Structural basis of long-term potentiation in single dendritic spines

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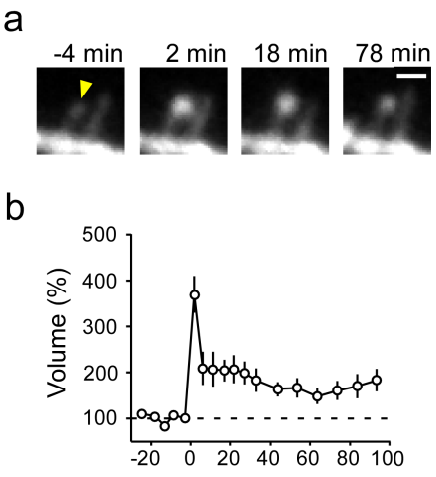
Supplementary Information: Spine enlargement

The increases in fluorescence of spines by repetitive uncaging of MNI-glutamate observed in eGFP transfected neurons (Fig. 1a) could similarly be detected in CA1 pyramidal neurons loaded with calcein by a short whole-cell perfusion (Supplementary Fig. 1a and b, *n* = 11). These data show that fluorophore diffusion/expression is not rate limiting for eGFP-labelled neurons. The apparent changes in spine fluorescence cannot be due to change in the position of spines, because we found only increases but not decrease in fluorescence after stimulation, and because the images were stacked with a distance of 0.5 μm far below the axial resolution of our imaging (2.1 μm), and hence spine fluorescence should not be affected by spine position.

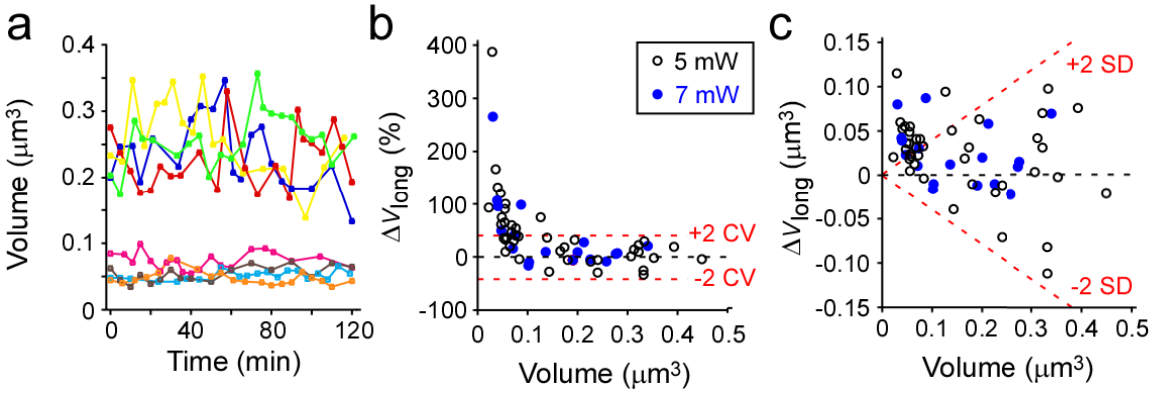
Supplementary Figure 1 Spine-head enlargements induced by repetitive uncaging of MNI-glutamate in hippocampal CA1 pyramidal neurons that were loaded with calcein (1 mM) with whole-cell perfusion for 10 s. A patch-pipette was withdrawn after the loading to prevent washout of plasticity. **a**, Time-lapse images of dendritic spines. The arrowhead indicates the spot of two-photon uncaging of MNI-glutamate. Calcein was excited at 830 nm. Images were stacked along the *z*-axis. Scale bar, 1 μm. **b**,



Averaged time courses of calcein fluorescence of spine head during two-photon uncaging of glutamate (n = 11). Error bars represent ±s.e.m.

Fluorescence of dendritic spines shows spontaneous fluctuations (Supplementary Fig. 2a) due to spontaneous morphological changes or "motilities" (Ref. S1). The absolute amplitudes of fluctuation in fluorescence were proportional to the spine volumes, as the coefficient of variation (CV) of the fluctuation was similarly estimated as 0.21 ± 0.01 (n = 19) and 0.21 ± 0.03 (n = 17) for large (>0.1 μm^3) and small (<0.1 μm^3) spines, respectively. The fluctuation was partly dependent on NMDA receptors; CV was reduced to 0.15 ± 0.01 (n = 23) in the presence of Mg^{2+}. Amplitudes of AMPA currents also fluctuated with a CV of 0.15 ± 0.02 (n = 8) in the presence of Mg^{2+}. Supplementary Fig. 2b shows the distribution of spine enlargement vs. spine volumes, illustrating the fact that enlargement was seldom induced when spine head volume was >0.1 μm^3 (Supplementary Fig. 2b). Moreover, long-lasting enlargement was absent in large spines even with a laser power of 7 mW that triggered release of two times, ~$(7/5)^2$, larger amount of glutamate (Supplementary Fig. 2b, blue dots).

The absolute changes in fluorescence after stimulation were positive in most small spines (Supplementary Fig. 2c), while were random and their amplitudes were within the range of spontaneous fluctuation in large spines. The mean changes in volume in large spines was 0.010 ± 0.009 μm^3 (mean ± s.e.m., n = 31) which was smaller than that of small spines of 0.038 ± 0.005 μm^3 (n = 28, P<0.01). Thus, large spines were less susceptible to the structural plasticity even in terms of the absolute change in the volume.



Supplementary Figure 2 Long-lasting alterations in spine-head volumes. **a**, spontaneous fluctuation in fluorescence of representative large (above) and small (below) spines in the absence of Mg^{2+} in the external medium. Different colors denote data from different spines. **b**, **c**, Long-lasting spine-volume changes induced by repetitive uncaging of MNI-glutamate in spines with various initial volumes. Changes in volume were quantified by % changes relative to the initial volume (**b**) or by absolute volume in μm^3 (**c**). ΔV_{long} was obtained from the values between 70 to 100 min after stimulation and 0 to 20 min before stimulation. Red dashed lines represent the two times SD levels estimated during spontaneous fluctuation shown in (**a**), assuming that SD of fluctuation is proportional to the spine volumes. Black and blue dots represent the experiments where repetitive uncaging was effected at power of ~5 mW and 7 mW, respectively.

Supplementary Methods

Estimation of spine-head volume

Spine-head volume was estimated from the total fluorescence of the spine head in the *z*-stacked fluorescence images, which was calibrated with the use of a sphere-like large spine in the same dendritic field, assuming that eGFP homogeneously filled the volume of dendrites. In the stacked image of the large spine, the one-dimensional fluorescence profile crossing the center of the head, $f(r)$, was measured, and fitted by the following equation:

$$F(r,R) = \frac{A}{(2\pi)^{3/2}\sigma_x^2\sigma_z} \iiint_{x^2+y^2+z^2<R^2} dxdydz \int dz' \, e^{-\left[\frac{(x-r)^2}{2\sigma_x^2}+\frac{y^2}{2\sigma_x^2}+\frac{(z-z')^2}{2\sigma_z^2}\right]}$$

where A represents the intensity at the center (r = 0) of the unit sphere, R the radius of the spine head, and σ_x (0.19 μm) and σ_z (0.91 μm) the standard deviations of the Gaussian approximation of the focal volume of two-photon excitation at 910 nm estimated with a bead. The estimation can be applied for spines in slice preparations, because spatial resolution of two-photon microscope shows no degradation even in highly turbid medium (Ref. S2). *F(r, R)* was numerically obtained with Mathematica4 software (Wolfram Research, Champaign, Illinois) to find the value of $2R$ that fits with full-width-at-half-maximal (FWHM) diameter of $f(r)$. The spine-head volume of large spines was then estimated as $\frac{4}{3}\pi R^3$. Our estimation in the diameter of

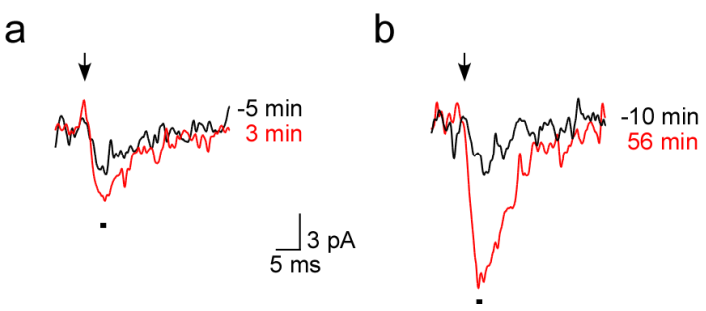
spine head (Fig. 1e) was confirmed using fluorescent beads with diameters of 0.415 μm and 0.50 μm (Molecular Probes), which were estimated with our method as 0.40 ± 0.02 μm (mean ± s.e.m., *n* = 5) and 0.51 ± 0.01 μm (*n* = 9), respectively.

Supplementary Methods References.

(S2) Dong,C.Y., Koenig,K. & So,P. Characterizing point spread functions of two-photon fluorescence microscopy in turbid medium. *J. Biomed. Opt.* **8**, 450-459 (2003).

When the cells were whole-cell clamped in the perforated-patch mode, spine enlargement was less pronounced even in small spines (Fig. 4a) and almost absent in large spines (Fig. 3b; 4b), in comparison with non whole-cell clamped cells (Fig. 2a). This was the case even when uncaging was effected in the absence of Mg^{2+} in perforated-patch clamped cells (data not shown). We therefore think that the structural plasticity was weakened by whole-cell perfusion even though the perforated-patch mode was utilized.

The time courses of glutamate-induced currents were not significantly altered by the induction of spine enlargement (Supplementary Fig. 3a, b) as in the case with LTP. The times-to-peak were 4.3 ± 0.3 ms and 4.1 ± 0.3 ms (7 of open circles in Fig. 4b, which showed spine enlargement and current potentiation above two SD values of volume and current fluctuation, respectively) before and after the induction, respectively. Half decay was achieved at 6.6 ± 1.4 ms and 5.8 ± 0.8 ms (*n* = 7), before and after the induction, respectively. These data speak against, though not completely exclude, the possibilities that the enhancement of glutamate sensitivity by repetitive uncaging of MNI-glutamate may involve increases in the volume of synaptic cleft or decreases in activities of glutamate transporters.



Supplementary Figure 3
Glutamate induced currents recorded from spines shown in Fig. 3a (a) and Fig. 3b (b) before (black) and after (red)

induction of spine enlargement. Arrows represent the time of uncaging. Horizontal bars show the time period when current amplitudes were measured.

Supplementary Information Reference.

(S1) Dunaevsky,A., Tashiro,A., Majewska,A., Mason,C. & Yuste,R. Developmental regulation of spine motility in the mammalian central nervous system. *Proc Natl Acad Sci U S A* **96**, 13438-13443 (1999).