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Presynaptic ultrastructural plasticity along CA3→CA1 axons during LTP in Mature Hippocampus

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Abstract

In area CA1 of the mature hippocampus, synaptogenesis occurs within 30 min after the induction of LTP; however, by 2 hr many small dendritic spines are lost, and those remaining have larger synapses. Little is known, however, about associated changes in presynaptic vesicles and axonal boutons. Axons in CA1 *stratum radiatum* were evaluated with three-dimensional reconstructions from serial section electron microscopy at 30 min and 2 hr after induction of LTP by theta-burst stimulation (TBS). The frequency of axonal boutons with a single postsynaptic partner was decreased by 33% at 2 hr, corresponding perfectly to the 33% loss specifically of small dendritic spines (head diameters <0.45 μ m). Docked vesicles were reduced at 30 min and then returned to control levels by 2 hr following induction of LTP. By 2 hr there were fewer small synaptic vesicles overall in the presynaptic vesicle pool. Clathrin-mediated endocytosis was used as a marker of local activity, and axonal boutons containing clathrin-coated pits showed a more pronounced decrease in presynaptic vesicles at both 30 min and 2 hr after induction of LTP relative to control values. Putative transport packets, identified as a cluster of less than 10 axonal vesicles occurring between synaptic boutons, were stable at 30 min but markedly reduced by 2 hr after the induction of LTP. APV blocked these effects, suggesting that the loss of axonal boutons and presynaptic vesicles was dependent on NMDA receptor activation during LTP. These findings show that specific presynaptic ultrastructural changes complement postsynaptic ultrastructural plasticity during LTP.

Keywords

synaptic vesicles; axonal boutons; vesicle pools; docked vesicles; serial section electron microscopy; 3-D reconstruction; hippocampal slice

The relative presynaptic and postsynaptic contributions to long-term potentiation (LTP) are a topic of considerable investigation (Kerchner and Nicoll, 2008; Lisman and Harris, 1993). Quantal analyses (Bolshakov et al., 1997; Choi et al., 2003; Malgaroli and Tsien, 1992; Sokolov et al., 2002; Stevens and Wang, 1994), optical imaging of vesicles labeled with FM dyes or fluorescently-tagged proteins (Bayazitov et al., 2007; Ratnayaka et al., 2012; Ryan et al., 1996; Zakharenko et al., 2003; Zakharenko et al., 2001), and optical imaging of calcium influx into postsynaptic spines following stimulation of paired neurons (Emptage et al., 2003; Enoki et al., 2009; Ward et al., 2006) all provide supportive evidence for a presynaptic role during LTP. In addition, new puncta can form during LTP along axons of

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cultured neurons (Antonova et al., 2001; Antonova et al., 2009; Bozdagi et al., 2000), and vesicles can be added to existing boutons to form new release sites (Kim et al., 2003). However, it has also been demonstrated that strictly postsynaptic changes, such as the insertion of AMPA receptors at silent and active synapses and the outgrowth of new spines, can sustain LTP for up to 1 hour (Isaac et al., 1995; Kauer et al., 1988; Kerchner and Nicoll, 2008; Kullman, 1994; Liao et al., 1995; Manabe and Nicoll, 1994; Matsuzaki et al., 2004; Muller and Lynch, 1988; Nicoll and Malenka, 1995; Shi et al., 1999). Recent work has shown rapid movement of extrasynaptic vesicles between boutons located more than 20 μm apart over a period of minutes both *in vivo* (Herzog et al., 2011) and *in vitro* (Darcy et al., 2006; Staras et al., 2010; Westphal et al., 2008). However, it is not known whether this mobilization of vesicles between axonal boutons is involved in the synaptic plasticity associated with LTP. Hence, many questions remain regarding the coordination of presynaptic and postsynaptic plasticity during LTP, especially at longer times after induction and in the mature hippocampus.

In mature hippocampal area CA1, 30 min after the induction of LTP with theta-burst stimulation (TBS) there is local synaptogenesis as evidenced by an increase in non-synaptic protrusions both from the shaft of the dendrite and the head and neck of other spines (Bourne and Harris, 2011). However, by 2 hr, 33% of small dendritic spines (head diameters $<0.45 \mu\text{m}$) are lost. This loss is coupled to a corresponding enlargement of synapses on the remaining dendritic spines, which scales perfectly such that the summed synaptic area per unit length of dendrite is the same at all times after the induction of LTP (Bourne and Harris, 2011). It is known from past *in vivo* studies that the number of presynaptic vesicles scales with the surface area of the postsynaptic density (Harris and Stevens, 1989; Harris and Sultan, 1995; Lisman and Harris, 1993; Schikorski and Stevens, 2001), thus one might expect the number of vesicles to increase with LTP. However, prior single section EM analyses of hippocampal dentate gyrus and area CA1 suggested that presynaptic vesicles numbers are reduced shortly after induction of LTP (Applegate et al., 1987; Fifkova and Van Harreveld, 1977; Meshul and Hopkins, 1990). Numerous studies suggest LTP has distinct underlying mechanisms at 30 min and 2 hr (Abraham and Williams, 2003; Raymond, 2007; Reymann and Frey, 2007). Hence, we wanted to learn whether changes in presynaptic structure accompany the postsynaptic changes in synapse number and size at these functionally distinct times following the induction of LTP. We demonstrate through three-dimensional reconstructions from serial section EM robust changes in axons, presynaptic vesicles, and extra-synaptic vesicles that correspond to the postsynaptic ultrastructural plasticity previously demonstrated during LTP in the mature hippocampus.

Materials and Methods

Preparation and recording from acute hippocampal slices

Hippocampal slices were prepared from 7 young adult male Long Evans rats aged 56–65 days old (weighing 263–361 g). All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee and the Institutional Biosafety Committee and followed the National Institutes of Health guidelines for the humane care and use of laboratory animals. Animals were anesthetized with halothane and decapitated, and slices were prepared at room temperature ($\sim 25^\circ\text{C}$) using artificial cerebrospinal fluid (ACSF) containing (in mM): 116.4 NaCl, 5.4 KCl, 3.2 CaCl_2 , 1.6 MgSO_4 , 26.2 NaHCO_3 , 1.0 NaH_2PO_4 , and 10 D-glucose. Slices were transferred in less than 5 min to nets positioned over wells containing ACSF at the interface of humidified O_2 (95%) and CO_2 (5%); slices then were maintained at 32°C for at least 3 hr in these *in vitro* chambers prior to experimentation. For the APV experiments ($n=2$ slices from 2 animals), 50 μM D-2-amino-5-phosphonopentanoic acid (Sigma-Aldrich, St. Louis, MO) in ACSF was transferred into the wells at the end of the 3 hour pre-incubation period and remained for the duration of

the experiment. Two concentric bipolar electrodes (Fred Haer, Brunswick, ME) were located 300–400 μm on either side of a single extracellular recording electrode placed in the middle of *stratum radiatum* (glass micropipette filled with 120 mM NaCl) for a total separation of 600–800 μm (Figure 1A). Extracellular field potentials were recorded using IGOR software, and stimulations were alternated between the electrodes once every 2 min at a 30 second interval between electrodes (Bourne and Harris, 2011). Prior work has shown that optimal synaptic ultrastructure, which is comparable to perfusion-fixed hippocampus *in vivo*, is preserved for many hours *in vitro* under these conditions of slice preparation and maintenance (Bourne et al., 2007).

LTP paradigm

To begin, a stimulus/response curve was acquired for both stimulating electrodes at the two sites in the middle of *s. radiatum* in each slice; then a stable baseline was established at ~50% maximal response for 30 min (1 pulse every 2 min) (Figure 1B–D). Theta burst stimulation (TBS: 8 trains of 10 bursts at 5 Hz of 4 pulses at 100 Hz delivered 30 seconds apart, requiring 3.5 min in total) was delivered to one stimulating electrode at the same stimulus intensity as baseline. The positions of the control and TBS electrodes were alternated between the CA3 and subicular sides of the recording electrode, counterbalancing across experiments. Responses from both stimulating electrodes were monitored (1 pulse every 2 min) for 30 min or for 2 hr after the end of the first train of TBS for the LTP and APV experiments (Figure 1B–D). The delivery of TBS resulted in robust potentiation relative to control (% baseline $\pm$ SD: 30 min LTP: 234 ± 22 ; 30 min Control: 103 ± 4 ; 2 hr LTP: 170 ± 32 ; 2 hr Control: 96 ± 5) that was blocked in the presence of 50 μM APV (% baseline $\pm$ SD: TBS-APV: 88 ± 6 ; Control-APV: 109 ± 11). The slices were then fixed by immersion in mixed aldehydes (6% glutaraldehyde and 2% paraformaldehyde in 100 mM cacodylate buffer with 2 mM CaCl_2 and 4 mM MgSO_4) during 10 seconds of microwave irradiation to enhance diffusion of the fixative to the middle of the slice with a final temperature less than 35°C, and then post-fixed in the same fixative overnight at room temperature (Jensen and Harris, 1989). After fixation, slices were processed, ultra-thin sectioned, and imaged for serial section transmission electron microscopy as previously described (Bourne and Harris, 2011).

Three-dimensional reconstructions and measurements

Serial sections were cut from a small trapezoid positioned in the middle of the region of optimal tissue preservation, ~100 μm from the slice air surface, and mounted on Pioloform-coated slot grids (Synaptek, Ted Pella Inc.). Sections were counterstained with saturated ethanolic uranyl acetate followed by Reynolds lead citrate for 5 min each. Sections were imaged, blind as to condition, on a JEOL 1230 electron microscope with a Gatan digital camera at 5000x magnification.

The serial sections were aligned and axons were traced using the RECONSTRUCTTM software (available for free download at <http://synapses.clm.utexas.edu> (Fiala, 2005)). Section dimensions were calibrated using a diffraction grating replica (Ernest Fullam, Inc., Latham, NY) imaged with each series, and the cylindrical diameters method was used to compute section thickness by dividing the diameters of longitudinally sectioned mitochondria by the number of sections they spanned (Fiala and Harris, 2001a). The computed section thicknesses ranged from 38–60 nm and were not correlated with condition.

The area of a cross-sectioned synapse was calculated by measuring its length on each section and multiplying by section thickness and the number of sections traversed. Enclosed areas of *en face* and obliquely sectioned PSDs were measured on the section they appeared

and added to the area computed for the cross-sectioned portions of that synapse. Vesicles were counted in presynaptic boutons; in addition, axons were traced across their lengths and vesicles were counted in at least two neighboring axonal boutons. See figure legends for relevant N's. Adobe Photoshop (San Jose, CA) was used to adjust the brightness and contrast of the electron micrographs in the figures.

Statistical analyses

Statistical analyses were done in the STATISTICA software package (StatSoft, Tulsa, OK). Two-way analysis of variance (ANOVA) was used to evaluate differences in axonal bouton frequencies at the TBS relative to control sites of stimulation for LTP and APV conditions, and at each time point with slice as the second variable (Figure 3, 7). Changes in vesicle number per axonal bouton were analyzed using a nested hierarchical ANOVA with axon nested in condition and experiment (Figures 4–6). For all analyses, data are plotted as means overlaid with individual data points. Then, to visualize the relative differences between LTP and control values, individual measurements were subtracted from the mean control value within each slice and plotted as the mean relative change ($\bar{x}$ mean, $\pm$ S.E.M) for each condition and time point.

Results

TBS or control stimulation was delivered at electrodes positioned on either side of a recording electrode positioned in the middle of *s. radiatum* of area CA1; the site of TBS nearer to CA3 or subiculum was counterbalanced across experiments such that each slice provided both conditions (Figure 1A). Hippocampal slices were rapidly fixed at 30 min (Figure 1B) or 2 hr after induction of LTP with TBS (Figure 1C) (Bourne and Harris, 2011). To control for differential amounts of presynaptic activation, TBS and control stimulation were repeated in the presence of 50 μ M APV, which blocked induction of LTP (Figure 1D).

Serial section electron microscopy and three-dimensional reconstruction were used to examine synaptic vesicles in 772 individual excitatory boutons. Complete axonal segments (192) were reconstructed for a subset of those boutons. Presumed glutamatergic excitatory axons were identified by the relatively round, clear vesicles contained in them and their association with dendritic spine synapses that had a thickened postsynaptic density (Figure 2). Axonal boutons could be classified as single synaptic boutons (SSBs) if they had one postsynaptic partner (Figure 2A), multisynaptic boutons (MSBs) if they had two or more postsynaptic partners (Figure 2B), nonsynaptic boutons (NSBs) if they had more than 10 vesicles in a cluster but no postsynaptic partners (Figure 2C). Representative three-dimensional reconstructions illustrate the heterogeneity of axonal boutons along single axons (Figure 2D–G). The frequency of mitochondria within axonal boutons was ~50% across all time points and conditions (30 min Control = 51% $\pm$ 4.8; 30 min LTP = 46% $\pm$ 8.6; 2 hr Control = 50% $\pm$ 6.4; 2 hr LTP = 53% $\pm$ 0.8; APV Control = 45% $\pm$ 1.2; APV-TBS = 56% $\pm$ 4.5).

Previously, we determined that small dendritic spines (with head diameters $<0.45 \mu$ m) were decreased in number by 33% at 2 hr after the induction of LTP in these same slices (Bourne and Harris, 2011). Hence, we wanted to determine whether axonal boutons were also lost or if they simply became devoid of postsynaptic partners, so we performed an unbiased 3D brick analysis (Fiala and Harris, 2001b). A 3.5 μ m $\times$ 3.5 μ m sampling frame was positioned over the sample area on 50 serial sections, which then contained the largest axonal boutons (Figure 3A). All boutons contained within the grid or touching the 2 inclusion lines (green) were counted. A total of 1,334 excitatory axonal boutons were followed through the image volumes (including the area surrounding the images), and the adjacent serial sections (up to 100), to identify them as SSB, MSB, or NSB. If the boutons contacted one of the two

exclusion lines (red) of the grid, they were not included (Figure 3B). This gave an unbiased estimate of number per volume of each type of bouton (Fiala and Harris, 2001b). The density of each bouton type was calculated by dividing the total number of boutons by the total brick volume.

At 2 hr, but not 30 minutes, after the induction of LTP, SSBs were selectively lost ($p < 0.05$), while there was no significant change in the frequency of MSBs or NSBs at either time point (Figure 3C, D). Delivery of TBS in the presence of APV prevented this significant decrease in SSBs (APV Control: $SSB = 2.04 \pm 0.11$; $MSB = 0.54 \pm 0.08$; $NSB = 0.58 \pm 0.02$; APV-TBS: $SSB = 1.72 \pm 0.13$; $MSB = 0.2 \pm 0.11$; $NSB = 0.73 \pm 0.13$). These findings suggest that the SSBs were the original presynaptic partners of the lost small spines and that an NMDA-dependent process was involved in this presynaptic change.

Next, we investigated whether the vesicle composition of the axonal boutons was altered during LTP. Each bouton contains vesicles of varying release competence (Dobrunz, 2002; Murthy et al., 1997), and induction of plasticity can increase the fraction of vesicles available for release (Ratnayaka et al., 2012). As such, vesicles were classified as either docked, which are thought to be part of the readily releasable pool (Branco et al., 2010; Harris and Sultan, 1995; Schikorski and Stevens, 2001), or non-docked, which comprise the reserve vesicle pool (Figure 4A–B). At 30 min after the induction of LTP, the average number of docked vesicles per bouton was decreased ($p < 0.01$, Figure 4C, D). In contrast, the overall vesicle pool size did not differ significantly between conditions at 30 min (Figure 4E, F). By 2 hr, the average number of docked vesicles was similar to control levels (Figure 4C, D); however, the average size of the vesicle pool was significantly decreased ($p < 0.001$; Figure 4E, F). Delivering TBS in the presence of APV did not result in this long-lasting reduction in the number of docked or non-docked vesicles relative to control stimulation (APV-Control: Docked = 3.71 ± 0.3 , Non-docked = 311.39 ± 33.97 ; APV-TBS: Docked = 3.68 ± 0.4 , Non-docked = 363.51 ± 41.7). These findings suggest that the additional action potentials delivered during TBS were not sufficient to alter the vesicular composition of boutons and that the decrease in vesicles with LTP involved NMDA-receptor activation.

Synaptic vesicles in axonal boutons are recycled via endocytosis in order to sustain release (Haucke et al., 2011; Lou et al., 2012; Murthy and De, 2003; Ryan, 2006). Such a tight coupling between release and endocytosis suggests that anatomical evidence of endocytosis could serve to mark which boutons were undergoing presynaptic activity at the time of fixation. To investigate whether the change in number of docked and non-docked vesicles might be related to recycling, we focused on boutons that showed anatomical evidence of recycling via clathrin-coated pits (CCPs), an early stage of endocytosis. During control stimulation alone, clathrin-coated pits (CCPs; Figures 5A, 6A, C) were observed in 17% of boutons (Figure 5B), and boutons with a CCP had more docked vesicles (Figure 5C) and larger vesicle pools (Figure 5D) than boutons without a CCP.

CCPs were also observed in some presynaptic boutons during LTP (Figure 6B, D). The decrease in docked vesicles only reached statistical significance at 30 minutes in boutons without a CCP ($p < 0.01$; Figure 6E, F). The number of vesicles in the vesicle pool was significantly reduced at both 30 min and 2 hr during LTP for axonal boutons with a CCP ($p < 0.05$); by 2 hr, the vesicle pools were also smaller in boutons without a CCP ($p < 0.01$, Figure 6G, H). There were no significant differences in the number of docked or non-docked vesicles when TBS was delivered in the presence of APV, regardless of the presence or absence of a CCP (APV-Control: Docked: +CCP = 3.57 ± 0.42 , -CCP = 3.79 ± 0.4 , Non-docked: +CCP = 406 ± 79.17 , -CCP = 266.33 ± 31.6 ; APV-TBS: Docked, +CCP = 4.78 ± 0.91 , -CCP = 3.21 ± 0.4 , Non-docked, +CCP = 453.87 ± 100.03 , -CCP = 324.43 ± 40.7). Thus, while boutons with evidence of recycling had a more pronounced reduction in their

vesicle pool at 30 min and 2 hr after the induction of LTP, the longer lasting decrease was also observed in boutons without a CCP.

Vesicles are not restricted to presynaptic axonal boutons. Small “transport packets” of vesicles trafficking between boutons may also play an important role in synaptic plasticity (Ahmari et al., 2000; Sabo et al., 2006; Ziv and Garner, 2004) as key components of the vesicle “superpool” (Darcy et al., 2006; Staras et al., 2010; Westphal et al., 2008). Here we evaluated whether this inter-bouton transport might be altered during LTP by quantifying changes in the smallest NSBs, those containing 10 or fewer vesicles, and classified as transport packets (Figure 7A, arrow). Interestingly, 3% of these transport packets showed evidence of endocytosis (Figure 7B–C), suggesting that some of the vesicles could be release competent during transit. At 30 min, there was no significant difference in the number of transport packets between conditions; however, by 2 hr nearly all transport packets had been eliminated ($p < 0.01$; Figure 7D, E). In the presence of APV, transport packet frequency did not change with control or TBS stimulation (APV-Control = 21.64 ± 4.21 % per axon; APV-TBS = 14.75 ± 4.01 % per axon). These data suggest a decrease in the inter-bouton trafficking of synaptic vesicles occurs by 2 hr after induction of LTP in the mature hippocampus.

Discussion

Our findings show that significant presynaptic structural plasticity occurs during LTP induced by TBS. A decrease in docked synaptic vesicles at 30 min was followed by a more widespread reduction of vesicles in the pool, and a loss of presynaptic boutons by 2 hr. Small transport packets of vesicles were nearly eliminated by 2 hr after induction of LTP, suggesting that either they were incorporated into presynaptic boutons or their contents were released in transit. Blocking NMDA receptors during delivery of TBS prevented the loss of presynaptic boutons and the reduction of both docked and non-docked presynaptic vesicles, and retained transport of vesicles between boutons. Thus, LTP-specific changes occur in the vesicle composition of presynaptic boutons along CA3–CA1 axons of the mature hippocampus.

The loss of SSBs at 2 hr after induction of LTP is consistent with previous reports of increased turnover and dynamic remodeling of axonal boutons during synaptic plasticity in cultured neurons (Antonova et al., 2001; De Paola et al., 2003; Nikonenko et al., 2003). Boutons in the hippocampus and cortex remain dynamic into adulthood (De Paola et al., 2003; Gogolla et al., 2007; Umeda et al., 2005), and the presence of mobile transport packets or “orphan” boutons in cultured neurons suggests that release sites can form and disassemble quickly (Krueger et al., 2003). The decrease in SSBs at 2 hr after induction of LTP, without a corresponding increase in NSBs or MSBs, suggests that boutons were completely disassembled rather than converted to another type when small spines were eliminated. In fact, the 33% loss of SSBs corresponds perfectly with the 33% decrease in small thin spines discovered previously (Bourne and Harris, 2011), and thus the SSBs were likely the presynaptic partners of the eliminated spines. These data also suggest that synapses are cohesive units and elimination of a postsynaptic spine during LTP triggers a corresponding disassembly of components in associated presynaptic axonal boutons. It further shows that axonal boutons along axons, i.e. *en passant* boutons, differ in their dynamics from terminal boutons, such as those found at the neuromuscular junction. While loss of a terminal bouton causes retraction of the axon (Bishop et al., 2004), loss of *en passant* SSBs is synapse specific and does not cause axonal retraction in mature hippocampus.

The number of vesicles docked at the active zone is thought to be indicative of release probability (Branco et al., 2010; Harris and Sultan, 1995; Schikorski and Stevens, 2001). Individual presynaptic boutons of CA3–CA1 axons can contain more than a thousand vesicles, yet less than 5% of all vesicles are docked and available for release (Harris and Sultan, 1995). In addition, the release probability of docked vesicles is heterogeneous across hippocampal boutons (Dobrunz and Stevens, 1997; Murthy et al., 1997), and minimal stimulation usually yields release of just one vesicle (Hanse and Gustafsson, 2001; Hjelmstad et al., 1997; Stevens and Wang, 1995). These observations raise the question: why are there so many vesicles in the pool? One possibility is that multivesicular or enhanced exocytosis of the readily releasable pool during synaptic facilitation (Christie and Jahr, 2006) or LTP (Bayazitov et al., 2007; Bolshakov et al., 1997; Enoki et al., 2009; Sokolov et al., 2002; Zakharenko et al., 2001) results in vesicle reduction, which would be consistent with observations from cultured neurons where more vesicles were shown to be released and recycled during LTP (Ratnayaka et al., 2012). However, clathrin-mediated endocytosis can recycle fully collapsed vesicles within ~60 seconds (Rizzoli and Jahn, 2007), and the observation of CCPs suggests that exocytic/endocytic coupling was intact during LTP in the mature hippocampus. While an increase in full fusion events with LTP could saturate the endocytic machinery, a delay in recycling in a subset of boutons is unlikely given that endocytosis kinetics appear to vary little from bouton to bouton (Armbruster and Ryan, 2011).

Although LTP may not alter the kinetics of the release and recycling of the readily releasable pool, it could have an impact on the reserve vesicle pool. Recently, it was discovered that the reserve pool (comprising ~80% of vesicles) was selectively involved in spontaneous release (Chung et al., 2010; Fredj and Burrone, 2009; Sara et al., 2005). This pool of vesicles has a slightly different complement of proteins including the v-SNARE VAMP7 rather than VAMP2 (Hua et al., 2011). VAMP7 preferentially binds to AP-3, an adaptor protein important for synaptic vesicle formation from endosomes (Blumstein et al., 2001; Faundez et al., 1998; Glyvuk et al., 2010) that is distinct from AP-2, which operates at the plasma membrane to recycle the readily-releasable pool (Di Paolo G. and De Camilli P., 2006; Kim and Ryan, 2009). Vesicles expressing VAMP7 localize to the reserve pool and only undergo spontaneous release (Hua et al., 2011). Interestingly, the recycling of VAMP7 vesicles is much slower than the VAMP2 vesicles that comprise the readily releasable pool (Hua et al., 2011); hence, the reserve pool could be reduced if spontaneous release increased with LTP. Previous reports that mini-EPSP frequency increases with LTP (Bekkers and Stevens, 1990; Malgaroli and Tsien, 1992; Wiegert et al., 2009) support this idea that the reserve pool may be mobilized to enhance spontaneous release during synaptic plasticity.

Another possible explanation for the reduction in vesicles with LTP is that a non-recyclable fraction of the vesicle pool contains elements to expand the active zone rather than neurotransmitter. Calcium channel accumulation at active zones is an essential regulator of neurotransmitter release (Graf et al., 2012; Holderith et al., 2012; Hoppa et al., 2012; Yao et al., 2009). The abundance of calcium channels at the synapse is determined by the size of the active zone (Holderith et al., 2012) and through interactions with proteins such as RIM, an organizer of the presynaptic release apparatus (Graf et al., 2012), and α_2 , a subunit associated with voltage-gated calcium channels (VGCCs) (Hoppa et al., 2012). In *Drosophila*, a transmembrane calcium channel named Flower was identified in synaptic vesicles at the neuromuscular junction (Yao et al., 2009). Upon stimulation, fusion of the Flower-containing vesicles added calcium channels to the presynaptic membrane where they regulated resting intracellular calcium levels. While the majority of the Flower protein was removed with endocytosis, some of the protein remained in the presynaptic membrane. Thus, perhaps LTP recruits the exocytosis of vesicles that contain components of the active zone, such as calcium channels, to regulate dynamically the release properties of individual

boutons. Future studies including immuno-labeling of the vesicle pool will help elucidate the fraction of vesicles having functions beyond neurotransmitter release.

A superpool of vesicles has recently been discovered that can mobilize between neighboring boutons and participate in release (Darcy et al., 2006; Staras et al., 2010; Westphal et al., 2008). Recycling has been observed among vesicles in transport packets along axons of cultured neurons (Ratnayaka et al., 2011). Here we discovered the first evidence for this extrasynaptic release and recycling in mature hippocampal (CA3–CA1) axons. In addition, the small transport packets were nearly eliminated by 2 hr after the induction of LTP, but not at 30 min. These data support findings from cultured neurons suggesting that the early stage of enhanced release with plasticity (<30 min) did not require extrasynaptic vesicle trafficking (Ratnayaka et al., 2012). However, by 2 hr after the induction of LTP, the widespread decrease in vesicle number appears to have recruited vesicles from transport packets to supplement their vesicle pools.

These results provide new evidence for coordinated pre- and postsynaptic structural scaling during LTP. We show that the elimination of small dendritic spines (Bourne and Harris, 2011) had a parallel loss of SSBs. There were fewer vesicles in the remaining boutons suggesting elevated exocytosis, which may include increased spontaneous release of neurotransmitter and/or fusion of vesicles containing elements to expand the active zone. Elimination of transport packets further suggests that traveling vesicles of the “superpool” were either captured by the remaining boutons or released during transit. Thus, induction of LTP results in dramatic ultrastructural remodeling of axons in area CA1 of the mature hippocampus. Future research is needed to reveal variation in the content of the vesicle pools and whether they are replenished at later stages of LTP.

Acknowledgments

JNB and KMH designed the experiments. JNB performed the experiments. JNB and MAC analyzed the images with confirmation from KMH on representative samples. JNB, MAC, and KMH analyzed the data and wrote the paper. We thank Libby Perry and Robert Smith for technical support with EM processing and imaging. This work was supported by NIH Grants NS21184, MH095980, and NS074644 to KMH.

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Figure 1.

Site-specific LTP in hippocampal slices from adult rats is blocked by APV. (A) A single recording electrode (arrow) was positioned in the middle of *s. radiatum* midway between two stimulating electrodes (dark posts). Scale bar = 500 μm. Theta burst stimulation (TBS) was delivered to one of the stimulating electrodes at time 0 (arrow) to induce LTP (dark gray diamonds), and baseline stimulation was maintained at the other electrode (light gray diamonds). The responses were monitored for (B) 30 min (n=3 slices from 3 animals), or (C) 2 hr (n=2 slices from 2 animals) after the delivery of the first theta burst stimulation. (D) TBS delivered in the presence of 50 μM APV did not induce LTP (n=2 slices from 2 animals). Example waveforms for each time (insets in B–D) are an average across the entire pre-TBS and post-TBS responses at the control (light gray) and TBS (dark gray) sites. Scale bars = 5mV/5ms. (Adapted from (Bourne and Harris, 2011)).

Figure 2.

Heterogeneity of individual axonal boutons and axonal segments in area CA1 of the mature hippocampus. (A) An EM and reconstruction of a single synaptic bouton (ax) filled with vesicles (green arrows) synapsing with the PSD (red arrow) on a dendritic spine (sp). (B) An EM and reconstruction of a multisynaptic bouton (MSB) and both postsynaptic spine partners. (C) An EM and reconstruction of a nonsynaptic bouton (NSB). (D–G) Three-dimensional reconstructions of axons from each condition. Axon = light blue, non-docked vesicles = green, docked vesicles = blue, mitochondria = purple, PSD = red. Scale cube = $0.125 \mu\text{m}^3$.

Figure 3.

Single synaptic boutons are reduced at 2 hr after LTP induction. (A) A sampling grid overlaid on an EM. Boutons within the grid or touching the green lines were included in the brick analysis, while boutons touching the red lines were excluded. Scale bar = 1 μm . (B) Example of a brick in 3-D with stamped boutons reconstructed as spheres. (C) The average frequency of SSBs, MSBs, and NSBs for each condition. The distribution of the data is indicated by the open circles and the means are represented by the superimposed black lines. 30 min data: $n=3$ bricks each for Control and LTP; 2 hr data $n=4$ bricks each for Control and LTP. (D) Brick analyses of the neuropil volume revealed a loss of SSBs 2 hr after induction of LTP ($p<0.05$) and no significant change in MSBs or NSBs (mean $\pm$ s.e.m.). There was no significant change in the frequency of any boutons at 30 min after LTP induction.

Figure 4.

Decrease in docked vesicles at 30 min after LTP followed by reduction in vesicle pool by 2 hr. (A) EM and (B) 3-D reconstruction of a dendritic spine (sp) and presynaptic bouton (ax) with the docked vesicles stamped in blue, the vesicle pool stamped in green, and PSD outlined in red. Scale bar = 0.25 μm . Scale cube = 0.0156 μm^3 . (C) A graph of the average number of docked vesicles for each condition (black lines) with the distribution of the data indicated by the open circles. (D) The number of docked vesicles per presynaptic bouton decreased at 30 min after induction of LTP ($p < 0.01$, mean $\pm$ s.e.m.). (E) Graph representing the average number of non-docked vesicles (black lines) for each condition with the distribution of the data indicated by the open circles. (F) By 2 hr after LTP, the vesicle pool was significantly smaller ($p < 0.01$, mean $\pm$ s.e.m.). 30 min Control: N=49 boutons; 30 min LTP: N=59 boutons; 2 hr Control: N=237 boutons; 2 hr LTP: N=312 boutons.

Figure 5.

Clathrin-coated pits (CCPs) occur in presynaptic boutons with more docked vesicles and larger vesicle pools under control conditions. (A) An EM of a control bouton with a CCP. (Scale bar = 0.5 μm .) (B) The two sets of control data were combined from the 30 min and 2 hr experiments, and revealed overall, that 17% of boutons contained a CCP (N=48 boutons with a CCP and 238 boutons without a CCP). (C) Boutons with a CCP had significantly more docked vesicles and (D) larger vesicle pools than boutons lacking a CCP.

Figure 6.

The number of vesicles was smaller in boutons with clathrin-coated pits (CCPs) at both 30 min and 2 hr after LTP induction. (A) An EM of a postsynaptic spine synapsing with a presynaptic axon from the 30 min Control condition. Non-docked vesicles of the pool are stamped in green. Scale bar = 0.25 μm . (B) EM of a presynaptic bouton from the 2 hr LTP condition with 2 CCPs colored orange (see large black arrows, scale bar = 0.25 μm). (Inset, Magnified CCP, scale bar = 0.1 μm .) (C) 3-D reconstruction of spine (yellow), PSD (red), non-docked vesicles (green), and a CCP (orange) from (A). (D) Reconstruction from (B) with CCPs in orange. Scale cube = 0.00156 μm^3 for C and D. (E) A graph of the average number of docked vesicles in axonal boutons with and without a CCP (black lines) across all conditions. Individual data points are represented by open circles. (F) At 30 min after LTP, the significant decrease in docked vesicles was observed in boutons without a CCP ($p<0.01$, mean $\pm$ s.e.m.). (G) A graph of the average number (horizontal black lines) of non-docked vesicles in boutons with and without a CCP across all conditions. The distribution of the data is represented by the open circles. (H) A significant reduction (mean $\pm$ s.e.m.) in vesicle pool size was observed in boutons with a CCP at 30 min ($p<0.05$) and 2 hr ($p<0.05$). 30 min Control: +CCP, N=16 boutons, -CCP, N=33 boutons; 30 min LTP: +CCP, N=20

boutons, -CCP, N=39 boutons; 2 hr Control: +CCP, N=32 boutons; -CCP, N=205 boutons;
2 hr LTP: +CCP, N=43 boutons; -CCP, N=269 boutons.

Figure 7.

Transport packets are substantially reduced at 2 hr after induction of LTP. (A) Reconstruction of axons from control and 2 hr LTP conditions. The control axonal bouton containing 7 vesicles and no postsynaptic partners was considered to be a “transport packet” (black arrow). Axon = light blue, vesicles = green, mitochondria = purple, PSD = red. Scale cube = $0.125 \mu\text{m}^3$. (B) An EM of a transport packet with an omega figure indicating potential for release at a nonsynaptic site along an axon. Scale bar = $0.25 \mu\text{m}$. (C) A small fraction of transport packets contained a CCP (N=2 transport packets with a CCP and N=71 transport packets without a CCP). (D) Average percentage of boutons along individual reconstructed axonal segments that were transport packets (black lines) with one data point per axon (open circles); for each condition, most axons had no transport packets as illustrated by the concentration of data points at zero. (E) The percentage of boutons along a reconstructed axonal segment that were transport packets (mean $\pm$ s.e.m.) was significantly reduced at 2 hr after induction of LTP ($p < 0.05$), but not at 30 min. 30 min Control: N=21 axons, 77 boutons; 30 min LTP: N=21 axons, 78 boutons; 2 hr Control: N=52 axons, 118 boutons; 2 hr LTP: N=58 axons, 126 boutons.

Table 1

Delivery of TBS in the presence of 50 μM D-APV had no significant effects on all but one of the quantified parameters (*). Results are shown as mean $\pm$ standard error, or as correlations (r) and significance of the correlation within condition.

Experimental Condition	APV-Ctrl	APV-TBS	p-values
N (animals, axons, boutons)	2 animals, 20 axons, 62 boutons	2 animals, 20 axons, 53 boutons	
SSBs ($\#/\mu\text{m}^3$)	2.04 ± 0.11	1.72 ± 0.13	0.20
MSBs ($\#/\mu\text{m}^3$)	0.54 ± 0.08	0.2 ± 0.11	0.13
NSBs ($\#/\mu\text{m}^3$)	0.58 ± 0.02	0.73 ± 0.13	0.36
Docked Vesicles	3.71 ± 0.3	3.68 ± 0.4	0.95
Vesicle Pool	311.39 ± 33.97	363.51 ± 41.7	0.24
Docked Vesicles CCP-	3.79 ± 0.4	3.21 ± 0.4	0.31
Docked Vesicles CCP+	3.57 ± 0.42	4.78 ± 0.91	0.01*
Vesicle Pool CCP-	266.33 ± 31.6	324.43 ± 40.7	0.30
Vesicle Pool CCP+	406 ± 79.17	453.87 ± 100.03	0.10
% Transport Packets/Axon	21.64 ± 4.21	14.75 ± 4.01	0.24
Total Vesicles PSD area	$r=0.61, p<.001$	$r=0.84, p<.001$	
Docked Vesicles PSD area	$r=0.74, p<.001$	$r=0.79, p<.001$	