SCAMβ was predicted to be discarded. Therefore, mutant mice selectively lacking S-SCAMα had been expected. The targeted and wild-type alleles can be differentiated by Southern blotting after XbaI and EcoRI digestion using the 5′ inner and 3′ outer probes (Fig. 1C and D).

The chimeric male mice were mated with C57BL/6 female mice to generate heterozygotes. S-SCAMα+/− mice appeared normal and were fertile. Heterozygotes were mated to generate homozygotes. Of 926 pups born, S-SCAMα+/+, S-SCAMα−/−, and S-SCAMα−/+ pups were 233:479:214, respectively, suggesting that the F2 mice genotypes exhibit a Mendelian ratio of 1:2:1. The anti-MAGI-2 antibody detected three bands in brain lysates of S-SCAMα−/+ and S-SCAMα−/− mice, while the largest band was missing in S-SCAMα−/− mice (Fig. 1E, top blot, arrows). The second and third bands in brain lysates had the same sizes as S-SCAMβ and -γ expressed in COS-7 cells. The immunoblotting using the S-SCAMα-specific antibody revealed that the amount of S-SCAMα decreased in S-SCAMα−/− mice (Fig. 1E, bottom blot). No signal was detected in S-SCAMα−/− mice. Thus, as expected, mutant mice express S-SCAMβ and -γ but lack S-SCAMα. S-SCAMα−/− mice breathe and feed normally and are indistinguishable from the wild-type mice and the heterozygotes for the first 12 h after birth. However, mutant mice die within 24 h, suggesting that S-SCAMα is essential for postnatal viability. Despite the decreased expression of S-SCAMα, the heterozygote mice do not show remarkable abnormalities for up to 2 years after birth. Thereby, in this study, we have focused on S-SCAMα−/− mice.

**Primary cultured hippocampal neurons from S-SCAMα mutant mice show elongated dendritic spines.** Brain development at the gross anatomical level seems normal (data not shown). By electron microscopy of the cerebral cortex, the postsynaptic density and synaptic vesicles were observed at birth (Fig. 2A). No significant difference was observed between mutant mice and wild-type mice. To further assess synaptogenesis, we prepared primary neuronal cultures from hippocampus. Neurons from mutant mice extended neurites as well as those of wild-type mice (data not shown). To evaluate the formation of excitatory synapses morphologically, postsynaptic (PSD-95 and NR1) and presynaptic (synapsin) marker proteins were immunostained in neurons from wild-type and homozygote mice at DIV 14 (Fig. 2B). As S-SCAM is also localized at inhibitory synapses, VGAT and synaptophysin were immunostained to evaluate the formation of inhibitory synapses (Fig. 2B). The quantitative results are summarized (Table 1). The ratios of PSD-95-positive puncta versus VGAT-positive puncta are 1.88 ± 0.70 for wild-type neurons and 2.06 ± 0.8 for mutant neurons. These results indicate that there is no significant alteration in excitatory and inhibitory synaptic formation at the morphological level. However, at 14 DIV, dendritic spines of neurons from mutant mice were more elongated compared with those of the wild-type mice (Fig. 2C). These elongated spines formed synapses with the axon as those in wild-type neurons did. As described in Materials and Methods, we assessed the length of the spine neck and the shape of the spine head separately and found that the primary abnormality resides in the length of spines (Fig. 2D). No significant difference was detected in the shape and size of terminal head portions (Fig. 2E and F). Wild-type and mutant neurons had 11.0 ± 2.3 and 10.5 ± 2.0 dendritic spines per 30 μm, respectively, and there was no significant difference in the spine density. To gain insight into spinogenesis at the early phase, we compared filopodia at 9 DIV in wild-type and mutant neurons. The length of filopodia also increases in mutant mice, suggesting that spine formation is affected at the early phase (Fig. 2G). Interestingly, the density of filopodia is larger in mutant neurons. Wild-type and mutant neurons have 8.3 ± 2.5 and 13.4 ± 2.8 filopodia per 30 μm, respectively.

**Reintroduction of S-SCAMα corrects the abnormality in the spine length.** To confirm that the abnormality in the spine length is indeed attributable to the loss of S-SCAMα, we expressed Myc-tagged S-SCAMα (Myc-S-SCAMα), -β, and -γ using Sindbis virus. With reintroduction of S-SCAMα, the length of dendritic spines shortened (Fig. 3A and B). Myc-S-SCAMβ and -γ had no effect. The quantification data support
the hypothesis that the expression of S-SCAMα, but not of S-SCAMβ or γ, rescues the abnormality (Fig. 3C).

The amount of active RhoA decreases in cortical neurons from S-SCAMα mutant mice. Rho family proteins are key molecules to regulate spine morphology (11, 32, 43, 46, 49, 50). The mutations of several regulators and target proteins of RhoA family proteins cause abnormal spine morphology and result in mental retardation in human patients (34). Therefore, we examined whether the regulation of Rho family proteins alters in S-SCAMα−/− mice. We used GST-rhotekin-RBD and GST-PAK-CRIB to isolate active RhoA, Rac1, and Cdc42. The amount of GTP-bound RhoA protein was about 50% lower in the mutant mice than in the wild-type mice, while no significant difference was detected for Rac1 and Cdc42 (Fig. 4A and B). We next expressed constitutively active RhoA, Rac1, and Cdc42 in wild-type and mutant neurons to compare the effects. The expression of the constitutively active RhoA dramatically reduced the number and the length of dendritic spines in wild-type neurons (Fig. 4C, Myc-RhoAV14). In mutant neurons, the constitutively active RhoA exhibited a similar effect, but the spines with almost normal length were also detected (Fig. 4C, Myc-RhoAV14, and D). In contrast, the constitutively active Rac1 induced the formation of lamellipodium-like veils along the dendrites in both wild-type and mutant neurons (Fig. 4C, Myc-Rac1V12). The constitutively active Cdc42 decreased the number and the length of dendrites, and stubby type protrusions appeared in both wild-type and mutant neurons (Fig. 4C, Myc-Cdc42V12). The results of quantitative analysis support the hypothesis that the ectopic expression of the constitutively active RhoA in mutant neurons shifts the spine length toward the normal level (Fig. 4E). This result suggests that the disorder of RhoA activation is implicated in the abnormal spine morphology in S-SCAMα−/− mice and that the machinery downstream of Rho activation to regulate the spine morphology is intact in mutant mice.

ROCK is implicated in S-SCAMα-dependent regulation of spine morphology. ROCK plays a pivotal role to regulate the actin cytoskeleton in the Rho-mediated signaling pathway (37). Consistent with the previous report, the treatment with ROCK inhibitor (Y27632) induced the elongation of spine necks in wild-type neurons, which is similar to the phenotype of mutant neurons (Fig. 5A). To test whether ROCK plays a role in the rescue by S-SCAMα in mutant neurons, Myc-S-SCAMα was expressed in mutant neurons treated with Y27632. Under this condition, S-SCAMα failed to correct abnormal spine morphology (Fig. 5B). Conversely, expression of the catalytic domain of ROCKII (ROCK-CAT) shortened spine neck length as the expression of constitutively active form of RhoA (Fig. 5C). These findings further support the hypothesis that in mutant neurons, RhoA activation is impaired and results in the dysfunction of regulation of dendritic spine cytoskeleton.

The signaling mediated by NMDA receptors is involved in the rescue by ectopic expression of S-SCAMα. The signaling of NMDA receptors is implicated in RhoA activation (24, 41, 50). The treatment of wild-type neurons with AP-5 induced the elongation of spine necks (Fig. 6A). As S-SCAM interacts with NMDA receptors, we tested whether NMDA receptor signaling is involved in S-SCAMα-dependent regulation of spine morphology. Myc-S-SCAMα was expressed in mutant neurons with the treatment of AP-5. Under this treatment, S-SCAMα did not correct spine morphology in the mutant neurons (Fig. 6B). AP-5 treatment did not interfere with the effect of ectopic expression of constitutively active RhoA in mutant neurons (data not shown). The results imply that S-SCAMα activates RhoA in response to NMDA receptor signaling or that S-SCAMα itself activates NMDA receptors and triggers the NMDA receptor-dependent RhoA activation. We next examined whether the blockade of various NMDA receptor signaling pathways attenuates the effect of S-SCAMα in mutant neurons. NMDA receptor signaling is mediated by calcium/calmodulin (CaM) kinase, ERK, and tyrosine kinase pathways. Both the CaM kinase inhibitor KN-62 and the Src family kinase inhibitor PP2 blocked the rescue effect of S-SCAMα, but the ERK inhibitor U0126 exhibited no effect (Fig. 6C). These results also support the hypothesis that NMDA receptor signaling is involved in the regulation by S-SCAMα of spine morphology.

NMDA fails to activate RhoA in dendritic spines in mutant neurons. We next wanted to compare the NMDA-induced RhoA activation in mutant and wild-type neurons. We first tested whether RhoA activation is detectable in the pull-down assay using GST-rhotekin-RBD. NMDA was applied to cortical neurons from wild-type mice, and the pull-down assay was performed using the neuron lysates. NMDA changes the actin cytoskeleton in dendritic spines, and it should affect the amount of GTP-bound RhoA, Rac1, or Cdc42. However, we could not clearly demonstrate the activation of RhoA in this assay, although ERK activation was detected (Fig. 7A). We also performed the pull-down assay using GST-PAK-CRIB, but the activation of Rac1 or Cdc42 was not shown either. In the pull-down assay, we use the neuron lysates and do not evaluate RhoA, Rac1, and Cdc42 separately from the cell bodies and from the neurites. Thereby, we next tried to detect in situ the GTP-bound form of RhoA protein using GST-rhotekin-RBD. The sensitivity of this assay is low but enables us to analyze whether RhoA is activated in dendrites. At the basal level, GTP-bound RhoA was only slightly detected in wild-type neurons (Fig. 7B, 0 min). NMDA treatment decreased the staining with rhodamine-phalloidin in spines and induced the retraction of spines in wild-type neurons (Fig. 7B, 5 min and 10 min). At the same time, GTP-bound RhoA came to be detected. The treatment with C3 exoenzyme abolished the signal, supporting the hypothesis that it reflects the active RhoA-like proteins (Fig. 7B, bottom row). In contrast, in mutant neurons, spines did not show retraction and the staining with rhodamine-phalloidin remained in dendritic spines after the NMDA treatment (Fig. 7C). GTP-bound RhoA did not increase in response to NMDA in these neurons. In the quantitative analysis, consistent results were obtained (Fig. 7D). Furthermore, under treatment with KN-62 and PP2, NMDA did not cause the spine retraction and the RhoA activation in dendrites, while U0126 did not interfere with the effect of NMDA (Fig. 8A and B). Together with the previous results (Fig. 6), the data imply that the same signaling pathways play a role in the regulation of spine morphology by S-SCAMα and in the NMDA-induced RhoA activation in dendrites.

High-potassium-induced RhoA activation and NMDA-induced Rac1/Cdc42 activation are maintained in mutant neurons. We next examined whether NMDA-dependent RhoA activation is specifically impaired in mutant neurons. Treat-
FIG. 6. NMDA receptor signaling is necessary for S-SCAMα to rescue the abnormal spine morphology in mutant neurons. (A) AP-5 causes the elongation of spines in wild-type (+/+) neurons. Neurons from wild-type mice were treated with AP-5 (500 μM) at 14 DIV and fixed. Close-up views show the representative spines of the mock-treated and AP-5-treated neurons. In the cumulative frequency plot of spine length; closed circles depict the mock-treated cells and open circles depict AP-5-treated cells. (B) Myc-S-SCAMα was expressed in mutant (−/−) neurons mock treated or treated with AP-5. AP-5 (500 μM) abolishes the rescue by S-SCAMα (SCα) in mutant neurons. Close-up views show the representative spines of neurons expressing Myc-S-SCAMα mock treated or treated with AP-5. In the cumulative frequency plot of spine length, closed circles depict mock-treated cells and open circles depict AP-5-treated cells. (C) Myc-S-SCAMα was expressed in mutant neurons treated with KN-62 (10 μM), PP2 (10 μM), or U0126 (20 μM). KN-62 and PP2, but not U0126, abolish the rescue by S-SCAMα (SCα) in mutant neurons. Close-up views of dendritic spines of neurons expressing Myc-S-SCAMα treated with KN-62, PP2, or U0126. Bars, 10 μm in the low-magnification images and 1 μm in the close-up views. Cumulative frequency plots were obtained as described in the legend to Fig. 2.
FIG. 7. RhoA is activated in dendrites in response to NMDA in wild-type neurons, but not in mutant neurons. (A) Cortical neurons from wild-type mice were treated with NMDA (100 μM) for the indicated periods. GTP-bound RhoA, Rac1, and Cdc42 were precipitated from cortical
neurons using GST-rhotekin-RBD and GST-PAK-CRIB domain as described in Materials and Methods. GTP-bound and total RhoA, Rac1, and Cdc42 were detected by immunoblotting. The neuron lysates were also immunoblotted by anti-ERK1/2 and anti-phospho-ERK1/2 (P-ERK1/2) antibodies to show that NMDA was active on neurons. Although ERK1/2 phosphorylation is detected, the NMDA-induced activation of RhoA, Rac1, or Cdc42 is not detectable in the pull-down assay. (B) GTP-bound RhoA in wild-type (+/+ ) neurons. GTP-bound RhoA was evaluated in wild-type neurons using GST-fused rhotekin RBD in situ 0, 5, and 10 min after 100 μM NMDA application. GST-rhotekin-RBD was stained with anti-GST antibody (green). Synapsin (blue) was immunostained as a synaptic marker, and actin (red) was visualized with rhodamine-phalloidin. After NMDA treatment, spines are retracted and rhodamine-phalloidin staining decreases in spines, while the signals of GST-rhotekin-RBD increase. C3 exoenzyme (50 μg/ml) abolished the signals detected by GST-rhotekin-RBD, supporting the hypothesis that they reflect RhoA-like proteins (bottom row). Bar, 10 μm. (C) GTP-bound RhoA in mutant (−/− ) neurons. GTP-bound RhoA was evaluated in mutant neurons in situ 0, 5, and 10 min after NMDA (100 μM) application. GTP-bound RhoA is not increased with NMDA treatment, and rhodamine-phalloidin staining is detected in dendritic spines (white arrowheads). Bar, 10 μm. (D) Quantification of GTP-bound RhoA in wild-type (+/+ ) and S-SCAMα-deficient (−/− ) neurons. The bars indicate the amounts of GTP-bound RhoA at 0, 5, and 10 min after treatment with NMDA. NMDA induces a significant increase of GTP-bound RhoA in wild-type neurons but not in mutant neurons. Error bars indicate standard errors of the means. Values that are significantly different (P < 0.001 by t test) from the control value (0 min) are indicated (***).

FIG. 8. KN-62 and PP2, but not U0126, block NMDA-induced spine retraction and RhoA activation in dendrites. (A) Neurons were treated with NMDA (100 μM) in the presence of KN-62, PP2, or U0126. GTP-bound RhoA was detected as described in the legend to Fig. 7. GST-rhotekin-RBD was stained with anti-GST antibody (green). Synapsin (blue) was immunostained as a synaptic marker, and actin (red) was visualized with rhodamine-phalloidin. NMDA fails to trigger the spine retraction and to activate RhoA in dendrites in the presence of KN-62 or PP2. U0126 does not block the effect of NMDA. Bar, 10 μm. (B) Quantification of GTP-bound RhoA in wild-type neurons treated with various reagents. Bars indicate the amounts of GTP-bound RhoA 0, 5, and 10 min after treatment with NMDA. The NMDA treatment does not induce a significant increase of GTP-bound RhoA in the presence of KN-62 or PP2. Error bars indicate standard errors of the means. Values that are significantly different (P < 0.001 by t test) from the control value (0 min) are indicated (***).
FIG. 9. The high-potassium treatment activates RhoA in dendrites of mutant neurons, and NMDA induces the activation of Rac1 and Cdc42 in mutant neurons. (A) Neurons from wild-type (+/+) and S-SCAM-deficient (−/−) mice were treated with 90 mM KCl for the indicated times.

(B) Neurons from wild-type (+/+) and S-SCAM-deficient (−/−) mice were treated with 50 μM NMDA for the indicated times.

(C) Graph showing the GTP-Rho signal intensity in neurons treated with KCl for 0, 1, or 3 min.

(D) Graph showing the GTP-Rac1/Cdc42 signal intensity in neurons treated with NMDA for 0 or 5 min.
ment with 90 mM KCl induced the significant activation of RhoA in mutant neurons, although the activation was smaller than in wild-type neurons (Fig. 9A and C). The signals detected by GST-P AK-CRIB were also significantly increased with the NMDA treatment in mutant neurons, but the activation was smaller (Fig. 9B and D). These data suggest that the machineries to activate RhoA under the high-potassium treatment and to activate Rac1 and Cdc42 under the NMDA treatment are maintained in mutant neurons, although they are partially impaired.

Reintroduction of S-SCAMα recovers RhoA activation in response to NMDA in mutant neurons. We subsequently tested whether S-SCAMα recovers NMDA receptor-dependent RhoA activation in mutant neurons. In S-SCAMα-expressing mutant neurons, spines retracted and RhoA was activated after the application of NMDA (Fig. 10A and C). The expression of S-SCAMβ had no effect (Fig. 10B and C). Thus, S-SCAMα is essential for NMDA receptor activation to induce spine retraction and RhoA activation in dendrites.

S-SCAMα is not necessary for NMDA receptor-dependent ERK activation, PI3K activation, or calcium flux. In the last set of experiments, we examined whether S-SCAMα is also necessary for NMDA receptor-dependent signals other than RhoA activation. The phosphorylation of ERK1/2 and Akt was induced by NMDA in S-SCAMα-deficient cortical neurons as well as in wild-type neurons (Fig. 11A). Since the phosphorylation of Akt was blocked by wortmannin, this finding supports the hypotheses that the phosphorylation of Akt depends on PI3K and that PI3K is activated by NMDA normally in these mutant neurons. Finally, we investigated the ability of NMDA receptors to mobilize calcium in S-SCAMα-deficient cortical neurons. We could not detect a significant difference in the NMDA-induced calcium increases in mutant and wild-type neurons (Fig. 11B and C).

**DISCUSSION**

S-SCAMα is a scaffold protein localized at both excitatory and inhibitory synapses (16, 42). S-SCAMα-deficient mice are born at the predicted Mendelian frequency and appear indistinguishable from wild-type mice. However, they do not survive the first day after birth. This indicates that S-SCAMα is essential for the development of the brain after birth and is not substituted by other molecules, including S-SCAMβ and γ, which are expressed in these mutant mice. These mice feed and breathe normally, and further studies are necessary to find out the direct cause of the neonatal death. We have here focused on the analysis of primary cultured neurons from mutant mice to elucidate the S-SCAMα-specific function at the cellular level.

The Rho family of GTPases and related molecules are important to regulate spine formation and maintenance (11, 32, 43, 46, 50). Among them, RhoA is one of the most extensively studied. The effects of RhoA manipulation on spine morphology have been investigated in a variety of rodent neuronal culture systems, and in all cases, introduction of constitutively active RhoA decreases spine density and length, indicating that RhoA has a negative effect on spine formation and maintenance (32, 46). Conversely, inhibition of RhoA using C3 exoenzyme increases the density and length of spines of some mouse cortical and hippocampal pyramidal neurons in organotypic slices (46). In addition, the effects of RhoA activity on spine number and morphology are shown to be mediated by the effector ROCK (32, 37, 49). In hippocampal neurons from S-SCAMα-deficient mice, dendritic spine necks are elongated. Although the spine density is normal at 14 DIV, the abnormal shape of spines led us to suspect that RhoA activation is impaired in mutant mice. Consistently, the pull-down assays using rhokin-RBD and PAK-CRIB demonstrate that RhoA activation is suppressed in cortical neurons from mutant mice, whereas Rac1 activation and Cdc42 activation are normal. Expression of constitutively active RhoA shifts spine neck length toward the normal level, and the inhibitor of ROCK blocks the rescue by S-SCAMα. All these data support the hypothesis that RhoA activation in mutant neurons is impaired.

NMDA receptor activation alters the actin cytoskeleton in dendritic spines. It causes the loss of F-actin and spine retraction, and it also stabilizes the dendritic spines (1, 13). These spine changes are thought to be correlated to synaptic plasticity (14, 15, 19, 21, 51). Spine retraction may reduce the surface receptors and the signaling molecules that are localized in the vicinity of synapses. It may also affect the geometry that controls the efficiency of calcium signaling. Importantly, our data support the hypothesis that S-SCAMα plays a role in the regulation of the actin cytoskeleton by NMDA receptors. In the presence of AP-5, S-SCAMα fails to correct the spine morphological abnormality. The inhibition of NMDA signaling by a CaM kinase inhibitor, KN-62, or by a Src family kinase inhibitor, PP2, also blocks the effect of S-SCAMα on spine morphology in mutant neurons. The results of the in situ RhoA
GTPase assay demonstrate that RhoA activation in response to NMDA treatment is impaired in mutant neurons, while RhoA activation in response to the high-potassium treatment is intact. These findings suggest that S-SCAM is essential to mediate the activation of RhoA in response to NMDA signaling. In contrast, NMDA-induced ERK activation, Akt phosphorylation, or calcium influx is not impaired in mutant neurons, suggesting that S-SCAM is not involved in all the signals triggered by NMDA. We found that the RhoA activation can be induced by the high potassium in mutant neurons but is slightly lower than in wild-type neurons. We consider that under the conditions of high-potassium treatment, RhoA activation is mainly mediated by the NMDA receptor-independent pathway but that the NMDA receptor signaling also partially contributes to RhoA activation, which is impaired in mutant neurons. A recent report shows that high-potassium treatment increases membrane-bound active RhoA in dendritic shafts in rat hippocampal neurons (41). In those experiments, spines drastically retract and GTP-RhoA is detected within the shaft in the immunofluorescence assay. In that re-

FIG. 10. (A) NMDA triggers the accumulation of GTP-bound RhoA in mutant neurons expressing Myc-S-SCAMα (white arrowheads). GTP-bound RhoA was detected as described in the legend to Fig. 7. GST-rhotekin-RBD was stained with anti-GST antibody (green). Synapsin (blue) and Myc tag (red) were immunostained. (B) Expression of Myc-S-SCAMβ (Myc-SCβ) does not recover the NMDA-induced RhoA activation in mutant neurons. Bar, 10 μm. (C) Quantification of GTP-bound RhoA in mutant neurons expressing Myc-S-SCAMα or Myc-S-SCAMβ. Bars indicate the amounts of GTP-bound RhoA at the basal level and after treatment with NMDA. NMDA treatment induces a significant increase of GTP-bound RhoA in Myc-S-SCAMα-expressing mutant neurons. Error bars indicate standard errors of the means. Values that are significantly different (P < 0.001 by t test) from the control value (0 min) are indicated (**).
GTP-bound RhoA can be detected only in the in situ assay

and spine density. The fact that NMDA-induced increase of GTP-bound RhoA is not involved in the regulation of neurite growth suggests that the population of RhoA activated by NMDA stimulation is rather small and that not all of the RhoA proteins in neurons are regulated by NMDA receptor signaling. It is also necessary to consider that neurite growth is determined at the early phase of the neuron development ahead of synaptogenesis and that the expression of S-SCAMα increases during synaptogenesis. S-SCAMα may not be involved in the regulation of RhoA that determines neurite growth. The observation that the number of filopodia at 9 DIV is larger in mutant neurons can lead us to another speculation. Various signals are implicated in neurite growth and spine formation. As S-SCAM interacts with a wide variety of synaptic components, the deficit of S-SCAMα may affect some other signal than RhoA activation, which cancels out the consequence caused by impaired RhoA activation.

Moreover, even if we discuss only the regulation of RhoA, S-SCAMα is likely to play a versatile role. In our experiments, the NMDA-induced increase of GTP-RhoA is undetectable in the pull-down assay using cortical neurons, suggesting that the NMDA-induced increase is spatially or temporally limited. Then, why is the difference detectable in the amount of GTP-RhoA at the basal level for wild-type and mutant neurons as shown in Fig. 4A? We speculate that the difference detected in the pull-down assay may reflect the amount of GTP-RhoA not in dendritic spines but in other parts of neurons. As the localization of S-SCAMα is not restricted to excitatory synapses, it is not surprising that S-SCAMα also regulates the amount of GTP-RhoA in neurons through a NMDA receptor-independent pathway. The impairment of such a pathway results in the decrease of GTP-RhoA at the basal level, which can be detected in the pull-down assay.

Which molecules are involved in the S-SCAMα-dependent RhoA activation? This is the most intriguing question. Many regulators for Rac1 and Cdc42 exist in dendritic spines, some of which are involved in the regulation of RhoA (21, 34, 43). Because the activation of Rac1 and Cdc42 in response to NMDA application is not as significantly suppressed as that of RhoA in mutant neurons, we have focused on RhoA-specific regulators. There are three kinds of regulators for Rho family of GTPases, GDP dissociation inhibitor (GDI), GTPase-activator protein (GAP), and GDP/GTP-exchange factor (GEF) (12). There is no report to illustrate the relationship between RhoGDI and NMDA receptors. RICS/p250GAP/p200RhoGAP/GRIT is a GAP identified by several groups, and there are conflicting reports about which Rho family of GTPases are regulated by this GAP (29, 31, 33, 36). One group argues that the GAP regulates RhoA and is involved in NMDA receptor activity-dependent actin reorganization (33). Kalirin is a dual RhoGEF that has two GEF domains for Rac1 and RhoA (26, 38). There are several splicing variants. Among them, Kalirin-7 is the most abundant in brain and importantly interacts with S-SCAM. Kalirin-7, however, regulates only Rac1. Kalirin-12, which regulates both Rac1 and RhoA, in turn lacks the putative S-SCAM-interacting PDZ-binding motif. Overexpression of kalirin causes simplification of the dendritic arbor, suggesting that kalirin rather limits RhoA activity. Rho-specific GEF Lfc is translocated to spines following activation of NMDA receptors and interacts with neurabin to regulate spine morphology (40). These GAP and GEF proteins might be involved in the S-SCAMα-dependent RhoA activation, but so far no

![FIG. 11. S-SCAMα is not necessary for NMDA receptor-dependent ERK activation, PI3K activation, or calcium flux. (A) Cortical neurons from wild-type (+/+) and mutant (−/−) mice were treated with NMDA (100 μM) for the indicated periods. The cell lysates were immunoblotted by anti-ERK1/2 (ERK1/2), anti-phospho-ERK1/2 (P-ERK1/2), anti-Akt (Akt), and phosphorylation site (Thr at position 308)-specific anti-Akt (P-Akt) antibodies. (B) Pseudocolor representation of relative fluo-4 fluorescence intensities 1 min following the application of mock or 100 μM NMDA in wild-type (+/+) and mutant (−/−) neurons. The pseudocolor calibration scale for the signal intensity is shown on the right. Bar, 10 μm. (C) Quantification analysis of fluo-4 fluorescence signal intensity in wild-type (+/+) and mutant (−/−) neurons. The intensity for the mock treatment is set at 1. Gray bars indicate the relative intensity for NMDA-treated neurons. The results of three independent experiments were analyzed, and error bars indicate standard errors of the means.](http://mcb.asm.org/)

port, the researchers demonstrate using biochemical pull-down and coimmunoprecipitation assays that active RhoA increases in whole cells but decreases in synaptosomes and propose a model that the activation of NMDA or AMPA receptors results in the redistribution of RhoA from ionotropic receptors to metabotropic receptors.

The previous reports reveal that RhoA activation reduces neurite extension and spine density (24, 46). However, S-SCAMα-deficient neurons do not show enhanced neurite extension or increased spine density. This suggests that there are distinct populations of RhoA proteins in neurons and that RhoA, whose activation in response to NMDA depends on S-SCAMα, is not involved in the regulation of neurite growth and spine density. The fact that NMDA-induced increase of GTP-bound RhoA can be detected only in the in situ assay
direct evidence has been obtained. As S-SCAMβ and γ cannot substitute for S-SCAMα, the N-terminal S-SCAMα-specific region may interact with one of these regulators or with some molecule, such as a kinase that activates or inactivates the regulators. Disappointedly, a molecule that interacts with the N-terminal region of S-SCAMα, including PDZ0, has not yet been identified. The identification of such a protein-protein interaction mediated by S-SCAMα-specific region will shed light on a molecular link between NMDA receptor activity and regulation of spine morphology.

ACKNOWLEDGMENTS

We thank S. Narumiya, K. Kaibuchi, and T. Tominaga for the plasmids. This study was supported by grants-in-aid for Scientific Research and Priority Areas and Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. K.S., A.K., and S.O. are supported by the Tokyo Medical and Dental University 21st century COE program “Brain Integration and Its Disorders.”

REFERENCES


