Research report

Release of synaptic zinc is substantially depressed by conventional brain slice preparations

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Abstract

Research on synaptically-released zinc is frequently done in vitro with acute brain slice preparations. We show here the in vitro hippocampal slice preparation has two major pitfalls for zinc research. First, up to 50\% of the synaptic zinc is lost during slice cutting and/or the first 10 min of slice incubation, with the losses being most pronounced on the edges of the slice. Second, the release of the remaining zinc from a slice is substantially depressed (up to 50\%) at the low temperatures (32\C) typically used for brain slice studies. In concert, these effects reduce zinc release about 75\% in vitro, compared to in vivo. Implications for research on synaptically-released zinc are discussed.

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1. Introduction

Historically, in vitro investigations of synaptically-released zinc have yielded results that varied with choice of research paradigm. Specifically, whereas studies using pharmacologic mimicry (adding exogenous Zn\textsuperscript{2+}) have consistently revealed substantial and reliable effects of the Zn\textsuperscript{2+} [8,25,30,32], studies using pharmacologic blockade (blocking the endogenous Zn\textsuperscript{2+} signal) have typically failed to find a consistent effect of the blockade [6,12,13,14,28,31,32].

One explanation of this difference could be that synaptic release of endogenous zinc is absent or minimal under typical in vitro brain slice conditions. Indeed, although a temperature, Ca\textsuperscript{2+}, and impulse-dependent release of zinc from boutons has been shown in the brain slice preparation [4,18], recent attempts to replicate and extend these early findings have yielded only marginal ‘release’ of zinc [6,20].

In the present work we tested the hypothesis that zinc release might be suppressed under ‘standard’ in vitro slice conditions. Two factors were considered (i) the potential loss of vesicular zinc induced by the tissue slicing and (ii) the potential suppression of zinc release by the relative hypothermia (30–32\C) that is now typically used for brain slice preparations [20,32].

To measure zinc release, we used the strategy of measuring the level of residual synaptic zinc left in a slice after various in vitro zinc-releasing treatments. The gas-autometallographic (gas-AMG) technique [11] was used to stain vesicular zinc, and densitometry was used for quantification.

Parts of this paper have been published previously in abstract form [16].
2. Materials and methods

2.1. Hippocampal slice preparation

Experiments were performed on transverse hippocampal slices taken from 5 to 6-week-old Wistar male rats. In all, 40 hippocampal slices and 10 additional hippocampi taken directly from the intact animal (‘freshly frozen’ see below) were studied quantitatively. After halothane (3.5%) inhalation in the anesthetic box, rats were perfused with cold ACSF (4°C, NaCl was substituted by 190 mM sucrose) by intracardially for 1 min [1,26]. After perfusion, rats were decapitated, and hippocampi were dissected out and placed in cold (4°C) artificial cerebrospinal fluid (ACSF). Transverse slice (400 μm) were cut with a vibratome (Campden Vibraslice, model # 752) and placed on a nylon mesh in an incubation chamber. The ACSF temperature in the incubation chamber was gradually increased from 4°C to 20°C for >30 min. The slices were kept at 20°C in oxygenated (95% O₂, 5% CO₂) ACSF for 30 min before stimulation. The gas mixture was also bubbled through the water bath in the chamber and vented over the chamber. The standard ACSF comprised (in mM) 130 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 20 NaHCO₃, 2 ascorbic acid and 10 D-glucose. The high potassium (K⁺) ACSF contained 23.5 mM KCl [4].

Of the total 40 hippocampal slices (400 μm) used in the experiment, 10 were taken directly from the vibratome cutting bath (within 10 min of cutting, below 4°C) and frozen for subsequent analysis of vesicular zinc. The remaining 30 slices were moved forward to preincubation at 20°C in normal ACSF with 5 mM CaEDTA.

2.2. High K⁺ evoked zinc release

All K⁺ stimulation experiments consisted of 15 min incubation at the target temperature (26°C (n=10), 32°C (n=11), or 39°C (n=9)), 15 min incubation with high K⁺ (23.5 mM), followed by 30 min washout in normal ACSF, all maintained at target temperature. CaEDTA (5 mM) was present in the bath for blocking zinc reuptake for the entire hour. At the end of experiments, slices were quickly frozen by CO₂ gas for cutting and gas-AMG staining. The membrane impermeable disodium-calcium salt of EDTA (Ca-EDTA, Sigma, ED₂SC) was used to prevent loss of calcium ions while allowing zinc ion chelation from the extracellular fluid. The effect of the CaEDTA is governed by the affinities of the chelator for the divalent cations [24]: the affinity for zinc is very high (KD for Zn-EDTA is ~10–15) whereas the affinity for Ca²⁺ is lower (KD=10–7,3) and that for Mg²⁺ lower still (KD=10–5.4). Accordingly, when added as the calcium complex, CaEDTA will remove zinc (higher affinity) but not Mg²⁺ (lower affinity) or Ca²⁺ (ion swapping only). In the absence of greater numbers of competing zinc ions, Cu²⁺ and Fe³⁺ would also be chelated by CaEDTA.

2.3. Fresh frozen hippocampal section preparation

For the purpose of measuring the normal concentration of vesicular zinc in the hippocampal formation, ten 5- to 6-week-old male rats were sacrificed with halothane and decapitated, and those brains were quickly removed and frozen on a CO₂ gas freezing stage, and cut in a closed-top cryostat at 30 μm. The first section was collected at 3.6 mm caudal to Bregma, then every 3rd section was saved until 10 were obtained. After cutting, the sections were thawed onto glass slides, dried, then stored (~80°C) for subsequent exposure to gaseous H₂S.

2.4. Autometallographic zinc staining (AMG)

AMG staining was performed by the postmortem-biopsy H₂S method [11]. Briefly, the freshly-cut sections were thawed onto cleaned glass slides for a few seconds then returned to the −9°C cryostat chamber. For S²⁻ exposure, the sections were transferred directly to a −17°C chamber which was filled with H₂S gas [11]. After exposing the sections to H₂S gas for 60 min to precipitate the free Zn²⁺ as ZnS clusters or crystallites, the slides were developed by the silver lactate AMG developer [9,10] for 60 min. All sections were counterstained with toluidine blue. After rinsing and dehydration, the sections were mounted using DePex mounting medium.

2.5. Optical density measurements of zinc staining

After gas-AMG staining, sections were mounted in the microscope, transilluminated with a fixed intensity of white light (tungsten), and individual images were captured by CCD camera, digitized, and stored. Every section that was obtained from an individual brain slice (typically 10–15) was used for quantification. Two images were captured from each section, one including the hilus of the dentate gyrus and adjacent CA1 of the hippocampus, the other including CA3a (Fig. 1). The zones were digitized as follows: reference a square of stratum lacunosum-molecular-away the middle of the blade of the dentate gyrus; CA1, the same square zone, moved into s. radiatum (SR), adjacent to the reference zone; hilus, the same square, moved top the CA3c-hilar confluence; CA3, the same square centered over s. lucidum in CA3a-b (SL). Optical Density was calculated conventionally (O.D. = (log₁₀ [incident light/transmitted light]), with ‘incident light’ taken as the intensity of light transmitted through the zinc-free reference zone (in lacunosum-molecular-away from the lateral perforant path innervation), and ‘transmitted light’ taken as the raw intensity reading for individual samples.
different regions lost between 45% and 60% of the total vesicular zinc by the time they were taken out of the vibratome collection tray for subsequent testing (Fig. 1). More zinc was lost from the margins of the tissue than from the core (Fig. 2).

Tests of temperature effects showed that the magnitude of depolarization-induced release varied monotonically with temperature. At 26°C, the mossy fiber neuropil lost 36% to 42% of the vesicular zinc, whereas at 39°C roughly twice as much (67–79%) of the vesicular zinc was released (Figs. 1, 3). To visual inspection, sections incubated for 1 h in normal K⁺ showed no apparent loss of zinc at any temperature tested (data not shown).

4. Discussion

The use of densitometry with silver-stained tissue as a measure of vesicular zinc rests on two assumptions. The first assumption is that the silver staining actually stains vesicular zinc. This is a relatively safe assumption in the presynaptic boutons in all of the hippocampal regions under consideration [11, 23].

The second assumption is that the optical density (OD) values are at least monotonically related to the actual amount of vesicular zinc in the tissue. This assumption also appears to be supportable, given that the regional OD data correlate highly with prior microfluorimetric measurements of regional vesicular zinc concentrations [15]. Specifically, the relative amounts of vesicular zinc found in a prior fluorimetric study (CA1, 13, Stratum lucidum 75, hilus 81) correlate highly (r=0.99) with the relative abundance found in the present work by OD (CA1 0.12, stratum lucidum 0.65, hilus 0.70).

Another issue affecting the estimation of vesicular zinc concentration from the O.D. of the staining is the matter of tissue swelling, which would dilute the tissue concentration of vesicular zinc. The amount of slice swelling can be substantial in some conditions [2, 21], but the osmolarity of the medium used in the present experiments (with 10 mM glucose) is generally associated with only a 10–15% increase in tissue volume (tissue water) [5]. Thus, not more than 15% of the reduction in O.D. of zinc staining should be attributable to slice swelling.

Accepting OD levels as indicative of vesicular zinc levels (+15%) leads to the conclusion that very little—perhaps only 1/4th or less of the normal vesicular zinc can be released from presynaptic boutons in a conventional, 32°C acute brain slice preparation. This may explain, for example, why Budde and colleagues [6, 20] could only obtain modest and inconsistent synaptic release of zinc in their prior work, whereas studies done at normal rat body temperature either in vivo [3, 7, 19] or in vitro [17, 18, 27] obtained consistent and vigorous release.

Moreover, if synaptic release of endogenous zinc is
Fig. 2. Zinc Loss From Slice Edges. The percent intensity of staining of vesicular zinc (optical density) is shown at 3 different layers. Hippocampal slices were cut with vibratome for in vitro use, then collected immediately and frozen (30 μm, within 10 min). Slices were cut into 8–13 sections. Total OD determinations have been done from 381 slice images. ‘Edge’ refers to the first 2 sections on the top of the slices and the last 2 on the bottom of the slices, which were averaged together (Hilus, n=47; CA1, n=46; CA3, n=40). ‘ Intermediate’ refers to the next 2 sections from the top and the next 2 sections from the bottom of the slices, which were averaged together (Hilus, n=47; CA1, n=46; CA3, n=40). The ‘middle’ refers to the remaining section’s average on the core of the slice (Hilus, n=37; CA1, n=39; CA3, n=39). Intensity of zinc staining in edge is lower than middle of slices about 33–50%. All of the values are relative to 100% for ‘middle’. A. Hilus, hippocampal hilus mossy fiber area; B, SL of CA3, Stratum Lucidum of Cornu Ammonis area 3; C, SR of CA1, Stratum Radiatum of Cornu Ammonis area 1. D represents schematic drawing of OD measured layers; e, ‘edge’, i, ‘intermediate’ and m, ‘middle’. Bars show one S.E.

spotty and inconsistent in the typical acute brain slice paradigm (as shown here), this may explain prior failures to find a role for synaptically-released zinc with the brain slice preparation. If little or no endogenous zinc is released in the brain slice paradigm, then blocking that signal with a chelator would be expected to have little effect. This is what Easley et al. [13,14] found when using DEDTC or TPEN to chelate zinc in slices maintained at 30–32°C. Although an effect of chelation on high-frequency synaptic driving was inferred, essentially no effect whatever was observed on single-pulse monosynaptic transmission at zinc-containing synapses. Doller and Crawford likewise found essentially no effect of zinc chelation (dithizone) on synaptic transmission in hippocampal slices [12]. Smart and colleagues have also had mixed success in demonstrating effects of zinc-signal blockade in vitro. Some zinc binding agents have produced effects on post-synaptic electrophysiology at 30°C [31,32] whereas others inexplicably have not [32]. Budde et al. also observed only inconsistent and transitory effects on synaptic transmission [6].

The present results suggest that acute brain slice preparations may be poorly suited for research on roles of endogenous synaptic zinc. Possibly, incubating slices in media in which the free zinc is held constant by a suitable zinc buffer would allow the slices to replenish vesicular zinc pools by the use of demonstrated zinc uptake processes. However, another strategy might be to use organotypic cultured brain slices, which, if collected from suitably mature rat pups will contain richly-laden zinc-containing boutons and will release zinc vigorously [17,27].

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Lacunosum-Moleculare, Hilus, SL of CA3, and SR of CA1. 234.

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