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Studies of the functional and morphological changes of the long-lasting synaptic suppression after repetitive LTD inductions.

繰り返し LTD 誘発後の長期持続的シナプス弱化現象の機能的および形態的変化の解析

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要旨

記憶は動物の生存にとって重要で、脳の主要な機能な一つである。行動上の記憶は、事象の経験後即座に成立する「獲得」の相と、それを長期に維持する「固定」の相との、少なくとも 2 つの相に分けられる。それに対応する細胞レベルの現象として、既存の神経回路で即時にシナプスの伝達効率が変化して情報流路が変わる「短期可塑性」の相と、その後にシナプス自体の新生・廃止を含む構造変化で情報流路が維持される「長期可塑性」の相が想定されている。

これまで、記憶の細胞レベルの研究では、成体げっ歯類脳から作成した海馬切片で、強いシナプス活動後にシナプス伝達効率が増大する LTP (Long-Term Potentiation) と、中程度の活動後に伝達効率が減少する LTD (Long-Term Depression) が詳しく解析され、その分子基盤までほぼ解明されている。これらの現象は、既存のシナプスで即時に起こる短期可塑性現象であり、行動上の「獲得」相に対応すると考えられるが、「固定」相をも解析できるかについては意見が分かれる。LTP/LTD には、確かにシナプスの肥大・縮小という構造変化が伴うが、これが長期可塑性に直結するかは未詳である。いいかえると、長期可塑性の機構は適切なモデル系がなく、解析が進んでいない。

冨永ら（2002）は、長期間の観測が可能な安定培養下の海馬切片を用い、次のような現象を見出した。1 回誘発した LTP は 24 時間以内に消失してしまうが、LTP を適切な時間間隔をおいて 3 回以上繰り返し誘発すると、シナプスの新生を伴う新たな伝達強化現象が起こり、数週間以上持続した。これを RISE (Repetitive-LTP-Induced Synaptic Enhancement) とよぶ。また、篠田ら（2005）は、LTD の繰り返し後にシナプスの廃止を伴う長期的シナプス弱化現象が起こることを見出して LOSS (LTD-repetition-Operated Synaptic Suppression) と名付けた。この 2 つの対称的な構造可塑性現象は、行動上の記憶「固定」相に対応する細胞レベルの可塑性機構の解析モデル系になる可能性がある。

大江ら（2013）は、海馬 CA1 領域の錐体細胞の一部が蛍光タンパク質を発現するマウスから培養切片を作成し、RISE 生起時の樹状突起棘（シナプスの後構造）の動態を経時的に追跡した。その結果、棘は常時発出と退縮の「ゆらぎ平衡」状態にあるが、刺激後にまず発出・退縮とも上昇する「ゆらぎ増大」期、ついで退縮率が先に刺激前レベルに戻る「ゆらぎバイアス」期、そして発出率も戻る「ゆらぎ再平衡」期を経て確率論的に (stochastically) 増加することが明らかになった。またこれらの動態は既存棘密度の低い樹状突起部分に限って起きていた。

RISE と LOSS は、さまざまな点で鏡像的な現象である。そこで私は、本論文の第 1 章で、LOSS 生起時の棘動態も RISE 生起時と同様な確率論的経過をとるかについて追跡した。その結果、意外なことに、3 回目の LTD 誘発刺激の後すぐに棘退縮率が上がり、棘数減少が始まった。また、この変化は既存棘密度と無関係にすべての樹状突起部分で起きていた。すなわち、これらの点において、RISE と LOSS は鏡像的ではなかった。

しかし、この知見は新たな問題を生む。構造変化は急速に起こるのに、機能変化（シナプス後電位の減弱）は緩徐に進行する。この時間的乖離をどのように説明するかという問題である。私は本論文の第 2 章で、想定可能な 3 つの仮説、「1. 未熟で非機能的な棘が先に退縮する」、「2. サイレントシナプスが先に退縮する」、「3. シナプス前構造を伴わないいわゆる orphan spine が先に退縮する」を立て、当否を検討した。その結果、未熟な棘とされる切株型、細棒型シナプスは減っておらず、仮説 1 は支持されなかった。また、LTP 誘発規模は不変で、仮説 2 も支持されなかった。しかし、シナプス前構造染色の結果、初期にシナプス前構造は減少しておらず、仮説 3 を支持する結果がえられた。

さらに私は、LOSS 現象に伴う一連の形態変化をもたらす細胞内信号について、初期的な薬理学的検討を加えた。阻害剤による LOSS 生起抑制実験の結果から、calcium/diacylglycerol-dependent protein kinase (PKC) および calcium/calmodulin-dependent protein kinase (CaMKII) の関与が示唆された。これらの結果も第 2 章に記載した。

以上の解析結果は、行動上の記憶の「固定」過程の細胞基盤を解析する上で、とくにシナプス廃止による神経回路改編の機構を解析する上で、新たな知見を加えるものである。

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総論

記憶は動物の生存にとって最も重要な機能の一つであり、脳の主要な機能である。記憶の細胞レベルの基盤として、「シナプス可塑性」が想定されている。これは神経細胞間の結合であるシナプスの伝達効率が活動依存的に変化する現象であり、外部からの入力によって引き起こされる。

記憶形成には、即座に形成される短期的な相である「獲得」と、その情報を保存するための長期的な相である「固定」の二つの相に分けることができる。これらの相に対応する細胞レベルの現象は、「獲得」では既存のシナプスでの伝達効率の変化（増強または減弱）、「固定」ではシナプス構造自体の新生または廃止であると想定されている。

記憶の細胞レベルの研究の多くは、成体げっ歯類脳より作成した海馬切片を用いて行われてきた。たとえば高頻度の刺激を加えることによって既存のシナプスの伝達効率が増大する LTP(Long-Term Potentiation)が、中程度の刺激により伝達効率が減少する LTD(Long-term Depression)が誘発されることが明らかになり、その分子機構も概略解明されている。これらの現象は、既存シナプスの調節として即時に成立する点から、「獲得」の基盤現象としての資格を満たすが、これがそのまま長期化するか、いいかえると「固定」過程に直結するか否かは不明だった。LTP/LTD には、当該シナプスの肥大や縮小という形態変化が伴うことが報告されているが、その肥大／縮小形態が長期持続するかも不明である。つまり、「固定」の機構は未だ解明されたとはいえない。

当研究室では、この問題に答えるために、長期間の実験観測が可能な培養下の海馬切片を用いて研究を行ってきた。その結果、LTP/LTD は、シナプス電位についても形態変化についても 24 時間以上は持続せず、消滅してしまうことが分かった。

私たちの日常の記憶・学習経験でも動物の行動実験でも、記憶・学習の長期化には繰り返しが重要である。そこで、培養海馬切片に LTP を 24 時間間隔で 3 回、繰り返し誘発した。すると LTP 自体は消失した後に、ゆっくりと伝達強度が再増大し、その増大状態は数週間以上持続した。このとき新規のシナプス形成が起こっていた。この現象を RISE (Repetitive-LTP-Induced Synaptic Enhancement) とよぶ。また、LTD を繰り返し誘発すると、LTD 自体は消失した後に、シナプスの廃止を伴う長期持続的な減弱が起こった。この現象を LOSS (LTD-repetition-Operated Synaptic Suppression) とよぶ。これらの現象は、記憶の「固定」過程に想定されていた構造変化現象であり、実際に長期持続することから、「固定」過程の in vitro 再現と考え、解析を進めてきた。

これまでに、RISE と LOSS には多くの共通点、そして対称的（symmetric）な点が存在することが分かっている。共通点として、まず成立に 3 回の繰り返しの刺激が必要かつ十分な点である。どちらの現象も 2 回の刺激では成立せず、4 回以上繰り返しても増強・減弱がそれ以上大きくなることはない。またどちらも成立にタンパク質合成が必要である。次に、成立に時間を要する点である。どちらの現象も、3 回目の刺激による LTP/LTD はいっ

たん消滅し、その後数日経過したのちに再びシナプス間の伝達強度が増大・減少し、その状態が長期にわたって持続する。

逆に対称的な点としては、変化の方向が正反対な点である。RISE ではシナプス新生を伴い伝達強度が上昇するが、LOSS では対照的にシナプス廃止を伴って伝達強度が低下する。さらに、成立に関与する信号分子に関しても対照的である。RISE では神経細胞の軸索・樹状突起の伸長や神経細胞死防止に関与するとされる脳由来神経栄養因子 BDNF とその同受容体 TrkB の信号経路が介在するが、LOSS では軸索・樹状突起の短縮や神経細胞死誘発に関与するとされる proBDNF－p75NTR の信号経路が介在する。

当研究室の大江らは、RISE 誘発時の樹状突起棘（スパイン；興奮性シナプスのシナプス後構造）の動態に注目した研究を行った。海馬 CA1 領域の錐体細胞の一部にランダムに蛍光タンパク質が発現した遺伝子組み換えマウスを用い、RISE 刺激誘発後のスパインを観察した。その結果、RISE によるスパイン新生は以下のような段階を踏んで進行することが分かった。通常の状態でもスパインは常に発出と退縮を繰り返している動的平衡状態にあるが、RISE 生起刺激後、それらの割合が共に上昇する（raised fluctuation phase）。次に退縮率のみ先に元のレベルに戻るため、結果的にスパイン密度は上昇する(biased fluctuation phase)。しかし、数日後には発出率も元のレベルまで戻って、新しい動的平衡状態に入り高スパイン密度の状態が維持される(re-balanced phase)。加えて、これらのスパイン密度増加は、既存のスパイン密度が低い樹状突起領域で優先的に発生していることが分かった。

この結果から新たな問いが生まれる。このスパイン動態においても、ほかの多くの点と同様に RISE と LOSS は対称的なのだろうか。

本研究の第 1 章では、LOSS 誘発後のスパイン動態に注目した。LOSS でも RISE と同様に、段階的なスパイン廃止が行われているのだろうか。また、既存のスパイン密度に依存して LOSS の発生する樹状突起領域に差があるのだろうか。先の実験と同様の手法を用い解析した結果、RISE とは異なり、LOSS では 3 回目の LTD 誘発刺激の翌日にはスパイン退縮率が上昇し、シナプス密度減少が開始された。すべての観察期間においてスパイン発出率の上昇は見られなかった。つまり RISE とは異なり raised fluctuation phase は存在しなかった。また、スパイン密度減少は既存スパイン密度に依存せず、どの樹状突起領域でもスパイン密度減少が見られた。以上の結果より、スパイン動態に関しては、RISE と LOSS は対称的ではないことが分かった。

また、細胞内シグナルの活性化も形態変化と同様、刺激後の早い段階で活性化されることが報告されている。当研究室の桜木らの研究で、LOSS 生起には、proBDNF-TrkB の経路が活性化されることが分かっている。しかし、3 回目の LTD 誘発から proBDNF の合成・分泌までの間はどのようなシグナル経路が働いているのだろうか。第 2 章前半ではこの問題について初期的な検討を試みた。私は、LTD 誘発のために刺激した mGluR のシグナル経路が即座に誘発される LTD だけでなく、その後に誘発される LOSS のトリガーとしても機能しているという仮説を立て、その検証を行った。その結果、mGluR の下流でタンパク

質合成の活性化に寄与する PKC 信号系の活動が必要なことが分かった。また、スパイン廃止を起こす実行経路はどのようなものだろうか。スパインの形態変化にはスパイン内部のアクチン細胞骨格の再編成が想定される。同じく第 2 章ではこれにも注目し、細胞骨格結合タンパク質の関与を調べた。阻害剤を用いた実験により、LOSS によるスパイン廃止では細胞骨格の安定性を制御する CaMKII の関与が示唆された。

しかし、同時に新たな疑問が生じた。LOSS によるシナプス伝達強度の低下は約 1 週間後から始まった。ところが本実験の結果ではスパインの減少はもっと早く始まっている。つまり、スパイン数は減少しているにも関わらず、伝達強度は変化していない時期があることになる。そこで第 2 章後半では、二つの減少の開始時期の乖離の原因を解明するべく、以下の 3 つの仮説を立てた。(1) 通常の状態でもスパインには大きく成熟したスパインと、未熟で伝達に関与しないスパインに分類することができる。その中で、LOSS では未熟なスパインが優先的に廃止されるため、シナプス伝達の変化が遅れるという可能性。(2) 海馬のシナプスには、通常のシナプス伝達に用いられる AMPA 受容体を持たず、伝達に関与しないサイレントシナプスと呼ばれるものが相当数存在する。LOSS ではこれらが優先的に廃止されるため、伝達の変化が遅れるという可能性。(3) スパインにはシナプス前構造を伴って機能的シナプスを形成しているものと、オーファンスパインと呼ばれるシナプス前構造を持たず機能的シナプスを成さないスパインとが存在する。LOSS ではこのオーファンスパインが優先的に除去されている可能性。以上の 3 つの仮説をそれぞれ検証し、その結果仮説 3 を支持する実験結果が得られた。LOSS 生起刺激後にはオーファンスパインがまず廃止され、その後に機能的なスパインが廃止されることで、形態面と機能面の乖離が起きる可能性が高い。

本学位研究で、私は、記憶固定過程の細胞基盤であるシナプスの構造可塑性の一つの表現型であるシナプス廃止について、細胞レベルの機構解析を行い、繰り返し LTD 誘発後のスパインの動態、シナプス応答とスパイン動態との関係、細胞内信号経路の一端を明らかにした。今後、RISE を解析モデル系として用いたシナプス新生と、LOSS を解析モデル系として用いたシナプス廃止のさらなる機構解析により、記憶の固定過程の細胞機構の理解が進むことが期待される。

General introduction and summary

Memory is one of the most important functions for animals to survive and is the major function of the brain. The cellular basis of memory is assumed to be synaptic plasticity, activity-dependent modifications of synaptic transmission strength leading to the alteration of information flow through neural circuits in the brain.

The process of memory formation *in vivo* is divided to two phases: the memory "acquisition" phase which occurs instantaneously using existing neural circuits; and the memory "consolidation" phase which occurs after the acquisition phase to fix the acquired information. Corresponding to these two phases of the memory *in vivo*, synaptic plasticity on the cellular level is assumed to have two phases: the short-term phase which occurs instantaneously by post-translational modification in the pre-existing synapses; and the long-term phase which converts the short-term phase to a long-lasting one by translational modification that leads to structural changes including the formation of new synapses and the elimination of existing synapses.

The majority of studies of synaptic plasticity have been performed using thin slices of the hippocampus acutely prepared from adult animal brains. A strong (*i.e.* of high-frequency) neural activity leads to an increase in synaptic transmission efficiency in the pre-existing synapses, and a moderate (*i.e.* of medium-frequency) neural activity results in a decrease in transmission efficiency. These phenomena, called LTP (Long-Term potentiation; although it is undetermined how long it lasts) and LTD (Long-Term Depression), respectively, served as useful *in vitro* model systems for the analyses of the acquisition phase of memory. By the end of the 20th century, the gross outlines of LTP and LTD were revealed as externalization and internalization, respectively, of neurotransmitter receptor molecules in existing synapses. However, whether these phenomena are truly long-lasting or not, in other words, whether they lead to structural changes that explain the consolidation phase of memory, remains unclear, since the acute hippocampal slices do not hold for more than several hours. Morphological changes that may suggest the initiation of structural plasticity, enlargement and shrinkage of synapses, are known to accompany LTP and LTD in fact. But the longevity of these morphological changes is unknown, meaning that the cellular bases of memory consolidation still remain unrevealed.

The organotypic slice cultures of rodent hippocampus, prepared from neonate brain and grown to maturation by two weeks of culture, reproduce the neural circuits equivalent to those in the brain *in vivo* and allow truly long-term examinations for

weeks and months. By using the cultured slices, Tominaga-Yoshino *et al.* (2002) revealed that the changes in synaptic transmission efficiency and spine shape accompanied by LTP or LTD did not last for more than 24 hours.

From our daily experience as well as from behavioral experiments of learning and memory in animals, we know that the repetition of performance and training is essential for fixation of learning/memory. Hinted by this fact, the authors induced LTP repeatedly more than 3 times in the hippocampal slice and found that, after the disappearance of LTP, a different type of strengthening of synaptic transmission developed slowly. This enhanced state lasted for more than weeks, accompanied by formation of new synapses. They named this phenomenon RISE (Repetitive-LTP-Induced Enhancement).

Shinoda *et al* (2005) found an opposite phenomenon: LTD inductions repeated more than 3 times brought about a weakening of synaptic transmission accompanied by elimination of synapse after the disappearance of LTD itself. This is named LOSS (LTD-repetition-Operated Synaptic Suppression). These phenomena are the types of long-lasting structural plasticity, assumed as the cellular basis of memory consolidation. Regarding these phenomena *in vitro* models for the analysis of memory consolidation process *in vivo*, the laboratory I belong to has been exploring the cellular mechanisms underlying RISE and LOSS.

It has been revealed that there are many common and symmetric aspects between RISE and LOSS. As common aspects, 3 repetitions of stimulation are necessary and sufficient to induce RISE and LOSS: both are not produced by 2 repetitions, but 4 repetitions produced no further changes. Protein syntheses are necessary to produce RISE and LOSS. Both phenomena develop slowly: there are apparently "latent" periods before the changes in synaptic strength take place.

As symmetric aspects, directions of changes in RISE and LOSS are opposite, accompanied by synapse formation in RISE and synapse elimination in LOSS. Critical cytokines that mediate RISE and LOSS are contrasting. A BDNF-TrkB (Brain-Derived Neurotrophic Factor-Tyrosine Receptor Kinase type B) pathway, which is known to play roles in the extension of axon/dendrite and in the suppression of neuronal death, is involved in RISE, whereas a proBDNF-p75^{NTR} (precursor of BDNF-p75 NeuroTrophin Receptor) pathway, which is known to play roles in the retraction of axon/dendrite and in the promotion of neural apoptosis.

Oe *et al.* (2013) focused on the behavior of dendritic spines, the postsynaptic structure of excitatory synapse) during the development of RISE. Using transgenic mice which express YFP (Yellow Fluorescent Protein) in a limited population of CA1 pyramidal

neurons of the hippocampus, they concluded that RISE proceeded along the stochastic process as follows: 1) even without stimulation, the dendritic spines are constantly generated and retracted, but both rates are balanced ("balanced fluctuation phase"); 2) after repeated inductions of LTP, both the rates of spine generation and retraction increased, but both are still balanced resulting in no net increase in spine number ("raised fluctuation phase"); 3) then, the rate of spine retraction returns to the pre-stimulus level earlier than that of spine generation leading to a net increase in spine number ("biased fluctuation phase"); 4) finally, the rate of spine generation returns to the pre-stimulus level to regain balance so that the increased spine number is maintained ("re-balanced phase"). Additionally the authors found that the above stochastic process (phases 2-4), occurred limitedly in the dendritic segments having low pre-existing spine density.

From these results a new question arises: does the decrease in synapse number in LOSS proceed in stochastic process same as that in RISE. In the first Chapter of this thesis, I focused on this question by examining the spine dynamics during the development of LOSS. Contrary to the expectation, the rate of spine retraction was elevated soon after the repeated inductions of LTD, resulting in a rather quick decrease in spine number. RISE and LOSS were not symmetric in this respect. In addition, the reduction in spine number occurred irrespectively to the pre-existing spine density. RISE and LOSS were not symmetric in this respect, either.

In addition to morphological change, previous report showed that signaling pathways which lead to LOSS is activated quickly after 3rd LTD induction. In the Chapter 2, I tried to get clues for the subcellular/molecular bases of LOSS. As explained above, Sakuragi *et al.* (2013) showed that the proBDNF-TrkB pathway works to produce LOSS. Then, what signaling pathway leads to the synthesis and secretion of proBDNF after the repeated inductions of LTD.

I assumed that a signaling pathway, which is downstream of mGluR activation that is performed to induce LTD, would also trigger to produce LOSS. In other words, PKC (Calcium/diacylglycerol-dependent Protein Kinase) would have two roles; a quick one to induce LTD and a slow one to trigger LOSS. Application of a PKC inhibitor actually blocked the development of LOSS.

An additional question addressed in the Chapter 2 is what machinery brings about the elimination of spines? I also focused on this signaling pathway and examined the involvement of several actin binding proteins. The experiment using inhibitor indicated that CaMKII, which regulates spine stability, is involved in spine density decrease after repetitive LTD inductions.

Then, another new question arises. As explained above, there is a latent period between the LOSS-producing stimulus and reduction in synaptic strength. In case of RISE, the latent period was explained by the presence of "raised fluctuation phase" with which no net increase in spine number was coupled. But in case of LOSS, what is the cause of this disparity of onsets of structural change (spine number decrease) and functional one (EPSP decrease).

In the second Chapter, I addressed this question by testing three possible hypotheses: 1) there are mature/functional and immature/non-functional synapses by nature and the immature ones disappear at first resulting in the delayed onset of functional change; 2) there are silent and active synapses by nature and the silent ones disappear at first; 3) there are spines coupled with presynaptic counterparts and those without (orphan spines) by nature and the ones without disappear at first. I obtained the results indicating possibility that the orphan spines disappear preferentially at the beginning of the development of LOSS, supporting the hypothesis 3.

In this thesis, I analyzed the cellular mechanisms underlying the synapse elimination, which is a form of structural synaptic plasticity, using a model phenomenon called LOSS, the synapse number reduction after the repeated inductions of LTD in the slice cultures of rodent hippocampus. Together with the mechanistic analyses of RISE, a contrasting form of structural synaptic plasticity, the understanding of the cellular bases of memory consolidation process should be advanced.

Chapter1

Spine dynamics after repetitive LTD induction that leads to long-lasting synaptic weakning.

第 1 部 : 繰り返し LTD 誘発後の長期持続的シナプス弱化におけるスパイン動態

1-1. Introduction

Making memory is an essential function for all animals to live. In our brain, there are an astronomic number of nerve cells called neurons and they make huge neural circuits. The cellular basis of memory is assumed to be synaptic plasticity which is the change of synaptic transmission in the neural circuit due to its neural activity. Memory is stored in the neural circuit through at least two distinct processes, acquisition and consolidation. In addition, it is also assumed that acquisition is based on enhancement/depression of synaptic transmission and consolidation is based on formation/retraction of synapse compartment. The cellular mechanisms underlying acquisition are well understood by analyzing LTP (Long-Term Potentiation: Bliss *et al.*, 1973) and LTD (Long-Term Depression: Lynch *et al.*, 1977) using freshly prepared brain slices. Contrastingly, those underlying consolidation are largely unknown because long term recording or examination of the neurons in the brain slices (Wong *et al.*, 1999) is difficult due to their short viability.

Organotypic brain slice cultures prepared from rodent newborn pups and maintained until construction of neural circuits equivalent to those of native brain (Stoppini *et al.*, 1991) is desirable for testing the longevity of LTP and LTD. Tominaga-Yoshino *et al.* (2002) and Shinoda *et al* (2005) revealed that singly-evoked LTP and LTD do not sustain over 24 hours (Tominaga *et al.*, 2002; Shinoda *et al.*, 2005).

Hinted from the fact that repetition is very important for learning (Gerfen et al., 2006; Schacher et al., 1993; Whishaw et al., 2004), repetitive LTP inductions were applied to the cultured hippocampal slices and it was revealed that 3 times repeated LTP inductions with 24 hours' intervals led to a long-lasting (over few weeks) enhancement of synaptic transmission accompanied by an increase of dendritic spine density (Oe *et al.*, 2011; Oe *et al.*, 2013; Tominaga-Yoshino *et al.*, 2002; Tominaga-Yoshino *et al.*, 2008; Urakubo *et al.*, 2006). Similarly to the repetitive LTP, repeated LTD inductions led to a long-lasting synaptic suppression coupled by a decrease of spine density (Egashira *et al.*, 2010; Kamikubo *et al.*, 2006; Shinoda *et al.*, 2005). From the fact that those long-lasting phenomena developed after the third LTP/LTD had disappeared and that the long-lasting synaptic transmission changes were brought about by morphological changes, the repetitive LTP/LTD were judged to produce qualitatively distinct phenomena from LTP/LTD per se. We assumed that these phenomena are the reproduction of memory consolidation processes and named RISE and LOSS, respectively.

One of the most important differences of RISE/LOSS from LTP/LTD is the change in

the number of synapses per se, since LTP/LTD is the modification of transmission efficacy of readily existing synapses. It is known that, in the steady-state conditions, the dendritic spines, the postsynaptic structure of excitatory synapse, are constantly generated and retracted and these levels are balanced (Trachtenberg *et al.*, 2002; Yasumasu *et al.*, 2008). In the previous-studies of my laboratory, Oe *et al.* reported that there are three phases to lead to a spine density increase after repetitive LTP inductions (Oe *et al.*, 2013). After the third LTP induction, at first, the rates of both spine generation and retraction, increase (raised fluctuation phase). At this phase, total spine density does not change. This phase is followed by a biased fluctuation phase where spine retraction rate returns to the basal level. Then spine generation rate exceeds retraction rate so that the total spine density increases at this phase. In the final phase, spine generation rate returns to the baseline so that the spine density becomes stable at the heightened level. Through these three steps, spine density is increased gradually and remains long term in the high density after repetitive LTP inductions.

RISE and LOSS have apparently symmetric aspects including requirement of 3 times repetition, slow occurrence, long lasting nature accompaniment of structure changes, involvement of neurotrophin signaling (Egashira *et al.*, 2010; Sakuragi *et al.*, 2013). Then, one question arises, whether spine dynamics after repetitive LTP and LTD are also symmetric or not. How is dendritic spine density reduced after the repetitive LTD inductions? To answer this question, I performed time-lapse observation for the change of spine dynamics after repetitive LTD inductions. As a result, against expectation, in terms of the spine dynamics, RISE and LOSS are not symmetric. The spine density increase in RISE is regulated by both of spine generation and retraction rates, the spine density decrease in LOSS after repetitive LTD is resulted from the increase in spine retraction rate alone.

1-2. Results

Confirmation of chemical LTD and spine density decrease after repetitive LTD inductions.

Since I use here a transgenic mouse clone expressing fluorescent protein in the hippocampal neurons, I need to confirm that LTD 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. 1-1a. This is important since test pulses, a component indispensable in some LTD protocols, were not included in the present protocols.

Next, I confirmed the induction of long-lasting synapse elimination equivalent to LOSS, consistent with previous findings in rat slice cultures after 3 repeated inductions of LTD (Fig. 1-1b–d). This structural plasticity is produced after 3x LTD inductions, but not after 1x or 2x LTD inductions (supplementary Fig.S1-1).

Spine dynamics after repetitive LTD

In the previous examination of RISE, 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 LTP induction (raised fluctuation phase). However, in the present spine elimination, the spine number (density) had already decreased by the 4th day after the 3rd LTD induction (Fig. 1-1d; 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. 1-2a). In parallel to this, the disappearance of pre-existing spines was significant during the first 4 days after the 3rd LTD induction (Fig. 1-2b 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. 1-2c), reflecting the restoration of equilibrated fluctuation.

Decrease of spine density begins without raised fluctuation phase

This suggestion was confirmed by chasing of the spines in a short interval. Within one day after the 3rd LTD induction, the spine retraction rate was larger than the generation rate (Fig. 1-3a, b and d) resulting in a decreased number of spines (Fig. 1-3c). The dendrites directly underwent into the biased fluctuation phase, indicating that RISE and LOSS are asymmetric in this aspect of spine dynamics. My observations also confirm the absence of biased fluctuation and thus no change in spine number after a

single LTD induction (Fig. 1-3b–e).

Reduction of spine density occurs uniformly on the neuron

Oe *et al* reported that the increase in spine number occurs preferentially in the dendritic segments having low pre-existing spine density (Oe *et al.*, 2013). The segments having high spine density do not experience raised fluctuation. As seen in Fig. 1-4ab, 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.

To confirm whether decrease of spine density occurs uniformly on the neuron, I examined the correlation between the spine density change and distance from cell body using immuno-labeled slices which were fixed after 4th-observation at PS day 20 (Fig. 1-4c). There was no correlation (Fig. 1-4de) and this result supports that decrease in spine density induced by LOSS occurs uniformly on the neuron.

1-3. Discussion

RISE and LOSS appeared 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 not symmetric in the spine dynamics leading to the final effects. Instead of 3 phases of spine dynamics in RISE (Oe *et al.*, 2013) (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.

If neurons are killed due to some negative effects accompanied by repetitive LTD inductions, a decrease in synapse density is inevitable as a result of reduced number of input fibers. But Kamikubo *et al.* (2006) confirmed that the number of neurons did not change after repeated LTD inductions, indicating that the decrease in spine density is not the result of input fiber reduction. Recently I and collaborators monitored metabolic changes of the cultured brain slice, using high performance liquid chromatography (Okahashi *et al.*, 2015). For activity, neurons import glucose and make energy through glycolysis and TCA cycle. The patients of Alzheimer disease show a decreased glucose consumption rate, indicating lowered neuronal activity (Winkler *et al.*, 2015). Additionally, lowered activity of neurons is often coupled with increased lactate release (ibid). But here, the rates glucose consumption and lactate release were unchanged after the repetitive LTD inductions (supplementary Fig.S1-2), indicating that the decreased spine density in LOSS was not the result of lowed neuronal viablilty.

Omission of the "raised fluctuation phase" in LOSS is might be reasonable in logics. In RISE, for the site where a new synapse is formed, there has been no promise to form synapse there. Hence, the dendrite need to depend on fluctuation to form new synapses blindly and to let some of them survive. Contrastingly, in LOSS, there is no need to depend on fluctuation, since the target of elimination already exists.

Recently, Ramiro-Cortés and Israely (Ramiro-Cortés and Israely., 2013) reported that a single induction of LTD led to spine elimination in slice cultures from the mouse hippocampus. Since their results are contradictory to my laboratoy's previous findings, I re-examined whether a single LTD would cause immediate morphological changes. As shown in Supplementary Fig. S1-3, I confirmed the previous results of decrease in spine number only after 3× LTD inductions. The reason for this discrepancy remains unclear,

but one possibility may be differences in the age of cultures (Muller *et al.*, 1993), since Ramiro-Cortés' experiments used cultures of 8–11 days *in vitro*, which are more immature than those used in our experiments.

The present results appear to contradict the findings of Wiegert and Oertner (Wiegert and Oertner., 2013), 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 the 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 LTD. As reported previously (Kamikubo *et al.*, 2006), however, long-lasting decrements in synaptic strength and synapse density are resulted not only from repeated LTD 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 LTD. I propose that RISE and LOSS should serve as the models *in vitro* for the cell biological analyses of repetition-dependent memory consolidation.

1-4. Materials and Methods

Organotypic slice culture of the mouse hippocampus

Organotypic slice cultures of the hippocampus were prepared from the Thy1-YFP H line mice (Feng *et al.*, 2000). 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 (Stoppini *et al.*, 1991; Tomunaga-Yoshino *et al.*, 2002). 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 LTP and LTD induction

For the long-term maintenance of aseptic conditions, LTD was induced by chemical means (LTD) as reported previously (Kamikubo *et al.*, 2006). 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 LTD 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 LTD was induced 1 day later, in the same manner, and the third LTD was induced 1 day after the second LTD. Cultures treated so were referred to as 3× LTD. Control specimens were prepared either by inducing LTD only once (1× LTD) or by repeating the same procedure with a medium containing no DHPG (3× mock).

Microscopic morphometry

Following the protocol described previously (Oe *et al.*, 2013), 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, I 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 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. I acquired 12 serial images (24 scans including Kalman noise reduction) at 0.75-μm Z-axis steps (9 μm Z-thickness in total).

With the present 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, I 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. I 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, I 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 photodynamic damage to the cell was remarkable in such a protocol. From the requirement of long-term

examination, I made compromise to use 2-dimensionally projected images, in which protocol I 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 (Dumitriu *et al.*, 2011). 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 the present preparation (probably because of well-matured cultures), I excluded filopodia-like protrusions by following conventional criteria: head diameter ≤1.2 × neck diameter or neck length ≥ 3 × neck diameter (Lai *et al.*, 2012). 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 the 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.

Immunohistochemical staining

At PS day 20, cultured slice prepared from Thy-1 YFP mice was fixed after 4th observation with 4% paraformaldehyde in phosphate-buffered saline for 24 hours at 4℃. After treatment with 1% Triton X-100 (Sigma), the slice was exposed sequentially to a primary antibody (anti-green fluorescent protein rabbit IgG, 1:200 dilution, 48 hours, 4℃; life technologies, USA) and to a secondary antibody (Alexa 488 conjugated anti-rabbit IgG, 1:200 dilution, 48 hours, 4℃, life technologies, USA). Observation was performed in the similar setup with time-lapse imaging as described above.

Statistical analysis

All figures include indication of the means±standard errors of means. For comparison of two groups, Welch's paired t-test assuming non-identical variance was applied. For

the comparison of ≧3 samples groups, ANOVA followed by Bonferroni's test was applied. Statistically significant differences are shown as * (for $p < 0.05$), ** (for $p < 0.01$), or *** (for $p < 0.001$).Exact P values are indicated in the figure legends.

1-5. Figures and Legends

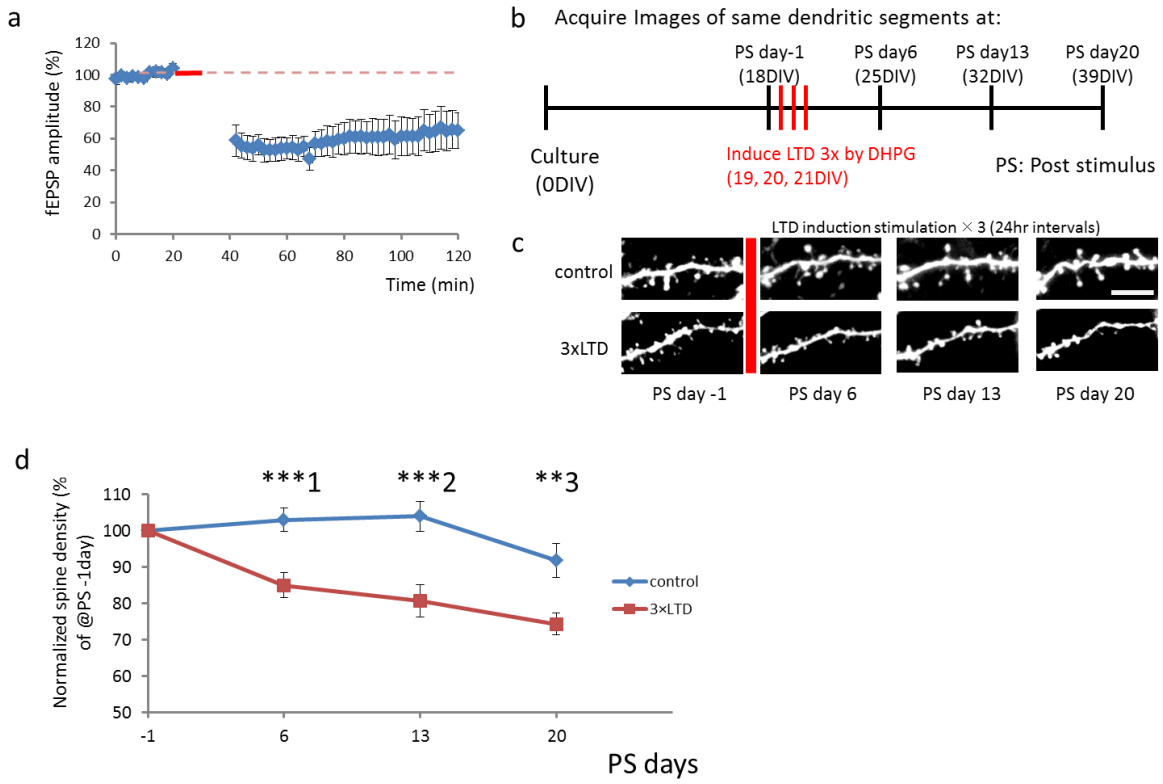


Fig.1-1 | Induction of chemical LTD and establishment of long-lasting synapse reduction by repetitive inductions of chemical LTD.

(a) LTD induced by exposure of DHPG (50μM, 10min). n=5.

(b) Timeline of experimental procedure.

(c) Representative images of time-lapse imaging. Scale bar indicates 5μm.

(d) Spine density change after repetitive LTD inductions. 3 times repetitions of LTD decrease spine density (control, n=20; 3xLTD, n=23; p=2.3x10-7 for ***1, 1.4x10-10 for ***2 and p=5.7x10-7 for **3 in 2-factor factorial ANOVA followed by Bonferroni' test.)

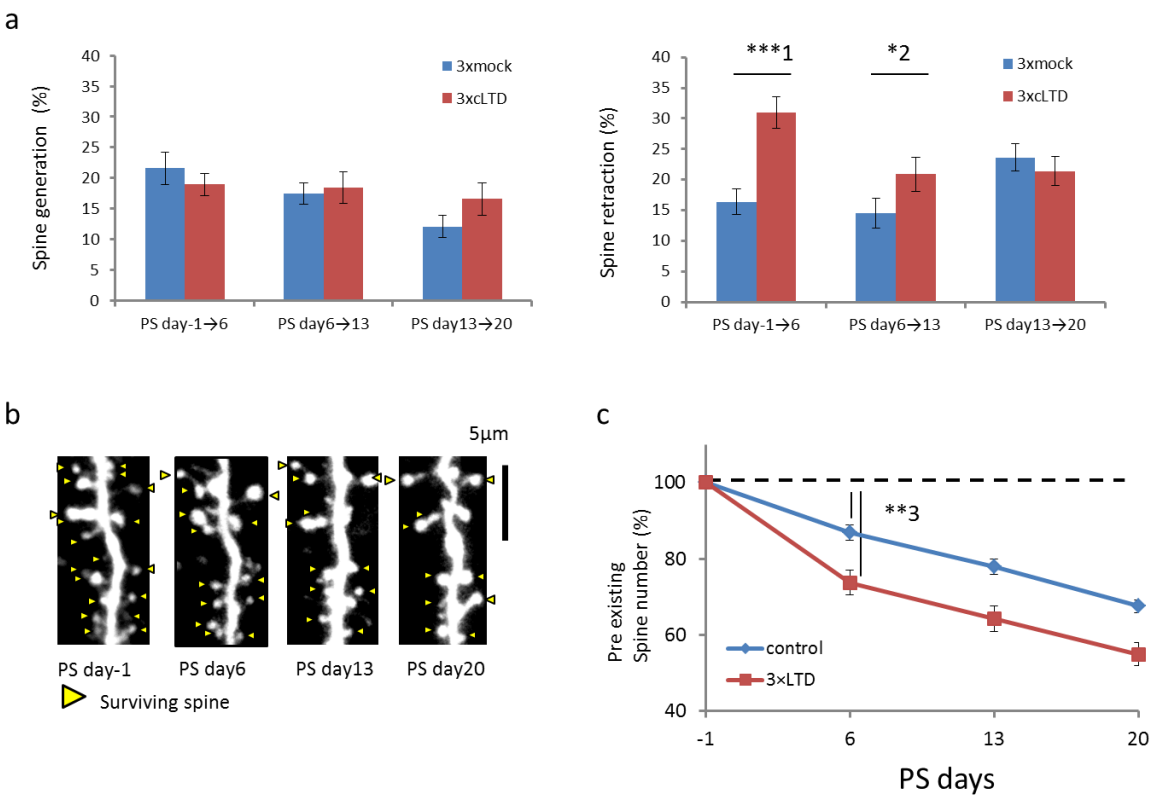


Fig.1-2| Dendritic spine dynamics after 3 inductions of LTD.

(a) Spine generation and retraction rates during indicated periods, after repetitive LTD inductions. Retraction rate was significant increase at PS day 1-6 and 6-13, though generation rate did not change (control, n=20; 3xLTD, n=23; p=8.7x10-5 for ***1 and p=0.047 for *2 in paired t-test).

(b) Representative images of time-lapse imaging. Yellow arrow heads indicate spines which exist at PS day -1 and remain at each observation days. Scale bar indicates 5μm.

(c) The number of spines which exist at PS day -1 and remain at PS day 6, 13, 20. Spine number was decreased significantly in 3xLTD at PS day 6 comparing control group. After PS day 6, disappearance of spine occurred in similar rate in both control and 3xLTD (control, n=19; 3xLTD, n=19; p=4.9x10-12 for ***3 in 2-factor factorial ANOVA followed Bonferroni's test).

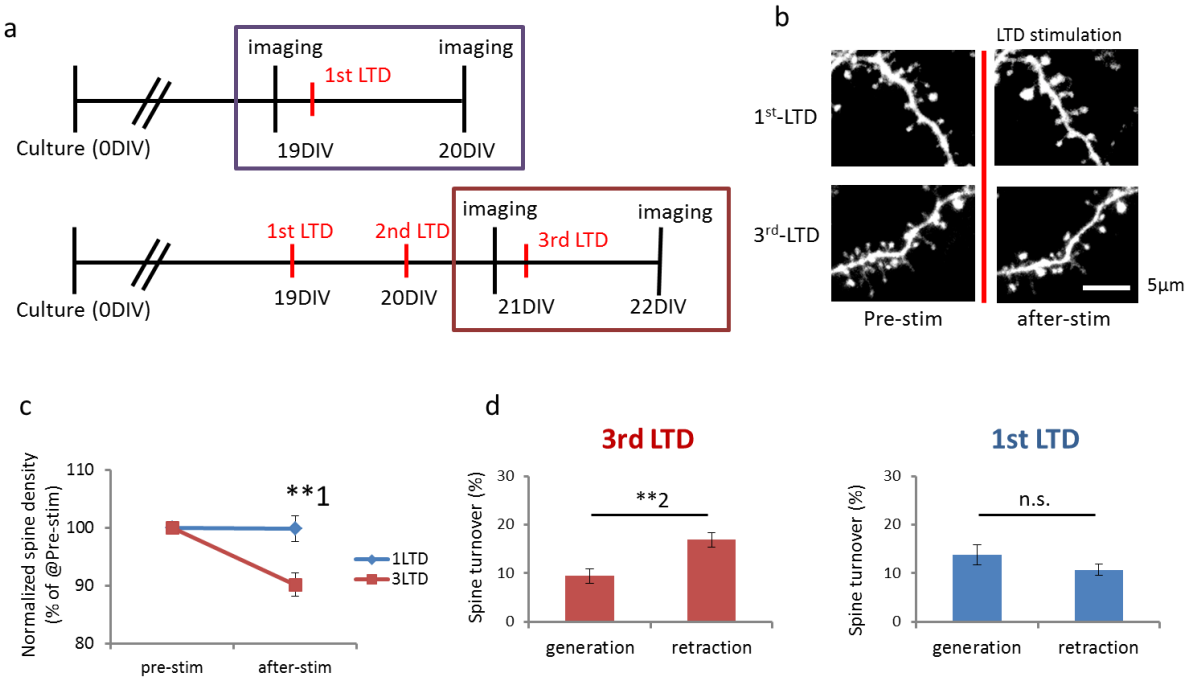


Fig.1-3| Absence of "raised fluctuation phase" during reduction of spine density as compared by observation of 1-day interval.

(a) Timeline of experimental procedure. Images was obtained 2 times. 1st imaging was made 1-4 hours before LTD inducing stimulation and 2nd imaging was made 24 hours after 1st imaging.

(b) Representative images of time-lapse imaging. Scale bar indicates 5μm.

(c,d,) Spine density turnover rate after 1st LTD and 3rd LTD. 3rd LTD decreased spine density and increase retraction rate though single LTD did not (1LTD, n=26; 3LTD, n=27; p=2.3x10-3 for **1 and p=1.1x10-3 for **2 in paired t-test).

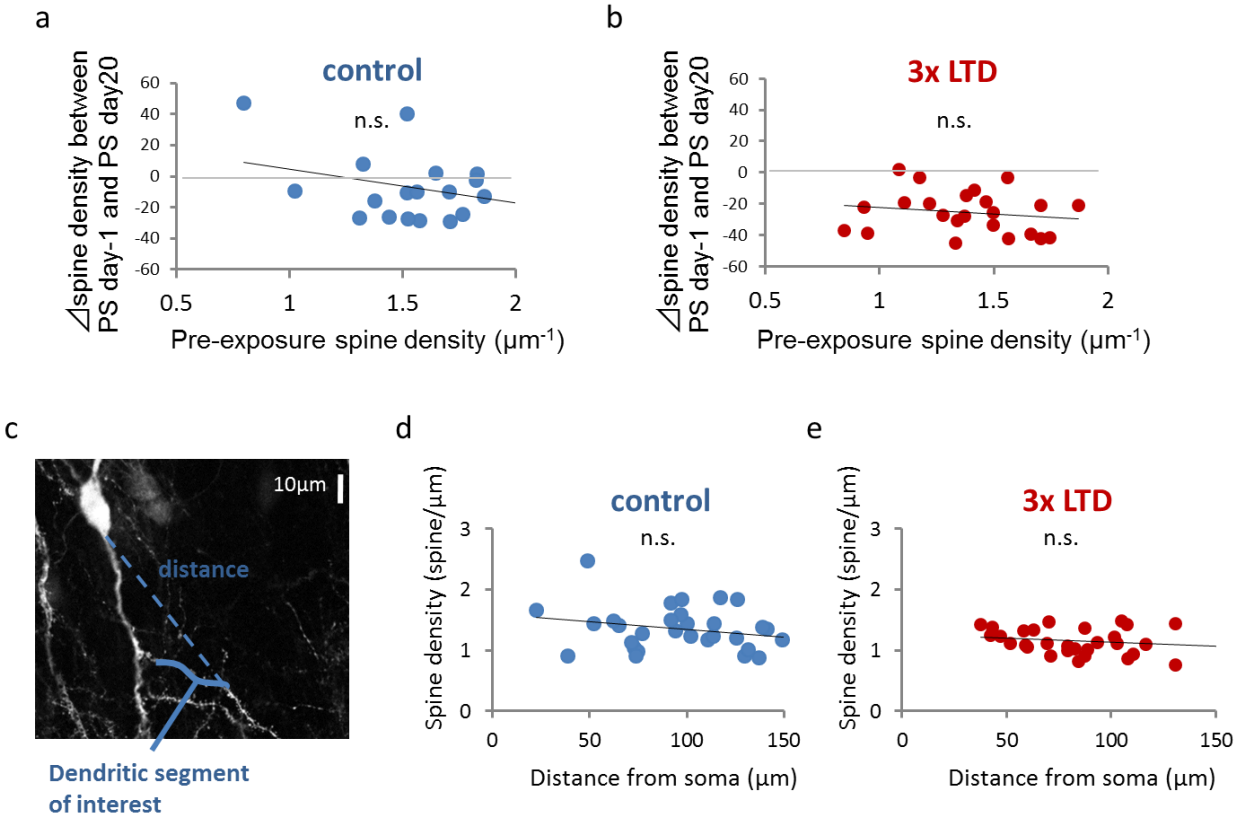
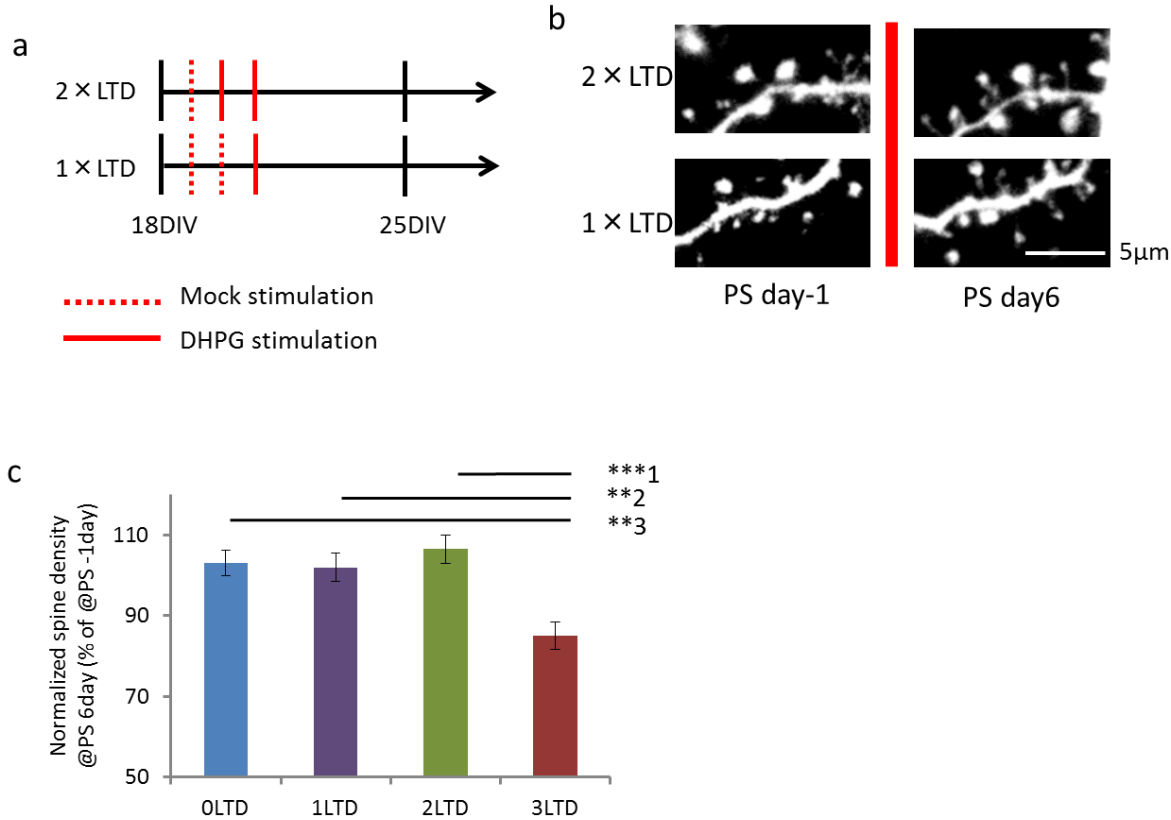


Fig.1-4| Spine retraction induced by repetitive LTD occurs uniformly on the neuron.
(a,b) Correlation between pre-existing spine density and spine density change induced by repetitive LTD. There was no correlation. Positive values of vertical axis indicate spine density increase and negative values indicate spine density decrease.
(c) Representative images of fixed slice to measure the distance between the soma and dendritic segment of interest. The distance from soma was measured between base of primary dendrite on soma and end of dendrite which is the most distant side. Scale bar indicates 10μm.
(d,e) Correlation between distance from soma and spine density (spine/μm). There was also no correlation (control, n=34 ; 3xLTD, n=30).

1-6. Supplementary figures and legends

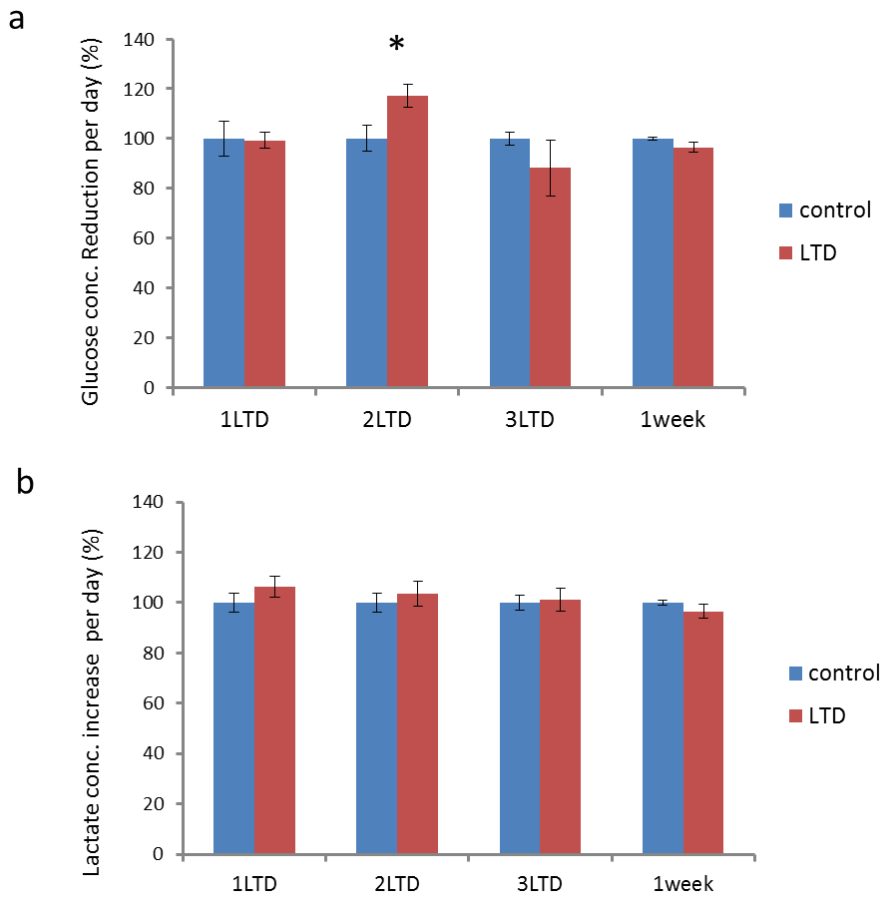


Supplementary Fig.1-1 | Requirement of repetitive stimulation to induce spine density decrease.

(a) Timeline of examination. In 2xLTD group mock-LTD-LTD were performed and in 1xLTD group mock-mock-LTD were performed.

(b) Representative images of time-lapse imaging. Scale bar indicates 5μm.

(c) Spine density change at PS day 6. Significant decrease of spine density was observed in 3LTD compared with others (0LTD, n=20; 1LTD, n=17; 2LTD, n=19; 3LTD, n=23; p=5.3x10-3 for ***1, p=9.0x10-3 for **2, p=2.4x10-3 for **3 in one-way ANOVA followed by Bonferroni's test).



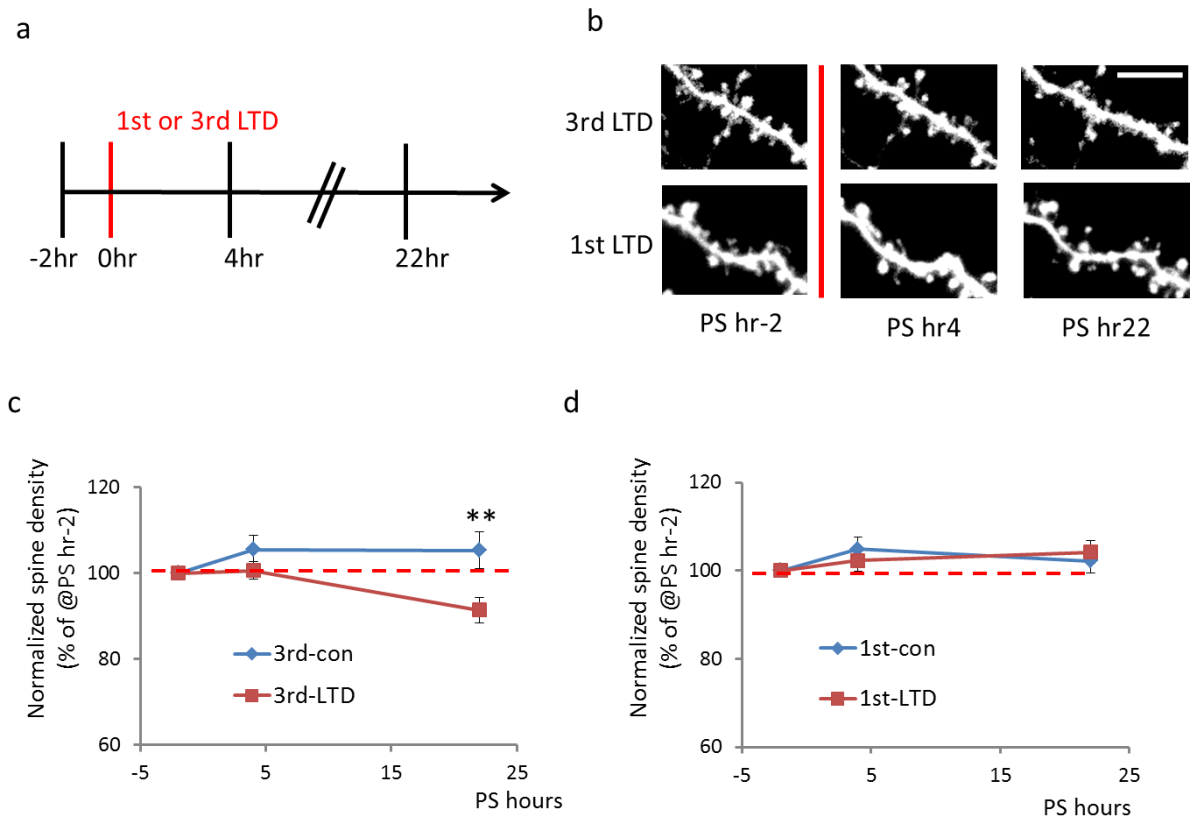
Supplementary Fig.1-2| Spine density decrease induced by repetitive LTD is not reduction of neuron's condition.

In this figure, hippocampal slice cultures prepared from SD rats were used. Glucose and Lactate concentrations in the cultured medium were measured using HPLC. Cultured medium was obtained 24 hours after medium change or DHPG exposure.

(a) Reduction of glucose concentration in the cultured medium during indicated periods. After 2LTD, slight increase was observed, however no reduction was induced (control, n=6 ; LTD, n=6 ; p=0.032 in paired t-test).

(b) Increase of Lactate concentration in the cultured medium. Metabolic change of slice culture was not observed.

*HPLC analysis was performed by Nobuyuki Okahashi (Laboratory of Metabolic Engineering, Graduate School of Information Science and Technology, Osaka University)



Supplementary Fig.1-3 | Start timing of spine retraction after 3rd LTD induction.

(a) Timeline of experimental procedure.

(b) Representative images of time-lapse imaging. Scale bar indicates 5μm.

(c) Spine density change after 3rd LTD. In the 3rd-con specimen, LTD-LTD-mock stimulation were performed and imaging was performed before and after mock stimulation. Significant decrease was observed at 22hr in 3rd LTD specimen though at 4hr no change was observed (3rd-con, n= 22; 3rd-LTD, n=24; p=2.7x10-6 for ** in 2-factor factorial ANOVA followed by Bonferroni's test).

(d) Spine density change after 1st LTD. No significant change was observed in both specimens (1st-con, n=16; 1st-LTD, n=26)

Chapter 2

Apparent disparity between the structural change (dendritic spine decrease) and the functional change (EPSP reduction) in the development of LOSS

第 2 部：繰り返し LTD 誘発後の構造的変化（樹状突起棘密度減少）と機能的変化（興奮性シナプス電位縮小）との時間的乖離

2-1. Introduction

The slowly-developing and long-lasting structural synaptic plasticity phenomena found in the stably cultured rodent hippocampal slices, RISE and LOSS, incremental and decremental forms, respectively, may be useful in vitro models for the cellular and subcellular analyses of memory consolidation processes. In case of RISE, slow development is explained by an apparent "latent period" in which dendritic spines are both actively generated and retracted so that no net increase in spine density is resulted (presence of a so-called "raised fluctuation phase"). In case of LOSS, however, as explained in the previous chapter, the retraction of spines occurs quickly after the LOSS-producing stimulus without the presence of raised fluctuation phase. Nevertheless, the decrement of EPSP becomes apparent slowly. In other words, RISE and LOSS are symmetric in a functional aspect but not in a structural aspect.

In addition to morphological change, it was reported that signaling pathways are also activated quickly after 3rd LTD induction. Previous studies reported the requirement of protein synthesis after each LTD induction and the involvement of proBDNF (Egashira *et al.*, 2010; Sakuragi *et al.*, 2013), the precursor of brain-derived neurotrophic factor that is relevant in neurite retraction and cell death (Lu *et al.*, 2002). Additionally, activation of pro BDNF occurs 6 hour after 3rd LTD induction though it disappears 24 hour later, indicating that signaling pathways which induce LOSS are quickly activated. What does activate protein synthesis after 3rd LTD induction? In this chapter, I tried to get clues for the cellular signaling events which lead to LOSS. As to the signal initiating the protein synthesis, I applied several inhibitors and found that an application of a PKC (calcium/diacylglycerol-dependent protein kinase) inhibitor 3 hours after the 3rd LTD induction, but not that of a PKA inhibitor, prevented the decrease of spines. This result suggests that PKC activation downstream of mGluR leads to the protein synthesis. In addition to protein synthesis, morphological changes should be accompanied by the rearrangement of cell cytoskeleton. Therefore I also focused on cellular mechanisms for the rearrangement of cell cytoskeleton. As to the signal for the rearrangement of cytoskeleton, an inhibitor of CaMKII (calcium/calmodulin-dependent protein kinase type II) was applied. The result of blockage of the spine density decrease suggests that CaMKII regulates the rearrangement of cytoskeleton leading to spine elimination.

Then a new question arises. What is the gap between the structural and functional decrements (quick spine decrease and slow EPSP decrease) in LOSS.

I assume three hypotheses that may explain that gap: 1) there are mature/functional

and immature/non-functional synapses by nature (Knott *et al.*, 2006; Nagerl *et al.*, 2007) and the immature ones disappear at first; 2) there are silent and active synapses by nature (Issac *et al.*, 1995; Liao *et al.*, 1995; Durand *et al.*, 1996) and the silent ones disappear at first ; 3) there are spines coupled with presynaptic terminals and those without by nature (Nagerl *et al.*, 2007; Arellano *et al.*, 2007) and the ones without disappear at first.

The hypothesis 1 may be tested by measurement of the size of spines, when based on a generally accepted theory that synapses with mushroom-shaped spines are mature/functional and those with stubby- and thin-shaped spines are immature/non-functional (Beltra'n-Campos *et al.*, 2010; Holtmaat *et al.*, 2005; Kasai *et al.*, 2003; Parnass *et al.*, 2000). The hypothesis 2 may be examined by the inducibility of LTP, since LTP occurs by turning silent synapses to active one (Isaac *et al.*, 1995) and thus LTP should become smaller or absent when silent synapses disappear in the early phase of LOSS. The hypothesis 3 may be tested by estimating the density of presynaptic terminals, since it should not change if the spines without presynaptic terminals disappear selectively at first.

By a series of examination, the results supporting the hypotheses 1 and 2 were not obtained, whereas the results consistent with the hypothesis 3 were obtained. Although the possibilities other than above 3 hypotheses have not been excluded yet, I suppose the hypothesis 3 is the most plausible explanation for the gap between the quick structural change and slow functional alteration in the development of LOSS.

2-2. Results

Activation of PKC is necessary to induce spine retraction.

Similar to morphological change, quickly activation of protein synthesis and increment of pro BDNF were reported. In this chapter, at first, I investigated the signaling pathway which leads protein synthesis. In this study, I used DHPG, a Group I mGluR agonist, to induce chemical LTD. Previous study indicated that DHPG has two pathways to reduce the synaptic transmission (Snyder *et al.*, 2001). One is the PI3 pathway that activates Ca^{2+} release from internal storage and leads to internalization of AMPA receptors. The other is the PKC pathway that activates protein synthesis to prolong the state. Thus I assumed that PKC pathway is also relevant to the elimination of spines. To examine this assumption, I used a PKC inhibitor, Go-6983. A 24-hr incubation with Go-6983 was begun 3 hours after the 3rd LTD induction. This delay was intended to avoid the interference with LTD itself (Egashira *et al.*, 2008). Imaging was performed at PS day-1 and PS day6. I found that no significant difference is observed between the control and 3x LTD+Go-6983 groups, indicating that PKC activation is needed to reduce spine density (Fig. 2-5ab). As a negative control experiment I applied a PKA inhibitor, H-89. Spine density decrease was not influenced, indicating that the PKA pathway is not concerned (Fig. 2-5cd).

CaMKII regulates spine retraction after repetitive LTD inductions.

I examined the signaling pathway which leads to rearrangement of cell cytoskeleton. After activation of mGluR induced by DHPG application, an increase in cytoplasmic Ca^{2+} concentration occurs. Elevated Ca^{2+} would activate CaMKII which regulates the stability of cytoskeleton and spine morphological change (Hudmon *et al.*, 2002). To examine the involvement of CaMKII activation in the spine elimination of LOSS, a CaMKII inhibitor, KN-62 was used and time-lapse imaging was performed. Similar to the examinations of PKC and PKA, CaMKII inhibitor was applied for 24 hours beginning at 3 hr after the 3rd LTD induction. The spine density decrease was blocked by KN-62 (Fig. 2-6), indicating that CaMKII is involved in the spine density decrease after repetitive LTD inductions.

Confirmation of the slow change in synaptic strength after repetitive induction of LTD

In the previous studies which examined functional change in LOSS, hippocampal slice cultures prepared from rats were used. Although the possibility is low, but the slow development of EPSP decrease would be specific to the slice cultures prepared from rat,

since the structural examination in the previous chapter was done using the slice cultures prepared from mice. So, at first, I tried to confirm the slow development of EPSP decrease in mouse slice cultures. When the maximal fEPSP amplitude was measured at PS day (poststimulus day = the day after the third induction of LTD) 6, no difference was observed yet between the control (mock-stimulated) slices (the decrement of EPSP has not begun). But when the maximal fEPSP amplitude was compared at PS day 20, a significant difference was recognized between the control and 3x LTD-induced slices (Fig. 2-1ab). Hence, taken together with the results shown in the previous chapter, the gap between quick structural change and slow functional change was confirmed in the hippocampal slice cultures derived from the mouse.

Thin and stubby spines remain during LOSS development

To explain the gap between the spine decrease and the EPSP reduction, I assumed three hypotheses: 1) there are mature/functional and immature/non-functional synapses by nature (Knott *et al.*, 2006; Nagerl *et al.*, 2007) and the immature ones disappear at first; 2) there are silent and active synapses by nature (Issac *et al.*, 1995; Liao *et al.*, 1995; Durand *et al.*, 1996) and the silent ones disappear at first ; 3) there are spines coupled with presynaptic terminals and those without by nature (Nagerl *et al.*, 2007; Arellano *et al.*, 2007) and the ones without disappear at first.

The first hypothesis may be tested by measurement of the size of spines, when based on a generally accepted theory that synapses with mushroom-shaped spines are mature/functional and those with stubby- and thin-shaped spines are immature/non-functional (Beltra'n-Campos *et al.*, 2010; Holtmaat *et al.*, 2005; Kasai *et al.*, 2003; Parnass *et al.*, 2000).

According to a standard criterion I classified the 3 types: a) a mushroom spines having a large head (>0.4 um); b) a thin spine having small head (<0.4 um) and a shaft; c) a stubby spine having no neck (head-neck ratio <1:1)(Fig. 2-2ab) using Neuronstudio software (Dumitriu *et al.*, 2011). Remember that the absolute number of spines were smaller in the 3x LTD group than the control (3x mock-stimulated) group at PS day 6, the day of examination. So I showed the results in two ways: the first way was to show percentage of each type spine (Fig. 2-2c), and the second way was re-calculation to make the total spine number of 3x LTD group 0.85% of that of the control group (Fig. 2-2d: actual spine densities of each spine subtype were shown in supplementary Fig.S2-1) .

Unexpectedly, the result was that the mushroom spines, but not the thin and stubby spines, decreased the number preferentially (Fig. 2-2d). 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, I classified living spines (compromising in unideal image definition) at PS day 2 (2 hours before the 3rd LTD) 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. S2-2).

Silent synapses remain during LOSS development

The second hypothesis is that there are silent and active synapses by nature (Issac *et al.*, 1995; Liao *et al.*, 1995; Durand *et al.*, 1996) and the silent ones disappear at first. This hypothesis can be tested by the inducibility of LTP, since LTP is known to occur by turning silent synapses to active one (Issac *et al.*, 1995; Liao *et al.*, 1995; Durand *et al.*, 1996) and thus LTP should become smaller or absent when silent synapses disappear in the early phase of LOSS.

As shown in Fig. 2-3, the magnitude of LTP was unchanged at PS day 6 in comparison to the day-matched control (3x mock-stimulated) slices. This result indicates that silent synapse did not disappear selectively by the LOSS-producing stimulus.

Presynaptic structure decrease late after repetitive LTD inductions.

The third hypothesis is that there are spines coupled with presynaptic terminals and those without (so-called "orphan" spines) by nature (Nagerl *et al.*, 2007; Arellano *et al.*, 2007) and the ones without disappear at first.

This hypothesis should be tested by estimating the density of presynaptic terminals, since it should not change if the spines without presynaptic terminals disappear selectively at first.

To count the presynaptic terminals, I stained the cultures with an antibody to synaptophysin, the synaptic vesicle protein. At PS day 6, when the decrease spine density is already apparent, the density of pre-synaptic terminal remained unchanged (Fig. 2-4). Although the presynaptic sites visualized here are not necessarily excitatory, the delayed onset of presynaptic change is in line with this hypothesis.

2-3. Discussion

The laboratory I belong to has revealed symmetric nature of two forms of structural plasticity, RISE and LOSS. Both of them require three inductions of short-term plasticity, LTP and LTD. They develop slowly after the third LTP/LTD disappear. They last longer than 2 weeks after their full development. They are accompanied by structural changes, the formation/elimination of synapses. They require closely related cytokines, BDNF for RISE and proBDNF for LOSS (Sakuragi *et al.*, 2013).

However, in the first chapter of this thesis, I revealed that the dynamics of dendritic spines was not symmetric between these two forms of structural plasticity. The increase in spine density in RISE is delayed due to the presence of a phase of increased but balanced fluctuation. To the contrary, the spine density decreases quickly in case of LOSS. But EPSP amplitude is reduced after a considerable delay. So I have to figure out the mechanism underlying the apparent gap between the quick structural change and the slow functional alteration.

Here I assumed three hypotheses that are more-or-less plausible. The first one is that the immature synapses (represented by thin and stubby spines) would be lost preferentially so that the EPSP is affected very little at first. But the result of examination and classification of spine shapes was opposite unexpectedly. The mushroom spines decreased the number preferentially. The hypothesis was not supported.

The second hypothesis is that the silent synapses disappear at first. The silent synapses are the synapses that have NMDA receptors but not AMPA receptors so that they contribute little to usual synaptic transmission. Since LTP is the phenomenon that turns the silent synapses to active ones through externalization of internally stored AMPA receptors, the hypothesis can be tested by testing the inducibility of LTP. If the silent synapses would be lost at first, magnitude of LTP should be reduced. But it was not the case. To say strictly, it is desirable to quantify the NMDA and AMPA components of EPSP by pharmacological means, since NMDA receptors do contribute to EPSP as its slow component. Disappearance or no disappearance of silent synapses should be estimated by the change or no change in NMDA component in EPSP.

The third hypothesis is that the orphan spines are lost at first. Orphan spines are the ones without presynaptic counterparts and thus do not contribute to EPSP. In the literature such synapses are ~3.6% in CNS synapses (Arellano *et al.*, 2007). By examining the presynaptic terminals stained with anti-synaptophysin antibody, the decrease in presynaptic sites did not show a decrease at PS day 6, the time-point

postsynaptic sites already showed decrease. This result was confirmed by immnostaining/counting using an antibody to bassoon, which is a presynaptic active zone protein (tom Dieck *et al.*, 1998) and thus an independent presynaptic marker (Fig. S2-3). Therefore, this hypothesis is the only one that does not contradict the observed gap between quick structural change and slow EPSP change. Criticism is possible, of course, since synaptophysin and bassoon do not selectively mark excitatory terminals: if inhibitory presynaptic terminals outnumber the excitatory terminals and if they do not decrease so soon, the hypothesis 3 does not hold.

So far I have tested three hypotheses. There are many other hypotheses worth testing. They include: hypothesis 4) a parallel decrease of excitatory and inhibitory synapses so that no net change of postsynaptic potential appears soon; hypothesis 5) spines are quickly lost but the presynaptic terminals that lost postsynaptic counterparts would translocate to the neighboring synapses so that EPSP should be uninfluenced. The presence of synapses that have multiple presynaptic terminals attached to a single spine is reported (Knott *et al.*, 2006; Nageral *et al.*, 2007).

The hypothesis 4 is not supported by the result of counting presynaptic sites, but the hypothesis 5 is not excluded at present. Electron microscopic examination coupled with three-dimensional reconstitution should be necessary to prove/disprove this hypothesis.

Kamikubo *et al.* (Kamikubo *et al.*, 2006) showed that LOSS could be produced by repeated inductions of LTD, induced by chemical means other than the exposure to DHPG. Those are the exposure to mild concentration of NMDA (N-methyl-D-aspartate) cause and DHO (dihydroouabain; an Na+/K+ ATPase inhibitor). Although the resulted LTD is the same, the underlying mechanisms are thought distinct.

DHPG-dependent LTD is mediated by phospholipase C activation followed by PKC activity that phosphorylates AMPA receptor at the site different from that in case of LTP. NMDA-dependent LTD is mediated by a medium-level Ca^{2+} influx followed by a protein phosphatase activity that dephosphorylates AMPA receptor and TARP (transmembrane AMPA receptor regulatory protein), as the reverse of LTP (Lee *et al.*, 1998). DHO-dependent LTD is mediated by presynaptic depolarization followed by a sustained glutamate release and a sustained Ca^{2+} influx into postsynaptic spines via voltage-gated Ca channels (Reich et al., 2004). The latter two LTD are thought to be independent of PKC. Then does PKC play critical roles in the NMDA-produced LOSS and DHO-produced LOSS similarly to the DHPG-produced LOSS studied here?

It is an important issue in considering the signaling pathway of LOSS and I like to test that question pharmacologically. Note, however, that medium rises in Ca^{2+}

concentration in the spine cytoplasm are occurring in all cases that may activate PKC. In other words, PKC is still eligible as a "final common path" to lead to spine elimination.

I suggest here that CaMKII may also play an important role to produce LOSS. It may sound oddly, however, since CaMKII is generally thought to be a positive (*i.e.* incremental) regulator of synaptic plasticity. LTP is mediated by CaMKII that phosphorylates AMPA receptor to make its dwelling time on the postsynaptic membrane longer (Hudmon et al., 2002; Lisman et al., 2002). CaMKII also phosphorylates TARP to facilitate its AMPA receptor recruiting activity. Those effects result in an increase in the number of AMPA receptor on the postsynaptic membrane (Colbran and Brown., 2004). A transgenic mouse line devoid of CaMKII cannot induce LTP (Silva *et al.*, 1992). Tominaga-Yoshino *et al.* (Tominaga-Yoshino *et al.*, 2008) showed that CaMKII participates also in RISE, since a CaMKII inhibitor (H-89) applied after the 3rd LTP blocked the establishment of RISE. So, the participation of CaMKII in both RISE and LOSS may be puzzling.

Kawaai *et al.* (Kawaai *et al.*, 2010) proposed a scheme of "actin-cytoskeleton regulatory network" working in RISE production. Cofilin, one of the actin-associated proteins, facilitates actin polymerization in its phosphorylated form, whereas it facilitates actin depolymerization in its dephosphorylated form (Bamburg *et al.*, 1999; Pollard et al., 2003). Phosphorylation of cofilin is mediated by a protein kinase called LIMK and dephosphorylation is mediated by a protein phosphatase called slingshot (Aizawa *et al.*, 2001; Niwa *et al.*, 2002). CaMKII activates LIMK by phosphorylation and thus facilitates actin polymerization to lead to RISE. However, CaMKII also regulates the expression of a variety of genes relevant to actin dynamics including cofilin itself. Newly synthesized cofilin is yet non-phosphorylated so that the balance of phosphorylated cofilin and non-/dephosphorylated cofilin may lean toward depolymerization of actin. Thus the results may reverse whether CaMKII activates LIMK or activates cofilin expression. It is necessary to analyze quantitatively the balance of phosphorylated and non-/dephosphorylated cofilins.

I examined the kinase pathways for the LOSS production in the present study. As explained above, however, the phosphatase pathways may also play key roles. Especially in the case of LOSS produced by the repetition of NMDA-dependent LTD, possible participation of phosphatase cannot be overlooked. I propose pharmacological examinations using okadaic acid, calyculin A (both inhibitors for PP1 and PP2A), cantharidin (a PP2A-specific inhibitor) or deltamethrin (a PP2B-specific inhibitor) for deeper discussion.

2-4. Materials and Methods

Preparation of organotypic slice cultures of the mouse hippocampus, induction of LTD by chemical means, microscopic morphometry and statistical analyses were performed by the same procedures as those described in Chapter 1.

Application of inhibitor

To block the activation of PKC, PKA and CaMKII, Go-6983 which is PKC inhibitor, H-89 which is PKA inhibitor and KN-62 which is CaMKII inhibitor was used. The slice culture at PS day 2, 3 hours later after 3rd LTD was exposed to the culture medium containing 100nM Go6983, 1 μM H-89 or 10 μM KN-62. New culture was introduced 3 hours later, in order to dilute the inhibitor, followed by another replacement with culture medium.

Electrophysiology

Extracellular recording of CA1 neurons activity was performed conventionally (Sakuragi *et al.*, 2013). Briefly, the slice cultures were transferred to a recording chamber and a glass microelectrode filled with ASCF (input residence, 5-10MΩ) was inserted into the CA1 pyramidal cells' somatic payer for recording. A monopolar electrode was placed in the CA3 cells' somatic layer for stimulation. Test stimulation pulses of 100 μs duration were delivered every 30 sec to record field EPSP.

The maximal field EPSP amplitude recorded from the somatic layer was taken as an index for synapse number. The logic for this is explained in the Muller *et al* (Muller *et al.*, 1993). To obtain this value an increasing series of stimulation current pulses were given to obtain a saturation level of field EPSP. The recording electrode was then shifted to a new site within the CA1 somatic layer. By such recording from ≥3 CA1 sites, the maximum value of the maximal field EPSP was adopted as a representative value of that slice.

To induce LTP, a stimulation current chosen was that induced 50-60% of saturation level of field EPSP. After field EPSP was recorded stably, a tetanic stimulation (100 Hz, 1 sec) was delivered. Recorded EPSP amplitudes were binned for 1.5 min periods and plotted.

Fluorescent dye injection

The culture prepared from the YFP-expressing mouse used for live examination of spine dynamics in Chapter 1 does not have sufficient

definition for the determination of spine shapes. To classify the spine shapes, I used intracellular injections of a fluorescent dye, Lucifer yellow lithium salt (LY: Nacalai Tesque, Inc., Kyoto, japan), were performed at PS day6 as reported previously (Pace *et al.*, 2002). Briefly, the cultured slice prepared from wild type C57BL/6 mouse pup was fixed mildly with 2% paraformaldehyde dissolved in a balanced salt solution, and a glass micropipette filled with 8% LY was inserted slowly into the CA1 pyramidal cell layer. Pyramidal cell was recognized by its cell-shape. A negative current (1-3 nA) was applied for 3-5 min through the micropipette. The slice was then fixed overnight with 4% paraformaldehyde.

2-5. Figures and Legends

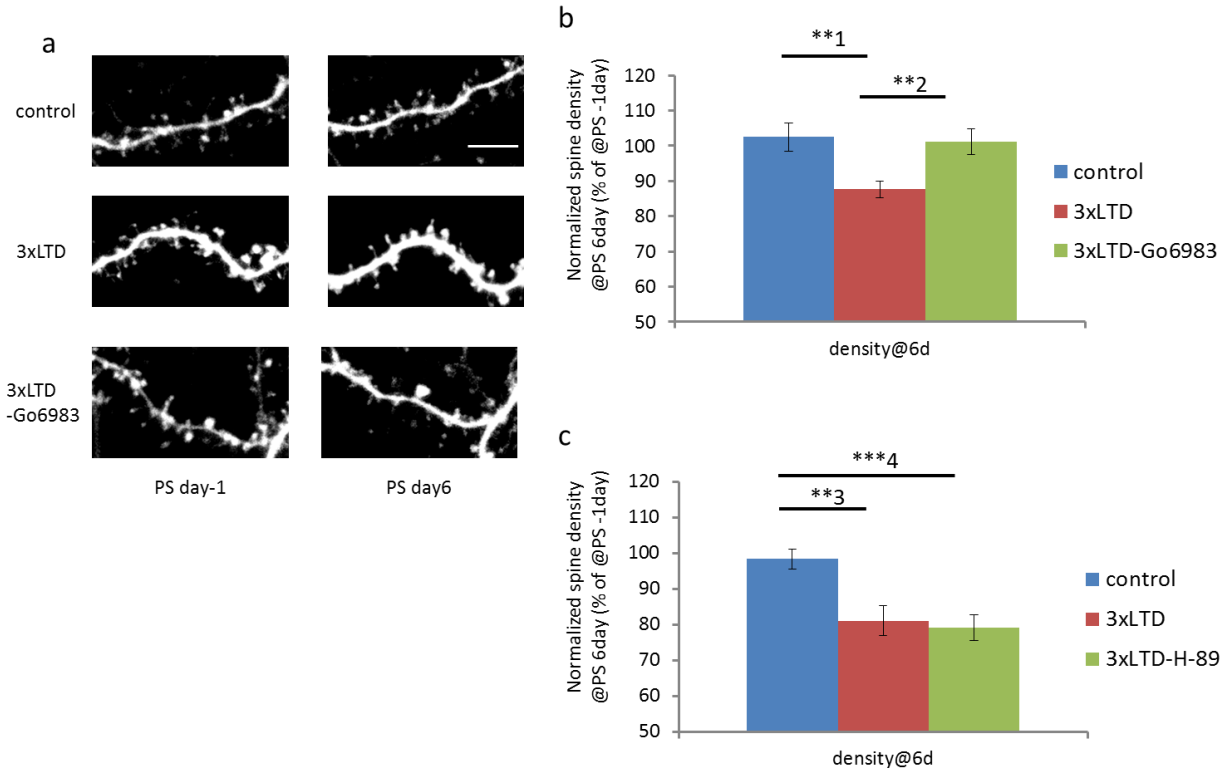


Fig.2-1| Activity of PKC, not PKA, is necessary for spine elimination induced by repetitive LTD inductions.

(a)Representative images of time lapse imaging. Scale bar indicates 5μm.

(b) Spine density change after repetitive LTD inductions and application of PKC inhibitor, Go6983. Go6983 blocks decrease in spine density induced by repetitive LTD inductions (control, n=15; 3xLTD, n=19; 3xLTD-Go6983, n=18; p=6.1x10-3 for **1 and p=9.5x10-3 for **2 in one-way ANPVA followed by Bonferroni's test.)

(c) Spine density change after repetitive LTD inductions and application of PKA inhibitor, H-89. H-89 does not block spine density decrease (control, n=15; 3xLTD, n=12; 3xLTD-H-89, n=14; p=4.6x10-3 for **3 and p=6.2x10-4 for ***4 in one-way ANPVA followed by Bonferroni's test.)

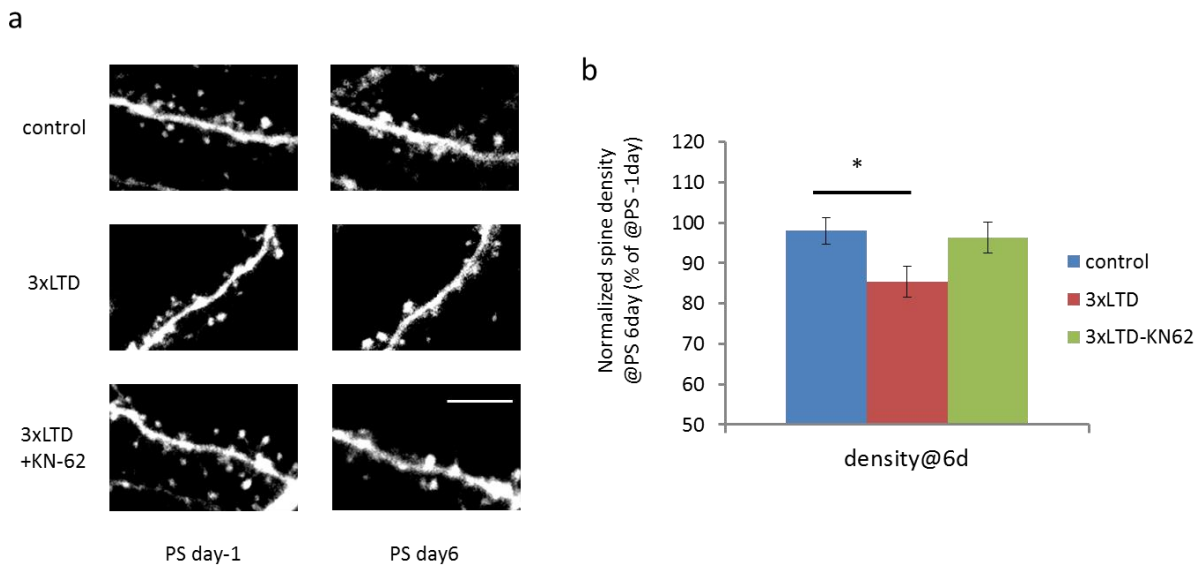


Fig.2-2| Requirement of CaMKII activity for spine retraction after repeated LTD inductions.

(a)Representative images of time-lapse imaging. Scale bar indicates 5μm.

(b) Spine density change after repetitive LTD inductions and application of CaMKII inhibitor, KN-62. Application of KN-62 blocks decrease in spine density induced by repetitive LTD inductions (control, n=19 ; 3xLTD, n=16 ; 3xLTD-KN62, n=17 ; p=0.049 for * in one-way ANOVA followed by Bonferroni's test.)

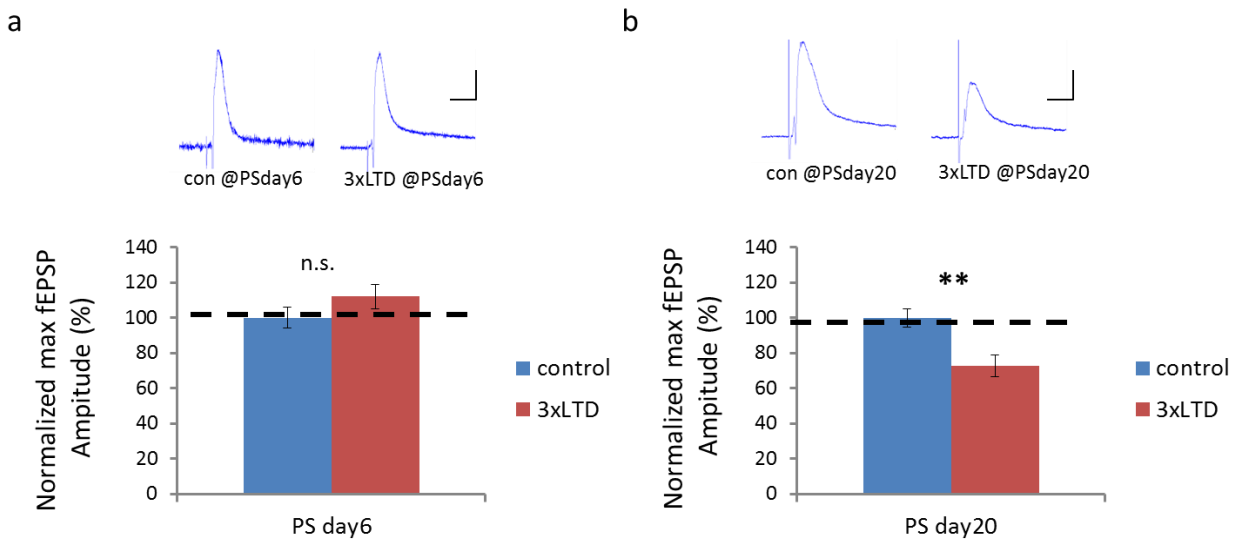


Fig.2-3| Confirmation of fEPSP change after repetitive LTD inductions.

(a)The synaptic strength, as indicated by the maximal fEPSP, is shown together with representative recording at PS day6. The scale bars are 2mV(vertical) and 20ms(horizontal). (control, n=15; 3xLTD n=15)

(b) Maximal fEPSE is shown together with representative recording at PS day20. the scale bars are 2mV(vertical) and 20ms(horizontal). (control, n=21; 3xLTD n=21 ; $p=1.7\times10^{-3}$ for ** in paired t-test)

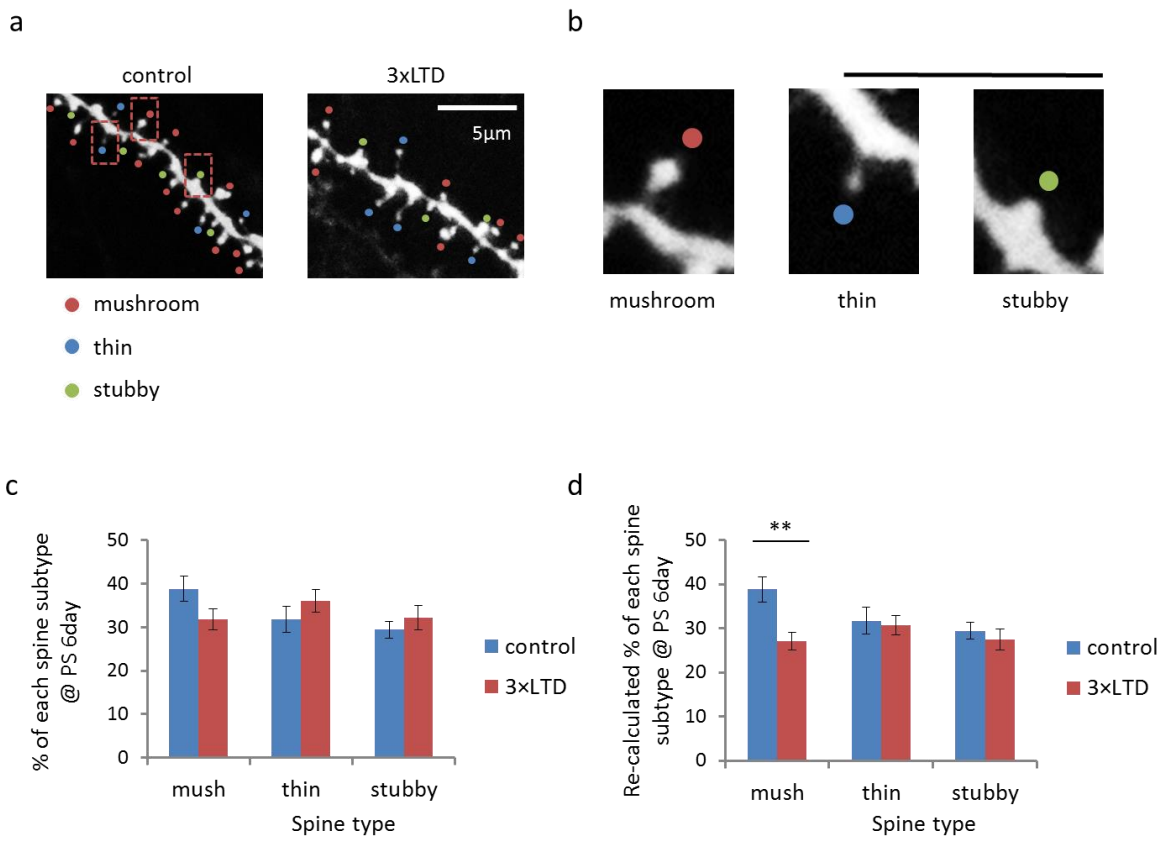


Fig.2-4 | Difference of stability among the spine subtypes.

(a,b) Representative images of spines classified into 3 subtypes. Scale bars indicate 5 µm.

(c,d) Spine density change of each subtypes at PS day6. (c) shows percentage of each spine subtype (each spine subtype density/ total spine density). (d) shows re-calculated percentage of spine density. At PS day 6, total spine density of 3xLTD group is 0.85% of that of the control group. Note the reduction of mushroom type spines in 3xLTD group compared to control group(control, n=28; 3×LTD, n=26; p=1.7×10-3 for ** in paired t-test).

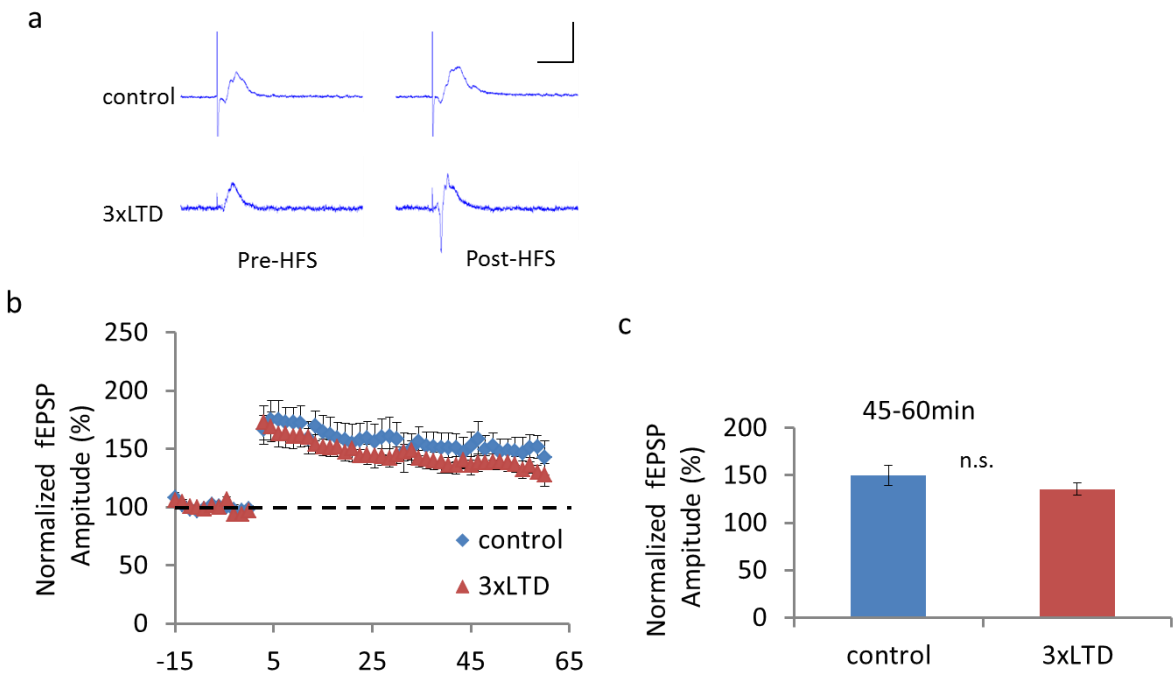


Fig.2-5| Inducibility of LTP induced by high frequency stimuli after repetitive LTD inductions.

(a)Representative traces of maximal fEPSP before and after high frequency stimulation. LTP was recorded at PS day3-5. Scale bars are 2mV(vertical) and 20ms(horizontal).

(b) LTP of CA3-CA1 synapses evoked by high frequency stimulation in control and 3×LTD. Repetitive LTD do not change the inducibility of LTP (control, n= 6; 3×LTD, n=7).

(c) Quantitative analysis of fEPSP for minutes 45-60.

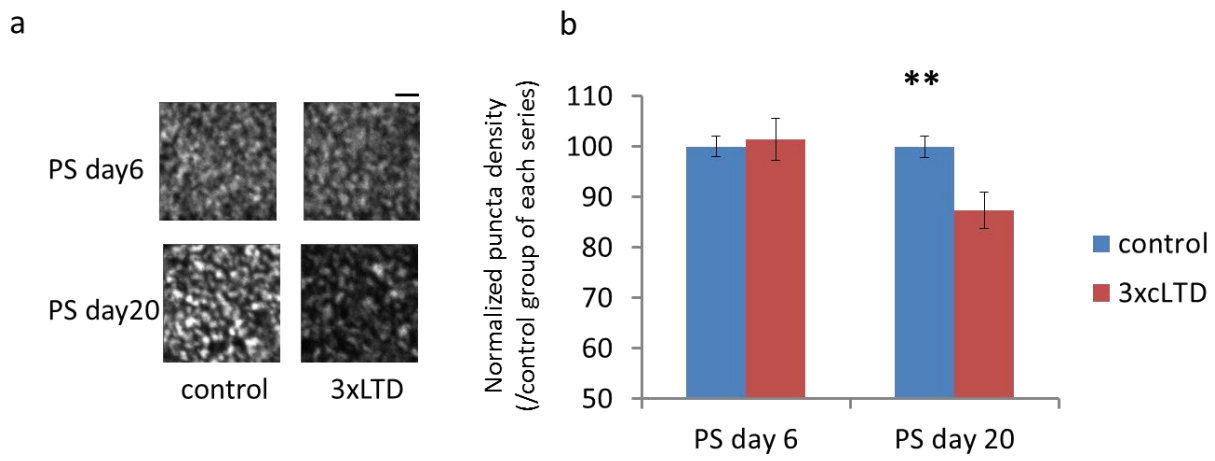
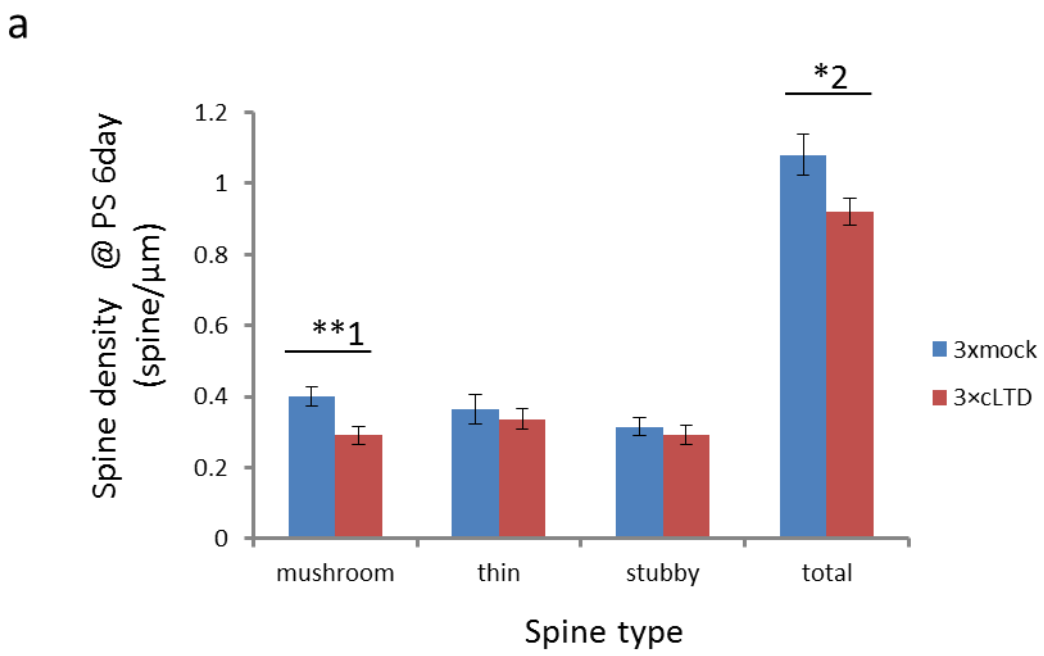


Fig.2-6| Synaptophysin-immunopositive puncta examined at PS day6 and PS day20 after repeated LTD stimulations.

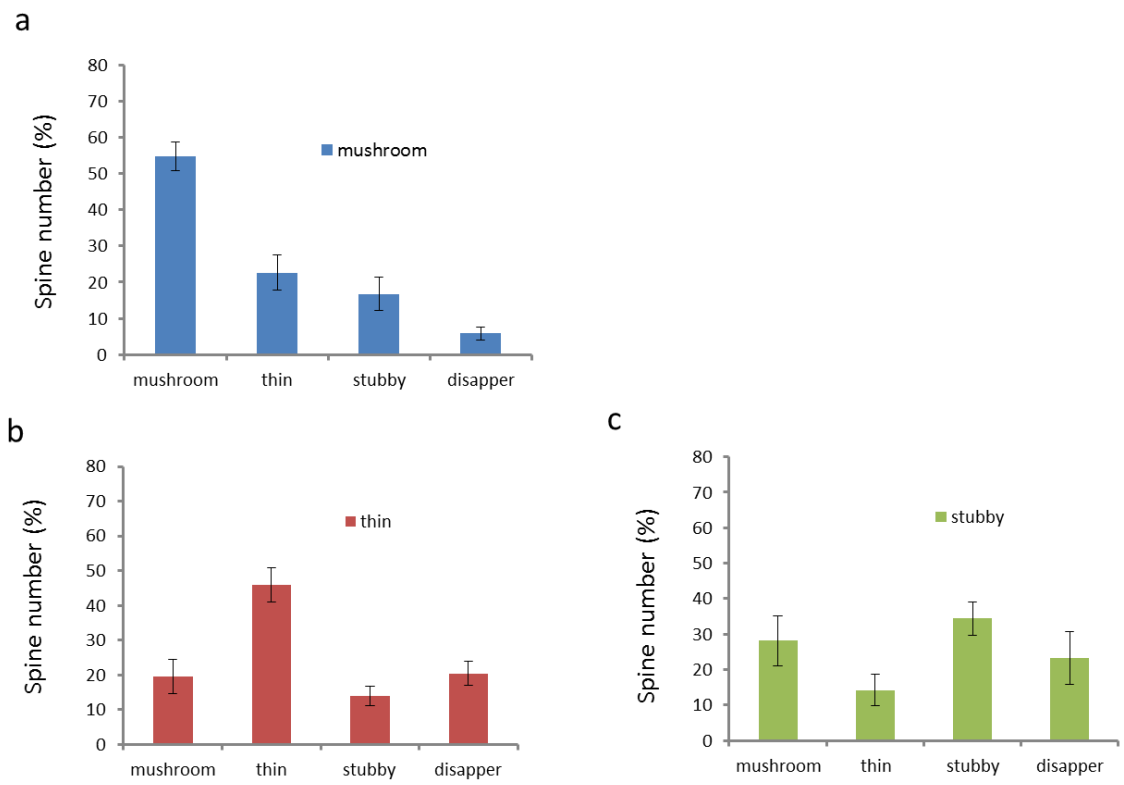
(a)Representative fluorescent images of synaptophysin puncta. Scale bar indicates 2μm. (b) Mean density of synaptophysin immunopositive puncta. The decrease of puncta density occur at PS day20 though density of puncta do not change at PS day6 (control at PS day6, n=45; control at PS day20, n=45; 3×LTD, n=42; 3×LTD, n=39; p=4.2×10-3 for ** in paired t-test).

2-6. Supplementary Figures and legends



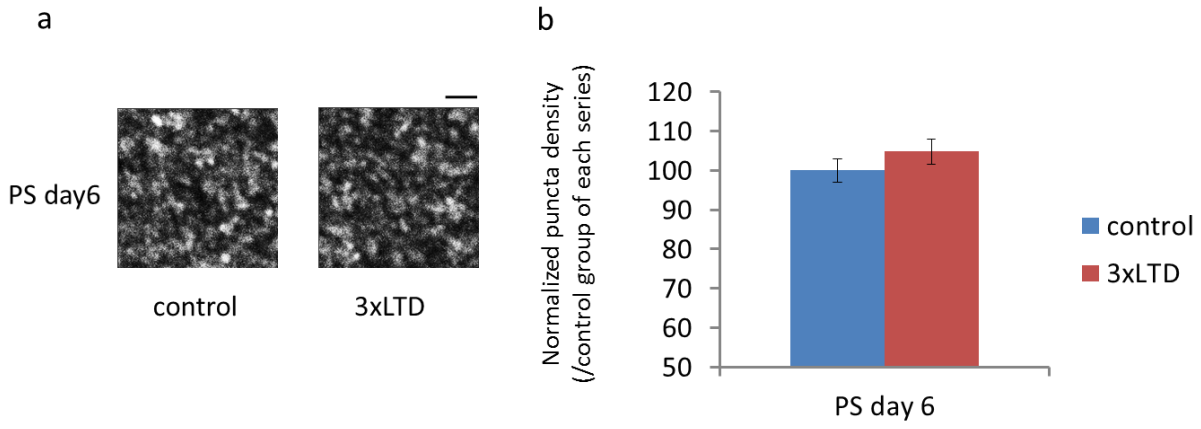
Supplementary Fig.2-1 | Difference of stability among the spine subtypes.

(a) Spine density change of each subtypes at PS day6. Note the reduction of mushroom type spines (control, n=28; 3×LTD, n=26; p=5.3×10-3 for **1, p=0.027 for *1 in paired t-test).



Supplementary Fig.2-2| Fate of individual spines with shapes classified before stimulation.

In this examination, I classified the spine type in living samples at PS day2 (2 hours before the 3rd LTD induction) and examined outcomes for each spine at PS day3 (22hours after the 3rd LTD stimulation). In contrast to the examination of fixed/due-injected samples (Fig.2-2), the classification on living/endogeneously fluorescent samples examined here would include some uncertainty due to weaker contrast. Note, however, that the mushroom type spines are not necessarily retracted. The spines examined are 289 from 9 neurons of 9 independent cultures. Timeline of examination is the same as that in Fig.2-4.



Supplementary Fig.2-3| Bassoon-immunopositive puncta analysis at PA day6 after repetitive LTD inductions.

(a) Representative images of immunolabeled bassoon puncta. Scale bar indicates 2μm.

(b) Quantitative analysis of bassoon immuopositive puncta. Consistent with analysis of synaptophysin, bassoon puncta density do not change at PS day6 (control, n=29; 3×LTD, n=21).

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Publications and Presentations

Original article

Sho Hasegawa, Shigeo Sakuragi, Keiko Tominaga-Yoshino, Akihiko Ogura
Dendritic spine dynamics leading to spine elimination after repeated inductions of LTD.
Scientific Reports 5, 7707 doi: 10.1038/srep07707 (2015)

International scientific meetings

Sho Hasegawa, Yuki Oe, Keiko Tominaga-Yoshino, Akihiko Ogura
Asymmetric dendritic spine dynamics in the apparently symmetric long-lasting synaptic plasticity phenomena after repeated LTP/LTD inductions
Society for Neuroscience 2014 ポスター発表　演題番号 : E39-518.04 （サンディエゴ　コンベンションセンター）11 月 12 日　2013 年

Domestic scientific meetings

長谷川翔、大江祐樹、冨永（吉野）恵子、小倉明彦

Dendritic spine dynamics in the long-lasting synaptic suppression after repetitive LTD induction.

第 90 回生理学会大会　ポスター発表　Program:2PK-043（タワーホール船堀、東京都） 3 月 28 日　2013 年

長谷川翔、大江祐樹、冨永（吉野）恵子、小倉明彦

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ポスター発表　Program : P2-2-5 （国立京都国際会館、京都府） 6 月 21 日　2013 年

長谷川翔、冨永（吉野）恵子、小倉明彦
繰り返し LTD 誘発後の長期持続的シナプス減弱（LOSS）生起時の樹状突起棘の動態
生理研研究会「シナプス・神経ネットワークの機能ダイナミクス」　口頭発表（生理学研究所、愛知県）
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Intracellular signaling pathway and dynamics of long-lasting dendritic spine retraction after repetitive LTD inductions.
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Other's article

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