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Regulation of dendritic spine morphology by an NMDA receptor-associated Rho GTPase-activating protein, p250GAP

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Abstract

The NMDA receptor regulates spine morphological plasticity by modulating Rho GTPases. However, the molecular mechanisms for NMDA receptor-mediated regulation of Rho GTPases remain elusive. In this study, we show that p250GAP, an NMDA receptor-associated RhoGAP, regulates spine morphogenesis by modulating RhoA activity. Knock-down of p250GAP increased spine width and elevated the endogenous RhoA activity in primary hippocampal neurons. The increased spine width by p250GAP knock-down was suppressed by the expression of a dominant-negative form of RhoA. Furthermore, p250GAP is involved in NMDA receptor-mediated RhoA activation. In response to NMDA receptor activation, exogenously expressed green fluorescent protein (GFP)-tagged p250GAP was redistributed. Thus, these data suggest that p250GAP plays an important role in NMDA receptor-mediated regulation of RhoA activity leading to spine morphological plasticity.

Keywords: dendritic spine, NMDA receptor, p250GAP, RhoA, spine morphogenesis.

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Dendritic spines are specialized protrusions that receive the majority of excitatory synaptic input in the CNS. Spines are highly plastic, changing their shape and size in response to synaptic activity (Bonhoeffer and Yuste 2002; Kennedy *et al.* 2005; Matus 2005; Segal 2005; Tada and Sheng 2006). Long-term potentiation is associated with an increase in spine number or spine size (Matsuzaki *et al.* 2004; Nagerl *et al.* 2004; Okamoto *et al.* 2004), while long-term depression results in shrinkage or retraction of spines in the forebrain (Nagerl *et al.* 2004; Okamoto *et al.* 2004; Zhou *et al.* 2004). Drugs that inhibit spine motility interfere with long-term potentiation (Fischer *et al.* 1998). These findings argue that synaptic activity-regulated morphological plasticity of spines plays an important role in the functional plasticity of synaptic transmission that underlies learning and memory formation (Fischer *et al.* 1998; Matsuzaki *et al.* 2004; Nagerl *et al.* 2004; Okamoto *et al.* 2004; Zhou *et al.* 2004).

The *N*-methyl-D-aspartate subtype of ionotropic glutamate receptor (NMDA receptor) plays a critical role in the activity-dependent morphological plasticity of spines (Kennedy *et al.* 2005; Matus 2005; Segal 2005). Given that the morphological plasticity of spines depends on reorganization of actin

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Abbreviations used: 4-AP, 4-aminopyridine; APV, 2-amino-5-phosphonovaleric acid; DIV, days *in vitro*; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; GFP, green fluorescent protein; LTP, long-term potentiation; TTX, tetrodotoxin.

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cytoskeleton (Newey *et al.* 2005; Tada and Sheng 2006), the NMDA receptor is likely to regulate spine morphological plasticity by modulating the activity of regulators of actin cytoskeleton. As the key regulators of dynamics of actin cytoskeleton, Rho family GTPases (RhoA, Cdc42, and Rac1) are the most likely link between NMDA receptor activity and spine morphological plasticity (Newey *et al.* 2005; Tada and Sheng 2006). The activities of Rho family GTPases are modulated positively by guanine nucleotide exchange factors (GEFs) and negatively by GTPase-activating proteins (GAPs) (Lamarche and Hall 1994; Hall 1998). Several RhoGEFs and RhoGAPs have been found to regulate spine morphogenesis (Penzes *et al.* 2003; Govek *et al.* 2004; Nishimura *et al.* 2005; Ryan *et al.* 2005; Tolias *et al.* 2005; Van de Ven *et al.* 2005; Zhang *et al.* 2005). Among these, only Rac1-GEF, Tiam1 is shown to couple the NMDA receptor activity to the regulation of Rho GTPases leading to spine morphological plasticity (Tolias *et al.* 2005). To date, the molecular mechanisms that link NMDA receptor activity to the activity of Rho family GTPases, especially RhoA, remain unclear.

In our previous study, we identified p250GAP as a novel NMDA receptor-interacting RhoGAP (Nakazawa *et al.* 2003; Taniguchi *et al.* 2003). p250GAP was also identified as Grit/p200RhoGAP/RICS/GCGAP by other groups (Nakamura *et al.* 2002; Moon *et al.* 2003; Okabe *et al.* 2003; Zhao *et al.* 2003). p250GAP promotes GTP hydrolysis of Cdc42 and RhoA *in vitro* (Nakazawa *et al.* 2003). Within mature neurons, p250GAP is concentrated in post-synaptic densities and interacts with the NR2B subunit of the NMDA receptor (Nakazawa *et al.* 2003). However, the role of p250GAP in the NMDA receptor complex has remained unknown. In this paper, we show that p250GAP regulates spine morphogenesis through its RhoGAP activity for RhoA. p250GAP is involved in NMDA receptor-mediated RhoA activation. Moreover, NMDA receptor stimulation leads to redistribution of p250GAP. Taken together, these data suggest that p250GAP plays an important role in NMDA receptor-mediated regulation of RhoA activity leading to spine morphological plasticity.

Materials and methods

Generation of recombinant sindbisviruses, lentiviruses, and adenoviruses

Sindbisviral vectors were kindly provided by Dr Pavel Osten (Northwestern University, Chicago, IL, USA). Generation of recombinant sindbisviruses expressing GFP-tagged p250GAP was performed as described previously (Kim *et al.* 2004). Lentiviral vectors were kindly provided by Dr David Baltimore (California Institute of Technology, Pasadena, CA, USA). For generation of the p250GAP shRNA construct, two complementary DNA oligos containing 2935–2953 nucleotide positions of murine p250GAP coding sequence (GenBank accession no. NM177379) (5′-accggtacaagaagcaccaagtattcaagagatacttggtgcttcttgtacttttttc-3′ and 5′-tcgagaaaaagtacaagaagcaccaagtatctcttgaatacttggtgcttcttgtac-3′) were synthesized, annealed, and inserted between *Bbs*I and *Xho*I sites of pBS-1 vector (Qin *et al.* 2003). Generation of recombinant lentivirus expressing shRNA against p250GAP was performed as described previously (Lois *et al.* 2002; Qin *et al.* 2003). Recombinant adenoviruses expressing a dominant-negative form of RhoA (RhoN19) together with GFP under the control of a CAG promoter were constructed as previously described using Adenovirus Expression Vector Kit (Takara, Ohtsu, Japan) (Niwa *et al.* 1991).

Cell culture and infection

Hippocampal cultures from 17-day-old embryonic mice were prepared as described previously (Okabe *et al.* 1999). High-density cultures of cortical neurons were prepared as described (Nakazawa *et al.* 2006). For morphometric and biochemical analysis, neurons were infected with recombinant sindbisviruses, lentiviruses, and adenoviruses at 14 DIV (days *in vitro*), 7 DIV, and 12 DIV and maintained for additional 12–15 h, 6 days, and 1 day, respectively. Experiments with animals were carried out in accordance with the guideline for animal use issued by the Committee of Animal Experiments, Institute of Medical Science, University of Tokyo.

Morphometric measurements

Neurons were labeled with DiD (Molecular Probes, Eugene, OR, USA) and imaged with a Bio-Rad Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA, USA). Each image was a z-series projection of approximately 10 images (0.5 μm depth interval). To determine the length of a spine, the distance from the protrusion's tip to the dendritic shaft was measured. To measure the width of a spine, the maximal length of the spine head perpendicular to the long axis of the spine neck was measured.

Preparation of lysates, immunoprecipitation, and immunoblotting

Preparation of cell lysates, immunoprecipitation, and immunoblotting were performed as described previously (Nakazawa *et al.* 2001, 2006). For quantification, bands for specific immunocomplexes were analyzed with NIH Image software.

Antibodies

Polyclonal antibodies against p250GAP, NR2B, and PSD-95 were described previously (Nakazawa *et al.* 2003). The commercially available antibodies used in this study were anti-Myc (9E10), anti-RhoA, anti-Rac1, and anti-Cdc42 antibodies (BD Biosciences, San Jose, CA, USA; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Plasmids

The expression plasmids pEFBOS-myc-RhoA (wild-type and active), Rac1 (wild-type and active), and Cdc42 (wild-type and active) were kindly provided by Dr Yoshimi Takai (Kobe University, Japan).

RhoGAP assay

The activities of RhoA, Rac1, and Cdc42 were measured by pull-down methods as described previously (Nakazawa *et al.* 2003).

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Pharmacological treatment of neurons

Cortical neurons were infected with lentiviruses and adenoviruses at 7 DIV and 12 DIV, respectively. Neurons were pre-treated with 1 μM tetrodotoxin at 12 DIV and were stimulated with 50 μM bicuculline (Sigma–Aldrich, St Louis, MO, USA) and 500 μM 4-aminopyridine (4-AP) (Sigma–Aldrich) or NMDA (Sigma–Aldrich) at 13 DIV and then cells were lysed.

Time-lapse imaging and image analysis

For time-lapse imaging, hippocampal neurons (12–14 DIV) were infected with sindbisviruses expressing GFP-tagged p250GAP and then incubated with 1 μM tetrodotoxin for 12–15 h. Live cells were placed in a chamber containing Tyrode's solution (in mM: 119 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 HEPES (pH 7.4), 30 glucose) for 30 min before the experiment and then stimulated with 50 μM bicuculline (Sigma–Aldrich) and 500 μM 4-aminopyridine (4-AP) (Sigma–Aldrich). Time-lapse imaging and image analysis were performed as described previously (Kuriu *et al.* 2006). Relative intensity was calculated from the fluorescence intensity in the spine head and the underlying dendritic shaft (Ackermann and Matus 2003).

Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was done using Student's *t*-test. Differences with $p < 0.05$ were considered as significant.

Results

Knock-down of p250GAP increased spine width

We previously reported that a novel brain-enriched Rho-GAP, p250GAP, accumulates in spines (Nakazawa *et al.* 2003). p250GAP may regulate spine morphogenesis, because Rho family small GTPases play important roles in the regulation of spine morphology (Kennedy *et al.* 2005; Newey *et al.* 2005; Tada and Sheng 2006). To investigate the role of p250GAP in spine morphogenesis, we used lentivirus-based RNA interference (RNAi) to knock-down the expression of endogenous p250GAP in cultured hippocampal neurons. A 19-nucleotide sequence corresponding to nucleotides 2935–2953 of the murine p250GAP sequence dramatically reduced the expression of endogenous p250GAP in hippocampal neurons without affecting the expression of other proteins such as PSD-95 and NR2B (Fig. 1a and data not shown). The control virus did not affect expression of endogenous p250GAP (data not shown). With the p250GAP knock-down, we observed a significant increase in spine width (control, 0.60 ± 0.02 μm; p250GAP knock-down, 0.95 ± 0.03 μm; $p < 0.05$, Student's *t*-test) (Fig. 1b–d). A slight increase observed in spine length by p250GAP knock-down was not significant (control, 1.14 ± 0.02 μm; p250GAP knock-down, 1.24 ± 0.03 μm; $p > 0.09$, Student's *t*-test) (Fig. 1b, e, and f). p250GAP knock-down had no obvious effect on dendritic branching patterns and spine

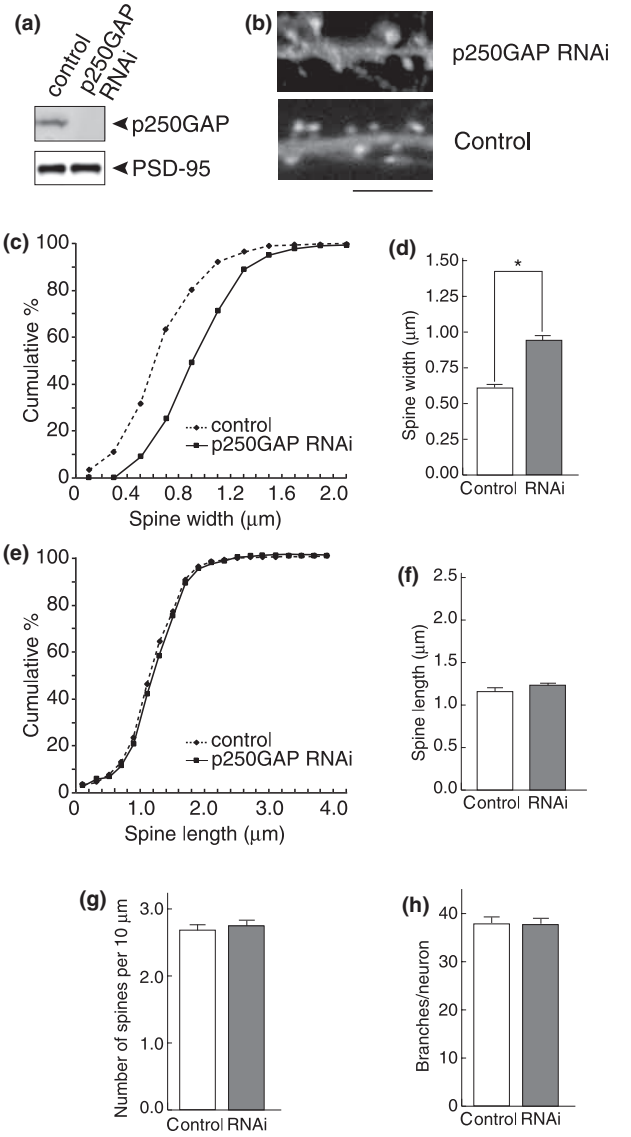

Fig. 1 Knock-down of p250GAP increases spine width. (a) Reduced expression of endogenous p250GAP in p250GAP shRNA-infected neurons. Neurons were infected with p250GAP shRNA expressing and control lentiviruses at 7 DIV and lysed at 13 DIV. Lysates of neurons were immunoblotted with antibodies against p250GAP and PSD-95. Note that the expression of PSD-95 was unaltered in p250GAP shRNA-expressing neurons. (b) Hippocampal neurons were infected with p250GAP shRNA expressing lentiviruses (upper) and control lentiviruses (lower) at 7 DIV and maintained for additional 6 days. Neurons were visualized by a lipophilic dye, DiD. Scale bar, 5 μm. Cumulative distribution of spine width (c), quantification of spine width (d), cumulative distribution of spine length (e), and quantification of spine length (f) for the cells described in (b) (13 DIV; control, $n = 355$ spines; p250GAP RNAi, $n = 415$ spines). (g) Densities of spines (13 DIV; control, $n = 80$ cells; p250GAP RNAi, $n = 80$ cells). (h) Number of branches per neuron (13 DIV; control, $n = 80$ cells; p250GAP RNAi, $n = 80$ cells). All data are presented as the mean ± SEM. *$p < 0.05$, Student's *t*-test.

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density (Fig. 1g and h). Therefore, the effect of p250GAP knock-down appeared to be selective for spine morphogenesis.

Over-expression of p250GAP decreased spine width and increased spine length

We next examined 'gain-of-function' phenotypes in cultured hippocampal neurons infected with recombinant sindbisviruses expressing GFP-tagged p250GAP. Neurons expressing GFP-tagged p250GAP showed filopodia-like spines those are longer and thinner than those of neurons expressing GFP (Fig. 2a). Quantification of these changes showed that over-expression of p250GAP decreased spine width (control, 0.71 ± 0.03 μm; p250GAP over-expressed, 0.53 ± 0.03 μm; $p < 0.05$, Student's *t*-test) (Fig. 2b and c) and increased spine length (control, 1.39 ± 0.03 μm; p250GAP over-expressed, 2.09 ± 0.02 μm; $p < 0.05$, Student's *t*-test) (Fig. 2d and e). Over-expression of p250GAP had no obvious effect on dendritic branching patterns and spine density (Fig. 2f and g).

p250GAP promoted GTP hydrolysis on RhoA in dissociated neurons

Cdc42, Rac1, and RhoA play distinct function in spine morphogenesis (Newey *et al.* 2005). p250GAP promotes GTP hydrolysis of Cdc42 and RhoA *in vitro* and binds to active Cdc42 and RhoA *in vitro* (Nakazawa *et al.* 2003 and Supplementary material Fig. S1). We next examined the specificity of the GAP activity of p250GAP *in vivo* using dissociated hippocampal neurons. To examine this, we again used an RNAi approach in dissociated neurons. GTP-bound active RhoA was precipitated from cell lysates with the RhoA binding domain of Rhotekin, an effector protein of RhoA that binds GTP-bound active RhoA but not GDP-bound inactive RhoA. Likewise, the levels of active Cdc42 and Rac1 were examined using the Cdc42/Rac1 binding domain of p21 activated kinase (PAK). With the p250GAP knock-down, we observed a significant increase in RhoA activity (165 ± 15% of control, $p < 0.05$, Student's *t*-test) (Fig. 3a). By contrast, we did not find significant changes in Cdc42 and Rac1 activities in p250GAP knock-down neurons compared with control neurons (Fig. 3b and c). These data indicate that RhoA, but not Cdc42 and Rac1, is a substrate of p250GAP in dissociated neurons.

Expression of a dominant-negative form of RhoA suppressed the increase in spine width in p250GAP knock-down neurons

To confirm that p250GAP affects spine width by acting on RhoA signaling pathway, we tested whether inhibition of RhoA pathway can rescue the increase in spine width that are caused by p250GAP knock-down. For these experiments, we used a dominant-negative form of RhoA (RhoDN). We found that an ectopic expression of RhoDN suppressed the increase in spine width in p250GAP knock-down neurons (Fig. 3d). Together with our observation that the lack of the GAP activity of p250GAP for Cdc42 and Rac1 in dissociated hippocampal neurons (13 DIV) (Fig. 3b and c), these results

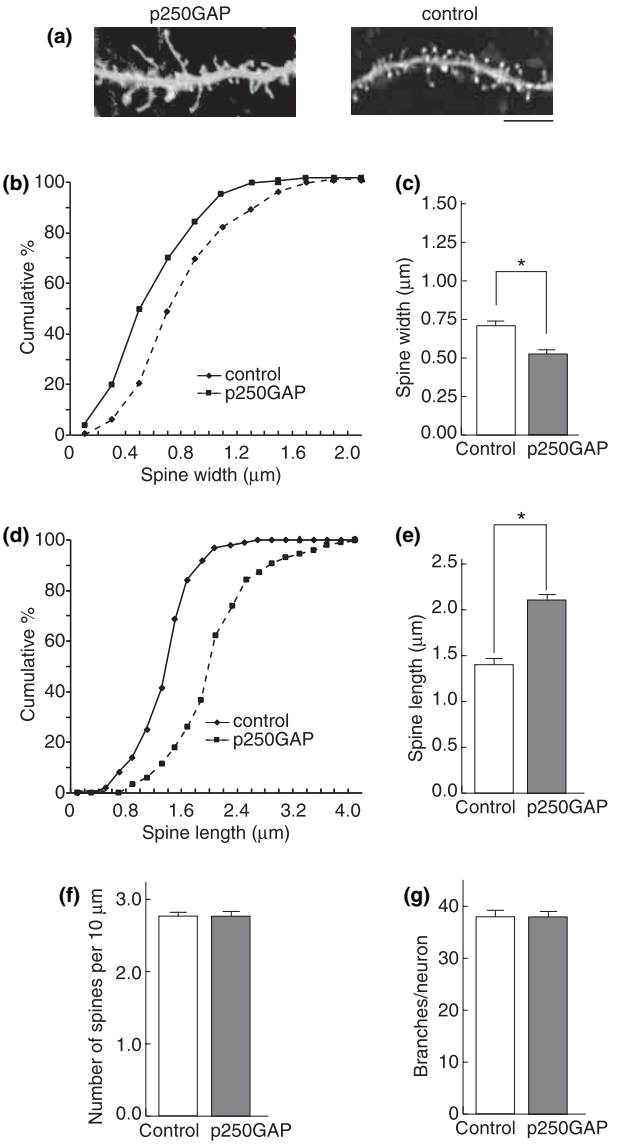

Fig. 2 Over-expression of p250GAP increases spine length and decreases spine width. (a) Hippocampal neurons were infected with recombinant sindbisviruses expressing GFP-p250GAP (left) and GFP (right) at 14 DIV and maintained for additional 12–15 h. Neurons were visualized by a lipophilic dye, DiD. Scale bar, 5 μm. Cumulative distribution of spine width (b), quantification of spine width (c), cumulative distribution of spine length (d), and quantification of spine length (e) for the cells described in (a) (15 DIV; control, $n = 310$ spines; p250GAP, $n = 367$ spines). (f) Densities of spines (15 DIV; control, $n = 70$ cells; p250GAP RNAi, $n = 70$ cells). (g) Number of branches per neuron (15 DIV; control, $n = 70$ cells; p250GAP RNAi, $n = 70$ cells).All data are presented as the mean ± SEM. *$p < 0.05$, Student's *t*-test.

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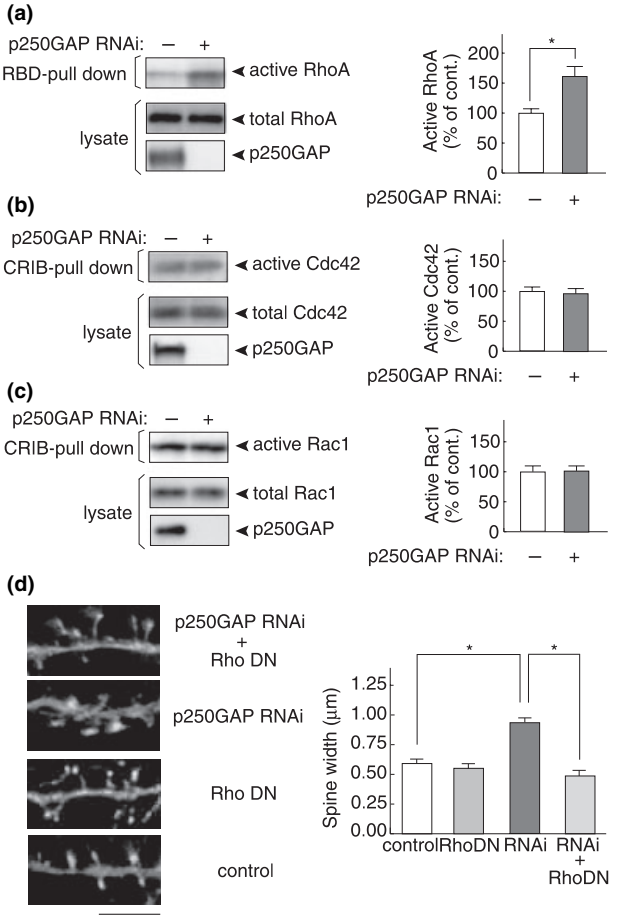

Fig. 3 p250GAP promotes GTP hydrolysis on RhoA. (a) Elevated level of active RhoA in p250GAP knock-down neurons. Neurons were infected with p250GAP shRNA expressing and control lentiviruses at 7 DIV and maintained for additional 6 days. RhoA activity was assessed by RhoA-binding domain (RBD) pull-down assay (left). Quantification of RhoA activity (right). Data are mean ± SEM (control, $n = 6$; p250GAP RNAi, $n = 6$). *$p < 0.05$, Student's *t*-test. (b and c) The activity of Cdc42 (b) and Rac1 (c) in p250GAP knock-down and control neurons. Neurons were infected with p250GAP shRNA expressing and control lentiviruses at 7 DIV and maintained for additional 6 days. Cdc42 (b) and Rac1 (c) activity was assessed by Cdc42/Rac1-binding-domain (CRIB) pull-down assay (left). Quantification of Cdc42 (b) and Rac1 (c) activity (right). Data are mean ± SEM (b, control, $n = 4$; p250GAP RNAi, $n = 4$; c, control, $n = 3$; p250GAP RNAi, $n = 3$). (d) Ectopic expression of a dominant-negative form of RhoA (RhoDN) suppresses the increase in spine width by p250GAP knock-down. Hippocampal neurons were infected with p250GAP shRNA expressing lentiviruses (at 7 DIV) and RhoDN expressing adenoviruses (at 12 DIV) (left, top), p250GAP shRNA expressing lentiviruses and control adenoviruses (left, middle-upper), control lentiviruses and RhoDN expressing adenoviruses (left, middle-lower), and control lentiviruses and adenoviruses (left, bottom). Scale bar, 5 μm. Quantification of spine width (13 DIV neurons) (right). All data are presented as the mean ± SEM (control, $n = 90$ spines; RhoDN, $n = 105$ spines; p250GAP RNAi, $n = 103$ spines; p250GAP RNAi+ RhoDN, $n = 115$ spines). *$p < 0.05$, Student's *t*-test.

suggest that p250GAP regulates spine morphogenesis by modulating RhoA activity.

p250GAP is involved in NMDA receptor-mediated activation of RhoA

The finding that p250GAP interacts with the NMDA receptor (Nakazawa *et al.* 2003) led us to examine whether p250GAP plays a role in NMDA receptor-mediated RhoA regulation (Schubert *et al.* 2006; Iida *et al.* 2007). NMDA receptor-mediated RhoA regulation is important for spine morphological plasticity that is thought to play a crucial role in synaptic strength. Application of 50 μM bicuculline with 500 μM 4-AP enhances synaptic transmission and increases the spontaneous firing rate and is associated with global calcium transients caused by calcium flux through synaptic NMDA receptors (Hardingham *et al.* 2002; Kuriu *et al.* 2006). We used this synaptically evoked stimulation to examine the role of p250GAP. We found that the bicuculline/4-AP stimulation increased the level of GTP-bound active RhoA in control neurons (206 ± 25% of mock-stimulated neurons, $p < 0.05$, Student's *t*-test) (Fig. 4a, lane 1 vs. lane 2). This RhoA activation was blocked by application of the NMDA receptor antagonist, 2-amino-5-phosphonovaleric acid (APV) (Fig. 4a, lane 2 vs. lane 3), suggesting that NMDA receptor-mediated calcium influx-induced RhoA activation. In addition, this bicuculline/4-AP stimulation-mediated RhoA activation declined to basal level after 30 min (Fig. 4b). We next examined the effect of p250GAP knock down on the NMDA receptor-mediated RhoA activation. As described above (Fig. 3a), RhoA was activated in p250GAP knock down neurons under unstimulated condition (Fig. 4a, lane 1 vs. lane 4). In these RhoA-activated p250GAP knock down neurons, in contrast to the case with control neurons, the same bicuculline/4-AP stimulation failed to induce further increase in the active RhoA level (Fig. 4a, lanes 4 vs. 5). Although the data suggest that p250GAP knock down occludes NMDA receptor-mediated RhoA activation, it is possible that the lack of further RhoA activation in p250GAP knock down neurons is because RhoA activation reaches almost maximum level in this assay system. To examine the possibility that RhoA activation reached almost maximum by p250GAP knock down, we applied various doses of NMDA to cultured neurons (Fig. 4c). The level of active RhoA in the 50 μM NMDA condition reached approximately 300% of the control level (Fig. 4c), indicating that RhoA activation did not reach maximum by p250GAP knock down (approximately 200% of the control level; Fig. 4a and c). Although RhoA activity did not reach maximum, the bicuculline/4-AP stimulation failed to induce further increase in the active RhoA level in p250GAP knock down neurons (Fig. 4a, columns 4 vs. 5), indicating that p250GAP knock down occluded bicuculline/4-AP stimulation-mediated RhoA activation. Similarly, p250GAP knock down occluded 10 μM NMDA-induced RhoA activation

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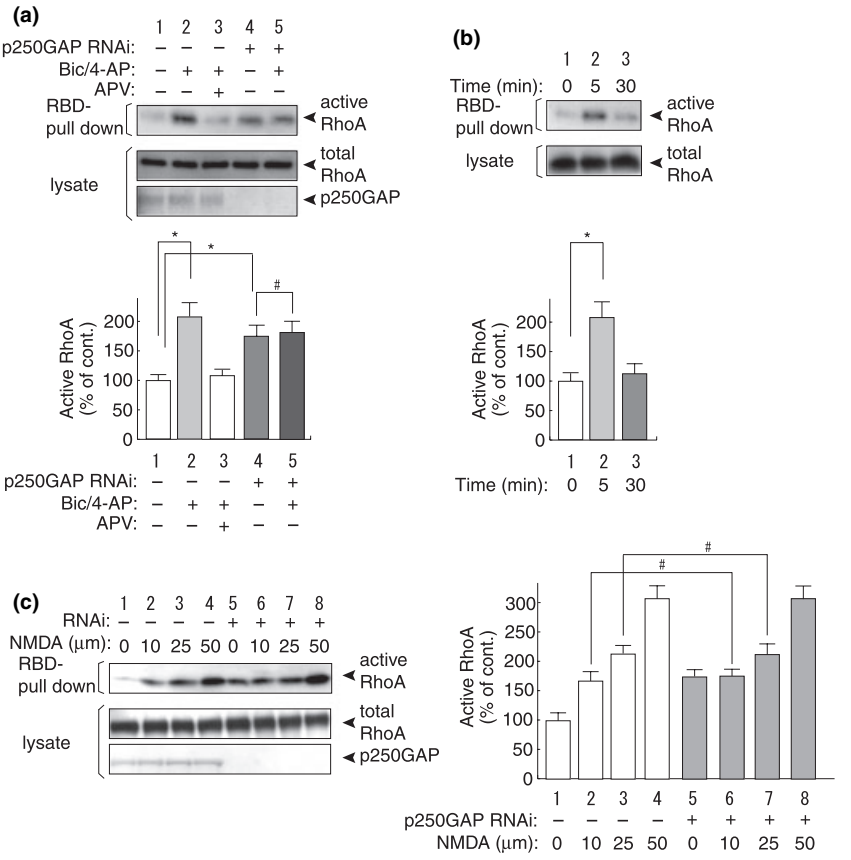

Fig. 4 p250GAP occludes NMDA receptor-mediated RhoA activation. (a) Cortical neurons were infected with p250GAP shRNA expressing and control lentiviruses at 7 DIV and stimulated with 50 μM bicuculline (Bic) and 500 μM 4-AP for 5 min at 13 DIV. RhoA activity was assessed by RhoA-binding-domain (RBD) pull-down assay. As shown in Fig. 3a, RhoA was activated in p250GAP knock-down neurons compared to control neurons under unstimulated condition (upper, lane 1 vs. lane 4). The Bic/4-AP stimulation failed to induce a further increase in active RhoA in p250GAP knock-down neurons (upper, lane 4 vs. lane 5). Quantification of RhoA activity (13 DIV neurons) (lower). Data are mean ± SEM ($n = 5$). *$p < 0.05$, #$p > 0.6$, Student's *t*-test. (b) The Bic/4-AP-mediated RhoA activation was declined to basal level after 30 min. Cortical neurons were stimulated with 50 μM Bic and 500 μM 4-AP for 5 or 30 min at 13 DIV. RhoA activity was assessed by RhoA-binding-domain (RBD) pull-down assay (upper). Data are mean ± SEM ($n = 4$) (lower). *$p < 0.05$, Student's *t*-test. (c) Cortical neurons were infected with p250GAP shRNA expressing and control lentiviruses at 7 DIV and stimulated with indicated NMDA doses for 5 min at 13 DIV. RhoA activity was assessed by RhoA-binding-domain (RBD) pull-down assay (left). Quantification of RhoA activity (13 DIV neurons) (right). The level of active RhoA in the 50 μM NMDA condition reached approximately 300% of the control level, indicating that RhoA activation did not reach maximum by p250GAP knock down (approximately 200% of the control level). See text for detail. Data are mean ± SEM ($n = 5$). #$p > 0.6$, Student's *t*-test.

(Fig. 4c, columns 5 vs. 6). In the 25 μM NMDA condition, RhoA activity increased only ~ 35% from the non-stimulated condition in the p250GAP knock down neurons without reaching to the maximal level. Since 25 μM NMDA was able to induce ~ 110% increase in the active RhoA level in the control neurons, active RhoA level in the p250GAP knock down neurons should have been higher if NMDA receptor-mediated RhoA activation is not dependent on p250GAP. Taken together, these results confirm that p250GAP is involved in NMDA receptor-mediated RhoA activation. Given that p250GAP negatively regulates RhoA activity, these results suggest that NMDA receptor stimulation suppresses the activity of p250GAP toward RhoA.

Shaft-to-spine redistribution of p250GAP by synaptic activity

Within spines, the actin cytoskeleton is highly dynamic and is regulated by synaptic activity (Fischer *et al.* 1998; Star *et al.* 2002; Carlisle and Kennedy 2005; Newey *et al.* 2005). It has been reported that NMDA receptor stimulation induces redistribution of F-actin-associated proteins such as Cortactin to regulate local actin cytoskeleton (Hering and Sheng 2003; Iki *et al.* 2005). We examined whether p250GAP changes its distribution in response to synaptic activity using the bicuculline/4-AP stimulation. Time-lapse confocal microscopy was performed on GFP-tagged p250GAP expressed by recombinant sindbisvirus in live hippocampal neurons

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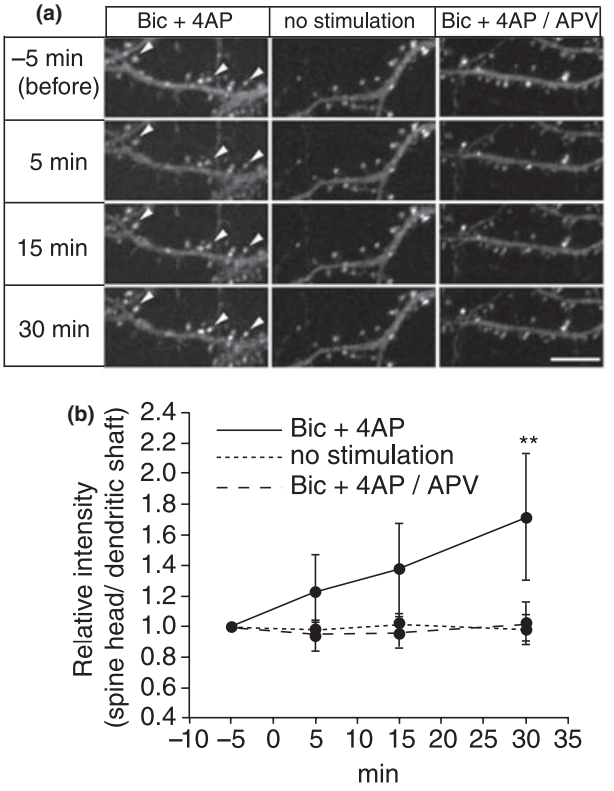

Fig. 5 NMDA receptor-mediated redistribution of p250GAP from dendritic shafts to spines in response to synaptic activity. (a) Time-lapse images of neurons expressing GFP-tagged p250GAP before and after application of bicuculline (Bic)/4-AP. The redistribution of GFP-tagged p250GAP was blocked by the addition of the NMDA receptor antagonist, APV. Scale bar, 5 μm. (b) Quantification of the change in fluorescence of GFP after Bic/4-AP stimulation. Relative intensity was calculated from the fluorescence intensity in the spine head and the underlying dendritic shaft (Ackermann and Matus 2003). Data are mean ± SEM (control, $n = 60$ clusters from three cells; Bic/4-AP, $n = 100$ clusters from five cells; Bic/4-AP+APV, $n = 80$ clusters from four cells). $^{**}p < 0.05$, Student's *t*-test.

(12–14 DIV) where most actin in dendritic spines is highly dynamic (Star *et al.* 2002). In unstimulated condition, an ectopically expressed GFP-tagged p250GAP was distributed within dendritic shafts and spines (Figs. 5a, center and 5b). Thirty-minutes after bicuculline/4-AP stimulation, GFP-p250GAP fluorescence intensity increased in spines and decreased in the dendritic shafts (Fig. 5a, left and 5b), indicating that GFP-tagged p250GAP was redistributed from dendritic shafts to spines. To assess the possibility that NMDA receptor activity regulates the distribution of GFP-tagged p250GAP, we used a selective NMDA receptor antagonist, APV. Application of APV completely blocked the activity-dependent redistribution of GFP-tagged p250GAP (Figs. 5a, right, and 5b). These results show that NMDA receptor activity regulates the redistribution of GFP-tagged p250GAP from dendritic shafts to spines.

Discussion

p250GAP is concentrated in post-synaptic densities and interacts with the NR2B subunit of the NMDA receptor (Nakazawa *et al.* 2003). However, the role of p250GAP in neurons has remained unclear. In this study, we have found that p250GAP regulates spine morphogenesis by modulating RhoA activity (Figs 1–3). We have also found that knock-down of p250GAP occludes the NMDA receptor-mediated RhoA activation (Fig. 4) and that GFP-tagged p250GAP is redistributed from dendritic shafts to spines in response to NMDA receptor activity (Fig. 5). These findings suggest that p250GAP provides a link between NMDA receptor activity and spine morphological plasticity that are thought to play a crucial role in synaptic functions.

Recent studies have shown that dendritic spines are highly dynamic, changing their size and shape in response to synaptic activity. The NMDA receptor regulates activity-dependent spine morphological plasticity by modulating the activity of Rho GTPases (Bonhoeffer and Yuste 2002; Wong and Ghosh 2002; Matus 2005; Segal 2005; Tada and Sheng 2006). However, little is known about the mechanisms involved in the regulation of the activity of Rho GTPases, especially RhoA in spines. Our biochemical experiments indicate that a substrate of p250GAP is RhoA (Fig. 3a). Given that p250GAP is concentrated in post-synaptic densities (Nakazawa *et al.* 2003), p250GAP may suppress a local RhoA activity in spines. Our data suggest that NMDA receptor stimulation suppresses the activity of p250GAP toward RhoA (Fig. 4a and c). We have found that NMDA receptor stimulation by NMDA (10 μM, 5 min) induces dissociation of p250GAP from the NR2B subunit of the NMDA receptor (Supplementary material Fig. S2). Given that RhoA as well as p250GAP associates with the NMDA receptor complex in spines (Nakazawa *et al.* 2003; Schubert *et al.* 2006), the dissociation of p250GAP from the NMDA receptor complex may lead to detachment of p250GAP from its substrate, RhoA. Accordingly, the activity of p250GAP toward RhoA may be suppressed downstream of NMDA receptor activation. Alternatively or in addition, CaMKII activated by Ca^{2+} entry through the NMDA receptor may inactivate p250GAP, because the GAP activity of p250GAP is inhibited in the presence of CaMKII *in vitro* (Okabe *et al.* 2003).

In addition to the regulation of p250GAP within spines, p250GAP is redistributed from dendritic shafts to spines 30 min after NMDA receptor stimulation (Fig. 5). Spines are continually changing their shape under homeostatic control (Wong and Ghosh 2002; Yuste and Bonhoeffer 2004; Matus 2005; Segal 2005), suggesting that the activity of Rho GTPases are also homeostatically controlled. Although the significance of the redistribution of p250GAP remains unclear, it is possible that the redistribution of p250GAP from dendritic shafts to spines might be important for

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preventing excess activation of RhoA in spines after NMDA receptor activation (Fig. 4b). Such homeostatic control of p250GAP might be important for neurons to be functionally continuing.

Previous studies have found that an ectopic expression of a dominant-negative form of RhoA increases spine density and length (Tashiro *et al.* 2000). On the contrary, a restrictive inhibition of Rho kinase, a downstream effector of RhoA, in spines induces localized spine retraction (Tashiro and Yuste 2004; Schubert *et al.* 2006). Although the level of active RhoA is reduced in S-SCAMα (an NMDA receptor-associated scaffolding molecule)-deficient neurons, these neurons do not show increased spine density (Iida *et al.* 2007). Therefore, the function(s) of RhoA may be different depending on its localization, upstream regulators, and downstream effectors. We found that RhoA activation by p250GAP knock-down does not decrease spine density and length (Fig. 1), while over-expression of constitutive active RhoA decreases spine density and length (Nakayama *et al.* 2000; Tashiro *et al.* 2000). These phenotypic differences may be explained by spatially restricted regulation of RhoA by p250GAP: p250GAP may regulate RhoA specifically in spines, because p250GAP is enriched in spines and associates with the NMDA receptor (Nakazawa *et al.* 2003). Alternatively, it is likely that the level of active RhoA is higher in neurons over-expressing active RhoA than in neurons with reduced p250GAP levels, resulting in the different phenotypes. In addition, some specific RhoA effectors present in spines may be involved in the p250GAP-mediated regulation of spine morphogenesis.

Although previous studies have found that p250GAP is also active on Cdc42 as well as RhoA *in vitro* (Nakamura *et al.* 2002; Moon *et al.*, 2003; Nakazawa *et al.* 2003; Okabe *et al.* 2003; Zhao *et al.* 2003), no change in the activity of GTP-bound active Cdc42 was detected in p250GAP knock down neurons (Fig. 3b). Given that, in contrast to RhoA, Cdc42 is barely detectable in postsynaptic densities where p250GAP is highly localized (Nakazawa *et al.* 2003; Schubert *et al.* 2006), p250GAP may be inaccessible to Cdc42 and thus be inactive on Cdc42 in neurons used in this study.

To our knowledge, p250GAP is the first RhoGAP shown to be involved in the NMDA receptor-mediated RhoA regulation. We have found that although 10 μM NMDA, which is enough to activate RhoA in control neurons, failed to activate RhoA, 50 μM NMDA was able to activate RhoA (Fig. 4c, columns 5 vs. 8), indicating that, in addition to p250GAP, other RhoA regulators such as RhoA GEF is involved in NMDA receptor-mediated RhoA activation in the 50 μM NMDA condition. Several RhoA regulators such as Oligophrenin-1 (RhoA GAP) and Lfc (RhoA GEF) have been implicated in spine morphogenesis (Govek *et al.* 2004; Ryan *et al.* 2005). These regulators may function at downstream of the NMDA receptor in different ways depending on stage of development, cell type, and cellular context, such as the level of NMDA receptor activation.

Cdc42 and Rac1 as well as RhoA regulate spine morphogenesis: Cdc42 and Rac1 promote the growth of spines (Nakayama *et al.* 2000; Tashiro *et al.* 2000; Irie and Yamaguchi 2002; Tashiro and Yuste 2004). Several RhoGEFs and RhoGAPs, including Kalirin (Rac1 GEF), Tiam1 (Rac1 GEF), PIX (Rac1 GEF), Intersectin (Cdc42 GEF), and α1-Chimerin (Rac1 GAP), have been implicated in spine morphogenesis (Penzes *et al.* 2003; Nishimura *et al.* 2005; Tolias *et al.* 2005; Van de Ven *et al.* 2005; Zhang *et al.* 2005). Given that the cross-talk between Rho family GTPases and the resulting balance between RhoA, Rac1, and Cdc42 activities influence spine morphogenesis (Newey *et al.* 2005), further analyses of the functional link between various regulators of Rho family GTPases are needed to clarify the mechanisms that underlie spine morphological plasticity.

In the present study, we suggest that p250GAP is involved in the NMDA receptor-mediated regulation of RhoA activity leading to spine morphological plasticity. Recent studies have shown that cognitive deficits in humans can be attributed to impaired Rho family GTPase-mediated spine morphogenesis (Newey *et al.* 2005). For instance, of 13 genes involved in nonspecific X-linked mental retardation, three encode regulators and downstream effectors of Rho family GTPases (Govek *et al.* 2004). Therefore, further elucidation of the roles of p250GAP in spine morphological plasticity may provide therapeutic methods for mental retardation.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1 p250GAP associates active RhoA and Cdc42 *in vitro*.

Fig. S2 p250GAP dissociates from the NR2B subunit of the NMDA receptor following NMDA receptor stimulation.

This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1471-4159.2008.05335.x (This link will take you to the article abstract).

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