Neuroscience 141 (2006) 2097–2106

DENSE CORE VESICLES RESEMBLE ACTIVE-ZONE TRANSPORT VESICLES AND ARE DIMINISHED FOLLOWING SYNAPTOGENESIS IN MATURE HIPPOCAMPAL SLICES

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Abstract—Large dense core vesicles (~100 nm) contain neuroactive peptides and other co-transmitters. Smaller dense core vesicles (~80 nm) are known to contain components of the presynaptic active zone and thought to transport and deliver these components during developmental synaptogenesis. It is not known whether excitatory axons in area CA1 contain such dense core vesicles, and whether they contribute to synaptic plasticity of mature hippocampus. Serial section electron microscopy was used to identify dense core vesicles in presynaptic axons in s. radiatum of area CA1 in adult rat hippocampus. Comparisons were made among perfusion-fixed hippocampus and hippocampal slices that undergo synaptogenesis during recovery *in vitro*. Dense core vesicles occurred in 26.1±3.6% of axonal boutons in perfusion fixed hippocampus, and in only 17.6±4.5% of axonal boutons in hippocampal slices ($P<0.01$). Most of the dense core vesicle positive boutons contained only one dense core vesicle, and no reconstructed axonal bouton had more than a total of 10 dense core vesicles in either condition. Overall the dense core vesicles had average diameters of 79±11 nm. These small dense core vesicles were usually located near nonsynaptic membranes and rarely occurred near the edge of a presynaptic active zone. Their size, low frequency, locations, and decrease following recuperative synaptogenesis in slices are novel findings that merit further study with respect to small dense core vesicle content and possible contributions to synapse assembly and plasticity in the mature hippocampus.

Key words: synaptic vesicles, presynaptic active zone, brain slice, CA1, dendritic spine, serial section electron microscopy.

Little is known about the mechanisms of presynaptic assembly on mature neurons. Rapid proliferation of dendritic spines during estrous, long-term potentiation (LTP), and slice preparation involves the formation of new synapses on pre-existing axonal boutons, resulting in more multiple synapse boutons (Fiala et al., 2002; Kirov et al., 1999; Toni et al., 1999; Yankova et al., 2001). Under these circumstances, most of the machinery for the release of presynaptic vesicles is already assembled presynaptically. What is missing is the active zone. Evidence from cultured hippocampal neurons shows that small dense core vesicles (DCVs) may provide rapid delivery of pre-assembled active zones to new presynaptic sites in response to a postsynaptic signal (Ahmari et al., 2000; Vaughn, 1989; Ziv and Garner, 2004). These DCVs have been referred to as "piccolo/bassoon transport vesicles" because they contain the proteins that are required for the assembly of presynaptic active zones on developing neurons (Shapira et al., 2003; Zhai et al., 2001).

Nothing is known about the distribution of small DCVs at mature hippocampal CA1 synapses, and whether it is altered during synaptogenesis and plasticity. Hence, it was of interest to learn whether DCVs might also be available to provide preassembled components to synaptic active zones in the adult nervous system. DCVs may also contain neuropeptides or growth factors (Torrealba and Carrasco, 2004; Wu et al., 2004), which influence synaptogenesis. During the preparation of hippocampal slices from mature animals, presynaptic axons discharge many vesicles, glia loose glycogen granules, dendritic microtubules are lost, dendrites become swollen and dendritic spines and synapses are diminished (Fiala et al., 2003; Kirov et al., 2004; Lipton et al., 1995). The neurons then undergo a remarkable recovery once the slices are maintained *in vitro* in a life-support chamber for about 3 h. Much of the cellular disruption is normalized relative to hippocampus fixed by intravascular perfusion, and dendritic spines with synapses proliferate (Kirov et al., 1999, 2004). Evidence is accumulating that exposure to cold during slice preparation may trigger the spine loss and subsequent proliferation during re-warming (Fiala et al., 2003; Kirov et al., 2004; Roelandse and Matus, 2004). This rather rapid synaptogenesis on mature neurons suggests that local dendritic and axonal mechanisms are required for the formation of new synapses.

Local dendritic protein synthesis and actin-based mechanisms could supply a new dendritic spine and postsynaptic density (PSD) (Brunig et al., 2004; Martin, 2004; Smart and Halpain, 2000; Steward and Schuman, 2001). A local store of DCVs in presynaptic axons, perhaps piccolo/bassoon transport vesicles, may help to generate new presynaptic

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Abbreviations: ACSF, artificial cerebrospinal fluid; ASD, adjusted synaptic density; DCV, dense core vesicle; MSB, multisynaptic bouton; n_{sect}, number of sections; PSD, postsynaptic density; SA, sample area; SSB, single-synapse bouton; SSV, small synaptic vesicle.

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doi:10.1016/j.neuroscience.2006.05.033

active zones. If DCVs are involved in synapse formation in the mature brain, then their contents may be released during robust synaptogenesis. Consequently, fewer DCVs should be evident in those axons that have recently undergone slice-induced recuperative synaptogenesis. We examined this possibility by evaluating the distribution and frequency of DCVs in perfusion-fixed and recovered hippocampal slices.

EXPERIMENTAL PROCEDURES

DCVs were quantified in presynaptic boutons through serial section electron micrographs from four mature male rats of the Long-Evans strain, two each from perfusion fixed and hippocampal slices (see Table 1 in, Kirov et al., 1999). Methodological issues relevant to the present analyses are briefly described here; Kirov et al. (1999) should be consulted for the complete details. In addition, updated methods are posted on our website called "SynapseWeb" at synapse-web.org. All experiments conformed to the guidelines of the U.S. National Institutes of Health and were reviewed by the Institutional Animal Care and Use Committee at Children's Hospital, Boston. The number of animals used and their suffering were minimized.

Intracardiac perfusions with fixative were done under pentobarbital anesthesia (80 mg/kg) and started within one minute after opening the chest cavity to minimize hypoxia–ischemia. For one animal, the fixative contained 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 2 mM $CaCl_2$ and 4 mM $MgCl_2$ at pH 7.4. (There was a misprint in the previously published methods section stating 1 mM $CaCl_2$ and 2 mM $MgCl_2$, respectively). Fixative was delivered to the body at 37 °C and 4 p.s.i. backing pressure of 95% O_2 and 5% CO_2. The other animal was perfusion fixed with 6% glutaraldehyde to match the fixation concentration used in the slices.

Hippocampal slices were prepared from rats that were first anesthetized with 80 mg/kg pentobarbital to mimic the "pre-fixation" conditions of the perfusion protocol. Slices were cut at 400 μm thickness and received into ice-cold artificial cerebrospinal 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 at pH 7.4 and bubbled with 95% O_2–5% CO_2). Slices were transferred via the blunt end of a micropipette to a net at the interface of ACSF and humidified 95% O_2–5% CO_2 and maintained at 32 °C for 9–10 h *in vitro*. Physiological recordings were made to demonstrate neuronal responsiveness in the slices, and then slices were fixed during 8 s of microwave irradiation in mixed aldehydes (2% paraformaldehyde and 6% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 and containing 2 mM $CaCl_2$ and 4 mM $MgCl_2$), and stored overnight in fixative at room temperature (Jensen and Harris, 1989).

Perfusion-fixed hippocampus and hippocampal slices were both subsequently fixed in reduced osmium (1% OsO_4 with 1.5% $K_4Fe(CN)_6$) followed by 1% OsO_4, to enhance membranes; then exposed to 1% aqueous uranyl acetate and dehydrated and embedded in epoxy resins and hardened at 60 °C for 48 h prior to collecting serial thin sections. The serial thin sections were stained with ethanolic uranyl acetate followed by Reynolds' lead citrate. Serial sections were photographed in the middle of stratum radiatum at a location about 150–200 μm from the CA1 pyramidal cell body layer. These protocols produced well-stained and readily identifiable DCVs (Figs. 1 and 2). All series were coded and analyzed blind as to condition.

Three-dimensional reconstructions and analyses were performed using the software entitled "Reconstruct" (developed by Dr. John Fiala (Fiala, 2005), and freely downloaded from http://synapses.bu.edu). The vesicles were marked with a circular stamp that was adjusted to match the circumference (C) of the vesicle at its widest diameter if it happened to span more than one section. The vesicles were assumed to be approximately spherical so that the average diameter was computed as ($d=C/\pi$). The distances of DCVs to synaptic and non-synaptic plasma membranes were measured within or across serial sections as needed, using the z-trace tool in Reconstruct.

Adjusted synaptic densities (ASD, # synapses/100 μm^3) were computed by feature (e.g. ASD of synapses with DCVs) as described in Kirov et al. (1999) based on the following computations: $ASD=(n_{syn}/HNA)\times mean(1/n_{sect})\times(1/st)$. Synapse number ($n_{syn}$) was computed by counting the number of PSDs in the sample area (SA). Synapses crossing two of the four lines that defined the sample frames on individual sections and one of the bounding faces of the cube were excluded, as a physical dissector to quantify synapse profiles, and minimize counting bias (Fiala and Harris, 2001b). Synapses were counted if the PSD was evident on the sample section and if the presynaptic vesicles occurred on the sample section or on an adjacent section. Synapse density is markedly influenced by elements occurring nonuniformly in the SA (i.e. myelinated axons, cell bodies, and large dendrites with section profiles >0.94 μm^2) so the areas of these elements were measured and subtracted from the SA to obtain the homogeneous neuropil area (HNA). PSDs have different shapes and sizes, and the probability of capturing them on a single section differs in proportion to the number of sections (n_{sect}) they occupy. Thus, the n_{sect} of each synapse occupied was counted, and the average n_{sect} was computed for each group. The mean inverse of the n_{sect} per condition was used to adjust for any differences in viewing probability (Fiala and Harris, 2001b). Sampling is also affected by section thickness which was obtained for each series by measuring the diameters (d) of longitudinally sectioned mitochondria, counting the number of serial sections they occupied (n), and computing section thickness (st) as: $st=d/n$ (Fiala and Harris, 2001a).

Excel software (Microsoft, Redwood, CA, USA) was used to organize the data. Statistica (StatSoft, Tulsa, OK, USA) was used to obtain means and standard deviations to perform statistical analyses, *t*-tests and the Kolmogorov-Smirnov statistic to compare distributions (criterion $P<0.05$) and graph the data.

RESULTS

DCVs had similar appearances in both preparations whether 2.5% or 6% glutaraldehyde was used and whether the fixation was facilitated by microwave irradiation to speed diffusion, as required for the immersion fixation in slices (Figs. 1 and 2). Upon viewing through many serial EM sections, DCVs could be distinguished along the axons of perfusion-fixed hippocampus (Fig. 1) and hippocampal slices (Fig. 2). DCVs occurred among the small synaptic vesicles (SSVs) in some presynaptic axonal boutons (Figs. 1a–d; 2a–f). Occasionally, a DCV was observed in the vicinity of a synaptic active zone (Fig. 1a, b) or at its edge (Fig. 2a, b). More often they were located near extrasynaptic plasma membrane in a presynaptic axonal bouton (Fig. 1c, d; 2c-f); or in the regions between synaptic boutons along the axons (Fig. 1e, f; 2 g, h). Depending on the plane of view, tiny projections called "spicules" (Vaughn, 1989) could be discerned along the surface of some DCVs (Fig. 2b, f).

Five series were analyzed from perfusion fixed hippocampus (two from one animal and three from the other) and six series were analyzed from the hippocampal slices (three from each animal). Each PSD on the central reference section of the series was viewed through serial sec-

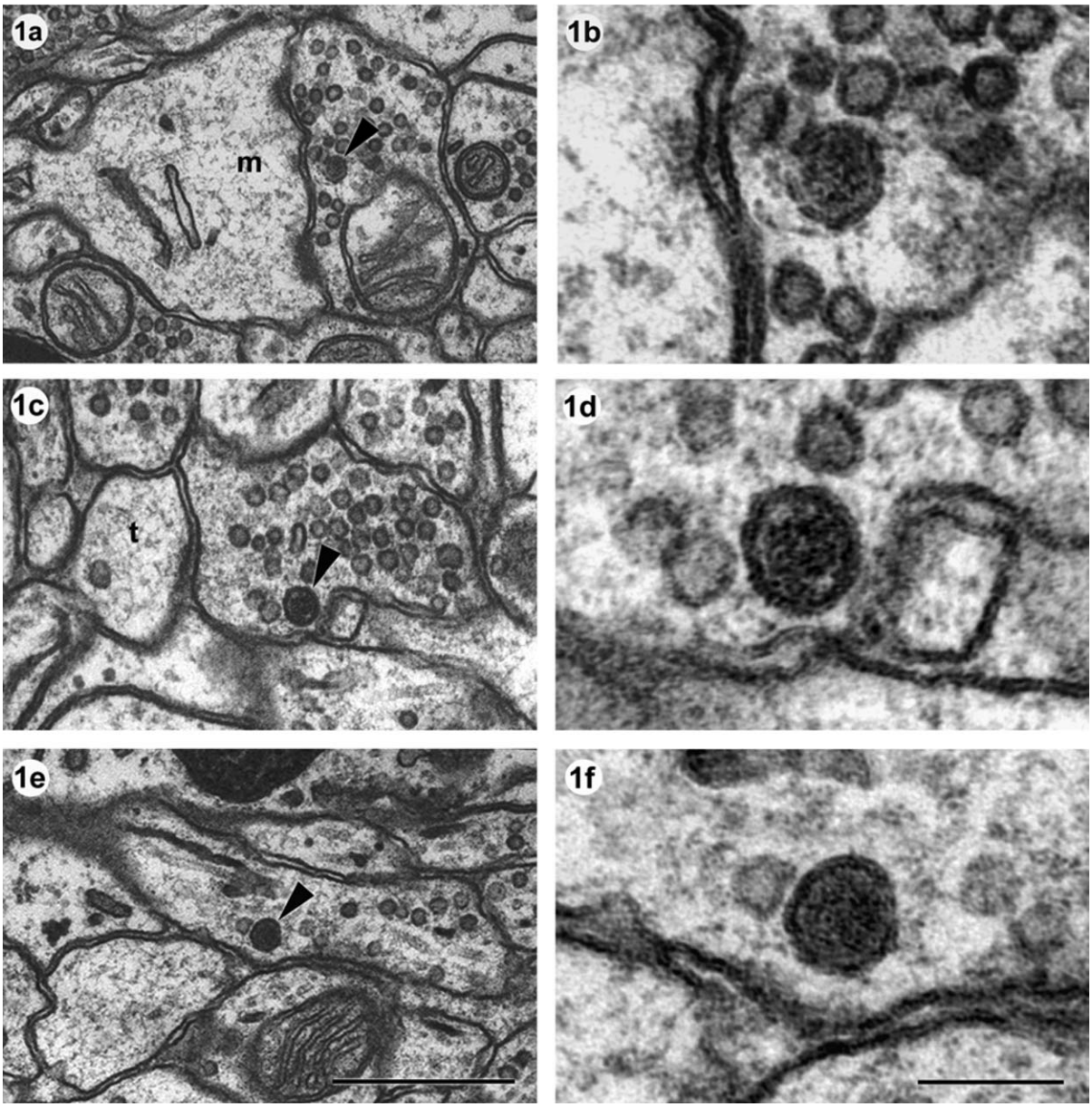

Fig. 1. DCVs (arrows) in stratum radiatum of hippocampal area CA1 in perfusion fixed brain. (a, b) DCV located in a presynaptic bouton of an axon synapsing with a mushroom-shaped dendritic spine (m) and perforated PSD. (c, d) DCV located near the nonsynaptic plasma membrane of a presynaptic bouton that synapses on a thin dendritic spine (t). A macular PSD was located on adjacent serial sections. (e, f) DCV found in an "inter-bouton" region, presumably being transported along the length of an axon. The adjacent clear vesicles are freely floating, not located in a presynaptic bouton. Scale bar=0.5 μm in e for a, c, e. Scale bar=0.1 μm in f for b, d, and f, which are higher magnification views of the DCVs in a, c, and e, respectively.

tions to identify whether its presynaptic bouton contained one or more DCVs. Three segments of axons that were of approximately equal length were reconstructed in three dimensions to illustrate the varied distribution of DCVs (Fig. 3). Boutons containing DCVs formed synapses with dendritic spines of diverse morphologies (e.g. thin, mushroom, stubby, branched) having either macular or perforated PSDs. No significant differences were detected regarding the presence of DCVs across these various spine and synapse morphologies. Individual synaptic boutons contained from 0 to nine DCVs. Most of the DCV-containing boutons (70%) had a single DCV (Fig. 1, Fig. 3a), while 30% had two or more DCVs per bouton (Fig. 2e, 2f, Fig. 3b, 3c).

A total of 257 DCVs were analyzed in 671 presynaptic boutons of asymmetric, presumably excitatory synapses. DCVs were larger with average diameters of 79.2±11 nm than neighboring SSVs in the same boutons with average diameters 46.6±5 nm in both perfusion-fixed brain and hippocampal slices ($P<0.00001$, Fig. 4). In both conditions, DCVs were located closer to the nonsynaptic plasma membrane than to the presynaptic active zone (K-S sta-

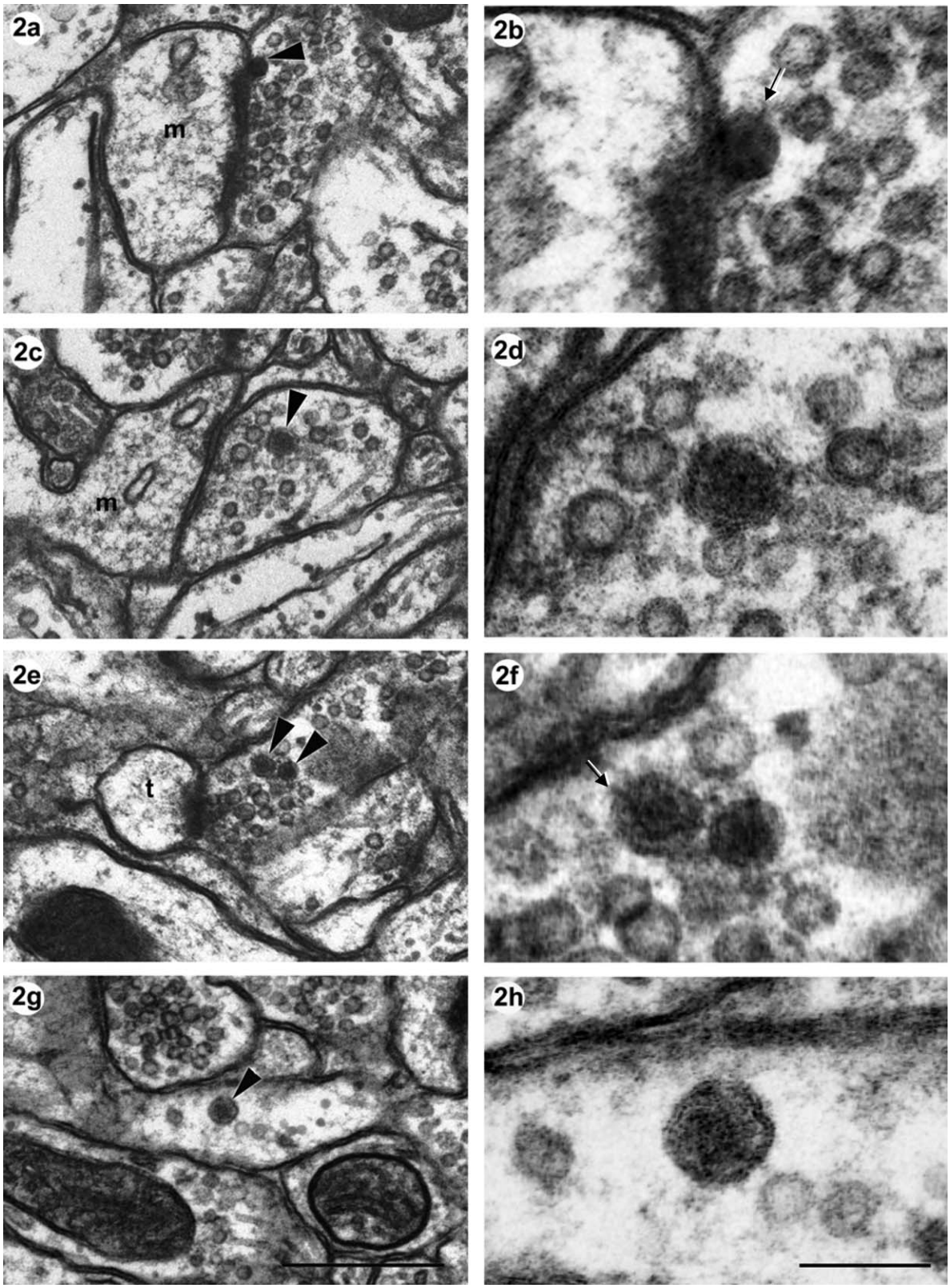

Fig. 2. DCVs (arrows) found in recovered hippocampal slices. (a, b) Rare, morphologically docked DCV at the edge of a presynaptic active zone (SAZ) at mushroom-shaped dendritic spine (m). (c, d) DCV is approximately equidistant between the SAZ and the nonsynaptic membrane of a presynaptic bouton which synapses on a mushroom-shaped dendritic spine (m) that has a perforated synapse. (e, f) Synaptic bouton of a thin dendritic spine (t) with two DCVs located closer to the nonsynaptic membrane than the SAZ. (g, h) DCV is in an inter-bouton region along the length of the axon. Tiny dense-staining protrusions called "spicules" (arrows in b, f) were clearly observed projecting from the membrane of some DCVs. Scale bar=0.5 μm in g for a, c, e, and g. Scale bar=0.1 μm in h for b, d, f, and h which are higher magnification views of the DCVs in a, c, e, and g, respectively.

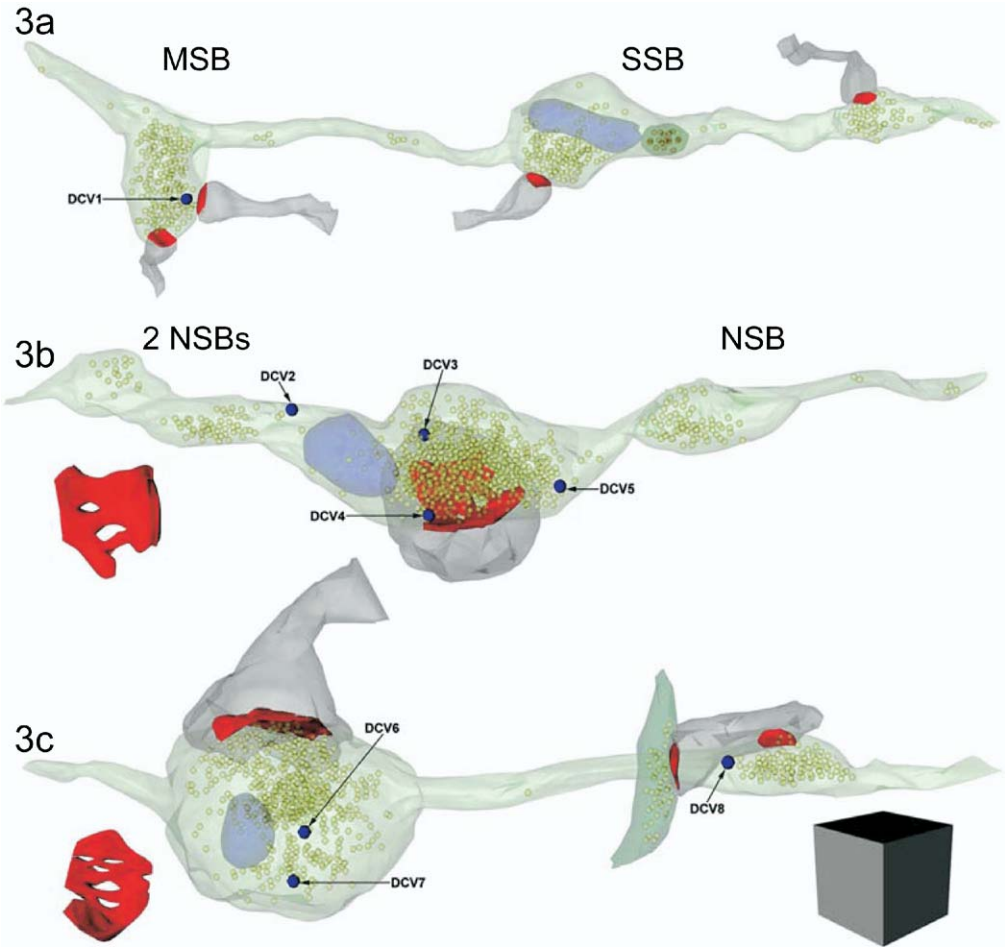

Fig. 3. Reconstructions of three axonal segments in s. radiatum of hippocampal area CA1 were completed. The reconstructed axons are illustrated as pale green with DCVs (dark blue), SSVs (yellow), mitochondria (pale blue), and dendritic spines (gray) with PSDs (red). (a) Axon with a multiple synapse bouton (MSB) and two single synapse boutons (SSB). A DCV is in the MSB but not the other synaptic boutons. A multivesicular body (dark green) is near the mitochondrion in the central bouton. All of the spines along this segment are thin with small macular PSDs. (b) Axonal segment having one large bouton that synapses with a mushroom spine and perforated PSD (inset). Along this axonal segment there are also three nonsynaptic boutons (NSB) or "orphan sites" (Krueger et al., 2003), each containing small clear vesicles (yellow). Four DCVs were identified as indicated, with one in an NSB and three in the large SSB. (c) An axon with two synaptic boutons. One of these is a large SSB containing two DCVs and synapsing with a mushroom spine that has a perforated synapse (inset). A second smaller bouton contains one DCV. This bouton makes a synapse on the neck of a thin spine. That thin spine also formed a second synapse on its head with a different axon (dark green). Measurements for each DCV were (diameter, distance to the nonsynaptic plasma membrane, distance to nearest presynaptic active zone in nanometers): DCV1 (74, 14, 270), DCV2 (66, 19, 769), DCV3 (64, 224, 224), DCV4 (73, 9, 9), DCV5 (82, 99, 316), DCV6 (94, 196, 1095), DCV7 (87, 20, 218), DCV8 (61,431,1709). Scale cube=0.5 μm^3.

tistic, $P<0.00001$). In fact, most of the DCVs were located within a single vesicle diameter of the non-synaptic plasma membrane (<50 nm; Fig. 5a) but many vesicle diameters away from the presynaptic active zone (Fig. 5b). In several cases, even when the DCVs were within two vesicle diameters of the presynaptic active zone, they were still closer to the non-synaptic membrane (inset, Fig. 5b). When compared across perfusion and slice conditions, DCVs did not differ significantly in their distances from the plasma membrane ($P=0.31$) or presynaptic active zones ($P=0.24$).

The relative ASDs (see Experimental Procedures) of DCV-containing presynaptic boutons were computed and corrected for neuropil heterogeneity and differences in viewing probabilities. In perfusion-fixed hippocampus, 26.1±3.6% of the axonal boutons contained one or more DCVs, whereas in slices, 17.6±4.5% of the boutons contained one or more DCVs. Comparison of these ASDs revealed the difference to be statistically significant (Fig. 6a, $P<0.01$). The DCVs found in slices were slightly smaller on average than those in perfusion-fixed hippocampus (Fig. 6b). Subtle differences in tissue shrinkage between slices and perfusion fixed brain might have been responsible for this size difference; however, this explanation seems unlikely because the SSV diameters were the same size in both conditions (Fig. 6b). Alternatively, more of the large DCVs may have been released during synaptogenesis in the slices or some of the DCVs may have released only part of their contents during a "kiss and run" event (Rutter and Tsuboi, 2004) in the slices.

Finally, we evaluated the probability of DCVs occurring in various types of presynaptic boutons in both perfusion-fixed and in hippocampal slices. First we confirmed earlier

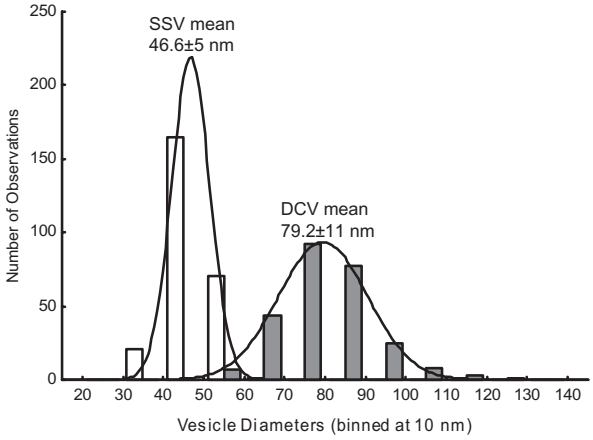

Fig. 4. Neighboring SSV diameters were smaller than DCVs ($n=257$ measurements of each type of vesicle, $P<0.00001$). All data from the perfusion-fixed and slice conditions are graphed together. (See also Fig. 6 for differences in DCV mean diameters.)

findings (Kirov et al., 1999) that synaptogenesis in hippocampal slices results in more multisynaptic boutons (MSBs, Fig. 7a). New MSBs might have formed via the addition of synapses to existing single-synapse boutons SSBs (i.e. SSB to MSBs conversion during synaptogenesis). However, the frequency of SSBs did not decrease in slices (Fig. 7a, $P=0.27$). This observation suggests that SSBs were not the only source of new synapses. New synapses may also form at pre-existing nonsynaptic boutons (e.g. dcv2 of Fig. 3b), which would replenish the SSB population that became MSBs. New SSBs may also lose DCVs, consistent with the quantitative data showing fewer DCV-containing SSBs in slices relative to perfusion fixed brain ($P<0.05$ Fig. 7b). Fewer MSBs contained more than one DCV in slices than perfusion fixed hippocampus, consistent with loss of DCVs when new synapses were added to axonal boutons in slices (Fig. 7c, $P<0.001$).

DISCUSSION

These findings provide the first three-dimensional quantification of DCVs in mature hippocampal axons. The DCVs occurred in 20–30% of the axonal boutons, most having just one DCV, and none were observed having more than 10 DCVs per fully reconstructed bouton. DCVs co-mingled with smaller clear vesicles in both synaptic and nonsynaptic boutons. They were occasionally positioned at the edge of an existing active zone, but usually DCVs were located closer to non-synaptic plasma membranes suggesting the possibility of selective targeting to the inner surface of the plasma membrane of boutons. DCVs also occurred in the inter-bouton segments of these mature axons. Their low frequency suggests that the contents of these DCVs are not intended for secretion of neurotransmitters or neuropeptides, which occur primarily in larger DCVs and at a higher frequency per bouton in other systems (Torrealba and Carrasco, 2004). Instead the small DCVs in these mature CA1 radiatum axons may deliver preassembled components to establish new active zones, like those found at newly forming synaptic sites along developing axons in culture (Ahmari et al., 2000; Roos and Kelly, 2000; Ziv and Garner, 2004). DCVs are synthesized in the soma and transported by slow anterograde mechanisms into axons; hence recovery of DCVs in distal axonal boutons may require a day or more. On a time scale of hours to days, changes in their frequency or location are thus sensitive indicators of altered function (Pierce et al., 1999). The reduced number of boutons containing DCVs in mature hippocampal slices is consistent with a role for DCVs in synaptogenesis in the mature hippocampus. Their size and distribution are consistent with the hypothesis that these small DCVs are available to transport preassembled components to active zones. Further experiments are needed to assess the composition of these DCVs and whether they contain piccolo, bassoon, and other substances involved in the construction of the presynaptic active zone.

The diameter of the DCVs in mature hippocampal axons averaged 80 nm, which is equal to DCVs in developing hippocampal axons in culture (Ahmari et al., 2000; Ziv and Garner, 2004). Vaughn (1989) first suggested that DCVs may carry presynaptic active zones in packets and described spicules emerging from DCVs as a possible source of the active zone; similar spicules were observed on some of the DCVs shown here in mature CA1 axonal boutons. The surface of DCVs in cultured hippocampal axons has been found to be immunolabeled with antibodies to piccolo and bassoon, proteins that are important for the assembly of the presynaptic active zone (Shapira et al., 2003; Zhai et al., 2001). These DCVs also occur at a low frequency of about two to five per bouton (Bresler et al., 2004); similar to the frequency observed here in mature CA1 radiatum axons. New experiments will be needed to determine whether the small DCVs in these mature axons also contain piccolo, bassoon or other components of the active zone.

DCVs were first identified as membrane-bound organelles containing a dense granular substance in glutaraldehyde and osmium fixed tissue of adrenergic terminals in the autonomic nervous system (Peters et al., 1991). At ~80 nm the DCVs described here are similar in size to the norepinephrine and serotonin containing DCVs, however, these secretory DCVs occur in a much higher frequency at hundreds to thousands per axonal bouton (Buma and Roubos, 1986; Zhu et al., 1986; De Biasi and Rustioni, 1988; Peters et al., 1991; Cheng et al., 1995). DCVs with diameters larger than 100 nm have been found to contain neuroactive peptides such as growth hormones, orexin, dynorphin or neuropeptide Y (e.g. (Lowe et al., 1988; Parker et al., 1998; Torrealba et al., 2003; Valentino et al., 2001)). Although the peptidergic DCVs are co-localized with SSVs, many of these large DCVs are released at nonsynaptic sites and act via volume transmission through extracellular space (Martin, 2003). Large DCVs (80–120 nm) that are immunoreactive for neuropeptides Y and dynorphin modulate the release of neurotransmitter presynaptically in the hippocampus and are found in septal cholinergic axons on proximal CA1 dendrites as well as in the mossy fiber boutons on proximal CA3 dendrites (Pickel et

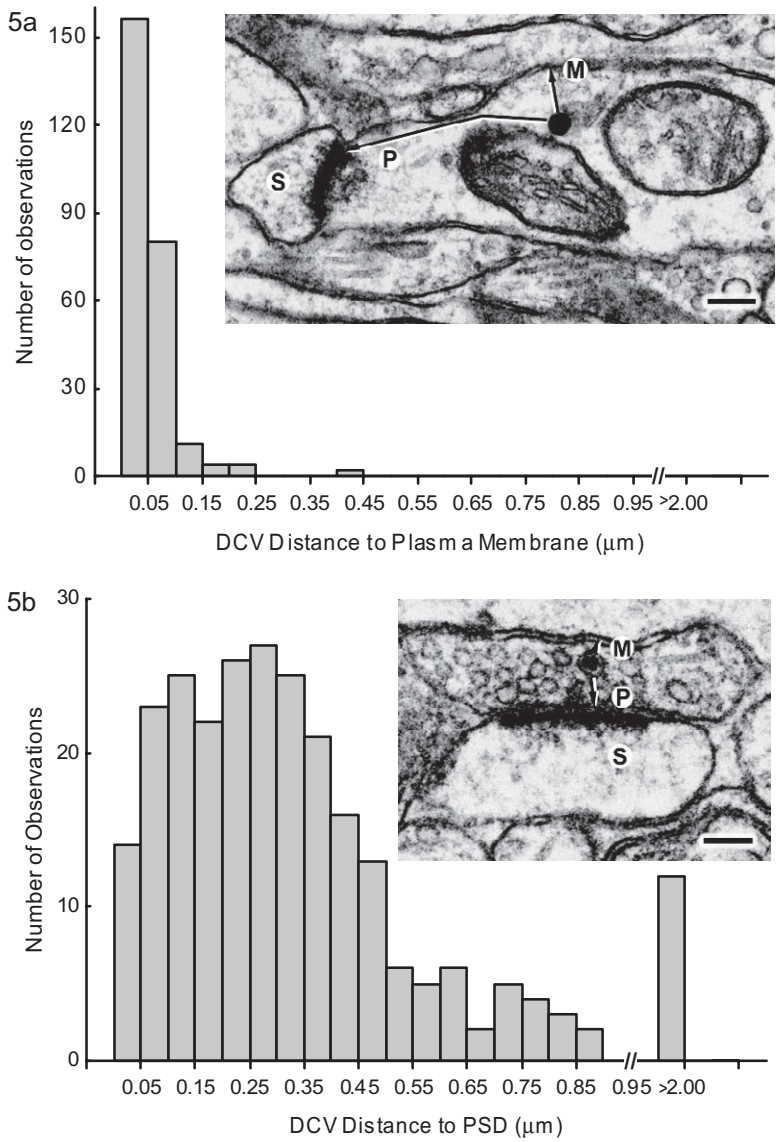

Fig. 5. Location of DCVs within synaptic boutons relative to the nonsynaptic plasma membrane (M, insets) and the synaptic active zone, as defined by the nearest edge of the PSD (P, insets). (a) Most of the DCVs were located within 50 nm of the nonsynaptic plasma membrane. (b) In contrast, most of the DCVs were located more than 100 nm away from the synaptic active zone. There were no significant differences between the perfusion-fixed and slice conditions for these measurements so all of the data are graphed together.

al., 1995; Pierce et al., 1999). DCVs in the hilus and mossy fibers occur at a frequency of more than 10 per thin section, for a total of hundreds to thousand per synaptic bouton (Chicurel and Harris, 1992; Torrealba and Carrasco, 2004). Moreover, the large DCVs appear to occur at this high frequency in every synaptic bouton of those systems. In contrast, neuropeptide Y immunoreactive axons in hippocampus, and glutamatergic axon terminals in cortical and thalamus are presented in the literature as not containing many DCVs (Pickel et al., 1995; Torrealba and Carrasco, 2004), similar to those measured here in s. radiatum of area CA1.

During development, dendritic spines evolve from filopodial outgrowth, migration of synapses along a filopodium toward the dendritic shaft, and subsequent enlargement of spine heads with synapses (Fiala et al., 1998; Harris, 1999; Marrs et al., 2001; Petrak et al., 2005). If all DCVs were only involved in synapse formation at new presynaptic boutons, then we would have expected to see DCVs located only in nonsynaptic boutons or in boutons synapsing on filopodia, dendritic shafts or small stubby spines. Instead, DCVs were in boutons that synapsed with dendritic spines of all shapes and sizes, and some DCVs were situated at the edge of active zones. In addition, DCVs appeared to be lost from all types of presynaptic boutons. These locations support the hypothesis that DCVs not only create new synapses but can also add synapses to boutons with pre-existing synapses thereby creating more MSBs or enlarging existing synapses.

Previous analyses showed that the synaptogenesis in these slices primarily involved multiple synaptic boutons and

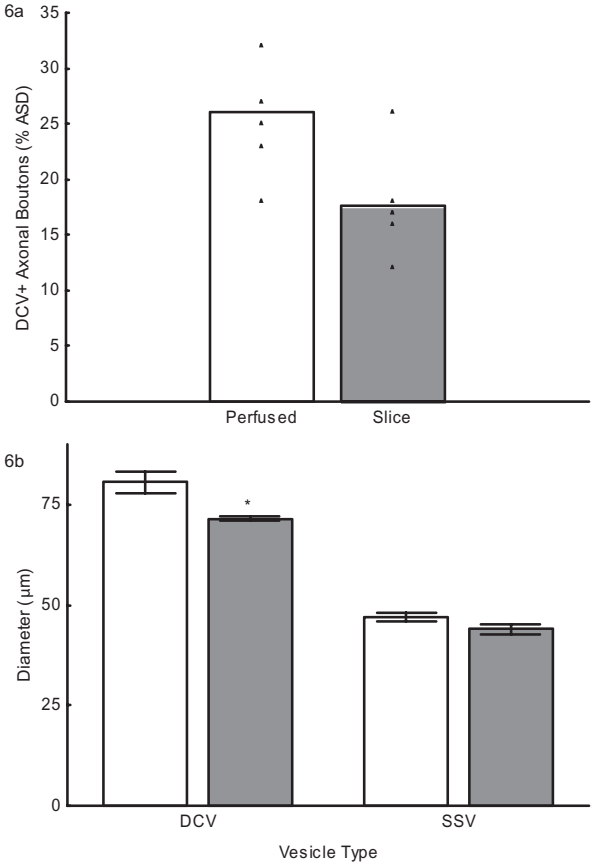

Fig. 6. Comparison of DCVs in perfusion fixed hippocampus (*in vivo*) and acute slices. (a) Comparison of the ASDs revealed a greater percentage of presynaptic boutons contained DCVs in the perfusion fixed hippocampus (n=463 boutons) than in the slices (n=207 boutons, $P<0.05$). (b) DCVs were slightly larger in perfusion fixed tissue than in slices ($P<0.01$) while SSV dimensions were comparable between the two conditions ($P=0.36$).

to a lesser extent single synapse boutons (Kirov et al., 1999). In a subset of the same slices, 4% of vesicle-containing boutons had no postsynaptic partners (Shepherd and Harris, 1998), namely "orphan sites" (Krueger et al., 2003). Due to their relatively low frequency, additional experiments will be needed to determine whether the frequency of orphan sites declines during synaptogenesis in the mature brain. Nevertheless, the observation of occasional orphan sites containing DCVs in mature axons (e.g. Fig. 3), suggests that they might be the source of new presynaptic boutons. There was also a decrease in the proportion of multiple DCV containing MSBs, consistent with the idea that loss of DCVs turned them into single DCV containing MSBs during recuperative synaptogenesis in slices. One might then predict that MSBs with one or zero DCVs should also have more PSDs than MSBs with multiple DCVs. Alternatively, SSBs with multiple DCVs could have become MSBs. It was not possible to address this question directly because most MSBs contain only two PSDs and only in rare instances are there three or more PSDs on a single MSB (Sorra and Harris, 1993). New imaging methods will be needed to monitor where DCVs are released and whether new active zones emerge at their full size with release of one or more DCVs and/or whether active zones are enlarged during DCV release at the edge of growing synapses.

Growth factors are released from glutamatergic synapses, but it is not clear whether they are released via DCVs or other mechanisms (Michael et al., 1997; Fawcett et al., 1997; Wang et al., 2003). For example, maintenance of septal input to hippocampal dentate granule cells is

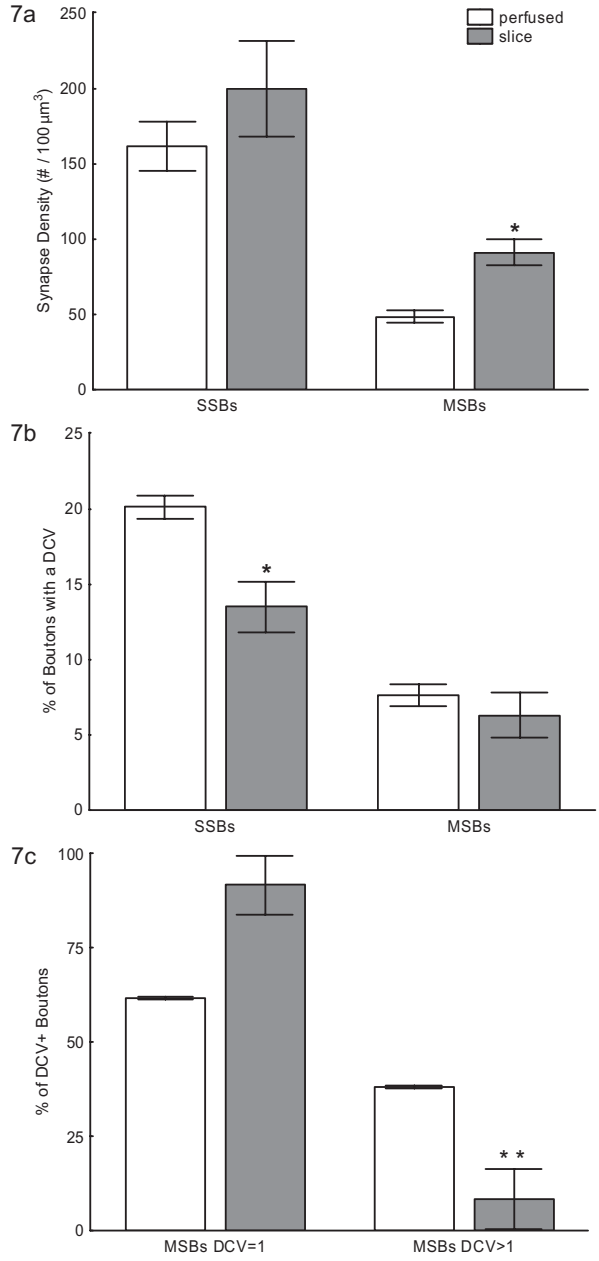

Fig. 7. Relative distribution of DCVs in SSBs and multiple synapse boutons (MSBs). (a) The adjusted density of synapses occurring on MSBs was higher in hippocampal slices than perfusion-fixed brain (* $P<0.05$). This graph is restricted to the subset of synapses analyzed here; and replicates results published in Kirov et al. (1999). (b) Fewer SSBs contained any DCVs in slices than in perfusion fixed brain (* $P<0.05$). (c) Fewer MSBs contained multiple DCVs in slices in comparison with perfusion fixed hippocampus (chi square ** $P<0.001$).

dependent on TrkA, and antibodies to TrkA are localized to cytoplasmic patches among synaptic vesicles and occasionally label DCVs (Barker-Gibb et al., 2001). At active glutamatergic synapses, the signaling cascades associated with glutamate receptors may compete with growth factors to stabilize spines as well as suppress new spine outgrowth (Mattson et al., 1988; Mattson, 1990; McKinney et al., 1999; Luthi et al., 2001; Schwyzer et al., 2002). Conversely, when glutamatergic transmission is suppressed, the growth factors may stimulate spine formation and synaptogenesis (Kirov et al., 1999). New experiments will be needed to determine whether the DCVs in mature hippocampal axons contain growth factors or if growth factors are restricted to cytoplasmic domains in these boutons.

Primary and secondary lysosomes also have dense cores (Peters et al., 1991). They are distinguished from DCVs in that their electron dense centers are less granular and extend to the edges of the vesicle where the surrounding membrane is often obscured. Lysosomes are also larger than the DCVs measured here, ranging in size from 100–200 nm. Lysosomes were not observed in this sample of synaptic boutons from mature hippocampal axons.

CONCLUSIONS

During the preparation of hippocampal slices, neurons undergo considerable reorganization, synapse loss, and subsequently exuberant synaptogenesis during recovery. This reorganization motivated a search for local presynaptic organelles that could be involved in rapid assembly of the presynaptic active zones. We elucidated characteristics of small DCVs in the presynaptic axons of the CA3 to CA1 synapse that might facilitate rapid synaptogenesis in the mature hippocampus. The relatively small size, sparse number, location typically near the plasma membrane, but away from existing active zones, and loss during synaptogenesis, suggest that these small DCVs might be transport vesicles involved in synaptogenesis. They might also secrete growth factors. Productive avenues for future research should reveal the composition of these DCVs in mature axons and whether they respond differentially to synapse formation, growth, or elimination during synaptic plasticity such as long-term potentiation, long-term depression, or learning and memory.

Acknowledgments—This work was supported by grants NS21184 and NS33574 from the National Institutes of Health and the Human Brain Project grant EB002170 to K.M.H. and NIH KO1MH02000 to S.K. We thank Craig Garner for his encouragement and for reading the manuscript, John Fiala for the Reconstruct software (available at http://synapses.bu.edu; or http://synapses.mcg.edu); Marcia Feinberg for serial sectioning, and Robert Smith and Cameron Slayden for technical assistance on the images.

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(Accepted 6 May 2006)
(Available online 22 June 2006)