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Polyribosomes Redistribute from Dendritic Shafts into Spines with Enlarged Synapses during LTP in Developing Rat Hippocampal Slices

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Summary

The presence of polyribosomes in dendritic spines suggests a potential involvement of local protein synthesis in the modification of synapses. Dendritic spine and synapse ultrastructure were compared after low-frequency control or tetanic stimulation in hippocampal slices from postnatal day (P)15 rats. The percentage of spines containing polyribosomes increased from 12% ± 4% after control stimulation to 39% ± 4% after tetanic stimulation, with a commensurate loss of polyribosomes from dendritic shafts at 2 hr posttetanus. Postsynaptic densities on spines containing polyribosomes were larger after tetanic stimulation. Local protein synthesis might therefore serve to stabilize stimulation-induced growth of the postsynaptic density. Furthermore, coincident polyribosomes and synapse enlargement might indicate spines that are expressing long-term potentiation induced by tetanic stimulation.

Introduction

Long-term potentiation (LTP) is an enhanced synaptic response that is thought to underlie some forms of learning and memory (Teyler and DiScenna, 1985; Davis and Squire, 1984; Guzowski et al., 2000). It can be induced by a variety of stimulation patterns and lasts for hours to days, depending on the age of the animal and the exact experimental conditions (Bliss and Collingridge, 1993). The enduring nature of LTP has prompted many investigations into whether an increase in synapse number or size serves to stabilize the potentiated response (reviewed in Yuste and Bonhoeffer, 2001). Studies in adult hippocampus provide conflicting results with either subtle or no changes in synapse number, shape, composition, or dimensions (e.g., Van Harreveld and Fifkova, 1975; Fifkova et al., 1982; Desmond and Levy, 1990; Lee et al., 1980; Chang and Greenough, 1984; Sorra and Harris, 1998). Recent studies from developing hippocampal neurons in organotypic slice cultures have consistently shown evidence for the formation of new dendritic protrusions after different forms of activation that induce LTP (Maletic-Savatic et al., 1999; Engert and Bonhoeffer, 1999; Toni et al., 1999; Fiala et al., 2002). Immature hippocampal neurons might be more susceptible to structural changes during LTP, or new spines might be more easily detected against a background of fewer spines (Harris et al., 1992; Fiala et al. 1998). Dendritic spines begin to be the dominant site of excitatory synapses, and tetanic stimulation begins to induce enduring LTP, lasting more than 4 hr in the hippocampus at P15 (Harris and Teyler, 1984; Jackson, et al., 1993), making it an appropriate age to investigate the role of tetanic stimulation in altering spine and synapse structure.

The location of polyribosomes in or near dendritic spines suggests that neurons may translate new proteins exactly where they are needed to modify synapses in response to potentiating stimuli (Steward and Levy, 1982; Steward, 1983; Steward and Schuman, 2001). New protein synthesis occurs in isolated hippocampal dendrites and synaptoneurosomes after depolarization (Rao and Steward, 1991; Torre and Steward, 1992; Weiler and Greenough, 1991), after exposure to brain-derived neurotrophic factor (BDNF; Kang and Schuman, 1996), and in response to a glutamate receptor agonist (Job and Eberwine, 2001a). Recent imaging studies show that mRNA localizes to synaptic sites on dendrites and initiates protein synthesis at synapses exposed to BDNF in cultured hippocampal neurons (Aakalu et al., 2001). Local protein synthesis appears to be involved in other forms of synaptic plasticity as well. Hippocampal long-term depression requires dendritic protein synthesis (Huber et al., 2001), and local translation is required presynaptically for sustained facilitation at sensory-motor neuron synapses in *Aplysia* (Martin et al., 1997).

Recent studies show that mRNAs are rapidly translocated to dendritic regions that have received high-frequency synaptic activation (Roberts et al., 1998; Steward et al., 1998). These mRNAs may be used in creating or maintaining stable changes in synaptic strength, such as LTP. An early short-term phase of LTP is protein synthesis independent, while the subsequent enduring phase requires new protein synthesis (Frey et al., 1988; Otani et al., 1989; Nguyen et al., 1994). Curiously, protein synthesis is needed at the time of induction, even though the protein synthesis-dependent component of LTP appears 1–2 hr later (Otani et al., 1989). In addition, dendrites severed from their cell bodies display only short-term potentiation, suggesting that mRNAs or proteins transported from the soma are needed to sustain LTP (Frey et al., 1989). It has not been reported whether enduring LTP requires new protein synthesis at P15, however, the culture experiments described above suggest that developing dendrites are capable of stimulation induced changes in protein synthesis.

High-frequency stimulation in the hippocampus has been shown to induce not only the transcription of the activity-regulated, cytoskeleton-associated protein Arc, but also the accumulation of its mRNA and protein in the stimulated lamina only (Steward et al., 1998). This finding suggests that stimulated synapses attract, capture, and translate mRNA. It is unclear at the light microscopic level whether the mRNA and proteins are associated with individual synapses or are collected throughout

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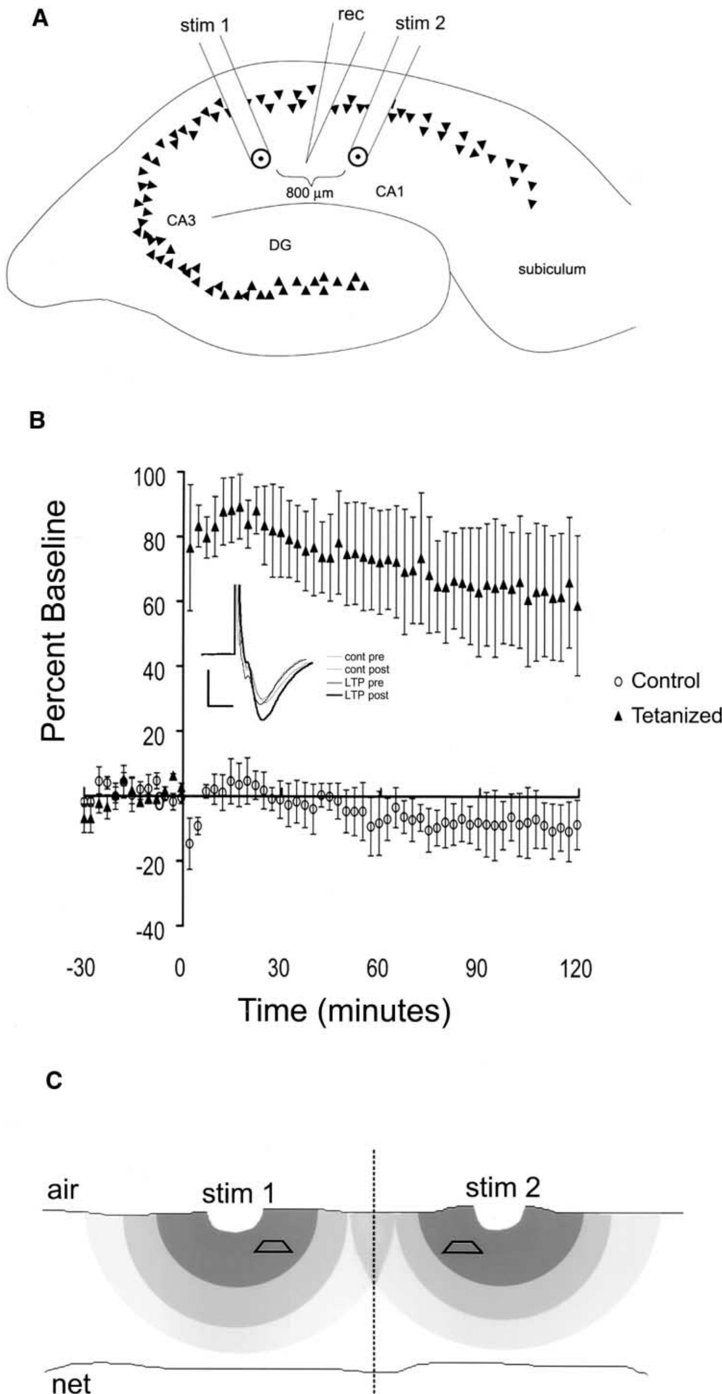

Figure 1. Site-Specific LTP in Hippocampal Slices

(A) Two stimulating electrodes were positioned approximately 800 μm apart in the middle of stratum radiatum of hippocampal area CA1 with a recording electrode positioned halfway between them.

(B) Tetanic stimulation was delivered to one of the stimulating electrodes at time 0 to induce LTP (triangles), and the control stimulating electrode (circles) received low-frequency stimulation. Example waveforms (inset) are each an average of the last ten pretetanus and last ten posttetanus responses at the control (cont) and tetanized (LTP) sites (scale 1 mv, 5 ms). Responses from each slice were averaged over 5 min intervals and then the mean ± SEM was graphed (n = 3 animals). The responses were monitored for 2 hr posttetanus prior to fixation.

(C) Diagrammatic view through the depth of a slice in the middle of stratum radiatum, from the air to the net surfaces, illustrating the falloff with distance in the density of synapses affected by each stimulating electrode. The slice was divided between the two stimulating electrodes (dotted line) so that the two regions could be separately sampled by serial electron microscopy (trapezoids).

the dendrites in the stimulated region. If high-frequency stimulation causes mRNA and newly synthesized proteins to accumulate at potentiated synapses, then we reasoned that dendritic spines should contain more polyribosomes after tetanization. We chose to investigate this hypothesis at 2 hr after tetanic stimulation induced LTP because it is long enough for newly synthesized mRNA to be transported from the soma into the dendrites (Knowles et al., 1996).

We used serial electron microscopy to test whether induction of LTP by tetanic stimulation causes a change in protein synthesis at synapses by identifying the location of every polyribosome in reconstructed hippocampal CA1 dendrites and dendritic spines. We found fewer polyribosomes in dendritic shafts and more in dendritic spines after tetanic stimulation than after control stimulation. In addition, the spines containing polyribosomes after tetanic stimulation had larger synapses, which might serve to sustain the potentiated response.

Results

Tetanic stimulation was compared to control low-frequency stimulation within hippocampal slices from three

15-day-old rats (Figure 1A). Electrophysiology demonstrated that site-specific LTP was induced only at the stimulating electrode used to deliver the tetanic stimulation (Figure 1B, see Experimental Procedures). Two hours after tetanic stimulation, the slices were fixed and prepared for EM. Based on the electrophysiology, the highest density of synapses receiving either control or potentiating stimulation should have been located near the control or tetanized sites, respectively. Serial thin sections were therefore cut 50–100 microns beneath each stimulating electrode (Figure 1C). Reconstructions of dendritic segments were used to determine whether polyribosomes, synapse number, and/or synapse size were altered 2 hr after tetanic stimulation.

Identification and Location of Polyribosomes

Dendrites and spines from the tetanized and control sites were examined through serial sections. Individual ribosomes were identified by size, opacity, and shape. The ribosomes had opaque centers that were about 10–25 nm in diameter and were surrounded by lighter, irregular edges. Polyribosomes were identified when three or more ribosomes occurred together. Polyribosomal arrangements included clusters, spirals, and staggered lines (Figure 2). A large polyribosome could span multiple serial sections, but most of the polyribosomes and individual ribosomes were contained in one section because section thickness averaged about 46 nm. The quantitative analyses were restricted to free polyribosomes because the presence or absence of ribosomes on the dark-staining membranous organelles could not be determined with certainty in these preparations. All identifications, counts, and measurements were done blind to experimental condition.

Polyribosomes were quantified in the base, neck, and head of dendritic spines (Figure 2A). The spine base location was defined as within 0.1 μm of a spine origin (Figures 2B and 2C). The spine neck was the region of relatively uniform width between the origin and the enlarged head of a spine (Figures 2D and 2E). All other polyribosomes were located in the dendritic shaft (Figures 2F and 2G). The number of ribosomes in a polyribosome ranged from 3–28 with an average of 8, consistent with findings from earlier studies (Steward, 1983; Steward and Reeves, 1988). The average number of component ribosomes per polyribosome was uniform across the four dendritic and spine locations and did not differ between the control and tetanized sites.

More Dendritic Spines Have Polyribosomes after Tetanic Stimulation

Reconstructions of dendrites from the control and tetanized sites illustrate representative distributions of polyribosomes (Figure 3). Analyses were from reconstructions of all the spiny dendritic segments of less than 1 μm diameter (mean 0.58 ± 0.03 μm) in each of the LTP and control sample volumes (8–12 dendrites per sample). The average dendritic segment length was 5.2 ± 0.1 μm, for a total of 263 μm from 51 segments (27 control and 24 tetanized). These segments had 284 complete dendritic spines (146 control, 138 tetanized). In addition, there were 62 incomplete dendritic protrusions that were truncated at the end of the series; these were excluded because the absence of polyribosomes or synapses could not be established. A total of 261 polyribosomes were included in the analyses below.

The number of spines containing polyribosomes in any of the three locations increased from 0.15 ± 0.05 per μm at the control sites to 0.41 ± 0.05 per μm at the tetanized sites ($p < 0.0001$). This represents an increase from 12% $\pm$ 4% of spines containing polyribosomes under control conditions to 39% $\pm$ 4% containing polyribosomes at 2 hr after tetanic stimulation had induced LTP (Figure 4A). Next, the frequency of polyribosomes per unit length of dendrite was determined, and their relative positions in spine bases, necks, or heads were calculated. Both spine bases and heads had more polyribosomes at the tetanized sites (Figure 4B). The trend toward more polyribosomes in the spine necks did not reach statistical significance.

Fewer Polyribosomes Occur in Dendritic Shafts after Tetanic Stimulation

This increase in spines containing polyribosomes might have simply resulted from a global upregulation of protein synthesis throughout the neurons at the tetanized sites, resulting in a uniform increase that also affected spines. To test this hypothesis, we determined whether the frequency of polyribosomes in the dendritic shafts also increased. The combined frequency of polyribosomes in both the shafts and spines was measured per unit length of dendrite, and no change was detected between the control (1.0 ± 0.2 polyribosomes per μm) and tetanized sites (1.1 ± 0.2 polyribosomes per μm; $p > 0.5$). Instead, the increase in polyribosomes per micron located in spines was accompanied by a commensurate loss from the dendritic shafts (Figure 4C). Correlations were performed to test whether the elevation in spine polyribosomes was specifically associated with a local loss from their dendritic shafts. Dendritic segments were excluded if none of the spines on them contained polyribosomes. Fewer polyribosomes were in the shafts as more spines along them had polyribosomes at the tetanized sites ($r = -0.62$, $p < 0.005$; $n = 19$), but not at the control sites ($r = 0.36$, $p = 0.19$; $n = 15$). These findings suggest that mRNAs might be selectively recruited into dendritic spines after tetanic stimulation at the expense of ongoing synthesis in the adjacent dendritic shafts.

Neither Spine Nor Shaft Synapse Number Was Changed after Tetanic Stimulation

There was no change in the density of dendritic spines between the control (1.2 ± 0.1 per μm) and tetanized sites (1.0 ± 0.1 per μm; $p > 0.2$; Figure 5A). Results from earlier studies of single sections in adult hippocampal area CA1 suggested a subtle increase in the frequency of dendritic shaft synapses after induction of LTP by high-frequency stimulation (Lee et al., 1980; Chang and Greenough, 1984). These authors acknowledged that the shaft synapses might have been on the aspiny dendrites of interneurons, though in single sections they were hard to distinguish. We readily distinguished the aspiny interneuron dendrites from the spiny pyramidal cell dendrites through serial EM sections. Our analysis of asymmetric shaft synapses was restricted to the spiny pyramidal cell dendrites where they might be involved in the formation of new dendritic spines

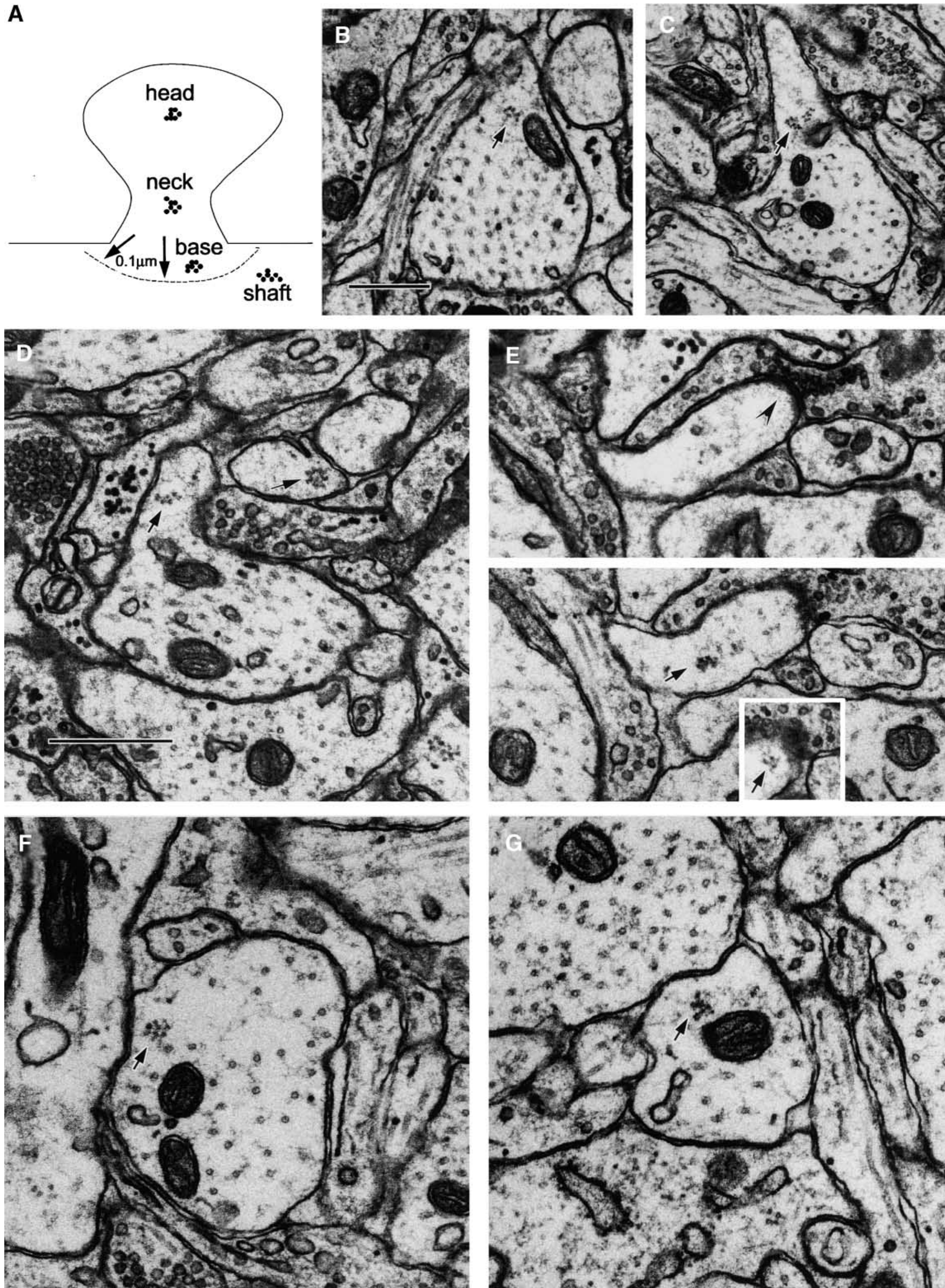

Figure 2. Locations and Arrangements of Polyribosomes in Dendrites and Spines

(A) Polyribosomes were located in the base (within 0.1 μm of a spine origin), neck, and head of spines and in the dendritic shaft.

(B and C) Polyribosomes at a spine base in a control sample (B) and at a spine base in an LTP sample (C).

(D) Staggered line arrangement of ribosomes in a spine neck (left arrow) and a spiral pattern of ribosomes in the head of a different spine (right arrow) from an LTP sample.

(E–G) In (E), three serial sections showing a spine head with a synapse and two polyribosomes in a LTP sample. Top, presynaptic vesicles and PSD (arrowhead) on one section; center, polyribosome in the spine head (arrow) in the next section; inset, PSD and another polyribosome (arrow) close to the synapse on the third section. Polyribosomes (arrows) in dendritic shafts (F) from an LTP sample and from a control sample (G). Scale bar: 0.5 μm in (B), (C), and (D)–(G).

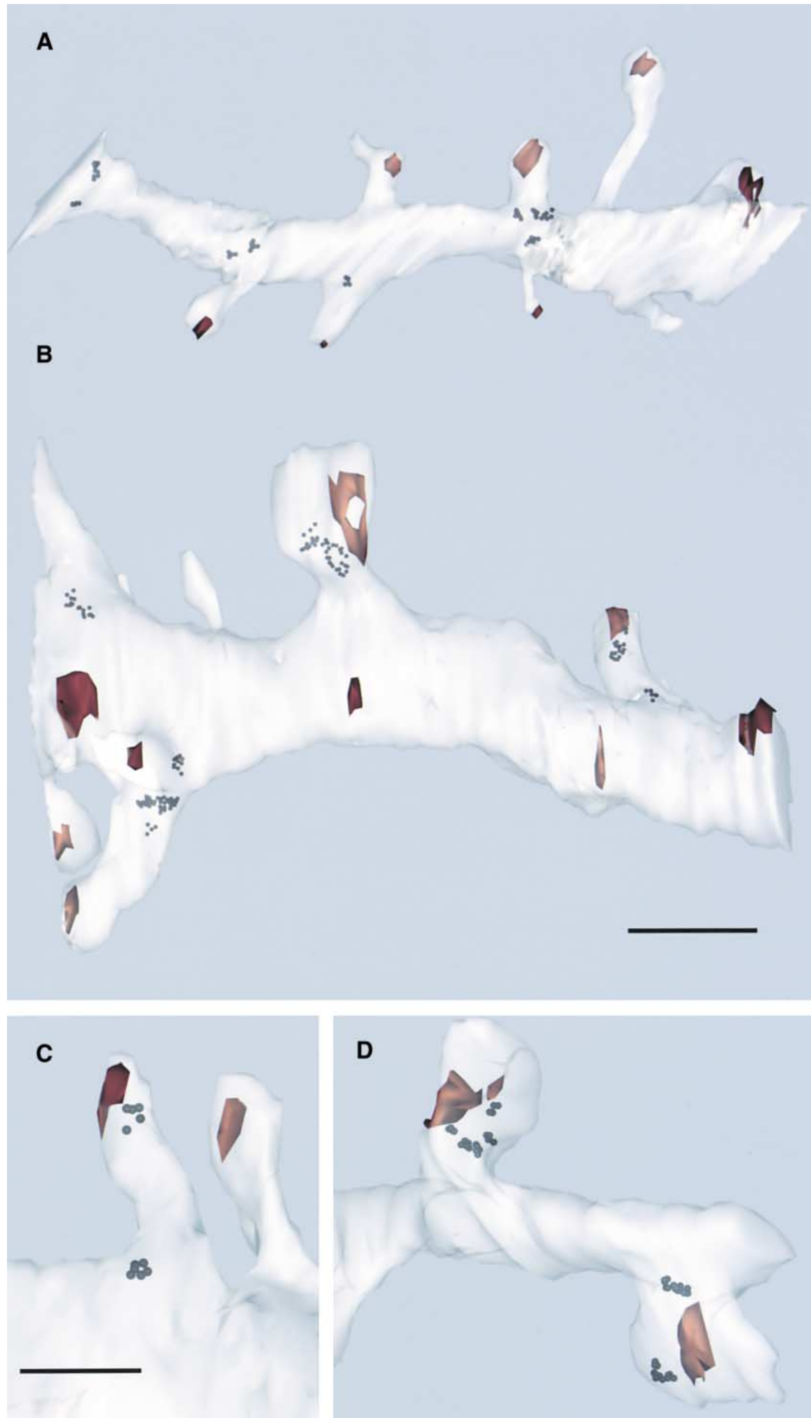

Figure 3. Three-Dimensional Reconstructions of Dendrites with Polyribosomes

(A) Dendritic segment from a control sample with most of its polyribosomes in the shaft. Each ribosome is represented by a black sphere, and PSDs are shown in red.

(B) Dendritic segment from an LTP sample with most of its polyribosomes in spines.

(C and D) Polyribosomes in spines from LTP samples.

Scale bar: 1 μm in (A) and (B) and 0.5 μm in (C).

(Harris, 1999; Marrs et al., 2001). There were only 44 shaft synapses across all conditions, and their frequency in the control (0.18 $\pm$ 0.03) and tetanized (0.14 $\pm$ 0.03) conditions did not differ significantly ($p > 0.3$; Figure 5B).

Polyribosomes Were Not Enhanced at Asymmetric Shaft Synapses

To determine whether the location of polyribosomes was shifted closer to synapses on the dendritic shaft,

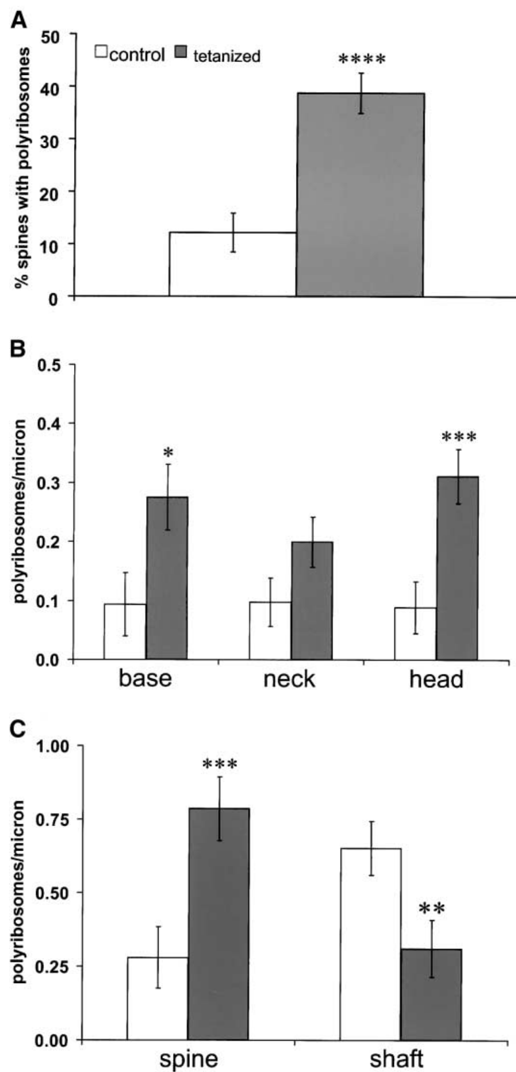

Figure 4. Redistribution of Polyribosomes from Dendritic Shafts to Spines after Tetanic Stimulation that Induced LTP

(A) The percentage of spines containing polyribosomes was greater at tetanized sites.

(B) The frequency of polyribosomes increased significantly in spine bases and heads, but not necks ($p = 0.09$).

(C) The increase in polyribosomes located in spines was accompanied by a commensurate loss from dendritic shafts where polyribosomes decreased from 0.65 ± 0.09 per μm in the controls to 0.31 ± 0.10 per μm at the tetanized sites. (p values are: $*p < 0.03$, $**p < 0.02$, $***p < 0.002$, and $****p < 0.0001$).

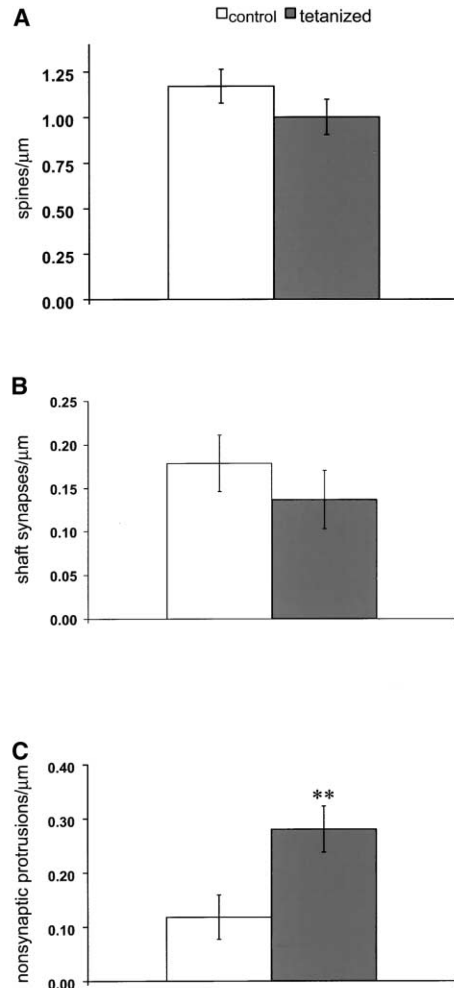

Figure 5. No Change in Synapse Number at Tetanized Sites

(A) Frequency of dendritic spines was the same at both sites.

(B) Frequency of asymmetric shaft synapses was the same at both sites.

(C) Nonsynaptic protrusions at control and tetanized sites ($**p < 0.02$).

we counted the polyribosomes located within 3 μm of a shaft synapse. This distance was chosen because none of the spines were longer than 3 μm. Of the 44 shaft synapses, 28 had polyribosomes within 3 μm, and there was no difference in frequency between the conditions ($p > 0.7$). Thus, tetanic stimulation does not appear to cause a shift in the location of polyribosomes toward shaft synapses, but only toward synapses occurring on dendritic spines.

Polyribosomes Were Not Enhanced in Nonsynaptic Dendritic Protrusions

Previous studies using two-photon microscopy showed the emergence of new dendritic protrusions beginning 30 min after induction of LTP (Engert and Bonhoeffer, 1999). It was not known, however, whether the new dendritic protrusions had synapses. Here, the frequency of dendritic protrusions without synapses was 0.12 ± 0.04 after low-frequency stimulation and 0.28 ± 0.04 per μm after tetanic stimulation ($p < 0.01$; Figure 5C). The percentage of nonsynaptic dendritic protrusions containing polyribosomes did not differ between the control ($12\% \pm 9\%$) and tetanized sites ($13\% \pm 7\%$; $p > 0.9$). Thus, although dendritic protrusions without synapses were present after tetanic stimulation in these slices, the nonsynaptic protrusions did not preferentially acquire polyribosomes. It is unknown whether polyribosomes would appear in these protrusions later if synaptic connections were to form on them.

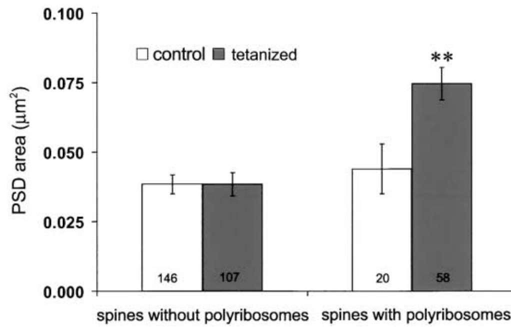

Figure 6. Synapses with Polyribosomes Were Larger during LTP

Synapses on spines without polyribosomes were the same size in both conditions and did not differ significantly from control synapses on spines with polyribosomes. Synapses on spines with polyribosomes were significantly larger during LTP than any of the other three categories (**$p < 0.02$; the number of synapses measured is listed for each condition, 19 control spines had multiple synapses: 11 with 2, 7 branched with 2, and 1 branched with 3 synpases; 21 LTP spines had multiple synapses: 11 with 2, 3 with 3, 6 branched with 2, and 1 branched with 3 synapses).

Synapses on Spines with Polyribosomes Are Larger after Tetanic Stimulation

One possible mechanism for the enhanced response during LTP is synapse enlargement (Bliss and Collingridge, 1993). To test this hypothesis, we measured the surface area occupied by the postsynaptic densities (PSD) through serial EM. The mean PSD area at the tetanized sites (0.056 ± 0.004 μm^2) was not significantly greater than at the control sites (0.041 ± 0.005 μm^2; $p = 0.073$). Perforated PSDs were infrequent in both the control condition (0.12 ± 0.04 per μm) and tetanized sites (0.12 ± 0.04 per μm, $n = 15$; $p = 0.99$), consistent with results from studies using theta-burst stimulation in organotypic slices (Toni et al., 1999).

There was an important distinction between spines with and without polyribosomes (Figure 6). Synapses on spines without polyribosomes were the same size in the control (0.038 ± 0.003 μm^2) and tetanized conditions (0.038 ± 0.004 μm^2). Similarly, in the control condition, synapses on spines with polyribosomes were not significantly different in size (0.044 ± 0.009 μm^2) from synapses on spines without polyribosomes in either condition. After tetanic stimulation, however, spines with polyribosomes had significantly larger synapses (0.075 ± 0.006 μm^2; $p < 0.02$), supporting the hypothesis that new protein synthesis in spines serves to enlarge specific synapses during LTP.

Discussion

The predominant location of polyribosomes shifted from dendritic shafts to spines 2 hr after induction of LTP. Furthermore, the spines that acquired these polyribosomes had larger synapses. Earlier studies have established that protein synthesis is needed to sustain LTP, supporting the hypothesis that new polyribosomes are in spines that are expressing LTP. Further study is needed to establish whether the shift in local protein synthesis demonstrated by polyribosomes results from high-frequency stimulation per se and is age-dependent or if it always occurs during LTP.

Importance of Three-Dimensional Reconstruction

The distribution and density of polyribosomes cannot be accurately mapped on single sections because spines occupy many sections, while polyribosomes typically occupy only one section. The profile of a spine or dendrite on a single section might not contain a polyribosome, while an adjacent section of the same structure does. Furthermore, if a spine enlarges and occupies more sections, the probability of detecting a polyribosome in a particular profile on one section decreases, even if more spines contain polyribosomes. Three-dimensional reconstruction from serial EM provides the analytical power needed to distinguish synaptic, dendritic, and organelle relationships in the neuropil and was required to map the elevation of polyribosomes and their association with synapse enlargement in a subset of dendritic spines after tetanic stimulation that induced LTP.

Role of Polyribosomes in Synapse Enlargement after Tetanus-Induced LTP

Protein synthesis may have distinct functions during the different phases of LTP. Immediately after induction, the potentiated response is likely carried by AMPA receptors, which are phosphorylated and inserted into synapses (reviewed in Luscher and Frerking, 2001). This process does not require protein synthesis. Nevertheless, when either mRNA or protein synthesis are blocked during the minutes preceding or immediately following induction, LTP lasts only about an hour before the response decays back to baseline (Otani et al., 1989; Frey et al., 1988; Nguyen et al., 1994). AMPA receptors are stabilized by cytoplasmic scaffolding proteins in the postsynaptic density (Kennedy, 2000; Sheng, 2001). If they are not anchored in place, they are subject to endocytosis or migration away from the synapse (Luscher and Frerking, 2001), events that would explain the decay of short-term LTP after an hour. Some of the mRNAs involved in synthesis of these scaffolding proteins, including αCAM Kinase II, PSD95, and MAPII, have already been found in dendrites (Wells et al., 2000). Furthermore, there is a large increase in the level of CAMKII mRNA in potentiated CA1 neurons (Mackler et al., 1992). Translation of these mRNAs near synapses would stabilize the new receptors and account for the enlarged PSDs visible in our material after tetanic stimulation that induced LTP (e.g., Ouyang et al., 1999; Steward and Halpain, 1999). Frey and Morris (1997) have proposed that stimulated synapses produce local “tags” that recruit newly synthesized macromolecules into dendritic spines. Tetanic stimulation triggers expression of immediate early genes, which could initiate synthesis of new mRNAs and proteins at the soma (Greenberg and Ziff, 2001; Casadio et al., 1999). These new mRNAs may then be captured by synapses and used to enlarge the potentiated PSDs.

Only 12% of control spines had polyribosomes, and these did not have statistically larger synapses than spines without polyribosomes. Polyribosomes have also

been described in P15 hippocampal dendritic spines in perfusion-fixed brain, although no comparable measurements of synapse area were made (Steward and Falk, 1991). Perhaps polyribosomes in the control spines are synthesizing proteins not preferentially destined for the PSD, but some other part of the spine.

Commensurate Loss and Gain of Polyribosomes in Dendritic Shafts and Spines

There was more than a 3-fold increase in the frequency of dendritic spines containing polyribosomes after tetanic stimulation. The increase was paralleled by a decline from the adjacent dendritic shafts, resulting in a constant frequency of about one polyribosome per micron length of dendrite. This observation is consistent with other studies showing that some mRNAs are increased during neuronal activation, while others are decreased (Mackler et al., 1992; Roberts et al., 1998; Scheetz et al., 2000; Krichevsky and Kosik, 2001), possibly maintaining a constant number involved in translation at any one time.

This shift in the local distribution of polyribosomes could reflect a change in the dynamics of assembly and disassembly of polyribosomes in dendrites and spines in response to synaptic activation. The increase in polyribosomes in spines could also result from dormant mRNAs becoming activated (Wells et al., 2001) or from mRNA being captured from the dendrites. The dendritic source could be local mRNAs or mRNA newly transported from the soma (Job and Eberwine, 2001b). As unmasked or newly delivered mRNA accumulates in the spines, a parallel degradation of mRNA could occur in the dendritic shafts as has been suggested for Arc mRNA, which becomes elevated in stimulated dendritic regions while decreasing in neighboring regions (Steward and Worley, 2001).

Dendritic spines rarely contain microtubules but do contain an abundance of actin (Chicurel and Harris, 1992; Harris, 1999; Kaech et al., 2001). The known mRNA transport proteins (e.g., Staufen) bind and transport along microtubules (e.g., Kohrmann et al., 1999; Tang et al., 2001) and are thus unlikely to be responsible for transport of mRNA from dendritic shafts into dendritic spines. Instead, transport and anchoring of mRNA in spines might be actin-dependent, as has been shown for other cell types (Takizawa et al., 1997; Long et al., 1997; Sundell and Singer, 1991; Yisraeli et al., 1990). Whatever the mechanism, the results suggest strict local control over the distribution and amount of protein translation in dendritic spines and shafts.

Are the Effects Age Dependent?

An open question is whether the redistribution of polyribosomes might represent an effect of high-frequency activation on developing neurons, rather than an effect of LTP. Steward and Falk (1991) report from single-section analyses on perfusion-fixed brain that a greater percentage of hippocampal dendritic spines have polyribosomes at P1–P10 than in adults. The percentage of polyribosome-containing spines reaches adult levels by P15, an age when LTP becomes enduring (Harris and Teyler, 1984; Jackson et al., 1993). Since there are more spines in adults, the absolute number of polyribosome-containing spines continues to increase. Thus, the occurrence of polyribosomes in dendritic spines is not strictly developmental.

Desmond and Levy (1990) gave repeated tetani to the perforant path in adult rats in vivo, and subsequent single-section analysis in the stimulated region of dentate granule cells revealed a decrease in the number of polyribosomes in dendritic shafts, consistent with the observations we report here for immature area CA1. It was not possible in the previous study (Desmond and Levy, 1990) to determine whether polyribosomes were commensurately increased in dendritic spines because serial sections are required. These observations suggest, however, that a similar redistribution of polyribosomes might also occur in the adult hippocampus during LTP.

Synapses and Protrusions Are Required for Redistribution of Polyribosomes

An analysis of nonsynaptic protrusions and dendritic shaft synapses made clear that the redistribution of polyribosomes required both dendritic protrusions and synapses (i.e., dendritic spines). Others have shown with live imaging that new dendritic protrusions emerge about 30 min after activation of synapses in organotypic slices (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999). There was not, however, sufficient resolution to determine whether the protrusions had synapses and what subcellular organelles were involved. Our observations suggest that dendritic protrusions resulting from tetanic stimulation might not have synapses. Unlike dendritic spines, these nonsynaptic dendritic protrusions did not collect proportionately more polyribosomes after tetanic stimulation. Nor were polyribosomes preferentially located near asymmetric shaft synapses. Dendritic spines, therefore, appear to be the preferred site of polyribosome aggregation after tetanic stimulation.

Conclusions and Future Directions

One possibility that emerges from the accumulated data is that dendritic protein synthesis serves to stabilize newly inserted glutamate receptors in the PSD during LTP. This explanation accounts for the decay of LTP if translation is inhibited, as well as our observation of enlarged PSDs and more polyribosomes in spines 2 hr after tetanization. Several questions remain. How soon after induction of LTP do polyribosomes appear in spines? Which mRNAs are enriched in dendritic spines? What localization signals are involved in transporting them there? Answers to these and related questions are crucial to understanding mechanisms of structural change during synaptic plasticity.

Experimental Procedures

Preparation and Recording from Acute Hippocampal Slices

Hippocampal slices were prepared from 15-day-old male Long-Evans rats according to standard procedures (Harris and Teyler, 1984; Sorra and Harris, 1998). All of the procedures followed the National Institutes of Health guidelines and undergo yearly review by the Animal Care and Use Committee. The slices were maintained at 32°C in an interface chamber with oxygenated artificial cerebral spinal fluid (ACSF) containing: 117 mM NaCl, 5.3 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 10 mM glucose (pH 7.4). Slices were allowed to recover for about an

hour before electrophysiological recordings were begun. Two concentric bipolar stimulating electrodes (25 μm pole separation; Fred Haer, Brunswick, ME) were placed in the middle of stratum radiatum and located 300–400 μm on either side of a single extracellular recording electrode (glass micropipette filled with 120 mM NaCl, Figure 1A). Preliminary experiments demonstrated that LTP was site specific as long as the two stimulating electrodes were separated by more than 400 μm, so we doubled this distance. Signals were filtered at 5 kHz, digitized at 29 kHz, and analyzed with the Scope software (RC Electronics, Santa Barbara, California). The slope (mV per ms) of the fEPSP was measured from the steepest segment of the negative field potential 400–600 μs after the stimulus artifact for the duration of the experiment. Test stimuli (0.1 ms) were given at 1 per 30 s, and all stimuli were at a constant intensity that produced a response with a slope in the range of 1–1.3 mV per ms during the pretetanus baseline.

Control and LTP Paradigms

During the establishment of a stable pretetanus baseline, the control electrode received a pair of low-frequency stimuli (LFS), delivered at 5 Hz for 20 s with a 20 s interval between them for a total of 200 pulses. This control LFS produced no enduring increase in the magnitude of the response (Figure 1B). At 5 min after the control LFS, the other electrode received a pair of tetanic stimuli (two trains at 100 Hz for 1 s with a 20 s interval). In other experiments, two additional pairs of control and tetanic stimuli were given at 10 and 20 min intervals to demonstrate that one pair was sufficient to saturate the LTP. The positions of the control and LTP electrodes were alternated between the CA3 and subicular side of the recording electrode in different experiments. Responses were monitored for 2 hr after the first tetanic stimulation. The slices were then fixed by immersion in mixed aldehydes (6% glutaraldehyde, 2% paraformaldehyde in 100 mM cacodylate buffer with 2 mM $CaCl_2$ and 4 mM $MgSO_4$) during 10 s of microwave irradiation to enhance diffusion of the fixative to the middle of the slice (final temperature less than 45°C) and then they were postfixed in the same fixative overnight (Jensen and Harris, 1989).

Processing for Serial EM

Slices were rinsed five times in buffer with repeated agitation during 1 hr. Each slice was manually trimmed under a dissecting microscope to the two regions containing each of the stimulating electrodes (see Figure 1). Slices were soaked in 1% osmium and 1.5% potassium ferrocyanide in 100 mM cacodylate buffer for 1 hr, rinsed five times in buffer during 1 hr, soaked 1 hr in 1% osmium, rinsed five times in buffer and two times briefly in water and graded ethanols through to propylene oxide, and then embedded in LX112 and cured for 48 hr (current methods using microwave-enhanced processing are also available at http://synapses.bu.edu/lab/howto/protocols/em_index.html). Serial thin sections were obtained near the LTP or control sites of stimulation by positioning a trapezoid 120–150 μm below the air surface of the slice 50–100 μm beneath and to one side of the center of the indentation left by the stimulating electrode (Figure 1C). Series were photographed on a JEOL 1200EX electron microscope at 10,000× magnification using a rotating holder.

Three-Dimensional Reconstructions

Series of 80–110 serial sections were digitized and aligned using SEM Align, and dendrites were traced using IGL Trace (Fiala and Harris, 2001a; http://synapses.bu.edu/tools/). Section dimensions were calibrated using a diffraction grating replica (Ernest Fullam, Inc., Latham, NY) imaged and scanned with the series. Section thickness was calibrated using the cylindrical diameters method, by dividing the diameters of longitudinally sectioned mitochondria by the number of sections they spanned (Fiala and Harris, 2001b). Section thickness ranged from 43–49 nm. Each experiment had a calibrated volume of approximately 250 μm^3 for each of the LTP and control conditions. Lateral dendritic segments (diameters <1 μm) traversing the middle of this volume were traced and measured using IGL Trace. Synapse area was computed by summing the length of the PSD × section thickness over all sections on which it appeared or by measuring the enclosed area of an en face PSD. Reconstructions of object surfaces were also produced by IGL Trace with subsequent rendering in 3D Studio MAX (Discreet Logic, Montreal, Canada).

Statistical Analyses

Statistical analyses were done in the STATISTICA software package (StatSoft, Tulsa, OK). All data are expressed as mean ± standard error of the mean. Synapse areas have a skewed distribution and, therefore, were normalized by the logarithm for statistical analysis, but the actual measurements are plotted and described. Previous studies from the hippocampus of 21-day-old and adult rats suggest that more spines and synapses occur in slices than in vivo (Kirov et al., 1999). The density of synapses from these P15 rat slices was somewhat higher than previously reported in perfusion-fixed brain from the same age (Harris et al., 1992). We controlled for potential slicing effects on polyribosome distribution or PSD area by reconstructing and comparing dendrites at control and tetanized sites within a slice. Then a two-way ANOVA was used to analyze for differences between the tetanized and control sites of frequencies or synapse areas with slice number as the second variable. A three-way ANOVA was used to analyze the combined effects of tetanic stimulation and polyribosome distribution on PSD area, with each slice as the third variable. Chi-square analysis was used for proportions, and correlations were used where indicated.

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