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# Nanoscale analysis of structural synaptic plasticity

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## Abstract

In the 1950's, transmission electron microscopy was first used to reveal the diversity in synaptic structure and composition in the central nervous system [1;2]. Since then, visualization and reconstruction of serial thin sections have provided three-dimensional contexts in which to understand how synapses are modified with plasticity, learning, and sensory input [3–17]. Three-dimensional reconstruction from serial section electron microscopy (ssEM) has proven invaluable for the comprehensive analysis of structural synaptic plasticity. It has provided the needed nanometer resolution to localize and measure key subcellular structures, such as the postsynaptic density (PSD) and presynaptic vesicles which define a synapse, polyribosomes as sites of local protein synthesis, smooth endoplasmic reticulum (SER) for local regulation of calcium and trafficking of membrane proteins, endosomes for recycling, and fine astroglial processes at the perimeter of some synapses. Thus, ssEM is an essential tool for nanoscale analysis of the cell biological and anatomical modifications that underlie changes in synaptic strength. Here we discuss several important issues associated with interpreting the functional significance of structural synaptic plasticity, especially during long-term potentiation, a widely studied cellular model of learning and memory.

## General Principles

Successful interpretation of structural synaptic plasticity using electron microscopy requires consideration of multiple factors. Especially important are the methods of tissue preservation, experimental preparation, LTP induction, and appropriate analysis.

### Tissue preservation

Rapid freezing, particularly combined with high-pressure, followed by freeze-substitution is considered to be the best method for preserving extracellular space, membrane structure, and molecular composition of the cytoplasm [18;19]. However, the amount of tissue with high quality freezing is limited to small patches located unpredictably throughout the region of interest. Outside these tiny patches of high quality preservation, the tissue is grossly distorted by ice-crystal artifact. Hence, this approach has not been widely adopted for experimental studies of ultrastructural synaptic plasticity where complete access to whole regions of interest is needed to trace dendrites and axons for sufficient length and volume to investigate changes in circuitry and structure. Currently, the gold standard for tissue preservation is rapid *in vivo* perfusion of fixatives at specific times following the induction of plasticity or consolidation of behavioral tasks (Figure 1A) [12–14;16;17]. However,

functional synaptic plasticity is widely studied in acute or organotypic brain slices and tissue culture where electrophysiological, genetic, or pharmacological manipulations can be restricted to specific regions of interest. In these preparations, perfusion-quality tissue preservation is routinely obtained by microwave-enhanced rapid fixation followed by routine tissue processing (Figure 1B)[3–6;10]. Recent findings also suggest that microwave-enhanced fixation and processing by freeze-substitution produces high quality images [18].

## Experimental preparation of brain slices

Although the brain slice preparation has proven invaluable for physiological studies of long-term potentiation (LTP) [20], one must be aware that differences in slice preparation and the recovery time before experimentation can have profound effects on baseline ultrastructure. For example, upon dissection of the hippocampus there is a synchronous release of synaptic vesicles, dendritic microtubules depolymerize, and glycogen granules disappear from astroglial processes [21–23]. Within 5 minutes of slice recovery *in vitro*, synaptic vesicles have recovered to levels found in the *in vivo* perfusion-fixed condition. Initially, microtubules re-polymerize in places where they do not normally occur *in vivo* (such as in dendritic spines), however by 3 hours of recovery *in vitro*, these aberrant microtubules disappear and dendritic microtubules reappear at their pre-dissection, perfusion-fixation frequency and lengths. Incubation of slices for 3 hours *in vitro* prior to experimentation allows all of the ultrastructural features to return to levels found in perfusion fixed brain *in vivo* [23]. This prior incubation time also facilitates the recovery of metabolic stability necessary for enduring LTP [24].

Fine processes of astroglia are retained in the vicinity of synapses in slices that are maintained at the interface of carbogen and artificial cerebral spinal fluid (aCSF) (compare Figure 1B to Figure 1A). In contrast, perisynaptic astroglial are effectively absent in slices that are submerged and superfused with aCSF (Figure 1C). The absence of astroglial processes gives the impression of more extracellular space in submersion-based slice preparations. In fact, their loss is of great concern for understanding mechanisms of structural synaptic plasticity because perisynaptic astroglial processes are linked to synaptogenesis and subsequent stabilization both during development [25;26], and in mature brain slices [11;27].

When the hippocampus is dissected and vibra-sliced under ice-cold conditions, and then rewarmed to physiological temperatures, there is a rapid and enduring proliferation of dendritic spines, at densities beyond those found in perfusion fixed brain [28–30]. If instead, the hippocampus is prepared quickly at warmer temperatures using a tissue chopper, the resulting number of spines is comparable to hippocampus that is perfusion-fixed *in vivo* [31] (Figure 2). Thus, warmer dissection and adequate recovery time *in vitro* provides acute hippocampal slices that are more comparable to *in vivo* fixed hippocampus and provide a better baseline from which to detect plasticity based changes in ultrastructure.

## LTP Induction paradigm

Another important consideration for evaluating LTP-mediated ultrastructural plasticity of synapses is the induction paradigm. Early studies used tetanic stimulation *in vivo* (100 Hz for 1 s) followed by instant brain removal and rapid freezing of a chopped surface and found evidence for spine swelling within minutes after the stimulation compared to control brains [32;33]. Others used tetanic stimulation and perfusion of chemical fixatives *in vivo* [34–36] or immersion fixation of brain slices [37], with limited success, although the induction paradigm was confounded by the limitations of single-section analyses discussed below. Brain slices have also been exposed to a cocktail of substances, i.e. chemical LTP, which

presumably enhances synaptic strength at all synapses in the slice, but surprisingly little net change in synapse ultrastructure was detected relative to control slices [8].

Theta burst stimulation (TBS), engages multiple plasticity mechanisms including back propagating action potentials, release of neurotrophic factors, activation of voltage gated calcium channels, and enhancement of presynaptic release; all of which are absent during tetanic stimulation [38–40]. TBS also better resembles the endogenous neuronal firing patterns in the hippocampus [41]. Induction of LTP with TBS in area CA1 of mature animals resulted in a pronounced turnover of synapses, protein synthesis-mediated enlargement of spines and synapses, and a coordinated loss of small spines that was perfectly counterbalanced by an enlargement of the remaining synapses [4]. As discussed below, these findings suggest that closer mimicking of the *in vivo* firing patterns results in more robust structural synaptic plasticity not previously found with tetanic- or chemically-induced LTP in brain slices.

### Analysis of ultrastructural synaptic plasticity

Sampling methods in EM can markedly influence the detection of structural synaptic plasticity. Complete reconstruction of all objects in the local neuropil provides the most precise quantification of changes in connectivity (number), size and composition of spines, synapses and associated structures. This approach, however, is very time-consuming [42]. Computer-assisted image analysis to automate alignment and segmentation of objects through sseM sections are being developed and hold promise for future application [43–45]. In the past, single-section analyses have dominated the field with limited results for two reasons: first because it is often impossible to identify a synapse or other structures on a single EM section (Figure 3), and second, because counts are biased due to differences in sizes of synapses and other structures, such that larger objects are more likely to be counted but less likely to be accurately identified. For example, single-section analyses have suggested more shaft and sessile spines form after induction of LTP in hippocampal area CA1 [36;37], although it is impossible to distinguish reliably the spiny dendrites of CA1 pyramidal cells from the non-spiny dendrites of inhibitory interneurons on individual sections used for those analyses. Stereological studies using a small number of serial EM sections have suggested an increase in the frequency of perforated synapses [46] and an increase in spine dimensions following the induction of LTP in the mature dentate gyrus [32;33;47]. Similarly, single section analyses following hippocampus-dependent learning found only transient [48;49] or subtle [50] changes in the number and morphology of synapses. However, the reported lack of profound changes with learning and plasticity may be confounded by missed and incorrectly identified synapses or a misrepresentation of their identity, dimensions and composition.

Until dense reconstruction of all structures in a potentiated network becomes routine [43–45], computer-assisted manual reconstruction of representative dendrites, axons, and perisynaptic astroglial processes in well-defined regions of interest provides the best alternative to evaluate ultrastructural synaptic plasticity. Sparse sampling from a well-defined subpopulation of dendrites ensures that comparable populations are evaluated across experimental conditions.

## Sample Results from ssEM that illustrate Ultrastructural Plasticity of Synapses

Synapses are dynamic structures surrounded by a complex neuropil including dendrites, axons, and astroglial processes. Synapses within a small area of neuropil can vary greatly in their size, shape, composition of subcellular organelles and access to perisynaptic astroglial

processes. All of these factors influence functional synaptic plasticity, hence it is important to identify their structural relationships accurately. Here we discuss representative examples where ssEM has served to disambiguate processes of synapse formation and growth during synaptic plasticity.

At present there are essentially two strategies amenable to investigating structural synaptic plasticity at the ultrastructural level, each with their strengths and caveats. One approach has been to image dendrites while inducing plasticity in organotypic slice cultures [51] or while eliciting stereotypical behavior in a well-known circuit, such as whisking and mouse barrel cortex [12–14]. Coincident changes in dendritic spine structure and physiology or behavior are tracked and approximate regions of interest are noted by gross landmarks such as blood vessels. The tissue is fixed and previously visualized cells are processed to visualize an electron dense marker, such as HRP enhanced antibodies to GFP. ssEM is then used to identify synapse locations on the previously visualized cells. An important caveat is that the electron dense product often obscures subcellular components that help to understand the local cell biology of the synapses under investigation.

The other approach has been to stimulate most or all synapses with electrical or pharmacological manipulations and perform statistics by comparing populations of synapses that underwent the differential activation [4;6–8;10;52–54]. This approach allows for a larger sample size but has the caveat of not knowing which individual synapses were potentiated. In addition, pre- and postsynaptic organelles, molecules, and electrical signals spread amongst neighboring synapses for 10's of microns following induction of LTP at an individual synapse [55–57]. Hence, whether individual synapses are visualized in the living state before and after induction of LTP or a large number of dendrites and synapses in the path of electrical or chemical induction of LTP are evaluated, one cannot be sure exactly which synapses mediated the LTP. Others have used calcium-oxalate in an attempt to identify where synapse-specific calcium influx might have occurred following induction of LTP [58]; however, the electron dense calcium-oxalate precipitate shows high background and only those spines containing smooth endoplasmic reticulum accumulated enough precipitate in either the control or LTP conditions. Hence, this approach to identify the potentiated synapses is biased because only about 10–15% of hippocampal dendritic spines contain SER [59]. Thus, it is not possible with current methods to define unequivocally which synapses were responsible for expressing LTP. Despite these caveats, it is reasonable to deduce that the structural changes are associated with LTP. The challenge ahead is to learn which structural changes are crucial for LTP.

## Dendritic Spines and Postsynaptic Densities

The use of ssEM has been essential to the assessment and identification of dendrites and spines that were first imaged with light microscopy either *in vivo* or in cultured neurons. GFP-labeled neurons in the rodent barrel cortex imaged *in vivo* following whisker trimming revealed an increase in the turnover of dendritic spines [12–14]. ssEM analyses of the imaged dendrites containing a GFP-directed antibody and HRP-reaction product revealed that the new spines formed synapses with presynaptic axons and that dendritic protrusions formed prior to synapses [12–14]. Combined light and ssEM imaging on cultured neurons revealed that Purkinje spines with synaptic contacts remain motile [60] and that induction of LTP in cultured hippocampal neurons resulted in spinogenesis that was followed by a delayed formation (>15 hrs) of synapses [61]. However, the long intervals between imaging sessions could have missed synapse turnover and other studies have suggested that synapse formation on new spines could happen on a much more rapid time scale [15;62].

Initial ssEM analyses found little or no change in synapse size or number during LTP [7;8;10]; however, warmer slice preparation and more robust statistical analyses later

revealed marked and balanced change in synapse size and number during LTP in mature hippocampal slices [4]. An increase in the turnover of spines was observed within the first 30 minutes after induction of LTP, as evidenced by an increase in excitatory shaft synapses, stubby spines and nonsynaptic protrusions. At 2 hours after LTP induction, small spines were lost and the remaining PSDs were enlarged (Figure 4A-E). The increase in PSD area was sufficient to compensate for the loss of spines such that the total summed PSD area per micron length of dendrite remained constant between control and LTP dendrites (Figure 4E, F). This structural synaptic scaling would have been missed with single section analyses or light microscopy, highlighting the essential need for ssEM to deduce structural plasticity, particularly in the mature hippocampus.

The “spine splitting” hypothesis is another example of the requirement for ssEM to elucidate mechanisms of structural plasticity. Ultrastructural analyses found an increase in multiple synaptic boutons (MSBs) following induction of LTP in area CA1 of organotypic slices, and led to the speculation that new spines may preferentially synapse with boutons that were strongly stimulated [54]. It was also suggested that the new spines were created through a process of “splitting” wherein a pre-existing spine split into two separate entities to increase synaptic density. However, ssEM and three-dimensional reconstructions revealed that the neuropil between neighboring spines from a single dendrite synapsing with the same presynaptic bouton contained numerous long axons that could not accommodate the extensive retractions and rearrangements that would have had to occur to allow the so-called spine splitting [5]. Instead, ssEM analyses revealed that these rare same-dendrite, multi-synaptic boutons likely form via new spine outgrowth which occurs within the first 5–30 minutes after the onset of LTP [4;5].

## Presynaptic Axons

More recently, ssEM has begun to elucidate structural changes among presynaptic axons and boutons during LTP and LTD. ssEM of organotypic slice cultures confirmed the formation of new presynaptic boutons with LTP [52] and that the varicosities that increased their turnover with LTD were indeed presynaptic boutons [51]. Multiple synaptic boutons (MSBs) have been considered a potential substrate for co-activation of multiple spines and have been proposed as a mechanism to coordinate plasticity between neighboring neurons [63]. However, they occur at a relatively low frequency in stratum radiatum of area CA1 [64;65] and immuno-labeling revealed that low levels of postsynaptic glutamate receptors on spines synapsing with MSBs suggests that simultaneous depolarization would not occur [66]. Thus, while light microscopy has revealed the temporal dynamics of some presynaptic changes, ssEM was needed to correctly identify presynaptic structures.

Presynaptic boutons have large pools of vesicles that are organized into functional categories depending on their probability of release [67]. ssEM has provided an essential component by describing the distribution of vesicles within boutons of Schaffer collaterals, mossy fibers, and the neuromuscular junction [64;68;69]. Work combining FM-labeling of recycling vesicle with ssEM has been used to describe the probability of release at synapses and to reveal the distribution of recycled vesicles within boutons [68;70–72]. The combined light-level and ssEM imaging of labeled vesicles also revealed the existence of a “superpool” of vesicles that are shared between neighboring boutons along an axon which expands the presynaptic release capacity well beyond the scope of individual synapses [55;73].

Questions remain as to the fate of the “superpool” vesicles with plasticity and whether the increase in presynaptic release observed with FM1-43 [39;40] corresponds to a decrease in synaptic vesicles. Single section analyses revealed a loss of vesicles within the first hour after LTP induction in both area CA1 [74] and the dentate gyrus [32], but a comprehensive

3-dimensional analysis of synaptic vesicle distribution during LTP is still missing. In the lateral amygdala, ssEM did not reveal a change in the overall number of vesicles with fear conditioning, although an increase in the ratio of PSD area to docked vesicle number was observed [16]. Thus, future studies with ssEM will be necessary to examine the timing and coordination of pre- and postsynaptic structural changes.

In addition to small synaptic vesicles, ssEM has characterized the frequency and distribution of small dense core vesicles (DCVs) [75], which are known to contain active zone proteins [76]. Induction of recuperative synaptogenesis during the rewarming of acute hippocampal slices prepared with ice-cold media resulted in a loss of DCVs, suggesting that they were used to create new active zones [75].

## Organelles

Local protein synthesis is required for maintenance of LTP [77] and is evidenced ultrastructurally by polyribosomes (Figure 3B, C). Polyribosomes occur every 1–2 microns along the lengths of both immature and mature hippocampal dendrites and thus intersperse among 4–10 dendritic spines [3;4;6]. Single section analyses suggested a decrease in polyribosomes at the base of spines in the mature dentate gyrus at various time points after LTP [78]. In contrast, ssEM demonstrated a redistribution of polyribosomes from dendritic shafts into spines with larger PSDs at 2 hours after induction of LTP by tetanic stimulation in hippocampal slices from young [6] and mature [3] rats. More recent work found a depletion of polyribosomes that was coupled with an enlargement of PSDs at 2 hours after induction of LTP by TBS, suggesting that mRNAs may have been “used up” to increase the size of the PSD [4]. Thus, ssEM is needed to detect the timing of formation and specific localization of polyribosomes.

Smooth endoplasmic reticulum (SER) enters a small percentage of spines in both the hippocampus and the lateral amygdala [17;59;79]. The spine apparatus, a specialization of SER found preferentially in large spines, is involved in the regulation of calcium and the synthesis of transmembrane proteins and is important for synaptic plasticity [80]. In the lateral amygdala, ssEM revealed that fear conditioning resulted in an enlargement of PSDs on a subset of spines lacking a spine apparatus [59]. Spines that contained a spine apparatus did not further enlarge with fear conditioning, suggesting a ceiling effect, but did decrease in size with safety learning. Thus, the presence of a spine apparatus appears to have an important role in regulating changes in synaptic size in the lateral amygdala.

Recycling or sorting endosomes occur in dendritic shafts at a rate of about one per 10–20 dendritic spines [9;79]. Endosomes are involved in recycling receptors and contributing membrane to spines for enlargement with LTP. Live imaging of cultured neurons found that induction of LTP resulted in the trafficking of recycling endosomes and vesicles into spines [9]. Quantification through ssEM of membrane surface area in these compartments within dendrites showed sufficient membrane was available to account for dendritic spine enlargement [9]. Together these findings suggest that investigation of dendritic segments through ssEM is necessary to elucidate the distribution and frequency of core dendritic resources that random sampling of individual dendritic spines would miss.

## Perisynaptic astroglial processes

Characterization of perisynaptic astroglia is particularly dependent of the use of ssEM because the smallest processes are not resolved at the level of light microscopy. ssEM revealed that fewer synapses had perisynaptic astroglia in recovered slices following recuperative synaptogenesis, and that synapses were larger when perisynaptic astroglia was present [11]. Interestingly, the amount of the synaptic perimeter surrounded by perisynaptic

astroglia and the distance between neighboring synapses was not proportional to synapse size, suggesting that both large and small synapses retain access to extracellular glutamate and secreted astroglial factors [11]. These findings suggest that as synapses enlarge and release more neurotransmitter, they attract astroglial processes to a discrete portion of their perimeters, further enhancing synaptic efficacy without limiting the potential for sharing extracellular resources with neighboring synapses. In addition, ssEM revealed a disruption in the 3-dimensional relationship between perisynaptic astroglia and synapses in the human hippocampus removed to alleviate severe epilepsy [27].

In addition to astroglia, the interaction of microglia with synapses in the visual cortex has also been described with ssEM [81]. During normal visual experience, most microglial processes interacted with multiple synapses and preferentially localized to small and transient dendritic spines. Light deprivation decreased the motility of microglial processes and changed their localization to the vicinity of a subset of larger dendritic spines that subsequently decreased in size. These changes were reversed upon light re-exposure. Thus, addition to the role of microglia in the immune response, they also involved in regulating spine size during visual experience.

## Conclusions

In the past 60 years, ssEM has evolved into a powerful tool with which to study the ultrastructural plasticity of synapses. Advances in technology have aided the rate at which data can be acquired and analyzed, leading to an expanding interest in mapping the neurocircuitry of the brain at the nanometer level, the so-called ‘connectome’ [82]. However, even knowing the location of every synapse in the connectome will not provide the complete answer because we need to know the functional state of each synapse, which can be further influenced by the ion channel composition in the nonsynaptic membrane of the postsynaptic dendrite or spine [83]. Detailed analysis through ssEM reveals invaluable information about the cell biological mechanisms that underlie the direction and degree of plasticity expressed by individual synapses. The finding of structural synaptic scaling during LTP emphasizes an important question of whether a few large synapses are actually more effective than many small synapses [4;84]. Elucidating ultrastructural relationships between neighboring synapses will help to reveal how the weight of synaptic responses is distributed and how it is modified during plasticity and learning.

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## Highlights

- Serial section electron microscopy (ssEM) is a necessary tool to study plasticity.
- Multiple experimental and analytical factors must be considered when using ssEM.
- ssEM has revealed at nanometer resolution changes in synapse size and composition.

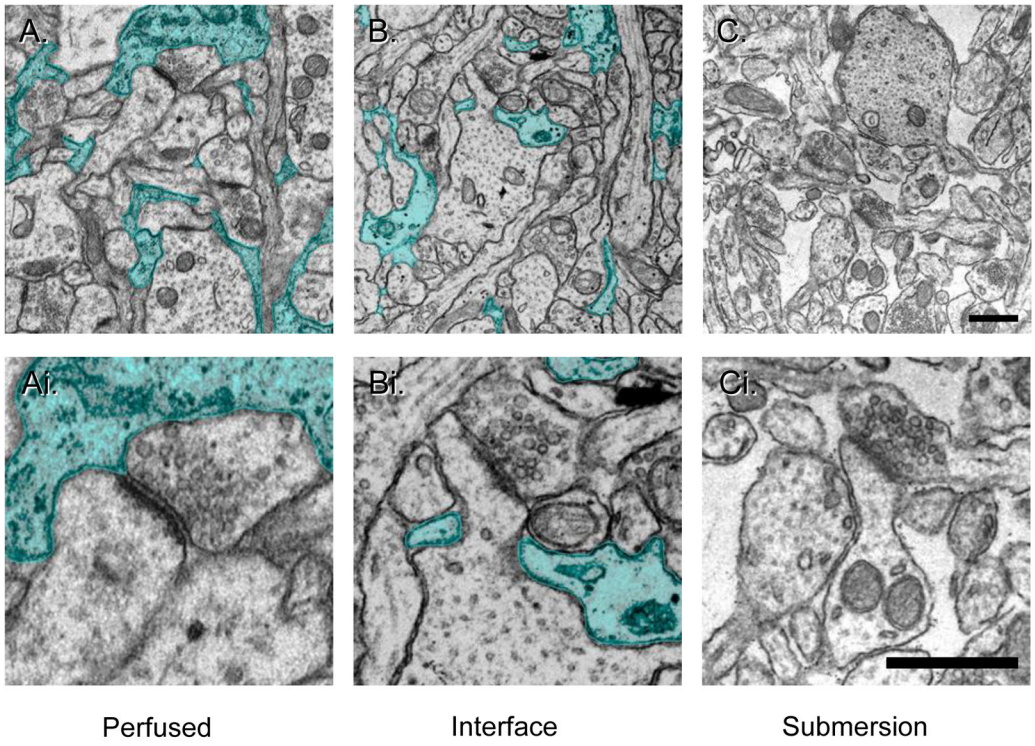


**Figure 1.**

Perisynaptic astroglial processes are intact in acute hippocampal slices maintained in an interface chamber, but are lost or retracted when slices are maintained under submersion conditions. Astroglial processes are colorized in blue. (A) EM from perfusion fixed hippocampus. (Ai) A region enlarged from A that highlights the presence of perisynaptic astroglial at a spine synapse. (B) EM from a hippocampal slice maintained in an interface chamber showing numerous astroglial processes. (Bi) Close up of (B) showing an astroglial process contacting the neck of a dendritic spine. (C) EM from a hippocampal slice recovered in a submersion chamber showing the absence of astroglial processes resulting in an expansion of extracellular space. (Ci) Close up of (C) demonstrating a dendrite and synapse with no perisynaptic astroglial processes. Scale bars = 0.5 microns.

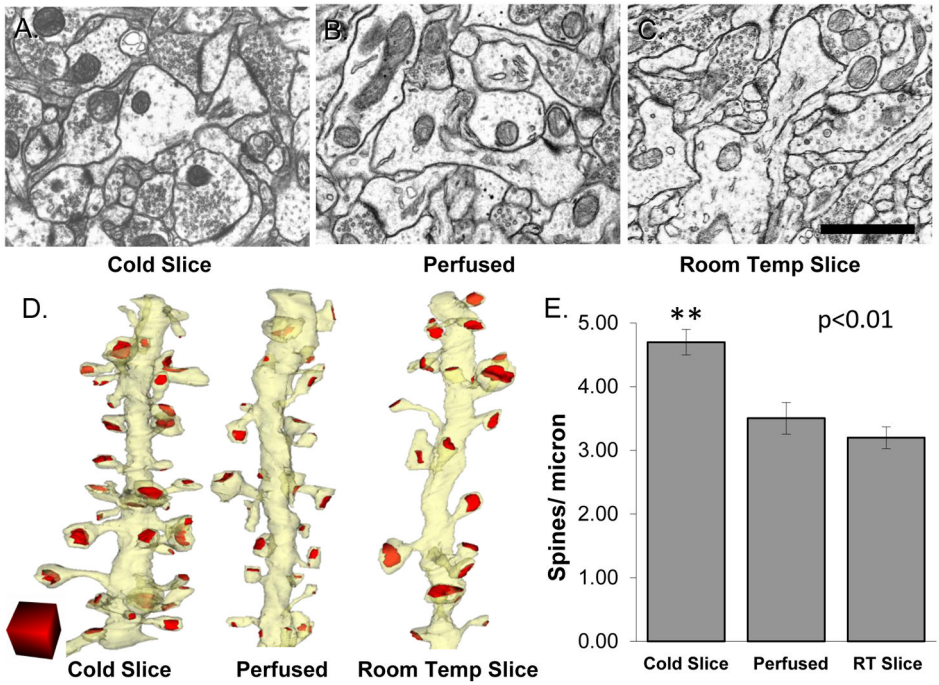


**Figure 2.**

Temperature during slice preparation ultimately influences total synapse number in the recovered slices. Tissue quality of hippocampal neuropil in stratum radiatum of area CA1 is comparable across (A) slices prepared under ice-cold conditions and maintained at ~32° C for 9–10 hr in an interface chamber, (B) hippocampus perfusion-fixed *in vivo*, and (C) in slices prepared at room temperature and maintained at ~32° C for 4.5–5.5 hr in an interface chamber. (D) Three-dimensional reconstructions of dendrites from each of the three conditions. Scale cube = 1 micron on a side. (E) Dendrites from ice-cold slice preparations had a higher spine density at 9–10 hr *in vitro*, compared to perfusion-fixed hippocampus and room temperature slices ($P<0.02$). Adapted from Bourne et al., 2007.

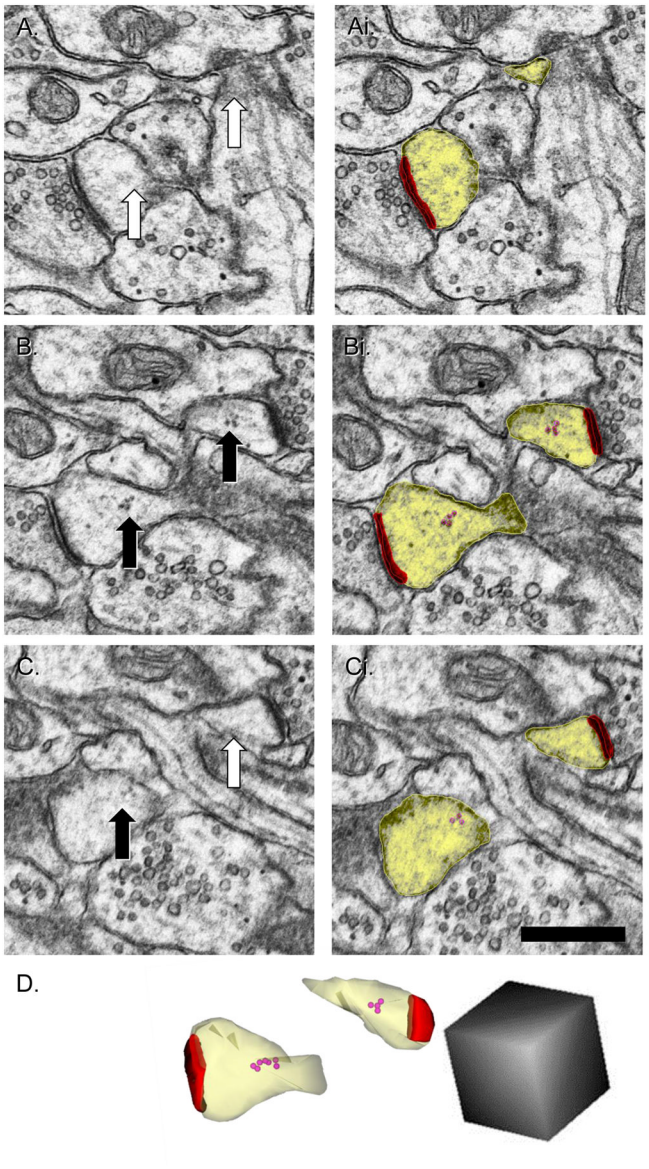


**Figure 3.**

Serial images through two spines illustrate the need for ssEM to identify the location of PSDs and polyribosomes. (A–C) Serial images illustrating the presence (black arrows) of polyribosomes on some sections and their absence in the same locations on adjacent sections (white arrows). (Ai-Ci) Same images with dendritic spines highlighted in yellow, PSDs in red and polyribosomes in magenta Scale bar = 0.5 microns for both columns. (D) Three-dimensional reconstruction of spines (yellow), PSDs (red) and polyribosomes (magenta). Scale cube = 0.5 microns on a side.

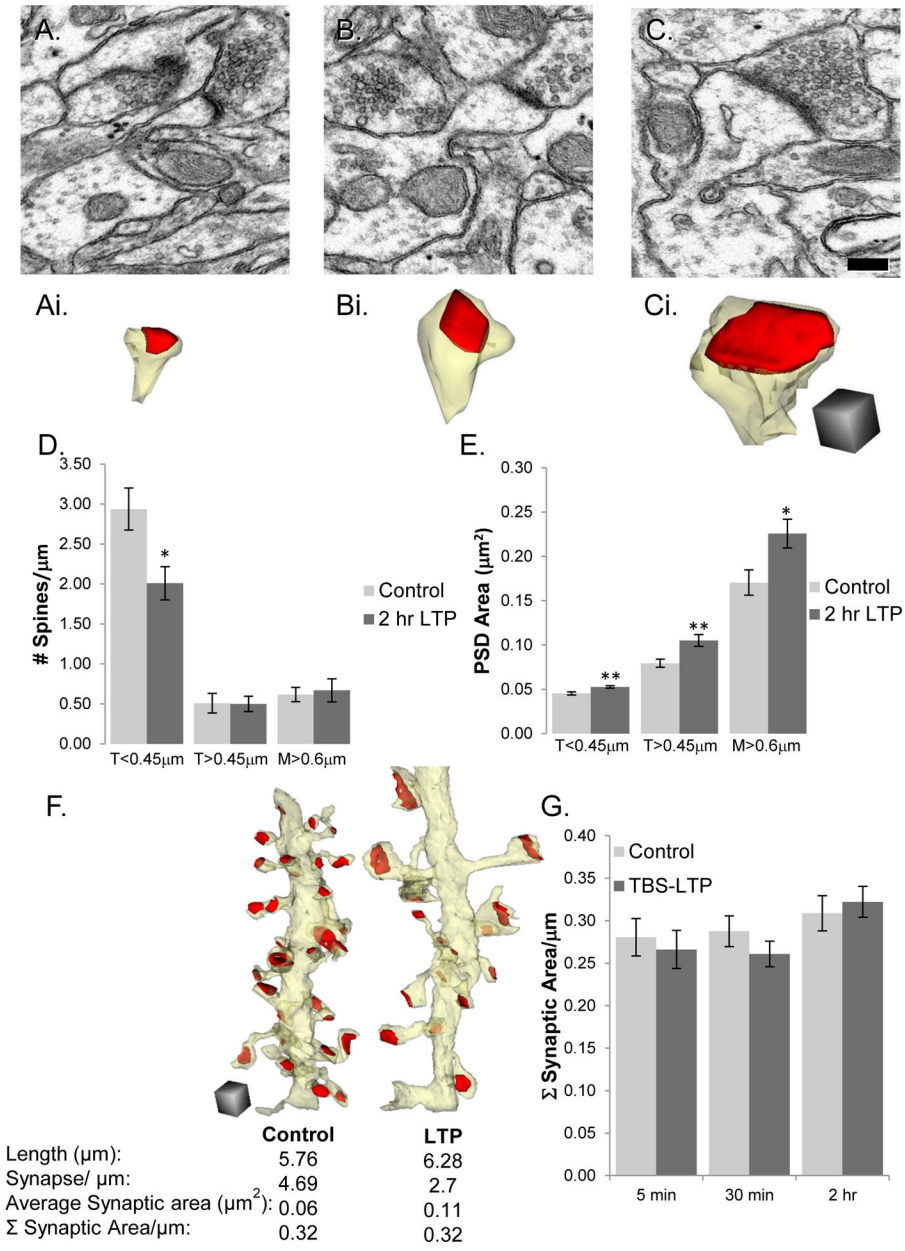


**Figure 4.**

Dramatic structural synaptic scaling revealed by ssEM following induction of LTP by TBS in the mature hippocampus. (A) EM and (Ai) reconstruction of a small thin spine (category T, head diameter <0.45 µm). (B) EM and (Bi) reconstruction of a medium thin spine (category T, head diameter >0.45 µm but <0.6 µm). (C) EM and (Ci) reconstruction of a mushroom spine (M, head diameter >0.6 µm). D. Small thin spines were significantly reduced in number ($p<0.05$) while (E) PSDs on all remaining spines were significantly enlarged (**$p<0.01$; *$p<0.05$). (F) Reconstructions of control and LTP dendrites demonstrate equal summed PSD area per micron length of dendrite, despite large differences in their average synapse size and density. Scale cube = 0.5 microns on a side. (G) Average summed PSD area per micron length of dendrite is the same for dendrites across all times in the control and TBS-LTP conditions. Adapted from Bourne and Harris, 2011.