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Dendritic spine dynamics leading to spine elimination after repeated inductions of LTD

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Memory is fixed solidly by repetition. However, the cellular mechanism underlying this repetition-dependent memory consolidation/reconsolidation remains unclear. In our previous study using stable slice cultures of the rodent hippocampus, we found long-lasting synaptic enhancement/suppression coupled with synapse formation/elimination after repeated inductions of chemical LTP/LTD, respectively. We proposed these phenomena as useful model systems for analyzing repetition-dependent memory consolidation. Recently, we analyzed the dynamics of dendritic spines during development of the enhancement, and found that the spines increased in number following characteristic stochastic processes. The current study investigates spine dynamics during the development of the suppression. We found that the rate of spine retraction increased immediately leaving that of spine generation unaltered. Spine elimination occurred independent of the pre-existing spine density on the dendritic segment. In terms of elimination, mushroom-type spines were not necessarily more stable than stubby-type and thin-type spines.

L earning and memory are fixed solidly and become long-lasting by repetition of experiences or tasks[1–3]. However, the cellular mechanisms underlying this repetition-dependent consolidation/reconsolidation of memory remain unclear. Using stably maintained organotypic slice cultures of the rodent hippocampus, we previously reported that 3 repeated inductions (but not a single induction), of chemically-evoked long-term potentiation (cLTP) led to a slowly developing long-lasting enhancement in synaptic strength, which was coupled with formation of new synapses[4–8]. We termed this phenomenon as RISE (*R*epetitive-*LTP*-*I*nduced *S*ynaptic *E*nhancement) and proposed its use as an *in vitro* model system for analyzing the cellular/molecular mechanisms of repetition-dependent memory consolidation.

An apparently opposite phenomenon, synaptic weakening accompanied by synapse elimination, was established after 3 repeated inductions of chemically evoked long-term depression (cLTD) and has been termed LOSS (*L*TD-repetition-*O*perated *S*ynaptic *S*uppression)[9–11]. The phenomena of RISE and LOSS are symmetric in various aspects including the requirement of 3 repetitions of stimulus, time course of development, long-lasting nature[4,9], and involvement of related neurotrophic molecules[12] (brain-derived neurotrophic factor in RISE and its precursor in LOSS).

The physiological role of synapse elimination in memory has not been fully understood to date. It is possible to assume that synapse formation corresponds to memory formation, while synapse elimination corresponds to forgetting. This assumption is relevant to a massive synapse loss in the brain of Alzheimer disease[13]. Memory reversal coupled with spine formation and elimination is reported[14]. It is also possible, however, to assume that both synapse formation and elimination contribute to memory[15]. According to Hebb's principle[16], memory is the alteration of the information flow path so that both synapse formation and elimination contribute to memory through rearrangement of the neural circuits. In fact, examples of memory formation through synapse elimination have been reported[17,18]. Thus, analyses of synapse elimination *in vitro* are necessary in memory research.

Recently, we examined the dynamics of dendritic spines during the development of RISE and found a sequence of distinct phases leading to an increase in spine density[19]. In the stable cultures, the dendritic spines of the CA1 pyramidal neurons are in the state of equilibrated fluctuation[20,21], where the spines constantly generate and retract but both processes are balanced. The RISE-producing stimulus (i.e., 3× cLTP inductions) leads spines into a raised fluctuation phase, in which the rates of both spine generation and retraction increase. The raised fluctuation phase is followed by a biased fluctuation phase, which is characterized by a net increase in spine number as the retraction rate returns to the pre-stimulus level before the generation rate does. Finally, the generation rate

returns to the pre-stimulus level, and thus the dynamic equilibrium is restored. This characteristic sequence occurs preferentially in the dendritic segments that are low in pre-existing spine density.

The above-mentioned phenomenological symmetry between RISE and LOSS suggests the possibility of stochastic behavior of dendritic spines during the development of LOSS similar to that during the development of RISE. The question we address in the study is whether or not the dynamics of spines also follows 3 phases (raised fluctuation followed by biased fluctuation and re-balanced fluctuation) during the development of LOSS. The presence or absence of the dependency on pre-existing spine density in LOSS is also questioned. In addition we investigated differences in the type of spines between the spines that remain and those that are lost.

Results

Since we use here a transgenic mouse clone expressing fluorescent protein in the hippocampal neurons, we need to confirm that cLTD can be induced by means of group I metabotropic glutamate receptor (mGluR) activation, without assistance of test pulses, in slice cultures prepared from the present mouse clone. This was confirmed as shown in Fig. 1a. This is important since test pulses, a component indispensable in some LTD protocols, were not included in our protocols.

Next, we confirmed the induction of long-lasting synapse elimination equivalent to LOSS, consistent with previous findings in rat slice cultures after 3 repeated inductions of cLTD[10] (Fig. 1b–d). This structural plasticity is produced after 3× cLTD inductions, but not after 1× or 2× cLTD inductions (Fig. 1d).

In our previous examination of RISE[19], rates of both spine generation and retraction were elevated and thus no net increase in spine number resulted until the 3rd day after the 3rd cLTP induction (raised fluctuation phase). However, in the present spine elimination, the spine number (density) had already decreased by the 4th day after the 3rd cLTD induction (Fig. 1d and e; indicated as PS day 6, since post-stimulus days were counted from the start of stimulation). During this period, the rate of spine retraction was elevated, but that of spine generation was unaltered (Fig. 2a). In parallel to this, the disappearance of pre-existing spines was significant during the first 4 days after the 3rd cLTD induction (Fig. 2b and c). These results suggest that the "raised fluctuation phase" is absent in the development of LOSS. After the first 4 days had passed, the disappearance rate was similar between stimulated and unstimulated cultures (Fig. 2c), reflecting the restoration of equilibrated fluctuation.

This suggestion was confirmed by chasing of the spines in a short interval. Within one day after the 3rd cLTD induction, the spine retraction rate was larger than the generation rate (Fig. 3a, b and d) resulting in a decreased number of spines (Fig. 3c). The dendrites directly underwent into the biased fluctuation phase, indicating that RISE and LOSS are asymmetric in this aspect of spine dynamics. Our observations also confirm the absence of biased fluctuation and thus no change in spine number after a single cLTD induction (Fig. 3b–e).

We reported that the increase in spine number occurs preferentially in the dendritic segments having low pre-existing spine density[19]. The segments having high spine density do not experience raised fluctuation. As seen in Fig. 4a, the decrease in spine number did not significantly correlate with the segment's pre-existing spine density. Therefore, RISE and LOSS are also asymmetric in this aspect of spine dynamics.

Depending on morphology, dendritic spines are classified into 3 types: mushroom, thin, and stubby. These represent the state of maturity, with the mushroom type being the most mature and stable[22–24]. We examined whether differences in stability existed among the spine types for the present structural plasticity. In Fig. 4b and c, we compared the distribution of spine types between
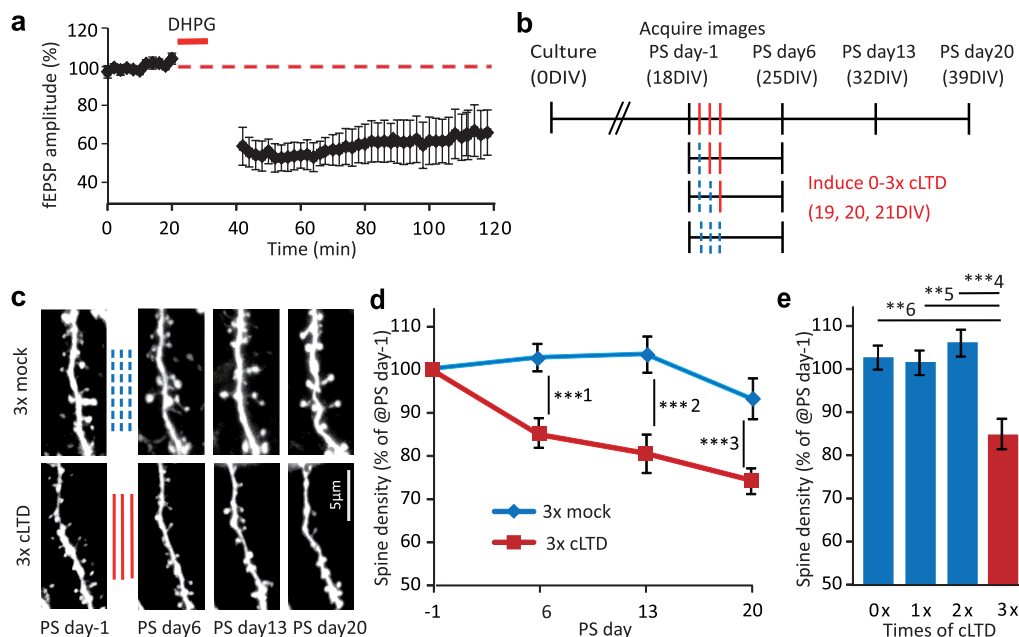

Figure 1 | Induction of chemical LTD and establishment of long-lasting synapse reduction by repetitive inductions of chemical LTD. (a) cLTD induced by exposure to DHPG (50 μM, 10 min). (b) Timeline of experimental procedures. The same dendritic segments were observed periodically using a confocal microscope. (c) Representative series of images. Each image is a reconstruction created by stacking images obtained at multiple focus planes, but we chose the segments running almost horizontally as possible. (d) Spine density as a function of number of days after repetitive induction of cLTD. The dendritic segments examined are 20 from 9 independent 3× mock cultures and 23 from 10 independent 3× cLTD cultures. P values are 2.3×10^{-7}, for ***1, 1.4×10^{-10}, for ***2 and 5.7×10^{-7} for ***3 in 2-factor factorial ANOVA ($F(1,41) = 17.7$, $P = 1.4 \times 10^{-4}$) followed by Bonferroni's test. Due presumably to an extended culture period and photodynamic damages, the spine density at PS day 20 was lowered. (e) The ineffectiveness of <3 × cLTD for synapse reduction as assayed at PS day 6 (4 days after the 3rd cLTD induction). The dendritic segments examined are 20, 17, 19, 23 from 9, 6, 9, 10 independent cultures for 0×, 1×, 2× and 3× cLTD induction groups, respectively. P values are 5.3×10^{-4} for ***4, 9.0×10^{-3} for **5 and 2.4×10^{-3} for **6 in one-way ANOVA ($F(3,75) = 8.6$, $P = 5.5 \times 10^{-5}$) followed by Bonferroni's test.
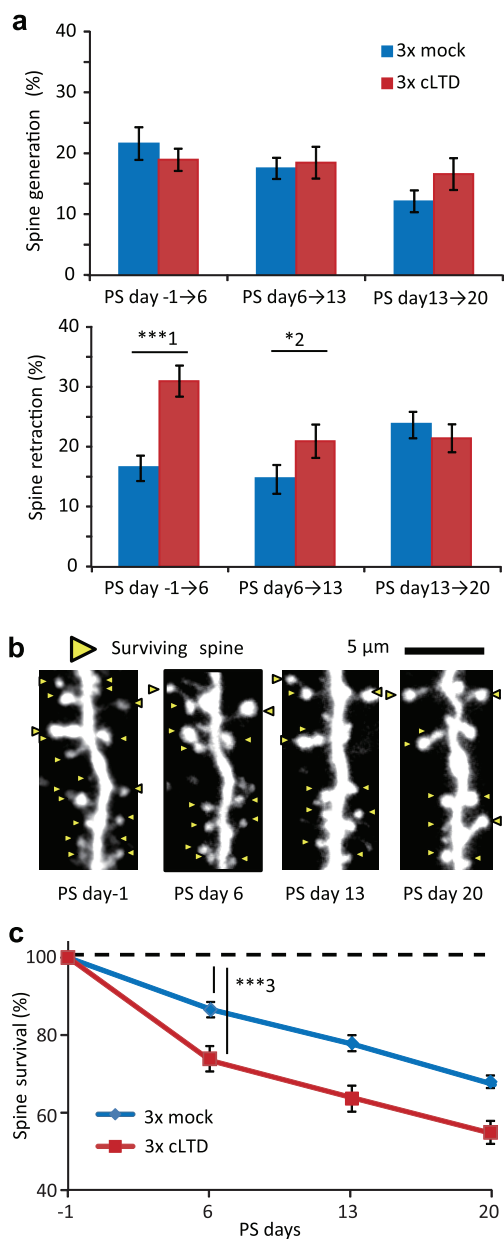
Figure 2 | **Dendritic spine dynamics (spine generation and retraction) after 3 inductions of cLTD.** (a) Spines generated and retracted during indicated periods, after 3 inductions of cLTD. Two consecutive images were compared, and both generated and retracted spines are identified and expressed as the percentages of the total number of spines that existed in the prior image. The dendritic segments examined are 20 from 9 independent 3× mock cultures and 23 from 10 independent 3× cLTD cultures. Significance values are $P = 8.7 \times 10^{-5}$, $t(40) = 4.4$ for ***1 and $P = 0.047$, $t(41) = 2.0$ for *2 in paired t-test. (b and c) Fate of individual spines that existed at PS day–1 (see Fig. 1b for timeline of experiment). Representative series of images is shown in (b). The plotting shown in (c) indicates the number of spines which were present at PS day-1 and remaining at PS day 6, 13 and 20. The spines newly generated after PS day-1 were not included. Note that disappearance of spines occurred immediately after the 3rd cLTD (PS day 6 means 4 days after the 3rd cLTD). After PS day 6, disappearance of spines occurred in a similar rate in both 3× mock and 3× cLTD groups. The dendritic segments examined are 19 from 6 independent 3× mock cultures and 19 from 8 independent 3× cLTD cultures. P value is 4.9×10^{-12} for ***3 in 2-factor factorial ANOVA ($F(1,36) = 14.0$, $P = 6.3 \times 10^{-4}$) followed by Bonferroni's test.

cells subjected to 3× cLTD and those exposed to a 3× mock stimulation (cells were injected with a fluorescent dye and examined after chemical fixation, which improved image definition). Contrary to our presumption, the mushroom type of spines decreased significantly.

This result can be interpreted in two ways; the mushroom type spines are retracted selectively, or, the stubby and thin type spines are retracted and the mushroom type spines are transformed into the stubby and thin type spines. To clarify this point, we classified living spines (compromising in unideal image definition) at PS day 2 (x hr before the 3rd cLTD) and observed each of the spines again 1 day later (at PS day 3). The disappearance of mushroom type spines was not significant in comparison with that of the stubby and thin types (Supplementary Fig. S1). This result supports the latter interpretation and indicate that mushroom type spines are not necessarily more stable than the other types of spines.

Discussion

Until the present study, RISE and LOSS were thought to be symmetric in various aspects, including the requirement of 3 repetitions, the time course of development, the long-lasting nature, and the involvement of related neurotropic molecules. However, the present study reveals that the phenomena are asymmetric in the spine dynamics leading to the final effects. Instead of 3 phases of spine dynamics in RISE[19] (raised fluctuation followed by biased fluctuation and re-balanced fluctuation), the dendritic spines decreased in 2 phases (biased fluctuation followed by re-balanced fluctuation). Nevertheless, the time courses of changes in synaptic strength in RISE and LOSS are apparently symmetric due to the delay of functional change from the morphologic change.

The present study demonstrates that the decrease in spine number begins immediately after the 3rd cLTD induction. However, according to our previous report, a decrease in population field EPSPs (excitatory postsynaptic potentials) became apparent 2 weeks after the LOSS-producing stimulus (3× cLTD) was applied[10]. Species differences between cultures from rats and mice could be relevant, so we examined field EPSPs in the present mouse cultures. Results confirmed that there was no decrease in field EPSP amplitude at PS day 6 (4 days after the 3rd cLTD induction) either in the present culture (Supplementary Fig. S3).

One possible explanation for the observed time gap is that the functionality of existing synapses should vary so that those of low or no functionality will be preferentially lost at first, leaving functional synapses intact. The functional synapses will begin to decrease ~10 days later. In the hippocampus *in vivo*, only a small fraction of existing synapses is functional[25]. During pruning of the climbing fiber synapses in the developing cerebellum, synapses of low functionality (synapses that evoke small EPSPs) are preferentially lost[26]. Note, however, that this explanation should not include the assumption that the spine types represent functionality, since the mushroom type of spines were not necessarily stable here (Fig. 4b and c).

A possible alternative explanation is that inhibitory synapses (not visualized here due to the absence of spiny structure) are eliminated in parallel with excitatory synapses, resulting in unchanged net field EPSPs. It is also assumable that the spines far from the somata would respond differently[27], since the present study examined only the first and second branches of apical dendrites. An explanation that the presynaptic terminals may remain longer than postsynaptic spines and activate nearby surviving spines is not eliminated either.

Omission of the "raised fluctuation phase" in LOSS is might be reasonable in logics. In RISE, there is no pre-determined reason for synapse formation at the site where a new synapse is formed. Hence, the dendrite needs to depend on fluctuation to form new synapses blindly and to let some of them survive. In contrast, during elimination there is no need to depend on fluctuation, as the target already exists.
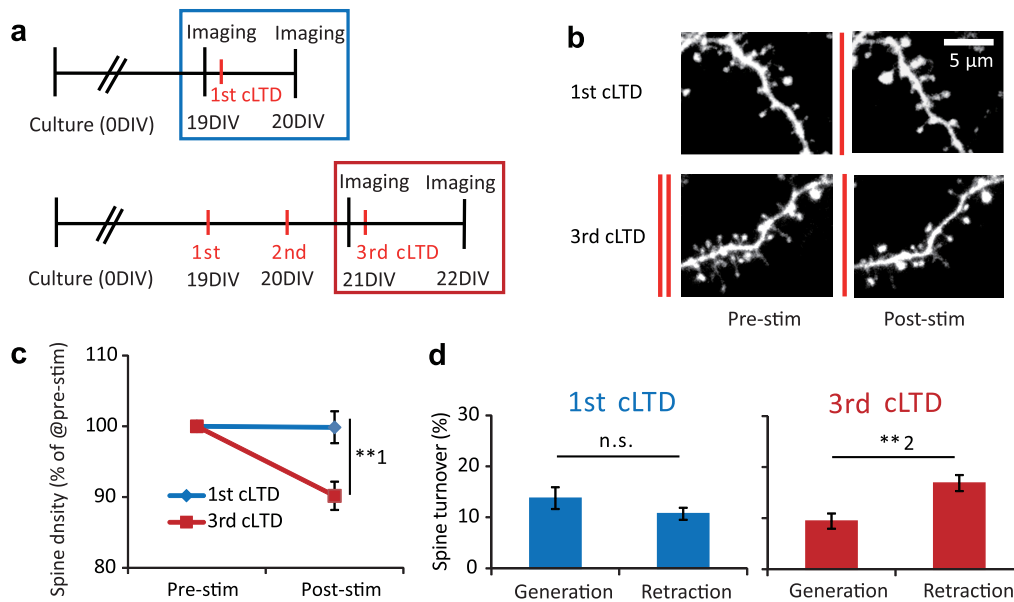
Figure 3 | Absence of the "raised fluctuation phase" during spine number reduction as confirmed by observation of 1-day interval. (a) Timeline of examination. The 1st imaging was made 1–4 hr (typically 2 hr) before cLTD induction and the 2nd imaging was made 24 hr after the 1st imaging. (b) Representative series of images. (c) Spine number reduction occurred within 1 day after the 3rd cLTD induction. (d) Dynamics of individual spines. Note that the disappearance of spines occurred only after the 3rd cLTD induction. In (c) and (d), the dendritic segments examined are 26 and 27 from 13 and 13 independent cultures for the 1st cLTD and the 3rd cLTD groups, respectively. Significance values are $P = 2.3 \times 10^{-3}$, $t(50) = 3.2$ for **1 and $P = 1.1 \times 10^{-3}$, $t(52) = 3.5$ for **2 in paired t-test.

Recently, Ramiro-Cortés and Israely[28] reported that a single induction of cLTD led to spine elimination in slice cultures from the mouse hippocampus. Since their results are contradictory to our previous findings, we re-examined whether a single cLTD would cause immediate morphological changes. As shown in Supplementary Fig. S4, we confirmed our previous results of decrease in spine number only after 3× cLTD inductions. The reason for this discrepancy remains unclear, but one possibility may be differences in the age of cultures[29], since Ramiro-Cortés' experiments used cultures of 8–11 days in vitro, which are more immature than those used in our experiments.

Our results appear to contradict the findings of Wiegert and Oertner[30], since a single optogenetically evoked LTD (oLTD) in CA1 pyramidal neurons resulted in elimination of some synapses. According to their report, the eliminated synapses were those with unstable responses, irrespective of spine type. Although some of our present findings are consistent with these results, the spine elimination after single oLTD induction is not concordant.

As far as the present results are concerned, criticism might occur that the spine elimination observed here would be a pharmacologic effect of repeatedly applied DHPG, apart from the consequence of repeatedly induced cLTD. As reported previously[10], however, long-lasting decrements in synaptic strength and synapse density are resulted not only from repeated cLTD inductions through mGluR activation but also from those through NMDA (N-methyl-D-aspartate) receptor activation or those through Na$^+$/K$^+$ ATPase inhibition. Thus it is likely that the present repetition-dependent slowly developing long-lasting structural synaptic plasticity is not the pharmacologic effect of repeated activation of mGluR but the consequence of repeated induction of cLTD. We propose that RISE and LOSS should serve as the models in vitro for the cell biological analyses of repetition-dependent memory consolidation.

Methods

Organotypic slice culture of the mouse hippocampus. Organotypic slice cultures of the hippocampus were prepared from the Thy1-YFP H line mice[31]. The mice (listed as B6.Cg-Tg (Thy1-YFP) HJrs/J; stock number 003782 in the supplier's catalogue) were

purchased from Jackson Laboratory (Maine). The cultures are prepared as previously reported[4,32]. Briefly, a newborn pup of either sex was anesthetized and sacrificed at postnatal day 7. The hippocampus from either side was isolated, and its dorsoventrally central 1/3 portion was cut into 250-µm thick slices with a McIlwain tissue chopper. Preliminary examinations indicated that sex and side of the hippocampus did not affect the results (Dr. R. Shigemoto, personal communication). Each slice was laid on a piece of polytetrafluoroethylene filter (~5 × 5 mm), which was placed in an insert of a Millicell CF (Millipore) multiwell dish.

The cultures were maintained in a humidified atmosphere of 34°C for 18 days before beginning experiments. Culture medium, composed of 50% minimal essential medium of Hanks' salt, 25% Hanks' basal salt solution, and 25% heat-inactivated horse serum (all from Gibco), was renewed every 3–4 days.

Chemical LTD induction. For the long-term maintenance of aseptic conditions, LTD was induced by chemical means (cLTD) as reported previously[10]. DHPG (3,5-dihydroxyphenylglycine), an agonist specific for group I metabotropic glutamate receptors, (Tocris, Bristol, UK), was dissolved in distilled water at 10 mM for storage. For cLTD induction, the slice culture at 19 days in vitro (DIV) was exposed to the culture medium (minus sera) containing 50 µM DHPG (prepared immediately before use). New culture medium was introduced 10 min later, in order to dilute the DHPG, followed by another replacement with culture medium. The second cLTD was induced 1 day later, in the same manner, and the third cLTD was induced 1 day after the second cLTD. Cultures treated so were referred to as 3× cLTD. Control specimens were prepared either by inducing cLTD only once (1× cLTD) or by repeating the same procedure with a medium containing no DHPG (3× mock).

Electrophysiology. Extracellular recordings of CA1 neurons' activity were performed conventionally[12]. Briefly, slice cultures were transferred to a recording chamber and a glass microelectrode filled with ACSF (input resistance, 5–10 MΩ) was inserted into the CA1 pyramidal cells' somatic layer for recording. A monopolar electrode was placed in the CA3 cells' somatic layer for stimulation. Test stimulation pulses of 100 µs were delivered every 30 sec to record field EPSP amplitude. Data were binned for 2-min periods and plotted.

To estimate the total synaptic strength of the CA1 cells, using the same experimental configuration as above, we delivered a 0.4-mA stimulus current, which evoked fEPSP of saturated amplitude, meaning that practically all inputting CA3 axons were activated. For each slice, this test was performed at more than 3 sites in the CA1 area, and thus-obtained maximum value was adopted as the slice's representative value.

Microscopic morphometry. Following the protocol described previously[19], laser confocal microscopy was applied to the first branch (emerging from the dendritic shaft) or the second branch (emerging from the first branch) of the apical dendrites of CA1 pyramidal cells located within 150 µm from the soma. To minimize the effect of culture medium exchange on spine dynamics, we fixed the timing of medium renewal at 3 days prior to observation, except for observations at 1-day interval where medium was changed 2 days prior to the first observation. The observation date was
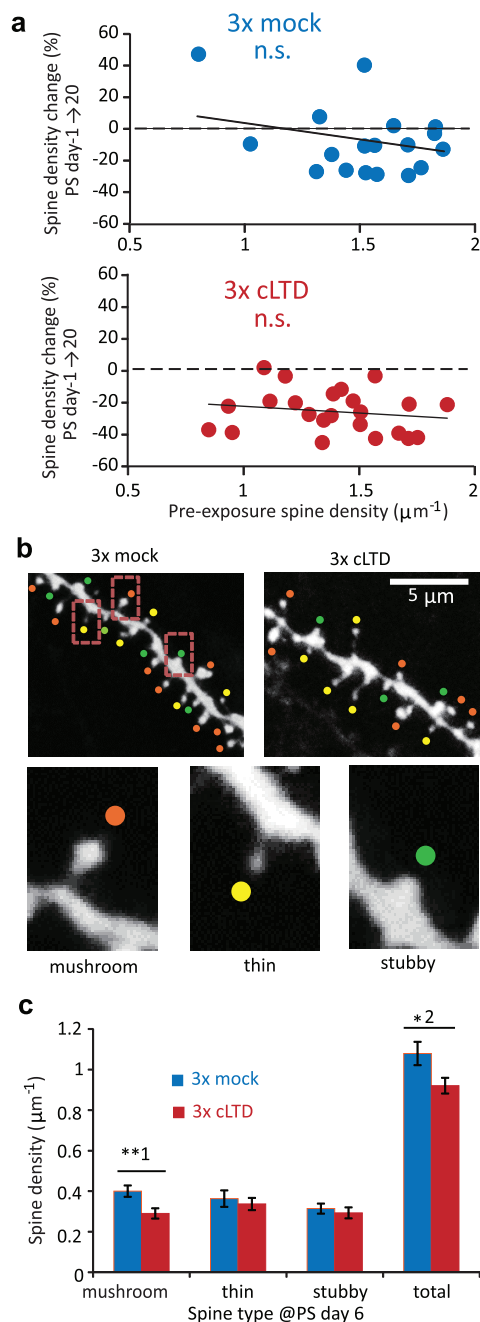
Figure 4 | Factors that may affect the fate of dendritic spines.
(a) Dependency on pre-existing density of the dendritic segments. Spine number increments depended on pre-existing density after 3× cLTP. However, there was no correlation between spine number reduction and pre-existing spine density. Changes of 0 to 60% indicate increases and changes of 0 to −60% indicate decreases. Regression lines were drawn, although they were statistically insignificant. R (regression) and F (variance) values are $R = 0.355$, $F = 0.125$ for the 3× mock group and $R = 0.173$, $F = 0.431$ for the 3× cLTD group. (b and c) Dependency on the spine shapes. The spines are classified into 3 types. Representative images are shown in (c). In this examination, the cultures were fixed and the cells were injected with a fluorescent dye. Thus, comparisons are made between spine type distributions of 3× mock and 3× cLTD groups as shown in (d). Note the reduction in mushroom type spines. In (d), the dendritic segments examined are 28 and 26 from 9 and 10 independent cultures for the 3× mock and 3× cLTD groups, respectively. Significance values are $P = 5.3 \times 10^{-3}$, $t(52) = 2.9$ for **1 and $P = 0.027$, $t(46) = 2.3$ for *2 in paired t-test.

determined beginning on the day of first exposure to DHPG as post stimulus (PS) day 0, so the day before the first DHPG application is referred to as PS day−1.

A laser-scanning confocal optics single photon system (Olympus FV300) was mounted onto an Olympus IX71 inverted epifluorescence microscope equipped with a 60× water immersion objective lens (UplanSApo60, NA 1.20). A glass-bottom dish (Matsunami) was placed on a temperature-controlled stage (Tokai-hit, set at 34°C), within which the filter piece carrying a cultured slice was placed. A 50-μl aliquot of the original culture medium was transferred onto the top of the slice to prevent drying, and no medium perfusion was made during image acquisition. To minimize fluorescence photo bleaching, laser intensity was limited to 1–3% of maximum. Acquisition conditions were unchanged over different days of observation.

On the occasion of first imaging (at PS day−1 typically), a lowly magnified image was acquired in order to facilitate target cell identification in later examinations. A region of interest (ROI) of 60 μm × 60 μm was set approximately at the center of CA1 stratum pyramidale. In the center of the ROI, a YFP-positive pyramidal neuron was captured. We acquired 12 serial images (24 scans including Kalman noise reduction) at 0.75-μm Z-axis steps (9 μm Z-thickness in total).

With our optical system, 1 μm in the X-Y plane corresponds to 17 pixels. In typical examinations, identical ROIs were imaged 4 times during the examination periods (of 21 days). After all image acquisition, we extracted out of each ROI a dendritic segment running nearly horizontally for a length of >10 μm throughout the observation period and stacked into a 2-dimensional plane.

It is arguable that the spines protruding vertically would not be captured in thus-obtained 2-dimensional images. We know that three-dimensional reconstruction is desirable for the analysis of spine dynamics. However, to reconstruct a sufficiently fine three-dimensional image including vertically protruded spines, we must obtain serial images of 15-μm thickness (i.e. 9 μm plus 6 μm, assuming maximal spine length 3 μm up and down) at 0.25 μm Z-axis steps. This means that 60 images (120 scans including Kalman noise reduction) must be taken each time of examination. Preliminary examination revealed that the fluorescence bleaching as well as photo-dynamic damage to the cell was remarkable in such a protocol. From the requirement of long-term examination, we made compromise to use 2-dimensionally projected images, in which protocol we could minimize the bleaching and cell damage. Hence, the spines protruding vertically from dendritic shaft were not captured so that the numbers of spines listed here might include underestimation. It is unlikely, however, that the vertically protruded spines would be selectively influenced by stimulation. The underestimation, if any, should be common in both stimulated and control specimens, and thus the comparison between the specimens should be valid.

Analyses were performed using ImageJ, following the conventional protocols[33]. Spines were counted by a cell counter plugin and the lengths of dendrites were measured using a segmented line tool from a raw picture, under blind conditions. By comparing two images, a spine recognized in the present image but not in the previous one was referred to as "generated" and a spine recognized in the previous image but not in the present one was referred to as "retracted". A spine displaced laterally within 1 μm in appearance was classified as identical. Although filopodia were rare in our preparation (probably because of well-matured cultures), we excluded filopodia-like protrusions by following conventional criteria: head diameter ≦1.2 × neck diameter or neck length ≥ 3 × neck diameter[14]. The rates of spine generation and retraction were represented as percentages of the total spine number in the segment under examination. Spine stability was determined by examining the spines existing at PS day−1, which were calculated as a percentage of remaining spines.

Imaging procedures (from removal from to return to our incubator) were completed within 30 min (including laser illumination for 5 min at maximum). The slice was returned to the original Millicell filter insert to continue culturing.

Fluorescent dye injection. Intracellular injections of fluorescent dye were conducted following the methods of Pace et al.[34] with slight modifications. The cultured slice was mildly fixed with 2% paraformaldehyde in a balanced salt solution, and a glass micropipette filled with 8% Lucifer yellow Li salt (LY; Nacalai Inc., Kyoto, Japan) was inserted slowly into the CA1 pyramidal cell layer. Penetration of the micropipette into the pyramidal cell was recognized by the sudden emergence of cell-shaped fluorescence. A negative current (1–3 nA) was applied through the micropipette for 3–5 min. The LY injection was performed on 1 or 2 cells for each slice. The slice was then fixed overnight with 4% paraformaldehyde.

Statistical analyses. All figures include indication of the means ± standard errors of means. For statistical comparisons of two sample groups, Student's (in cases of identical variances) or Welch's paired t-tests (in cases of non-identical variances) were applied. For comparisons of ≥3 sample groups, ANOVA followed by Bonferroni's tests were applied. For comparisons of datasets obtained by repeated measurements on the same specimens, 2-factor factorial ANOVA followed by Bonferroni's tests were applied. Statistically significant differences are shown as * (for $P < 0.05$), ** (for $P < 0.01$), or *** (for $P < 0.001$). Exact P and F values are indicated in the figure legends.

Animal care. The study was carried out in accordance with the Regulation on Animal Experiments of the Animal Experiments Committee of Osaka University. The protocol was approved by the Committee for Animal Experiments of Osaka University Graduate School of Frontier Biosciences (No. 12-027).

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Author contributions

K.T.-Y. and A.O. designed the experiments; S.H. and S.S. conducted the research; K.T.-Y. instructed the experimenters in the procedures; and A.O. wrote the paper.

Additional information

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