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

Sho Hasegawa, Shigeo Sakuragi, Keiko Tominaga-Yoshino & Akihiko Ogura

Laboratory of Synaptic Plasticity, Osaka University Graduate School of Frontier Biosciences, Yamadaoka 1-3, Suita 565-0871 Osaka, Japan.

Memory is fixed solidly and become long-lasting by repetition of experiences or tasks1–3. However, the cellular mechanisms underlying this repetition-dependent consolidation/reconsolidation remains 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 synapses4–8. We termed this phenomenon as RISE (Repetitive-LTP-Induced Synaptic Enhancement) 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 (LTD-repetition-Operated Synaptic Suppression)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 nature4,9, and involvement of related neurotrophic molecules12 (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 disease13. Memory reversal coupled with spine formation and elimination is reported14. It is also possible, however, to assume that both synapse formation and elimination contribute to memory15. According to Hebb’s principle16, 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 reported17,18. 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 density19. In the stable cultures, the dendritic spines of the CA1 pyramidal neurons are in the state of equilibrated fluctuation20,21, 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...
after 1 dendritic segments examined are 20 from 9 independent 3 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 confocal microscope. (c) Representative series of images. Each image is a reconstruction created by stacking images obtained at multiple focus planes, but by exposure to DHPG (50 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 cLTD10 (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 RISE19, 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 density19. 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 stable22–24. 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 2.3×10−5, 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 = 1.4×10−5) 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 = 5.5×10−3) followed by Bonferroni’s test.
cells subjected to $3 \times \text{cLTD}$ and those exposed to a $3 \times \text{mock}$ 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 (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 ($3 \times \text{cLTD}$) was applied. 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. During pruning of the climbing fiber synapses in the developing cerebellum, synapses of low functionality (synapses that evoke small EPSPs) are preferentially lost. 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.

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 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 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, 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, 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 cLTD 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, however, long-lasting decrements in synaptic strength and spine 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. The mice (listed as B6.Cg-Tg(Thy1-YFP)Hrfs/J, stock number 003782 in the supplier’s catalogue) were purchased from Jackson Laboratory (Maine). The cultures are prepared as previously described.

Chemical LTD induction. For the long-term maintenance of aseptic conditions, LTD was induced by chemical means (cLTD) as reported previously. 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. 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 EPSP 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, 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 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 protrusions by following conventional criteria: head diameter $\leq 14 \mu m$, neck diameter $\leq 1.2 \mu m$ neck diameter or neck length $\geq 3 \times$ neck diameter. 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 then fixed with 4% paraformaldehyde.

Fluorescent dye injection. Intracellular injections of fluorescent dye were conducted following the methods of Pace et al. 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 was inserted 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 (−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 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 $\geq 3$ 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.

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