Introduction

The mechanisms underlying functional diversity among excitatory synapses are poorly understood. In the hippocampus, synaptic regions of the brain stand out for their high content in synaptic zinc, which may thus be involved in synaptic function. The relative number, chemical nature and transmitter receptor profile of synapses that sequester vesicular zinc are largely unknown. To address this, we combined pre-embedding zinc histochemistry and post-embedding immunogold electron microscopy in rat hippocampus. All giant mossy fibre (MF) terminals in the CA3 region and 45% of boutons making axosomatic synapses in stratum radiatum in CA1 contained synaptic vesicles that stained for zinc. Both types of zinc-positive boutons selectively expressed the vesicular zinc transporter ZnT-3. Zinc-positive boutons further immunoreacted to the vesicular glutamate transporter VGLUT-1, but not to the transmitter γ-aminobutyric acid. Most dendritic spines in CA1 immunoreacted to α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR) subunits GluR1–3 (∼80%) and to N-methyl-D-aspartate receptor (NMDAR) subunits NR1 + NR2A/B (∼90%). Synapses made by zinc-positive boutons contained 40% less AMPAR particles than those made by zinc-negative boutons, whereas NMDAR counts were similar. Further analysis indicated that this was due to the reduced synaptic expression of both GluR1 and GluR2 subunits. Hence, the levels of postsynaptic AMPARs may vary according to the presence of vesicular zinc in excitatory afferents to CA1. Zinc-positive and zinc-negative synapses may represent two glutamatergic subpopulations with distinct synaptic signalling.

Materials and Methods

Perfusion and Staining for Zinc

All procedures followed present regulations for animal care and handling and were approved by the University of Barcelona Ethical Committee. Male Wistar rats (250–400 g) were used. Two rats were perfused under anaesthesia (chloral hydrate, 600 mg/kg), first with heparinized saline, then with 200 ml of 0.1% Na2S, 2.5% glutaraldehyde, 0.002% CaCl2 in 0.1 M phosphate buffer (PB; pH = 7.4) and finally with 400 ml of 2.5% glutaraldehyde, 1% formaldehyde, 0.2% picric acid and 0.002% CaCl2 in PB, at a rate of 50 ml/min. Na2S renders zinc precipitates that become visible by silver amplification. The brains were taken out, postfixed, frontally cut on a Vibratome at 50 µm, rinsed in PB and processed free-floating for the visualization of zinc (Danscher, 1981). Briefly, sections were developed in a solution containing gum arabic, citrate buffer, hydroquinone and silver lactate for 75 min in the dark at 26°C.

Osmium-free Postfixation and Epon Embedding

Following staining for zinc, slices were processed as described previously (Phend et al., 1995). Briefly, they were rinsed in PB and immersed in 0.1 M maleate buffer solutions (MB; pH = 6) containing 1% tannic acid, 1% uranyl acetate and 0.5% iridium tetrabromide, then in 30, 50 and 70% glycerol (10, 20, 30% in TM). The rostral hippocampus was cut out, rinsed in 0.1 M Tris–maleate buffer (TM; pH = 7.4) and cryoprotected in 400 ml of 2.5% glutaraldehyde, 0.2% picric acid and 0.002% CaCl2 in PB, at a rate of 50 ml/min. Na2S renders zinc precipitates that become visible by silver amplification. The brains were taken out, postfixed, frontally cut on a Vibratome at 50 µm, rinsed in PB and processed free-floating for the visualization of zinc (Danscher, 1981). Briefly, sections were developed in a solution containing gum arabic, citrate buffer, hydroquinone and silver lactate for 75 min in the dark at 26°C.

Freeze-substitution and Acrylic Embedding

Four rats were perfused with saline followed by 500 ml of 4% formaldehyde, 0.1–0.2% glutaraldehyde, 0.2% picric acid and 0.002% CaCl2 in PB. The brains were postfixed and cut, as above. Slices were rinsed in 0.1 M Tris–maleate buffer (TM; pH = 7.4) and cryoprotected in glycerol (10, 20, 30% in TM). The rostral hippocampus was cut out, frozen in liquid propane, freeze-substituted in methanol containing 0.5% uranyl acetate and embedded in LocrylicHM20 (van Lookeren et al., 1991). Another two rats were processed as above, except that fixation was preceded by the addition of 0.1% Na2S and 0.2% glutaraldehyde in PB. Brain slices were silver-amplified as above.

Cortical regions of the brain stand out for their high content in synaptic zinc, which may thus be involved in synaptic function. The relative number, chemical nature and transmitter receptor profile of synapses that sequester vesicular zinc are largely unknown. To address this, we combined pre-embedding zinc histochemistry and post-embedding immunogold electron microscopy in rat hippocampus. All giant mossy fibre (MF) terminals in the CA3 region and 45% of boutons making axosomatic synapses in stratum radiatum in CA1 contained synaptic vesicles that stained for zinc. Both types of zinc-positive boutons selectively expressed the vesicular zinc transporter ZnT-3. Zinc-positive boutons further immunoreacted to the vesicular glutamate transporter VGLUT-1, but not to the transmitter γ-aminobutyric acid. Most dendritic spines in CA1 immunoreacted to α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR) subunits GluR1–3 (∼80%) and to N-methyl-D-aspartate receptor (NMDAR) subunits NR1 + NR2A/B (∼90%). Synapses made by zinc-positive boutons contained 40% less AMPAR particles than those made by zinc-negative boutons, whereas NMDAR counts were similar. Further analysis indicated that this was due to the reduced synaptic expression of both GluR1 and GluR2 subunits. Hence, the levels of postsynaptic AMPARs may vary according to the presence of vesicular zinc in excitatory afferents to CA1. Zinc-positive and zinc-negative synapses may represent two glutamatergic subpopulations with distinct synaptic signalling.

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Carlos Balet Sindreu1,3, Hélène Varoqui2, Jeffrey D. Erickson2 and Jesús Pérez-Claussell1

1Departament de Biologia Cellular, Facultat de Biologia, Universitat de Barcelona, ES-08071 Barcelona, Spain.
2Neuroscience Center, University of Louisiana Health Sciences Center, New Orleans, LA 70112, USA and 3Anatomisk Institut, Neurobiologi, Aarhus Universitet, DK-8000 Aarhus C, Denmark

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Antibodies
Rabbit GluR1 (3 µg/ml), GluR2/3 (3 µg/ml), NR2A/B (4 µg/ml) and mouse GluR2 (4.5 µg/ml) antibodies (Chemicon, Temecula, CA), rabbit GABA antibody (0.05 µg/ml; Sigma, St Louis, MO), rabbit NR2A and NR2B antibodies (2.5 µg/ml; Sigma-RBI), mouse NR1-pan antibody (5 µg/ml; Pharmingen, San Diego, CA) and rabbit VGLUT-1 antibody at a dilution of 1:1000 (Varoqui et al., 2002) were used. The specificity of NR2A and NR2B antibodies was confirmed by immunoblotting of rat cortical synaptosomal fractions (data not shown). Rabbit ZnT-3 antibody (1:100) (Palmiter et al., 1996) was a kind gift from Dr T.B. Cole (University of Washington) to Dr G. Danscher (Aarhus University).

Post-embedding Immunolabelling
Ultrathin sections were picked up on formvar-coated gold grids, washed in Tris-buffered saline (50 mM Tris–HCl, 0.3% NaCl; TBS; pH = 7.6) and incubated in 1% sodium borohydride/TBS (10 min, room temperature), then in 0.005% Tergitol NP-10 in TBS (TBST), followed by primary antibodies in TBST containing 2% serum (overnight at 36°C). They were rinsed in TBST (4 × 5 min) and TBST pH 8.2 (2 × 10 min), then incubated in secondary antibodies, goat anti-rabbit or goat anti-mouse IgG or F(ab), fragments coupled to 10 or 15 nm colloidal gold particles (British BioCell International), diluted 1:30 in TBST pH 8.2 with 0.5% BSA (2 h, 36°C), rinsed, immersed for 5 min in TBS/2% glutaraldehyde, jet-washed and counterstained.

Image Acquisition and Data Analysis
Electron micrographs were recorded by a CCD camera (Gatan Inc.). We checked the penetration of the silver amplification solution into the slice. Thin sections were cut transversal to the Vibratome slice and adjacent micrographs were taken from each section at ~100 µm from the CA1 pyramidal layer. The penetration of the developer began to decrease at 4 µm under the slice surface, so that the frequency of ZN+ boutons shown decreased gradually (P < 0.05, n = 10 experiments, four slices). Hence, analysis in subsequent experiments was restricted to this 4 µm band. ZN+ boutons in CA1 were quantified by random systematic sampling along the axis of apical dendrites. Perforated spines and dendritic shafts were excluded. In the stratum lucidum, only giant MF terminals were analysed to avoid contamination from spiny nonpyramidal neurons (Soriano and Frotscher, 1993). Based on previous findings (Matsubara et al., 1996; Fabian-Fine et al., 2000; He et al., 2000), synapses immunopositive for glutamate receptors were defined as those presenting one or more gold particles located as far as 45 nm from the inner leaflet of the postsynaptic membrane associated with the PSD. To compare the efficiency of immunolabelling in each protocol, we calculated the average number of gold particles that labelled the CA1 stratum radiatum after incubation with GluR1 + GluR2/3 antibodies. In conventional epon material, efficiency of immunolabelling amounted to 7 ± 2 gold particles/µm² (area examined = 87 µm²). When combined with staining for zinc, the efficiency remained similar: 10 ± 4 particles/µm² (area = 148 µm²; P = 0.3) and was highly specific (only 0.3 ± 0.1 particles/µm² in the absence of primary antibody; area = 39 µm²; P < 0.0001). Similar values were found in Lowicryl-embedded CA1 (10 ± 1 AMPAR particles/µm²; area = 35 µm²), Lowicryl-embedded CA1 stained for zinc (9 ± 2 particles/µm²; area = 58.2 µm²) and in a previous work (Racca et al., 2000).

Differences between means were assessed with the Mann–Whitney U-test. Significance was set at 0.05. Values are given as means ± SD unless stated otherwise. Relative frequencies were obtained from samples of similar but not identical size, hence the arcsine of the square root of each value was calculated to homogenize variances and data were compared with the Wilcoxon signed ranks test. Observed frequencies were fit to a Poisson distribution by the least-squares method.

Results

ZN+ Boutons in the Hippocampus Form a Subpopulation of Synapses
Staining for vesicular zinc was intense in the neuropile of CA3 and CA1 regions, but absent in cell body layers, the stratum lacunosum-moleculare and the medial perforant-path of the dentate gyrus (Fig. 1A). At the ultrastructural level, zinc ions (detected by silver grains) were associated with round, clear synaptic vesicles in boutons that formed asymmetric synapses with dendritic spines. In the stratum lucidum in CA3, all large MF boutons were ZN+. In the stratum radiatum in CA1, 38 ± 1% (n = 174) of axospinous boutons were identified as ZN+ in single sections. This proportion remained constant between 100 and 300 µm from the pyramidal cell layer. The mean postsynaptic density (PSD) length (Table 1) or gross bouton morphology did not differ between ZN+ and zinc-negative (ZN–) synapses.

To test whether the lack of staining for zinc in the remaining boutons in CA1 was due to technical limitations, boutons were sampled through five serial sections to increase the efficiency of detection. In these conditions, 46 ± 2% of boutons reacted for vesicular zinc (n = 77). In addition, 87 ± 2% of the profiles from identified ZN+ boutons were labelled for zinc (n = 52 ZN+ boutons, data from a different sample; three to five serial sections). Thus, ZN+ boutons were underestimated in only ~6% of cases in single sections, a proportion far from that of ZN–boutons (~55%).

To confirm the histochemical results, we performed immunogold staining for the obligatory vesicular zinc transporter ZnT-3 on thin sections. As shown in Figure 1C1, C2, ZnT-3 particles were consistently found in ZN+ boutons, whereas virtually all ZN– boutons were devoid of immunostaining. Most ZnT-3 particles overlaid synaptic vesicles (Fig. 1F). Results are summarized in Figure 1I. As a positive control, heavy ZnT-3 staining was found at MF to pyramidal CA3 synapses (Fig. 1B). Altogether, data strongly pointed to two separate populations of afferent boutons (ZN+ /ZnT-3+ versus ZN–/ZnT-3– ) making synapses onto CA1 spines.

Expression of VGLUT-1 at ZN+ Boutons
To provide evidence relative to the transmitter content of ZN+ boutons, we studied the presence of the vesicular glutamate transporter isoform VGLUT-1 (which is mostly telencephalic) and the inhibitory transmitter γ-aminobutyric acid (GABA). Both ZN+ and ZN– boutons showed strong immunoreactivity for VGLUT-1 (Figs. 1D, E, F), the majority of which concentrated over synaptic vesicles and, to a lesser extent, on junctional membranes (Fig. 1G). VGLUT-1 particles were frequently associated with the luminal face of the vesicular membrane and seemed to distribute evenly over the vesicle pool. Notably, some vesicular VGLUT-1 particles were apposed to medium-sized (30–50 nm) silver grains in ZN+ boutons (Fig. 1H).

Immunoreactivity for GABA left all axospinous boutons unstained, but was abundant in symmetric synapses on shafts (Fig. 1J), in a few postsynaptic dendrites and in mitochondria (Fig. 1K).

Co-localization of Postsynaptic Ionotropic Glutamate Receptors with ZN+ Terminals
The identification of two axospinous populations of glutamatergic boutons in CA1 prompted us to analyse the postsynaptic expression of glutamate receptors relative to ZN+ and ZN– boutons. The efficiency and specificity of immunolabelling was unaffected by zinc histochemistry (see Material and Methods). Furthermore, the mean number of synaptic AMPAR (GluR1 + GluR2/3 antibodies) and NMDAR (NR1 + NR2A/B or NR1 + NR2A + NR2B antibodies, pooled data) particles coincided with that found in conventional immunocytochemical studies (Table 1) (Rubio and Wenthold, 1997; Kharazia and Weinberg, 1999; Petralla et al., 1999). Closer examination revealed the typical
pattern of AMPAR labelling: 69% of particles (from a sample of 490 particles) associated with the postsynaptic membrane or density; 11.2% on presynaptic terminals, most of which were on synaptic vesicles; and 19.8% localized to the cytoplasm or extrasynaptic membrane of the spine. Except for the synaptic cleft, extracellular compartments were virtually unlabelled. Several synapses were immunopositive in single sections stained for zinc: 64 ± 6% (n = 273) and 66 ± 4% (n = 250) of synapses were labelled after incubation with NMDAR or AMPAR antibody mixtures, respectively. When analysed through two adjacent sections, the percentage of labelled synapses increased to 92 ± 2% for NMDARs (n = 108) and to 79 ± 5% for AMPARs (n = 105; different from NMDAR, P < 0.05).

NMDAR (Fig. 2A–D) and AMPAR (Fig. 2F–I) particles were found along the PSD of spines contacted by either ZN+ or ZN– boutons. Receptor particles and zinc precipitates were sometimes apposed to one another at individual ZN+ synapses (Fig. 2D,G). Similar proportions of ZN+ and ZN– synapses were immunolabelled for NMDAR (P = 0.13) and AMPAR subunits (P = 0.8), respectively (Table 1).

In CA3, all large MF boutons were profusely labelled for vesicular zinc and commonly enveloped immunostained thorny excrescences. As in CA1, both NMDAR (Fig. 2E) and AMPAR (Fig. 2L) particles were localized over the postsynaptic membrane and density of spines.

Further supporting the specificity of double staining, results were reproduced in lightly fixed, Lowicryl-embedded CA1 region: GluR2/3 protein and zinc ions showed post- and presynaptic distributions, respectively, and frequently co-localized to single synapses, although tissue preservation was largely compromised (Fig. 2J,K).

**Lower Amounts of AMPAR, but not NMDAR, Immunoparticles at ZN+ Synapses**

Synaptic immunostaining was quantified at 200–300 µm from the pyramidal cell layer in CA1. The mean number of NMDAR particles was similar at ZN+ and ZN– synapses (Table 1; particles per labelled synapse, P = 0.9), as were the frequency distributions of NMDAR particles found in the two populations (Fig. 3A). In contrast, AMPAR particles were significantly less abundant (~4%) at ZN+ synapses than at ZN– synapses (particles per labelled synapse, P < 0.01; Table 1). The frequency distribution of AMPAR labelling was much more skewed towards lower values at ZN+ synapses (Fig. 3B; skewness factor for ZN+ = 1.6; skewness factor for ZN– = 0.8; Shapiro–Wilk test). The distinct content in AMPAR particles was confirmed in an additional sample of synapses reacted through two serial sections: the mean number ± SEM of particles per synapse was
Figure 2. Expression of AMPA- and NMDA-receptor subunits at ZN+ synapses in CA1 and CA3 hippocampal regions. Gold particles (10 nm) coding for NR1 + NR2A/B (A–E) and GluR1–3 (F–I) subunits localized to asymmetric synapses made by ZN+ (b+) or ZN– (b–) boutons in CA1 stratum radiatum (A–D, F–K) and CA3 stratum lucidum (E, J). Particles located as far as 45 nm away from the inner face of the postsynaptic membrane (2G, dashed lines) were considered ‘synaptic’ (black arrowheads) and beyond, ‘extrasynaptic’ (white arrowheads). (A–D) Co-localization of postsynaptic NMDAR label and vesicular zinc at single spine (s) synapses. (E) A large MF terminal heavily stained for vesicular zinc and contacting two thorny excrescences (t), of which only one is NMDAR-immunoreactive. (F–I) Co-localization of postsynaptic AMPAR label and vesicular zinc at single synapses. Note in (G) a silver grain (solid arrow) by the presynaptic membrane and opposite two AMPAR particles. Scale bar = 50 nm, m, pre- and postsynaptic membranes; b, bouton; c, synaptic cleft; d, postsynaptic density. (J, K) Synapses in CA1 that were cryoembedded in Lowicryl and immunoreacted for GluR2/3. (J) Samples not developed for zinc; (K) double staining for zinc and GluR2/3. Note the poor structural preservation and difficulty of identifying synaptic vesicles in both cases. (L) A giant MF terminal is stained for vesicular zinc and contacts several AMPAR-immunoreactive thorny excrescences. Scale bars = 0.2 µm.

Table 1

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<tr>
<th>GluR1–3</th>
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<th>ZN+ (CA1)</th>
<th>ZN+ (CA3)</th>
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<td>Synapses examined</td>
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<td>121</td>
<td>118</td>
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<td>Immunolabelled synapses (%)</td>
<td>67 ± 10</td>
<td>60 ± 11</td>
<td>67 ± 8</td>
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<td>Particles/labelled synapse</td>
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<td>216 ± 22b</td>
<td>309 ± 32b</td>
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<td>7.1 ± 1.3bc</td>
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<th>ZN+ (CA1)</th>
<th>ZN+ (CA3)</th>
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<tr>
<td>Synapses examined</td>
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<td>147</td>
<td>110</td>
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<tr>
<td>Immunolabelled synapses (%)</td>
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<td>Particles/labelled synapse</td>
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<td>10.4 ± 1.1b</td>
<td>9 ± 2b</td>
<td>4.91 ± 0.18bc</td>
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* Difference between ZN– and ZN+ synapses in CA1.
* Difference between CA3 and CA1 regions.
* Difference between AMPAR and NMDAR label.
and was also lower than that of NMDARs in CA1 (P < 0.05). As synapses were compared at the same distance from the pyramidal cell layer, data were not affected by the distance-dependent increase in dendritic AMPARs (Andrasfalvy and Magee, 2001). In CA3, the linear density of NMDAR particles at MF synapses was markedly lower than that of AMPARs (P < 0.05; Fig. 4Ce). Similarly, 54 ± 8% of ZN− synapses (n = 94) reacted for GluR2, but only 37 ± 8% of ZN+ synapses did (n = 129; P < 0.05; Fig. 4D–F). Immunoreactive ZN+ synapses tended to have fewer particles for both GluR1 (1.4 ± 0.2 particles at ZN+ synapses and 1.7 ± 0.4 at ZN−; P = 0.4) and GluR2 (1.4 ± 0.1 particles at ZN+ synapses and 1.6 ± 0.3 at ZN−; P = 0.3), but differences were not statistically significant.

### Discussion

The main finding of the present experiments is that, in the stratum radiatum in CA1, two populations of glutamatergic boutons making morphologically similar synapses can be differentiated based on their vesicular zinc content and ZnT-3 expression. Furthermore, the levels of postsynaptic AMPARs, but not of NMDARs, correlate with the presynaptic bouton type. Thus ZN+ synapses showed a lower AMPAR content than ZN− synapses.

**ZN+ Boutons in the Hippocampus: Abundance and Neurochemistry**

The presence of histochemically chelatable zinc, corresponding to vesicular zinc, in the CA1 region is well established (Slomianka, 1992; Sanchez-Andres et al., 1997). However, the incidence of vesicular zinc among defined boutons has been unexplored. We identified vesicular zinc in ~45% of the boutons making axosominal synapses in the intact CA1 stratum radiatum. Despite the low number of silver grains per bouton profile, due to the small bouton size in this region, staining for zinc (or the lack of it) was consistent in serial sections. Furthermore, zinc stain was accompanied by ZnT-3 immunoreactivity of synaptic vesicles, in contrast to ZN− boutons. This provided independent evidence that zinc histochemistry accurately identifies vesicular zinc, even in small cortical boutons. Complementing these data, all large MF boutons in CA3 stained for zinc and ZnT-3. These findings strongly advocate a diversity of zinc content among synapses based on the differential expression of ZnT-3. Whether this presynaptic diversity reflects the heterogeneous origin of the parent cell bodies or an as yet overlooked diversity among

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**Figure 3.** Frequency distribution of NMDAR and AMPAR particles obtained from single section analysis of ZN+ and ZN− synapses in CA1. The frequency (%) of synapses labelled at the postsynaptic membrane or density (PSD) is plotted against the number of gold particles. (A) Immunolabelling for NR1 + NR2A/B is similarly distributed in ZN+ and ZN− synapses. (B) Immunolabelling for GluR1–3 at ZN+ synapses is skewed to lower values than at ZN− synapses. There was a higher fraction of ZN+ synapses stained with only one particle (P < 0.02) and a lower fraction with three (P < 0.05) and four particles (P < 0.02). (C) Observed distributions for AMPAR labelling at both ZN+ and ZN− synapses fit well the Poisson distributions expected for the corresponding mean (particle per synapse) values (ZN+ = 1.74 ± 0.3; ZN− = 1.05 ± 0.2). Data are expressed as mean ± SEM.

**Figure 4.** Immunolabelling for GluR1 and GluR2 subunits in epon-embedded, stratum radiatum in CA1. Examples of ZN+ and ZN− synapses labelled with gold particles coding for GluR1 (15 nm) in (A–C) or for GluR2 in (D–F) (D and F, 10 nm; E, 15 nm). Black arrowheads, synaptic particles; white arrowheads, extrasynaptic particles. Scale bar = 0.2 µm.
CA3 pyramidal cells is currently under investigation. Functional experiments in vitro have failed to detect zinc release (Howell et al., 1984) or a post-synaptic effect of endogenous zinc (Lu et al., 2000) in CA1. Since vesicular zinc is widespread in this region, the discrepancy may be explained by (i) the substantial depletion of endogenous zinc observed in in vitro preparations (Suh et al., 2000), (ii) the presence of contaminant zinc, which hinders the interpretation of negative results (Paoletti et al., 1997) and (iii) the distribution of vesicular zinc to a subset of boutons, as shown here. Hence, our data encourage the reassessment of the synaptic actions of zinc in the CA1 region.

All ZN+ boutons stained for VGLUT-1 and lacked GABA, confirming their excitatory nature (Martinez-Guijarro et al., 1991; Beaulieu et al., 1992). The hypothesis that vesicular zinc is synaptically released together with glutamate to modulate postsynaptic responses is based on the assumption that both are contained in the same synaptic vesicle, at least in synapses where exocytosis is quantal. We observed several silver grains overlying or contained within synaptic vesicles that were juxtaposed to VGLUT-1 immunoparticles. It is possible, then, that ionic zinc and glutamate co-exist in a number of vesicles. About 25% of VGLUT-1 labelling was also found on pre- and postsynaptic junctional membranes. This may correspond to VGLUT-1 from docked presynaptic vesicles, but also to some VGLUT-1 expressed on the plasma membrane, as suggested elsewhere (Belloccchio et al., 1998).

**Expression of AMPARs at ZN+ Synapses**

The majority of dendritic spines in CA1 and CA3 reacted for AMPAR and NMDAR subunits after being processed for the visualization of zinc. The percentages of immunolabelled synapses are similar to those described in single sections of epon-embedded sensory cortex (Kharazia and Weinberg, 1999), although equivalent studies in the hippocampus are not available. Percentages increased when synapses were immunoreacted in paired sections to levels consistent with those found in Lowicryl (Nusser et al., 1998; Takumi et al., 1999; Racca et al., 2000). AMPAR label was not detected in ~20% of spines, which might correspond to functionally ‘silent’ synapses at resting membrane potentials or to synapses with AMPARs expressed at levels below the detection limit of the immunogold method (Groc et al., 2002). The subsynaptic distribution of AMPARs matched that reported in synapses of CA3 radiatum (Fabian-Fine et al., 2000). Likewise, in MF synapses the density of AMPARs was higher than that of NMDARs, coincident with a previous study (Takumi et al., 1999) and in agreement with electrophysiological data (Weiskopf and Nicoll, 1995). AMPAR counts, however, were not higher than in CA1, in contrast with other work (Nusser et al., 1998; Takumi et al., 1999). Differences in the antibody mixtures used here (GluR1–3 antibodies) and elsewhere (pan-AMPAR + GluR2/3 and GluR1–4 antibodies) may account for this discrepancy and possibly reflect a distinct AMPAR subunit enrichment at MF synapses. Altogether, data confirm the good performance of the double staining method used here.

Gold particles located over the postsynaptic junctional membrane most likely identified subunits that were assembled into synaptic receptors. This conclusion is based on the correlation between the number of receptor particles and the amplitude of miniature synaptic currents reported elsewhere (Nusser et al., 1997; Golshani et al., 2001). Thus specific immunolabelling for glutamate receptor subunits provided strong anatomical–molecular evidence supporting the expression of functional glutamate receptors at ZN+ synapses.

The population of ZN+ synapses presented, on average, significantly lower AMPAR staining than the ZN– population. In principle, reduced GluR1–3 labelling can be caused by either a lower receptor number or a different subunit composition of receptors. Immunoprecipitation studies indicate that these synapses form most of the receptor complexes expressed by CA1 pyramidal neurons, with little presence of GluR4 (Wenthöld et al., 1996). We found that GluR1 and GluR2 subunits were similarly widespread at ZN– synapses in CA1, much like combined GluR1–3. However, both GluR1 and GluR2 were detected in substantially fewer ZN+ synapses, again in very similar proportions. Hence, these comparisons reveal that ZN+ synapses most likely express lower levels of AMPAR than ZN– synapses, rather than being enriched in different subunits. Differential AMPAR expression according to these different subtypes may explain, at least in part, the variability in AMPAR content that is not due to differences in synaptic size (Nusser et al., 1998). On top of this, synaptic activity may modulate receptor levels within a given range (Heyen et al., 2000). It remains to be determined whether ZN+ and ZN– boutons have different exo/endocytotic properties, which are key to all forms of synaptic plasticity that require high-frequency presynaptic use.

**Expression of NMDARs at ZN+ Synapses**

Exogenously added zinc inhibits the NMDAR component of synaptic responses (Westbrook and Mayer, 1987). Double labelling showed that NMDARs were found on the majority of ZN+ synapses in CA1 and CA3 regions, suggesting that the synaptic co-release of zinc may mediate a similar inhibition in vitro. There is strong evidence for such an interplay at MF synapses, where endogenous zinc tonically inhibits postsynaptic NMDARs (Vogt et al., 2000), but not in CA1 (Lu et al., 2000). Conversely to functional results, the linear densities of NMDAR particles were clearly higher at CA1 synapses than at MF synapses (Table 1). Other factors, such as subunit-specific receptor affinity (Chen et al., 1997; Paoletti et al., 1997), carboxyl-tail interactions (Yamada et al., 2002), or peak concentration of released zinc may thus contribute to the inhibition of NMDARs by zinc.

In conclusion, due to the presence of vesicular zinc in about half of spine synapses and the associated selectivity of glutamate receptor content, synaptic transmission and plasticity in CA1 neurons may be considerably affected by the particular excitatory input.

**Notes**

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Address correspondence to Carlos Balet Sindreu, Departament de Biologia Cellular, Universitat de Barcelona, Av. Diagonal 645, ES-08071 Barcelona, Spain. Email: carbalet@bio.ub.es.

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