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Synaptic release of zinc from brain slices: Factors governing release, imaging, and accurate calculation of concentration

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Abstract

Cerebrocortical neurons that store and release zinc synaptically are widely recognized as critical in maintenance of cortical excitability and in certain forms of brain injury and disease. Through the last 20 years, this synaptic release has been observed directly or indirectly and reported in more than a score of publications from over a dozen laboratories in eight countries. However, the concentration of zinc released synaptically has not been established with final certainty. In the present work we have considered six aspects of the methods for studying release that can affect the magnitude of zinc release, the imaging of the release, and the calculated concentration of released zinc. We present original data on four of the issues and review published data on two others. We show that common errors can cause up to a 3000-fold underestimation of the concentration of released zinc. The results should help bring consistency to the study of synaptic release of zinc.

Keywords: Synaptic release; Zinc; Cerebrocortical neurons

1. Introduction

Ever since Haug suggested that certain synaptic vesicles are rich in "free" (rapidly-exchangeable) zinc (Haug, 1967), it has been proposed that the zinc would be released along with other contents of the synaptic vesicles. Such release was first shown in two reports 1984, which established that zinc release is impulse- and calcium-dependent (Howell et al., 1984; Assaf and Chung, 1984).

A variety of indirect demonstrations of zinc release have been adduced since, including the finding that vesicular zinc is present in axons of control animals, but absent in animals that have been subjected to intense and/or prolonged activation of the axons just prior to staining for zinc (Haug et al., 1971; Sloviter, 1985; Frederickson et al., 1988; Suh et al., 2000a). Another indirect replication is found in the results of experiments in which a zinc-containing pathway is activated, and some of the observed effects of activation are then blocked by blocking (typically chelating) the released zinc (Vogt et al., 2000; Molnar and Nadler, 2001; Smart et al., 2004).

Direct demonstrations of zinc release have included the release of total elemental zinc as well as the release of the free zinc ion. The total zinc release studies have been done using microdialysis in the intact brain (Charton et al., 1985; Aniksztejn et al., 1987; Itoh et al., 1993; Takeda et al., 1999; Minami et al., 2002) as well as fraction collection from brain slices in vitro (Howell et al., 1984; Assaf and Chung, 1984).

Another approach has shown that zinc labeled in situ in the vesicles is released (along with the label) over time (Perez-Clausell and Danscher, 1986) and more rapidly over time dur-

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ing stimulation (Kay et al., 1995; Budde et al., 1997; Varea et al., 2001; Quinta-Ferreira and Matias, 2004). Release of free zinc has been studied by microdialysis in the intact brain (Frederickson et al., 2005a) and by fluorimetric imaging of zinc release from brain or retinal slices (Thompson et al., 2000; Li et al., 2001; Ueno et al., 2002; Takeda et al., 2005; Qian and Noebels, 2005; Komatsu et al., 2005; Redenti and Chappell, 2005).

The amount of zinc that can be released from presynaptic terminals by electrical stimulation is an issue not yet fully resolved. One clear fact is that some cytoarchitectonic regions of the brain, such as the neuropil of the lateral amygdala, subiculum, and the mossy fiber neuropil of the hippocampus, contain from 200 to 600 μM of rapidly-exchangeable zinc. This has been shown by instrumental analyses indicating, for example, that the mossy fiber neuropil has from 70 to 200 ppm (dry weight) of "extra" zinc that is above and beyond what adjacent gray matter contains, which averages 70 ppm (Frederickson et al., 1983). This "extra" 70–200 ppm zinc, which corresponds to about 200–600 μM of zinc in the wet tissue, is definitely concentrated in the zinc-rich axons, as is shown by two observations. First, it is anatomically co-extensive with the axons (Frederickson et al., 1983; Wensink et al., 1987), and, second, in genetic knock-out mice that lack vesicular zinc influxers (ZnT-3 knockouts), the enrichment is not present (Linkous et al., 2006).

Most investigators who have measured the synaptic release of "free" Zn^{2+} from the densely innervated mossy-fiber neuropil have found that around 1–10% of the free zinc in the vesicles can be released, resulting in 1–10 μM "puffs" of free zinc being detected by fluorescent imaging techniques (Thompson et al., 2000; Li et al., 2001; Ueno et al., 2002; Komatsu et al., 2005; Takeda et al., 2005; Redenti and Chappell, 2005; cf. Kay, 2003). The present work was undertaken in search of greater understanding of the factors that govern the final estimates of the concentration of zinc released.

2. Methods

2.1. In the laboratory of NeuroBioTex Inc.

Timed pregnant rats were obtained from Sprague Dawley, and allowed to deliver in the animal colony. The pregnancies were timed to provide 1 or more litters of pups reaching the ages of 16, 23, 30, 37 and 90 days of age all on the same day so that they could be killed and brain processed all on the same day. In all the total number of pups that were processed was 16 day 5, 23 day 6, 30 day 4, 37 day 6. In addition, a group of adult rats (200–350 g) was killed.

Rats were killed with an overdose of halothane, decapitated, and brains were quickly removed, frozen on a liquid CO_2 freezing stage, then wrapped in cold Saran Wrap, enclosed in a small air-tight plastic jar, and stored at −30 °C. Within 2 months of freezing, brains were removed in "squads" (one of each age) and processed side-by-side through cutting (30 μm frozen; horizontal), staining (30 μL of TSQ), clearing (60 μL of glycerine), coverslipping, and imaging. The sections selected for processing were standardized by counting every section from the first tissue contact, and taking the pre-set section numbers determined in earlier studies of complete serial sections through the pup brains of all ages used. Imaging was done using a large-format UV fluorescent transilluminator with the section lying atop the illuminator and photographed from above through a UV blocking and a 505 nm band pass filter. Camera focus, zoom, exposure time, aperture, and all compensation controls were locked in manual, unchanged settings throughout the entire project.

2.2. In the laboratory of Cellular Neurobiology at the University of Valencia

Sections were processed only from 16-day-old and 90-day-old rats. Briefly, rats were killed by an overdose of chloral hydrate and decapitated. Then brains were removed, and 400 μM slices were obtained using a Mc Ilwain chopper. Sections were collected into ice-cold ACSF. Slices of each animal were separated into four different groups. Sections were incubated for 15 min in either (i) ACSF, (ii) ACSF and CaEDTA (50 μM), (iii) ACSF and 50 mM K^+ and (iv) ACSF plus CaEDTA and K^+. For zinc quantification, TSQ was introduced into the slice chamber (after incubations, at a concentration of 30 μg/ml TSQ in ACSF for 10 min) and the slices were imaged with a high sensitivity camera (Hamamatsu C4880-80) and Aquacosmos software for analysis.

2.3. In the laboratory of Jae-Young Koh

Wistar rats, 16, 23, 30, 37 and 90 days of age ($n = 6$ for each age) were studied. Coronal brain sections of 10 μm thickness were obtained with a cryostat, and incubated for 30 min in 25 μM Zinquin in PBS. Zinquin fluorescence intensity in the dentate hilus was measured with the fluorescence microscope using a computer-assisted image analysis program (Image-Pro, Media Cybernetics, Silver Spring, MD). To determine specific zinc fluorescence, background fluorescence in the adjacent white matter was subtracted from that in the dentate hilus. Specific fluorescence values were normalized to the mean specific value of 90-day-old rats as 100%.

2.4. In the laboratory of Michal Hershfinkel

Transverse slices were prepared from newborn mice postnatal days 8–14 (young, $n = 20$) or 40–45 days old mice (young adult, $n = 20$) as previously described (Schipke et al., 2001). Slices were sectioned at 4 °C and then allowed to equilibrate in Ca^{2+} free ACSF, for 10–30 min at 25 °C, to minimize spontaneous activity. Slices that were equilibrated for longer periods did not show significant zinc release, possibly due to depletion of synaptic zinc. Slices were then mounted on a microscope stage chamber and immediately before the stimulation the perfusion was stopped. All slice manipulation was done using ACSF that did not contain PO_4, because it precipitates zinc. The cell impermeable acid form of ZnAF2 (4 μM) or Newport Green (2 μM) was applied into the chamber and within 30 s a current was applied using a bipolar tungsten electrode, stimulating at