



Structural LTP: from synaptogenesis to regulated synapse enlargement and clustering

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Nature teaches us that form precedes function, yet structure and function are intertwined. Such is the case with synapse structure, function, and plasticity underlying learning, especially in the hippocampus, a crucial brain region for memory formation. As the hippocampus matures, enduring changes in synapse structure produced by long-term potentiation (LTP) shift from synaptogenesis to synapse enlargement that is homeostatically balanced by stalled spine outgrowth and local spine clustering. Production of LTP leads to silent spine outgrowth at P15, and silent synapse enlargement in adult hippocampus at 2 hours, but not at 5 or 30 min following induction. Here we consider structural LTP in the context of developmental stage and variation in the availability of local resources of endosomes, smooth endoplasmic reticulum and polyribosomes. The emerging evidence supports a need for more nuanced analysis of synaptic plasticity in the context of subcellular resource availability and developmental stage.

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Introduction

Analysis of LTP provides a powerful window into cellular mechanisms of learning. Hence, LTP is mostly studied in the hippocampus, a brain region required to form memories. The importance of prior activation history and specific induction paradigms are increasingly emphasized to understand mechanisms of LTP [1–3]. Dendritic spines are tiny protrusions that stud the surface of dendrites and host most of the excitatory synapses throughout the brain. The importance of context arises even when single spine synapses are potentiated by glutamate uncaging [3]. Most experiments image changes in spine structure as a proxy for synapse growth, and usually end within an hour after

onset of potentiation. Such experiments have revealed exquisite detail about molecular and cellular mechanisms controlling spine structural plasticity during the early phase of LTP. Here we consider more enduring structural LTP in the context of developmental stage and availability of local resources.

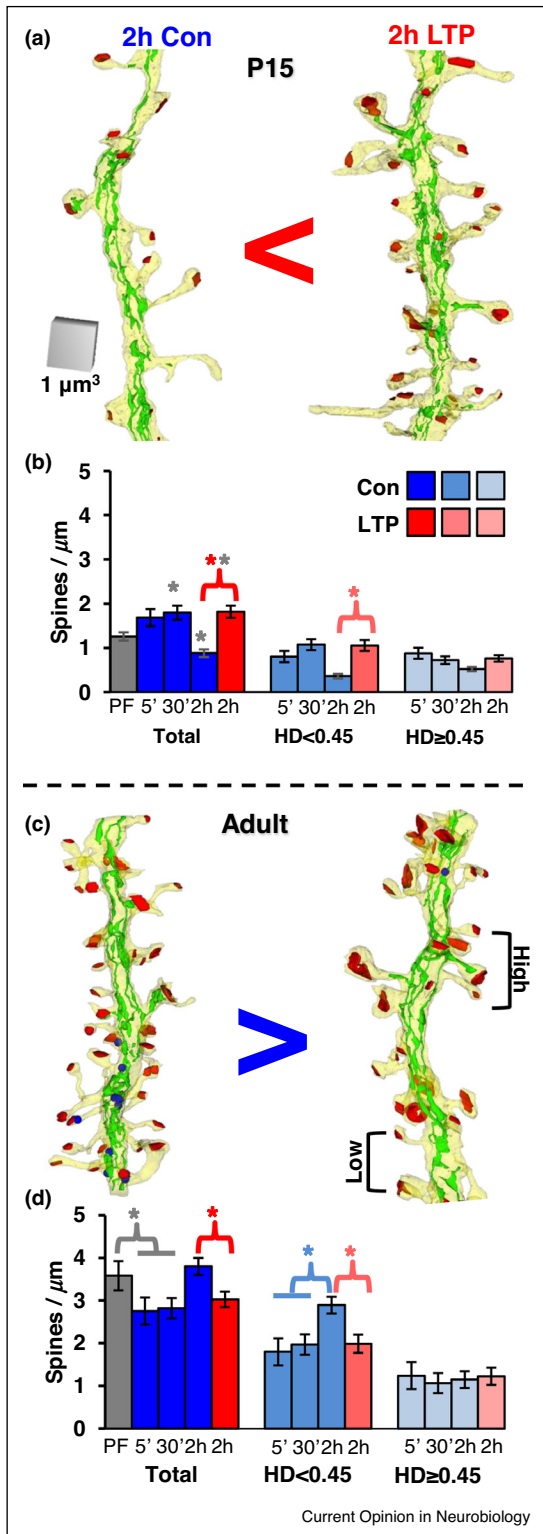
LTP enhances synaptogenesis at P15 but stalls spine outgrowth in adults

To investigate enduring LTP, hippocampal slices are prepared, allowed to rest for 3–4 hours, and then test pulses are delivered at a frequency of one per 2 min for 30–40 min to establish baseline response. Then LTP is induced with a pattern of theta-burst stimulation (TBS) that fully saturates LTP [4,5]. The number and frequency of test pulses is matched in control and LTP conditions for varying times post-TBS. Three-dimensional reconstruction from serial section electron microscopy (3DEM) obtained at different times post-TBS provides time-series snapshots of the underlying structural plasticity.

In stratum radiatum of rat hippocampal area CA1, 3DEM shows that spine density reaches about a third of adult levels by postnatal day (P)15 (Figure 1). Prior work shows this density reaches ~80% of adult levels one week later at P21 [6]. Thus, P15 is an age when the rate of natural synaptogenesis is high. In P15 rat hippocampal slices, control test pulses markedly reduce spine outgrowth over time (Figure 1b). The TBS counteracts inhibited spine outgrowth and the resulting LTP enhances synaptogenesis by 2 hours (Figure 1b), but not at 5 or 30 min after TBS. The LTP-related synaptogenesis adds small dendritic spines, while the density of large spines remains essentially stable across time for both the LTP and control conditions. At P15, synapse dimensions on the LTP-related new spines are comparable to those on control small spines. Synapse dimensions on large spines are also comparable across perfusion-fixed, control, and LTP conditions [5,7].

The effects of control stimulation and TBS in adult rats (P60–75) are opposite from those found at P15 (Figure 1c,d). Relative to perfusion-fixed brain, spine density is initially reduced in slices from adult hippocampus. Over time, delivery of control test pulses results in recovery of small spines. TBS stalls the small spine recovery while the density of larger, presumably more stable, spines is unchanged (Figure 1d). These findings show profound developmental differences in the response of hippocampal neurons to saturating induction of LTP.

Figure 1



LTP enhances synaptogenesis at P15 but stalls spine outgrowth in adults. **(a)** 3DEMs of dendrites from oblique dendrites in s. radiatum of hippocampal area CA1 at P15 representing the 50th percentile rank. Spine density in the 2-hour (2 hour) control is less than (red <) the 2 hour LTP condition. **(b)** Quantification of spine density (spines/ μm)

Resource dependent synapse enlargement and synaptogenesis

Multiple subcellular resources contribute locally to structural LTP. Smooth endoplasmic reticulum (SER) is a continuous internal membrane system that extends from the cell body into dendrites and into some spines. The SER regulates calcium and the synthesis and trafficking of lipids and proteins [8]. In locations where the SER elaborates, ER exit sites abound and can deliver resources of membrane and proteins to synapses [9**]. The spine apparatus is a structure elaborated from SER into membrane sheets separated by dense plates containing the actin binding protein synaptopodin. In addition to its SER-related functions, the spine apparatus also may acquire Golgi-like properties that could provide post-translational modification of transmembrane proteins, although this function appears to be lacking in young dendrites [10].

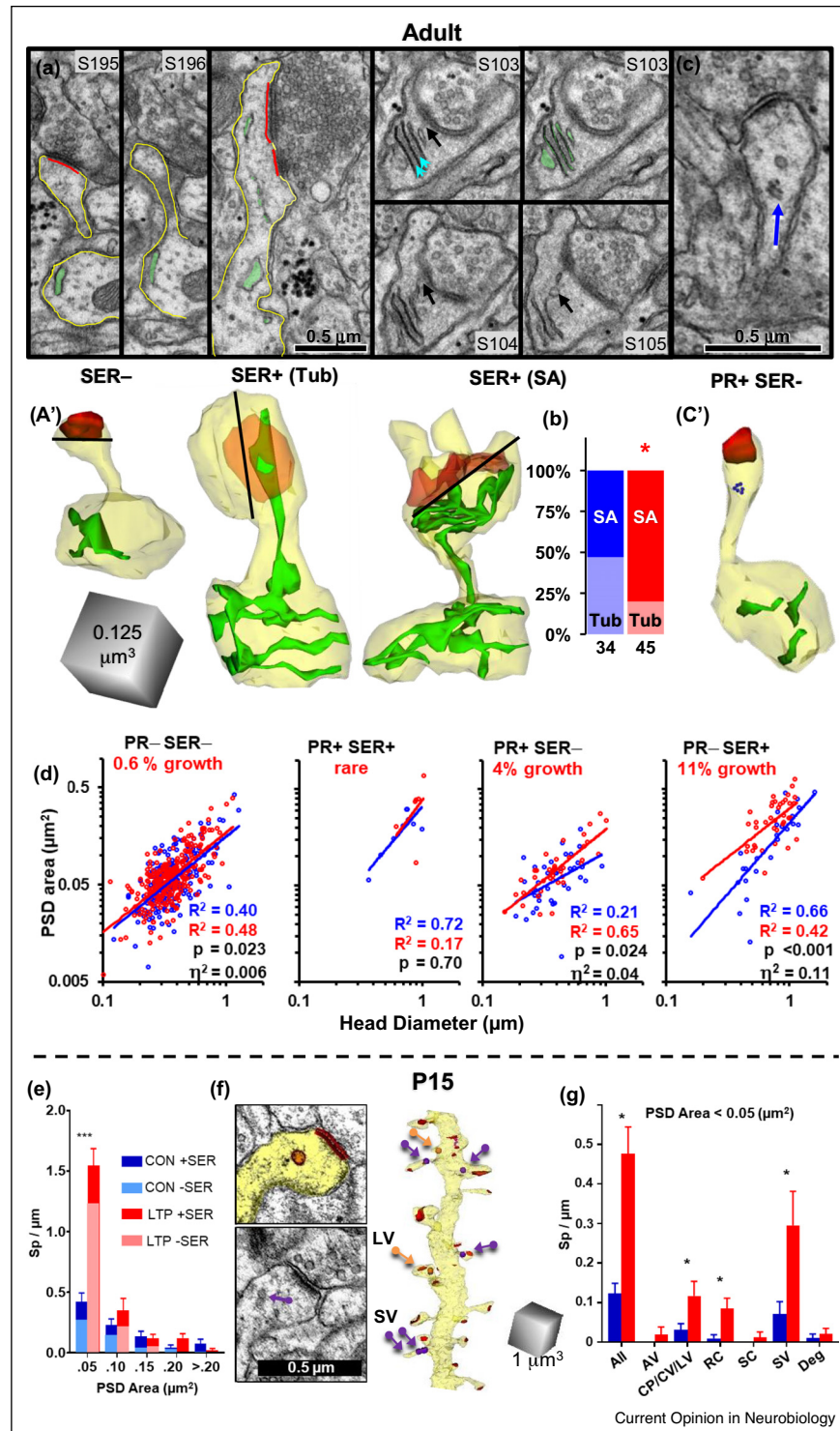
Local protein synthesis is another critical resource for structural LTP and is evidenced in electron microscopy by the presence of monosomes, polyribosomes (PR), or rough endoplasmic reticulum, all of which can be found 'in dendrites and spines. <https://synapseweb.clm.utexas.edu/141-dendritic-spines-25> Local protein synthesis is required for normal synaptogenesis during development and for enduring LTP and learning [11,12]. The PR are more readily identified in 3DEM than monosomes or RER; hence, their quantification provides a conservative estimate of local protein synthesis in various locations at the time when the tissue was fixed.

Endocytic, secretory, and recycling components also contribute to LTP under age and time-dependent constraints [7,9**]. These subcellular structures are highly dynamic with rapid rates of turnover. Thus, their presence or absence relative to time post-TBS is also a conservative reflection of their roles in spine formation and regulated synaptic growth.

In adults, only 10–15% of dendritic spines in s. radiatum of hippocampal area CA1 contain a tubule of SER or a

length of dendrite in s. radiatum of perfusion-fixed hippocampus (PF), and after 5 min (5'), 30', and 2 hour in the control or LTP conditions. **(c)** 3DEMs of dendrites from adult hippocampal s. radiatum representing the 50th percentile rank. **(d)** Plots show spine density in the 2 hour control is greater than (>) in the 2 hour LTP condition. Comparing PF between ages in (b) and (d) reveals natural age-dependent synaptogenesis. In all graphs, data were controlled for spine head diameter (HD). The LTP effects were not evident at 5 or 30 min; hence, these data are not plotted here, for simplicity, but they are available in the original publications. (Yellow – dendrite, green – smooth endoplasmic reticulum, red – excitatory postsynaptic density surface area. Gray asterisks indicate significant ($p < 0.05$) differences between the indicated condition and PF; red asterisks show significant LTP effects. The medium blue asterisk in D, shows significant differences between time points for small spines. Adapted from Refs.: Bourne and Harris [4]; Bell *et al.* [28]; Watson *et al.* [5]; Kulik *et al.* [7]).

Figure 2



Role of SER and polyribosomes in supporting synaptic growth after LTP. **(a)** Electron micrographs and (A') 3DEMs of dendritic spines without SER, with a simple tubule of SER, or a fully elaborated spine apparatus (SA). **(b)** Following LTP in adults, the frequency of SER containing spines does not change; however, there is a significant shift from a single tubule (T) to the SA form of SER (*, $p < 0.5$, $n =$ number of spines in each condition). **(c)** Electron micrograph and (C') 3DEM of spine containing a polyribosome, but no SER. **(d)** The LTP-related synapse enlargement is minimal in spines lacking PR or SER, is greater on spines that retain PR, and is greatest on spines containing SER. Each graph illustrates the actual PSD areas, controlled for head diameter, and plotted on a log-normal scale, with correlation values (R^2), and results of ANCOVA (p values

fully elaborated spine apparatus (Figure 2a) [13]. There is no change in this overall low frequency of spines that contain SER. By 2 hours after induction of LTP more of the SER-containing spines have acquired a spine apparatus (Figure 2b). The relative decrease in spines containing a single tubule of SER suggests that the spine apparatus could be elaborated locally in a spine from a single tubule of SER following LTP [14].

The frequency of PR-containing spines (Figure 2c) also changes over the time course of LTP in adult hippocampus. The PR + spine frequency is elevated at 5 and 30 min after TBS; however, by 2 hours post-TBS PR + spines are reduced relative to controls at the same time point [13]. This effect is dependent on the induction protocol, because the PR remain elevated in spines for at least 2 hours following tetanus-induced LTP in adult hippocampus [15]. Growth in the postsynaptic density (PSD) surface area was greatest on spines that contained SER, regardless of whether PR were present in the spine (Figure 2d). This growth was not limited by spine head size. Although PR and SER were rarely captured in the same spine, their spine synapses were as large as the spines containing SER alone in both control and LTP conditions (Figure 2d). More work is needed to determine whether monosomes or RER are differentially expressed across time following induction of LTP, which could reflect synthesis of different populations of proteins [16**].

The SER rarely occurs in electron micrographs of dendritic spines from developing neurons. This rare occurrence might reflect the highly dynamic state of SER making quick visits without stopping to stabilize a tubule or form a spine apparatus [17]. At P15, the LTP-enhanced synaptogenesis involves formation of spines that lack SER (Figure 2e) [7]. Instead the new small spines contain more secretory compartments, especially large and small vesicles (Figure 2f,g). These vesicles are likely derived from ER exit sites or recycling endosomes [7,9**].

At P15, the PR are elevated for at least 2 hours after TBS saturated LTP, especially at the base of dendritic spines [11]. PR frequency is also high in spines that form during control stimulation in adult hippocampus. These results suggest that highly dynamic, age-dependent, and state-dependent utilization of local resources supports synaptogenesis during LTP in developing neurons or recovering adult slices, and the enlargement of synapses following LTP in adults.

Maturation of homeostasis and spine clustering

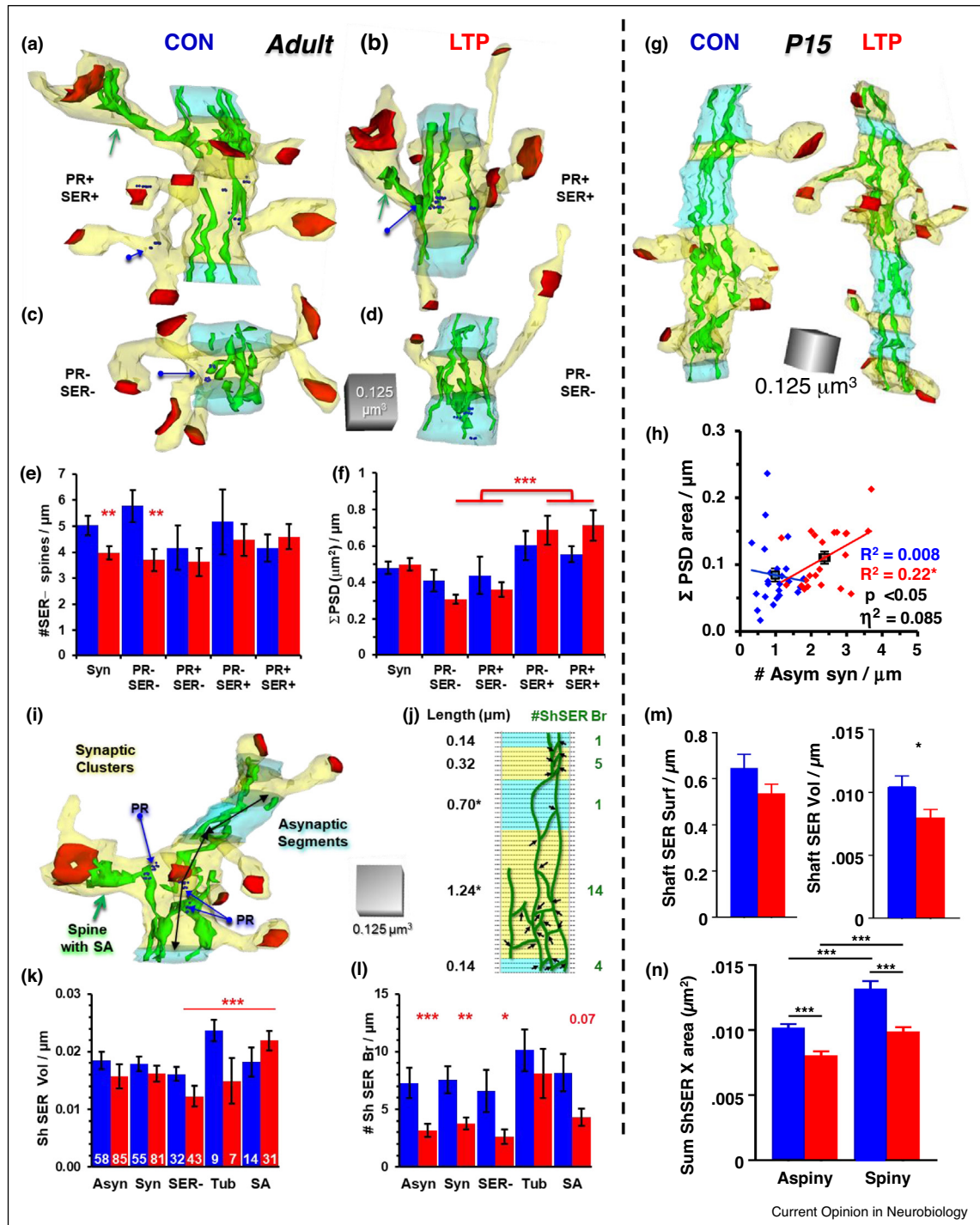
Recent experiments using optogenetics, live imaging, and computational models suggest that clusters of spines cooperate to enhance the efficacy of particular inputs during plasticity and learning [18*,19**,20,21*,22–24]. The redistribution of subcellular resources could be critical in determining where such spine clustering hotspots arise. During LTP, do the enlarging synapses on SA-containing spines sequester resources and prevent neighboring spine outgrowth, or do they share with neighbors and deprive distant spine outgrowth?

To answer this question, clusters are defined by the overlapping origins of spines and shaft synapses (Figure 3). The spine/synapse clusters are surrounded by synaptic dendritic regions (>120 nm) without intervening spine origins or shaft synapses. In adults, some clusters contain resource rich spines (Figure 3a,b), while other clusters have no SER or PR in the spines, only in the dendritic shaft (Figure 3c,d). In adults, spine outgrowth is stalled in synaptic clusters that have no resource-rich spines (PR–SER– spines, Figure 3e). Clusters having at least one SER + spine recover the same spine density as controls (Figure 3e). Total synaptic weight is measured as the summed PSD surface area across all synapses per unit length of dendrite in the cluster. In adults, the total synaptic weight is balanced across all synaptic clusters (Figure 3f). However, total synaptic weight is elevated following LTP, in synaptic clusters that had SER + spines (Figure 3f). Thus, in adults, LTP engages a homeostatic process that enlarges some synapses, creates spine clusters around them, and stalls distant spine outgrowth to balance total excitatory synaptic input along the dendrite [13]. At P15, the LTP-enhanced synaptogenesis results in a greater total synaptic weight per length of dendrite (Figure 3g,h). Together these findings suggest that LTP preserves the normal process of synaptogenesis in the developing system but encounters a profound regulation of total synaptic weight as the potentiated synapses enlarge in adults. The regulation occurs at a distance from the enlarging spine, which shares its resources locally to create a cluster of stronger dendritic spines.

The synaptic crosstalk between the LTP-enlarged spines that preserves and strengthens its neighbors in a cluster could be mediated by the SER in the dendritic shaft via a local spread of calcium release from the stores [25,26]. To test this hypothesis using 3DEM in adult hippocampal slices, the volume of SER is

(Figure 2 Legend Continued) and effect sizes, η^2). (e) At P15, most of the new spines produced 2 hours after LTP induction have small synapses and contain no SER. (f) 3DEM of dendritic segment from P15 illustrating secretory compartments increase in spines after LTP. (g) At P15, the increased secretory elements are primarily small vesicles (sv) or recycling compartments (RC) while some are also coated pits (cp) coated vesicles (cv) or large clear vesicles (LV). Amorphous vesicles and degradative structures are not elevated significantly.

Figure 3



Resource regulation of spine clusters. **(a)–(d)** Representative dendritic segments from adult hippocampal slices under control and LTP conditions. Resource rich clusters have spines that contain PR or SER and resource poor clusters have no PR-containing or SER-containing spines. Each reconstruction is at about the 50th percentile rank within condition by spine density within the synaptic cluster (yellow) which is surrounded by an asynaptic region (light blue that is at least 120 nm long, and averages 250 nm in both control and LTP conditions). **(e)** The density of spines without SER is reduced overall in the synaptic clusters (Syn) following LTP, and this effect only occurred in clusters that lacked resource-rich spines (** $p < 0.01$). **(f)** Summed PSD area is balanced across all synaptic clusters and is greater following LTP in clusters that have resource-rich spines (** $p < 0.001$). **(g)** Representative dendritic segments from P15 hippocampal slices under control and LTP conditions. **(h)** Synaptogenesis following LTP increases the mean (black squares) summed PSD area in proportion to the increase in spine

measured and normalized across the dendritic shaft length of the synaptic clusters and asynaptic segments (Figure 3i). Shaft SER complexity was determined by counting the total number of branches in each segment (Figure 3j). Although shaft SER volume was similar over all asynaptic and synaptic clusters, it was greatest in synaptic clusters that had at least one spine with a spine apparatus, especially following LTP (Figure 3k). At P15, the SER surface area and volume in the dendritic shaft is also reduced 2 hours post-TBS (Figure 3m). When the SER complexity is measured as the summed cross-sectional area (X-sect) in each cluster, both aspiny and spiny segments show a decreased complexity following LTP (Figure 3n). At first glance the similar outcomes for shaft SER appear to conflict with the opposite outcomes for spinogenesis at P15 and synapse enlargement in adults. At both ages, a drop in SER complexity and associated ER exit sites could reflect production of vesicles that would support spine outgrowth at P15 and synapse enlargement in adults, with developmental shifts in the specific cargoes being targeted following LTP.

Silent formation and enlargement of synapses

Curiously, synaptogenesis and synapse enlargement appear to be silent at P15 and adult hippocampus. Enhanced synaptogenesis with LTP (P15) or recovery of spines during control stimulation in adults are both silent. This conclusion is obvious from looking at the time course of spine formation during control stimulation or LTP relative to the physiological response across time during LTP experiments (Figure 4a). In adults, if the spines that recovered in response to control stimulation were active, then the physiological response should climb as the spine number increases over hours. Instead, the physiological response to test pulses is stable for hours. Following TBS, the level of potentiation is fully saturated by 5 min; however, both synaptogenesis at P15 and synapse enlargement in adults does not occur at 5 or 30 min but is observed instead at 2 hours, yet the potentiated response remained stable. The quiet spinogenesis is not surprising, because newly formed spines typically do not contain AMPA receptors and the unsilencing of synapses by their addition has long been an integral mechanism of LTP in young hippocampus (Figure 4b) [27]. However, it is perhaps more surprising that enlargement of the PSD surface area in adults is also not observed at 5 or 30 min when LTP is saturated, but takes time, during which the physiological response is stable. This silent PSD enlargement is not due to the

absence of postsynaptic receptors but instead to the absence of presynaptic vesicles that creates a silent zone across from the PSD (Figure 4c) [28].

Presynaptic axons track postsynaptic changes

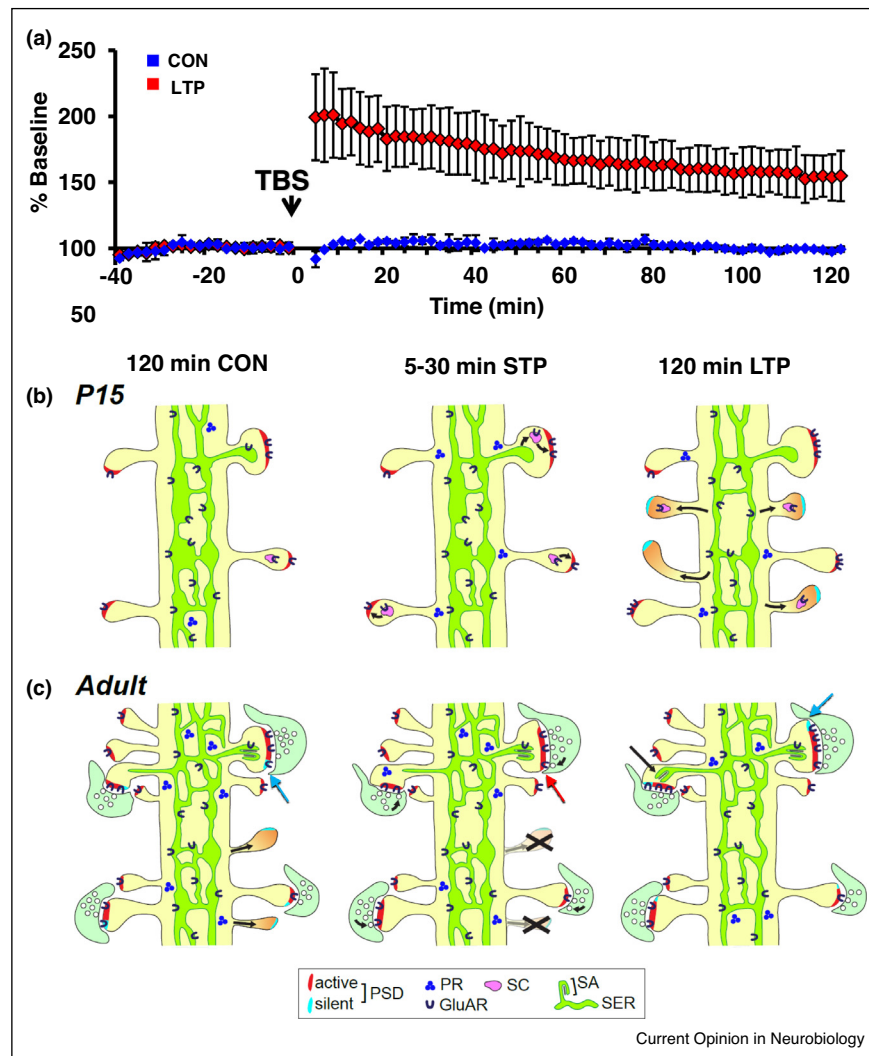
Presynaptic plasticity is also developmentally regulated by LTP [28–30]. At P15, more presynaptic boutons form to accommodate the LTP-induced synaptogenesis. In adults, fewer presynaptic boutons accompany stalled spine outgrowth after LTP. At both ages, a drop in presynaptic vesicles remains for at least 2 hours after TBS-induction of LTP, especially in boutons with mitochondria [29]. This drop could reflect the elevated recycling of presynaptic vesicles detected 30 min post induction of LTP [31]. However, recent findings suggest that the vesicle surface area associated with this drop provides enough membrane to account for an LTP-associated growth in presynaptic bouton surface area [32]. These findings suggest that a pool of presynaptic vesicles are available to maintain the well-known coordination between presynaptic and postsynaptic dimensions throughout life. It will be interesting to learn whether the presynaptic effects, specific to nascent zone formation and axon expansion, might in turn influence spine cluster formation after LTP.

Other considerations

Several other factors may contribute to the maturation of homeostasis and dendritic spine clustering. We focused here on the extent to which dendritic shaft SER and the associated ER exit sites may serve to define regions of dendritic spine clustering. The post-LTP spread of numerous other molecules may be restricted to individual spines or short regions of the dendritic shaft [2,33,34]. Differential expression of calcium-permeable AMPA receptors could influence the range over which a calcium influx may enhance spinogenesis following LTP during development [25]. Improved methods are needed to identify the specificity of LTP expression among the spines in and outside the clusters [35]. It will be interesting to know how local resource availability influences outgrowth and stabilization of dendritic spines *in vivo* during learning [18*,19**,36]. Perisynaptic astroglia, microglia, and local inhibition may also serve to control the maturation and location of dendritic spine clustering [37,38,39*]. Ultimately, all of these resources speak to mechanisms that may regulate information content at synapses throughout the brain [40].

(Figure 3 Legend Continued) density, with an overall effect size of 8% (η^2). (i)–(j) Calculating the number of SER branches per synaptic cluster or asynaptic segment length in adults. (k) The volume of SER in the dendritic shaft increases following LTP in those clusters that had a spine with a spine apparatus (SA). (l) Overall, the number of SER branches in the dendritic shaft decreases following LTP in adults but is retained in the clusters that contain spines with SER tubules (Tub) or SA. (m) At P15, Shaft SER volume decreased as does the (n) complexity of shaft SER in both aspiny and spiny segments. (Adapted from Refs: Chirillo *et al.* [13]; Watson *et al.* [5]; and Kulik *et al.* [7]).

Figure 4



Model for silent synaptogenesis and synapse enlargement. **(a)** Saturation of LTP and stable control responses. **(b)** During STP at P15, GluAR are added to existing PSDs. By 120 min during LTP, new GluAR-lacking spines emerge (orange spines with light blue PSDs). **(c)** In adults, some spines have GluAR-containing portions of the PSD, that are never-the-less silent because there are no presynaptic vesicles opposed to those zones (light blue zones in PSD, at 4 spines with presynaptic axonal boutons also illustrated; for simplicity, the other presynaptic axons are not illustrated at P15 or in adults). Zones of the PSD with presynaptic vesicles are red, being both presynaptically and postsynaptically active. In adults, the new spines that emerge during control stimulation lack GluARs. Induction of LTP blocks spine outgrowth (X's) and fills presynaptic zones with vesicles (red arrow). By 120 min, new PSD areas are added that lack presynaptic active zones (blue arrow) (b and c adapted from Kulik *et al.* [7]).

Conclusion

Despite dramatic structural plasticity, and daily turnover of synaptic proteins, memories stored in synapses show remarkable tenacity. Synapse stabilization appears to require reactivation, especially during sleep [41,42]. Failure of synapses to form, grow, or remodel is likely responsible for many developmental and age-related disorders. [43]. It remains unclear whether dendritic spine loss is a cause or consequence. Observing that dendrites retain immature varicosities and filopodia in developmental disorders is not sufficient to explain the cause. Dendrites

are almost spine-free in seizure disorders, which may reflect homeostatic down regulation of excitatory input. However, the remaining spines host multiple synapses, suggesting they try to compensate for input loss. A disruption in spine structure could undo critical biochemical compartmentation needed to isolate calcium-intense reactions from the dendritic shaft to avoid disruption in microtubules and trafficking of organelles and proteins. Synapses on spines that contain a spine apparatus undergo the most enlargement following LTP and these spines are preferentially reduced in Alzheimer's disease.

Knowing whether dendritic spine responses are a cause or consequence is fundamental to deciding whether to target presynaptic, postsynaptic and/or perisynaptic glial components. Here we show evidence supporting the need for more nuanced analysis of synaptic plasticity in the context of subcellular resource availability and developmental stage.

Conflict of interest statement

Nothing declared.

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