



Published in final edited form as:

Nature. 2011 April 7; 472(7341): 100–104. doi:10.1038/nature09823.

Local, persistent activation of Rho GTPases during plasticity of single dendritic spines

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Abstract

The Rho family of GTPases play important roles in morphogenesis of dendritic spines^{1–3} and synaptic plasticity^{4–9} by modulating the organization of the actin cytoskeleton¹⁰. Here, we monitored the activity of Rho GTPases, RhoA and Cdc42, in single dendritic spines undergoing structural plasticity associated with long-term potentiation (LTP) using 2-photon fluorescence lifetime imaging microscopy (2pFLIM)^{11–13}. When long-term volume increase was induced in a single spine using 2-photon glutamate uncaging^{14,15}, RhoA and Cdc42 were rapidly activated in the stimulated spine. These activities decayed over ~5 min, and were then followed by a phase of persistent activation lasting more than 30 min. Although active RhoA and Cdc42 were similarly mobile, their activity patterns were different. RhoA activation diffused out of the stimulated spine and spread over ~5 μm along the dendrite. In contrast, Cdc42 activation was restricted to the stimulated spine, and exhibited a steep gradient at the spine necks. Inhibition of the Rho-Rock pathway preferentially inhibited the initial spine growth, whereas the inhibition of the Cdc42-Pak pathway blocked the maintenance of sustained structural plasticity. RhoA and Cdc42 activation depended on Ca^{2+} /calmodulin-dependent kinase (CaMKII). Thus, RhoA and Cdc42 relay transient CaMKII activation¹³ to synapse-specific, long-term signalling required for spine structural plasticity.

Previous studies using 2pFLIM and 2-photon glutamate uncaging revealed the spatiotemporal dynamics of signalling proteins CaMKII and HRas in single spines undergoing structural plasticity and LTP^{12,13}. CaMKII activation is restricted to spines, and decays rapidly with a time constant of ~10 s¹³. In contrast, HRas activity spreads from the stimulated spines along dendrites and into surrounding spines over ~10 μm ¹². However, in order to achieve long-lasting, spine-specific plasticity, there should also exist signalling pathways that relay compartmentalized signalling in the time scale of minutes to hours. Rho GTPases may constitute such signalling, because they play important roles in regulating

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Supplementary Information accompanies the paper

H.M. and R.Y. designed the experiments, H.M. performed the experiments and data analysis. H.W. performed electrophysiological experiment, H.M. and R.Y. wrote the paper.

actin cytoskeleton^{3,16}, which is essential for spine-specific, long-term structural and functional plasticity^{14,17}.

To measure activation of Rho GTPases in single dendritic spines, we developed fluorescence resonance energy transfer (FRET)-based sensors optimized for imaging under 2pFLIM using a design similar to a previously developed HRas sensor¹¹. The RhoA/Cdc42 sensors consist of two components: RhoA/Cdc42 tagged with monomeric enhanced green fluorescent protein (mEGFP) and their binding partner, Rho GTPase binding domain (RBD) of Rhotekin/Pak3, doubly tagged with mCherry (mCherry-RBD-mCherry) (Supplementary note). When mEGFP-Rho GTPase is activated, mCherry-RBD-mCherry binds to mEGFP-RhoA/Cdc42, causing FRET between mEGFP and mCherry (Supplementary Fig. 1, 2). These sensors were verified to be specific and sensitive under 2pFLIM (Supplementary note).

Using these sensors, we measured the activity of RhoA and Cdc42 during spine structural plasticity associated with LTP (Figs. 1, 2 and 3). Pyramidal neurons in the CA1 region of cultured hippocampal slices were ballistically¹⁸ transfected with the RhoA or Cdc42 sensor, and the FRET signal was imaged under 2pFLIM. The spine volume was monitored using the red fluorescence of mCherry-RBD-mCherry (Supplementary Fig. 3)¹². To induce structural plasticity in a single dendritic spine, we applied a low frequency train of two-photon glutamate uncaging pulses (30 pulses at 0.5 Hz) to the spine in zero extracellular Mg^{2+} (Ref 13,14,19). The spine volume increased rapidly by ~300% following glutamate uncaging (transient phase) and relaxed to an elevated level of 70–80% for more than 30 min (sustained phase) (Figs. 1d, 2d)^{12–14}. The time course of spine enlargement in neurons expressing the FRET sensor was similar to that in neurons expressing only EGFP (Fig. 4)¹⁴, suggesting that the overexpression of FRET sensors causes almost no effects on spine structural plasticity (Supplementary note).

Under the basal condition, there was no correlation between the activity of Rho GTPases and spine volume (Supplementary Fig. 4). When spine structural plasticity was induced, both RhoA (Fig. 1a, b) and Cdc42 (Fig. 2a, b) were activated rapidly within ~30 s in the stimulated spines. The activation decayed over ~5 min, followed by sustained activity lasting more than 30 min. RhoA activation spread into the dendrites over several micrometers (Fig. 1a–c, 3a, b), and slightly invaded surrounding spines (~25% of the stimulated spines). In contrast, Cdc42 activation was restricted to the stimulated spines (Fig. 2a–c, 3c, d). For both RhoA and Cdc42, the gradient at the spine necks was maintained more than ~30 min (Fig. 1b, c, 2b, c).

Next, we pharmacologically identified the signalling pathways that activates RhoA and Cdc42. Inhibition of NMDA receptors with 2-amino-5-phosphonopentanoic acid (AP5, 50 μ M) abolished activation of RhoA (Fig. 1b, c) and Cdc42 (Fig. 2b, c) as well as spine enlargement (Figs. 1d, e, 2d, e)¹⁴, indicating that RhoA and Cdc42 are activated by Ca^{2+} through NMDA receptors. The CaMKII inhibitor KN62 is known to strongly inhibit sustained spine enlargement, but has significantly less of an effect on transient spine enlargement (Figs. 1d, e, 2d, e)^{13,14}. KN62 (10 μ M) partially inhibited RhoA and Cdc42 activation during the transient phase, and more strongly during the sustained phase. (Figs.

1b, c, 2b, c). Expression of autocamtide CaMKII inhibitor peptide 2 (AIP2) also inhibited spine volume change and Rho GTPase activation in a similar manner (Supplementary Fig. 5). These results suggest that RhoA and Cdc42 are downstream of CaMKII.

We next characterized the spatial profile of RhoA and Cdc42 activities along dendrites as a function of the distance from the stimulated spines (Fig. 3). RhoA activity showed a relatively small gradient between the stimulated spines and dendrites, and spread along the dendrites. The length constant of spread along the dendrite was 4.5 μm (Fig. 3a, b), a value similar to that for HRas (~10 μm)¹². In contrast, Cdc42 activation was restricted to the stimulated spines (Fig. 3c, d), showing a spatial pattern similar to that of CaMKII¹³. A small fraction of Cdc42 activation spread into the dendrite and decayed sharply with a length constant of ~1.9 μm (Fig. 3c, d). These experiments were performed at room temperature (25–27°C), but similar results were also obtained at near-physiological temperature (32–34°C; Supplementary Figs. 6, 7).

To test whether the difference in the degree of the compartmentalization of RhoA and Cdc42 is due to a difference in their mobility^{12,13} we measured spine-dendrite diffusion coupling using photoactivatable GFP (paGFP)²⁰ fused to Rho GTPases. Following photoactivation of paGFP in a spine, the fluorescence intensity in the spine decayed due to the diffusion of paGFP-Rho GTPases out of the spine with a time constant of ~3 s for wild-type and ~6 s for their constitutively active mutants (Fig. 3eh). These values are ~10 times larger than the decay time constant of cytosolic paGFP (~0.4 s) and similar to that of a constitutively active HRas mutant (~6 s) (Fig. 3e–h)¹². The difference between wild-type Rho GTPases and constitutively active mutants presumably reflects the difference in the fraction of the protein bound to the plasma membrane, as active Rho GTPases are localized on the plasma membrane²¹. There was only a small fraction (10–20 %) of fluorescence remaining at 20 s after photoactivation (Fig. 3e–h), suggesting that no major immobile fraction of RhoA or Cdc42 exists in spines. Thus, Cdc42 is as mobile as RhoA and HRas, yet only Cdc42 shows the compartmentalized activity.

Next, to elucidate the roles of Rho GTPase activation in spine structural plasticity, we measured the spine volume change under the inhibition of Rho or Cdc42 signalling (Fig. 4a–j). Downregulation of RhoA and RhoB with short-hairpin RNA (shRNA) decreased the transient volume change, but did not appreciably affect the sustained volume change (Fig. 4a, i, j). In contrast, shRNA against Cdc42 decreased the sustained volume change, but not the transient volume change (Fig. 4e, i, j). The phenotypes caused by these shRNAs were rescued by co-expressing shRNA-resistant mEGFP-Rho GTPases (Fig. 4b, f, i, j), indicating that the effect of the shRNAs are specific and the mEGFP-RhoA and mEGFP-Cdc42 used in the FRET sensors are functional as endogenous proteins. Because downregulation of proteins with shRNA is partial (Supplementary Fig. 8) and requires relatively long time (4 days), we also inhibited Rho and Cdc42 signalling by expressing mCherry-C3 transfarase (C3), a Rho inhibitor²², and the Cdc42 binding domain of Wasp tagged with mCherry (Wasp)²³, respectively, for shorter time (24 hours). Rho inhibition with C3 inhibited both transient and sustained phases (Fig. 4c, i, j), showing stronger effects than shRNA (Fig. 4a, i, j). Cdc42 inhibition with Wasp inhibited the sustained phase but not the transient phase (Fig. 4g, i, j), consistent with the shRNA result (Fig. 4e, i, j). Thus, our data suggest that Rho

signalling is required for the transient phase and likely the sustained phase of spine enlargement, while Cdc42 signalling is required for the sustained phase. Neither C3 nor Wasp affected CaMKII activation (Supplementary Fig. 9), indicating that there is no feedback signalling from Rho and Cdc42 to upstream Ca^{2+} and CaMKII. C3 and Wasp also inhibited synaptic potentiation induced by pairing postsynaptic depolarization (0 mV) and 2-photon glutamate uncaging (Supplementary Fig. 10)^{13,14}, suggesting that Rho and Cdc42 are important for the functional plasticity as well as the structural plasticity of spines.

Amongst known effectors of Rho and Cdc42, Rock and Pak are two kinases that can be activated respectively by these GTPases^{24–26}. We tested if they are required for structural plasticity through acute (30–40 min) application of specific pharmacological inhibitors. Inhibition of Rock with Glycyl-H1152 (GH1152; 2 μM)²⁷ suppressed both transient and sustained volume change (Fig. 4d, i, j) similarly to Rho inhibitor C3 (Fig. 4c). In contrast, inhibition of Pak with IPA3 (100 μM)²⁸ decreased the sustained volume change selectively without changing the transient volume change (Fig. 4h, i, j) similarly to inhibition of Cdc42 signalling (Fig. 4e, g). Taken together with the results from Rho/Cdc42 inhibition (Fig. 4a–j), our data implies that the Rho-Rock pathway is required for both transient and sustained phase, while the Cdc42-Pak pathway is required for the sustained phase of the structural plasticity but not for the transient phase (Fig. 4k).

In this study, we visualized RhoA and Cdc42 activation in single dendritic spines undergoing structural plasticity associated with LTP^{12–15,19}. The time course of their activation was similar to that of the volume change: rapid activation was followed by persistent activation lasting more than 30 min (Fig. 4l). As expected from its high mobility (Fig. 3), RhoA spread into dendrite upon activation (Fig. 1a–c)¹³. However, the activity invasion into adjacent spines was relatively small (25% of the stimulated spines, Fig. 1b, c) and was not sufficient to produce plasticity (Fig. 1d, e). In contrast with the diffusive pattern of RhoA activity, Cdc42 activity was restricted to the stimulated spines (Fig. 2, 3). The compartmentalization of Cdc42 activity is not due to the limited diffusion of active Cdc42, because active Cdc42 is as mobile as RhoA and HRas (Fig. 3e–h). Since the high spatial gradient of Cdc42 between the stimulated spines and dendrite was maintained for more than 20–30 min (Fig. 2b, c, 3d), Cdc42 must be continuously activated at the stimulated spines during plasticity, and inactivated immediately after diffusing out of the spines. The short length constant of Cdc42 in the dendrites (1.9 μm , Fig. 3) also supports the fast inactivation of Cdc42 in the dendrite: the inactivation time constant τ is related to the length constant L and the diffusion constant $D \sim 0.6 \mu\text{m}^2$ (Ref 12) as $\tau = L^2/D$, and thus is ~ 6 s for Cdc42 and ~ 30 s for RhoA, compared to 200–300 s for HRas¹².

Our results further indicated that RhoA and Cdc42 activation is CaMKII-dependent, and activation lasts for more than 30 min (Fig. 1, 2). The previous imaging study suggested that CaMKII activity decays with a time constant of ~ 10 s (Fig. 4k, l)¹³, thereby integrating NMDA receptor-evoked Ca^{2+} transients that last for ~ 0.1 s^{13,29}. Localized, persistent activation of RhoA and Cdc42, which peaks in between CaMKII activation and the volume change (Fig. 4l), relays the transient CaMKII signalling¹³ to long-term spine structural plasticity (Fig. 4k). In particular, as both CaMKII^{13,14} and Cdc42 exhibit spine specific activation and required for the maintenance of plasticity (Fig. 4e, g, i, j), the NMDA

receptor-CaMKII-Cdc42-Pak pathway (red line in Fig. 4k) constitutes the spine specific signal transduction spanning the time scale from sub-second to more than 30 min to cause sustained structural and functional spine plasticity (Fig. 4k, l).

Methods Summary

Hippocampal cultured slices were prepared from postnatal day 6–7 rats as described³⁰. Neurons were sparsely transfected with Rho GTPase FRET sensors using ballistic gene transfer¹⁸ at days in vitro 10–14, and imaged 2–4 days after transfection. Rho-GTPase activity was measured using 2pFLIM (green) and spine volume change was monitored by measuring the fluorescence intensity of mCherry-RBD-mCherry (red) in spines using regular 2-photon microscopy (Supplementary Fig. 3)^{12,13}. Most of the imaging experiments were performed at room temperature (25–27°C).

Supplementary Material

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Acknowledgments

We thank Drs. K. Hahn, G. Bokock, A. Aplin, S. Soderling, M. Matsuda, and Y. Hayashi for plasmids and reagents, S.-J. Lee for CaMKII activation data, S. Soderling, S. Raghavachari, L. van Aelst, Y. Hayashi, H. Kasai, and M. Ehlers for discussion, and H. Hedrick and M. Patterson for comments on the manuscript. We also thank A. Wan for preparing cultured slices and D. Kloetzer for laboratory management. This study was funded by Howard Hughes Medical Institute, National Institute of Mental Health, National Institute of Neurological Disorders and Stroke, National Institute of Drug Abuse, Alzheimer's Association and the Japan Society for the Promotion of Science (H.M.).

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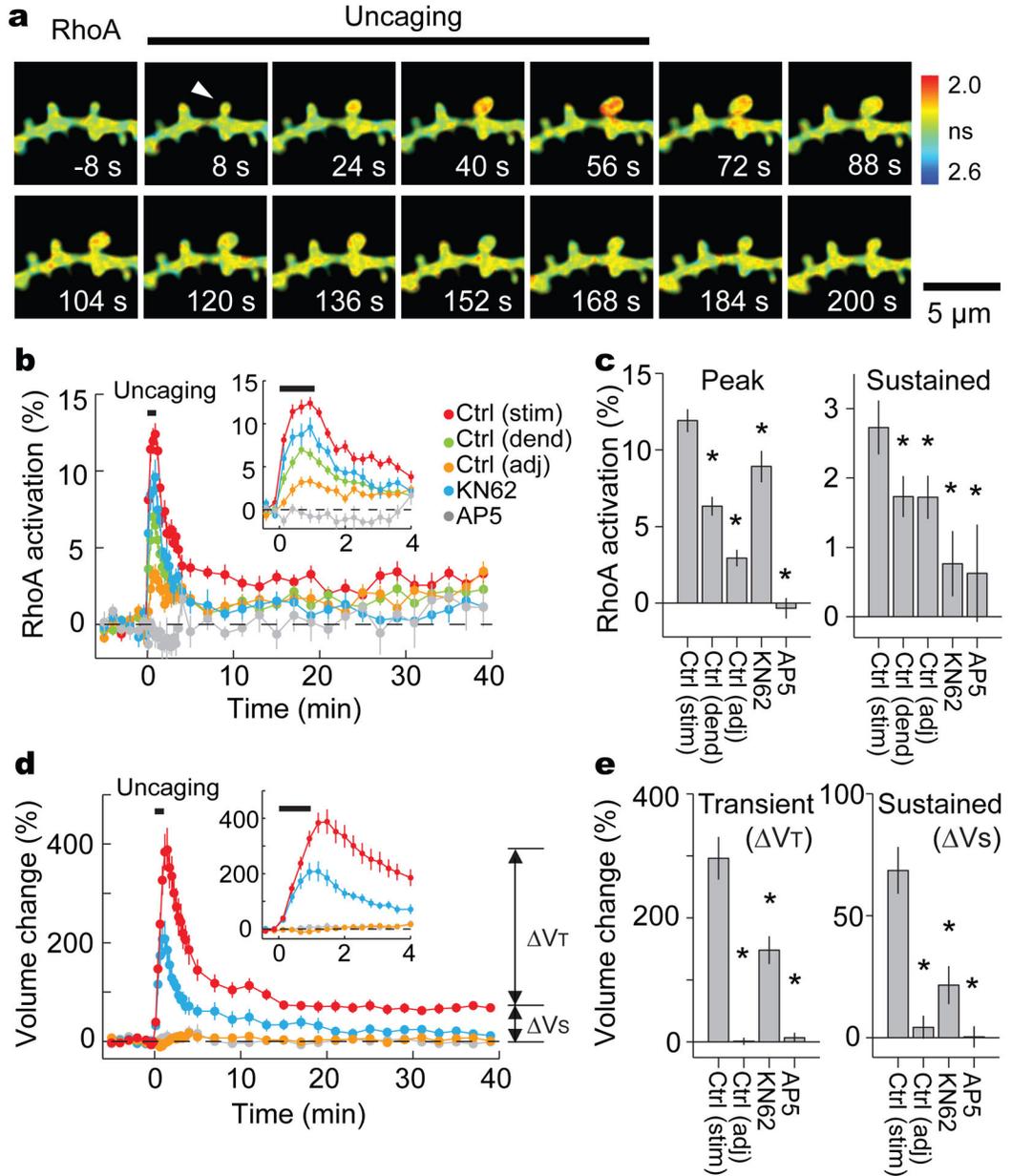


Fig. 1. Spatiotemporal dynamics of RhoA activation during long-term structural plasticity induced in single spines

a, Fluorescence lifetime images of RhoA activation during spine structural plasticity induced by 2-photon glutamate uncaging. Arrow heads indicate the stimulated spine. Warmer colours indicate shorter lifetimes and higher RhoA activity. Scale bar, 5 μ m.

b, Time course of RhoA activation measured as a change in the fraction of mEGFP-RhoA bound to mCherry-RBD-mCherry in stimulated spines (stim), the dendritic shaft beside the stimulated spines (dend; within 1 μ m), and adjacent spines (adj; between 3–5 μ m of the stimulated spines). Data using pharmacological inhibitors (Ctrl, control condition; KN62, CaMKII inhibitor; AP5, NMDA receptor inhibitor) are also shown. Inset: closer view. The

numbers of samples (spines/neurons) are 35/29 for stimulated spines and dendrites, 29/26 for adjacent spines, 16/10 for KN62 and 8/5 AP5. Error bars are s.e.m.

c, Transient (averaged over 16–64 s) and sustained (averaged over 20–38 min) RhoA activation. Stars denote statistically significant difference (< 0.05) from the value in the stimulated spines under control condition. Wilcoxon signed-rank test was used for dendrites and adjacent spines, and ANOVA followed by post-hoc tests using the least significant difference was used for experiments with pharmacological inhibitors.

d, Averaged time course of spine volume change in the same experiments in **b**.

e, Transient (volume change averaged over 1.5–2 min subtracted by that over 20–38 min) and sustained volume change (volume change averaged over 20–38 min).

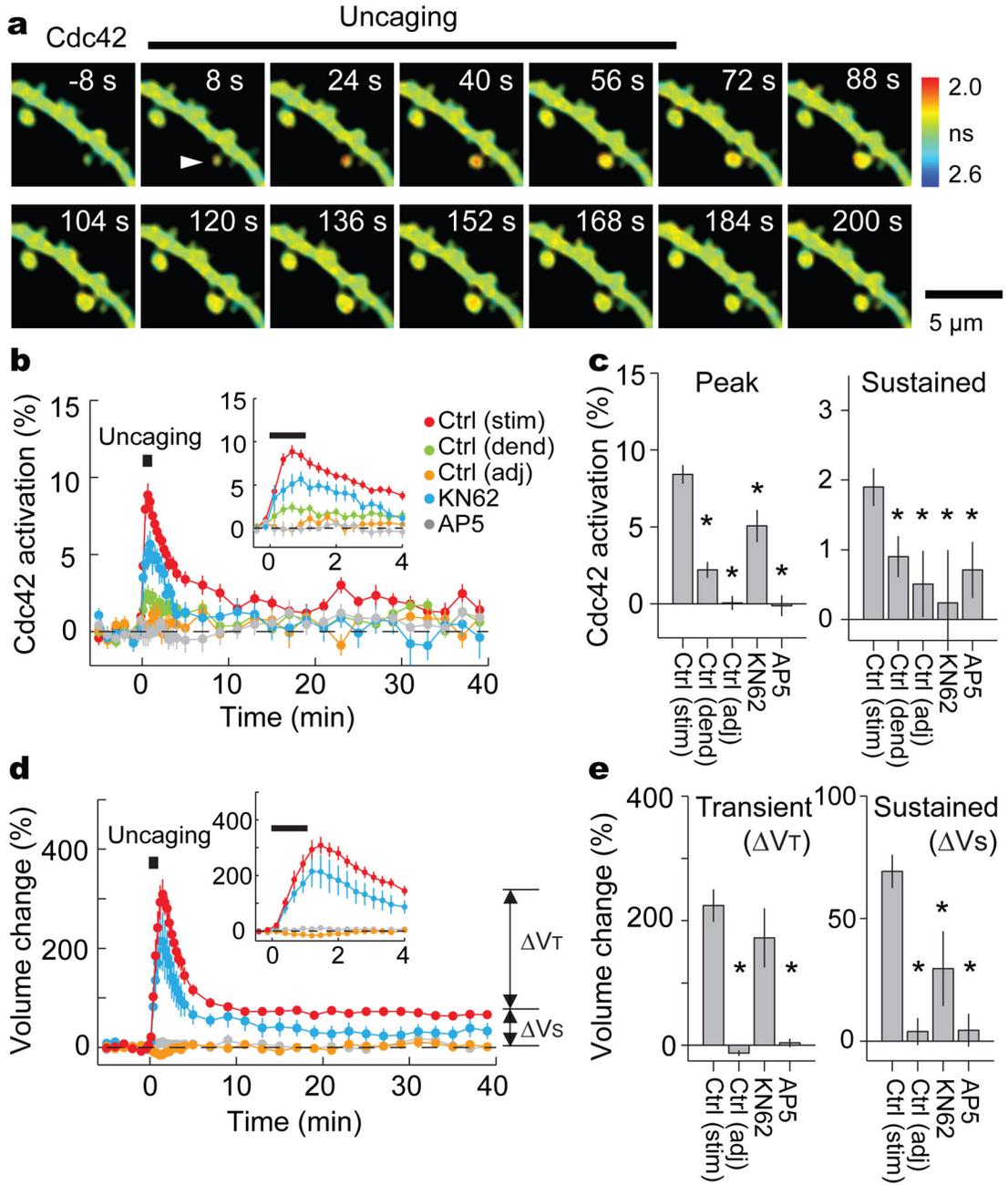


Fig. 2. Spatiotemporal dynamics of Cdc42 activation during long-term structural plasticity induced in single spines

The same experiments and analyses as in Fig. 1 except for measuring Cdc42 activity instead of RhoA activity. The numbers of samples (spines/neurons) are 33/28 for stimulated spines and dendrite, 33/28 for adjacent spines, 11/6 for KN62 and 12/8 for AP5.

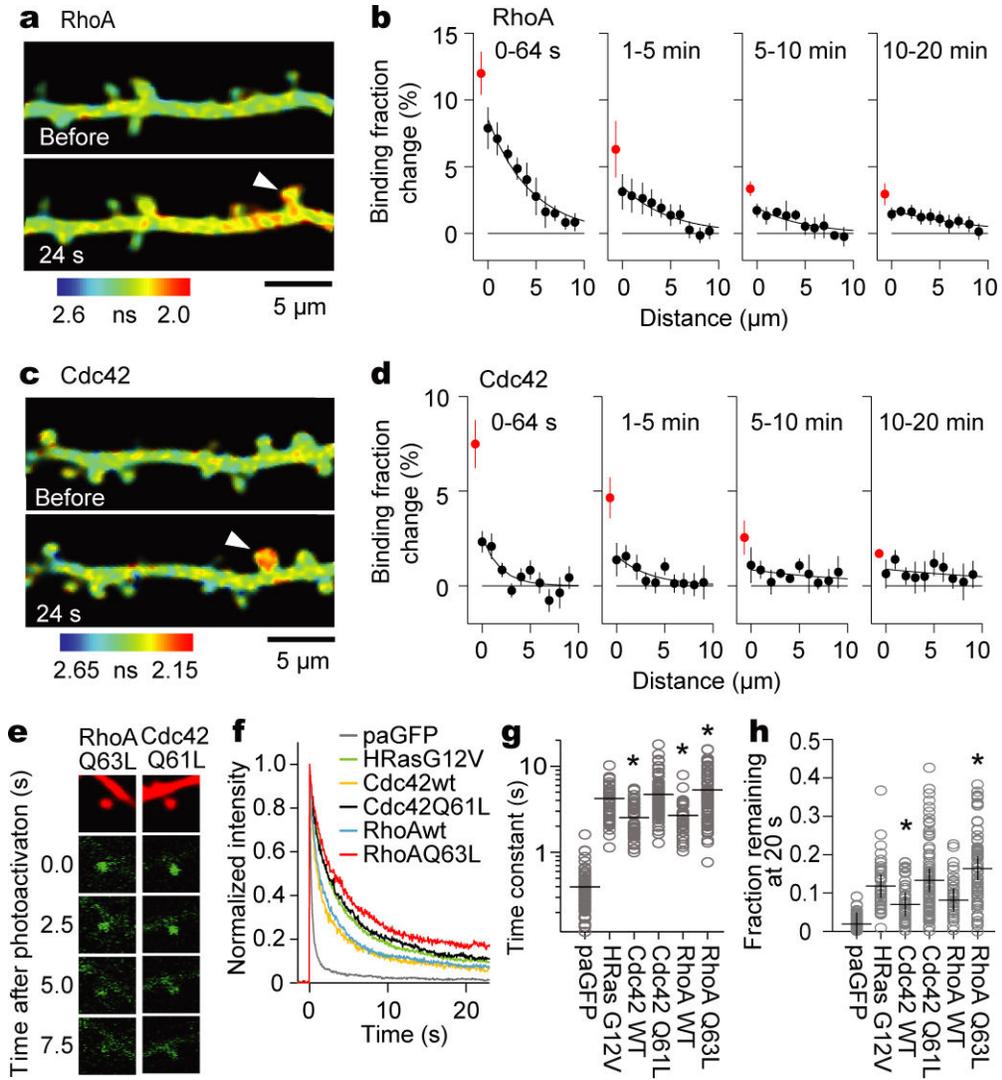


Fig. 3. Spatial profile of RhoA and Cdc42 activities

a, Fluorescence lifetime images of RhoA activity before and after glutamate uncaging. Arrow heads indicate stimulated spine.

b, Averaged spatial profile of RhoA activation. Red circles indicate the activity in the stimulated spine, and black circles indicate the activity in dendrite, plotted as a function of the distance along the dendrite from the simulated spine. The number of samples (dendrites/neurons) is 20/18.

c, Fluorescence lifetime images of Cdc42 activity.

d, Averaged spatial profile of Cdc42 activation. The number of samples(dendrites/neurons) is 30/26.

e, The fluorescence images of paGFP-RhoA (left) and paGFP-Cdc42 (right) after spine-head photoactivation (green, paGFP-Rho GTPases; red, tandem mCherry).

f, Averaged timecourse of fluorescence decay in spines after photoactivation of paGFP tagged proteins in the spines. The fluorescence intensity was normalized to the peak. The numbers of samples (spines/neurons) are 63/6 for paGFP, 38/4 for paGFP-HRas (G12V),

41/5 for paGFP-Cdc42 (WT), 83/9 for paGFP-Cdc42 (Q61L), 40/4 for paGFP- RhoA (WT) and 79/10 for paGFP-RhoA (Q63L). HRas (G12V), RhoA (Q63L) and Cdc42 (Q61L) are constitutively active mutants.

g,h, Decay time constants (**g**) and the fraction remaining at 20 s (**h**) of paGFP fluorescence in the photoactivated spines. Horizontal bars, mean.

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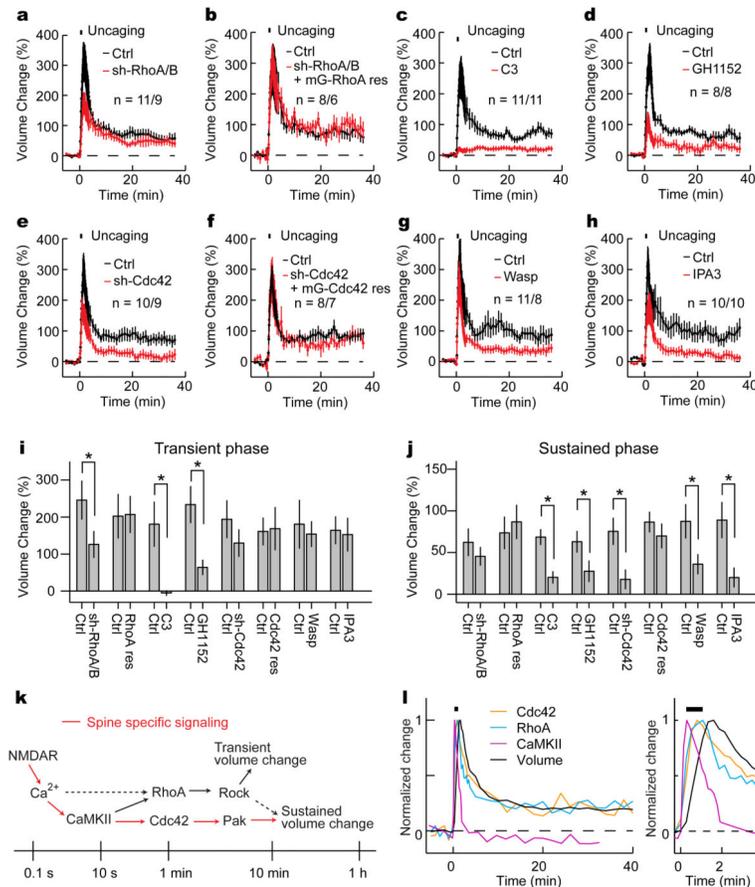


Figure 4. The effect of Rho GTPase inhibition for structural plasticity of spine head enlargement

a-h, Averaged time course of spine volume change in stimulated spines in neurons under manipulations of Rho GTPase signalling. Neurons were transfected with shRNAs against RhoA and RhoB (sh-RhoA/B) and mEGFP (**a**), sh-RhoA/B, mEGFP-shRNA resistant RhoA (mEGFP-RhoA res) and tandem mCherry (**b**), mCherry-C3 (C3) and mEGFP (**c**), mEGFP (**d**, **h**), shRNA against Cdc42 (sh-Cdc42) and mEGFP (**e**), sh-Cdc42, mEGFP-shRNA resistant Cdc42 (mEGFP-Cdc42 res) and tandem mCherry (**f**) or mCherry-Wasp(210–321) (Wasp) and mEGFP (**g**) (red). Paired control experiments (black) were performed in the same batch of slices using a scrambled shRNA instead of targeted shRNAs (**a**, **e**), mEGFP alone (**b**, **f**) or mCherry instead of C3 and Wasp (**c**, **g**). Pharmacology experiments (**d**, **h**) were performed before (paired control, black) and 30–40 min (red) after applying drugs to the bath. Fluorescence intensity of mEGFP (**a**, **c**, **d**, **e**, **g**, **h**) or tandem mCherry (**b**, **f**) was used to measure the spine volume change. The numbers of samples (spines/neurons) are indicated in the figures (same numbers for control and experiment groups).

i, Transient volume change (volume change averaged over 1.5–2 min subtracted by that averaged over 20–36 min). Stars denote statistical significance ($p < 0.05$, paired t-test).

j, Sustained volume change (volume change averaged over 20–36 min).

k, A model of Cdc42 and RhoA activation.

I, Superimposed time courses of spine volume change and activation of RhoA (Fig. 1b), Cdc42 (Fig. 2b) and CaMKII13 in spines undergoing structural plasticity. The time courses were normalized to the peak. Right, closer view.

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